Synthesis of 2′-Modified RNAs as Substrates Towards the Inhibition of tRNA 2′-Phosphotransferase (Tpt1)

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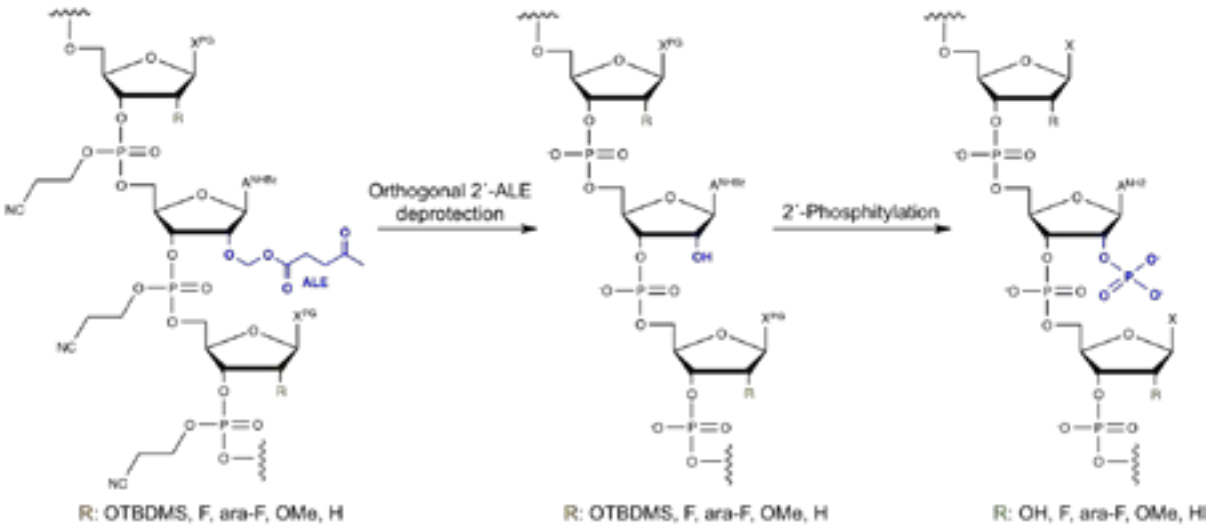
tRNA 2′-Phosphotransferase (Tpt1) is an essential agent of fungal tRNA splicing that removes the 2′-monophosphate (2′-PO_4) at the splice junction generated by fungal tRNA ligase. It does so *via* a two-step mechanism wherein an internal RNA 2′-PO_4 is transferred to NAD^+ to form mature 2′-OH RNA and ADP-ribose-1″,2″-cyclic phosphate. Our interest in Tpt1 arises from the fact that, while Tpt1 activity is essential for fungal growth, it is inessential in exemplary bacterial and mammalian taxa. As such, Tpt1 is seen as an attractive antifungal target since inhibitors of the enzyme should block the growth of fungal pathogens while sparing the human host. In light of this, we have implemented an optimized automated synthetic route for the synthesis of a series of chemically modified RNA substrates that mimic the splice junction. This presentation will cover the synthesis of these oligonucleotides and their effectiveness as Tpt1 substrates and inhibitors.



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Disulfide-unit conjugation enables ultrafast cytosolic internalization of antisense DNA and siRNA

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Development of intracellular delivery methods for antisense DNA and siRNA is important. Previously reported methods using liposomes or receptor-ligands have a problem that oligonucleotides take several hours or more to reach the cytoplasm due to long-time residence of oligonucleotides at endosomes. In this study, we clarified that oligonucleotides modified with low molecular disulfide units at the terminus reaches the cytoplasm 10 minutes after the administration to cultured cells. This rapid cytoplasmic internalization of disulfide-modified oligonucleotides suggests the existence of an uptake pathway other than endocytosis. In fact, the mechanistic analysis revealed that the modified oligonucleotides are efficiently internalized into the cytoplasm through disulfide exchange reactions with the thiol groups on the cellular surface. Because our approach solves several critical problems with the currently available methods for enhancing cellular uptake, including toxicity, undefined molecular composition, inefficient endosomal escape, and serum stability, this method may be an effective approach in the medicinal application of antisense and RNAi methods.

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003

LNA-rich oligonucleotides delivered by gymnosis down-regulate Huntingtin expression; effect of different LNA nucleotide patterns.

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Huntington disease (HD) is a neurodegenerative disease caused by CAG trinucleotide expansion at exon 1 of the Huntingtin gene (*HTT*). The consequence of this expansion is the formation of mutant *HTT* transcript and protein. Recent reports have confirmed formation of toxic RNA foci. Decreasing the levels of transcript and protein would be a very useful therapeutic option. Gymnotic delivery (naked delivery) of oligonucleotides (ON) is considered a more physiological and preferred approach compared to transfection. Transfection vectors are often associated with low efficacy or high cytotoxicity.
Most of the current literature focuses on targeting the mutant mRNA or protein. However, we are interested to test LNA/DNA ONs complementary to the *HTT* gene. We have previously shown that we could down-regulate both *HTT* mRNA and protein expression by targeting DNA[1]. In here we have studied the efficacy of such ON when delivered via gymnosis. Additionally, our investigation included different lengths of ON with different LNA nucleotide patterns.

The results display down regulation of *HTT* mRNA with the different lengths of ON tested. We have reported before that lengths lower than 12 mers are non-effective[1]. The mRNA levels were decreased up to 60% with some of the tested ONs. Interestingly, the effect was shown with longer ON (45 mer) with no observed toxicity. Lowering the content of LNA below certain limits decreased the effects dramatically regardless of the length of the tested ON. Additionally, different LNA nucleotide patterns did not show significant impact on the results obtained as long as the LNA content is unchanged.

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Using Lipid Nanoparticles to Efficiently Deliver CRISPR/Cas9 for Genome Editing in Rodents and Non-Human Primates

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There are multiple options to deliver CRISPR/Cas9 genome editing components to target cells *in vivo*. Of these, non-viral systems, specifically, lipid nanoparticles (LNPs) offer a number of advantages over viral-based systems. Apart from the cargo, the primary components for these types of LNPs include neutral lipid, cholesterol, PEG lipid and ionizable lipid. Previous research has demonstrated that ionizable lipids play an important role in cellular uptake and endosomal escape of LNP components.
Here, we present the development of an LNP-mediated delivery system for cargo comprising Cas9 mRNA and chemically synthesized sgRNA, and show that this system is capable of producing significant editing of the transthyretin (TTR) gene in the liver of rodents and non-human primates (NHPs). Following a single dose of LNP-delivered Cas9 mRNA/TTR sgRNA in both mice and rats, we achieved editing levels that resulted in > 90% reduction in circulating serum protein, which was sustained for at least 12 months in mice. Separately, in NHPs we achieved a therapeutically meaningful level of TTR protein reduction that correlated with robust and significant liver editing following single and multiple injections of LNPs. We have identified that RNA cargo optimization, including a novel sgRNA modification pattern and mRNA optimization to increase Cas9 protein expression, was the key to significantly improve the potency of the LNPs. This work demonstrates that our LNP system can achieve clinically viable levels of *in vivo* editing and reduction of TTR serum protein, highlighting the potential of this method for effective therapeutic delivery to the liver.

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005

Conversion of RNA aptamer into hybrid modified DNA aptamers provide for prolonged stability and enhanced antitumor activity

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AXL overexpression in epithelial ovarian cancer, the most lethal gynecological malignancy, is associated with reduced overall survival. Therefore, there is a need for novel therapeutic strategies for silencing and blocking the AXL signalling pathway. Several AXL inhibitors have been described in the literature, including small molecule kinase inhibitors or monoclonal antibodies. The issues with use of small molecules and antibodies as drugs are their side effects due to non-specificity; and the difficulties in manufacturing antibodies.
Aptamers, synthetic single strand oligonucleotides, are similar in their functionality to antibodies; have minimal side effects; and thus hold a high promise as therapeutics. The typical issues to use aptamers, as therapeutic agents, are the stability and bioavailability. Here, we present the translation to RNA aptamer to modified hybrid aptamers, targeting p-AXL. Based on the comparative analysis, a library of 17 hybrid DNA aptamers, differing in the position of dithio-modifications within sequence, was screened and characterized the best aptamer candidates to target p-AXL.
Here we demonstrate the use and improvement of aptamer specificity, bioavailability and antitumor activity in their effective silencing of the p-AXL target by the chemical modifications of existing aptamers for p-AXL. There is an immediate unmet need for novel therapeutics for the safe and effective treatment of OC and other cancers. Results obtained so far by us provide strongest evidence and lay the foundation for the next stage of development to advance towards the translation of the aptamer candidates and companion biomarkers to the clinic.

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2'OmethylRNA *EFG1* antisense oligomer to control *Candida albicans* filamentation

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Antisense oligomers (ASO) and their analogues have been successfully utilized to silence gene expression for the treatment of many human diseases, however the control of yeast´s virulence determinants have never been exploited before. In this sense, this work is based on the key hypothesis that if a pathogen's genetic sequence is a determinant of virulence, it will be possible to synthesize a nucleic acid mimic based on antisense therapy (AST) that will bind to the mRNA produced, blocking its translation into protein and consequently reducing the pathogen virulent phenotype. *EFG1* is an important determinant of virulence that is involved in regulation of *Candida albicans* switch from yeast to filamentous form. Thus, our main goal was to design and synthesize an ASO targeting the *EFG1* mRNA and to validate its *in vitro* applicability in order to control *C. albicans* filamentation.
For that, an ASO against *EFG1* was designed, including 2'OMethylRNA chemical modification, through bioinformatic tools. The fluorescence in situ hybridization (FISH) was performed and demonstrated that the ASO was able to penetrate *Candida* cell wall with high sensitivity and specificity. To determine the effect of anti-*EFG1* 2'OMe, the gene level expression, protein translation inhibition and reduction of filamentation reduction were analysed. The results show that the anti-*EFG1* 2'OMe oligomer was able to significantly reduce the levels of *EFG1* gene expression (around 58%) and of Efg1p protein translation (approximately 56%), as well as effectively prevent filamentation of *C. albicans* cells (by 80%). Moreover, it was verified that anti-*EFG1* 2'OMe keep the efficacy in different simulated human body fluids.

Undeniably, this work provides potentially valuable information for future research into the management of *Candida* infections, regarding the development of a credible and alternative method to control *C. albicans* infections, based on AST methodology.

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Networking to improve the Delivery of Antisense RNA therapeutics

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The main hurdle for the efficacy of antisense oligonucleotides (ASOs) seems to be their deficient delivery to target tissues but, while translational research on ASO is surging, very little is known about the mechanisms by which ASOs are taken up by different tissues and specific cells. Regarding delivery, the ASO field is fragmented, with researchers in academia and industry working in isolation on specific diseases, generally focusing on therapeutic effects in target tissues.

The European Cooperation in Science and Technology (COST) Association is the longest-running framework supporting trans-national cooperation (www.cost.eu). COST Actions, once approved, are not restricted to the original applicants: in fact, they are open to anyone with a legitimate interest to join the network. Funding is used for workshops, training schools, short scientific missions, and dissemination activities. We represent a COST Action (Delivery of Antisense RNA ThERapeutics-DARTER, www.antisenserna.eu) that aims to use networking and capacity building in the field of nucleic acid therapy delivery to allow RNA-targeting nucleic acid drugs to reach their full potential and become a mainstream therapeutic option.

DARTER will act until October 2022 through three working groups with research objectives (delivery strategies, model systems, safety and toxicology) and one capacity building group (stakeholder communication) with the shared objective of achieving consensus on protocols and assessment of ASO delivery and toxicology and training new researchers within a cooperative research framework. In addition, this new Action will have a particular focus on sharing negative results: a session on negative results will be included in each workshop and training school. This new network is currently comprised of more than 220 researchers from 27 European countries, plus 3 non-European ones (USA, Canada, and China) and is open to new collaborators.

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First-in-Human Study for Targeted Delivery of CD40 siRNA Complexed with β-Glucan to Antigen Presenting Cells

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Development of a delivery system has been an obstacle for advancement of oligonucleotide therapeutics. NapaJen Pharma has been developing a unique delivery system for oligonucleotides utilizing the β-glucan schizophyllan (SPG). SPG receptor Dectin-1 is expressed on myeloid cells, including monocyte/macrophage and neutrophil lineages [1]; therefore, it is anticipated that oligonucleotides complexed with SPG could be selectively delivered into cells expressing Dectin-1. We previously reported that approximately 8% of human peripheral blood mononuclear cells (PBMCs) express Dectin-1, including $CD14^+$ monocyte and conventional dendritic cells (cDCs)[2]. Selective delivery of siRNA/SPG into Dectin-1-positive PBMCs in a dose-dependent manner was confirmed while sparing Dectin-1-negative cells, such as T and B cells. Supported by these findings, NJA-730, a CD40 siRNA complexed with SPG, has been developed as an immune suppressing agent. Further, the *CD40* gene-silencing activity of SPG complex NJA-730 was confirmed in a preclinical study using non-human primates [2]. PBMCs obtained from the monkeys 24 hours after intravenous administration of NJA-730 demonstrated the expected cleavage products via 5'RLM-RACE analysis, indicating NJA-730 *in vivo* activity.

A first-in-human phase 1 study to evaluate the safety and tolerability of NJA-730 was conducted in 72 healthy male subjects. The study consisted of a single ascending dose (SAD) and a multiple ascending dose (MAD) portion. NJA-730 was administered via intravenous infusion over a 30 minute duration. For the SAD study portion, NJA-730 doses (oligonucleotide basis) starting at 5 μg/subject (~0.07 μg/kg) up to 600 μg/subject (~8.6 μg/kg) were investigated in a total of seven cohorts. No dose-limiting toxicity (DLT) was observed. n the MAD study portion, 300 or 600 μg/subject NJA-730 was administered every other day for 6 days. NJA-730 intravenous administration up to 600 μg/subject every other day for 6 days was safe and well tolerated. No serious or severe adverse events were reported at the end of the study within a 55 day follow-up period. The *CD40* gene-silencing activity of NJA-730 was evaluated by 5'RLM-RACE using PBMC samples and will be discussed further.

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Efficient target gene suppression effect of double stranded DNA structure type of oligonucleotide.

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Antisense oligonucleotides (ASOs) are promising therapeutic approach to target intracellular RNAs. Successes on the development of ASO therapeutics for spinal muscular atrophy, Duchenne muscular dystrophy or familial amyloid polyneuropathy predict a promising future for patients. Indeed, multiple clinical trials using ASO therapies are currently on going. Previously, we developed a DNA/RNA heteroduplex oligonucleotide (HDO (cRNA)), in which a complementary strand consisting of RNA (cRNA) was hybridized to the ASO. We presume that cRNA strand of HDO is recognized by RNase H1 and cleaved, consequently the parent ASO is released. Toc-HDO (cRNA), which was conjugated tocopherol to the complementary strand of HDO, showed higher target gene suppression in mouse liver compared to the parent ASO. (Nishina K., et al. Nat. Commun. 2015)

Here, we replaced complementary RNA strand to DNA strand, developed DNA/DNA double stranded oligonucleotide (HDO(cDNA)) and then evaluate its effect. ASO duplexed with a tocopherol-conjugated complementary DNA (Toc-HDO(cDNA)) , was also significantly more potent at reducing the expression of the target mRNA in mouse liver than parent ASO.

Then, we estimated the degradation pattern of the complementary strand over time in the mouse liver by Northern blot. This revealed that the cDNA was degraded more quickly than cRNA, suggesting that enzymes other than for RNase H1, such as DNase, are involved in the degradation of cDNA of HDO.

We have discovered that the DNA/DNA double stranded oligonucleotide highly increases the gene suppression effect of single stranded ASO. To further investigate the differences in immune response, stability in blood, and mechanism of action will be required.

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010

Improved Safety & Tolerability Profile with Ligand-Directed Delivery of Antisense Oligonucleotides in Humans: An Integrated Comparison of Parent 2'-*O*-Methoxyethyl Chimeric ASOs to the GalNAc$_3$-Conjugates

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Receptor-mediated delivery of ligand-conjugated antisense oligonucleotides (LICAs) are revolutionizing antisense therapeutics. Immediate utility has been found with the triantennary *N*-acetylgalactosamine (GalNAc$_3$) moiety for molecular targets expressed by liver hepatocytes, via the asialoglycoprotein receptor. In humans, GalNAc$_3$-conjugated phosphorothioate (PS) modified 2'-*O*-methoxyethyl (2'MOE) antisense oligonucleotides (ASOs) are up to 30-fold more potent than the parent ASO, with ED_{50s} ranging from 4-10 mg/wk. This increase in potency has led to lower and less frequent dosing, and a consequent reduction in systemic exposure. Here we report the results from an integrated safety and tolerability assessment of placebo-controlled data available from phase 1 dose-ranging trials in healthy volunteers for 4 GalNAc$_3$-conjugated ASOs compared to the respective parent ASOs. Safety profiles of the parent and GalNAc$_3$-conjugated ASOs were compared by the incidence of signals in standardized laboratory tests, and by the mean test results as a function of dose level over time. Tolerability profiles were compared by the assessment of rate per injection of local cutaneous reactions at the injection site (LCRIS) and flu-like reactions (FLRs), as well as by the incidence of dose discontinuations. This analysis included 195 subjects (71 parent ASO, 21 placebo; 79 GalNAc$_3$-conjugated ASO, 24 placebo), assigned to receive multiple subcutaneous doses of study drug over a 4 to 6-week treatment period at doses ranging from 50 to 450 mg/wk for parent ASOs and 10 to 120 mg/wk for the GalNAc$_3$-conjugated ASOs. There were no confirmed incidences of abnormal laboratory tests for liver function, and kidney functional tests were unremarkable. Mean ALT, creatinine and platelet levels all remained within the range of normal over time for both the parent and conjugate groups. GalNAc$_3$-conjugated ASOs demonstrated an average 30-fold reduction in the mean per injection rate of LCRIS compared to parent ASOs (28.6%, vs 0.9% conjugate). The incidence of FLR was <1% in the parent ASO group, vs 0% conjugate. Three subjects discontinued dosing in the parent ASO group, whereas all completed treatment in the GalNAc$_3$-conjugated ASO group. Although total exposure is limited in these trials, the results from this integrated comparison indicate a marked improvement in the safety and tolerability of GalNAc$_3$-conjugated ASOs compared to the unconjugated parent ASOs.

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Backbone-modified locked nucleic acids for therapeutic antisense applications

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Oligonucleotides with artificial backbones which mimic the natural phosphodiester linkage often show enhanced stability toward nucleases and have found applications in synthetic biology, nanotechnology, and gene synthesis. Whilst the incorporation of charge neutral analogues reduces the overall negative charge, these linkages typically reduce the thermal stability of the corresponding duplex, limiting their potential as therapeutics. In contrast, incorporation of conformationally restrained locked nucleic acid (LNA) into DNA improves the thermal stability of DNA:RNA duplexes. Therefore, we sought to combine the favourable properties of charge neutral linkages with those of LNA to create a new type of antisense oligonucleotide.

We have developed efficient strategies to prepare oligonucleotides containing multiple charge neutral linkages flanked by LNA on their 5′-side, 3′-side, or on both sides. The resulting oligonucleotides were found to bind their RNA targets with higher specificity and affinity that the corresponding unmodified DNA and have enhanced stability towards enzymatic degradation. We believe that these properties, along with the overall reduced anionic charge, make this class of oligonucleotides promising therapeutic agents and we are now carrying out cellular uptake and gene inhibition studies. I will present the synthesis, thermal and nuclease stability studies, along with crystal structures of the most promising candidates, and discuss preliminary data from exon skipping cell assays.

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Redox-sensitive meta-stable three-way nucleic acid junctions for multivalent delivery of therapeutics

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The burgeoning field of nucleic acid nanotechnology has produced over the last decade a significant number of functional RNA/DNA nano-devices for a variety of applications including diagnostics and therapeutics. Among others, nanostructures that deliver siRNAs have been developed by many groups and used to silence one or multiple target genes. However, *in vitro,* delivery of siRNAs using nucleic acid nanostructures appear to be less efficient compared to other means such as lipid transfection. For example, lipofectamine-transfected individual siRNAs can effectively silence genes at sub-nanomolar concentrations while existing nucleic acid nanostructures tend to require significantly higher concentrations. We hypothesize that one issue affecting potency is that the attachment of siRNAs to larger nanostructures can interfere with processing by the RNA interference (RNAi) pathway.

To test this hypothesis, we have developed two different compact three-way junctions (3wj) held together by Watson and Crick base-paring and disulfide crosslinking. Under non reducing conditions such as those encountered in the extracellular environment, the junctions are highly stable. Under reducing conditions that cleave disulfide bonds, tethered siRNAs are rapidly released allowing efficient RNAi loading. Optimized assembly procedures allow facile and efficient assembly of junctions with different siRNAs and delivery ligand combinations. When delivered with lipofectamine, siRNA attached to the junction showed identical potency compared to free siRNAs indicating that the junctions do not compromise RNAi processing. Our poster will discuss these results and additional biological data.

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Bioconjugated Oligonucleotides: Recent Developments and Therapeutic Applications

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Oligonucleotide-based agents have the potential to treat or cure almost any diseases, and are one of the key therapeutic drug classes of the future. Bioconjugated oligonucleotides, a subset of this class, are emerging from basic research and being successfully translated to the clinic. Two major approaches are used for inhibiting specific genes using oligonucleotides: antisense DNA (ASO) and RNA interference (RNAi). Recent developments in bioconjugated oligonucleotides include those possessing GalNAc, cell-penetrating peptides, α-tocopherol, aptamers, antibodies, cholesterol, squalene, fatty acids or nucleolipids. These novel conjugates provide a means to enhance tissue targeting, cell internalization, endosomal escape, target binding specificity, resistance to nucleases, and more. Some bioconjugated oligonucleotides are approved for the patient use or in clinical trials. Bioconjugation chemistry is at the centerpiece of this therapeutic oligonucleotide revolution and significant opportunities exist for development of new modification chemistries, for mechanistic studies at the chemical−biology interface, and for translating such agents to the clinic.

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014

Investigation of alternative scaffolds for systemic delivery of small interfering RNAs

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Small interfering RNAs (siRNAs) are a promising class of novel RNA-directed therapeutics. While exerting their gene-silencing activity similar to micro RNAs (miRNAs) by recruiting the RNA induced silencing complex (RISC) toward mRNA targets, siRNAs are capable of inducing Argonaute 2 (AGO2) mediated target RNA cleavage.
Although highly efficient *in vitro*, systemic delivery turned out to be the major hurdle for clinical applications. Patisiran was the first siRNA receiving FDA approval in August 2018[1], nearly 20 years after the discovery of RNA interference[2]. Current clinically relevant siRNAs depend on lipid nanoparticle formulation or heavy chemical modification involving complex targeting moieties and a variety of non-natural building blocks[3], each posing a specific risk when exposed to the human metabolic machinery.

In previously published work we showed that the stereochemical distribution of PS linkages in the RNA backbone can be influenced through the choice of activator during chemical synthesis of PS siRNAs and in turn biophysical properties, stability toward nucleases and activity in cells can be modulated[4]. Building on these results, we scaled up the chemical synthesis and characterized a full PS siRNA targeted against the oncoprotein Lin28B in terms of cellular activity, nuclease resistance and biodistribution in nude mice. We are currently investigating additional naturally occurring chemical modifications to further improve the pharmacokinetic properties of such siRNAs.

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Identification of small activating RNAs that can upregulate immune activation targets for cancer immunotherapy

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Small activating RNAs (saRNAs) are short double strand oligonucleotides designed to specifically upregulate target genes leading to new mRNA and protein production. We have investigated whether this technology could be applied to a number of immune stimulatory genes and thus be used for cancer immunotherapy.

saRNAs (human and mouse cross reactive) were designed to IL-23A, IL-36g, OX40L, UCP2 and APLNR using a proprietary algorithm that designs the saRNA sequences to bind to long non-coding RNA +2000 to -2000 nucleotides from the transcriptional start site. The top 5-10 saRNA sequences from the algorithm for each target were synthesised using mUmU overhangs at the 3' ends to help impart stability and reduce non-specific immunogenicity. The saRNAs were screened in human (A549 and HepG2) or murine (CT26, BNL.1ME and RAW264.7) for upregulation of mRNA levels of the target genes by qPCR.

The saRNA algorithm identified active saRNAs for all 5 targets leading to upregulation of target mRNA at 48-72hrs post transfection compared to oligonucleotide transfection controls. The levels of mRNA upregulation for the top saRNA sequence from this screen were: IL23A (4.9 fold), IL-36g (3.5 fold), OX40L (6.7 fold), UCP2 (1.9-fold) and APLNR (5.4-fold). The specificity of the most active saRNA for the targets was confirmed by mutation of 2-3 nucleotide residues in the seed portion of the sequences (nucleotides 2-8 in the anti-sense strand). These seed mutant saRNAs all had reduced induction of target mRNA compared to the parent saRNA molecules confirming the nucleotide sequence dependence of the mRNA upregulation. The presence of increased target protein in saRNA transfected cells was confirmed either by immunofluorescence, ELISA or western blot.

In summary, saRNA technology has been applied to 5 different immune targets leading to specific up-regulation of target mRNA and protein. Delivery of these double strand saRNAs to the tumour either via intra-tumour injection or systemic delivery represents a promising novel therapeutic approach for cancer immunotherapy.

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016

Characterizing the inhibition potential of DNA aptamers on α-synuclein fibril formation

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Parkinson's Disease (PD) is one of the most prevalent neurodegenerative disorders and is commonly characterized by the selective degeneration of dopamine neurons and the formation of intraneuronal inclusions, known as Lewy bodies, through α-synuclein (α-Syn). Since α-Syn has proven to be a reliable therapeutic target for the treatment of PD, a simple method utilizing the specific detection of monomeric α-Syn through the use of aptamers has been established. Five 66-base aptamers labelled ASYN1-5 were previously selected using monomeric α-Syn. In this study, the aptamers high binding affinity and specificity for α-Syn was characterized using electrochemical impedance spectroscopy (EIS), with recorded K_D values in the nanomolar range. Subsequent *in vitro* studies were performed in order to evaluate each aptamers ability to block fibril formation. Each aptamer was incubated with α-Syn monomeric protein specially formulated to generate pre-formed fibrils and incubated for seven days. Fibril formations were observed using TEM and characterized with ImageJ where each aptamer was scored on its ability to block aggregation. All five aptamers, with emphasis on ASYN2, could inhibit the formation of α-Syn aggregates at molar ratios (ASYN: α-Syn) of 10:1 and as low as 1:1. These aptamers present a high therapeutic potential towards the management of progression of fibril formation in PD.

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017

Natural exon skipping reveals that antisense oligonucleotide-mediated exon skipping should be directed at the recessive type of dystrophic epidermolysis bullosa

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Dystrophic epidermolysis bullosa (DEB) is a devastating genetic blistering disease affecting skin and mucous membranes. DEB is caused by pathogenic variants in the *COL7A1* gene encoding type VII collagen, and can be inherited dominantly or recessively. The structure of *COL7A1* makes it an attractive candidate for exon-skipping therapy. Recently, we demonstrated promising proof-of-principle for antisense oligonucleotide (AON)-mediated exon-skipping as a systemic therapeutic approach for DEB. However, it is unclear what phenotypic effect may be expected from exon-skipping and which patient groups may benefit the most.
To answer these questions, we studied new clinical and molecular data on seven patients from the Dutch EB registry and reviewed the literature on pathogenic *COL7A1* variants inducing 'natural exon-skipping'.
Results: We found that the natural skipping of certain exons led to disease in a heterozygous state, while the skipping of other exons only led to disease if combined with a pathogenic variant on the other *COL7A1* allele. The dominant DEB phenotypes associated with heterozygous exon-skipping could not be distinguished from dominant phenotypes caused by heterozygous *COL7A1* variants not inducing exon-skipping. Phenotypes associated with recessive exon-skipping mutations were, however, on average relatively mild in the spectrum of recessive DEB.
In conclusion, for dominant DEB, AON-mediated exon-skipping is unlikely to make a clinical difference. In contrast, we anticipate that exon-skipping has the potential to induce a clinically relevant improvement of the devastating recessive DEB phenotype, especially the types caused by bi-allelic null variants.

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018

Evidence for an Intracellular Depot that Contributes to the Extended Duration of Activity of GalNAc-siRNA Conjugates

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Advances in siRNA delivery and safety during the past two decades enabled approval of the first RNAi therapeutic, with several additional candidates directed against multiple liver targets currently in late stage clinical trials. We sought to understand the reasons behind the extended duration of activity seen for GalNAc-siRNA conjugates in the clinic by studying intracellular trafficking in preclinical models. Using primary hepatocytes we show rapid clathrin-mediated internalization of GalNAc-siRNA conjugates, intracellular trafficking through various subcellular compartments and lysosomal accumulation within hours of dosing. We hypothesized that siRNA is slowly released from an intracellular depot to sustain the long duration of activity seen *in vivo*. We will share several lines of evidence in support of this hypothesis, including 1) Release of siRNAs from acidic compartments using endolytic peptides administered weeks after initial siRNA dosing in mice boosts knockdown. 2) Newly translated, tagged-Ago2 can load functional siRNA weeks after dosing in mice. 3) Identical siRNAs delivered via LNP have shorter duration than when delivered subcutaneously. These data, along with additional evidence, support the hypothesis that extended duration of activity may be due to a slow release of functional siRNA from an intracellular storage depot.

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Stereopure oligonucleotides that promote *USH2A* exon skipping for the treatment of Usher syndrome type 2A

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Wave Life Sciences has developed PRISM™, our proprietary discovery and drug development platform that enables us to generate stereopure oligonucleotides in which the chiral configuration of backbone-modified oligonucleotides is precisely controlled at each linkage. PRISM combines our unique ability to construct stereopure oligonucleotides with a growing knowledge of how the interplay among oligonucleotide sequence, chemistry and backbone stereochemistry impacts key pharmacologic properties. With PRISM, we are designing and advancing stereopure oligonucleotides for the potential treatment of rare, inherited retinal diseases.

Usher syndrome type 2A is most commonly caused by mutations in the *USH2A* gene that disrupt production of usherin protein and photo transduction in rods and cones of the retina. Patients experience progressive vision loss that begins in adolescence or adulthood. One approach to treat retinal manifestations of this syndrome is to develop therapeutic exon-skipping oligonucleotides that cause the splicing machinery to skip over the disease-causing mutations in *USH2A* transcripts and restore production of functional usherin to photoreceptor cells. Our preclinical data have demonstrated that stereopure oligonucleotides developed with PRISM exhibit superior potency and durability as compared to stereorandom oligonucleotides and can be optimized to minimize immune activity. A single intravitreal injection of stereopure oligonucleotide targeting the long non-coding RNA *Malat1* in the eye of non-human primates (NHP) resulted in greater than 95% knockdown of *Malat1* in the retina for at least four months. Based on these data, we are working to develop candidates that could achieve a therapeutic effect with only a few doses per year. We provide an update on our efforts to develop stereopure exon-skipping oligonucleotides that target *USH2A*. We show that stereopure oligonucleotides can be optimized to promote potent exon skipping in cellular models under free-uptake conditions (gymnotic delivery). We also present data demonstrating target engagement *ex vivo* in NHP and human eyes.

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020

Therapeutic potential of antagomiR-23b for treating Myotonic Dystrophy

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Myotonic Dystrophy type 1 (DM1) is a life threatening and chronically debilitating rare genetic disease that originates from expansion of a non-coding "CTG" repeat in the *DMPK* gene. The expansion becomes pathogenic when *DMPK* transcripts contain 50 or more repetitions due to the sequestration of the muscleblind-like (MBNL) family of proteins. Depletion of MBNLs causes alterations in splicing patterns in transcripts such as chloride channel 1 (*CLCN1*) and insulin receptor (*INSR*), which ultimately lead to clinical symptoms including myotonia, insulin resistance, and muscle weakness. We previously found that miR-23b directly represses translation of MBNL1 and 2 in DM1 patient myoblasts and the HSA^{LR} mouse model. Thus, antisense technology ("antagomiRs") blocking these miRNAs boosts MBNL1 and 2 protein levels. Here we provide new data to describe the time course of effect intensity in response to administration of antagomiR-23b as a treatment in HSA^{LR} mice. We show that the effect of a single injection of antagomiR-23b in HSA^{LR} mice is not significantly different using subcutaneous or intravenous administration. Subcutaneous administration of a single injection of antagomiR-23 up-regulates the expression of MBNL proteins and rescued splicing alterations, grip strength, and myotonia, in a dose-dependent manner with minimal effects on blood biochemistry. Although the most important effects of the antagomiR in terms of MBNL protein upregulation were found 4 days after the injection, the effects of the treatment on grip strength and myotonia were still notable after 45 days. The pharmacokinetic and pharmacodynamic data obtained provide further evidence that miR-23b could be a valid therapeutic target for DM1 and establish a benchmark against which to compare antagomiRs with alternative chemistries in search of a lead compound for first human administration.

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A novel therapeutic aptamer targeting LAG3 for immuno-oncology therapy

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Immune checkpoint molecules are often activated in cancer, leading to suppression of anti-tumor immune responses. Immune checkpoint inhibitor therapies thereby provide effective long-term treatment for a variety of cancers. However, only a subset of cancer patients was found to respond to these treatments. It is therefore of great interest to develop combination therapy by inhibiting multiple immune checkpoint targets to improve response rates of immuno-oncology therapy. To achieve this goal, many efforts have been made in utilization of new entity of therapeutic molecules. As one of potential cancer immune-therapeutic entities, aptamers possess many advantages such as high specificity and affinity, easy to be production and more cost effective over commonly used antibodies.

Lymphocyte-activated gene 3 (LAG-3) is mainly expressed on activated T cells, natural killer cells, B cells and plasmacytoid dendritic cells, and acts as a brake in maintaining immune homeostasis and preventing immune over-activation. Previous study indicated that LAG-3 antagonistic antibody significantly enhanced the efficacy of anti-PD1-based immunotherapy. In this study, a novel LAG-3 aptamer with high affinity and specificity had been identified from a high variety of library. Tetrameric LAG-3 aptamer, MBS921, was further constructed with a palindrome bridge. Antagonistic efficacy of MBS921 was 5 times enhanced compared to monomeric LAG-3 aptamer with IC50 from 1.4 μM to 92nM in Jurkat cell reportervsystem. Surprisingly, the syngeneic mouse model indicated that only single injection of MBS921 was sufficient to augment the maximum anti-tumor effect of PD-L1 antibody, and therefore a hit-and-run therapeutic strategy was suggested for LAG-3 antagonist.

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022

Development of OLX10020, a cell-penetrating asymmetric siRNA for age-related macular degeneration (AMD) treatment

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Age-related macular degeneration (AMD) is defined as an abnormality of the retinal pigment epithelium (RPE) that leads to degeneration of the overlying photoreceptor in the macular and consequent loss of central vision. Advanced AMD leads to loss of vision and can be classified into two categories: Geographic Atrophy (GA), which is characterized by atrophy of the RPE, and Choroidal Neovascularization (CNV; also known as wet AMD), which is due to the abnormal growth of blood vessels. Anti-angiogenic therapies targeting vascular endothelial growth factor A (VEGFA) have proven to be highly effective in treating neovascular AMD. To date, however, there is no approved treatment available that can significantly slow the progression of GA and improve the clinical outcome. OLX10020 is a cell-penetrating asymmetric siRNA (cp-asiRNA) targeting a novel undruggable gene involved in GA. It has demonstrated excellent activities in both Alu RNA-induced and $NaOI_3$-induced mouse GA models. Furthermore, OLX10020 has been proved to be effective in wet AMD animal models, indicating that it is a potential first-in-class drug that can treat both GA and wet AMD.

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023

Gapmers targeting DUX4 as a therapeutic strategy for facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common inherited muscular dystrophies with an incidence of 1:8,000 to 1:20,000. Studies showed that FSHD is caused by aberrant expression of double homeobox 4 (DUX4) due to epigenetic changes of the D4Z4 macrosatellite repeat region at chromosome 4q35. The aberrant expression of DUX4 misregulates downstream genes and pathways, which leads to muscle pathologies and weakness. To date, there is no effective treatment for FSHD. The goal of this study is to evaluate antisense oligonucleotide strategies to reduce the pathogenic DUX4 mRNA in affected muscles and improve muscle pathology and function using an FSHD mouse model. LNA and 2'-MOE gapmers were delivered by either intramuscular injections (i.m.) or subcutaneous injections (s.c.) to the DUX4-expressing FLExDUX4 mouse model. Our results showed that i.m. injections (20ug) of gapmers into the tibialis anterior muscles significantly reduced DUX4 transcripts in the muscles of the FLExDUX4 mice. Short-term treatment by s.c. injections (20mg/kg) of the gapmers reduced DUX4 expression in the muscles. In a long-term 10-week trial, s.c. injections (20mg/kg) twice a week significantly reduced DUX4 expression. In addition, muscle function measured by grip strength testing showed functional recovery after the treatment. Muscle fibrosis was also reduced by the treatments. Our findings showed that the gapmers targeting DUX4 significantly reduced DUX4 transcripts. The reduction of DUX4 was accompanied by recovery of muscle functional deficits and improvement of muscle pathology in the FLExDUX4 mice. The data support the use of gapmers as a viable therapeutic approach for FSHD.

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024

Performance of TsiRNA, STsiRNA and their cholesterol derivatives in vitro and in vivo.

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Novel type of interfering RNAs - trimeric-siRNA (TsiRNA) and suplamolecular trimeric siRNA (STsiRNA) were proposed and studied. Selectively modified 63 bp tsiRNAs induced more effective RNAi at lower concentrations than classical 21 bp siRNA. Selective 2'-O-Me modification pattern defines the action in a Dicer independent or Dicer dependent mode. Nuclease-resistant 63-bp TsiRNA comprising in one duplex the sequence of siRNAs targeting mRNAs of *MDR1*, *LMP2*, and *LMP7* genes is able to suppress the expression of all the target genes independently and with high efficiency, acting via a Dicer-dependent mechanism. These results remove the length limits for the design of RNAi effectors. The conjugation of siRNA to the molecules, which can be internalized into the cell by natural transport mechanisms, is a promising approach for the delivery of siRNA into the cells. Previously, we showed, that 5'-cholesterol-siRNA exhibits enhanced accumulation in lungs, liver, kidney and xenograft KB-8-5 tumor and target gene silencing. The results showed that increasing the length of the RNA duplex in such a conjugate increases its biological activity when delivered using a transfection agent. However, the efficiency of accumulation in human drug-resistant KB-8-5 cells in vitro in a carrier-free mode was reduced as well as efficiency of target gene silencing. In in vivo experiments on healthy and KB-8-5 xenograft tumor-bearing SCID mice, cholesterol-TsiRNAs demonstrated a similar biodistribution pattern with more efficient accumulation in organs and tumors of mice than cholesterol-conjugated canonical siRNAs; however, this accumulation did not provide a silencing effect. In order to overcome this limitation, we have constructed a new type of RNA interference inductors - STsiRNA, which represents a complementary complex consisting of 3 molecules of the antisense strand and two different one-and-a-half sense strands containing LNA modifications to ensure duplex stability. It was shown that, unlike TsiRNA, STsiRNAs effectively accumulate in the cells of a drug-resistant KB-8-5 tumor and suppress the expression of the target gene which may be associated with higher flexibility of suplamolecular duplex. The use of supramolecular complexes allows to attach ligands with different functionalities in the composition of different oligoribonucleotides, and to combine inhibitors of several therapeutic genes in one STsiRNA with enhanced tumor-accumulation properties.
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A Fluorescent Cytosine Analog in Gapmer Technology: Precise tracking by Stealth Labeling of Antisense Oligonucleotides

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Over the past 20 years the activity around oligonucleotides within the pharmaceutical industry has intensified significantly as new oligo based drugs enter the market. Many techniques to determine the properties of the oligos of interest have been developed, such as knockdown efficiency via qPCR, internalisation efficiency with commercially available kits and cell localisation with various dying protocols. It is also very common to make use of commercial fluorescent dyes such as AlexaFluor, Bodipy and cyanine based dyes to visualise trafficking inside cells during imaging studies. However, these fluorescent dyes may change the overall properties of the oligo of interest since they are usually quite large and also contain multiple charges that may effect permeability, uptake, endosomal escape, trafficking inside the cell, accumulation in different cell compartments as well as the interaction with its target.
Recently, our lab has made good use of fluorescent base analogs (FBAs) in several FRET studies of DNA and RNA and we recognized that these FBAs could also prove useful in imaging studies of oligos without affecting the knockdown or internalisation efficiency. To establish if results generated with commercial dyes are reliable, we decided to synthesize a number of naked and conjugated oligo gapmers containing either Cy3 or a fluorscent cytosine analog.
Our results will be disclosed in full for the first time during this OTS meeting.

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026

Antisense oligonucleotide-mediated splicing modulation of Amyloid Precursor Protein as treatment for D-CAA

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Dutch- Cerebral Amyloid angiopathy (D-CAA) is a rare autosomal dominant disease characterized by cerebrovascular deposition of the amyloid beta (Aβ) peptide. Aβ is produced after the enzymatic cleavage of Amyloid Precursor Protein (APP). The disease is caused by a missense mutation on chromosome 21 in the amyloid precursor protein (APP) gene, resulting in a glutamine for glutamic acid substitution (NP_000475.1:p.Glu693Gln). It is hypothesized that the loss of a negatively charged amino acid causes accelerated Aβ oligomerization. Together with impaired clearance, this leads to Aβ deposition mainly around the cerebral vessel walls making the blood vessels more prone to breakage. Our therapeutic strategy involves the use of antisense oligonucleotides (AONs) to target exon 17 in the APP pre-mRNA that contains the mutation and amyloidogenic region. This leads to splice switching, resulting in a new APP isoform that lacks the amyloidogenic region.

In vitro AON-induced APP exon 17 skipping was investigated in patient-derived fibroblasts, induced Pluripotent Stem cells (iPSCs) that were reprogrammed from the patient fibroblasts and differentiated to neurons and a neuroblastoma cell line (SH-SY5Y). In vivo APP exon skipping was investigated in wt-mice following intracerebroventricular administration of AON. Analysis was done on RNA and protein levels. The levels of Aβ40 were quantified with Aβ40 ELISA in cell culture media of iPSC-induced neurons treated with 100nM of AON.

We successfully designed an AON that induced efficient exon skipping on RNA and protein level both *in vitro* and *in vivo*. Preliminary results of ELISA on the cell culture medium of iPSC-induced neurons showed reduction in the Aβ40 levels of AON-treated cells versus non-treated that was persistent four days after AON transfection.

These results illustrate the potential of using an AON as therapy for D-CAA. Future studies include generation of isogenic iPSC lines with CRISPR/Cas9 and 3D disease modeling with cerebral organoids.

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A novel lipid-based nanoparticle formulation mediating safe and robust functional delivery of siRNA/miRNA

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Mounting evidence suggests that small interfering RNA- (siRNA) and microRNA- (miRNA) based therapies hold great promise for development of therapeutic modalities. However, despite the early promise and exciting potential, several specific technical barriers (e.g., extracellular and intracellular stability as well as effective and safe delivery) still need to be overcome. While unmodified RNAs are rapidly degraded in circulation, chemically modified versions hardly penetrate cells and require an additional delivery system to allow intracellular molecular effects. New strategies overcome the core obstacles to cellular delivery of siRNAs/miRNAs which include: (i) ribonuclease- (RNase) mediated degradation; (ii) short biological half-life; (iii) lack of endosomal escape; (iv) lack of tissue targeting; (v) inefficient biodistribution; and (vi) side effects. Despite significant efforts and progresses in chemical modification and cellular delivery for therapeutic siRNAs/miRNAs, only one drug (patisiran, ONPATTRO) has been approved by the FDA. InteRNA Technologies has pursued new approaches to identify robust drug delivery modality for its drug candidate, INT-1B3 (miR-193a-3p mimic) which is expected to enter soon into first-in-human clinical evaluation. During the last decade, InteRNA has tested various delivery technologies based on *in vivo* screening in which formulated siRNA targeting reference gene *HPRT-1* was administered (bolus *i.v.*) to human melanoma A2058 tumor-bearing immune compromised mice. Tissue samples from spleen, liver and tumor were evaluated for tissue distribution and pharmacodynamic (target engagement) effects. In addition, safety was assessed by cytokine expression in blood, and blood chemistry. Interestingly, based on this initial evaluation, InteRNA has identified a novel lipid-based nanoparticle (LNP) formulation which safely and effectively delivered the siRNA cargo to target tissues, in particular beyond delivery to the liver. This formulation was ultimately selected for further development, and after fine tuning became a key component of INT-1B3 (formulated 1B3). In parallel, InteRNA has developed an initial *in vitro* experimental approach and tested various chemical modifications on the miRNA molecule to improve metabolic stability and target recognition. Efficacy of INT-1B3 has been evaluated upon systemic administration in experimental tumor-bearing mouse models. Significant tumor growth inhibition was demonstrated at well-tolerated doses in a large panel of human and syngeneic tumor models. A full PK/PD and safety profile has been assessed in mice, and optimization of large-scale production of drug product has allowed IND-enabling GLP-Tox studies in rats and *non*-human primates to be initiated to support upcoming clinical development.

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Isoform-switching siRNAs efficiently correct Tau isoforms imbalance in a cellular model of Tauopathy

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Tauopathies are neurodegenerative diseases marked by the abnormal processing of microtubule-associated protein tau and its accumulation as insoluble neuronal deposits. Tau, encoded by the MAPT gene, regulates several neuronal functions, such as neurite outgrowth, microtubule dynamics and axonal transport. The adult human brain contains equal amounts of tau isoforms with three (3R) or four (4R) repeats of microtubule-binding domains, originated from the alternative splicing of exon 10 (E10) in the MAPT transcript. Several tauopathies are associated with imbalances of tau isoforms due to splicing shortfalls. Selective degradation of E10-containing MAPT mRNA isoforms is, in principle, possible by the use of exon-specific siRNAs. However, very few examples of successful exon-specific siRNAs are available in the literature. This might be due to the fact that secondary siRNAs can be produced by RNA-dependent RNA Polymerases in several organisms and cell types, which would generically silence all mRNA splicing isoforms. Moreover, siRNAs are known to function also in the nucleus and might target the nascent pre-mRNA, resulting in a decrease of all different splicing isoforms of the target mRNA. Here, we evaluate fourteen E10-targeting siRNAs for their efficiency in reverting the inclusion of E10 in MAPT transcripts and identify three effective siRNAs. We validated the three siRNAs in human neuronally differentiated cell models of tauopathy that produce abnormal excess of 4R tau. Our results suggest a promising potential for the use of RNA interference in human neurodegenerative diseases.

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Synthesis of Small Molecule-siRNA Conjugates to Improve siRNA Activity

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Aberrant gene expression is a hallmark of disease and targeting messenger RNA through RNA interference is a promising approach to reduce the expression of deleterious proteins. However, some of the current problems associated with using oligonucleotides as therapeutics include off-target toxicity, chemical lability, and poor cellular delivery. Many of these challenges are attributed to the native negatively charged phosphodiester backbone of the oligonucleotide. As such, some of our current interest in overcoming these obstacles involves chemical modification of the backbone with neutral unnatural functional groups. In particular, we have interest in designing and modifying siRNAs bearing non-natural aromatic functionalities. In addition, we have interest in chemically-tagging siRNAs with small molecules in order to improve the structure-activity profile of the siRNA. This poster will focus on two main types of small molecule-siRNA conjugates. The first will focus on the photoresponsive molecule azobenzene, and how its incorporation within siRNAs can lead to controlled gene silencing of targets. The other type of small molecule-siRNA conjugate will focus on using folic acid as a method to improve siRNA delivery in cellular systems. These developments will allow for future small molecule-siRNA chemical investigations that are substrates within the RNAi pathway.

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A new platform for the direct profiling of nucleic acids in biofluids

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Circulating nucleic acids such as microRNAs, cDNAs, lncRNAs and circRNAs have been identified as potential biomarkers for early detection, prognosis and prediction of several diseases. However, their use in clinical settings has been hindered by the absence of appropriate detection techniques. Most of the current technologies require complex protocols, not yet able to deliver robust and cost-effective assays in the field of clinical diagnostics. Here, we describe the development of an innovative platform for profiling circulating nucleic acids. The platform is composed of a novel silicon photomultiplier-based reader in conjunction with a chemical-based method for nucleic acid detection. Nucleic acids profiling without extraction, pre-amplification or pre-labeling of the target is now possible. In this work, we designed and synthesized a set of reagents that combined the chemical-based method with a chemiluminescent reaction, which is detected using a novel, compact silicon photomultiplier-based reader. Known concentrations of hsa-miR-21-5p spike-ins were used to determine the platform sensitivity which was calculated as 4.7 pmol/L. The platform was also successfully validated for the direct detection of hsa-miR-21-5p in eight non-small cell lung cancer plasma samples. Levels of plasma hsa-miR-21-5p expression were also analyzed via gold-standard TaqMan RT-qPCR. The combination of a unique chemical-based method for nucleic acid detection with a novel silicon photomultiplier-based reader created an innovative product (ODG platform) with diagnostic utility, for the direct qualitative and quantitative analysis of nucleic acids in biological fluids.

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Considerations for the Development, Scale-up and Manufacturing of mRNA Therapeutics

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Recently, there has been significant interest in the use of messenger RNA (mRNA) as an *ex vivo* and *in vivo* therapeutic. Since mRNA is expressed in the cytoplasm it may be particularly useful for improving gene expression in difficult-to-transfect non-dividing cells. In contrast to plasmid or viral vectors, there is no risk of insertional mutagenesis or subsequent oncogenesis upon mRNA transfection and the transient nature of mRNA expression is desirable for genome editing (CRISPR/ Cas Systems, ZFNs and TALENs) and vaccines. In each case, the goal is to produce a synthetic RNA that mimics a natural mRNA.

Many Biotech, Biopharmaceutical, and Pharmaceutical companies have initiated programs to investigate mRNA therapeutic applications. Their target centric research has identified thousands of potential mRNA candidates, but many companies struggle with determining the optimum path forward to progress identified candidates through the drug development process. Contract Development and Manufacturing Organizations (CDMOs) like TriLink BioTechnologies with focused expertise in mRNA and nucleic acid manufacturing optimization can greatly assist both virtual and established companies achieve their goals.

Multiple compound attributes and manufacturing parameters must be determined, optimized, and rigorously tested. It is essential to consider sequence design, raw material identification and sourcing, and manufacturing processes that are inherently scalable. Critical decisions must be made about:

» Project management
» Technology transfer process
» Transcription optimization
» Purification optimization
» Process scale-up
» Analytical development

We will provide a broad roadmap for the application of these principles to the design and manufacturing of novel mRNA therapeutics. Data from the development, optimization, manufacturing, and scale-up of mRNA will be presented.

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Amelioration of Mas-Related G-Protein-coupled Receptor X2-mediated itch and reduced mast cell degranulation by a single-stranded oligonucleotide

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Itch is a large problem in numerous skin disorders. The Mas-related G protein-coupled receptor X2 (MRGPRX2) has been shown to modulate itch by inducing non-IgE-mediated mast cell degranulation and the subsequent release of endogenous inducers of pruritus. Various substances collectively known as basic secretagogues, which include inflammatory peptides and certain drugs, can trigger MRGPRX2, thereby inducing pseudo-allergic reactions characterized by histamine release and inflammation. Here, we investigated the capacity of an immunomodulatory single-stranded oligonucleotide (ssON) to inhibit itch and mast cell degranulation. We show that intradermal injection of ssON in mice is able to inhibit itch induced via the basic secretagogue compound 48/80 by reducing acute mast cell degranulation in skin. Further, ssON demonstrates a capability to inhibit MRGPRX2 activation in vitro. We reveal that mast cell degranulation and calcium influx in MRGPRX2-transfected HEK293 cells induced by certain basic secretagogues are effectively inhibited by ssON in a dose-dependent manner. Since there is a need for new therapeutics targeting MRGPRX2-mediated activation of mast cells, ssON could be used as a prospective drug candidate to ameliorate itch in particular pathological settings.

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Enabling synthesis of nano-constructs for oligonucleotide therapeutics

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Oligonucleotide (ON) delivery to specific cell and/or body target sites has been one of the main limitations in ON therapeutics. Conjugation of natural or modified ON to different (bio)chemical moieties can improve existing oligonucleotide properties or provide new ones. One of the main challenges in developing ON conjugates apart from selecting a suitable functionality and conjugation site is development of proper linker. A functional linker should have suitable length and flexibility as well as a suitable chemical ´handle´ to enable selective conjugation. Here, we have developed two different types of linkers, which can be synthesized through a common intermediate. Conjugation to a construct is allowed either by amide bond formation, or by copper-catalyzed ´click´ chemistry. Both linkers we developed are compatible with automated ON synthesis using phosphoamidite chemistry and even multiple labeling to the same ON chain can be enabled. A PAMBA (4-((2-(prop-2-yn-1-yloxy)acetamido)methyl)benzoic acid) fragment in one of our linkers allows 1,3-dipolar cycloaddition to an azide and provides sufficient stability for automated synthesis. The backbone of the linkers gives the possibility to incorporate multiple linkers and at different positions in the ON-conjugate. In addition, we investigated the possibility to use a cyclooctyne containing linker, which could allow strain-promoted ´click` reactions. The stability of compounds containing the cyclooctyne fragment was studied under different conditions and the stability was found to be a major limitation for use of this type of linker in ON-conjugates.

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Controlling chirality of phosphorothioates in Antisense Oligonucleotides does not enhance potency or duration of effect in the CNS

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Gapmer antisense oligo nucleotides (ASOs) constitute the most widely studied class of therapeutic oligonucleotides. These are comprised of a central gap region of 7-12 DNA nucleotides flanked by wings of 2-5 modified nucleotides that enhance affinity for complementary RNA and stability towards nuclease mediated digestion. Most commonly, to improve oligo pharmacokinetic properties, the oligonucleotide phosphodiester backbone is replaced with the phosphorothioate linkage. This generates a new chiral phosphorous center at each linkage. Historically, these were introduced as racemic mixtures, and as such gapmer ASOs are not discreet chemical entities, but ensembles of diastereomers.

Recently, the formation of stereo-controlled oligonucleotide PS junctions has become synthetically tractable. This has allowed for the formation of ASO ensembles highly enriched in a single stereoisomer. We have used this technology to study the role of PS chirality on ASO pharmacokinetic and pharmacodynamic properties, including; duration of action, half-life, potency and ability to control RNase H1 cleavage. We found that controlling PS chirality in the MOE wings or in the DNA gap did not enhance ASO potency or duration of effect in the CNS relative to their stereorandom counterparts. Complete details including sequence, design of ASOs and lessons learned will be presented.

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Sequence Specific DNA-Polymer Conjugates and Their Applications in Drug Delivery

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Nucleic acid therapeutics, such as small interfering RNA (siRNA) and antisense oligonucleotides (AON), offer unique potential for gene therapy because of their effectiveness and directed silencing of the targeted gene of interest. However, significant challenges have impeded their translation into clinical applications, such as instability in biologically relevant media, off-target effects, and poor cellular uptake.

An attractive solution is the use of drug delivery nanomaterials that protect and deliver the oligonucleotide drugs to their desired target site, such as liposomes and polymeric nanoparticles. However, the approval of these drug delivery materials has been slow due to many hurdles blocking their translation from lab prototypes to actual clinical applications. Most synthetic carriers are a mixture of polydisperse molecules that are not precisely controlled in size, shape, and composition. This leads to heterogeneity in properties, toxicity, and off target effects which are highly undesirable.

DNA nanotechnology offers a very promising alternative, which utilizes nucleic acids as a material to build nanostructures that act as targeted drug carriers. This is due to the ease of manipulating DNA's structural parameters (size, shape, rigidity, functionalization) as well as chemical composition. These structures are monodis
perse, bio-degradable, non-toxic, and can themselves be therapeutic.

An especially powerful DNA functionalization is the attachment of hydrophobic polymers, which has led to the emergence of a new class of amphiphilic DNA block copolymers. We have developed a highly efficient solid-phase method to generate monodisperse and sequence-defined DNA-polymer conjugates.[1] Based on this method, we have designed a range of molecules and vehicles with various functionalities and applications in drug delivery, with total control over their properties.[2,3,4,5]

In this work, we will be highlighting the importance of this sequence-specific DNA-polymer conjugate method, which has allowed us to fabricate multiple systems for therapeutic applications. Specifically, we will be focusing on two systems developed from this method and their drug delivery applications: spherical nucleic acids (SNA) and albumin-binding Dendritic-DNA (DDNA) molecules. Studies such as characterization, stability, cell work, including gene silencing and conditional drug release, as well as *in vivo* investigations will be discussed.

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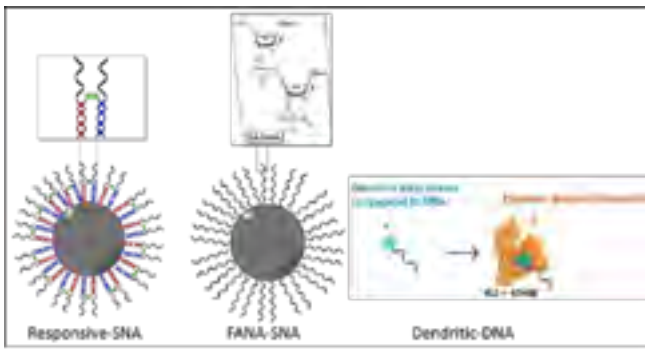

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Validated extracellular miRNA quantification in blood samples using RT-qPCR

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Extracellular microRNAs (miRs) have been proposed as important blood-based biomarkers for several diseases [1,2]. Despite the high number of miR biomarker studies, the specificity and reproducibility of those studies is missing [3]. Therefore, the standardization of pre-analytical and analytical methods is urgently needed. Here, we first validated miR analysis for RNA isolation and miR quantification by quantitative polymerase chain reaction (RT-qPCR) based on Good laboratory practice (GLP) principles. In our study, we identified and solved several pitfalls from handling to normalization strategy in the analysis of extracellular miRs that lead to inconsistent and non-repeatable data. In addition, we have for the first time defined quality controls to verify the reliability of extracellular miR analytics for biomarker studies in blood samples.

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Evaluation of CD39 and CD73 as targets for immunotherapy of cancer using LNAplus™ antisense oligonucleotides

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The adenosine axis is an important mechanism that allows tumors to escape from immunosurveillance. The ectonucleotidases CD39 and CD73 that act in concert to convert extracellular immune-stimulating ATP to immunosuppressive adenosine and the adenosine A2A receptor have emerged as promising targets to combat immunosuppression through the adenosine axis. We showed previously that LNA-modified antisense oligonucleotides (ASOs) targeting CD39 revert immunosuppression and result in potent antitumor immune activity. In the present study we performed a thorough analysis of the adenosine axis using LNA-modified ASOs targeting CD39 and CD73 as well as small molecule inhibitors targeting the A2AR in human PMBC and T cells.
Knockdown efficacy of ASOs on mRNA and protein level was investigated in primary human T cells. CD39 and CD73 activity was evaluated by measuring levels of adenosine triphosphate (ATP), adenosine monophosphate (AMP) and adenosine in cell supernatants. As functional readout we analyzed effects of extracellular ATP, AMP or the non-degradable adenosine analogue 5′-N-Ethylcarboxamidadenosine (NECA) on activated T cells.
Unformulated CD39- and CD73-specific ASOs achieved potent target knockdown on mRNA and protein level in primary human T cells. Furthermore, degradation of extracellular ATP or AMP was significantly blocked by CD39- or CD73-specific ASOs, respectively, while formation of adenosine was suppressed. Supplementation of cell culture medium with ATP impaired proliferation and viability in T cells expressing CD39 or CD73. Notably, ASO-mediated knockdown of CD39 or CD73 reversed this inhibitory effect of ATP. Furthermore, treatment with AMP resulted in decreased proliferation of activated T cells that could be reverted by ASO-mediated suppression of CD73 but not of CD39. Strikingly, we did not observe decreased T cell proliferation in presence of NECA and small molecule inhibitors targeting A2A receptors could not revert the suppression of T cell proliferation in presence of high levels of extracellular ATP or AMP arguing for other factors than adenosine being responsible for this effect.
Taken together we have shown that targeting the ectonucleotidases CD39 and CD73 by ASOs is a promising approach for treatment of cancer. We found fundamental qualitative differences compared to targeting the adenosine axis on the level of the A2AR.

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RNA synthesis by chemical ligation for mRNA therapeutics

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Recently, mRNA therapeutics have attracted attention as new oligonucleotide medicines, where therapy is performed by administering mRNA to cells and expressing desired proteins or bioactive peptides. There are several advantages in mRNA therapeutics compared with the conventional oligonucleotide therapeutics; 1) Contrary to the method such as RNAi or antisense methods, which suppress of the target protein expression, target gene expression can be enhanced. 2) Direct mechanism of protein expression by only translation step, 3) No need to enter in the nucleus for efficacy and immediate effect. 4) No concern about the integration of the administered oligonucleotides into the genome. mRNA therapeutics have attracted high expectations because of these superior features.

One of the major hurdles in realizing mRNA therapeutics is the synthesis of mRNA. Since it is a drug molecule, high chemical homogeneity is required, and in order to further improve the stability and physical properties of mRNA, synthetic methods capable of freely incorporating unnatural nucleic acids are required. For synthesizing mRNA, transcription with RNA polymerase is generally used, but the above-mentioned requirements are not satisfied.

Based on the above backgrounds, we developed a chemical ligation reaction that enables the chemical synthesis of mRNA. In this method, synthesis of mRNA is performed by sequentially ligating RNA fragments that are synthesized by an oligonucleotide synthesizer. In this presentation, we will report the chemical synthesis of mRNA and the evaluation of its translational ability.

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Disruption of the LIN28/Pre-let-7 axis through short oligonucleotides

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Most antisense and RNAi technologies available today aim to correct cancers and genetic diseases at the mRNA level. The proposed project aims to interfere with LIN28, a possible therapeutic oncogene target, through the use of a short oligonucleotide. LIN28 is an RNA binding protein expressed in ESCs, with multiple roles in development and disease. It is involved in many biological processes, including development, reprogramming, pluripotency, metabolism, tissue regeneration, and tumorigenesis. Humans express two isoforms of LIN28, LIN28A and LIN28B, which bind to the let-7 primary and precursor microRNAs through bipartite recognition, close to the Dicer cleavage site, impeding let-7 biogenesis.[1] It has been shown that in cancer, the tumor suppressor function of let-7 is abrogated by overexpression of LIN28. High concentration of LIN28 associates to progress of human malignancies, indicating LIN28 is acting as an oncogene.[2] The NMR structure shows that the two zinc knuckle domains of LIN28 are essential to establish a selective binding with pre-let-7 miRNAs.[3] The structure reveals that each zinc knuckle recognizes an AG dinucleotide separated by a single nucleotide spacer. We designed a series of short modified oligonucleotides (7-11mers) able to target the binding site of LIN28 with the aim to decrease and eventually inhibit the binding of the let-7 precursors.
The project investigates in specific the design of a new drug lead based on a modified 7/11 mer modified RNA sequence binding to LIN28. The Oligo carries a specific moiety, a short peptide, able to recruit the ubiquitin–proteasome system to achieve targeted degradation of LIN28A.[4] We are currently testing the oligonucleotide constructs in different cancer lines and analyzing the ternary complex LIN28A:Oligo:E3 through biophysical techniques. The nature of the new oligo construct makes the new class of oligo a potent drug and represents an innovative design, with great potential for pharmaceutical development.

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Differential expression of myelination-related transcripts during motor neuron neurodegeneration in a mouse model of spinal muscular atrophy

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Spinal muscular atrophy (SMA) is a devastating paediatric neuromuscular disease and the leading cause of premature children mortality due to a genetic defect, with an incidence of 1:6,000-11,000 live births. It is mainly characterized by the specific degeneration and cellular death of lower alpha motor neurons (MN) in the ventral spinal cord and in the brain stem.

The timing of SMN deficiency is crucial in SMA pathogenesis. Nusinersen, a splice-switching oligonucleotide, has been FDA- and EMA-approved for clinical use. In the absence of neonatal screening for SMA, it is often administered after onset of symptoms. Elucidating the transcriptional differences occurring at pre-symptomatic and symptomatic stages of SMA, and comparing these to oligonucleotide-treated samples, can help elucidate the gene networks involved in MN degeneration. This investigation could further lead to the discovery of pharmacologically relevant molecule(s) that could be used in combination with splice-switching oligonucleotide therapies.

We performed a study of the transcriptomic changes in the spinal cord between pre-symptomatic (post-natal day 2, PND2) and symptomatic (PND7) Taiwanese SMA pups. Tissues were collected from WT or SMA mice, at PND2 and PND7, either not treated, or treated at PND0 and PND2 with Pip6a-PMO (a splice-switching morpholino conjugated to a peptide to enhance the delivery) or Pip6a-scrambled_PMO as controls. MN bodies were isolated from the adjacent white matter by laser capture microscopy. RNA was extracted and transcriptomics studies were performed with Affymetrix® microarrays.

Amongst the transcripts differentially regulated at pre- and post-symptomatic stages, candidates for further studies include myelination-related transcripts *Plp1* and *Mobp*. Understanding their role and the mechanisms of their transcriptomic changes during motor neuron degeneration can offer further insight both in the fundamental mechanisms at play, and at the identification of new molecular targets to enrich the therapeutic arsenal available for SMA.

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Functional Disruption of a Disease Modifier Gene Using Antisense Oligomers: A Potential Molecular Therapy for *PRPF31*-associated Retinitis Pigmentosa 11

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Introduction. Heterozygous mutations in pre-mRNA processing factor 31 (*PRPF31*) cause retinitis pigmentosa 11 (RP11) and lead to retinal cell death and blindness. RP11 features incomplete penetrance within affected families, due to variable expression levels of functional PRPF31 from the remaining healthy allele. CCR4-NOT transcription complex subunit 3 (CNOT3) is a negative transcriptional regulator that binds to the promoter and inhibits *PRPF31* transcription, and is found at higher levels in RP11 patients, compared to asymptomatic family members carrying the same *PRPF31* mutations. **Objective.** It is hypothesized that lowering CNOT3 activity will enhance transcription of the remaining healthy *PRPF31* allele and improve pre-mRNA splicing function to prevent or delay RP11 disease progression. This study aims to modulate CNOT3 expression and function in order to upregulate functional PRPF31 using antisense oligomers (ASOs). **Results.** ASOs were designed to target selected CNOT3 exon(s) for exclusion during pre-mRNA processing, predicted to result in a frame shift and downregulation of CNOT3 expression, in patient retinal pigment epithelium (RPE) derived from induced pluripotent stem cells. As a consequence of CNOT3 knockdown, PRPF31 expression was upregulated 1.8-fold and 1.2-fold at the mRNA and protein levels, respectively. Alternatively, ASO-induced exclusion of in-frame exon(s) encoding essential functional domains induced exon skipping in a dose-dependent manner, and maintained *CNOT3* transcript at near-normal levels, whereas the truncated CNOT3 protein isoform(s) showed reduced nuclear localization in RP11 cells. PRPF31 mRNA and protein increased up to 2.2-fold and 1.6-fold, respectively, which is above the predicted therapeutic threshold. **Conclusions.** This study provides ASO sequences to downregulate or inhibit function of CNOT3, allowing *PRPF31* transcription to increase to a level expected to provide functional benefit. Transcriptome analysis and cellular function assays will be used to assess pre-mRNA splicing function resulting from increased PRPF31 expression in RP11 patient retinal cells.

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Localized hepatic delivery of a Natural ligand of PD-L1 by HBV-specific TCR redirected T cells.

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Increased PD-L1 expression in chronic HBV infected livers contributes to the inability of PD-1$^+$ HBV-specific T cells to clear infected hepatocytes. Therapy with monoclonal antibodies blocking PD-1/PD-L1 interaction has the potential to revert HBV-specific T cell hypo-responsiveness, yet at the risk of triggering systemic side effects.
We hypothesized that engineered HBV-specific TCR-redirected T cells could be utilized to deliver a natural ligand of PD-L1 in close proximity to HBV-infected targets, and hence modify the liver microenvironment. Our strategy is to modify T cells to redirect their specificity (with the HBV-specific TCRs) and to concomitantly allow them to secrete the soluble isoform of PD-1 (using Antisense OligoNucleotides, AONs).

We therefore designed AONs altering the splicing of PD-1 pre-mRNA by skipping the exon coding for the transmembrane domain. This should result in a decreased expression of membrane-bound PD-1 and in an increased secretion of the soluble isoform of PD-1 by engineered T cells.
We demonstrated that AONs–TCR double-transfected T cells express an HBV-TCR and they are functionally similar to traditional TCR-redirected T cells, but secrete higher quantities of soluble PD-1 after TCR-mediated specific activation. The functionality of AONs-TCR-redirected T cells was tested in a 3D *in vitro* model and in *in vivo* models against a hepatoma cell line expressing HBV antigens. Functional assays in the 3D model showed an increased efficiency by sPD-1-producing TCR-redirected T cells, also in the presence of PD-L1 expressing monocytes. In addition, sPD-1-producing TCR-redirected T cells were more effective in reducing the growth of hepatoma cells expressing HBV antigens and showed higher activation of global intra-lesional T lymphocytes in both immune-deficient and humanized murine models.
In conclusion, we developed a method to improve the effectiveness of TCR-redirected T cells *in situ*, converting PD-1 into a soluble isoform with the potential to mask PD-L1 sites on cognate targets.

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Beyond antisense therapy: using oligonucleotides to control gene expression by targeting cellular IRESs

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For antisense therapy, oligonucleotides have been developed to inhibit translation using two different approaches: enhancing the degradation of the targeted mRNA or blocking the translation machinery. Our aim goes beyond this: using oligonucleotides to control gene expression by either decreasing or increasing translation levels by modifying the secondary structure of cellular internal ribosome entry sites (IRESs).

We have focused our research on the BAG-1 IRES. Being aware of the controversy surrounding the existence of cellular IRESs, we have first verified the presence of an IRES in the p36 BAG-1 5' UTR. We then designed a pool of oligonucleotides targeting different regions of the BAG-1 IRES and generated a luciferase based method to quickly assay the effect of different oligonucleotides on IRES activity. The most promising oligonucleotides were modified in different ways to increase their activity and stability in cells.

We currently have some candidate oligonucleotides that have proven to modify translation *in vitro* and/or *in cellulo* and are carrying out further experiments to improve their activity.

Controlling translation initiation has a direct result on the efficiency of protein synthesis. Understanding the events in this process could lead to the discovery of new therapeutic targets, and thereby, the development of new therapies. In this way, these oligonucleotides could prove to be a treatment for diseases associated with an inappropriate amount of functional proteins.

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CRISPR interference as treatment strategy for Collagen VI Myopathy

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Collagen VI congenital muscle dystrophy (Col VI-CMD) is a spectrum disorder caused by mutations in *COL6A1*, *COL6A2* and *COL6A3*. The spectrum expands from the mildest and late onset form which is called Bethlem Myopathy (BM, OMIM # 158810) over intermediate phenotypes to the most severe and early onset form which is called Ullrich congenital muscular dystrophy (UCMD, OMIM #254090). So far, there is no treatment for Col VI-CMD.
Since heterozygous null mutations in *COL6A2* are nonpathogenic, allele-specific knock-down of dominant-negative mutations has to be investigated as a possible treatment strategy. However, the first and only approach for *Col6A2* dates back to 2012 and aimed at a variant in Intron 9 (c.954 + 17_954 + 22del28) leading to Ullrich CMD.
Our aim is to examine the allele-specific knockdown of a group of pathogenic variants leading to substitutions of the essential Glycine residues in triple helical repeats (Gly-X-X). The project was started with fibroblasts of a patient with the de novo pathogenic variant p.Gly283Glu and treatment with a variant-specific siRNA. A cell culture assay for siRNA transfection and induction of Collagen VI secretion into the extracellular matrix (ECM) was established. Changes due to the treatment with siRNA were detected on RNA level via real-time quantitative PCR and visualized via immunofluorescence staining.
To broaden our translational research pipeline a new approach called CRISPR interference is used utilizing a nuclease-dead Cas9 coupled to transcriptional repressors in combination with gRNAs. Potential target sites (GH21J046078, GH21J046091, GH21J046096 and GH21J046108) were Sanger sequenced to identify heterozygous common variants, because heterozygosity is essential for allele-specificity (Figure 1). To phase variants with the pathogenic *COL6A2* variant, genomic DNA was sequenced using an Oxford Nanopore MinION.
The first result using the new approach will be presented.

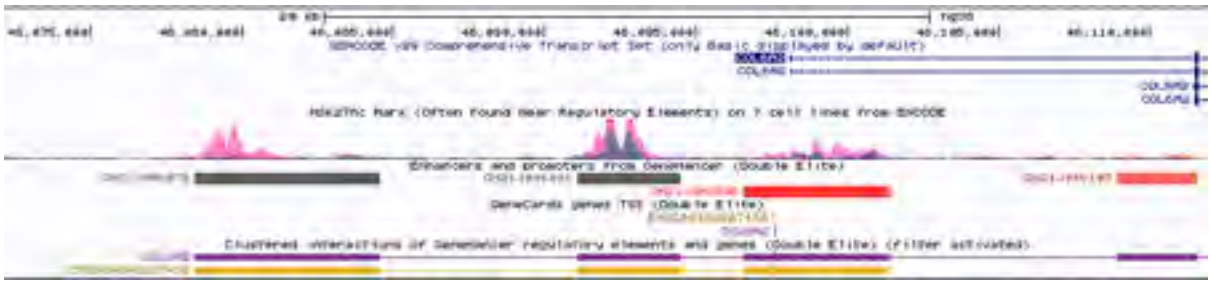

Figure 1: Regulatory Elements for COL6A2 shown in UCSC Browser

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Antisense oligonucleotide conjugates for erythropoeitic protoporphyria

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Erythropoeitic protoporphyria (EPP) is a genetic disorder affecting in average 1/100.000 individuals and causing light photosensitivity in patients who quickly develop skin irritation upon exposure blue light coming from natural or artificial sources [1]. EPP onset requires two independent genetic events on both alleles of the ferrochelatase (*FECH*) gene affecting the production of FECH protein [2]: in one allele, a non-sense or missense mutation prevents the synthesis of the functional enzyme; in the other, an intronic single nucleotide polymorphism (SNP) causes aberrant splicing of the pre-mRNA. Low levels of FECH lead to accumulation of its photoreactive substrate protoporphyrin IX (PPIX) in erythroid cells in the bone marrow and in the blood, as well as in the liver and the spleen.

We are investigating several strategies to restore FECH production. One strategy to treat EPP is to use splice-switching oligonucleotides (SSOs) - oligonucleotides specially designed to bind the pre-mRNA and sterically force the production of a functional *FECH* pre-messenger RNA. An active sequence was identified *in vitro* after screening and tested *in vivo* in an in-house disease model [3], where it was able to partially restore correct splicing in the liver and the spleen but failed to have an effect in the last disease-relevant tissue, bone marrow. Several peptide- and lipid- conjugates were therefore designed for an improved bone marrow vectorization and were tested *in vivo*, where one conjugate was able to improve the amount of correct FECH correct messenger RNA. In a parallel but independent manner, we are screening several approved small molecule drugs known to impact heme biosynthesis pathway, hoping to identify candidates able to directly increase FECH protein production.

Finally, we are working within this project with a technique developed for chemically modified oligonucleotides – the chemical-ligation qPCR (CL-qPCR) [4] – to quantify the amount of SSOs delivered within the cells *in vitro* or in tissues *in vivo*. The technique is currently being exploited as a platform to investigate the effect of oligonucleotide chemistry, length or conjugation on uptake, subcellular localization and biological activity.

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Azobenzene moieties as selective and tunable controls for siRNA activity

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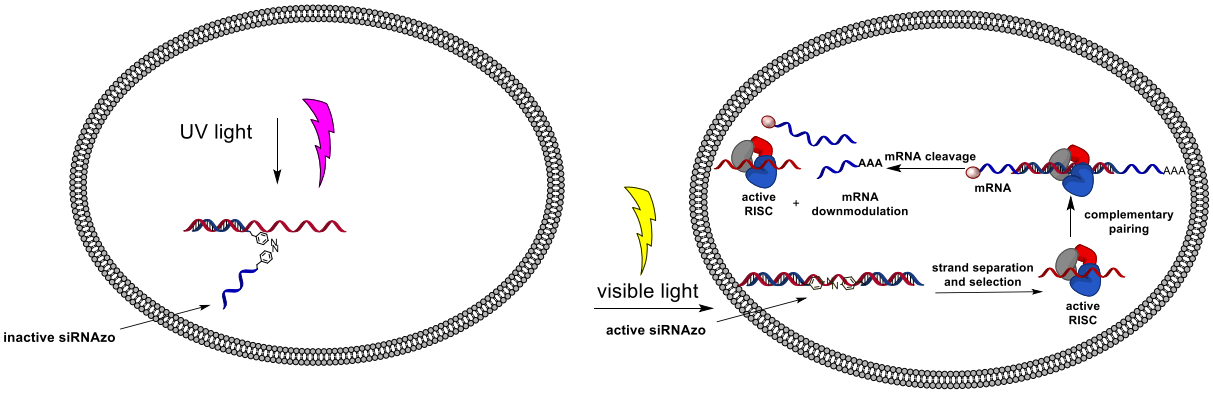
Utilization of the RNA interference pathway in order to disable unwanted gene expression in an aging western population has been of great interest recently. Using siRNAs as therapeutics however can be challenging, due to poor cell membrane permeability, off target effects, self degradation and endogenous RNases. Previously, we demonstrated addition of a photoswitchable azobenzene into specific sites of the siRNA is able to provide knockdown of exogenously added firefly luciferase, while simultaneously addressing several of the above problems with siRNA therapeutics.[1] We demonstrate that inactivation of the siRNA with UV light (before transfection), and reactivation with visible (after transfection) is possible. We also further explore the reversible *in vivo* activation and inactivation of the azobenzene containing siRNAs, where by exposing the cells to UV and visible light after transfection is able to control the activity of the siRNA. An additional chemical modification of the azobenzene moiety was synthesized in order to further explore the photo-isomerization properties at wavelengths in the visible region of the electromagnetic spectrum. The ability to inactivate the siRNA after deployment into the tissue would have many advantages, including more precise spatial and temporal control of the siRNAs. Since siRNAs specifically target mRNAs in the cell, these findings can easily be adapted for use in specific cancers, which could have targetable mRNAs, or even as an effective means of gene silencing for genetic disorders which can be difficult to treat with conventional therapeutics. With these advancements, we are looking into the development of a dual siRNA system, which could be used to selectively study gene expression on multiple targets simultaneously.



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In vivo tissue delivery of antibody-antisense morpholino conjugates in a mouse model of spinal muscular atrophy

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Drug delivery for neurodegenerative diseases is made difficult by the need to pass through the blood brain barrier (BBB) and blood spinal cord barrier (BSCB). The currently available antisense oligonucleotide (ASO) treatment of spinal muscular atrophy (SMA) circumvents the BSCB by administering directly into the spinal column (intrathecal). The hugely successful nusinersen, an ASO targeting survival motor neuron 2 in patients with SMA, has extended survival and welfare for many children. However, the intrathecal administrations requires patients to undergo a lifetime of this risky procedure. Therefore, a vehicle to carry ASO into the brain and spinal cord when systemically administered is an optimal way to meet the needs of all SMA patients.

We have recently discovered PMOs directly conjugated to an antibody targeting the transferrin receptor (anti-TfR) efficiently targets cells in the spinal cord. The transferrin receptor is the most widely studied pathway for transport of antibody-based drugs across the BBB. TfR is expressed on the luminal side of brain capillary endothelial cells, binds to iron laden transferrin and translocates it into the brain parenchyma. Studies into antibody delivery of ASOs to the brain is limited and typically do not report the cellular biodistribution within the brain and spinal cord.

Here we show that directly conjugated ASOs to anti-TfR improves the levels of SMN throughout the spinal cord and brain. In addition the highest level of anti-TfR accumulates within astrocytes in the spinal cord. Antibody-ASO conjugates provide a new and exciting way forward for ASO treatment in SMA and many other neurodegenerative diseases treatable with ASOs such as Huntington's disease, Alzheimer's disease and ALS.

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Hexose potentiates peptide-conjugated morpholino oligomer efficacy in cardiac muscles of dystrophic mice in an age-dependent manner

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Insufficient delivery of oligonucleotides to muscle and heart remains a barrier for clinical implementation of antisense oligonucleotide (AO)-mediated exon-skipping therapeutics in Duchenne Muscular Dystrophy (DMD), a lethal monogenic disorder caused by frame-disrupting mutations in the *DMD* gene. We previously demonstrated that hexose, particularly glucose:fructose (GF) significantly enhanced oligonucleotide delivery and exon-skipping activity in peripheral muscles of *mdx* mice; however its efficacy for the heart remains limited. Here we show that co-administration of GF with peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO, namely BMSP-PMO) induced approximately 2-fold higher level of dystrophin expression in cardiac muscles of adult *mdx* mice compared to BMSP-PMO in saline at single injection of 20 mg/kg, resulting in evident phenotypic improvement in dystrophic *mdx* hearts without any detectable toxicity. Dystrophin expression in peripheral muscles also increased. However, GF failed to potentiate BMSP-PMO efficiency in aged *mdx* mice. These findings demonstrate that GF potentiates oligonucleotide activity in *mdx* mice in an age-dependent manner and thus have important implications for its clinical deployment for the treatment of DMD and other muscular disorders.

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Tolerance for bulges in mature miRNAs, siRNA duplexes and target-bound guide strands

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Small non-coding RNAs are key players in gene regulation. Whereas miRNAs act as endogenous regulators of gene expression, siRNAs are well established as molecular biology tools and emerging as therapeutic agents. The siRNA guide strand is typically designed to form perfectly complementary duplexes both with the passenger strand and the target mRNA transcript. In contrast, nature designs eukaryotic miRNAs to contain elements that interrupt the perfect duplex, for example mismatches or bulges. Here, we tested if bulges of the guide strand are accepted in siRNA duplexes. Bulges of different lengths were introduced into siRNA duplexes by deleting one or more nucleotides from the passenger strand. Bulge tolerance at different positions was examined by systematically walking the bulge through the siRNA duplex. *In vitro* data were retrieved with unmodified and modified siRNAs of different nucleobase sequences. We also evaluated *in vivo* activity of selected, bulge-containing GalNAc-siRNA conjugates. Taken together, we find restrictions on bulge length and position in functional siRNAs and compare our insights to observations in the miRNA field. Finally, we asked if guide strand bulges may also form upon target RNA binding and evaluated this as a possible off-target risk.

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Absolute quantification of siRNA delivery and single cell knockdown kinetics

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For siRNA therapeutics, reaching tissues beyond the liver and getting macromolecular siRNAs into the cytosol of target cells are major issues. With current delivery strategies, only a small fraction of delivered siRNA is believed to reach the cytosol, while most siRNA is trapped within the endosomal system. A key issue complicating efforts to enhance the delivery efficiency of various siRNA delivery agents is a lack of tools to determine the absolute efficiency of a delivery strategy. In addition, the relationship between the amount of delivered siRNA and knockdown is unknown.

We have refined a previously developed method to detect the cytosolic delivery of fluorescently labelled siRNA, to allow detection of minute cytosolic siRNA release amounts. Airyscan based confocal imaging of live-cells was employed to monitor cytosolic release of Alexa Fluor 647 labelled siRNA (siRNA-AF647) delivered with a widely used commercial transfection lipid. Cytosolic release events were detected with an automatic algorithm flagging sudden increases of cytosolic siRNA-AF647 fluorescence.

Sensitivity and specificity of the detected release events were determined by correlating flagged events with an independent method to detect siRNA release using a galectin membrane damage sensor. By a combination of automatic detection and manual quality control, around 90% sensitivity and >90% specificity, was achieved for the detection of cytosolic release events in cells incubated with siRNA concentrations down to a few hundred pM. Incubating HeLa cells stably expressing a short half-life eGFP protein with small amounts of siRNA targeting eGFP, allowed determination of single-cell kinetics of siRNA mediated knockdown in the range from a few hundred to several thousand cytosolic siRNA molecules, demonstrating a dose-response relationship with respect to both knockdown induction and duration.

Here, we present a practical method to directly measure the absolute amount of cytosolic siRNA delivery. Furthermore, the establishment for the first time of a dose-response relationship for cytosol-delivered siRNA and knockdown kinetics will aid substantially in the development and characterization of diverse novel delivery strategies.

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SNAP®-ADAR mediated RNA-Editing in HepG2 cells

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The targeted delivery of therapeutics is still a very challenging part of modern therapeutic development and clinical research, whereby the receptor-mediated uptake became a promising approach. Especially the asialoglycoprotein receptor (ASGP-R), became very popular for the targeted uptake of oligonucleotides into hepatocytes. The abundantly expressed receptor is a hepatocyte-specific membrane-bound lectin receptor and is responsible for removing glycoproteins from mammalian serum by receptor mediated endocytosis. Its high affinity towards compounds with terminal *N*-acetyl galactosamines (GalNAc) is also providing a promising approach for the uptake of oligonucleotides[1],[2]. Especially the uptake of antisense oligonucleotides (ASO), which induce RNAi-based gene silencing, became a strategic approach to target liver disease affected mRNAs and proteins[3],[4]. With respect to artificial SNAP®-tagged ADAR systems[4], as well as the RESTORE-based recruitment of endogenous ADAR[5], the GalNAc mediated endocytosis of guide RNA's could also provide a beneficial opportunity to target naturally ASGP-R expressing cell lines. Therefore, this poster presentation should give an overlook about the development of a SNAP®-ADAR expressing HepG2 cell line, and the SNAP®-ADAR based A-to-I editing of a particular housekeeping gene. Based on this research, the ASGP receptor mediated uptake of GalNAc conjugated gRNA's into HepG2 cells, will be part of the further research.

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Evaluation of the Effect of siRNA Chemical Modifications on Next Generation Sequencing Library Preparation

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Modern therapeutic siRNAs are heavily chemically modified in order to stabilize them against nucleases and phosphatases, as well as the highly acidic environment of late endosomes and lysosomes. Common nucleotide modifications include 2′-Ome and 2′-F as well as 5′-vinyl phosphonate and phosphorothioate. Modified siRNAs are also often conjugated to chemical moieties in order to facilitate delivery into cells. These modifications and conjugates can complicate the extraction of modified siRNAs from tissues or cells and interfere with assays commonly used to detect small RNAs. It is important to be able to quantify the relative levels of modified siRNA and endogenous small RNAs in order to determine relative RISC occupancy and measure any effect by the modified siRNA on the cellular miRNA profile. Cloning modified siRNA guide strands for sequencing is less efficient than cloning unmodified small RNAs and this bias must be accounted for when using next generation sequencing to measure modified siRNAs.

The chemical modifications in modified siRNAs allow them to be selectively sequenced. Treating libraries with RNase and phosphatase during preparation removes endogenous small RNAs and only clones modified RNA. This method allows for detection of siRNAs in tissues with high sensitivity and confidence and with extremely low levels of contaminating sequencing reads from endogenous RNAs. This method will be useful when measuring modified siRNAs whose sequence is present in the endogenous small RNA pool.

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Delivery of therapeutic RNAs to mammalian mitochondria

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Mitochondria rely on the import of certain nuclear-encoded macromolecules to carry out biochemical processes like replication, translation, and membrane-mediated metabolic activities. Protein import in mitochondria has been well-documented and its mechanisms have been well-characterized. Nuclear-encoded RNAs have also been found to have important functions in the mitochondria. Observations that nuclear-encoded RNAs have a distinct presence in mitochondria suggest that efficient RNA import pathways have evolved for mitochondria. However, many of the key mechanisms involved with RNA import into mitochondria have yet to be defined.

We sought to understand RNA import pathways for mitochondria by identifying RNA sequence motifs, as well as modifications and necessary RNA-binding proteins, that would enable RNAs to traffic from the nucleus or cytoplasm to the mitochondrial matrix in mammalian cells. We have identified sequences from endogenous non-coding RNAs that enable the trafficking of exogenous RNA cargos to mitochondria in an initial screen. Using MRP and RNase P as known RNA targets, we optimized a mitochondrial fractionation- and quantitative PCR-based assay to determine the enrichment of nuclear-encoded RNA sequences in mammalian mitochondria. RNA immunoprecipitation was also used to detect non-mitochondrial encoded RNAs in mitochondria and to verify sequences found with fractionation-based methods. These strategies will be used in future studies to screen more RNA sequence-structure motifs and assess their functions in mitochondrial RNA import.

In addition to understanding endogenous systems of transport, we sought to develop artificial systems for transporting RNAs to mitochondria by re-engineering the localization patterns of RNA-binding proteins (RBPs). RBPs relocated to mitochondria were examined for their ability to bring specifically-bound RNAs to mitochondria. These artificial systems would enable the delivery of RNAs to mitochondria for gene therapy applications. RNA-based therapeutics are promising avenues in the future of gene therapy. In developing RNA-based therapeutics to correct genetic defects in mitochondria, it will be paramount to understand the cellular mechanisms for transporting nuclear-encoded or exogenous RNAs to mitochondria and to have strategies to manipulate them.

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Pre-clinical testing of a novel antisense oligonucleotide targeting CD49d inflammation in mdx mice

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Duchenne muscular dystrophy (DMD) is a X-linked inherited disease caused by mutations in the dystrophin gene. DMD affects approximately 1 in 3500 boys, and results in muscle weakness causing loss of ambulation (walking ability), and premature death as a result of cardiac and respiratory failure.

The pathogenesis of DMD is well understood - with a breakdown in muscle fibres caused primarily by the disruption of dystrophin, a key cytoskeletal protein that provides structural support between muscle fibres. The loss of dystrophin results in an increase in susceptibility to muscle damage and breakdown. This muscle breakdown causes an influx of inflammatory cells that attempt to remove damaged muscle, however muscle is typically replaced by fat and fibrotic (scar) tissue. Deterioration in DMD patients is most obvious in the skeletal muscles, initially affecting the legs, and upper limbs, and spreading to respiratory muscles and finally the heart. While a number of different treatments have been developed for DMD, a cure has yet to be achieved.

We aim to investigate the use of a novel therapeutic agent, ATL1102, as a treatment for patients with DMD. ATL1102 is an antisense oligonucleotide that selectively targets the RNA of human CD49d, the alpha chain of adhesion molecule VLA-4. VLA-4 is a key inflammatory protein present in patients with inflammatory diseases, including multiple sclerosis (MS) and asthma. In DMD, CD49d is more highly expressed in the T cells (CD4 and CD8) of patients with more severe and progressed disease, making it a potential therapeutic target.

Using the mdx mouse, a model of DMD, we have begun to test the efficacy of an antisense oligonucleotide to CD49d and assessed its effects on inflammation. It is hoped that this novel antisense oligonucleotide treatment will protect dystrophic muscles from the damaging inflammatory effects associated with repeated rounds of muscle breakdown to prevent the loss of muscle function in mdx mice. The mouse studies will enable us to assess the effect of this novel antisense on inflammatory T cell and macrophage infiltrates within the muscle as well as perform ex vivo muscle function analyses to examine strength and eccentric induced muscle damage.

ATL1102 is currently under phase 2 clinical trials in non-ambulant DMD patients. This study will complement the analysis of ATL1102 in DMD patients and provide further insight into how this novel oligonucleotide effects disease progression and development.

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Development of a novel siRNA delivery system for cancer treatment using a tight junction opening peptide modified tumor penetrable liposome

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This study aimed to develop a novel siRNA delivery system for cancer treatment, an siRNA carrier capable of delivering siRNA widely to tumor tissue using a tight junction opening peptide (AT1002), cytoplasm sensitive peptide and PEG-liposome. Its penetration and anti-proliferative effects in A549 human lung carcinoma cells in a spheroid culture system, and its tumor accumulation, penetration and anti-tumor effects after intravenous administration were evaluated. AT1002 modified siRNA loaded liposomes (AT1002-liposome) were prepared by post-inserting stearic acid modified AT1002 into DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) based liposomes, which displayed physical properties suitable for systemic delivery a high siRNA-encapsulation rate. A spheroid culture system was generated by culturing A549 cells in low-adsorptive U-bottom plates for five days. On evaluating the penetration in the spheroid of A549 cells using fluoresce-labeled siRNA and lipids, AT1002-liposomes showed remarkable permeability and cellular uptake ability compared with other groups. Furthermore, the anti-proliferative effect of AT1002-liposomes encapsulating PLK-1-targeting siRNA (siPLK-1) on A549 spheroid was evaluated. Consequently, the spheroid volume and the number of spheroid-forming cells transfected naked siPLK-1 were similar to those in the control groups. On the other hand, the AT1002-liposomes were observed remarkable anti-proliferative effects, owing to their enhanced intra-tumoral penetration through the AT1002 modification. And then, the AT1002-liposomes were intravenously administered to A549 tumor-bearing mice (siRNA: 0.5 mg/kg) using fluorescence labeled siRNA and their tumor accumulation was observed using an IVIS. The AT1002-liposomes highly accumulated at the tumor site. Additionally, cryosections of a tumor site observed by a confocal laser-scanning microscopy at 6 h after intravenous administration. It was demonstrated that distribution and penetration of AT1002-liposome was significantly and widely improved in the whole tumor site. Finally, anti-tumor effects of A549 tumor-bearing mice were evaluated by intravenous administration of siPLK-1-encapsulated in AT1002-liposomes (siRNA: 0.5 mg/kg). AT1002-liposomes were significantly inhibited the increase in tumor volume compared to other groups. In addition, siPLK-1 loaded AT1002-liposomes reduced the PLK1 mRNA levels in the tumor site. These results were suggested that the AT1002-liposomes is a potential siRNA delivery system for cancer treatment because of its high accumulation and penetration at the tumor tissue.

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Induction of cellular immunity of nucleic acid-antigen peptide conjugate

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Immunotherapy has become one of the attractive strategies for antitumor treatment. The induction of tumor-specific cytotoxic T-lymphocytes (CTLs), which recognize and eradiate tumor cells, is essential for the treatment of tumors. Antigen-presenting cells (APCs) such as macrophages and dendritic cells play a crucial role in the initiation of T cell immune responses. Inflammatory signals by adjuvant molecules induce mature APCs, followed by enhanced expression of major histocompatibility complex (MHC)-I and II molecules and costimulatory molecules. APCs present the internalized antigenic peptides on either an MHC class I molecule to induce $CD8^+$ T-cell responses or an MHC-II molecule to induce $CD4^+$ T-cell responses. However, the addition of antigen alone does not induce a sufficient immune responses to attack cancer cells. In this study, we prepared peptide-nucleic acid conjugates which deliver the bound peptide to APCs and evaluated the immune responses when immunized in combination with an adjuvant.

C57BL/6 mice weighing 16-21 g (7-8 weeks old) were intradermally administered the conjugate with adjuvant, or peptide only at a dose of 50 ng in a peptide amount. After 1 week, mouse splenocytes were stimulated with the peptide at 10 μg/ml for 24 hours. The supernatants were subjected to ELISA for mouse IFN-γ. The cells were plated at 1.5×10^5 cells/well in 48-well plates, and added peptides (or the conjugates) at 50ng of peptide dose. After incubation overnight, the cells were washed with PBS three times, and fixed with 4% paraformaldehyde. The cells were stained with PE-labeled anti-mouse $OVA_{257\text{-}264}$ (SIINFEKL) peptide bound to $H\text{-}2K^b$ antibody, and the fluorescence intensity of the cells was observed using a flow cytometer.

50 ng of the peptide conjugate could induce a large amount of IFN-γ production, while naked treatment and administration of mixture of adjuvant and the peptide did not cause any IFN response. The antigen presentation level by the conjugate was almost the same as that by the peptide only. Therefore we can conclude that the peptide of the conjugate can be presented on cellular surface even after conjugation with nucleic acid.

Our conjugates can be a candidate to improve the cancer vaccine efficacy.

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Preparation of Fibroblasts with Patient-relevant Mutations

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Nonsense mutations are the most prevalent patterns of germline and somatic mutations in patients with retinoblastoma. This study aimed to investigate the therapeutic potential of the genome editing-based technology in the restoration of *RB1* gene transcripts and retinoblastoma protein in cells containing nonsense mutations in the *RB1* gene of patients with retinoblastoma. We prepared skin fibroblast cells with nonsense mutations in the *RB1* gene mimicking those of retinoblastoma tumors by genome editing based on activation-induced cytidine deaminases. Treated cells were cultured by single cell and each single cell-derived clone were used for further experiments. Then, the cells were treated with adenine base editors targeting nonsense mutations. The efficacy of genome editing was evaluated with Western blotting and functional assays including analyses on cell growth. We obtained the cells with nonsense mutations in the *RB1* gene. Adenine base editors effectively recovered the expression of retinoblastoma protein on Western blotting. Also, the treatment with adenine base editors restored normal cellular function including the control of cellular growth. We expect that this genome editing-based technology can help to recover *RB1* gene transcripts and retinoblastoma protein in retinal cells containing nonsense mutations. It might be of therapeutic potential in the treatment of patients with retinoblastoma or people at risk.

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SNA modification on siRNA and anti-miRNA oligonucleotide for improvement of their performance

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RNA interference (RNAi) is an endogenous gene silencing system in which microRNAs (miRNAs) inhibit gene expression via sequence specific interaction with mRNA. It has therapeutic potential to treat human disease. Development of siRNA, which can exogenously induce RNAi-mediated knock down of the disease-related gene expression and antisense oligonucleotide against disease-related miRNA (anti-miRNA oligonucleotide, AMO) has attracted attention.

For practical use of siRNA and AMO, they must be resistant to degradation by endogenous nucleases. Although chemical modifications have increased the stability of siRNA and AMOs, these modifications often have negative effects on activity. Recently we have developed artificial nucleic acids: SNA that stably hybridized with DNA and RNA. SNA has several advantages of high nuclease resistance, synthetic facility, and so on due to their acyclic nature. Here, we demonstrate an improvement of the performance of siRNA and AMO with high activity and high nuclease resistance by utilizing the SNA (Fig. 1).[1,2] The high potency of the SNA modifications on siRNA and AMO suggests that these functional oligonucleotides will be useful as therapeutic reagents for control of endogenous gene and miRNA function in patients and as tools for investigating the role of RNAs in cells.

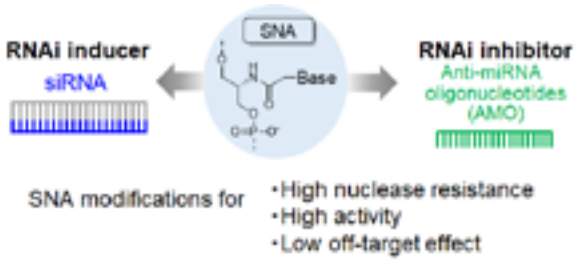

Figure 1. Introduction of SNA into siRNA or AMO to improve their performance

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L-type Calcium Channel Blocker Enhances Cellular Delivery and Gene Silencing Potency of Cell-Penetrating Asymmetric siRNAs

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The efficient delivery of small interfering RNAs (siRNAs) to the target cells is critical for the pharmaceutical success of RNA interference (RNAi) drugs. One of the possible strategies to improve siRNA delivery is to identify auxiliary molecules that augment their cellular uptake. In an effort to discover small molecules that enhance the potency of cholesterol-conjugated, cell-penetrating asymmetric siRNA (cp-asiRNA), herein, we performed a chemical library screening. Interestingly, three compounds identified from the screen share a common dihydropyridine (DHP) core and function as L-type calcium channel blockers (CCBs). Using confocal microscopy and quantitative analysis of small RNAs, we demonstrated that the L-type CCBs increased the endocytic cellular uptake of cp-asiRNA. Furthermore, these small molecules substantially improved the potency of cp-asiRNA, not only *in vitro* but also *in vivo* on rat skin. Collectively, our study provides an alternative pharmacological approach for the identification of small molecules that potentiate the effects of therapeutic siRNAs.

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Development of nucleic acid aptamer to capture exosomes

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Exosomes function as messengers responsible for intercellular communication, and are being investigated for use as disease biomarkers and drug discovery targets. Whereas, a simple and non-invasive purification method for exosomes has not been established. In order to solve this problem, we focused on nucleic acid aptamers. Nucleic acid aptamers are single-stranded nucleic acid molecules that can recognize and bind to the structure of a target molecule by forming a higher-order structure. They are created by the SELEX method, which includes the selection, amplification and purification steps. In this study, we tried to create anti-exosome nucleic acid aptamers by using polyethylene glycol (PEG) precipitation and size exclusion chromatography (SEC) in the selection step.
The initial library with a total length of 70 bases including a 30 base random region was incubated with exosomes purified by PEG precipitation from the culture supernatant of the A549 cell line. The selection of anti-exosome nucleic acid aptamers was performed by separating exosome-eluted fractions by SEC. After eight cycles of positive-selection and three cycles of negative-selection, we succeeded in obtaining five types of anti-exosome nucleic acid aptamers. These aptamers were found to have a binding activity to exosomes from the results of measurement with capillary electrophoresis. In addition, we succeeded in capturing exosomes by the aptamers immobilized on magnetic beads. In this presentation, the preparation and function of the anti-exosome nucleic acid aptamers will be shown in detail.

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Defining the Impact of RNAi in a Model Cell Line: Implications for the Regulation of Gene Expression by RNA

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RNA interference (RNAi) has the potential to be a potent regulatory mechanism in human cells. Synthetic RNAs can control gene expression in either the cytoplasm or nucleus and have been developed to be successful drugs. Endogenous miRNAs can control natural physiologic processes and disease. Despite two decades of study, however, the full scope of natural RNAi in cells and the extent of control of gene expression has remained obscure. Here we examined the ramifications of RNAi in a model colon cancer cell line, HCT116. Using eCLIP-seq, we defined locations within the transcriptome that were binding sites for argonaute 2 (AGO2). We used knockout cell lines for AGO1, AGO2, AGO1/2, and AGO1/2/3 to investigate how RNAi controls gene expression in HCT116 cells and how gene expression changes correlate with AGO2 binding to specific sequences. Quantitative mass spectrometry and protein titrations were used to estimate the numbers of AGO1 and AGO2 proteins per cell. RNA-seq was used to rank miRNAs in both cytoplasm and nuclei. We conclude that the protein and miRNA machinery of RNAi has a limited impact on regulation of gene expression in HCT116 cells. This limited impact may be due to a limiting pool of AGO proteins. Recent publication of a cell atlas of miRNA expression suggests that miRNA expression in HCT116 cells is typical. Our results suggest that the action of miRNAs in cell lines may be restricted to a relative handful of genes and that conclusions about endogenous roles in disease and normal physiology should be carefully justified.

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Neuroprotective effects of NXP031, AptaminC3T31 and ascorbic acid, in the MPTP-induced mouse model of Parkinson's disease

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Aptamers are short, single-stranded DNA or RNA oligonucleotides that are selected through systematic evolution to interact with any desired targets ranging from small molecules, proteins to cells with both affinity and specificity. Aptamers are promising drug candidates and are being increasingly employed in all phases of the drug discovery and development process. Parkinson's disease (PD) is a degenerative neurological disorder, which is characterized by the selective degeneration of dopaminergic neurons in the *substantia nigra*. Oxidative stress has been identified as one of the major contributors for the nigrostriatal degeneration in PD. Ascorbic acid is a well-known and vital antioxidant molecule and plays a key role in dopaminergic neuron differentiation. It is previously reported that ascorbic acid is associated with oligomeric α-synuclein indirectly and posttranslational α-synuclein modification caused by oxidative stress. However, ascorbic acid is easily oxidized to lose its antioxidant activity, limiting its efficacy as a therapeutic drug. To overcome this limitation, we have recently developed AptaminC3T31, a single-stranded DNA aptamer that binds to ascorbic acid with great specificity, reducing its oxidation and dramatically increasing its efficacy. NXP031 is an optimized formula of AptaminC3T31 and ascorbic acid for PD treatment. Therapeutic effect of NXP031 was tested by using 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced mouse model of PD. C57BL/6 mice were treated with intraperitoneal injection of MPTP at 20 mg/kg every 2 hours 4 consecutive times, causing acute degeneration of nigral dopaminergic neurons. 160 C57BL mice were randomly divided into 5 groups, consisting of control, MPTP treated, ascorbic acid plus MPTP treated, AptaminC3T31 plus MPTP treated, and NXP031 (4 mg AptaminC3T31/kg and 200 mg Ascorbic acid/kg body weight) plus MPTP treated. One day after MPTP treatment, NXP031 and ascorbic acid were intraperitoneally administered for 4 consecutive days, respectively. Next day, we conducted behavioral tests including pole test and rotarod test for evaluating motor dysfunctions. Tyrosine hydroxylase (TH) immunohistochemistry was performed and nigral dopaminergic neurons were counted by stereological analysis of TH-positive neurons. NXP031 treatment exhibited significant improvement of MPTP-mediated motor deficit. This result was supported by the observation of significant restoration of TH-positive dopaminergic neurons in the *substantia nigra* in the NXP031 treated group compared to the only ascorbic acid treated group. Here, we show that NXP031 protects nigral dopaminergic neurons from MPTP-mediated degeneration and results in a dramatic improvement of motor behavior, suggesting NXP031 a potential therapeutic for PD.

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Conquer refractory cancer through the inhibition of K-Ras or c-Myc signaling networks by replacement therapy with chemically-modified miRNA-143 or -145

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We have recently found a strong knockdown effect of K-Ras and c-Myc by microRNA (miR) replacement therapy[1]. The colon tumor specific downregulated miR-143 and miR-145 showed a potent anti-tumor effect through targeting K-Ras and c-Myc signaling networks.

When local treatment and intravesical administration of chemically modified miR-143 or miR-145 using unit polyion complex (uPIC) for colon cancer were performed, a remarkable therapeutic effect was shown. Mir-143 (CM-miR-143) silenced the expressions of such genes as Sos1,AKT, and ERK, as well as K-Ras. K-Ras inhibitor alone cannot suppress the networks completely. The extremely potent CM-miR-143 enabled us to understand K-Ras signaling networks and shut them down by combination treatment with this miRNA(CM-miR-143) and EGFR inhibitor in K-Ras-driven colon cell lines[1].

On the other hand, bladder cancer is one of the most difficult malignancies to control. Our study showed that CM-miR-145 was a potent anti-bladder cancer drug *via* inhibiting c-Myc, PI3K/AKT, and MAPK/ERK pathways *in vitro* and *in vivo*[2].

Currently, further research is ongoing for the purpose of developing clinical trials.

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Targeting the maladaptive ER-stress mediator Chop using LNAplus™ antisense-oligonucleotides in diabetic nephropathy

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Diabetic nephropathy (dNP) is the most common cause of end-stage renal disease worldwide. Established therapies like angiotensin-converting-enzyme inhibitors (ACEi) delay but fail to prevent dNP progression. Efficient therapies are lacking and there continues to be an unmet medical need in dNP. Recently, a maladaptive ER-stress response has been identified as an important factor in the pathogenesis of dNP. A key molecule conferring the renal maladaptive ER-stress response is C/EBP homologous protein (Chop). We hypothesized that Chop is a promising molecular target for novel dNP therapies. As Chop is an intracellular transcription factor it falls into the category "difficult to target" and represents an optimal target for antisense-oligonucleotides (ASOs).
We designed locked nucleic acid (LNA)plus™ -modified ASOs specific for mouse or human Chop using our Oligofyer™ bioinformatics system and screened them in mouse and human cell lines in vitro. For in vivo proof-of-concept experiments eight-week-old db/db mice were treated for eight weeks with 1 mg/kg of control or mouse Chop-specific LNAplus™ ASO as monotherapy or in combination with ACEi. We analyzed urinary output, urine albumin-to-creatinine ratio (UACR), histopathological damage, fibrosis and expression of ER-stress response factors at the end of the in vivo intervention.
Treatment of mice with the Chop-specific LNAplus™ ASO (but not with the control oligonucleotide) resulted in a Chop knockdown in kidneys of >80 %. Chop-specific ASO therapy significantly decreased UACR, urinary output and histopathological damage. Strikingly, the degree of tubular damage as well as renal fibrosis was significantly reduced when Chop-specific ASO therapy was combined with ACEi as compared to the monotherapy groups.
In conclusion, targeting the maladaptive ER-stress mediator Chop with LNAplus™ ASOs is a promising novel approach for the treatment of dNP that results in a therapeutic benefit on-top of currently available therapies. Further studies in mouse models of more advanced stages of dNP are ongoing.

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Compliant-ready Workflow for Mass Characterization of Oligonucleotide and Related Impurities

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There has been a significant increase in demand for analytical development of oligonucleotides. To date there is no compliant-ready software that can process oligonucleotide data. We have made improvements to our compliant-ready software, UNIFI, to allow for the confirmation of oligonucleotides and related impurities.

UNIFI Intact Protein Analysis within the Biopharma workflow has been enhanced to allow it to process 'Mass Only' targets. This has enabled the workflow to target any mass without the need for the user to specify a sequence. Deconvolution is performed using the existing MaxEnt1 for larger masses and introducing BayesSpray for use with lower masses. Custom modifications can also be added to the processing method to help identify potential oligonucleotide impurities.

The results of these improvements allow the user to specify 'Mass Only' target masses and to define and specify custom modifications. It also allows for the selection of regions of interest using retention time windows. A choice of deconvolution algorithms has been made available ensuring oligonucleotides can be processed more effectively based on the mass range. Additionally, both deconvolution algorithms consider backbone modifications, that is, whether the oligonucleotide is modified with phosphorothioate or non-modified phosphodiester backbone.

There has been an increasing demand of oligonucleotide characterization across all areas from research to drug production and manufacturing. Here we have introduced an improved compliant-ready workflow to allow mass characterization of oligonucleotides and related impurities.

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Discovery of gapmer antisense oligonucleotides targeting vascular endothelial growth factor-A: A combination study of three bridged nucleic acids such as AmNA, GuNA, and SCP.

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Vascular endothelial growth factor (VEGF) plays a critical role in tumor growth by promoting angiogenesis and by suppressing the function of cytotoxic T-lymphocyte. In fact, recent evidence suggests that inhibition of VEGF not only suppresses tumor angiogenesis but also improves tumor immune environment. Furthermore, the concept of combination therapy using inhibitors for VEGF and immune checkpoint has also been proved by recent clinical studies. However, conventional VEGF inhibitors shows several adverse events such as hypertension and acute renal failure. Therefore, there is a great demand for the development of a novel VEGF inhibitor with little adverse events. In this study, we determined the knockdown (KD) activity of antisense oligonucleotides (ASOs) targeting the VEGF mRNA, which are modified with three different bridged nucleic acids, such as amido-bridged nucleic acid (AmNA), guanidine-bridged nucleic acid (GuNA), and 2′-O,4′-C-spirocyclopropylene-bridged nucleic acid (SCP), all have a high nuclease resistance along with a high duplex-forming ability towards target RNA. Note that AmNA or SCP-modified ASOs are anticipated to reduce hepatotoxicity, one of important issue for ASO, while GuNA is expected to enhance cellular permeability of ASO.

AmNA-gapmer ASOs targeting mature VEGF mRNA were designed by the established algorithm, to obtain active compounds with minimal off-target risk. The KD activity of the ASOs was evaluated by the reduction of VEGF-A protein amount in the culture supernatant of A549 cell. After screening by enzyme-linked immunosorbent assay (ELISA), highly active top three ASOs were selected for further investigation. Then, we synthesized gapmer ASOs modified not only with AmNA but also with GuNA and SCP, subsequently examined their KD activity of VEGF-A mRNA in the A549 cell. In this presentation, we will discuss the KD activity of VEGF-A mRNA depending on the modification of ASO with the three bridged nucleic acids.

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Developing aptamer-conjugated contrast media for detection of blood clots in vascular diseases

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MRI and CT angiography are imaging techniques used to assess blood flow in the brain; however, neither can label a blood clot that can either disrupt flow or demonstrate where haemorrhage in the brain has occurred. To improve the diagnosis and treatment of brain vessel diseases such as stroke and aneurysms, we developed fibrinogen aptamer(FA)-driven contrast agents to enable the identification and labelling of blood clots. Fibrin was chosen as the target of interest as it is involved in blood clot formation and is a major element of conditions like stroke and aneurysms. Since FA was originally selected to bind fibrinogen, fibrin-binding validation was required. It was hypothesized that FA would retain some binding affinity towards the polymerized form, fibrin, given that enough of fibrinogen monomers would be available for FA to bind. To assess the affinity and selectivity of FA towards fibrin, FA was tagged with a green emitting fluorophore and fluorescence co-localization was monitored. FA was selective, and binding was immediate upon direct interaction, accumulating to a significant amount within minutes. Phantom MRI systems, utilizing gadolinium conjugates (Gd(III)-DOTA/NOTA-FA), were used to monitor contrast enhancement. Significant enhancement was observed when Gd(III)-DOTA/NOTA-FA contrast was incubated with fibrin powder. A larger synthetic fibrin clot was then produced and tested using the previously optimized conditions. It was found that FA not only retains its binding affinity towards the monomers of fibrinogen available on the surface but is also able to penetrate within the clot. Subsequent *in vitro* testing was preformed using clotted blood with incubations that were more clinically relevant. Visualizable contrast enhancement was observed after 45-minutes. In parallel, CT FA-formulations were tested, exhibiting mean contrast enhancement of 61.9 HU. *In vitro* testing utilizing clotted blood demonstrated visualizable contrast enhancement post an 18-hour incubation. *Ex vivo* CT scanning of isolated tissue was used to confirm the location of the clot. These novel conjugates can be used to deliver radiocontrast agents to the site of the blood clot formation for improvement of diagnostic and therapeutic applications of vascular diseases involving clotting.

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Observation of the siRNA delivery kinetics via nose-to-brain route combined with stearate- or polyethylene glycol-modified arginine-rich peptides using ex vivo fluorescent imaging.

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Nose-to-brain delivery is a highly versatile route, which, in combination with novel nucleic acid medicines being developed for treating intractable CNS diseases, is a promising approach for the treatment of disorders. Furthermore, basic peptide carriers may improve nose-to-brain drug delivery by their capability to increase the stability of the encapsulated nucleic acids against chemical and biological degradation. In this study, to investigate the nose-to-brain pathway using basic peptide carriers, the time-dependent distribution of siRNA in whole brain, nasal mucosa, and trigeminal nerve after intranasal administration with arginine-rich peptide carriers modified with hydrophobic or hydrophilic moiety. We used stearic acid (STR) as a hydrophobic moiety or polyethylene glycol (PEG) as hydrophilic moiety. To examine the siRNA delivery kinetics by nose-to-brain pathway with STR- or PEG-modified peptides, we observed extracted each tissue in rats following intranasal administration of fluorescein-labelled model siRNA (F-siRNA) by fluorescent imaging system.

Significantly stronger fluorescence was observed in the whole brain, nasal mucosa and trigeminal nerve following intranasal administration of F-siRNA with both peptide carriers than that of F-siRNA alone. In the group receiving the F-siRNA/STR-peptide complex, although the fluorescence intensity was weaker in the trigeminal nerves, very strong fluorescence of F-siRNA was observed in the nasal mucosa. In addition, strong fluorescence was observed in the olfactory bulb in the forebrain. In the group receiving the F-siRNA/PEG-peptide complexes, strong fluorescence was observed in the not only nasal mucosa and olfactory bulb but also trigeminal nerve. Furthermore, spreading of the fluorescence was observed after 30 min and 1 h in whole brain. Both peptide-based carriers have also demonstrated the ability to improve the delivery of model drugs from the nose to the brain. In addition, PEG-peptide can improve drug distribution throughout brain tissues after movement from the nose to the brain compared with STR-peptide. These results suggest that the enhancement of drug-delivery achieved via the nose-to-brain route may depend on the properties of the carriers.

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Treatment effects for cerebral ischemia-reperfusion injuries in rats using nose-to-brain delivery of anti-TNF-alpha siRNA with membrane-permeable polymer micelles

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The siRNA has been successfully demonstrated as effective therapeutics for central nerve systems (CNS) disorders by directly intracerebral injection *in vivo*. However, the blood-brain barrier limits the distribution of siRNAs by systemically administration to the CNS. We previously reported that a nose-to-brain delivery system combined with polyethylene glycol-polycaprolactone (PEG-PCL) polymer micelles conjugated with the cell membrane-permeable peptide, Tat (PEG-PCL-Tat), significantly improved siRNA distribution in the brain compared to either intranasal delivery of naked siRNA or intravenous delivery of siRNA with PEG-PCL-Tat.
In this study, to apply a nose-to-brain siRNA delivery with PEG-PCL-Tat to the novel therapeutic system for cerebral ischemia-reperfusion injuries, the treatment effects by intranasal administration of anti-TNF-alpha siRNA (siTNF-alpha) with PEG-PCL-Tat were examined using a transient middle cerebral artery occlusion (t-MCAO) rats.
The cerebral ischemia-reperfusion model rats used for this experiment were prepared by blocking the middle cerebral artery by insertion of a nylon suture for t-MCAO. Treatment was intranasal administered 30 min after the insertion of nylon suture and the blood flow was reperfused 2 hr after occlusion. At 22 hours after reperfusion, we assessed the following 4 points to determine the therapeutic effects: infarcted area, brain wet weight, amount of TNF-α production in the brain, and neurological score. Infracted areas were calculated from the triphenyltetrazolium chloride (TTC)-stained continuous coronal brain slices using the ImageJ analysis. The TNF-alpha production was determined by ELISA assay.
While infarcted area was observed over a wide range in the untreated group, the strongly shrinkage of infarcted area was observed in rats treated by intranasal administration of siTNF-alpha with MPEG-PCL-Tat micelles. In addition, the increases in brain wet weight caused by edema were significantly suppressed by intranasal administration of siTNF-alpha with MPEG-PCL-Tat micelles. As a result, TNF-alpha production in rats treated by intranasal administration of siTNF-alpha with PEG-PCL-Tat micelles was particularly lower in comparison to the untreated and naked siTNF-alpha treated rats. Furthermore, rats treated by intranasal administration of siTNF-alpha with PEG-PCL-Tat micelles exhibited particularly marked suppression of neurological score in comparison to rats in the both of untreated and naked siTNF-alpha intranasal administration group. These results indicate that the combining nose-to-brain delivery with MPEG-PCL-Tat micelles improved the therapeutic effects by siRNA for the cerebral ischemia-reperfusion injuries, suggesting that our nose-to-brain siRNA delivery system has a potential to become an effective siRNA therapeutic method for the various CNS disorders.

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Investigating the biodistribution of MTL-CEBPA reveals delivery of small activating RNA into CD34+ cells and different types of immune cells *in vivo*

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MTL-CEBPA is currently in Phase Ib clinical trials in patients with advanced hepatocellular carcinoma (HCC) (Clinical trial information: NCT02716012). MTL-CEBPA comprises a small activating RNA (saRNA) encapsulated inside a SMARTICLES® liposomal nanoparticle. The saRNA is an RNA duplex designed to specifically up-regulate endogenous CCAAT/Enhancer-Binding Protein alpha (CEBPA) via an AGO-mediated transcriptional activation mechanism. CEBPA is a leucine zipper protein which acts as a master regulator of liver homeostasis and multiple oncogenic processes including cell cycle control, proliferation and angiogenesis. CEBPA also regulates the characteristics of myeloid cells, influencing the functions of immune cells in blood and tumor microenvironment. We have previously showed that administrations of MTL-CEBPA in patients increased the CEBPA mRNA expression in white blood cells. Our preclinical studies also demonstrated that MTL-CEBPA modulated the properties of immune cells in the tumor microenvironment in syngeneic mouse tumour models, improved liver function and suppressed tumor growth in a rat hepatocellular carcinoma model.

Given that MTL-CEBPA can act on multiple tissues and cell types *in vivo*, this study aimed to extend our current knowledge on the biodistribution of MTL-CEBPA. We labelled our saRNA with a Cy3 fluorophore, encapsulated this Cy3-CEBPA within SMARTICLES® (MTL-Cy3-CEBPA). Following injections of MTL-Cy3-CEBPA in Wister rats, we observed uptake of the Cy3-CEBPA in different populations of immune cells in blood, bone marrow and spleen. Monocytes mediated a striking cargo uptake and CD34+ and dendritic cells in bone marrow also demonstrated a significant internalisation of the Cy3-CEBPA cargo. While macrophages in spleen had significant MTL-Cy3-CEBPA uptake, no uptake of MTL-Cy3-CEBPA was seen in lymphocytes. To conclude, MTL-Cy3-CEBPA can deliver saRNA to CD34+ cells and some myeloid cell populations but not lymphocytes. These data indicate that MTL-CEBPA is able to exert a modulatory influence on the immune system through alteration of CD34+ and myeloid cell activities.

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Fate of DNA Nanostructures in Biological Conditions

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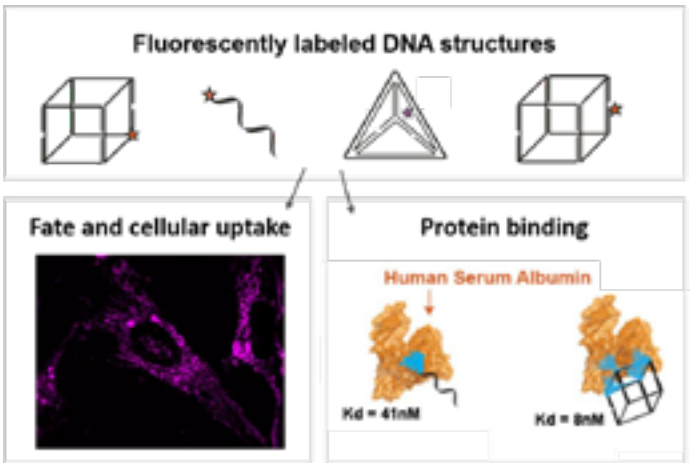
DNA-minimal nanostructures have been of great interest for biological applications such as bio-sensing or drug delivery, because of their low cost, high yielding assembly and retained functionality.[1] Yet, their behavior biological conditions remain ambiguous.

First, we will discuss the cellular uptake of DNA structures and DNA oligonucleotides in mammalian cancer cells.[2] Fluorescent dye labeling is one of the most used techniques to track their fate and cellular localization. Most studies have relied on attaching cyanine dyes (positively charged) to nanostructures, such as Cy3- and Cy5-. Yet, these dyes can direct the uptake of DNA strands themselves and interact strongly with mitochondria. We report that intracellular fluorescence, and even FRET signals, cannot be correlated with the cellular uptake of intact DNA structures. Live cell imaging revealed high colocalization of cyanine-labeled DNA oligos and nanostructures with phosphorylated small-molecule cyanine dyes, one of the degradation products from these DNA compounds. Nuclease degradation of the strands outside and inside the cell result in a misleading intracellular fluorescent signal.

Then, we will discuss strategies to tune DNA structures to improve their stability, cellular uptake and biodistribution *in vivo*.[3] We describe a strategy to engineer DNA structures with strong binding affinity to human serum albumin (HSA), the most abundant protein in the blood. HSA can hinder phagocytosis, is retained in tumors, and aids in cellular penetration. Conjugating dendritic alkyl chains to DNA creates amphiphiles that exhibit high-affinity binding to HSA. We also show that, in a site-specific manner, altering the number and orientation of the amphiphilic ligand on a DNA nanocube can modulate the affinity of the DNA cage to HSA. Complexation with HSA did not hinder the activity of silencing oligonucleotides inside cells, and the degradation of DNA strands in serum was slowed. We will also present preliminary results on cellular uptake and biodistribution in mouse models.



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Optimizing the guide RNA chemistry for the SNAP-ADAR system

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A-to-I-RNA editing is a very valuable approach for targeted non-synonymous and synonymous alteration of mRNA sequences. The SNAP-ADAR system is very powerful for targeted deamination in cell culture experiments, showing formidable efficiency and accuracy in targeting transcripts of endogenous genes. For *in vivo* application, further optimization is necessary. Therefore, we tested various modifications of the guide RNA, including backbone modifications, e.g. phosphorothioate, for stability in serum and the impact on the editing capacity of the ADAR isoforms. In addition, we explore the use of the SNAP-tag technology to recruit other base editors for expanding our repertoire of techniques.

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Discovery of anti-miR-17 oligonucleotide RGLS4326 for the treatment of autosomal dominant polycystic kidney disease

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Autosomal dominant polycystic kidney disease (ADPKD), caused by mutations in either *PKD1* or *PKD*2 genes, is among the most common human monogenetic disorders and the leading genetic cause of end-stage renal disease (ESRD). ADPKD is characterized by slowly progressive, bilateral enlargement of the fluid-filled polycystic kidneys, where increase in kidney volume ultimately causes ESRD in approximately 50% of ADPKD patients by age 60. Unfortunately, treatment options for ADPKD are limited.

MicroRNAs (miRs) are a class of non-coding RNA that play central roles in cell differentiation, proliferation and survival by binding to complementary target mRNAs, resulting in mRNA translational inhibition and eventual degradation. We have previously demonstrated that miR-17 is a promising drug target for treating ADPKD. Here we describe the discovery and preclinical evaluation of RGLS4326, a single-stranded, chemically-modified oligonucleotide designed to preferentially target the kidney and inhibit the pathologic functions of the miR-17 family of miRNAs in ADPKD.

RGLS4326 was discovered through screening a chemically-diverse library of anti-miR-17 oligonucleotides for optimal pharmaceutical properties. In preclinical studies, RGLS4326 potently inhibited miR-17 activity, displaced miR-17 from the translationally active high molecular weight polysomes, and de-repressed multiple miR-17 target genes. Importantly, RGLS4326 treatment reduced cyst growth in primary human ADPKD 3D-cyst cultures, and conferred efficacy in multiple mouse models of PKD following subcutaneous administrations. Taken together, our preclinical data support the clinical development of RGLS4326 for the treatment of ADPKD.

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Using lentivirally-expressed U7 snRNAs to screen for splicing modulating antisense oligonucleotides in cystic fibrosis organoids

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Cystic fibrosis (CF) is the most common monogenetic disorder in the Caucasian population, and is caused by mutations in the *cystic fibrosis transmembrane conductance regulator (CFTR)* gene. Approximately 12% of the reported CF-causing mutations are splicing mutations. These mutations generate aberrantly spliced pre-mRNA, leading to the production of incorrect mRNA transcripts and synthesis of non-functional CFTR.

Although splicing mutations compromise a significant group of all known CFTR mutations, therapeutic strategies targeting this class of mutations remain largely unexplored. Antisense oligonucleotides (AON) can sterically occupy aberrant splice sites and/or regulatory splicing elements, thereby restoring correct pre-mRNA splicing. This straightforward splicing modulation approach is an attractive therapeutic tool and has been successful for other diseases with the first therapies already having entered the market. Currently, AON-mediated splicing interventions do not exist for CF.

We initiated the development of a preclinical subject-specific AON development pipeline using CF patient-derived intestinal organoids cultures harbouring splicing mutations. We propose a novel screening approach using lentivirally expressed U7-small nuclear RNA (snRNA)-based AONs, to identify AON sequences that have the ability to restore aberrant CFTR splicing in patient-derived intestinal organoids. As proof of concept we investigated the splicing ability of this system in intestinal organoids using a validated control virus, expressing the previously published CypA-targeting U7-based AON sequence. Next, we performed an in silico coarse walk to identify possible AON splice modulating sequences along the 3272-26A>G splice mutation. These sequences will be, once cloned into the U7-snRNA-expressing lentiviral plasmids, used to generate AON sequence-specific lentivirus, which will be used for transformation into intestinal organoid. Splicing modulation in these organoids will be assessed by mRNA analysis of splice products and functional measurements using the forskolin-induced swelling (FIS) assay.

Here, we will present our results of this novel approach to identify and test AON sequences for splicing modulation approaches in CF organoids.

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Methodology Considerations for Circular RNA Knockdown

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Circular RNAs (circRNAs) are covalently closed RNA transcripts that are typically circularized from back-splicing events of their linear counterparts. The majority of identified circRNAs are exon-derived and predominantly reside in the cytoplasm. However, circRNAs can retain intron sequences that may be restrict their localization to the nucleus. From studying long non-coding RNAs (lncRNAs), we have found that antisense technology has higher activity in the nucleus while RNAi functions predominantly in the cytoplasm. Thus, the varying localization distribution of circRNAs may impact the optimal tool used in circRNA knockdown studies. Additionally, the different knockdown technologies or chemical modification strategies employed may have different levels of specificy for on-target (circle) vs off-target (linear) knockdown. The potency and specificity profiles of 2'OMe, 2'MOE, and LNA gapmer antisense oligonucleotides (ASOs), or 27mer Dicer-substrate siRNAs and 21mer siRNAs was compared for several circRNAs localized in either the nucleus or the cytoplasm. Site selection for knockdown reagents is confined to a small window surrounding the back-splice junction and shifting the placement of the site to enhance potency often causes undesired knockdown of the linear transcript, even in the presence of multiple mismatches. In examples where the sequence surrounding the back-splice junction was predicted to be suboptimal for designing potent knockdown reagents, we found that the ASOs were more tolerant and more likely to have effective knockdown than the RNAi reagents. However, if the sequence was algorithmically predicted to be a potent site for the RNAi reagents, they outperformed the ASOs. Interestingly, the RNAi reagents also performed well for the exonic/intronic circRNAs that are reported to be mostly retained in the nucleus. With these results, it may be necessary to compare both methods for any given circRNA to ensure the greatest knockdown potency while mitigating off-target knockdown of the linear parent RNA.

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CLiC single-molecule microscopy enables mechanistic insights for improved drug development

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Oligonucleotide interactions are at the root of all biological processes and thus play a major role in human health and disease. A number of therapeutic approaches are being developed to modulate our genes or their RNA products and treat disease at the source, including CRISPR/Cas9, RNAi, ASOs, etc. that rely on oligo-oligo- and oligo-protein interactions. Design of effective therapies hinges on a clear and thorough understanding of these interactions at the *single-molecule level*, such as drug-target reaction mechanisms and rates, and in particular the effects of small sub-populations and the natural heterogeneity of biomolecules.

Here, we describe our use of convex lens-induced confinement (CLiC) microscopy to start to peer into the mechanistic details of therapeutically-relevant oligonucleotide interactions. CLiC traps individual, freely-fluctuating biomolecules for single-molecule imaging in arrays of micro- or nanosized features for statistically relevant data collection. The molecules, and their response to added reagent molecules, can be followed in real time. For example, we uncovered surprising insights into the influence of physiologically-relevant, crowded environments over DNA higher-order structure that was not predicted by theory and that could be significant in the design of in vitro experiments. We are also examining mechanistic details of the CRISPR/Cas9 reaction and of ASO-RNA interactions that could enable design of guide- and drug oligos with improved target recognition for fewer off-target risks.

The detailed insights into drug-target interactions provided by CLiC could lead to more efficient drug research. The improved resolution could enable better predictions of drug success and provide valuable screening early in the drug development process.

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Selection of Aptamers against Chemokine Receptors and Characterization of Their Potential Functions in Signaling

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Chemokine receptors are a family of cell surface receptors that bind to chemokines and are part of the larger seven-transmembrane domain superfamily of G protein-coupled receptors (GPCRs). While normally expressed on the surface of leukocytes to mediate intercellular signaling and cellular trafficking during normal immune responses, these receptors are also aberrantly expressed on cancer cells. Aberrant expression of CXCR4 (C-X-C Chemokine Receptor 4) is an indicator for metastatic potential and poor prognosis for various cancers, including breast, prostate, lung, and pancreas. Likewise, CXCR7, is expressed throughout a similar range of cancers and, like CXCR4, can be induced by chemokine ligand CXCL12. Notably, it has been found that CXCR4 and CXCR7 can form heterodimers that can enhance cell migration *in vitro*, consistent with clinical observations that expression of both CXCR4 and CXCR7 is found in some highly metastatic cancers.

Aptamers are short oligonucleotides with high affinity for their targets; they also possess the ability to transport therapeutic RNA into cells – these functions make them ideal candidates for developing targeted therapy for chemokine receptor-overexpressing cancers. Currently, we are selecting RNA aptamers against CXCR4 and CXCR7 through SELEX (Systemic Evolution of Ligands by Exponential enrichment). In particular, we are selecting for aptamers that will bind with high specificity to: (1) CXCR4, (2) CXCR7 or (3) the CXCR4/CXCR7 heterodimers – these candidates will be characterized for functions using assays for chemokine receptor signaling and the wound-healing assays. We performed several rounds of selection to enrich for aptamers that are specific to CXCR4, CXCR7 or the CXCR4/CXCR7 heterodimers. Throughout the selection process, we performed high throughput sequencing and employed bioinformatics approaches to identify aptamer candidates for affinity testing.

Apart from selecting aptamers with high binding affinity, we are also exploring the functional properties of our aptamers through systematic screening. To the best of our knowledge, there no aptamers are known to functionally modulate the signaling of any chemokine receptor, so we are testing our aptamer candidates with a particular focus on this aspect of their functions. We are using two platforms for this purpose: (1) the PRESTO-Tango assay, for measuring β-arrestin recruitment and (2) the Ca^{2+} Influx assay. We are also using the wound healing and cell migration assays to assess the ability of our aptamers to reduce the invasiveness of cancer cells. With the functional characterizations mentioned above, we hope to broaden the therapeutic potentials of aptamers.

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Morpholino Oligomer-Induced Dystrophin Isoforms: Mapping the Functional Domains in the Distal Third of Dystrophin Gene

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The dystrophin protein plays a crucial role in maintaining sarcolemma stability during muscle contractions, and mutations which prevent the expression of a functional protein result in the progressive muscle wasting disorder, Duchenne muscular dystrophy (DMD). Antisense oligonucleotide-mediated manipulation of pre-messenger RNA splicing to by-pass DMD-causing mutations and restore some functional dystrophin expression has now entered the clinic for the most common type of *DMD* mutations: frameshifting genomic deletions flanking exons 45 (Casimersen, Phase 3 trials), 51 (Eteplirsen, conditional approval) and 53 (Golodirsen: under FDA evaluation). This strategy is based upon the genotype-phenotype correlations observed in the cases of Becker muscular dystrophy (BMD), where in-frame deletions of some of the dystrophin exons, especially in the central rod domain, result in internally truncated but semi-functional dystrophin protein isoforms. However, genomic deletions in the latter third of the dystrophin gene are very rare, and consequently BMD genotype-phenotype correlations cannot be made. Consequently, the amenability of mutations in the latter third of the *DMD* to exon skipping strategies remains unknown. In this study, dystrophin "BMD-like" isoforms were induced *in vivo* in mice after intraperitoneal injection of the peptide-conjugated phosphorodiamidate morpholino oligomers to skip in-frame exon blocks of 56+57 and 58+59. The isoform lacking exons 56+57 appears to be more functional than the isoform without exons 58+59, which was demonstrated by increased dystrophin expression and stabilized β-dystroglycan. Less muscle degeneration, less connective tissue infiltration and more normal looking muscle were observed in mice after the induction of the dystrophin isoform missing exons 56+57, indicating some functionality and therapeutic potential for DMD-causing mutations in these exons.

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Glycine enhances satellite cell proliferation , cell transplantation and oligonucleotide efficacy in dystrophic muscle

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The need to distribute therapy evenly systemically throughout the large muscle volume within the body makes Duchenne muscular dystrophy (DMD) therapy a challenge. Cell and exon-skipping therapies are promising but have limited effects, thus enhancing their therapeutic potency is of paramount importance to improve access in the clinic. Here we demonstrate that co-administered glycine improves phosphorodiamidate morpholino oligomer (PMO) potency in mdx mice with marked functional improvement and up to 50-fold increase of dystrophin in abdominal muscles compared to PMO in saline. Glycine boosts satellite cell proliferation and muscle regeneration by increasing activation of mammalian target of rapamycin complex 1 (mTORC1) and replenishing the one-carbon unit pool. The expanded regenerating myofibre population then results in increased PMO uptakes. Glycine also augments the transplantation efficiency of exogenous satellite cells and primary myoblasts in mdx mice. Our data provide evidence that glycine enhances satellite cell proliferation, cell transplantation and oligonucleotide efficacy in mdx mice, and thus has therapeutic utility for cell therapy and drug delivery in muscle wasting diseases.

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Targeted Delivery of miR-200c Inhibits EMT and Proliferation of Triple Negative Breast Cancer

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Epithelial to mesenchymal transition (EMT) plays a key role in cancer invasion and proliferation. Inhibiting cancer EMT is considered as a promising therapeutic approach for treatment of aggressive tumors. It has been shown that the microRNA miR-200 family has play a critical role in cancer EMT. However, miR-200 has not been investigated as a potential therapy for treating aggressive cancer, including triple negative breast cancer (TNBC). With no FDA approved targeted therapies, patients diagnosed with TNBC present a significantly worse clinical outcome when compared to those diagnosed with other types of breast cancer. Many types of TNBC cancer cells present low expression of epithelial markers, including miR-200, and high expression of the mesenchymal markers. Targeted delivery of miR-200 into TNBC cancer cells has the potential to inhibit EMT and to effectively treat TNBC. In this work, we have investigated the efficacy of targeted delivery of miR-200c, a member miR-200 family, for inhibiting EMT and treating TNBC in tumor models.

We have developed targeted nanoparticles using lipid ECO, a multifunctional pH-sensitive amino lipid developed in our lab, for tumor specific delivery of miR-200c in treating TNBC. The targeted nanoparticles (RGD-PEG-ECO/miR-200c) were formulated by self-assembly of REG-PET-MAL, ECO, and unmodified miR-200c in nuclease free water. We have shown in vitro experiments that the treatment of TNBC cancer cells with RGD-PEG-ECO/miR-200c resulted in significant upregulation of miR-200c and down-regulation of some mesenchymal markers, and consequently decreased cancer cell invasiveness. The treatment also inhibited tumor spheroid formation in 3D culture. Weekly systemic administration RGD-PEG-ECO/miR-200c nanoparticles at a dose of 1.0 mg-miR-200c/kg suppressed TNBC tumor proliferation with no overt side effects in mouse TNBC models. The tumor response has been non-invasively monitored by molecular imaging of a mesenchymal marker. Strong expression of the EMT marker was shown in the tumors before the treatment. Robust downregulation of the EMT marker was shown by molecular imaging after the treatment along with inhibition of tumor proliferation. In contrast, the tumors treated with non-specific miRNA maintained high expression of the EMT marker and fast tumor proliferation. These results have shown that targeted delivery of miR-200c has the potential to effectively inhibit EMT of TNBC cancer cells and prevent proliferation of TNBC tumors. The targeted nanoparticles RGD-PEG-ECO/miR-200c are a promising therapy for effective treatment of TNBC.

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Peptide nucleic acid-based artificial ribonucleases – studies aimed at potentiating PNAzymes for use in therapy

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Selective cleavage of specific RNA sequences is an important goal for nucleic acid chemistry, due to the need for nucleic acid manipulation in molecular biology research as well as for therapeutic intervention. Peptide nucleic acid-based artificial ribonucleases (PNAzymes) composed of a PNA backbone and a covalently linked metal chelate have an intrinsic ability to cleave RNA targets and have been shown to act in a catalytic fashion as artificial ribonucleases. PNA-neocuproine conjugates have shown particularly promising RNA cleavage kinetics when the PNA backbone forces the formation of a single-stranded bulge region in the RNA target due to partial complementarity. Cleavage of such RNA bulges can occur site-specifically in the presence of Cu^{2+} ions with half-lives down to 20-30 minutes, and highly site-selectively in the presence of Zn^{2+} ions with 7-8-hour half-lives. Further development of PNAzymes aims to achieve enzyme-like rates of catalytic cleavage preferably in the presence of Zn^{2+} ions which are more biocompatible. Here, we present new results from RNA cleavage with different PNA based artificial nucleases as well as on novel PNA conjugates where neocuproine has been replaced by different metal chelating groups.

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Non-clinical PK/PD assessment of STK-001, a clinical candidate for the treatment of Dravet syndrome

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Stoke Therapeutics is pioneering a new way to treat the underlying cause of severe genetic diseases by precisely upregulating protein expression. We are developing antisense oligonucleotide medicines that increase gene expression to treat genetic epilepsies and other severe monogenic diseases. STK-001 is an ASO that utilizes our TANGO platform and is being developed for the treatment of Dravet syndrome (DS). DS is a severe epileptic encephalopathy which is primarily caused by spontaneous, heterozygous loss of function mutations in the SCN1A gene that encodes the alpha subunit of the voltage-gated sodium ion channel $Na_V1.1$. STK-001 is designed to bind to the SCN1A pre-mRNA and redirect RNA splicing to decrease the amount of non-productive messenger RNA (mRNA) and increase the level of productive SCN1A mRNA, thus increasing the expression of the sodium channel $Na_V1.1$ protein. The targeted RNA splicing event in SCN1A is highly conserved across multiple species, including mouse, rat, non-human primates, and humans. The target sequence for STK-001 is also identical across species, facilitating cross-species assessment of PK/PD relationships in the CNS. Target engagement, pharmacology and biodistribution studies with STK-001 were performed in mice and rats.
Treatment with STK-001 using intrathecal administration (IT) in adult rats and intracerebroventricular (ICV) administration in neonate Dravet mice resulted in dose-dependent increases in $Na_V1.1$ protein in brain. Results from these studies will be presented.

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New probe design for covalent capture of miRNA targets in cells

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MicroRNAs (miRNAs) constitute a class of small, endogenous, noncoding RNAs (ncRNAs) and have a great influence on various processes within the cell. By base-pairing to their specific, partially-complementary sites located predominantly in the 3' untranslated region (3'-UTR) of the target messenger RNAs (mRNAs), miRNAs participate in the post-transcriptional regulation of gene expression. MiRNAs are responsible for controlling the expression of the majority of human protein-coding genes and their dysregulation has been related to many pathological processes and diseases.
MiRNAs can be considered as either therapeutic agents or therapeutic targets. In order to use miRNAs as therapeutic, in-depth understanding of their mechanisms of action is of great importance. One of the key challenges is the elucidation of miRNAs' targets, together with their sites of canonical and non-canonical interactions. Imperfect pairing between miRNAs and their target mRNAs in animals, as well as high false positive and false negative rates for current prediction algorithms, generate a need for experimental confirmation.
For this purpose, miRNA analogues bearing various cross-linkers can be applied. In the presence of cross-linkers a covalent bond between the miRNA and the mRNA target is formed, enabling elucidation and/or validation of the target site. The microRNA cross-linking and immunoprecipitation (miR-CLIP) approach developed in our group allows capturing predicted and unpredicted miRNA targets in cells employing pre-miRNAs site-specifically modified with biotin and trioxsalen (a psoralen derivative). We have previously shown that the bis-modified miR-106a probe is able to cross-link to complementary regions present in the mRNA targets, but the exact site of cross-linking has not been determined. In order to design a universal set of miR-CLIP probes, more knowledge about the course of the chemical transformations is required.
Herein, we present the results of the *in vitro* cross-linking assay with a set of synthesized trioxsalen-labelled miR-106a analogues. The results suggest that the cross-linking does not occur with a uridine base-paired with adenosine bearing trioxsalen moiety, as it was originally envisioned. This information is currently used to prepare optimized miR-CLIP probes for 3p and 5p miRNAs, with the ultimate goal to achieve effective miRNA-mRNA cross-linking independently of the miRNA sequence.
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Inhibition of IgE production upon treatment with antisense oligonucleotides targeting the secreted polyadenylation signal (sec-PAS)

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Immunoglobulins (Ig) are expressed either on the surface of B cells or as secreted antibodies by plasma cells that represent the final stage of B cell differentiation. In order to do so, different polyadenylation signals across the constant region of Ig heavy (IgH) chains are used: the membrane Ig polyadenylation signal (m-PAS) and the secreted Ig polyadenylation signal (sec-PAS).
Using an antisense oligonucleotide (ASO) hybridizing to the polyadenylation signal sequence of the transcript encoding the secreted form of IgE, we were able to induce significant inhibition of IgE. As a proof of concept, we found that passive administration of vivo-morpholino oligos (Gene Tools) targeting the IgE sec-PAS sequence induced a drastic decrease in IgE production in U266 cells (IgE-secreting human myeloma cell line). The particular configuration of the IGH locus makes it possible to mask the sec-PAS of a particular Ig subclass while promoting the use of an alternative polyadenylation signal encoding the membrane form of the corresponding Ig. In the case of IgE, a previous study demonstrated that membrane IgE expression has a pro-apoptotic effect in B cells (Laffleur et al, Cell Reports 2015). Consistent with this, marked apoptosis was observed in U266 cells treated with the ASO-IgE-sec-PAS, with only 20% viable cells after 48h treatment compared to ~70% with irrelevant ASO control. Next, we validated the efficacy of this antisense strategy in primary cells by using a mouse model expressing humanized IgE. Again, we observed a drastic decrease in IgE-secreting plasma cells while expression of the membrane IgE isoform was increased. The overproduction of allergen-specific secreted IgE is one of the established features of many forms of allergies including chronic allergic asthma or some food or skin allergies (Platts-Mills et al J Allergy Clin Immunol 2016). We also demonstrated that the targeting of sec-PAS specifically inhibited allergen-specific IgE production using several hybridomas expressing penicillin-, ovalbumin- and wasp venom-specific IgE (Marchalot et al, in preparation). Experiments are ongoing to provoke an isotype-specific inhibition of antibody production using ASO targeting the membrane or secreted poly-A signals of several Ig subclasses (i.e. IgM, IgG or IgA). Overall, these new antisense approaches targeting selectively each Ig isotype (patent EP19305716.3) could either inhibit B-cell receptor (BCR) expression and signaling (m-PAS) or decrease Ig secretion by plasma cells (sec-PAS), and hence, they should have broad clinical applications in BCR-driven lymphomas and antibody-mediated pathologies.

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A novel splicing regulatory element in *PAH* intron 11 and potential antisense based therapy for exon 11 skipping variants in phenylketonuria

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In the phenylalanine hydroxylase (PAH) gene, responsible for phenylketonuria (PKU), one of the most common inherited diseases of amino acid metabolism, exon 11 was recently identified as vulnerable due to a weak 3' splice site. Different exonic mutations affected exon 11 splicing by altering exonic splicing regulatory elements. The use of splice-switching oligonucleotides (SSOs) therapy targeting splice regulatory sites has allowed to successfully rescue aberrant splicing in a wide variety of diseases. We recently identified novel intron 11 regulatory elements that are involved in exon 11 definition, as revealed by the investigated pathogenic effect of variants c.1199+17G>A and c.1199+20G>C, identified in PKU patients. Both mutations cause exon 11 skipping in a minigene system. RNA binding assays showed strong binding of the U1-70K protein to the wt form that is lost with +17G>A and +20G>C variants. We performed deletion/point mutagenesis and overexpression of adapted U1snRNA to identify critical motifs involved in the regulation of correct splicing at the wt 5' splice site. The results indicate that U1snRNP binding at the intronic region is determinant for efficient exon 11 splicing. We also observed an increase of hnRNP H and hnRNP A1 binding in the variant +17G>A and +20G>C sequences. Surprisingly, the use of an SSO that targets the intronic sequence where both mutations are located results in recovery of correct splicing with exon 11 inclusion from the mutant pre-mRNAs. This suggests that binding of U1 to the cryptic splice site stimulates splicing by counteracting inhibitory binding of hnRNPH and hnRNPA1 to an intronic splicing silencer (ISS) in this region. We speculate that the SSO stimulates splicing by blocking binding of hnRNP H and hnRNP A1 to an ISS, thereby alleviating the need for U1 binding to the cryptic splice site. The same SSO also reverted the splicing defect caused by the disease-causing c.1144T>C exon 11 mutation, previously demonstrated to cause exon 11 skipping.
Our results provide insights into the mechanisms of PAH exon 11 recognition, pointing to the role of a critical ISS in intron 11 and demonstrate the potential of an SSO-based therapy targeting this ISS, to rescue exon 11 skipping defects induced by different exonic and intronic pathogenic variants.

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THE USE OF A MODIFIED U1 snRNA AS A THERAPEUTIC STRATEGY TO CORRECT A 5' SPLICE-SITE MUTATION IN MUCOPOLYSACCHARIDOSIS IIIC: *IN VITRO* STEPS TOWARDS AN *IN VIVO* APPROACH

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Genetic therapy directed towards the correction of RNA missplicing is being investigated not only at basic research level but even in late-stage clinical trials. Many mutations that change the normal splicing pattern and lead to aberrant mRNA production have been identified in Lysosomal Storage Disorders (LSDs). The Mucopolysaccharidosis IIIC (MPS IIIC) is a LSD caused by mutations in the *HGSNAT* gene, encoding an enzyme involved in heparan sulphate degradation. Splicing mutations represent one of the most frequent (~20%) genetic defects in MPS IIIC. Approximately 55% corresponds to 5' splice-site mutations which thus constitute a good target for mutation specific therapeutic approaches. Recently, we demonstrated in fibroblast cells that a modified U1snRNA vector designed to improve the definition of exon 2 5'ss of the *HGSNAT* can restore splicing impaired by the mutation c.234+1G>A (Matos *et al.*, 2014). Presently our goal is to evaluate *in vivo* the therapeutic potential of the modified U1snRNA by testing it in mice expressing the human splicing defect. For this, in a first step we tried to generate full-length splicing competent constructs of wild-type (wt) and c.234+1G>A *HGSNAT* by cloning the wt or the mutated *HGSNAT* splicing-competent cassettes into the pcDNA 3.1 backbone. According to the protocol reported by other researchers (Pinotti *et al.*, 2009), plasmid vectors will be used to promote transient expression of the human *HGSNAT* wt or mutant alleles in mice.
Here, we describe the cloning process followed to obtain the aforementioned splicing constructs. During the cloning steps different difficulties were found as, for example, in fragments amplification, ligation, and obtainment of bacterial transformants. Even so, positive bacterial colonies were obtained, selected, and amplified by colony PCR. However, DNA sequencing data showed the presence of different nucleotide point alterations in the obtained clones, invalidating its use for further steps. Therefore, plasmid constructs were ordered commercially. Now we are performing its transfection in COS-7 cells to confirm that they recapitulate the splicing process observed in wt and patient cDNA being thus ready to be expressed in mice to test the therapeutic effect of the modified U1snRNA.
This work shows the different steps and difficulties of the cloning process to obtain *HGSNAT* expression constructs towards testing of an in vivo U1snRNA therapeutic approach.

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Aptamers and DNAzymes for applications in human health

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Seminal work done by Thomas Cech and Sidney Altman, on the functional properties of RNA, earned them a Nobel Prize in Chemistry in 1989. This work inspired the field of functional nucleic acids, which has since found applications in oligonucleotide-based therapeutics. Aptamers and DNAzymes are two subclasses of functional nucleic acids. Where aptamers recognize and bind to a specific cognate target with high affinity and selectivity, DNAzymes exhibit catalytic properties activated by a specific target molecule, or cofactor. Researchers in the functional nucleic acids community have investigated the use of aptamers and DNAzymes in several brain and nervous system related maladies including but not limited to Alzheimer's disease, Parkinson's Disease, Huntington's disease, prion disease, multiple sclerosis, stroke and hemorrhage, and brain cancers. The work presented will highlight several examples of aptamers and DNAzymes for therapeutic and/or diagnostic applications related to the brain and human health. Specifically, the role of a dopamine binding aptamer in attenuating behavior, in rodent models of schizophrenia and addiction will be presented, and progress towards aptamers which influence protein aggregation in rodent models of Parkinson's disease will be discussed in detail.

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Nucleobase-modified XNA for Aptamer Selection

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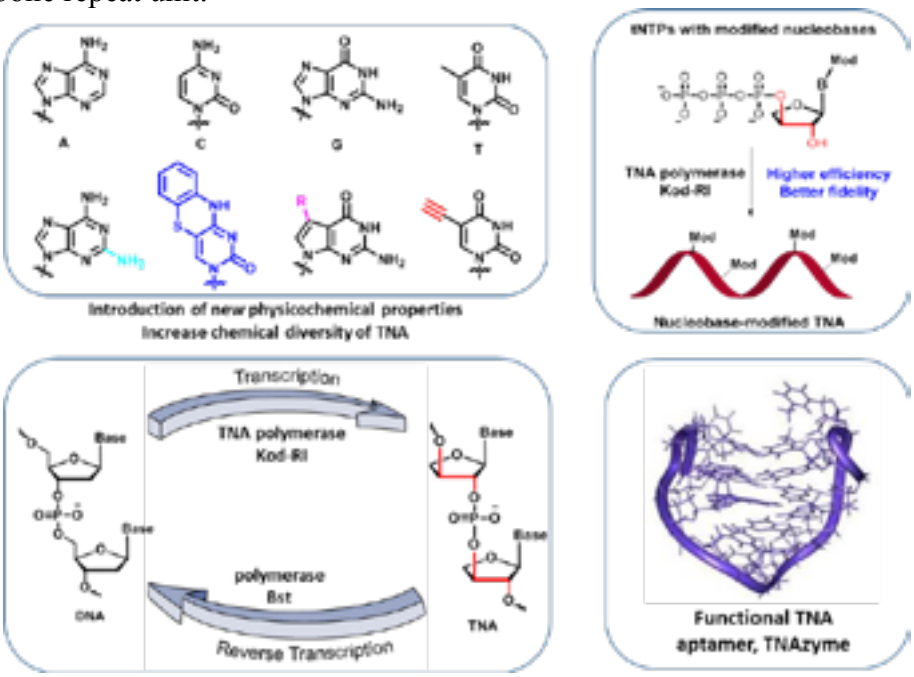
Xeno-nucleic acids (XNA), usually referred to nucleic acids with modified sugar backbones, are capable of undergoing Darwinian evolution to generate aptamers towards specific targets. There exist two main reasons for developing XNA polymers with modified bases: first, to overcome the inadequacies of engineered polymerases relative to natural ones for XNA synthesis; second, to endow XNA polymers with increased chemical diversity beyond canonical nucleobases. We are particularly interested in the development of nuclease-resistant aptamers based on α-threose nucleic acid (TNA) - a particular XNA with a shorter sugar backbone repeat unit.



We chemically synthesized many nucleobase-modified TNA triphosphates and studied the enzymatic replication of TNA polymers containing such modifications. All modified triphosphates are excellent substrates for engineered TNA polymerase Kod-RI. Particularly, substitution of tGTP for 7-deaza-modified-tGTP enabled the replication of TNA polymers with >99% overall fidelity. A TNA library containing the 7-deaza-modifications was used to evolve a biologically stable TNA aptamer that binds to HIV-RT with 10 nM affinity. We feel these modified bases could lead to the discovery of new XNA aptamers that function with superior activity relative to analogous polymers with unmodified bases.

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Optimizing RESTORE ASOs to Harness Endogenous ADARs for Therapy

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Site-directed RNA editing could be a safer alternative to genome editing. We recently published our RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing) approach. While there have been developed several strategies for site-directed RNA editing using ectopically expressed, engineered deaminase fusion proteins, RESTORE only requires an ASO guide RNA. Those ASOs are able to recruit endogenous human ADARs to elicit editing of arbitrary endogenous transcripts in a simple and programmable way. However, all artificial systems are complicated and all suffer from global off-target editing. In contrast, RESTORE ASOs displayed only very few off-target sites.

We now further optimized our RESTORE platform to achieve higher editing yields with shorter ASOs and without the use of interferon. We could not only successfully apply RESTORE in a panel of standard cell lines and primary cells, but also for several disease relevant mutations. Our optimized RESTORE 2.0 ASOs are not only shorter but exhibit enhanced stability in serum and cerebrospinal fluid. RESTORE thus represents an attractive platform for drug development.

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Identification of potent and specific antisense oligonucleotides for target gene knockdown using a high-throughput RT-qPCR based cellular screening platform

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While antisense oligonucleotides (ASOs) are in principle capable of efficiently silencing gene expression, *in silico* prediction of the potency of ASO sequences is challenging. Identifying potent ASOs often requires an *in vitro* evaluation of many candidates that typically tile the target RNA of interest. To enable such analyses, we have developed a comprehensive and high-throughput platform to screen hundreds of ASOs for on-target knock-down efficiency. We established a panel of 20 model cell lines that have high free-uptake capacity and cover the majority of the human transcriptome. For each cell line, we established optimal seeding densities to maximize ASO delivery efficiency. An appropriate screening cell line with high expression of the target gene is selected based on available RNA-sequencing data. Candidate ASOs are delivered to 96-well culture plates in a single dose and target gene expression is quantified by RT-qPCR on crude cell lysates using 5 independent assays and multi-gene normalization. These data typically reveal one or multiple hotspot regions in the target gene that are highly accessible for ASO mediated knock-down. Based on these regions, additional ASOs can be designed for secondary screens in order to further expand the set of potent ASOs. Hits identified from these screens are further validated in dose response experiments to narrow down the list of candidates. Candidate ASOs are subsequently annotated based on their off-target gene potential through a proprietary *in silico* prediction pipeline considering the number of mismatches and their position in the ASO sequence. As a result, potent and specific ASOs can be identified for any target gene of interest. To demonstrate the robustness and potential applications of our workflow, we have screened over 10,000 ASOs with different chemistries targeting 250 genes in multiple cell lines. Our data reveal differences in the potency of ASO chemistries, varying targetability across genes, and differences in target gene knock-down among qPCR assays that need to be considered when screening for potent ASOs.

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Investigation of Factors Potentially Modulating the Activity of Antisense Oligonucleotides Based on in House Screening Data

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Oligofyer™ an inhouse implemented pipeline is used for the design of antisense oligonucleotides (ASO) as potential drug candidates. The selection process is mainly based on the sequence specificity and for the development of ASOs the public databases from NCBI and ENSEMBL were crawled. Besides human mRNA and all associated intronic sequences, mice, rat and ape genomes were investigated was well to characterize each possible ASO according to its off-target profile and its potential cross-reactivity to other species. In order to improve our selection process regarding the activity of the ASOs we gathered all screening data from previous projects and investigated a series of distinct features. The aim was to see if there are factors or a certain combination of them that could lead to a prediction of activity.

At first the position of the ASO within the target sequence was investigated. That means whether ASOs binding to exonic / intronic regions, 5' / 3' UTR, or coding sequence show differences in activity. Additionally, we explored the effect of overall length of the target sequence (mRNA / pre-mRNA) on targetability.

Multiple ASO related properties were investigated, as well. Only ASOs containing locked nucleic acids (LNAs) in the gapmer format were considered for this study. Factors such as the length of the ASO, the length of the gap, the total amount of LNA modifications, the fraction of LNA-modified versus unmodified nucleotides, the overall GC content as well as the GC content within the gap and within the flanks were included in the analysis. Moreover, data correlating activity of ASOs between cell types within or between species will be shown. The results of these analyses are integral part of Oligofyer™ and are used to improve the selection process of ASOs with high potency and specificity.

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Defined Multimeric Oligonucleotides for Enhanced Therapeutic Effect.

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Modulating gene expression using therapeutic oligonucleotides has the potential to improve disease treatment and *in vivo* studies of complex biological processes. However, it remains difficult to deliver oligonucleotides in quantities sufficient to achieve a desired biological result into a particular cell or tissue type.

To address this issue, we have developed defined "multimeric" oligonucleotides, where a defined number of oligonucleotides (e.g., 3 or more siRNAs) are conjugated to a single targeting ligand.

The individual units can be synthesized using the normal procedures and then assembled into multimers via a novel thiol/maleimide intermediate and/or asymmetric annealing under neutral aqueous conditions at room temperature in high yield and purity. The linkages employed are sufficiently stable in serum to enable prolongued bioavailability but are readily cleaved after entry into the target cell.

The resulting multimers exhibit the same activity per unit of oligonucleotide, but enhanced activity per unit of ligand as the latter is enabling the uptake of multiple cargos by the target cell per ligand/binding event.

Further, large multimers are big enough to resist clearance via the kidney and in consequence have a greatly increased serum half-life, in the case of hexamers by a factor of 20 or more relative to the corresponding monomer. Such large multimers are therefore even more potent, not only delivering more cargo per ligand/receptor binding event but enabling many more such events, resulting in a synergistic enhancement in activity such that a single siRNA unit in a hexamer delivered via IV exceeded the reported activity of the same siRNA in monomeric form delivered via SC.

Here we present data obtained using a triantennary GalNAc ligand and three siRNAs targeting FVII, ApoB and TTR as a model system. All the methods described are compatible with a wide range of targeting ligands such as peptides, carbohydrates and aptamers, and can be used to deliver any combination of siRNAs, shRNAs, micro-RNAs, and ASOs.

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Enhancement of cancer vaccine by modification of antigenicity for cancer cells

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Introduction: In cancer immunotherapy, cytotoxic T cells (CTLs) induced by injection of cancer vaccine attack cancer cells. The CTLs attack cells with a cancer antigen on the surface. However, to escape from immunity, cancer cells spontaneously suppress the expression of cancer antigen that is essential for CTL recognition. For such cancer cells, CTL cannot attack. Therefore, if the cancer antigens are presented on the cellular surface, CTLs can attack the cancer cells again. However, in order to enable antigen presentation *in vivo*, it is necessary to deliver antigen specifically to cancer cells. It is known that CD44 which is one of hyaluronic acid (HA) receptors is excessively expressed on cancer cells. Therefore, HA chemically modified with the antigen is expected to specifically deliver the antigen to cancer cells via CD44. In this study, to restore the CTL response for cancer cells, we prepared the conjugate consisting of HA and antigenic proteins and evaluate the CTL response.

Methods: A carboxylic group on HA was modified with OVA by a dehydration condensation reaction using EDC and NHS. We evaluated the immune responses after mixing of splenocytes immunized with OVA and cancer cells treated with HA-OVA conjugates.

Results: HA, a linear polysaccharide, was formed by alternating D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) units. Because the glucuronic acid has a carboxylic acid, we tried chemical modification with ovalbumin (OVA) by a dehydration condensation reaction. The condensing agent used this time is 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and the additive was N-hydroxysuccinimide (NHS). The reaction was evaluated by UV measurement of gel permeation chromatography (GPC). The results are shown in Figure1. Since HA is no UV absorption and OVA has absorption at 280 nm, the reaction can be judged from the change in elution time of OVA. The peak for OVA is observed in 18 minute. After the reaction, the peak shifted to an earlier elution time around 14 minutes. Therefore, OVA increased the molecular weight by conjugating with HA. The splenocytes, which are immunized with OVA and adjuvant, mixed with cancer cells treated with HA-OVA conjugate secreted cytokines such as IFN-γ which shows Th1 type immune response. This result indicates that the replacement of weak antigenicity with strong antigenicity can be a novel strategy for cancer vaccine.

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Antisense Oligonucleotide-Associated Lysosomal Alterations

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Neuronal micro- and macro-vesicular vacuolation has been observed in a dose-dependent manner by histologic examination in non-human primates following repeat intrathecal administration of antisense oligonucleotides (ASOs). Immunostaining with anti-GFAP, -connexin 43, -IBA-1, and -MBP antibodies indicates there is no glial reaction to vacuolation or degenerative changes in vacuolated neurons. Closer examination with electron microscopy (EM) reveals numerous, and sometimes dilated, lysosomes containing stacked and/or reticulated membranous material.

A cellular model using induced pluripotent stem cell-derived motor neurons (iPSC-MNs) was established to investigate these lysosomal alterations and electron microscopy confirmed the dilated lysosome phenotype in a time- and dose-dependent manner at concentrations relevant to brain exposures achieved in *in vivo* studies. ASO exposure (up to 30 uM for up to 3 weeks) did not affect iPSC-MN viability as assessed by intracellular ATP levels. Our work aims to characterize the effects on the endolysosomal pathway after ASO administration and specifically to understand the mechanism of these lysosomal alterations. To this end we used liquid chromatography-mass spectrometry (LC-MS) as an unbiased approach to detect changes in global lipid composition. Briefly, iPSC-MNs were treated with ASOs for a prolonged period of up to three weeks, lysosomes were isolated by differential centrifugation, and LC-MS was performed to identify changes in lipid composition. Initial results show increases in bis(monoacylglycerol)phosphate, galactosylsphingosine, and triglycerides in lysosomes of iPSC-MNs treated with ASOs; however, it is unclear if these increases are simply due to the increase of lysosomal content observed upon ASO administration. Additional biochemical assays and confocal microscopy are being performed to investigate this question and asses overall cellular and lysosomal function.

Altogether, this work advances our understanding of the cellular consequences following prolonged ASO administration and may guide further studies to continue to characterize these effects.

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miR-29 Replacement as a Therapy for Multiple Fibrotic Indications

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miRNAs are small, non-coding RNAs that act as negative regulators of gene expression. The miR-29 family (miR-29a/b/c) targets multiple extracellular matrix proteins and profibrotic molecules, and is down-regulated during fibrotic disease, including scleroderma, idiopathic pulmonary fibrosis, and hepatic fibrosis in humans. A synthetic oligonucleotide mimic of miR-29b, remlarsen, has been previously shown to be pharmacodynamically active as measured by both target gene regulation and inhibition of fibrosis in rodent models and in skin wounds in a Phase 1 human clinical trial.

To assess the anti-fibrotic effects in corneal fibrosis, remlarsen was administered topically via eye drops in the setting of an alkali burn. Remlarsen was well-tolerated when administered twice daily for up to 28 days and resulted in a decrease in corneal hazing and scarring as assessed by clinical evaluation and histopathology following burn. Notably, remlarsen increased the corneal epithelial thickness, decreased the stromal thickness, and reduced the number of alpha-smooth muscle actin positive myofibroblasts in the corneal stroma, supporting miR-29 replacement as a therapeutic strategy to inhibit corneal fibrosis and scarring following injury or ulceration.

For internal organ fibrosis, an integrated approach was performed to optimize and develop next generation miR-29 mimics with targeted delivery capability and improved systemic stability. Lead compounds were advanced into mouse bleomycin or carbon tetrachloride (CCl_4) models to assess the anti-fibrotic effects of next-gen miR-29 mimics in pulmonary and hepatic fibrosis, respectively. Next-gen miR-29 mimics retained miRNA activity in Normal Human Lung Fibroblasts in vitro as determined by assessing regulation of direct targets (*COL1A1*), downstream targets (*ACTA2*), and a broader array of fibrotic genes. Further, next-gen miR-29 mimics blocked collagen production in a human precision cut lung slice model of induced fibrosis. In bleomycin-induced fibrosis in mice, next-gen miR-29 mimics dose-dependently blunted the induction of myriad pro-fibrotic genes that correlated to less collagen deposition as measured by quantitative histopathological analyses. Similar anti-fibrotic effects were observed in the CCl_4 model of hepatic fibrosis, where pro-fibrotic gene expression was blunted resulting in reduced collagen content.

Collectively, these data demonstrate the anti-fibrotic effect of miR-29 mimics across numerous fibrotic models and suggest broad application of miR-29 mimics for fibrotic indications.

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Knockdown of Host Factors by PPMO Inhibits Influenza Virus Infections in Vitro and in Vivo

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There is an *urgent unmet medical need* for new antivirals for treatment of influenza virus-infected patients. Annual influenza epidemics affect as many as 5 million people with up to 646,000 deaths worldwide. Host-directed antivirals are expected to provide a number of critical advantages over current drugs that target virally-encoded gene products. Specifically, knockdown of host factors can impede viral infections without rapid development of antiviral resistance. Host factors are genetically stable in comparison to viral genes, so this strategy is not expected to produce any obvious escape mechanisms. Additionally, targeting a cellular pathway that regulates replication or pathogenesis of multiple viruses offers the opportunity to develop a single antiviral agent that is useful against both known and emerging viruses.

Our strategy is to utilize peptide-conjugated morpholino oligomers (PPMO) for knocking down levels of host proteins that are critical for influenza A infection and replication. PPMO is a class of DNA-like cell-permeable antisense agents currently in clinical development for genetic diseases. TMPRSS2 is a cellular protease known to be important for influenza virus infectivity, while UBR4, a ubiquitin ligase, is thought to be important for replication. We have demonstrated that a PPMO targeted to TMPRSS2 was effective at inhibiting the cleavage of hemagglutinin of several influenza A viruses known to infect humans. Treatment of a Calu-3 airway epithelial cell line with the TMPRSS2-PPMO markedly reduced the growth and spread of H1N1 and H3N2 viruses at concentrations that were not cytotoxic to uninfected cells. In influenza-infected mice, knockdown of UBR4 by a UBR4-PPMO, administered intranasally, reduced virus growth in the lungs and increased overall survivorship of mice.

These studies further establish TMPRSS2 and UBR4 as important to viral replication and suggest these genes may serve as productive targets for development of PPMO-based antivirals against influenza virus infections.

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HIV-1 envelope T20-encoding Minicircle DNA elicits neutralizing antibodies and the potential use of Triplex Forming Oligonucleotides to produce high purity DNA vaccines against HIV-1

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Minicircles (MCs) are a class of non-viral vectors produced from a parental conventional plasmid. MC production occurs in bacteria by DNA recombination through sites flanking the expression cassette in the parental plasmid. The result of this recombination is a MC containing the gene of interest and the plasmid backbone (PB) containing bacterial elements. The T20 peptide was cloned into this novel class of non-viral vectors in order to develop and enhance the immune response against HIV antigen. T20 (enfuvirtide) is the first approved antiviral HIV-1 fusion inhibitor peptide-based drug for treatment of AIDS patients. Moreover, 3 other conventional plasmids expressing the envelope of HIV-1 subtype A, B and C, containing T20 in their gp41 region, were also evaluated (Hum Vaccin Immunother. 13:2849-58, 2017). Selected sequences of T20 both as a peptide and as an expression cassette enhanced HIV-specific antibodies and cellular responses. Thus, the T20 HIV-1 sequence can serve as a potential immunogen to provoke neutralizing antibodies against HIV-1. Further comparative studies will be using Triplex Forming Oligonucleotides (TFOs) to produce high quality MCs expressing T20 peptide. The procedure is based on the recognition of double stranded sequence in the parental plasmid by biotinylated TFOs and removing the formed triplex by streptavidin-coated magnetic beads. The TFOs are designed in a way to ensure high selectivity and affinity toward their target sequences in the parental plasmids, which can be obtained by using different TFO lengths and modifications.

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Development of Light-Inducible Oligonucleotides as Therapeutic Agents

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Photolabile protecting groups (PPGs) or so-called photocages have proven to be excellent tools to study, elucidate and control biological processes. Photolabile protecting groups have been attached to a variety of biologically active molecules like proteins, neurotransmitters, or oligonucleotides to temporarily block/inhibit their function. By absorbing light of a specific wavelength the PPG is cleaved and the function of the formerly photocaged compound is restored. This technique enables the spatio-temporal control of complex biological scenarios with light as a bioorthogonal trigger.
In the context of therapeutic oligonucleotides, local activation or delivery may be necessary to augment the biological function of antisense oligonucleotides (*ASOs*) in the target tissue, to reduce systemic toxicity or unwanted side effects. To that end, we were able to synthesize light-inducible antimiRs with modified oligonucleotides with photolabile protecting groups. We could successfully show the local down-regulation of antiangiogenic miR-92a expression in an *in vivo* setting in murine skin with UV irradiation of 385 nm.
Based on these findings we expanded this concept to other diseases and functional oligonucelotides (e.g. aptamers). For the treatment of acute myocardial infarction (*AMI*) it is necessary to regulate miR-34a expression in the myocardium, which is an well-perfused inlying organ. Therefore, we developed new photocleavable moieties, which can be post-synthetically attached to ASOs (*via CuAAC* click chemistry) and cleaved by irradiation with NIR-light (*near-infrared*). We are currently evaluating the therapeutic potential of those NIR light-inducible antisense oligonucleotides.

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A Phase 1 Study to Investigate the Absorption, Metabolism, and Excretion of [^{14}C]-Golodirsen Following a Single Intravenous Dose Administration in Healthy Male Subjects

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Introduction: Golodirsen is an investigational antisense oligonucleotide for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping. The absorption, metabolism, and excretion (AME) of golodirsen has not been characterized in humans.
Objectives: The primary objectives of this study were to determine mass balance and routes of elimination, assess the PK, and determine the urinary and fecal recovery of total radioactivity following a single dose of [^{14}C]-golodirsen. The secondary objectives were to characterize and identify major metabolites of golodirsen if any and evaluate the safety and tolerability of a single IV dose of [^{14}C]-golodirsen.
Material and Methods: This was a Phase 1, single-center, open-label, single dose, AME study. Eight healthy male subjects (mean age [range]: 36 years [24 to 51]) received a single 1800 mg IV dose of [^{14}C]-golodirsen (containing approximately 100 μCi of radiocarbon) administered via IV infusion on Day 1 (100 mL of solution infused over 60 minutes). Blood samples for plasma golodirsen concentrations and total [^{14}C]-radioactivity in whole blood and plasma were collected up to 216 hours (Day 10) after the start of infusion. Urine and fecal samples were collected pre-infusion and after the start of infusion up to 336 hours (Day 15) to determine golodirsen concentrations (urine only), total [^{14}C]-radioactivity, and/or for metabolite profiling.
Results: The AUC plasma golodirsen/total radioactivity ratio was close to 1, indicating golodirsen was the major drug-related material in systemic circulation. No metabolites were found in plasma and urine from the metabolite profiling characterization. The mean AUC blood to plasma ratio of 0.569 indicates that there is low association of golodirsen-related radioactivity with red blood cells. Urinary excretion was the primary elimination pathway for golodirsen, with essentially all the administered dose excreted in urine as unchanged drug. Over the 336-hour study, the mean total recovery of radioactivity in all excreta (urine and feces) was 89.7%, with urinary excretion accounting for nearly all of this and with only a negligible contribution from fecal excretion. No AEs were reported during this study. There were no clinically relevant findings noted in clinical laboratory evaluations, vital signs, ECGs, or physical examinations.
Conclusions: Golodirsen was eliminated predominantly by renal excretion and was metabolically stable. A single 1800 mg IV dose of [^{14}C]-golodirsen was well tolerated when administered to healthy male subjects in this study.

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Artificial Nucleic Acids Composed of Acyclic Backbone for Fluorescent Probe and Antisense Reagent

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Xeno nucleic acids (XNAs) are synthetic analogues of DNA and RNA carrying scaffolds different from natural D-ribose, which have attracted attention as a new nanomaterial for biological applications due to high nuclease resistance. We have developed two novel acyclic artificial nucleic acids (Fig. 1): *acyclic* D-threoninol nucleic acid (D-*a*TNA) and serinol nucleic acid (SNA). SNA recognized both DNA and RNA through base complementarity, whereas D-*a*TNA did not form stable duplexes with either DNA or RNA. In this study, we designed a molecular beacon composed of SNA (SNA-MB) for the detection of RNA in cell (Fig. 2), and synthesized acyclic L-threoninol nucleic acid (L-*a*TNA) for the strong hybridization with DNA and RNA.

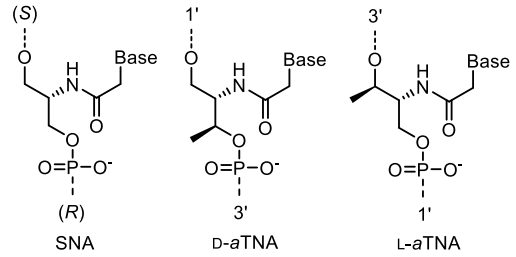

Fig. 1: Structures of artificial nucleic acids

Fig. 2: Schematic illustration of SNA-MB

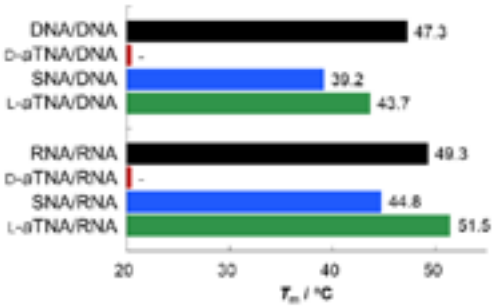

Fig. 3: T_ms of XNA with DNA and RNA

The SNA-MB detected its complementary RNA with extremely high sensitivity; the signal-to-background (S/B) ratio was as high as 930. We demonstrated that this probe selectively visualize target mRNA in fixed cells. Significantly high enzymatic resistance and sensitivity promises the use as a biological tool capable of visualizing RNA in living cells.[1]

The measurement of melting temperatures (T_m) of 15-nt sequences revealed that L-*a*TNA formed more stable duplexes with DNA and RNA than SNA did (Fig. 3). Such cross-pairing ability of L-*a*TNA with DNA and RNA as well as excellent solubility in aqueous solution allows versatile biological applications such as fluorescent probe and antisense drugs.[2]

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Invalidation of GSK3B in the Pancreatic Beta Cell as a Therapeutic Target for T2D

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Insufficient pancreatic β-cell mass or function is one of the main causes underlying diabetes mellitus. Glycogen synthase kinase 3 (*GSK3*) is a ubiquitously expressed serine/threonine kinase with important roles in the regulation of glycogen synthesis, protein synthesis, cell proliferation, cell differentiation and apoptosis. *GSK3* is encoded by two known genes, GSK3 alpha (*GSK3A*) and GSK3 beta (*GSK3B*), and small molecule inhibitors targeting both are known to induce micronucleation. Isoform specific small molecule inhibitors targeting GSK3B have demonstrated increased cell proliferation and expansion of islets mass in human and rodents. However, many of the inhibitors target dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) in addition to GSK3B making the data difficult to interpret (*Shen et al, Nature comms* 2015). GSK3B and/or DYRK1A have been proposed to induce β-cell proliferation by trapping nuclear factor of activated T cells (NFAT) (transcription factor) in the nucleus. Mice with β-cell overexpression of GSK3B have reduced β-cell mass and proliferation through modulation of PDX-1 stability, a protein required for the maintenance and survival of β-cells. Recently, Ämmälä et al. demonstrated conjugation of ASOs to glucagon-like-peptide-1 receptor (GLP1R) ligand can productively deliver ASO cargo to the insulin secreting β-cells, a cell type otherwise refractory to uptake. The doses needed for GLP1-conjugated ASOs to silence target genes in the β-cells are below what is needed to affect target gene expression elsewhere allowing for evaluation of specific inhibition in the β-cells. In lean C57B6 mice a single administration of GLP1-conjugated GSK3B ASO achieved an ED_{50}=*0.016μmol/kg* with no change to DYRK1A RNA expression. Here we demonstrate robust, specific *GSK3B* reduction in the β-cells after GLP1-conjugated GSK3B ASO treatment in *db/db* mice, a well-established T2D diabetic rodent model. In contrast to the SMI data, specific inhibition of *GSK3B* in β-cells worsened the health based on reductions in β-cell specific genes, *PDX1* and *MAFA*, and decreased the β-cell mass (2.9 ± 1.3mg GLP1-Con ASO vs 1.4 ± 0.6mg GLP1-GSK3B ASO). In addition, no effect on glucose or insulin was observed suggesting specific inhibition of *GSK3B* in the β-cell is not enough to preserve the function and increase β-cell survival. In summary, we have invalidated GSK3B as a therapeutic target for protecting the β-cell against glucotoxicity and T2D.

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PAH mRNA Treatment Reduced Plasma Phenyl in Phenylketonuria Mouse Model

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Phenylketonuria (PKU) is an autosomal recessive disorder caused by the deficiency of phenylalanine hydroxylase (gene name: *pah*), which catalyzes the hydroxylation of the aromatic side-chain of phenylalanine to generate tyrosine. Phenylalanine hydroxylase deficiency causes phenylalanine to accumulate in the blood and tissues to toxic levels. The brain is particularly sensitive to excessive phenylalanine levels, which can lead to irreversible intellectual disability, seizures, behavioral abnormalities and microcephaly if untreated. We designed and screened a series of codon-optimized *hpah* mRNAs *in vitro* for highest expression using primary human hepatocytes. In human hepatocytes, our codon-optimized *hpah* mRNA delivers 5-fold higher liver PAH protein levels relative to the endogenous PAH protein. We then formulated this *hpah* mRNA into a lipid nanoparticle delivery system, LUNAR®, in order to evaluate the pharmacology of expressed PAH protein in a mouse model of PKU carrying a point mutation in the *mpah* gene with deficient enzyme activity resulting in elevated plasma phenylalanine concentrations. A single systemic injection of LNP- *hpah* mRNA at 0.3 mg/kg reduced plasma phenylalanine concentrations back to control levels. In the mouse liver, we observed a dose-related correlation between *hpah* mRNA and hPAH protein levels, which also correlated with a reduction of circulating levels of plasma phenylalanine. After 2 weeks of repeated dosing of the LNP- *hpah* mRNA in PKU mice, the transient plasma phenylalanine lowering effect was similar between the first and the last doses. Importantly, there was no adverse events and no significant alteration in serum chemistry, including ALT/AST levels. In summary, delivering PAH mRNA to the liver utilizing a lipid nanoparticle formulation was able to induce hepatic PAH protein expression and reduce circulating plasma phenylalanine, and may be further developed as a potential therapy to alleviate the key symptom in PKU.

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Improving antisense oligonucleotide therapeutic applications: development of a novel antibody-mediated delivery system

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A number of therapeutic antisense oligonucleotides (AOs) applications have been developed as promising candidates for severe and devastating conditions such as Duchenne muscular dystrophy (DMD). DMD is a lethal, X-linked inherited disorder characterized by progressive muscle weakness, wasting and degeneration. AOs can be used to modulate dystrophin pre-mRNA splicing in a manner that restores the reading frame of the *DMD* transcript, producing a shorter but functional dystrophin protein (*approach approved by FDA in 2016*).

Nonetheless, one of the major challenges for successful AOs therapy in patients remains poor delivery and uptake in targeted tissues and cellular compartments. In order to address this issue, we designed and developed a novel antibody-mediated AO delivery system. A cell penetrating antibody was used as a carrier molecule for morpholino AOs (PMO) for targeted muscle specific delivery. We have explored several conjugation and purification strategies, and conducted a number of screenings *in vitro* to assess antibody-PMO conjugate functionality. Our preliminary results confirm that antibody-PMO conjugate retains its function to penetrate differentiated myotubes. However, pre-mRNA splicing might be affected by steric interference of the antibody on RNA splicing machinery. Consequently, we are currently developing an alternative conjugation approach to facilitate PMO release following targeted myotube penetration. If successful, this antibody-PMO delivery system could improve overall PMO efficacy in DMD and other muscle related disorders.

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A novel and translational role for autophagy in antisense oligonucleotide trafficking and activity

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Endocytosis is a mechanism by which cells sense their environment and internalize various nutrients, growth factors, and signalling molecules. This process initiates at the plasma membrane, converges with autophagy, and terminates at the lysosome. It is well-established that cellular uptake of antisense oligonucleotides (ASOs) proceeds through the endocytic pathway; however, only a small fraction escapes endosomal trafficking while the majority are rendered inactive in the lysosome. Since these pathways converge and share common molecular machinery, it is unclear if autophagy-related trafficking participates in ASO uptake or whether modulation of autophagy affects ASO activity and localization. To address these questions, we investigated the effects of autophagy modulation on ASO activity in cells and mice. We found that enhancing autophagy through small-molecule mTOR inhibition, serum-starvation/fasting, and ketogenic diet, increased ASO-mediated target reduction in vitro and in vivo in tissues with enhanced autophagy. Additionally, autophagy activation enhanced the localization of ASOs into autophagosomes without altering intracellular concentrations or trafficking to other compartments. These results support a novel role for autophagy and the autophagosome as a previously unidentified compartment that participates in and contributes to enhanced ASO activity. Further, we demonstrate non-chemical methods to enhance autophagy and subsequent ASO activity using translatable approaches such as fasting or ketogenic diet.

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Enhancement of Excess-electron-transfer Efficiency of a Pyrene-modified Oligonucleotide by Introducing a Methoxy Group on Pyrene

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DNA-mediated reductive electron transfer (ET) has been attracted as a powerful tool to repair damaged nucleobases, such as cyclobutane pyrimidine dimers (CPD) in DNA. Fluorescent molecules, such as pyrene, could inject an electron into DNA by photoexcitation. The electron is transferred to CPD through DNA; these electrons can cyclorevert the CPD. We have focused on 5-(pyrenylethynyl)-2'-deoxyuridine (^{Py}**U**) as an electron initiator. ^{Py}**U** has a stable excited state, and it could efficiently transfer one electron to CPD through double-stranded DNA. Although pyrene works as ET initiator, the intramolecular ET efficiency of unmodified pyrene is not enough for repairing the damaged nucleobases, and the pyrenyl radical cation oxidizes guanosine. Therefore, it is difficult to use the unmodified pyrene for repairing the damaged nucleobases. Herein, we synthesized a pyrene-modified nucleoside bearing an electron donor, methoxy or piperidinyl group, at pyrene moiety to improve ET property and successfully incorporated into oligonucleotides. These pyrene-modified nucleosides were also expected to reduce the oxidation potential of radical cation. We evaluated the electron transfer efficiency of the pyrene-modified nucleoside by using 5-bromouracil (^{Br}U) or CPD as indicators of one-electron reduction and found that the ET efficiency was up to five times higher than that of the nucleoside having unmodified pyrene. In addition, it did not oxidize the neighboring guanosine. Next, the pyrene-modified nucleoside was incorporated into triplex-forming oligonucleotides and its ET property was evaluated. As a result, very interestingly, ^{Br}U in the target double-stranded DNA was successfully reduced to give U. These results suggest that the novel pyrene-modified nucleoside could be useful for repairing the damaged nucleobases in genomic DNA.

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Unwinding-related Rapid Nuclear Inflow of DNA/RNA Heteroduplex Oligonucleotide; Unveiling Distinctive Intracellular Trafficking

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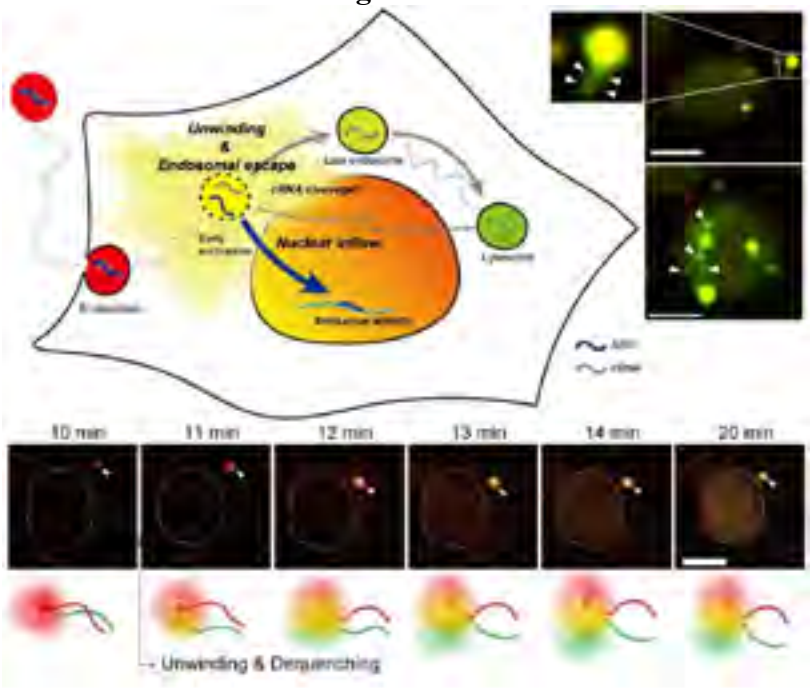
DNA/RNA heteroduplex oligonucleotide (HDO), composed of DNA/LNA antisense oligonucleotide (ASO) and complementary RNA, is a next generation antisense therapeutic agent. As well as improved delivery to target tissues, HDO intracellularly expresses higher gene silencing potency than parent ASO. In this study, we aimed to uncover intracellular trafficking mechanism of HDO. To reveal where and when HDO unwinds into antisense strands (AS) and complementary strands (CS), we performed live-cell time-lapse imaging and fluorescence resonance energy transfer (FRET) assays. HDO had the distinctive intracellular trafficking in comparison with single-stranded ASO. After the endocytosis, HDO unwound in the early endosomes, and both AS and CS were released into the cytosol. Most of the AS and part of the CS entered into the nucleus, and then, isolated AS showed its gene silence effect in the nucleus. A series of event; unwinding, release, and initiation of nuclear distribution occurred very rapidly (within 30 seconds) in this order. Appropriate degeneration of CS in lysosomes was also a necessary process to exert its potency. Understanding the unique intracellular mechanisms of HDO helps us design more efficient drugs, as well as bring us insight to the innate DNA / RNA cellular biology.

Figure. Proposed intracellular trafficking of HDO



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Inhibition of Respiratory Syncytial Virus infection by a Single-Stranded Oligonucleotide *in vitro* and *in vivo.*

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Respiratory Syncytial virus (RSV) utilizes clathrin-mediated endocytosis for viral entry. We recently revealed that a phosphorothioate single-stranded oligonucleotide (ssON) abolishes endocytosis of cargo taken up via the clathrin-mediated uptake route. There is a large unmet need for novel preventive and therapeutic strategies for RSV. Here, we investigated the capacity of ssON to inhibit RSV infection utilizing a GFP-expressing RSV allowing quantification by flow cytometry. We found that both concomitant and prophylactic ssON treatment significantly reduces the percentage of RSV infected A549 cells (lung epithelial cells). Furthermore, studies using an air-liquid interface cellular system show that concomitant ssON treatment effectively inhibits RSV infection in primary human airway epithelial cells as measured by RT-qPCR. Finally, using a recombinant RSV expressing luciferase as a reporter, we show that ssON is also effective *in vivo* in mice infected with RSV. Our data suggest that ssON has significant potential as a novel RSV antiviral agent.

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Neuroblastoma therapy using DNA aptamers for LPAR pathway control

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Neuroblastoma is the most common extracranial solid tumor of childhood and has a poor prognosis for patients with metastatic disease. Lysophosphatidic acid (LPA) is a known chemotactic molecule for cells and lysophosphatidic acid receptor 1 (LPAR1) has been shown to increase cell motility, invasion and metastasis and there has been a report of activating mutations of LPAR1 in neuroblastoma. Currently, LPAR-targeting drugs have not been US FDA approved and are not in clinical trials for cancer as there is a lack of cell-type specificity for the various agents that have been tested. While iRNAs (miRNAs or siRNA) clearly regulate multiple pathways integral to disease development and progression, the lack of safe and reliable means for specific delivery of aptamers to target tissues represents a major obstacle to their broad therapeutic application. In this work, we aim to develop aptamers that regulates LPAR signaling, and develop a system for efficient delivery of various iRNAs to cancer tissues. For this study, antibodies were used to validate the importing receptors for aptamer delivery in neuroblastoma and we synthesized and tested DNA aptamers for binding and internalization. We also examined the ability of LPA in regulating various neuroblastoma cell behaviors and profiled iRNA candidates in affecting LPA stimulation. In our review, these candidate iRNA were miRNA targeting let7g, STAT3, EGFR and Survivin.
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Characterizing Guide RNA 2'-Hydroxyl Ribose Requirements for CRISPR-Cas9 Gene Editing

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CRISPR (clustered regularly interspaced short palindromic repeat) RNAs and their associated Cas endonucleases are at the forefront of biotechnology and potentially the future of modern gene therapy. Many therapeutic applications will require making CRISPR enzymes more drug-like, especially through chemical modification of the guide RNA cofactor, such as to reduce its lability and immunogenicity or improve substrate specificity. Chemical substitution of all the RNA nucleotides in CRISPR RNA guides could establish principles for building more flexible or tunable drug-like CRISPR enzymes. It has been observed that maintaining A-form-like helical structure is critical for maintaining activity of CRISPR-Cas9. In addition, crystal structure data has predicted that the ribose 2'-hydroxyls of several residues make specific contacts with the Cas9 protein. These 2'-hydroxyls can be completely removed and provide robust cleavage activity *in vitro*, but they do not function for cell-based editing. To understand the nature of this discrepancy in enzyme activity, we are probing the chemical and structural requirement of these ribose 2'-hydroxyls by replacing them with chemically modified nucleotides that should support hydrogen bonding at or near the 2' position. We find that the availability and positioning of groups capable of hydrogen bonding, like the 2'-hydroxyl, are both important factors for enzyme activity *in vitro* and in cell-based gene editing. In addition to editing and cleavage assays, we have also performed ribonucleoprotein assembly, target DNA binding, and enzyme conformation studies to mechanistically characterize the 2'-hydroxyl or hydrogen bonding requirement. These results suggest an intimate co-evolution of the guide RNA and the Cas9 protein that may impact catalytic transition states necessary to achieve efficient cleavage of genomic DNA in cells.

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CORALL – The New Whole Transcriptome Library Prep with Precise End-to-End Coverage is an Efficient Solution for Low Input and FFPE RNA

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CORALL is Lexogen's new stranded total RNA library prep kit with excellent whole transcriptome coverage. CORALL enables streamlined generation of Illumina-compatible libraries within 4.5 hours, featuring seamless integration of Unique Molecular Identifiers (UMIs) and exceptional protocol-inherent strand specificity (>99 %). The fragmentation-free protocol uses Lexogen's proprietary Strand Displacement Stop and Ligation technologies to deliver complete transcript representation, including transcription start and end sites. CORALL has been tested with various RNA input types from human, mouse, mini-pig, hamster, plant to bacteria. In combination with Lexogen's RiboCop rRNA Deletion or Poly(A) Enrichment Kits CORALL can be used for RNA input as low as 1 ng as well as degraded and FFPE RNA samples. Here, we present the robustness and efficiency of CORALL Total RNA-Seq Library Prep over a range of input amounts, and for RNA isolated from mouse spleen FFPE samples.

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Accelerated Production of Nanoparticles for Use in Gene and Cell Therapies Using NxGen Microfluidic Technology

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Recent technological advances and clinical successes have led to unprecedented growth and promise in the field of genetic medicine. Notably, non-viral delivery of nucleic acids using nanoparticles is becoming a critical technology for the delivery of nucleic acid for parenteral gene therapies, genetic vaccines, and as non-viral transfection agents to engineer cell therapies. However, improved manufacturing methods are still required that is compatible with multiple formulations and scales easily from early discovery work to cGMP manufacturing for clinical trials.

Nanoparticle preparation through nanoprecipitation using microfluidic mixing has been a widely-used method with much of this work performed on the published staggered herringbone mixer (SHM) structure. We recently developed a novel, next-generation mixing structure (NxGen) which preserves time-invariant mixing conditions over a range of geometric configurations. This allows a mixer to be selected based on the single-mixer throughput required (0.2 to 20 L/h), thus enabling the predictable, simplified manufacturing of nanoparticles at any scale. In this work, we compare the bench-scale manufacture of lipid nanoparticles (LNP) encapsulating siRNA, mRNA and plasmids using both the NxGen and the classic SHM mixer on the NanoAssemblr Ignite system. The results indicate both mixers produce LNPs with identical properties under the same input conditions, demonstrating ease in translating nanoparticle production to NxGen to facilitate future scalability. We further compare performance of NxGen and classic SHM mixers for manufacturing a clinically-relevant LNP formulation encapsulating mRNA encoding erythropoietin (EPO). The results show that regardless of the mixer, the nanoparticles were identical in terms of physical properties and *in vivo* expression of EPO. Finally, data on the scale-up of LNPs is presented, demonstrating the capabilities of NxGen technology for producing large batches.

In conclusion, we have shown the rapid and robust manufacturing of different nucleic acid LNPs using the new NxGen microfluidic mixer. NxGen has now been implemented across a range of systems designed for every stage of development including cGMP production.

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Anchor peptide captures, targets, and loads exosomes of diverse origins for diagnostics and therapy

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Exosomes are circulating nanovesicular carriers of macromolecules, increasingly used for diagnostics and therapeutics. The ability to load and target patient-derived exosomes without altering exosomal surfaces is key to unlocking their therapeutic potential. We demonstrate that a peptide (CP05) identified by phage display enables targeting, cargo loading, and capture of exosomes from diverse origins, including patient-derived exosomes,through binding to CD63—an exosomal surface protein. Systemic administration of exosomes loaded with CP05-modified, dystrophin splice–correcting phosphorodiamidate morpholino oligomer (EXOPMO) increased dystrophin protein 18-fold in quadriceps of dystrophin-deficient mdx mice compared to CP05-PMO. Loading CP05-muscle–targeting peptide on EXOPMO further increased dystrophin expression in muscle with functional improvement without any detectable toxicity. Our study demonstrates that an exosomal anchor peptide enables direct, effective functionalization and capture of exosomes, thus providing a tool for exosome engineering, probing gene function in vivo, and targeted therapeutic drug delivery.

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Exclusive selectivity in RNA interference

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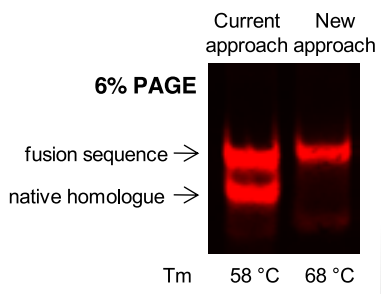
The prominent discoveries in molecular and cell biology offer improved opportunities to leverage genomic knowledge for both academic research and clinical application. Despite the massive global spend on biology-driven drug discovery and the technological advances, tackling the issue of undesired promiscuity of agents interacting with nucleic acids represents a persistent challenge. Although delivering authentic innovations today is more complex than ever, we anticipated to develop a reliable and precise technology to offer a helping hand in ***eliminating the*** current ***problem of non-selective recognition*** of nucleic acids.



Our findings within preclinical validation and molecular analyses using primary samples from patients with a fusion gene positive tumor provided early evidence that our ***patented technology provides exclusive discrimination*** between direct native homologues and target fusion sequences (Figure) ***that could not be achieved by today's hybridization approaches***. The proof of concept data revealing exclusive selectivity towards the target fusion sequence at the molecular level were moreover ***supported by*** the results from ***functional in vitro assays*** that showed ***unprecedented selective effect*** towards leukemic cells. At the same time, the progressivity beyond the state of the art arises also from the findings obtained by confocal microscopy, which revealed ***spontaneous cellular uptake*** of the RNA interfering agents during the tests.

The unmatched accuracy of the presented technology and the fascinating possibility of eliminating false positivity in diagnostics or off-target related side effects in therapy using our tool remains a potent stimulus to the continued exploration of this approach and may facilitate its implementation not only for research purposes, but principally into clinical practice.

Acknowledgement
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Exon skipping in the DMD pig, a large animal model for Duchenne muscular dystrophy

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Introduction: In Duchenne muscular dystrophy, molecular antisense-based approaches are increasingly being developed in recent years. In order to translate an optimised therapy into affected patients, these approaches can be investigated and verified in pig models.
Material: Myogenic primary cultures were isolated from our dystrophin deficient pig model (DMDpig), which carries the most common human mutation (deletion of exon 52 in the dystrophin gene). After differentiation *in vitro*, primary cells were transfected with OMeP (AON) and tricyclo-DNA (tcDNA) antisense oligonucleotides.
Results: In the cell culture model, both OMeP-AON and various tcDNA oligonucleotides efficiently restored a correct reading frame in the dystrophin transcript with skipped exon 51. RT-PCR analysis of the extracted RNA and direct sequencing showed an RNA splice product containing a direct transition from exon 50 to exon 53. Promising results in primary cell culture models can be extrapolated to local or systemic administration in the large animal model.
Discussion: Due to its proximity to the human organism, the DMDpig offers various possibilities for further preclinical research of promising individualized therapies. In the future, antisense oligonucleotide therapies may be assessed for toxicity, pharmacokinetic and pharmacodynamic parameters, restoration efficiency of a functional dystrophin protein, and finally, clinically relevant phenotype improvements in the large animal model.

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Enhanced combinatorial efficacy of RIG-I mediated immunotherapy and radiotherapy in malignant melanoma

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Radiotherapy is a well-established method in cancer treatment. While irradiation is known to induce cytotoxic DNA damage, recent studies have shown that it can also induce tumor-specific immunity. Although melanoma is generally considered to be highly radioresistant, combination therapies have lead to a reevaluation of its therapeutic application. One novel, promising approach in immunooncology is the selective activation of the anti-viral innate immune receptors, such as as the cytoplasmic RNA sensor retinoic acid-inducible gene-I (RIG-I). Activation of RIG-I in tumor as well as immune cells elicits a strong type I interferon-driven immune response and both direct and immune cell-mediated tumor cell death. Selective RIG-I ligands have recently entered clinical trials.

In this study, we investigated the effect of combining irradiation and RIG-I activation in a murine B16 melanoma model. We found that RIG-I-mediated immune activation induced cell-intrinsic expression of genes involved in the DNA damage response, cell cycle arrest and apoptosis. Concurrent 2 Gy low dose irradiation further enhanced the induction of RIG-I mediated tumor cell death in human and murine melanoma cell lines and the subsequent proliferation and activation of murine melanoma specific pmel-1 T-cells. By using CRISPR/Cas9-mediated p53 knockout B16.F10, we could show that synergistic tumor cell death and cell cycle arrest are dependent on the p53 status of the melanoma cells. However, enhanced T-cell activation in combinatorial therapy was not dependent on tumor-cell p53-status. Moreover, combinatorial intratumoral RIG-I activation and radiotherapy enhanced tumor recognition by immune cells *in vivo* and reduced tumor growth *in vivo* compared to monotherapies alone irrespective of the p53 status of the treated melanoma cells. Thus, although melanoma are considered radioresistant, combination of RIG-I activation with low-dose irradiation offers a promising approach to melanoma immunotherapy.

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Efficient Delivery of Antisense Oligonucleotides using Cell Penetrating Peptides Enables Potent, Durable Exon Skipping in Mouse and Human Disease Models

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Splice-switching antisense oligomer (AO) drugs have the ability to restore functional protein expression and ablate protein activity, expanding their therapeutic value across multiple indications that have previously been considered undruggable. The efficacy and safety profile of phosphorodiamidate morpholino oligomers (PMOs) is well established, however their therapeutic potential remains unrealized in many indications due to inefficient cellular uptake. Phylogica's Cell Penetrating Peptide (CPP) platform has identified a suite of CPPs with a high delivery/low toxicity profile across multiple tissues including the posterior segment of the eye and cardiac muscle. Phylogica's CPP-PMOs demonstrate efficient and durable exon skipping in mouse retinal cells compared to competitor strategies, following low dose treatment administered by intravitreal injection. These conjugates are also highly active in human retinal cells derived from patients with inherited retinal disease. Importantly, Phylogica's CPPs exhibit minimal toxicity following systemic delivery, permitting more clinically acceptable dosing regimes, and concomitant therapeutic benefit.

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Template-Independent, Enzymatic Synthesis of RNA Oligonucleotides

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Access to low-costing, error-free, and high-quality oligonucleotides at appreciable scales is relevant to the production and development of RNA-based therapeutics. Despite the rapidly growing market for RNA-based therapeutics and other emerging applications for RNA oligonucleotides, their chemical synthesis still relies on phosphoramidite chemistry. While reliable for short, unmodified standard RNA oligonucleotides, it is limited by cost, purity, and length. To overcome these limitations, we have developed an RNA oligonucleotide synthesis technique in which we employ an engineered enzyme to sequentially add ribonucleotide bases to a growing initiator strand in a template-independent manner. Extension reactions are limited to only an N+1 event through the use of reversible blocked ribonucleotides. The groups are removed using a mild deprotection scheme (**Figure 1**). This process is iteratively repeated until the desired RNA oligonucleotide sequence is synthesized. In addition to the standard ribonucleotide bases, we have shown that our engineered enzyme is compatible with a wide variety of non-standard nucleotides that can provide site-specific modifications to both the bases and backbone of a synthesized oligonucleotide.

Our enzymatic RNA oligonucleotide synthesis platform utilizes a heavily engineered mutant polymerase for the equal incorporation of all four natural nucleobases (A, U, G, C) without the need of a template sequence. Additionally, our enzyme is able to incorporate a wide variety of modified nucleotides such as pseudouridines, methylated bases. The employment of reversibly blocked nucleotides is crucial to controlling the template-independent terminal transferase activity of our enzyme to a single N+1 extension event. Additionally, our N+1 extension reactions are completed within 30 seconds of incubation and can be performed in both in bulk solution and on solid-phase surfaces (such as beads or plates). This makes our platform compatible with liquid handling robotics for parallelized RNA oligonucleotide synthesis or microfluidic formats due to the small volumes of our reactions (< 10 μL).

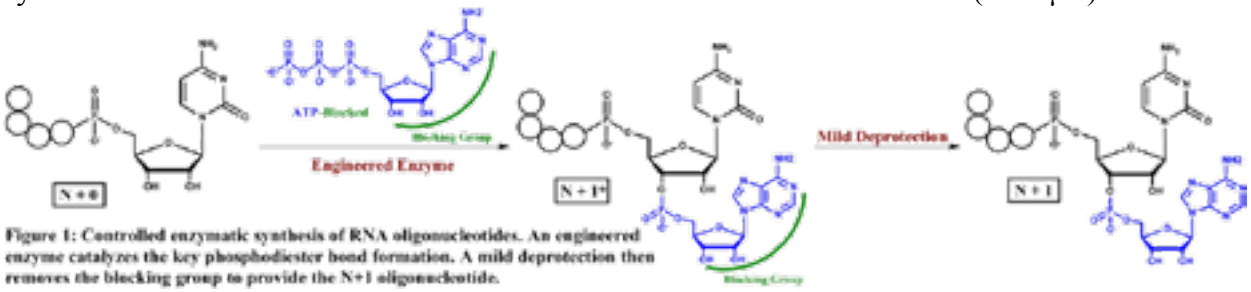

Figure 1: Controlled enzymatic synthesis of RNA oligonucleotides. An engineered enzyme catalyzes the key phosphodiester bond formation. A mild deprotection then removes the blocking group to provide the N+1 oligonucleotide.

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PTGER3 induces ovary tumorigenesis and confers resistance to cisplatin therapy through up-regulation Ras-MAPK/Erk-ETS1-ELK1/CFTR1 axis

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Inflammatory mediator prostaglandin E2–prostaglandin E2 receptor EP3 (PTGER3) signaling is critical for tumor-associated angiogenesis, tumor growth, and chemoresistance. However, the mechanism underlying these effects in ovarian cancer is not known. *Methods:* An association between higher tumoral expression of PTGER3 and shorter patient survival in the ovarian cancer dataset of The Cancer Genome Atlas prompted investigation of the antitumor effects of PTGER3 downmodulation. *PTGER3* mRNA and protein levels were higher in cisplatin-resistant ovarian cancer cells than in their cisplatin-sensitive counterparts. *Findings:* Silencing of PTGER3 *via* siRNA in cancer cells was associated with decreased cell growth and less invasiveness, as well as cell-cycle arrest and increased apoptosis, mediated through the Ras-MAPK/Erk-ETS1-ELK1/CFTR1 axis. Furthermore, sustained PTGER3 silencing with multistage vector and liposomal 2'-F-phosphorodithioate-siRNA–mediated silencing of PTGER3 combined with cisplatin resulted in robust antitumor effects in cisplatin-resistant ovarian cancer models. *Interpretation:* These findings identify PTGER3 as a potential therapeutic target in chemoresistant ovarian cancers expressing high levels of this oncogenic protein.

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Targeted Gene Regulation of a Proinflammatory Gene by Peptide Crosslinked Nucleic Acid Nanocapsules

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Biology has evolved the quintessential nanoscale assembly of nucleic acids, lipids and proteins in the form of a virus. Viruses are built from self-assembled peptide subunits surrounding charged nucleic acids, packaged tightly within a lipid-like envelope. To release nucleic acids in a specific environment, viral coat proteins are enzymatically degraded, in a location-specific manner, releasing contents into the surrounding environment. Our lab seeks to mimic not only the assembly, but the programmed disassembly of biomolecule-based nanomaterials through a combination of chemical crosslinking strategies and enzymatic assembly steps that can ultimately help control the delivery of therapeutic nucleic acids. Recently we have utilized our approach to deliver a therapeutic DNAzyme against the transcription factor target GATA-3 in vivo using a house dust mite mouse model. GATA-3 is an upstream regulator of inflammation pathways, particularly those associated with airway constriction diseases such as asthma. Using our hybrid DNA surfactant and a peptide-based self-assembly method, we have built a crosslinked micelle system that breaks down in response to the presence of various stimuli, much like a virus. For these studies we used the protease MMP9 as a trigger as it is also upregulated during airway inflammation. The nanocapsule displays highly specific responses in the presence of closely related proteases in vitro and in vivo the delivery platform shows an effective reduction in eosinophils and other airway disease biomarkers, indicating the promise of these nanocapsules for targeted and effective use both in vitro and in vivo.

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miR-7 levels contribute to the regulation of muscle catabolism in myotonic dystrophy type 1

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Unstable CTG expansions in the *DMPK* untranslated gene region are responsible for Myotonic Dystrophy type 1 (DM1) condition. Muscle dysfunction is one of the main contributors to DM1 mortality and morbidity. Pathways by which mutant *DMPK* trigger muscle defects, however, are not fully understood. We previously reported that *miR-7* was downregulated in a DM1 *Drosophila* model and in biopsies from patients. Here, using DM1 and normal muscle cells we investigated whether *miR-7* contributes to the muscle phenotype by studying the consequences of replenishing or blocking *miR-7* using modified oligonucleotides, respectively. Restoration of *miR-7* with agomiR-7 was sufficient to rescue DM1 myoblast fusion defects and myotube growth. Conversely, oligonucleotide-mediated blocking of *miR-7* in normal myoblasts led to fusion and myotube growth defects. *miR-7* was found to regulate autophagy and the ubiquitin-proteasome system in human muscle cells. Thus, low levels of *miR-7* promoted both processes and high levels of *miR-7* repressed them. Furthermore, we uncovered that the mechanism by which *miR-7* improves atrophy-related phenotypes is independent of MBNL1, thus suggesting that *miR-7* acts downstream or in parallel to MBNL1. Collectively, these results highlight a novel function for *miR-7* in muscle dysfunction through autophagy and atrophy-related pathways and support that restoration of *miR-7* levels is a candidate therapeutic target for counteracting muscle dysfunction in DM1.

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LNA-modified antisense oligonucleotides targeting host factor Niemann-Pick C1 potently reduce Ebola virus infection *in vitro*

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Ebola virus is the causative agent of Ebola virus disease, a severe, often fatal illness in humans. The virus belongs to the family of *Filoviridae* comprising a non-segmented negative-strand RNA genome and is a biosafety level 4 pathogen transmitted by contact with body fluids, fomites, and droplets from infected patients. Filoviruses are considered a significant threat to public health and global security because of their pandemic potential and the risk of being used as a bioweapon. Therefore, accelerated efforts in the development of therapeutics is a key objective in the filovirus research community, especially since the 2013–2016 EBOV disease epidemic in Western Africa. No vaccines or therapeutic agents with final US Food and Drug Administration (FDA) approval are currently available, and supportive care remains the standard for Ebola virus disease treatment.
Approaches directly targeting the virus are challenging due to the constant emergence of new variants as a result of the error-prone viral polymerase which enables incorporation of mutations facilitating resistance against antiviral drugs. This could be avoided by targeting host factors.

Interaction of the viral transmembrane glycoprotein (GP) with host factor Niemann-Pick C1 (NPC1) has been shown to be essential for cytoplasmic entry of filoviruses. Here, we selected the host factor *NPC1* as therapeutic target for suppression by locked nucleic acid-modified antisense oligonucleotides (LNA-ASOs).
Using the bioinformatics system Oligofyer™, a total of 36 ASOs was designed for the initial screen broadly covering the *NPC1* mRNA sequence. Avoidance of off-target gene suppression and enrichment of ASOs with high activity were key factors in the ASO-selection process. Screening of antisense oligonucleotides in human and murine cell lines led to identification of candidates with up to 94% knockdown efficiency and IC_{50} values in the submicromolar range. Selected candidate oligonucleotides efficiently suppressed NPC1 protein expression *in vitro* and did not show cytotoxic or unspecific immune stimulatory activity in cell-based assays. Treatment of Ebola virus infected HeLa cells with the most promising candidates resulted in significant virus titre reduction by more than 99%.
These results indicate that antisense oligonucleotides against *NPC1* are a promising therapeutic approach to treat Ebola virus infection.

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Centrally-modified folic acid-siRNAs exhibit enhanced gene-silencing activity and efficient receptor-mediated uptake in cancer cells

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RNAi applications have evolved from experimental tools to study gene function to the development of a novel class of therapeutics. Synthetic short interfering RNAs (siRNAs) are able to trigger the endogenous RNAi pathway, allowing for sequence-specific modulation of deleterious proteins. Despite advances in the field, there are still several limitations associated with the inherent nature of siRNAs such as low stability in serum, poor cellular uptake and off-target effects. One of the major hurdles in RNAi research has been the development of safe and effective delivery systems for siRNAs. Various approaches have been proposed, including the use of liposomes and nanoparticles. Despite these, however, delivery to target cells and tissues remains a challenge. In this work, we implement a receptor-targeting strategy to selectively deliver siRNAs to cancer cells utilizing folic acid as a ligand. Folic acid is able to bind cell-surface folate receptors with high affinity. These receptors have become important molecular targets for cancer research as they are overexpressed in numerous cancers, including breast, ovarian, breast and kidney cancers, despite being expressed at negligible levels in normal tissues. Literature reports show that folic acid-conjugation at the 5' or 3'-end of siRNAs allows for effective delivery into tumour cells but only with moderate gene-silencing activity against exogenous gene targets (~40-60% knockdown after 1 μM treatment).[1-3] We report the synthesis of siRNAs bearing folic acid modifications at different positions within the sense strand, with a particular focus on the central region which spans the cleavage site for the endonuclease Argonaute2. In the absence of a transfection carrier, these siRNAs were selectively taken up by cancer cells expressing folate receptors. We show that centrally modified folic acid-siRNAs display enhanced gene-silencing activity against an exogenous gene target (~80% knockdown after 0.75 μM treatment) and low cytotoxicity. In addition, these siRNAs achieved potent dose-dependent knockdown of endogenous bcl-2, an important anti-apoptotic gene.

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Spherical nucleic acids (SNAs) are versatile facilitators of oligonucleotide-mediated splice modulation

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Spherical nucleic acids (SNAs) are dense, radial arrangements of oligonucleotides around a nanoparticle core. Studies have shown the utility of SNAs for immune stimulation using CpG oligonucleotides and for gene knockdown mediated by antisense oligonucleotides or siRNA. Recently, SNAs have shown promise as effectors of splice modulation through delivery of splice-switching oligonucleotides (SSOs). This work investigates the suitability of SNAs broadly for splice modulation.

Alternative splicing of pre-mRNA leads to production of divergent mature mRNA products and is a major source of proteome diversity. Indeed, at least 70% of human genes are predicted to undergo alternatively splicing and it is estimated that 60% of human genetic diseases arise from mutations that effect splicing. Clearly, broadly applicable methods to influence splicing are of significant therapeutic value. Thus, when considering SNAs for splice modulation, it is critical to evaluate their efficacy across splicing contexts and targets.

SNAs were designed to facilitate exon skipping of signal transducer and activator of transcription 3 (STAT3), RE1 silencing transcription factor (REST), interleukin 17 receptor α (IL17RA), or IL1 receptor accessory protein (IL1RAP), and their efficacy was compared to that of linear SSOs. The results establish SNAs as comprehensively more effective (by 2- to 3-fold) at inducing preferential production of a natural transcript variant (IL17RA, REST), a novel, stable splice variant (IL1RAP), and a novel, frame-shifted variant that leads to nonsense-mediated decay (STAT3). SNAs exhibited this potency in several relevant cell lines and, in the case of IL17RA-targeted SNAs, in human skin biopsies. Additionally, survival of motor neuron 2 (SMN2)-targeted SNAs dramatically improved exon inclusion in human patient fibroblasts, exhibiting a more than 10-fold potency improvement over linear SSOs.

A unique feature of SNAs is that they can be designed to deliver multiple oligonucleotides to the same cell, enabling synergistic targeting of a single pre-mRNA with disparate SSOs or facilitating modulation of multiple therapeutic targets simultaneously by targeting different genes. Proof-of-concept work targeting two loci on the IL1RAP gene demonstrates the power of this approach. In this context, a bispecific SNA comprised of two SSOs targeting IL1RAP outperformed either SSO delivered on SNA individually.

In summary, the data reveal the potential of SNAs for mRNA reprogramming through splice modulation of pre-mRNA in a variety of modes, across therapeutic targets, and at multiple genetic loci. This establishes SNAs as potentially transformative therapies for diseases ranging from inflammatory skin conditions to cancer to rare neurological disorders.

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New receptor search for β-glucan/nucleic acid complex

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INTRODUCTION

AS-ODNs and short interference RNAs (siRNA) can silence specific gene. However, it's necessary for it to be delivered to target cells by a DDS (drug delivery system) carrier. We use the natural polysaccharide schizophyllan (SPG), as a carrier. We found that SPG forms a complex with single-stranded homo-polynucleotides and recognized by Dectin-1(β -glucan receptor) expressed on the cell surface [1,2]. Therefore, we have been conducting disease treatment targeting Dectin-1 expressing cells using SPG /DNA complex [3,4]. In previous study, we found cells that can obtain the cytostatic effect of Dectin-1 non expressing cells, and it has been shown that SPG /DNA complex may be recognized by receptors other than Dectin-1. Nowadays, polysaccharide receptors are known to be Dectin-1 and various others, but all of them have not been clarified. In this study, we clarify new receptors of SPG /DNA complex.

EXPERIMENT

We prepared human ovarian carcinoma cell line A2780 to observe the effect of the SPG/DNA complex. SPG/Alexa546-AS-ODN (SPG/A-AS) complex (25 nM) and dextran sodium sulfate (250 nM), which is a scavenger receptor inhibitor, were added to A2780 cells in 1 mL PBS. After mixing for 1 hour at 4°C, we evaluated by flow cytometry

RESULTS &DISCUSSION

We used dextran sulfate sodium because we guessed that the scavenger receptor was involved in the uptake of the SPG / DNA complex. Flow cytometry results indicate that the SPG /DNA complex is incorporated via a scavenger receptor, as the addition of dextran sulfate sodium reduced the fluorescence intensity from the SPG/A-AS complex. Based on this result, genetic analysis by microarray analysis showed that the scavenger receptor COLEC12 is highly expressed in A2780 cells. Next, when we silenced COLEC12 by siRNA, the SPG / DNA complex wasn't taken up.

CONCLUSION

In conclusion, it became clear that COLEC12 is a receptor of SPG / DNA complex. Furthermore, we believe that this is more than Dectin-1, and we can expect further breakthrough as a DDS carrier in the future.

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Peptide-mediated delivery of RNA

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According to the World Health Organization, in 2016 almost 40% of people over 18 were overweight[1]. However, most drugs currently available are aiming for a symptom treatment rather than treating its cause. This leads to undesirable side effects, as obesity is a risk factor for other diseases like type 2 diabetes and cardiovascular diseases[2].
This project aims to improve selectivity of drug targeting by fusing siRNA sequence to the neuropeptide Y (NPY). NPY receptor Y_1 is a well-known target for anti-obesity drugs[3,4], which can be specifically addressed with a modified NPY ([F^7, P^{34}]-NPY)[5]. Moreover, siRNAs are an already known tool in drug targeting. By combining [F^7, P^{34}]-NPY and siRNA, the drug molecule can be selectively transported to target cells expressing NPY receptor Y_1. A lysine linker non-covalently binds peptide and siRNA.
We have already shown that a complex formation takes place and is stable under MALDI-TOF conditions and during a gel shift assay. Next steps include the investigation of receptor activation and analysis of siRNA uptake.

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Improved Specificity of Conjugate siRNAs Through Chemical Modification

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During the lead discovery and selection process, a subset of GalNAc-siRNA conjugates do not pass the stringent safety criteria for nonclinical evaluation due to hepatotoxicity in the rat at suprapharmacological doses. Comprehensive mechanistic studies demonstrated that the observed hepatotoxicity can largely be attributed to RNA interference (RNAi)-mediated, hybridization-based off-target effects with little or no contribution from chemical modifications or the perturbation of RNAi pathways. To minimize the undesired miRNA-like off-target activity, we developed a novel design strategy termed ESC+, which may utilize a panel of chemical modifications aimed towards the thermal destabilization of base pairing between the seed region of the guide strand and off-target mRNAs. The enhanced specificity of ESC+ designs, which has been confirmed by off-target reporter assays as well as by RNASeq-based global transcriptome profiling, has translated to significant improvements in therapeutic index as observed in pre-clinical studies in rodents. One of the keys to enable this approach has been to identify chemistries and designs which can mitigate undesired miRNA-like off-target activity without compromising *in vivo* efficacy and durability. This ESC+ design strategy is now being applied to Alnylam's nonclinical programs and has shown successful translation of potency from rodents to non-human primates, with several ESC+ development candidates currently being evaluated in the clinic.

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Therapeutic Evaluation of Antisense Oligonucleotides based Multiple Exon Skipping Strategies in a Duchenne Muscular Dystrophy Patient.

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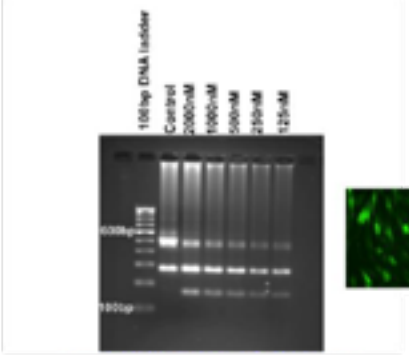
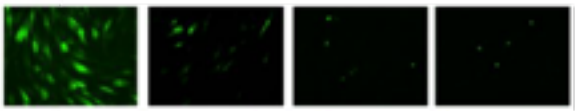
Duchenne muscular dystrophy (DMD) is caused by mutations in *DMD* gene. Multiple exon skipping, which is theoretically applicable to 80–90% of DMD patients in total, have been demonstrated in animal models, including dystrophic mice and dogs, using cocktail antisense oligonucleotides (AONs).

We have evaluated the therapeutic potential of AON mediated multiple exon skipping in a DMD patient having intra-exonic mutations in frame-shifting exons. The mutation was efficiently addressed *in vitro*, by skipping of two exons in the patient derived cells. *in vivo* safety studies showed NOAELs to be 50 mg/kg. Patient was clinically administered with a cocktail of two AONs (2.5 mg/kg) to evaluate the safety and efficacy.



Efficient Levels of Multiple Exon Skipping in Patient Cells



Fluorescence 6-FAM tagged AONS were Successfully transfected in Patient Cells

Safety and tolerability endpoints included observation for Adverse Events, physicDifferent laboratory assessments included hematology, clinical chemistry, biomarkers, urinalysis, coagulation, and immunology tests. All the above-mentioned parameters were assessed at Week 0, 13 & 26.

There were no adverse events related to injection site reactions. Overall clinical & physical parameters were improved in the patient, which is reflected with regards to stamina & movement. This study provides insights for further studies in the clinical program to optimize dosage regimen. The data from this study suggests that treatment with AON cocktails provides beneficial effects in slowing disease progression in the patient.

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Nucleic Acid Delivery System Based on Disulfide Unit

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Development of intracellular delivery methods for nucleic acid is important. Previously reported methods using liposomes or receptor-ligands have a problem that nucleic acid take several hours or more to reach the cytoplasm due to long-time residence of oligonucleotides at endosomes. In this study, we clarified that nucleic acid modified with low molecular disulfide units at the terminus reaches the cytoplasm 10 minutes after the administration to cultured cells. This rapid cytoplasmic internalization of disulfide-modified nucleic acid suggests the existence of an uptake pathway other than endocytosis. In fact, the mechanistic analysis revealed that the modified nucleic acid is efficiently internalized into the cytoplasm through disulfide exchange reactions with the thiol groups on the cellular surface. Because our approach solves several critical problems with the currently available methods for enhancing cellular uptake, including toxicity, undefined molecular composition, inefficient endosomal escape, and serum stability, this method may be an effective approach in the medicinal application.

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Stable Isotope labeling of Antisense Oligonucleotide and Applications in Imaging and Bioanalysis

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The renewed interest in oligonucleotides as a therapeutic modality necessitates the development of new analytical technologies to support fundamental research and drug development. In the realm of small molecule drugs stable isotope labelling is a staple technique for the quantification of molecules in biological fluids. We have recently shown a method for the "on synthesizer", late stage labeling of phosphonothioate oligonucleotides during chemical synthesis. This makes ^{34}S labeled oligonucleotides the new method of choice for the quantification of short therapeutic oligonucleotides within AstraZeneca.

Here we present the applications of ^{34}S stable isotope labeled oligonucleotides for mass-spectrometry based imaging which is combined with TEM imaging. A fully phosphorothioate modified oligonucleotide incorporating 15 ^{34}S labels was conjugated to a GLP1 peptide modified with diiodotyrosine. GLP1 conjugation was recently shown to increase oligonucleotide uptake in pancreatic beta cells by triggering receptor mediated endocytosis.

The knockdown efficiency, plasma stability and receptor-internalization of the construct was confirmed to be identical to a unlabeled control. HEK293 cells expressing the GLP1 receptor were incubated with the construct and fixed after 30 minutes. The Iodine labels and ^{34}S labels were simultaneously visualized by Nano secondary ion mass spectrometry (NanoSIMS). The peptide accumulated in endosomes at a concentration of 20-50 μM. Surprisingly the labeled oligonucleotides were present at a fraction of that concentration. Localization of the nucleotide in cytoplasm, endosomes and nucleus was confirmed by immunofluorescence staining.
We propose that within 30 mins of uptake the GLP1-peptide is cleaved from the oligonucleotide and the oligonucleotide escapes endosomes and is trafficked to the cytoplasm with significantly higher efficiency than expected.
Currently further investigation is underway to confirm this hypothesis and gain more insights into the early endosomal trafficking of oligonucleotide constructs.

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Myeloid Cell-Selective Delivery of miR-146a Inhibits NF-kB-Driven Inflammation and Leukemia Progression *in Vivo*

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The miR-146a is a negative feedback inhibitor of NF-κB signaling, a key signaling pathway driving inflammation as well as tumorigenesis. Despite therapeutic potential of miR-146a, the lack of efficient delivery strategies has so far hampered clinical translation of miRNA therapeutics. We previously described targeting of synthetic oligonucleotides (ONs) to immune cells via scavenger receptors (SR)/Toll-like receptor 9 (TLR9). Here, we adopted this approach for myeloid cell-selective NF-κB inhibition using a specific SR/TLR9- agonist conjugated to miR146a mimic (C-miR146a). Unlike an unconjugated miR-146a, C-miR146a was quickly internalized by target immune and leukemic cells. *In vitro,* C-miR146a reduced expression of two classical miR-146a target genes, *Irak1* and *Traf6*, and thereby inhibited NF-κB activation. Using *miR-146*-deficient mice, we verified that systemic administration of the mimic conjugate, dose-dependently restored miR-146a levels in bone marrow and splenic myeloid cells, thereby reducing IRAK1 and TRAF6 proteins for up to 48h. The repeated intravenous C-miR146a injections into *miR-146a*$^{-/-}$ mice alleviated myeloproliferation and hypersensitivity to endotoxin and bacterial challenge characteristic for these mice. Importantly, C-miR146a can also lessen severe inflammatory responses, such as cytokine release syndrome (CRS), in human immune system. Targeting human monocytes using C-miR146a, but not control C-scrRNA, prevented CRS triggered by CD19 CAR T-cell activity *in vitro* and in xenotransplanted B-cell lymphoma model by abrogating IL-1 and IL-6 release. Since miR146a also acts as a tumor suppressor, we investigated whether C-miR146a will suppress growth of human myelodysplastic or acute myeloid leukemia cells. In fact, C-miR146a administration thwarted human MDSL and HL-60 leukemia progression *in vitro* and *in vivo*. Overall, our results suggest that myeloid cell-selective miR146a delivery has potential for treating inflammatory and myeloproliferative disorders resulting from an excessive NF-κB signaling.

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Development of a sensitive and reliable LC-HRMS-based bioanalytical method for antisense therapeutics

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Antisense therapeutics (or antisense oligonucleotides, ASOs) are chemically synthesized single-stranded oligonucleotides, which account for the greatest proportion of clinically available oligonucleotide therapeutics. As for analytical methods for ASO bioanalysis, liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) has gained great attention in terms of direct and highly sensitive detection of original ASO molecules and their potential metabolites. However, appropriate extraction and analytical methods for LC-HRMS-based ASO measurement have not been well-examined so far. Therefore, using clinically available mipomersen as a model ASO, we aimed to develop a sensitive and reproducible bioanalytical method for LC-HRMS-based ASOs measurement. We first developed an analytical method for mipomersen using triethylamine and 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol. Using the method, we observed good linearity and inter-day reproducibility for mipomersen standard solutions (1-1000 ng/mL, r^2=0.993). Next, we established a Clarity OTX-based mipomersen extraction method from spiked rat plasma using ammonium acetate, resulting in >90% recovery rate. Using the current extraction and analytical methods, standard curve built by extracted mipomersen showed good linearity (0.5-250 ng/mL, r^2 = 0.997). Besides, ranges of accuracy and precision for QC samples (n=5, 5 concentrations) were 102.8-116.53% and 4.24-7.96%, respectively. Importantly, no carry-over peak of mipomersen was detected even after the injection of upper limit of quantification sample. Next, we evaluated the selectivity and matrix effect of our method using 6 different sources of rat plasma. It was found that there were no interfering signals as well as matrix effect for the measurement of mipomersen in the rat plasma. Moreover, the result of dilution integrity test demonstrated that both mean accuracy and precision of 10-fold and 100-fold diluted QC samples (n=6) were both within 15%. Furthermore, mipomersen showed good short-term (<24 hrs) stability in rat plasma stored at room temperature, 4 °C and -80 °C. Additionally, good long-term stability (>3 months) at -80 °C was also observed. Finally, we confirmed that our results met the criteria written in bioanalytical method validation draft guideline (M10) published by ICH in 2019. Taken together, in the current study, we developed a highly sensitive bioanalytical method for mipomersen, implying its potential application for bioanalyses of other ASOs.

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Development of cell-internalizing aptamers and investigation of the ASO efficacy of the aptamer-ASO conjugates

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Since it was reported that *N*-acetyl-galactosamine (GalNAc) improved antisense oligonucleotide (ASO) efficacy (T.P. Prakash, *et al., Nucleic Acids Res.*, 2014, 42, 8796.), ligand-ASO conjugates have been receiving a lot of attention. However, it is not easy to choose target membrane proteins and to obtain ligands which is suitable for ASOs delivery. We considered that cell-internalizing aptamers could be useful for ASOs delivery. In fact, nucleolin-targeted aptamers successfully delivered ASOs into cells and improved efficacy of ASOs (S. Hong, *et al., RSC Adv.*, 2016, 6, 112445.). To further improve ASO delivery, various membrane protein-targeted aptamers need to be developed to validate the effectiveness of the aptamer-ASO conjugates. However, conventional methods have limitations for this purpose. Aptamers are selected from library by Systematic Evolution of Ligands by EXponential enrichment (SELEX) method (C. Tuerk, *et al., Science*, 1990, 249, 505.). The SELEX method, which targets cells themselves, would be an effective way to obtain aptamers that bind to different membrane proteins.
In this research, we targeted human lung cancer cell line and developed cell-internalizing aptamers. When performing SELEX, we employed the modified nucleic acid with aromatic rings to increase the binding affinity of aptamers against target membrane proteins. The cell-internalizing ability of the generated aptamers were measured using real time PCR and confocal fluorescence microscopy. On the basis of the fluorescence observations, the generated aptamers likely internalized cells via endocytosis pathway. We also carried out proteome analysis to search the target proteins. Lastly, we attached an ASO at the 5' end of the aptamers and measured ASO efficacy. Unlike our expectations, the efficacy of ASO did not correlate with the cell-internalizing ability of aptamers. This might be due to difficulty of endosomal escape and accumulation in lysosome. In this presentation, we show the features of generated aptamers, the results of proteome analysis, and the efficacy of the aptamer-ASO conjugates.

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Guiding siRNA *in vivo* pharmacokinetics and ADME characterization…to make sense of it.

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After two decades teetering at the intersection of interest and practical reality, RNA interference (RNAi) based therapeutics are finally coming into their own. An important component in translating RNAi from a laboratory workhorse for generating knockout organisms, to a safe and effective treatment for human disease, has been the development of bioanalytical tools to precisely and accurately quantify oligonucleotide therapeutics *in vivo* and *in vitro*. To date, there has been lack of consensus and transparency around bioanalytical techniques utilized in the development of oligonucleotide therapeutics and we feel that this needs to be addressed.

Here, we describe an evolving suite of bioanalytical assays we have been developing to measure the pharmacokinetics (PK) of sense strand, antisense strand, antisense oligonucleotides (ASO) and intact siRNA-antibody conjugates in serum and tissue homogenate. Interpretation of PK and pharmacodynamics (PD) to inform PK-PD models for cross-species scaling and human dose projections requires an understanding of the absorption, distribution, metabolism, and elimination (ADME) properties of a therapeutic. To that end, we have developed assays to measure *in vitro* plasma protein binding (PPB) liver homogenate protein binding, and stability of the GalNAc/linker of GalNAc-siRNA conjugates in skin homogenate and serum.

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Metabolic and Energetic Benefits of microRNA-22 Inhibition

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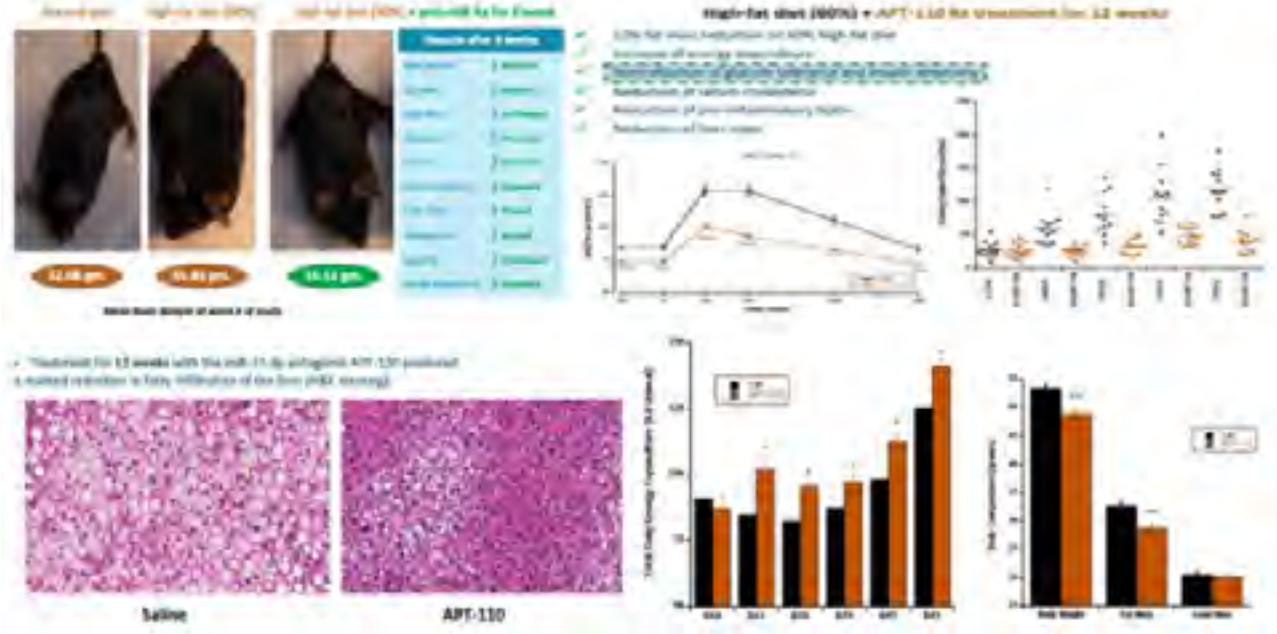
Diabesity and Non Alcoholic Fatty Liver disease (NAFLD) are growing pandemics with substantial health and financial consequences. We are developing microRNA-based drug candidates that increase lipid oxidation and energy expenditure to tackle metabolic diseases.

Phenotypic screening of miRNA antagomirs and agomirs in primary cultures of human subcutaneous adipocytes revealed that inhibition of microRNA-22-3p resulted in increased lipid oxidation, mitochondrial activity and energy expenditure. These effects may be mediated through activation of target genes like *KDM3A*, *KDM6B, PPARA, PPARGC1B and SIRT1* involved in lipid catabolism, thermogenesis and glucose homeostasis.

We investigated whether these in vitro findings could be replicated in vivo in diet-induced obese (DIO) mice of various ages. In DIO male C57Bl/6 mice fed a 60% fat diet, weekly s.c. injections of different miR22-3p antagomirs for 8 weeks produced a significant fat mass reduction, but no change of appetite nor body temperature. Insulin sensitivity, circulating glucose and cholesterol were also improved. A follow-up study exploring the metabolic and energetic effects of our first miR-22-3p antagomir drug candidate (APT-110) in DIO mice showed that weekly s.c. injections of APT-110 for 12 weeks produced a sustained increase of energy expenditure as early as Day 11 of treatment, a significant fat mass reduction, but no change of appetite, physical activity nor body temperature. Insulin sensitivity, glucose, cholesterol and leptin were also improved. Oral glucose tolerance tests were normalized. There was a dramatic reduction of liver steatosis at completion of the study after 3 months of active treatment. RNA sequencing by NGS revealed an activation of lipid metabolism pathways.

These original findings suggest that microRNA-22-3p inhibition could be an effective treatment of fat accumulation and related complex metabolic disorders like obesity and type 2 diabetes mellitus (the so-called diabesity) and NAFLD.



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Therapeutic oligonucleotides targeting DNA repeat expansions in Huntington´s Disease downregulate huntingtin gene expression in iPS cell-derived patient neuronal stem cells

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Huntington's disease (HD) is one of the most common trinucleotide-repeat neurodegenerative disorders. It affects the striatum, cerebral cortex and other subcortical structures in the brain leading to symptoms such as involuntary movement abnormalities, emotional disturbances and cognitive impairments. There is currently no cure. The conventional symptomatic treatments do not significantly alter the disease progression or increase the life expectancy, resulting in death 15-20 years post diagnosis. HD is caused by a CAG•CTG trinucleotide-repeat expansion in exon 1 of the huntingtin (*HTT)* gene leading to the formation of mutant HTT (muHTT) protein containing a polyglutamine tract. Besides toxicity of the mutated protein, there is also increasing body of evidence that mutHTT transcripts contribute to striatal and cortical atrophy. Thus, the reduction of both macromolecules would be the most beneficial form of treatment. We have previously used anti-gene therapeutic oligonucleotides targeting the repeat region in the *HTT* gene (Zaghloul EM et al., Nucl Acids Res, 45:5153-69, 2017). Now we investigated the effects of modified oligonucleotides during differentiation of HD-derived induced pluripotent stem cells (iPSCs) into neuronal stem cells (NSC). With naked delivery (gymnosis) of phosphorothioate LNA/DNA mixmers we achieved efficient downregulation of HTT mRNA and protein in both iPSCs and NSC.

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LNA-modified antisense oligonucleotides targeting NLRP3 for treatment of inflammatory and fibrotic diseases

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NLR family pyrin domain containing 3 (NLRP3) is a central component of the NLRP3 inflammasome complex and is predominantly expressed in immune cells, e.g. macrophages, but also in non-immune cells. The inflammasome complex is required for the proteolytic cleavage of pro-IL-1β and pro-IL-18 into the mature forms of these pro-inflammatory cytokines and a certain form of cell death, i.e. pyroptosis. In addition, NLRP3 contributes to fibrotic remodelling of tissues, in an inflammasome-independent manner. Since NLRP3 activity is involved in a broad range of inflammatory disorders like CAPS (cryopyrin associated periodic syndromes), chronic kidney disease, Alzheimer or liver fibrosis, it is a highly attractive therapeutic target. However, direct targeting of NLRP3 by antibodies is hampered by its intracellular localization while small molecule-based approaches lack specificity, resulting in toxic side effects. These limitations can be overcome by using antisense oligonucleotides (ASOs) directed against NLRP3.
We screened over 100 either mouse- or human-NLRP3 specific LNA-modified ASOs for target engagement after gymnotic delivery in relevant cell lines. The best human- or mouse-specific ASOs had half-maximal inhibitory concentrations (IC_{50}) in the submicromolar range and reduced NLRP3 mRNA expression by 80% to 90% in primary macrophages. Consequently, inflammasome-mediated IL-1β cleavage in activated macrophages was inhibited by NLRP3 specific ASOs. Finally, subcutaneous injection of healthy mice with a mouse specific candidate resulted in more than 50% reduction of NLRP3 mRNA in the kidney, liver and lymph nodes, while the control oligonucleotide had no effect.
Taken together, we identified NLRP3-specific ASOs that potently suppress target expression and downstream effects *in vitro* and show efficacy *in vivo*. While mouse-NLRP3 specific ASOs will be a valuable tool to demonstrate proof-of-concept in relevant pre-clinical models, the human-specific candidates might provide a new therapeutic modality to treat inflammatory and fibrotic diseases.

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Splice modulating antisense oligonucleotides to address COL3A1 mutations causing vascular Ehler Danlos syndrome

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Vascular Ehler Danlos syndrome or Ehler Danlos syndrome type IV (vEDS or EDS type IV) is a connective tissue disorder characterized by skin hyperextensibility, joint hypermobility and fatal vascular rupture. vEDS is caused by *COL3A1* mutations that disrupt collagen III expression. COL3A1is classified as fibrillar collagen, along with collagens I, II, V and XI in the extracellular matrix, particularly in the inner organs such as uterus, bowel, blood vessels, etc. To date, only symptomatic treatment for vEDS patients is available. vEDS patients with particular splice site mutations that lead to in-frame exon-skipping, with less collagen III secreted compared to normal fibroblasts, as assessed by immunofluorescence and western blot analysis. For example, vEDS patient fibroblasts carrying an IVS14-2G>A mutation in COL3A1 show exon 15 skipping and reduced COL3A1 secretion. In consistent with patient fibroblasts carrying a single base deletion (c.766delA) in exon 10, have the reading-frame disrupted and there was a reduction of secreted collagen III, presumably due to compromised COL3A1 homo-trimer fibril assembly. The aim of this study is to increase collagen III secretion in these patient fibroblasts using splice switching antisense oligonucleotides (AOs). We hypothesize that removal of the mutated exon from both the normal and disease causing *COL3A1* alleles would by-pass the mutation and produce COL3A1 isoforms capable of trimerization, thereby increasing collagen III secretion. Screening of three 2'O-methyl phosphorothioate AOs (2OMeAO) to skip exon 10 in patient fibroblasts did not show detectable exon 10 skipping when applied individually, but the combination of AO2 and AO3 induced 40% skipping of the targeted exon. These two AO sequences were resynthesized as phosphorodiamidate morpholino oligomers (PMOs) and induced 100% exon 10 skipping in both patient and normal fibroblasts. COL3A1 exon 15 was efficiently excised from the mature mRNA with 2OMeAOs and with the equivalent PMOs. Western blot was used to assess the collagen III secretion and immunofluorescence staining identified collagen III deposition in the extracellular matrix.

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Transcriptome-wide analysis reveals novel insights into tumor suppressor function of 1B3 (a chemically modified miR-193a-3p mimic)

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Background: Emerging data show that microRNA-193a-3p (miR-193a-3p) has a suppressive role in many cancer types and is often downregulated in tumors. Therefore, mimics of miR-193a-3p could be used as a novel therapeutic approach in oncology. Several miR-193a-3p targets have been described, but so far no transcriptome-wide search has been performed.

Methods: The non-small cell lung cancer (A549 and H460), hepatocellular cancer (HEP3B and HUH7), melanoma (A2058) and triple-negative breast cancer (BT549) human cell lines were transfected with 1B3 (miR-193a-3p mimic). Differentially expressed genes were evaluated by RNA sequencing, 24 or 72 h after transfection. These genes were mapped by Ingenuity Pathway Analysis (IPA).

Results: At 24 h post-transfection, 1B3 caused significant downregulation of 141 genes common to all six cell lines, of which 78% were predicted miR-193a-3p targets. IPA of genes differentially expressed at 24 h in each individual cell line showed a strong upregulation of the tumor suppressive PTEN pathway and downregulation of many oncogenic growth factor signaling pathways. At 72 h post-transfection, fewer differentially expressed genes were predicted miR-193a-3p targets and there was less overlap between cell lines, indicating more indirect target regulation at this later timepoint. Affected pathways at 72 h included cell cycle control and cancer signaling. Out of the 100 most significant biological functions that were changed by 1B3, those that were inhibited were related to cell survival, proliferation, migration or cancer, and those that were activated were related to cell death.

Conclusions: Our data demonstrate that 1B3 represents a potent tumor suppressor that directly and indirectly targets a wide variety of oncogenic pathways across cancer types. Therefore, introducing a miR-193a-3p mimic into tumor cells is a promising new strategy for cancer treatment.

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A novel ASO based approach to treat peripheral neuropathic pain

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Peripheral neuropathic pain affects about 8% of the population and is a major public health concern. There is an urgent need for novel therapeutic approaches since current treatments (opioids, repurposed anti-anxiolytics and anti-epileptics) cause serious side-effects and are only moderately effective in a minority of patients. We identified the NaK-ATPase modulator protein Fxyd2, expressed in specific subsets of somatosensory neurons of the dorsal root ganglia in rodents and humans. Analysis of null mutant *Fxyd2* mice in a rodent model of neuropathic pain showed that *Fxyd2* expression is necessary to maintain pain behavior.

Here, we tested the capacity of antisense oligonucleotides (ASOs) directed against *Fxyd2* mRNA to attenuate pain symptoms in different experimental models. Having identified a 20-mer ASO-Fxyd2, with strong inhibitory efficiency in a human embryonic kidney cell line, we then used this sequence to inhibit Fxyd2 expression in rat DRG neurons *in vivo*. Daily intrathecal injections of an ASO-Fxyd2, chemically modified for direct transfection without transfection reagent, in rat models of neuropathic and also chronic inflammatory pain, led to a complete attenuation of mechanical hypersensitivity. Injection of equivalent quantities of a control scrambled-ASO had no effect. Our results point to Fxyd2 inhibition by antisense oligonucleotides as a novel therapeutic approach to treating pain conditions.

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Deciphering the interactive network of the *DLK1-DIO3* locus in hematopoiesis and pediatric leukemia

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Non-coding RNAs (ncRNAs) have emerged as central regulators of chromatin and gene expression. The expression patterns of ncRNAs, especially long noncoding RNAs (lncRNAs), are highly cell-type specific, posing a novel window for targeted therapies in pediatric acute megekaryoblastic leukemia (AMKL). Our group previously profiled mRNAs, microRNAs, and lncRNAs in the human normal and malignant hematopoiesis, with a special focus on pediatric acute myeloid leukemia (AML). We discovered that the *DLK1-DIO3* locus was highly expressed in human CD34+ hematopoietic stem and progenitor cells (HSPCs), megakaryocytes (MKs), and AMKL.

To gain insight into the role of the *DLK1-DIO3* locus in normal haematopoiesis and to explore the therapeutic potential of this locus in AMKL, we performed ChIP-Seq for human HSPCs, MKs and monocytes, which showed cell type specific activating H3K3me3 and repressing H3K27me3 histone marks. RNA-seq data confirmed cell type specific expression of the *DLK1-DIO3* locus. The ncRNAs from this locus are transcribed as one huge polycistrone of which the transcription starts at the *MEG3* gene. Using Bisulphite sequencing, a significant correlation between *MEG3* expression and the methylation status of a CpG island downstream of the first exon of *MEG3* was revealed. When deleting the transcription start site of *MEG3* with a CRISPR-Cas9 vector containing two sgRNAs, proliferation of AMKL cell lines was impaired. Single cell clones from K562 and HEL cells showed a more mature phenotype upon deletion of *MEG3*.
In addition, the *DLK1-DIO3* locus contains the largest microRNA cluster in the human genome with 54 microRNA. Lentiviral expression of several highly expressed miRNAs of the *DLK1-DIO3* locus in CD34+ HSPCs resulted in accelerated megakaryocytes maturation *in vitro*. Demonstrating the importance of several microRNAs in the formation of MKs.

Using different approaches, we propose the *DLK1-DIO3* locus is an important regulator of megakaryopoiesis with different members controlling this process. Our studies provide the foundation for further investigations towards targeted therapies in AMKL.

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Antisense therapy against non-coding mitochondrial RNAs as potent therapy for prostate cancer.

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Prostate cancer (PCa) is one of the most common causes of male cancer-related death in western nations. In advances stages of disease the treatments are mostly palliatives. Therefore, there is a pressing need for a novel therapeutic strategies which will alter the natural history of prostate cancer.
Human cells express a family of noncoding mitochondrial RNAs (ncmtRNAs). Normal proliferating cells express both sense and antisense transcripts (SncmtRNA and ASncmtRNAs). However, tumor cells in culture or human biopsies express only the SncmtRNA and down regulate the ASncmtRNAs. This expression pattern is independent of tumor origin and distinguish tumor cell from normal cell. The interference of the few copies of ASncmtRNA with complementary antisense oligonucleotides, induce a massive cell death mediate by apoptosis.
The goal of this work was to evaluate the effect of antisense therapy in vitro, using four different cell lines. Antisense therapy, using an oligonucleotide complementary to ASncmtRNA (ANDES1537), induces a potent negative effect over proliferation rate in the four cell lines under study (PC3, LNCaP, 22Rv1 and VCaP). Depending of the cell line analyzed, we observed a differential effect in levels of protein involved in control of cell proliferation, as cyclins B1,D1 or antiapoptotic markers as survivin and BclxL.
The sphere formation assay identified the effect of any treatment over the viability cancer stem cells (CSC). We found that antisense therapy, affect severely the capacity of four cell lines to form spheres in vitro, suggesting that this treatment is a potent therapeutic tool.
The next step was to evaluate the effect of the therapy in a pre-clinical model. Therefore, 10 mice were subcutaneously injected with 5 x10^6 PC3 cells. When the tumor reach an average volume of 80-100 mm^3, 10 doses of 100 ug ANDES1537 were injected via intra-peritoneal every other day. We observed, that treatment induce a strong delay in tumor growth in vivo, suggesting that this antisense approach can be a novel a potent therapeutic tool for prostate cancer therapy.

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Development of GalNAc-conjugated saRNA targeting HNF4A for treatment of metabolic disease

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Hepatocyte nuclear factor 4 alpha (HNF4A) is a liver-enriched transcription factor and master regulator of hepatic function. HNF4A expression is downregulated in chronic liver disease, and the expression of HNF4A correlates with the level of liver dysfunction. We have developed a small activating RNA (saRNA) that upregulates the rodent HNF4A gene in primary hepatocytes in vitro and in vivo. In a rat high-fat diet model, HNF4A expression was downregulated compared to normal animals, and treatment with HNF4A saRNA complexed with a PAMAM dendrimer restored HNF4A expression to the level of normal animals. Intravenous injection of dendrimer-HNF4A saRNA significantly reduced the body weight, liver cholesterol, and blood glucose of high-fat diet rats.

For subcutaneous administration of HNF4A saRNA to treat chronic metabolic diseases such as non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH), we have developed GalNAc-conjugated HNF4A saRNAs for hepatocyte-specific delivery. Fully modified saRNA oligonucleotides activate HNF4A expression by transfection in primary rat hepatocytes in a dose-dependent manner. When conjugated to a triantennary GalNAc cluster, GalNAc-HNF4A saRNAs show dose-dependent upregulation of HNF4A and downstream target gene regulation by ASGR receptor-mediated uptake in primary rat hepatocytes.

When injected subcutaneously in normal mice, GalNAc-HNF4A saRNA conjugates upregulate HNF4A expression in the liver. Optimization of dose and schedule is ongoing, as well as efficacy in disease models. These data demonstrate that GalNAc-mediated delivery of saRNA is possible to upregulate hepatocyte-specific targets, and HNF4A is a promising target for treatment of NAFLD/NASH.

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Evaluation of Antisense Oligonucleotides Targeting Human mRNA Using Chimeric Mice with Humanized Liver

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Antisense oligonucleotides (ASOs) are molecules targeting gene sequences selectively, and it is difficult to predict the efficacy and safeness for human using general animal models. Thus, we focused on PXB-mice which are engrafted human hepatocytes as a model to predict the effects of ASOs on the human liver.
We administered GalNAc-conjugated or unconjugated ASOs, which were selected by in vitro screening, to PXB-mice at a dose of 1 - 100 mg/kg. The sequence of PCSK9-ASO targets human and mouse mRNA, and that of apoC-III ASO is human mRNA specific. Some days after the injection, we quantified target mRNA and conducted histopathological analysis. Besides, we observed time course of the gene silencing effect of the GalNAc-conjugated PCSK9-ASO. Next, the apoC-III targeting ASO were injected to monkeys at a single dose of 3 mg/kg to confirm whether the effect of the ASO on PXB-mice reflects that on a normal animal model.
As a result, the human mRNA silencing efficacy of both PCSK9-targeting ASOs and apoC-III-targeting ASO was observed in the liver of PXB-mice. However, the efficacy of PCSK9-ASOs in PXB-mice was lower than that in normal mice, and the effect reached a maximum ten days after the administration in PXB-mice, which was seemed to be later compared to normal mice. This result suggested that there are differences between the pharmacokinetics of ASOs in PXB-mice and that in normal mice. While the PCSK9 ASO acted to mouse and human PCSK9 mRNA, the apoC-III ASO showed human mRNA specific effect. Moreover, serum triglyceride levels that are a biomarker of apoC-III inhibitors were notably decreased in monkeys treated with this ASO. As regards the safeness, serum ALT levels of PXB-mice treated with PCSK9-ASOs at an excess dose dramatically elevated. On the other hand, the damage to human hepatocytes in the liver of PXB-mice was mild histopathologically. However, this reason was unclear, and additional experiments are needed to explain this result.
Based on the above, we anticipate PXB-mice will serve as a good model to predict the effects of ASOs on primates and be useful for screening of drug candidates.

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Block or Degrade? Balancing On- and Off-Target Effects of Antisense Strategies Against Transcripts with Expanded Triplet Repeats

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Antisense oligonucleotides (ASOs) have gained momentum as therapeutics for neuromuscular disorders. For microsatellite repeat expansion disorders such as Huntington's disease, *C9orf72*-amyotrophic lateral sclerosis and myotonic dystrophy, ASOs have not reached the clinic yet. Antisense therapies in repeat expansion disorders are based on preventing translation of expanded repeats or inhibiting sequestration of RNA-binding proteins. This can be achieved both by steric hindrance and by degradation of the transcript, and it is unknown which approach is superior.

We directly compared blocking ASOs with RNase H-recruiting gapmers in a human myotonic dystrophy type 1 cell model. Two target sequences were selected: the triplet repeat and a unique sequence upstream of the repeat. We assessed effects of the ASOs on transcript levels, ribonucleoprotein foci and disease-associated missplicing, and performed RNA sequencing to investigate on- and off-target effects on a transcriptome-wide level. The repeat blocking ASO was most effective in displacing MBNL1 protein and associated correction of splicing, and furthermore had the fewest off-target effects. By comparison, the off-target profile of the repeat gapmer discourages its further therapeutic development. Overall, our results demonstrate the importance of evaluating multiple read-outs of ASO activity and provide general principles for the safe and effective targeting of toxic repeat transcripts.

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LNP-*hagl* mRNA Reduced Liver Glycogen Accumulation and Decreased Hepatic Hypertrophy in a Mouse Model of Glycogen Storage Disease III

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Glycogen storage disease type III (GSDIII) is an inherited metabolic disorder caused by the deficiency of the glycogen debranching enzyme (GDE, gene name: *agl*), leading to an inability to completely catabolize glycogen to glucose. Deficiency in GDE results in pathological accumulation of limit dextrin in liver and muscle, and an increased risk for hypoglycemia, liver fibrosis, and myopathy. Here, we examined the pharmacology associated with treatment of a codon-optimized *agl* mRNA formulated into a lipid nanoparticle (LUNAR®) in an $AGL^{-/-}$ mouse model, which mimics the clinical, biochemical and pathological phenotypes of human GSDIII. We showed that a single systemic injection of LUNAR-*hagl* mRNA results in a reduction of liver glycogen and reduced hepatocyte vacuolization in a dose dependent manner. In $AGL^{-/-}$ mice maintained on a standard diet and ad hoc feeding, a single dose of 3 mg/kg mRNA treatment led to the reduction of liver glycogen as early as 24 hours post-treatment which was maintained for over 3 weeks. Our results demonstrate that treatment with LUNAR-*hagl* mRNA effectively rescued the liver phenotype in a mouse model of GSDIII and supports continued development of an LUNAR-*hagl* mRNA as a therapeutic approach for the treatment of GSDIII.

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Identification and functional analysis of potent TLR7- and TLR8-specific RNA oligonucleotide agonists for immunotherapy

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Immune sensing of nucleic acids by innate immune receptors such as TLR7 and TLR8 elicits potent immune responses. Both TLR7 and TLR8 are located in the endolysosome of certain immune cells. While TLR7 preferentially detects short dsRNA, TLR8 is activated by ssRNA and ssRNA cleavage products. Interestingly, TLR7 and TLR8 are phylogenetically similar but are expressed in different immune cell subsets. While TLR7 is primarily expressed in pDCs and B cells, TLR8 is highly expressed and functional in human primary monocytes. TLR7 activation in pDCs induced the secretion of high amounts of type I interferons. TLR8 activation in primary monocytes resulted in the secretion of Th1 inducing IL-12p70 and pro-inflammatory cytokines but not type I IFN.
Based on structural insight we designed an array of TLR7 and TLR8 specific RNA oligonucleotides. The minimal structural motif sufficient for full TLR7 activation is a single G•U wobble base pair within a non U-containing sequence context. Based on the structural requirements for TLR7 agonist activity, we were able to develop TLR8 selective RNA oligonucleotide agonists. Comparing our RNA oligonucleotide agonists to different small molecule agonists of TLR7 and TLR8 in vitro we demonstrate that our ligands were less toxic and more potent. Interestingly, the functional response to our TLR8 ligands not only substantially differed in the quantitative response but also exhibited a superior qualitative response pattern when compared to the respective small molecule TLR7/8 ligands. Because rodent in vivo models can not be applied for preclinical development of TLR8 RNA oligonucleotide agonists, ferrets (influenza) and woodchucks (hepatitis B) were identified as suitable animal models. Our results suggest that our TLR8 ligands due to their favorable functional characteristics are highly attractive compounds for the clinical development as anti-infectives and vaccine adjuvants as well as for cancer immunotherapy.

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Functional features defining the efficacy of cholesterol-conjugated, self-deliverable, chemically modified siRNAs.

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The use of heavily modified unformulated siRNA conjugates becomes a major mode in RNAi therapeutic development. The introduction of extensive chemical modifications is essential for conjugate-mediated delivery. Modifications have a significant impact on siRNA efficacy through interference with recognition and processing by RNAi enzymatic machinery, severely restricting the sequence space available for siRNA design and finding functional modified siRNA sequences is a daunting task requiring extensive screening. Many algorithms available publicly can successfully predict the activity of non-modified siRNAs, but the efficiency of these algorithms for designing heavily modified siRNAs has never been systematically evaluated experimentally. Here we screened several hundred cholesterol-conjugated siRNAs with extensive backbone modifications and developed a linear regression-based algorithm that effectively predicts siRNA activity using two independent datasets. We further demonstrate that predictive determinants for modified and non-modified siRNAs differ substantially. The algorithm developed from the non-modified siRNAs dataset has no predictive power for modified siRNAs and vice versa. In the context of heavily modified siRNAs, the introduction of chemical asymmetry fully eliminates the requirement for thermodynamic bias, the major determinant for non-modified siRNA efficacy. We further experimentally demonstrated the applicability of the developed algorithm to other modification patterns and evaluated the effect of the accessibility of the region 3' neighboring siRNA target site on siRNA efficacy. The development of the prediction algorithm for heavily modified siRNA conjugates opens a way for their adoption as research tool for ex-vivo and in-vivo functional genomics.

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Efficient delivery of RNAi to T-cells using hydrophobic siRNA conjugates.

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Cell-based therapies, especially those based upon the use of CAR T-cells, are quickly becoming one of the major approaches for cancer treatment. Although they have demonstrated high efficiency in treating hematological cancers, application of CAR T cell technology for the treatment of solid tumors has proven to be significantly more challenging. One of the major hypothesized reasons for the observed low efficacy of CAR T-cells in solid tumors are the inhibitory effects of the tumor microenvironment (TME). One approach to bypass the effects of the TME is to inhibit the functionality of immune checkpoints or of the pathways leading to immune suppression.
We demonstrate that the use of self-deliverable RNAi technology (sdRNAi), based upon the use of fully modified siRNA conjugates, is an efficient way to transfect T-cells and induce long-lasting suppression of gene expression. Transfection of T-cells is achieved via passive uptake of sdRNAs added to the cell culture media without the use of traditional transfection reagents or methods (i.e. electroporation). The use of sdRNAi results in nearly 100% transfection efficiency with up to 90% reduction of target gene expression lasting over multiple cell divisions.
We have successfully screened and selected sdRNAs targeting several immune checkpoints and have demonstrated simultaneous knockdown of multiple targets.
Our approach can be used as strategy to enhance the effect of CAR T-cells and other cell-based therapies as well to improve the manufacturing of therapeutic immune cells.

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Validation of mitochondrial nucleic acid therapy by the mitochondrial delivery of therapeutic RNAs

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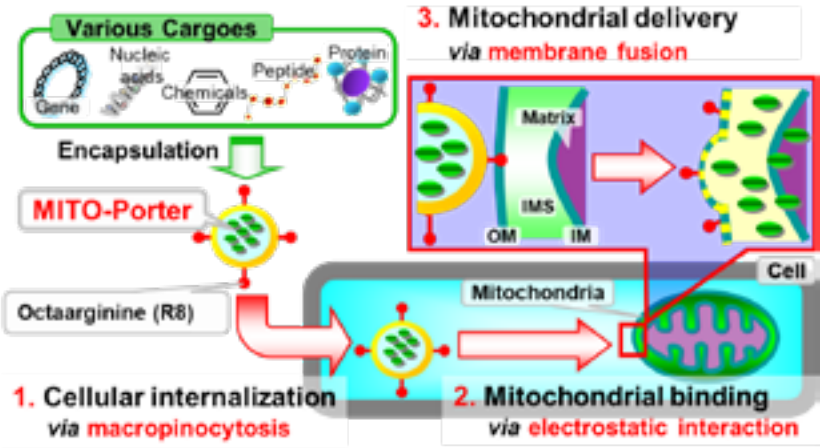
Mitochondria contain multiple mitochondrial DNAs (mtDNAs), with several thousand copies of mtDNA contained in one cell. Mitochondrial gene diseases are classified as homoplasmic mutations in which only mutant mtDNA exists and heteroplasmic mutations in which mutated mtDNA and normal mtDNA co-exist. In the case of heteroplasmic mutations, it has been reported that when the mutation rate of mtDNA exceeds a certain threshold value, disease symptoms associated with mitochondrial function develop. In this study, to validate a mitochondrial gene therapeutic strategy using cells derived from patients with a mitochondrial disease, primary cultured skin fibroblasts obtained from a skin biopsy of a patient with a mutation in mitochondrial RNA were examined. To date, it has been reported that several RNA sequences can be transported from the cytoplasm to mitochondria, but the efficiency of this transport is very low. In order to transport much larger amounts of RNA to mitochondria, a MITO-Porter, a liposome-based carrier for the mitochondrial delivery was developed in our laboratory, and was found to be a useful strategy for achieving mitochondrial delivery. The MITO-Porter is internalized into cells and delivers encapsulated molecules to mitochondria *via* membrane fusion, a process that is independent of its size and physical properties. Therefore, this system could be used for the direct mitochondrial transfection of nucleic acids. In this study, we report in attempts to deliver wild-type mitochondrial RNA using a MITO-Porter, in an attempt to decrease the mutation rate of RNA in mitochondria in a mitochondrial disease cell. The resulting mutant heteroplasmy levels were examined by an amplification refractory mutation system - quantitative PCR. We also investigated the influence of this on the mitochondrial respiratory activity associated with the decrease in the mutation rate.



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NOVEL DRUG DELIVERY SYSTEM FOR TARGETING EXOSOMAL MICRORNA

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Recently, mRNAs and microRNAs (miRNAs) have been identified in exosomes, which can be taken up by neighboring or distant cells. It has also been reported that such miRNAs (exosomal-miRNAs) regulates gene expression in the recipient cells. MiRNAs are a type of non-coding RNA that induce post-transcriptional gene silencing of their target genes and regulate a wide range of biological processes, including apoptosis, differentiation, metabolism, and cell proliferation. According to recent reports, the aberrant expression of miRNAs is associated with most pathological disease processes, including carcinogenesis. Therefore, circulating onco-miRs are considered as significant therapeutic targets for cancer therapy. However, there is no report to regulate the function of miRNAs in exosomes. In this study, we try to develop novel drug delivery system using anti-exosome antibody-oligonucleotide conjugates (ExomiR-Tracker) for functional inhibition of circulating miRNAs. The "ExomiR-Tracker" is the world's first innovative molecule which has targeting property for exosome-recipient cells and specifically deliver nucleic acid medicines to the target cells. We have found that ExomiR-Tracker can bind to the surface of exosomes and that the complexes are introduced into exosome-recipient cells then inhibit the activity of miRNA. We showed that ExomiR-Tracker can accumulate to not only primary cancer cells but also metastatic cancer cells after intravenous administration. Existing technologies have difficulties for introducing anti-miR into exosomes and extremely low possibility to deliver anti-miR to exosome-recipient cells after administration (ex. intravenous) into body. Development of inhibition technology against exosomal-miRNA has not achieved so far.

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Y-shaped block catiomer (YBC) forms "Unit Polyion Complex" with siRNA and ASO: A Novel DDS alternative to LNP

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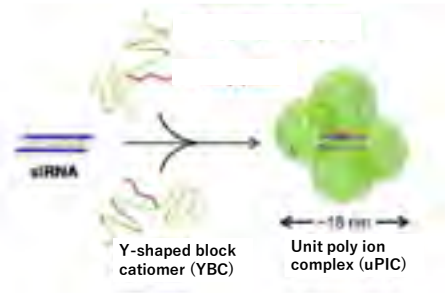
Delivery is a still key challenge to expand therapeutic application of oligonucleotides such as siRNA and antisense oligonucleotide (ASO). Various carriers utilizing cationic lipids have been developed and most of them encapsulated oligonucleotides into ~100-nm nanoparticles. They accumulate in liver well, but many technical problems remain for targeting other tissues. We have developed a novel approach to cancer-targeted oligonucleotide delivery using a Y-shaped block catiomer (YBC) comprising poly cationic amino acids and two-armed poly (ethylene glycol) (PEG)[1)]. The YBC and a single oligonucleotide are electrostatically bound to generate a dynamic ion-pair, termed as "unit polyion complex (uPIC)" (Figure). Owing to both improved stability in the bloodstream and small particle size (~18 nm), this uPIC efficiently delivers oligonucleotides into tumor or tumor microenvironment thus exerting potent anti-tumor activity in subcutaneously and orthotopically transplanted mice tumor models.

We are developing one siRNA and one ASO using this uPIC technology in oncology. The first program is a siRNA which targets PRDM14, a transcriptional factor, highly expressing in metastatic breast cancer cells [2)]. Intravenous injection of the PRDM14 siRNA/YBC-uPIC showed potent anti-tumor activity in several breast cancer mice models. We plan to commence an investigator-initiated Phase I clinical trial in early 2020.



The second program is the ASO/YBC-uPIC targeting TUG1 that is an oncogenic long noncoding RNA. TUG1 is highly expressed in glioblastoma (GBM) and is essential for tumor maintenance [3)]. In a mouse orthotopic model of patient-derived GBM, the TUG1 ASO/YBC-uPIC accumulated into the brain tumor tissue by intravenous administration and showed complete remission with long-term survival effects in the models[1)].

1. S. Watanabe, et al., Nature Commun., 10:1894, 2019.
2. H. Taniguchi, et al., Oncotarget. 8: 46856-46874, 2017.
3. K. Katsushima, et al., Nature Commun., 7:13616, 2016.

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Downregulation of the N-end rule pathway sensitizes cancer cells to chemotherapy *in vivo*

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The last decade has seen major advances in the treatment of cancer. Vaccines, CAR-T cells, checkpoint inhibitors and personalized medicine have redefined cancer therapy and offer promising solutions for previously untreatable diseases. However, some cancers, such as liver cancer, are refractory to immunotherapy, as demonstrated by the recent failure of Keytruda in a Phase III clinical trial in HCC patients (NTC02702401) and the development of new treatment options will rely on the identification of vulnerable molecular pathways and targets.

The N-end rule pathway is an emerging target for anti-tumor therapies, because of its capacity to positively regulate many hallmarks of cancer including angiogenesis, cell proliferation, motility and survival. Thus, inhibition of the N-end rule pathway offers the potential to be a highly effective anti-cancer treatment. Using a siRNA-mediated approach for selective downregulation of the four N-end rule-dependent ubiquitin ligases, UBR1, UBR2, UBR4 and UBR5, we demonstrated decreased cell migration and proliferation, and increased apoptosis in cancer cells. Chronic treatment with lipid nanoparticles (LNP) loaded with siRNA in mice efficiently downregulates the expression of Ubrs in the liver without any significant toxic effects but engages the immune system and causes inflammation. However, when used in combination with a chemotherapeutic drug, downregulation of the N-end-rule pathway E3 ligases successfully reduced tumor load by decreasing proliferation and increasing apoptosis in a mouse model of hepatocellular carcinoma, while avoiding the inflammatory response. Our study demonstrates that Ubr ubiquitin ligases of the N-end rule are promising targets for the development of improved therapies for many cancer types.

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Novel mesyl phosphoramidate antisense oligonucleotides as an alternative to phosphorothioates for efficient silencing of oncogenic microRNA in vivo.

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Forty years of studies proved that antisense oligonucleotides have great potential to target mRNAs of disease-associated genes and noncoding RNAs. Amongst vast number of oligonucleotide backbone modifications, the most widely used in research and clinic is phosphorothioate modification. However, along with merits, there are notable drawbacks of phosphorothioate oligonucleotides such as decreased binding affinity to RNA, reduced specificity and increased toxicity. We report herein on the synthesis and in vitro evaluation of a novel DNA analog, namely, mesyl (methanesulfonyl) phosphoramidate oligonucleotide (mesyl oligonucleotide). A novel type of DNA analogs which substitute mesyl group for the natural phosphodiester group in every internucleotidic position show significant advantages over often used DNA phosphorothioates in their RNA binding affinity, nuclease stability and specificity of their antisense action, which involves activation of cellular RNase H enzyme for hybridization-directed RNA cleavage. In vitro. Biological activity of the mesyl oligonucleotides was demonstrated with respect to pro-oncogenic miR-21, miR-155 and miR-17. A 22 – 24 nt anti-miR mesyl oligodeoxynucleotides specifically decreased levels of corresponding microRNA in melanoma B16 cells, induced apoptosis, reduced proliferation and impeded migration of tumor cells, showing superiority over isosequential phosphorothioate oligodeoxynucleotide in the specificity of its biological effect. Reduced overall toxicity compared to phosphorothioate and efficient activation of RNase H are the key advantages of mesyl phosphoramidate oligonucleotides, which may represent a new promising type of antisense therapeutics[1]. In vivo. The basic features of mesyl oligonucleotides applied as antitumor therapeutics were investigated. Evaluation of kinetics of tumor growth suppression, systemic toxicity, biodistribution and specificity of action as well as primary analysis of pharmacokinetic parameters of new analogs were performed in comparison with phosphorothioate oligonucleotides. Using drug-resistant model of human epidermoid carcinoma KB-8-5/SCID mice it was shown that peritumoral administration of anti-miR-21 mesyl oligonucleotide pre-complexed with folate-containing liposomes provides effective accumulation of therapeutic oligonucleotide in tumor tissue leading to 8-fold reduction in primary tumor growth. It was accompanied by significant increase in the expression of direct protein targets of miRNA-21 such as PTEN and PDCD4 in tumor tissue caused by manifold reduction in miRNA-21 level. No effects on the expression of other miRNAs were observed. Morphometric investigation of liver and kidneys as well as biochemical blood analysis demonstrated that mesyl oligonucleotides are devoid of pronounced toxicity in respect to liver and kidneys. The data obtained give evidence that mesyl-oligonucleotides represent novel vigorous implement of antisense technology.

[1.] Miroshnichenko S. K., et al., Proc. Natl. Acad. Sci U S A. - 2019. - V. 116. - P 1229–1234.
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Antisense oligonucleotides to target *SPTLC1* gene as a therapeutic strategy for hereditary sensory neuropathy type I

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Antisense oligonucleotide (ASO) therapy is successful in neuromuscular disorders, e.g. in Duchene muscular dystrophy and spinal muscular atrophy, by manipulating pre-mRNA splicing. In addition to the splice switching strategies (exon-skipping and exon-inclusion), allele-selective suppression by ASO has recently been investigated in some neurodegenerative genetic diseases and has shown its therapeutic potential. This strategy is potentially applicable to genetic disease caused by gain-of-function mutations. The selective binding of ASO to the mutant pre-RNA and RNA transcripts can activate RNase H activity and consequently induce target RNA degradation. The suppression of the mutant transcripts will diminish the dominant negative effect from the mutation and is hence expected to ameliorate disease symptom.

Hereditary sensory neuropathy type 1 (HSN1) is a rare peripheral neuropathy caused by dominant mutations in the *SPTLC1* gene. There is currently no effective treatment for this disease. Our clinical team at the National Hospital for Neurology and Neurosurgery and the MRC Centre for Neuromuscular Diseases at the University College London, has identified and been caring for the largest cohort of patients with HSN1 in the world. Moreover, we have a cohort within the United Kingdom where all the SPTLC1 patients have a common mutation p.C133W. This provides us the unique opportunity to develop ASO therapeutics by targeting this founder mutation for SPTLC1-related patients.

In this study, we have developed a new therapeutic approach using ASOs to selectively suppress the expression of mutant transcripts in the *SPTLC1* gene. We use patient's fibroblast cell line carrying the dominant p.C133W mutation as a cellular model. We have identified a number of promising lead ASOs which show the significant and specific suppression of the mutant transcripts.

Our study indicates that allele-specific gene silencing is a potential therapeutic strategy for SPTLC1-related HSN1. Further studies include the validation of the lead ASOs on 1) correcting the function of SPTLC1 gene in patient's iPSC–induced sensory neurons; 2) reducing the toxic metabolites in patient's fibroblasts, using the experimental platform established in our laboratories; and 3) in the p.C133W knock-in mouse model.

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Antisense Oligonucleotide Mediated Reduction of SPDEF Regulates Goblet Cell Formation in Multiple Murine Pulmonary Disease Models

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Many chronic pulmonary diseases, including cystic fibrosis (CF), asthma and chronic obstructive pulmonary disease (COPD), are characterized by excessive mucus production and increased numbers of goblet cells. SAM pointed domain-containing Ets transcription factor (SPDEF) is expressed in airway epithelial cells of the conducting airway and regulates goblet cell differentiation and mucus production. Overexpression of SPDEF in mouse lung causes goblet cell metaplasia while deletion of this gene resulted in the absence of goblet cells in the conducting airway epithelium. Here we assessed the effectiveness of mouse SPDEF specific antisense oligonucleotides (ASOs) in a β-ENaC mouse model of CF, a steroid resistant severe asthma (SA) model and a wood smoke (COPD) model. SPDEF ASO delivery to the lungs of mice in multiple models of pulmonary disease resulted in effective reduction of target mRNA. In the β-ENaC CF model with established lung disease, SPDEF ASOs reduced goblet cell metaplasia and mucus production, AHR and neutrophil recruitment to the airways. In the severe asthma model, SPDEF ASOs were also effective at reducing mucus genes, PAS-positive mucus and AHR. In the COPD model, the wood smoke but not filtered air induced SPDEF mRNA and other mucus genes which were all reduced by SPDEF ASO inhalation. In addition, AHR was improved. In summary, SPDEF inhibition by ASO reduced mucus production and prevented or reversed goblet cell metaplasia in CF, SA and COPD mouse models. These data demonstrate that ASO inhibition of SPDEF in airway could be an effective approach for the treatment of multiple pulmonary diseases with excess mucus.

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Impact of the Route of Administration on CNS-active siRNA Biodistribution in Large Animal Brains.

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Small interfering RNAs (siRNA), comprise a novel class of drugs that can be chemically engineered to silence any target gene and distribute to a variety of tissues. We recently identified a novel siRNA chemical scaffold that distributes throughout and silences target gene expression in the central nervous system of mice and non-human primates after administration via direct intracerebroventricular (ICV) injection: Di-siRNA (Alterman et al., Nature Biotech in press).

In mice, distribution of both ASOs and siRNAs is significantly affected by placement of administration, where ICV administration supports widespread brain distribution while intrathecal mostly delivers to the spinal cord and outside of the cortex. Given the smaller brain size and differences in CSF circulation dynamics relative to humans, the translatable potential of results generated in mice is limited. Clinical administration of oligonucleotides to CSF is possible via use of ommaya reservoir, an analog of ICV injection, or direct intrathecal injection. Thus, understanding the impact of route of administration on oligonucleotide distribution in larger brains is necessary.

To determine the impact of administration route on di-siRNA distribution, we compared four different administration methods in Dorset sheep: intra-striatal (IS, 1.6 mg), intracerebroventricular (ICV, 50 mg), intrathecal catheter to the cisterna magna (ITCM, 50 mg), and repetitive dosing into the lateral ventricle through an implanted reservoir device (RD, 4x25 mg weekly). All animals were taken down and distribution was evaluated 48-hours post final injection.

All routes of administration tested had no appreciable toxic effects and no changes from baseline chemistry or complete blood counts. As expected, the IS route resulted in distribution to the striatum only, supporting region-selective silencing. Interestingly, ICV and ITCM administration resulted in similar distribution and similar guide strand accumulation across all brain regions. Administration via implanted reservoir and repetitive dosing resulted in similar distribution to ICV and ITCM, however, we observed increased guide strand accumulation in all brain regions, specifically, deeper brain regions including the caudate, putamen, and hippocampus.

This is the first side-by-side studying comparing the impact of different routes of administration on di-siRNA distribution and accumulation in the CNS of large animals. These results suggest that placement of siRNA injection (ITC vs ICV) has minimal impact on the overall distribution. However, the use of loading dose might be beneficial for long-term silencing in the deeper brain structures. These results integrate basic science with novel clinical radiology and imaging techniques and have the potential to impact the planning and execution of preclinical and clinical studies using oligonucleotide therapeutics in the central nervous system.

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