Doctoral Thesis

Properties of polyamine-conjugated oligonucleotides

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Properties of polyamine-conjugated oligonucleotides

A thesis submitted to attain the degree of
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Abstract

Oligonucleotide drugs show great promise as a third major therapeutic platform after small molecules and biologicals, however their properties present great challenges with respect to both pharmacokinetics and pharmacodynamics. Numerous chemical modifications to the phosphate backbone, the ribose or the nucleobases have improved target RNA binding affinity, resistance to ubiquitously expressed nucleases as well as circulation time and conjugation with targeting moieties has assisted cell-type selective uptake. The biogenic polyamines spermine, spermidine and putrescine are known to preferably associate to RNA and to strengthen hybridization affinity. Mono- and polyaminogroups, which are positively charged at physiologic pH, have been appended to oligonucleotides in an effort to reduce electrostatic repulsion during hybridization to complementary RNA but proved little useful with respect to binding affinity.

Here, we present conjugation of polyamines to pyrimidines via a triazole linker, positioning the polyamine fragment in the major groove of an RNA duplex similar to binding of endogenous polyamines to structured RNAs in cells. Exceptional hybridization affinity increases of up to 12 °C for a single modification were observed. Surface plasmon resonance (SPR) studies showed that the increases derived predominantly from accelerated annealing rates $k_{on}$, predisposing polyamine-conjugation for use in oligonucleotides operating catalytic systems such as RNase H-competent gapmers. The high target binding affinity however failed to translate to enhanced potency in cellular assays. We discovered that tethering polycationic fragments to oligonucleotides reduced uptake in lipofection-based cellular assays, possibly due to a charge-mediated interference with the transfection process.

Our studies on a novel polyamine-oligonucleotide conjugate illustrate approaches to assess the potential of RNA modifications in vitro. We argue that binding kinetics are valuable information with respect to identification of a suitable target system and are more predictive for in vivo potency than standard melting temperature. Our results further suggest that when testing new oligonucleotide modifications in state-of-the-art transfection-based cellular assays, uptake efficiency should systematically be controlled, and that gymnastic delivery might represent an attractive alternative. Moreover, the triazole-linked polyamine fragments confer potentially useful properties for a wide range of pharmaceutical and diagnostic applications where increased annealing rates of oligonucleotides are advantageous.
Zusammenfassung


Abbreviations

A adenosine
ACN acetonitrile
AcOH acetic acid
Ago argonaute (protein)
APAO acetylpolyamine oxidase
ApoB apolipoprotein B
ApoC apolipoprotein C
ASO antisense oligonucleotide
BPS biphenylsulphonyl
BTT benzylthioutetrazole
C cytidine
CD circular dichroism
CMV cytomegalovirus
CNS central nervous system
CPG controlled pore glass (solid support)
CSO (1S)-(+)-(10-camphorsulfonyl)oxaziridine
CuAAC copper-catalyzed azide-alkyne cycloaddition
DCI 4,5-dicyanoimidazole
DIPEA N,N-diisopropylethylamine
DMAP dimethylaminopyridine
DMDF dimethylformamide
DMSO dimethylsulfoxide
DMT dimethoxytrityl
EDC N-ethyl-N'-(dimethylaminopropyl)carbodiimide
EPP erythropoietic protoporphyria
FECH ferrochelatase
G guanosine
GalNAc N-acetyl-galactosamine
HCV hepatitis C virus
HFIP hexafluoro-2-propanol
HPLC high pressure liquid chromatography
i.v. intravenous
ICAM-1 intercellular adhesion molecule 1
IRS-1 insulin receptor substrate 1
$K_a$ equilibrium association constant
$K_D$ equilibrium dissociation constant
$k_{off}$ dissociation rate constant
$k_{on}$ association rate constant
LC liquid chromatography
lncRNA long non-coding RNA
mC 5-methyl-cytidine
miRISC miRNA-loaded RNA-induced silencing complex
miRNA microRNA
MM12 MerMade12 synthesizer
MM192 MerMade192 synthesizer
MOE methoxyethyl
mRNA messenger RNA
MS mass spectroscopy
NHS N-hydroxysuccinimide
NMI N-methylimidazole
NMP N-methyl-pyrolidone
NMR nuclear magnetic resonance
1. Introduction

1.1. RNA as drug target

Currently, the majority of drugs on the market target proteins. Diseased conditions can be modulated by addressing specific proteins, whose malfunction or absence is critical in the pathogenesis, blocking, replacing or correcting their function. Most often, small molecules are used, inhibiting or activating receptors or enzymes. Some critical proteins can however not be targeted by small molecules due to lack of accessibility or selectivity of the targeting agent. Biologicals, which by now are a well-established class of therapeutic entities, permit almost ultimate specificity, however they suffer from unfavourable pharmacokinetics and immunogenic potential [1]. Still, there are disease modifying proteins that remain undruggable.

A step upstream of malfunctioning proteins in the central dogma of molecular biology is RNA: these include messenger RNA (mRNA) coding for proteins, ribosomal RNA (rRNA) and transfer RNA (tRNA) playing key roles in translation, microRNA (miRNA) involved in post-transcriptional regulation of gene expression and other non-coding RNAs. Targeting at the RNA level may virtually address any defective cellular function. A very limited range of small molecules is able to target RNA, mainly antibiotics binding bacterial rRNA, such as aminoglycosides, macrolides, clindamycin, linezolid or chloramphenicol. Biologicals can hardly reach intracellularly located RNA [2]. The molecular entities most suitable to selectively bind such a hydrophilic target are oligonucleotides, hybridizing with perfect or partial complementarity through Watson-Crick base pairing to their target RNA. This concept promises broad applicability owing to the universal code of 4 main units as well as full knowledge of genome sequences.

1.2. RNA derivatives as drug

After small molecules and biologicals, therapeutic oligonucleotides are about to establish themselves as an additional treatment option. Only recently 3 oligonucleotide drugs have been approved for the treatment of rare diseases: mipomersen [3], eteplirsen [4] and nusinersen [5]. Following their hyperbolic inception, failure and disappointment retarded development of oligonucleotide drugs, but after extensive technology maturation the new drug discovery platform is back on the road to success [6]. Their informational nature fits well into the postgenomic era of medicine [7,8]. The compelling benefit of oligonucleotide drugs is a conceptual separation of the pharmacophore, defined by its nucleotide sequence, and the dianophore (from the Greek dianomi, “distribution, delivery”) determining pharmacokinetics of a drug [8]. The dianophore can be optimized independently of the pharmacophore, playing on chemical structure and architecture such as sugar, backbone or base modifications or conjugation of a targeting ligand. The powerful advantages of the pharmacophore are, given a certain length of the nucleotide sequence, its very high target binding affinity and selectivity. With the human genome containing approximately 3 billion base pairs, the shortest sequence of \( N \) bases that is assumed to be unique is a 16mer, based on the expected occurrence of the sequence within the genome, which equals complexity/4\(^4\). The RNAome of a typical eukaryotic cell, reflecting the active genes, is considerably smaller in a sample cell than its genome, but is thought to cover nearly the entire genome at the level of a higher organism. A large proportion of cellular RNA are regulatory RNAs, probably more than those encoding proteins [9]. The function of long non-coding RNAs (lncRNAs) seems to be strongly determined by secondary structure rather than primary sequence [10]. Thus, lncRNAs might be less susceptible to targeting by hybridization-based drugs than coding RNAs. Though, even coding RNAs are folded to a large extent and associated with proteins, further restricting the number of accessible target sites. Despite the vast expansion of the theoretical target space in recent years, oligonucleotides much shorter than 16mers are considered specific. As an example, an oligonucleotides as short as 7 bases was suggested to allow for sufficient target selectivity due to limited accessibility of alternative target sites buried in folded RNA [11].
Natural RNA has however very unfavorable properties in terms of use as a drug. First of all, numerous defense mechanisms have evolved to protect mammalian cells from invading foreign RNA. Nucleases in the blood rapidly degrade foreign nucleic acids and therefore, unless chemically protected, nucleic acid drugs are cleared from the circulation very quickly [12]. Receptors of the innate immune system such as the toll-like receptors 3, 7 and 8 on the outside of cells or double-stranded RNA receptors on the inside of cells recognize and trigger elimination of foreign RNA [13]. Hence, susceptibility to endo- or exonucleolytic cleavage is an intrinsic problem of oligonucleotide drugs. Second, naked charged oligonucleotides are rapidly cleared from the circulation by the kidneys [14]. But most importantly, the biggest obstacle in successful delivery of therapeutic oligonucleotides is the lipid bilayer [6]. Oligonucleotides are big molecules and are very hydrophilic, therefore facing difficulties in crossing barriers such as a cellular membrane. Lipinski, medicinal chemist at Pfizer (at the time), once formulated the “rule of 5”, defining criteria which make a molecule a good drug candidate with respect to oral bioavailability [15]. His analysis revealed that absorption and permeation were more likely if a molecule had a molecular weight below 500 Da, its LogP (distribution coefficient) was below 5, the sum of its hydrogen bonding donors was below 5 and it had no more than 10 hydrogen bonding acceptors. Oligonucleotides violate all of these criteria. They enter cells via endocytosis and therefore need to make their way out from the endosomal compartment into the cytosol in a non-toxic way [16,17]. This is a major key technological problem to solve in oligonucleotide drug delivery. In theory, if the size of an oligonucleotide is decreased in order to alleviate challenging pharmacokinetics, target binding affinity is lost and needs to be compensated by affinity-enhancing chemistries. Therefore, to modulate properties of oligonucleotides, chemical modification is needed.

1.2.1. Medicinal chemistry of therapeutic oligonucleotides

A large number of chemical modifications on either the phosphate backbone, the sugar or the nucleobases have been developed to optimize target binding affinity, to increase nuclease resistance or to modulate pharmacokinetics and are summarized in a number of reviews [8,18-21] (Fig. 1). Nucleobase modifications can enhance target RNA hybridization affinity as well as base-pairing specificity. Modifications to the ribose predominantly address hybridization affinity and nuclease stability, while backbone modifications modulate nuclease stability as well as pharmacokinetic properties. Besides, conjugation of lipophilic or targeting moieties can alter protein binding and tissue distribution.

![Figure 1: Sites of chemical modification and examples of modifications used in clinically approved oligonucleotide drugs. Adapted from [22] and [23].](image)

The most common backbone modification is substitution of one of the non-bridging oxygens by a sulfur, resulting in a phosphorothioate (PS) linkage [24]. While the negative charge of the backbone is maintained, a chiral center is introduced at every internucleosidic linkage as the negative charge resides on the sulfur atom exclusively [24]. The two most important strengths of PS are that they increase resistance towards nucleases and their highly
favourable impact on oligonucleotide trafficking and uptake [25,26]. PS modification mediates protein binding, which prolongs the circulation time of PS-modified oligonucleotides before their excretion by the kidneys, but on the other hand may also result in sequence-independent effects [27,28]. Additionally, PS are compatible with RNase H cleavage. Their main drawback however is a decreased target binding affinity [28]. The two diastereomers arising from sulfurization have different advantages and drawbacks. The Sp PS diastereomer of DNA is highly stable against nucleases but has low binding affinity towards complementary RNA, while for the Rp isomer was found the opposite in terms of nuclease resistance and hybridization affinity [29,30]. As opposed to DNA, the Rp PS diastereomer in duplex RNA was shown to be more resistant and to protect siRNAs against nucleolytic degradation [31]. For effector functions including proteins such as RNase H (see below), it can be expected that precise patterns of alternating phosphate stereochemistry would result in diastereomers with optimal properties [32]. Even though the PS is one of the earliest modifications – PS DNA is called 1st generation modified oligonucleotides – it is still widely used in therapeutic oligonucleotides in the clinic. A number of other backbone modifications have been developed, some of which have clinical relevance and are discussed below (Fig. 2).

Thiophosphoramidates arose from phosphoramidates, replacing the 3’-oxygen of the PO linkage with a nitrogen [33]. They exhibit high binding affinity to complementary RNA but do not elicit RNase H cleavage. In contrast to phosphoramidates, thiophosphoramidates carry a sulfur in place of one of the non-bridging oxygens, which confers them interesting properties with respect to circulation half-life due to protein binding and increased stability. Thiophosphoramidates proved useful as antitelomerase agents in multiple myeloma and lymphoma models [34-36]. Phosphorodiamidates morpholinos (PMOs) represent an isostere of the sugar-phosphate backbone. The furanose has been replaced by a six-membered morpholino ring and the phosphodiester was changed to give a neutral phosphorodiamidate linkage [37]. PMOs have intermediate binding affinity but are extremely resistant to nucleases, on the other hand RNase H activity is not supported by this heavily modified backbone. Conjugation to positively charged peptides was used to enhance uptake efficiency of otherwise poorly penetrating oligomers despite their neutral character [38]. Thus, they are predominantly used in splice-switching applications such as for the treatment of Duchenne muscular dystrophy [39,40]. Even though structurally very different from PMOs, peptide nucleic acids (PNAs) display similar characteristic features to PMOs. Their heavily modified and neutral backbone, composed of a peptidic scaffold replacing the sugar-phosphate structure, is highly resistant to nucleases [41,42]. Watson-Crick base-pairing is maintained and yields good hybridization affinity. Penetration of cell membranes is a major obstacle and requires conjugation to uptake enhancing moieties, which also help to alleviate poor aqueous solubility and protein binding [43].

![Figure 2: Selected backbone modifications of therapeutic oligonucleotides.](image-url)
switch between A-form and B-form, but preferentially is in B-form. An A-form helix has a deep and narrow major groove and a wide and shallow minor groove, while a B-form helix has a wide but less deep major groove and a narrower minor groove [44]. 2'-substituents are situated in the minor groove of a double helix. Electron-withdrawing groups on the 2'-position bias the ribose towards C3' endo sugar puckering, leading to enhanced hybridization affinity to complementary RNA. 2'-modifications destined for the clinic include 2'-O-methyl (2'-OMe) [45], 2'-fluoro (2'-F) [46], 2'-O-methoxyethyl (2'-MOE) and the 2'-O, 4'-C-methylene linked bicyclic ribonucleotides called locked nucleic acid (LNA) [47,48] or constrained ethyl (cEt) [49], all of which display enhanced target binding affinity and reduced nuclease susceptibility (Fig. 3). The affinity ranking against complementary RNA is 2'-OMe < MOE < 2'-F << LNA = cEt, due to their varying conformational restriction in sugar puckering [19]. 2'-OMe, as first described by Inoue et al. [50], is often well tolerated owing to its small size and its natural occurrence and has highly beneficial effects on target RNA hybridization affinity, nuclease resistance as well as immune tolerance [8,51,52]. It has been widely used in various kinds of antisense applications such as in early attempts to create RNase H-competent gapmers [53,54] or to modulate mRNA splicing [55,56]. However, the most advanced 2'-OMe splice-switching oligonucleotide (SSO), drisapersen for the treatment of Duchenne muscular dystrophy, was discontinued early 2016 due to failure in meeting its primary endpoint in a phase III clinical trial [4,56]. As a successor of 2'-OMe, the 2'-methoxyethyl (MOE), is the most advanced 2'-modification by now and has entered the clinic in various oligonucleotide formats and for multiple indications [57,58]. Two out of three hybridization-based oligonucleotide drugs currently on the market are 2'-MOE substituted, mipomersen targeting ApoB for the treatment of familial hypercholesterolemia [3] and nusinersen used in spinal muscular atrophy [5]. Besides inducing C3' endo ribose conformation, it is assumed to form a shell of hydration covering the adjacent phosphate, which further rigidifies the A-form helix geometry [59]. Some of the early clinical candidates and with Pegaptanib even an approved drug contained 2'-F modified nucleosides. The conformation of the furanose ring is highly biased towards the C3'-endo sugar pucker and this most likely is the reason for increased RNA hybridization affinity, along with an enthalpy-driven positive effect on Watson-Crick base pairing as well as base stacking, since, in contrast to 2'-OH, 2'-OMe or 2'-MOE, fluorine does not participate in interactions with water molecules [60]. However, despite its very high binding affinity increase, this modification is now less often used, probably due to toxicological concerns [61]. The bicyclic LNA enforces a C3'-endo sugar pucker by permanently “locking” the ribose and thereby dramatically improves hybridization to complementary RNA [47,48,62]. It is most often used in chimeric oligonucleotides, interspersing LNA nucleotides at certain positions within a PS DNA oligonucleotide. LNA have found a multitude of both diagnostic and therapeutic applications [63], however, LNA-modified antisense oligonucleotides were associated with sequence-dependent hepatotoxicity [64,65]. This has stimulated efforts to synthesize and evaluate a wide range of bicyclic sugar analogs with the aim to improve the toxicity profile [66]. A resulting promising example is the S-cEt. Owing to its LNA-like enhancements in target RNA binding affinity, its even higher nuclease resistance and its favourable toxicity profile made S-cEt the next generation chemistry platform at Ionis Pharmaceuticals [66]. Due to the fact that 2'-substitution greatly reduces or fully abolishes RNase H cleavage gapmers have evolved, joining the advantages of high binding affinity and nuclease resistance by 2'-modified nucleosides in the terminal parts (“wings”) and RNase H compatibility with an internal DNA gap (see 1.2.2.1).
Watson-Crick base pairing takes place between nucleobases, restricting the scope for heterocycle modifications. The most popular sites for substitution are the 4- and 5-positions of pyrimidines and the 6- and 7-positions of purines, which are exposed to the major groove and at distance from the glycosidic bond, thus avoiding interference with base pairing, steric hindrance or any influence on the overall helix geometry. A large number of nucleobase modifications have been developed, mainly aiming at enhanced hybridization affinity due to additional hydrogen bonding or increased stacking interactions, or, alternatively, to modulate immunostimulatory properties of the oligonucleotide. However, very few of those ever made it to the clinic. The most prominent example is methylation at position 5 of pyrimidines, as for example in 5-methyl-dC (5mC), which increases duplex stability by about 0.5 °C per modification and avoids recognition of CpG motifs by toll-like receptor 9 [67,68]. In contrast to 5-methylated pyrimidines, 7-deazaguanosine has been shown to stimulate toll-like receptor 9, potentially advantageous in the context of cancer, infections or vaccines [69,70]. Among the many nucleobase modifications which have not entered the clinic two particular ones should be highlighted: 5-propynyl-uridine and the G-clamp, outstanding for their enormous binding affinity increase [71,72]. However, severe in vivo toxicity of 5-propynyl-U [73] and restricted cellular uptake of G-clamps [74] are prohibiting their use in therapeutic oligonucleotides (Fig. 4).

1.2.2. Mechanisms of action of therapeutic oligonucleotides
Typically, therapeutic oligonucleotides are targeting RNA. They may address pre-mRNA at the level of splicing or mature mRNA, if overexpressed and/or toxic, coding for a toxic protein or interfering with the regulation of other genes. Alternatively, they may also target miRNA or other non-coding RNAs. Mechanistically, therapeutic oligonucleotides either sterically block access of critical cofactors or catalyze enzymatic degradation of the target RNA. Besides, oligonucleotides may also directly target proteins. Numerous formats have been developed and will be presented in the following (Fig. 5). Of note, the first 3 formats (antisense oligonucleotides, siRNAs, ribozymes) operate a catalytic mechanism, while the last 3 (SSO, aptamers, antimirs) act through steric blocking.

Figure 3: Selected sugar modifications in therapeutic oligonucleotides (A) and their ribose conformation (B).

Figure 4: Selected nucleobase modifications.
1.2.2.1. RNase H-competent antisense oligonucleotides

Classical antisense oligonucleotides (ASOs) typically refer to inhibitors of mRNA translation, which recruit RNase H to endonucleolytically cleave the RNA strand of a DNA-RNA heteroduplex [75]. RNase H is an endonuclease, well conserved throughout evolution [76,77], with both nuclear and cytosolic location [78] (see 2.7.3.1). The enzyme is tolerant to PS substitution, however it requires a DNA stretch opposite to the cleavage site of the target RNA. This has lead to the so-called gapmer design, oligonucleotides of 15-20 nt in length with a middle part, called “gap”, consisting of 6-10 2’-deoxynucleotides, and a varying number of modified nucleosides at terminal positions (“wings”) to prolong circulation half-life [74]. Notably, ASOs have catalytic activity. Mipomersen, a 20mer 2’-MOE/DNA PS gapmer, is an approved drug for the treatment of familial hypercholesterolemia [3,79,80], and many more gapmers are currently in clinical trials (see 1.2.5).

1.2.2.2. siRNAs and RNAi

RNA interference (RNAi) is a process inhibiting gene expression intrinsic to eukaryotic cells and can be used as a valuable research tool as well as a therapeutic approach [81]. siRNAs are short 20-24 bp dsRNA, usually with 2 nt 3’-overhangs and a phosphorylated 5’-end. They are loaded into the RNA-induced silencing complex (RISC) and the guide strand mediates recognition of target mRNA, which is then degraded [82]. SiRNAs are unstable in serum, therefore a wide range of chemical modifications has been tested in order to improve stability and activity [83]. To date, therapeutic siRNAs have not entered the market due to more complex delivery issues than in the case of ASOs, but several candidates are in late stage clinical trials (see 1.2.5).

![Figure 5: Central dogma of molecular biology and intervention points of RNA therapeutics. SSO, splice-switching oligonucleotide. Adapted from [20].](image)

1.2.2.3. Ribozymes

Catalytic activity is not restricted to enzymes, but can just as well be carried out by ribozymes. Naturally occurring ribozymes mediate cleavage or ligation of phosphodiester bonds, while artificial ribozymes catalyze a broad variety of chemical reactions [84]. Thus, their conformational and functional flexibility has fueled the RNA world hypothesis.
1.2.2.4. Splice-switching oligonucleotides (SSOs)

Splice-switching oligonucleotides aim at restoring correct splicing by either inducing exon skipping or shifting the ratio between existing splice forms. An antisense oligonucleotide binds to a critical pre-mRNA site, thus altering interactions with splicing factors. Since the first report on splicing correction in a mutated thalassemic betaglobin pre-mRNA [85], this approach has been applied successfully in the treatment of genetic diseases such as Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA), among others, as exemplified on the two recently approved drugs eteplirsen [4] and nusinersen [5].

1.2.2.5. Aptamers

Aptamers are the nucleic acid analog to antibodies. They are molecular ligands prepared by systematic evolution of ligands by exponential enrichment (SELEX). A pool of chemically or biologically randomized sequences is run through an affinity column, whereupon eluted sequences are amplified by PCR, eventually randomized again and repeatedly submitted to the selection process. Originally developed to bind small molecules [86], aptamers are widely used for multiple applications now [87]. This molecular class is positioned at the interface between small molecules and biologicals, with the advantages of synthetic access, amenability to chemical modification for immobilization, labeling and increased stability.

1.2.2.6. miRNA-targeting oligonucleotides

MiRNA-targeting oligonucleotides aim at modulating the functions of endogenous miRNAs, a class of small non-coding RNAs involved in post-transcriptional regulation of gene expression [88]. In contrast to siRNAs, miRNAs generally bind to 3′-untranslated regions (UTRs) of target mRNAs through imperfect complementarity. They either sterically block mRNA translation or induce mRNA degradation in a similar fashion to siRNAs. Their mechanism of action and conventional sites of intervention for miRNA-targeting oligonucleotides are discussed below (see 1.2.3).

1.2.3. Targeting miRNA

MicroRNAs (miRNAs) are 20-22 nucleotide (nt) long non-coding RNAs and are key players in post-transcriptional gene regulation [89]. Misregulation of miRNAs is known to be involved in the pathogenesis of many human diseases such as cancer, metabolic or cardiovascular disorders, viral infections and numerous other severe diseases [90-94]. Thus, miRNAs represent an attractive target and have been addressed by three main approaches [95]: First, expression vectors, coding for an artificial mRNA containing multiple miRNA binding sites acting as “miRNA sponges” and sequestering endogenous miRNA. Their use has been limited to transgenic animals so far. Second, small molecules, identified from compound library screening in reporter assays. Their mechanism of action is unclear, possibly through transcriptional regulation of targeted miRNAs; their high EC50 (µM) represents a further limitation. Third, oligonucleotides, the most promising approach to date, especially antimirs directly blocking mature miRNA function. Their chemistry, their mode of action as well as their strengths and weaknesses are discussed in 1.2.3.2.

1.2.3.1. MiRNA biogenesis and function

Since their first discovery in *Caenorhabditis elegans* almost 20 years ago [96,97], miRNAs have been demonstrated to play crucial roles in almost every biological process, including embryonic development and the host response to pathogens. They are evolutionarily well conserved and their biogenesis is tightly controlled [98]. Acting through translational repression or mRNA destabilization, they can regulate multiple target transcripts simultaneously. A very large proportion of the transcriptome is subject to miRNA regulation [99].

A primary miRNA transcript (pri-miRNA) containing a stem-loop structure is generated by RNA polymerase II, often from intronic regions of coding genes [100,101]. In the nucleus, the pri-miRNA is digested to precursor miRNA (pre-miRNA) by the microprocessor complex containing Drosha and DGCR8 among others [102-104],
whereupon it is exported into the cytoplasm by the nuclear export protein exportin-5 [105]. Dicer and associated proteins such as TRBP further process the pre-miRNA, cleaving off the terminal loop region (TLR) and helping to load the mature double-stranded miRNA into the RNA-induced silencing complex (RISC) consisting of an Argonaute (Ago) protein and sometimes auxiliary proteins [106-110]. The passenger strand is expelled, while the active guide strand mediating target recognition is retained [111,112] (Fig. 6).

**Figure 6.** MiRNA biogenesis. RNA polymerase II transcribes miRNA genes, often located in introns. Pri-miRNAs are processed by Drosa/DGCR8, exported to the cytoplasm by exportin-5, where they are further digested by Dicer/TRBP and loaded into Argonaute proteins to form the RISC, the effector complex mediating translational inhibition.

Regulation of gene expression essentially relies on RISC, the effector complex composed of an Ago protein and a single-stranded guide RNA (ssRNA) at least. Ago proteins are well-conserved among kingdoms. Among human Ago proteins (Ago 1-4), Ago2 is the only one capable of target mRNA cleavage. It consists of four characteristic domains, the N-terminal domain (N), the PAZ (Piwi-Argonaute-Zwille) domain, the middle (MID) domain as well as the PIWI domain (P-element induced wimpy testes) (Fig. 7) [113]. The PAZ domain, also present in Dicer, associates to ssRNA with rather low binding affinity and in a sequence-independent manner, specifically recognizing the 3′-end of ssRNAs. The miRNA 5′-end is anchored via the 5′-phosphate at the MID and PIWI interface by a divalent cation, and the base of the 5′-terminal nt sitting in its binding pocket in the MID domain. The PIWI domain resembles RNase H. It carries a conserved catalytic site H-D-D-E and binds two divalent metal ions, cleaving target RNA opposite nt 10 and 11 of the miRNA. Bases of nts 2-6 are stacked, while helix 7 induces a prominent kink between nt 6 and nt 7 [111,114]. Most contacts of Ago2 to the guide strand are made through hydrogen bonds and charge-charge interactions to the RNA sugar-phosphate backbone, in particular in the minor groove [115]. Ago2 contacts to the seed paired target RNA consist in hydrogen bonds, charge-charge and van der Waals interactions to positions 2-7 of the duplex RNA. However, a very important mode of recognition seems to be shape complementarity to the minor groove of the guide-target duplex, except for nts 8-9, where Ago2 does not touch the minor groove. Beyond the miRNA binding sites, Ago2 does not interact with target mRNA [115].

Loading of RNA into Ago2 essentially stabilizes the architecture of the protein. Which one of the two strands is selected as guide strand is not entirely clear, a trend has been observed for the strand with the thermodynamically less stable 5′ end to be preferred [111]. Furthermore, there seems to exist a bias for a 5′-terminal U [116]. However, these observations are trends only, far from being rules.
Target recognition is mediated by the seed region, nts 2-8. The 3’ half of the guide is buried within Ago2 to prevent base-pairing with complementary targets. Nts 2-6 are pre-arranged in A-form to predispose for hybridization to complementary RNA. Initially, nts 2-5 nucleate and induce a conformational change within the RISC, further exposing nts 6-8 and 13-16 for recognition [115].

1.2.3.2. Targeting miRNA with oligonucleotides

Theoretically, miRNAs can be targeted anytime during their biogenesis, be it at the pri-, pre- or mature miRNA stage (Fig. 8). For pri- and pre-miRNA, which consist in hairpin-like structures, high affinity oligonucleotides are required to break up RNA secondary structure. Alternatively, looptomirs can bind the TLR, thereby preventing interactions of the loop with RNA-binding proteins (RBPs) [117]. However, two important considerations are that first, a large proportion of mature miRNAs are associated with Ago [118] and that second, RISC-loaded mature miRNAs are long-lived [119,120]. It follows that inhibition of pri- or pre-miRNA will have a delayed effect and that supposedly most efficient miRNA targeting is directed at Ago-bound miRNAs. To date, this is where most effort has been put in.

Antimirs associate with the target miRNA-Ago complex via base-pairing to the seed region. Hybridization of antimirs to their target miRNAs does not affect miRNA stability nor Ago association and was found to lead to increased abundance of target mRNA [118]. Two regions were shown to determine inhibitor specificity: Besides the seed (here, nts 3-8), an additional 3’-region proved critical, usually nts 13-18, in case of miR-122 nts 15-16, were shown to be of particular importance [121]. Mismatches outside of these regions were very well tolerated. Generally, a loose correlation of antimir activity and affinity was observed [122]. In some cases, additional parameters would govern miRNA inhibition [119]. For example, the modification pattern was shown to greatly affect the ability to target miRNA-Ago complexes. Compounds with identical numbers of sugar modifications could have drastically different antimir activity [118,119]. In addition, antimir chemistry was found to determine the fate of the target miRNA. High affinity modifications such as LNA or 2’-F/MOE were shown to sequester the target miRNA in a heteroduplex, while lower affinity oligos as for example 2’-OMe or 2’-MOE promoted miRNA degradation [123,124]. An interesting format for an antimir is the “tiny LNA”, a full LNA PS 8mer targeting the seed region [125]. Tiny LNAs were shown to be able to inhibit whole miRNA families with identical seed region. A tiny LNA targeting miR-122 had a very similar effect to 15mer miravirsen (SPC3649), including side-effects. If targeting any other region than the seed, tiny LNAs had no effect however. If shorter than 7 nt or in 2’-OMe chemistry it was inactive also, while mismatches or a length above 9 nt reduced potency. Tiny LNAs were found to
co-localize with Ago2-bound miRNA and were efficiently taken up in multiple organs and showed long-lasting effects [125].

![Image](image.png)

Figure 8. Sites of intervention of miRNA-targeting oligonucleotides. Looptomirs bind to the terminal loop region (TLR) of pri- or pre-miRNAs, antimirs associate with RISC-loaded mature miRNA.

1.2.4. Conjugation of therapeutic oligonucleotides for targeted delivery
As outlined above, pharmacokinetics of therapeutic oligonucleotides are challenging. They need to have an optimum size for a long circulation half-life as well as effective tissue penetration, ideally they have cell targeting properties to facilitate tissue accumulation or selective delivery, and finally they need to be able to escape the endosomal compartment to exert their function in the cytosol. A common strategy to add functionality to an oligonucleotide is to conjugate a dedicated moiety. Conjugates of oligonucleotides have been prepared most often to address pharmacokinetic issues, such as improvement of tissue distribution, cellular permeation, (sub)cellular targeting or modulation of protein binding to adjust circulation half-life [21].

1.2.4.1. Carbohydrates
The most advanced oligonucleotide conjugates are carbohydrate-siRNAs targeting the liver [126]. Developed by Alnylam for the treatment of transthyretin amyloidosis, hypercholesterolemia or hemophilia, 3 conjugates are actually in Phase II/III clinical trials. To the 3’-terminus of the sense strand are linked 3 molecules of N-acetylgalactosamine (GalNAc) via a triantennary spacer, thus representing an asialoglycoprotein receptor ligand (Fig. 9). The asialoglycoprotein receptor, mainly expressed on hepatocytes, binds galactose-terminated glycoproteins with high affinity and internalizes them via the clathrin-mediated pathway [127]. Robust gene silencing was achieved upon subcutaneous administration of GalNAc-siRNA conjugates [126].

1.2.4.2. Lipids
Cholesterol conjugated to the 5’- or 3’-terminus of the sense strand of an ApoB siRNA was shown to improve cellular uptake and pharmacokinetics of oligonucleotides [128,129]. The conjugates were associating with lipoproteins in the blood, prolonging their circulation time and mediating uptake into hepatocytes via lipoprotein
receptors [130]. Alternative lipid moieties conjugated to sense strands of siRNAs were fatty acids and α-tocopherol, both of which proved to enhance uptake into hepatocytes [130,131]. Conjugation of fatty acids to single stranded oligonucleotides and siRNAs further showed that, besides protein binding, the lipid moiety mediates association to cellular membranes, favouring cellular uptake [132,133].

**Figure 9:** Triantennary N-acetylglactosamine(GalNAc)-conjugated siRNA. Adapted from [21].

1.2.4.3. Small molecules
Another class of ligands for internalizing receptors are small molecules. Conjugates of selected small molecules were shown to increase activity of oligonucleotides in the absence of transfecting reagents. Among them, anisamide, a ligand for the sigma receptor, preferably expressed on tumor cells. Mono- and trivalent anisamide conjugated to the 3’-terminus of an SSO was shown to increase cellular uptake and biological activity [134]. Recent evidence however suggests that anisamide conjugates are not taken up by the sigma receptor since the latter may not be expressed on the cell surface [135,136]. Further examples are folate, targeting the folate receptor [137], or anandamide, targeting a cannabinoid receptor [138], both increasing activity of an siRNA when conjugated to the sense strand.

1.2.4.4. Peptides
Cell penetrating peptides (CPPs) are short basic amino acid-rich peptides able to enter cells and ship a cargo inside the cell [139]. They have been conjugated to numerous macromolecules including therapeutic RNAs such as siRNAs, ASOs and SSOS [140]. Most successful were SSO conjugates, especially uncharged morpholino (PMO) or peptide nucleic acid (PNA), used in the treatment of Duchenne muscular dystrophy. Both in cell culture and in mice substantially higher effects were obtained upon CPP conjugation [141,142].

Besides CPPs, cell-targeting peptides have been investigated for their ability to selectively deliver therapeutic oligonucleotides. Short peptides designed to bind receptors such as avβ3 integrin [143-146], gastrin-releasing peptide receptor (GRPR) [147] or insulin-like growth factor 1 receptor (IGF1R) [148,149] conjugated to SSOS or siRNAs have proven to increase cell-selective uptake or biological activity of their oligonucleotide cargoes.

1.2.4.5. Antibodies
Antibody-drug conjugates have proven useful in targeted drug delivery for cancer therapy [150,151]. Antibody-oligonucleotide conjugates are far from this success rate. Delivery of the constructs represents a major bottleneck, as they were efficiently internalized but not released from the endosomes [152,153]. When fused to positively charged protamine, which captures siRNA via non-covalent interactions and delivers it into cells, antibodies have demonstrated some success, supposedly due to enhanced endosomal release of the siRNA mediated by protamine [154,155].

1.2.4.6. Oligonucleotides
Aptamers are targeting moieties that can conveniently be fused to therapeutic oligonucleotides. They bind to cell surface receptors to either block the receptor or to enhance endocytosis of cargo molecules or both. As an
example, an siRNA fused to a prostate-specific membrane antigen (PSMA) aptamer inhibited growth of prostate cancer xenografts upon intratumoral injection [156]. Alternatively, the aptamer function can be used to block a receptor and as a targeting moiety at the same time: An aptamer-siRNA chimera inhibited HIV replication both by receptor blocking of the gp120-specific aptamer as well as by RNAi [157].

An alternative oligonucleotide-mediated targeted delivery strategy is the use of CpG motifs. Toll-like receptor 9 recognizes and internalizes CpG containing oligonucleotides, in addition to activating various immune responses. A CpG-siRNA conjugate aimed at Stat3-silencing, an immune suppressor, achieved an efficient antitumor response in mice in a synergistic way [158,159].

1.2.4.7. Polymers
Polymers are commonly used to modify the pharmacokinetic profile of a conjugated oligonucleotide. For example, PEGylation increases the size of the oligonucleotide, reduces renal filtration and thus increases the circulation half-life [160]. The leading polymer-oligonucleotide conjugates in clinical development are dynamic polyconjugates (DPCs). They are composed of several functional entities, each playing a role in oligonucleotide delivery. As an example, an amphipathic polymer able to disrupt cell membranes can act as a carrier, an acid-cleavable PEG shield may prevent untimely membrane disrupting abilities, GalNAc ligands may be appended to the carrier for targeting purposes as well as an siRNA, linked via a disulfide bond, to be released at the target site [161]. These constructs have allowed efficient delivery to hepatocytes and gene knockdown in mice [161].

1.2.5. Oligonucleotide drugs and therapeutic oligonucleotides in clinical trials
The productiveness of the field is currently growing, with major advances achieved in the last couple of years and several drug approvals to be expected in the coming years. A number of companies from small start-ups to large pharma companies dedicate big efforts to the transition of oligonucleotide drug candidates from the bench to the patient. In the following (see 1.2.5.3), oligonucleotide drugs approved to date will be presented along with a few promising candidates in late stage clinical trials. There are many more to come, including other disease indications, other molecular mechanisms or chemistries and the selection presented here is far from being complete. Rather, it should mention a few interesting examples to illustrate what is going on and where the challenges are.

1.2.5.1. Pharmacokinetics of oligonucleotides
Pharmacokinetics of oligonucleotides are largely determined by their chemistry and are rather independent of the sequence or mode of action [8,19]. Once they reach the blood, oligonucleotides generally distribute to tissues quite quickly within minutes to a few hours, where they are taken up into cells by endocytosis. The tissues with the highest concentrations are the liver, the kidney, the bone marrow, the cell body of adipocytes and lymph nodes [25]. Clearance half-lives are determined by plasma protein binding, predominantly depending on the backbone, and nuclease resistance, modulated by 2′-substitution. Ubiquitous endo- and exonucleases chop up nucleic acids, the resulting small fragments lose affinity to plasma proteins and are subsequently eliminated in urine. Oligonucleotides are not metabolized by the CYP450 liver enzymes and therefore have a very low risk of affecting the metabolism of simultaneously administered small molecule drugs. Subcutaneous (s.c.) or intravenous (i.v.) are the most common routes of administration [25]. Injection into the cerebrospinal fluid allows distribution in the central nervous system as due to their size and charge, nucleic acids are unable to cross the blood-brain barrier. Systemic absorption upon oral, intravitreal or pulmonary delivery is very low, generally below 1% [19]. Uncharged oligonucleotides such as morpholinos or peptide nucleic acids as well as unmodified siRNAs are rapidly cleared from the blood [25]. Thus, certain levels of both protein binding and nuclease resistance seem to be indispensable for successful tissue delivery.
1.2.5.2. Toxicology

Toxicities of oligonucleotide drugs can be attributed to either hybridization (and thus sequence) dependent or hybridization independent effects. Hybridization dependent toxicities are mainly off-target effects and their likelyness can be diminished by careful bioinformatic analyses to minimize potential targets with little or no mismatches. On-target toxicities can eventually be anticipated by comprehensive characterization of candidate drugs in preclinical models [19]. Hybridization independent toxicities are predominantly chemistry-driven and include interactions with proteins. Common examples are effects on coagulation, complement activation and immune cell activation, while sequence dependent immune cell activation has been observed as well [19]. Hepatotoxicity turned out to be a major issue for bicyclic sugar modifications, in particular LNA [64]. Best characterized oligonucleotides are first generation DNA PS and 2'-MOE modified ASOs owing to the large number of such compounds in clinical trials.

1.2.5.3. Oligonucleotide drugs and candidates in late stage clinical trials

The first oligonucleotide drug to be approved in 1998 was Fomivirsen (Vitravene®), a DNA PS 21mer for the treatment of cytomegalovirus(CMV)-induced retinitis in immunocompromised patients (HIV) [162]. The reason for its early success was likely the local delivery into an isolated compartment deprived from blood circulation and the immune system. Intravitreal injection allowed to circumvent tissue distribution, a major hurdle for oligonucleotide drugs. However, Fomivirsen is no longer on the market due to the fact that the development of the highly active antiretroviral therapy (HAART), state-of-the-art treatment for AIDS patients in western countries, drastically lowered the incidence of CMV infections. Other oligonucleotide drugs and candidates locally delivered to the eye include Pegaptanib and Aganirsen. Pegaptanib (Macugen®) is an aptamer targeting the vascular endothelial growth factor (VEGF) and is approved for the treatment of age-related (wet) macular degeneration, as an alternative to the fab fragment Ranibizumab (Lucentis®) or the less costly antibody Bevacizumab (Avastin®) used off-label for the same indication [163,164]. Aganirsen, a DNA PS 25mer in phase III, is administered as an eye drop and targets insulin receptor substrate-1 (IRS-1) to prevent corneal and retinal neovascularization [165].

Besides remedies for the eye, two candidate oligonucleotide drugs in the treatment of inflammatory bowel disease are likewise applied locally. Alicaforsen and Mongersen are DNA PS oligonucleotides in Phase III recruiting RNase H to degrade their target mRNA. Alicaforsen targets the mRNA of intracellular adhesion molecule-1 (ICAM-1) and is delivered by a nightly enema to treat pouchitis [166]. Mongersen, on the other hand, is taken orally by Crohn’s disease patients and addresses SMAD7, a protein involved in cell signaling and part of the TGFβ superfamily [167]. Its delivery system is not disclosed, yet it amazingly shuttles an intrinsically acid-labile and biologically rather unstable molecule to its site of action further down the digestive tract.

The first systemically delivered antisense drug on the market was mipomersen (Kynamro®), a 2'-MOE 20mer gapmer targeting apolipoprotein B (ApoB) and approved for the treatment of familial hypercholesterolemia in 2013 [3,79,80]. It is administered by weekly subcutaneous injections of 200 mg from pre-filled syringes. With the liver as a target organ, tissue distribution is still relatively straightforward. However, a 20mer second generation oligonucleotide that readily persists in the blood and is taken up by target cells followed by a clinically relevant physiologic effect represented an encouraging jump ahead for the field. Volanesorsen, currently in phase III, is another s.c. delivered 2'-MOE gapmer with a liver target, ApoC-III. High ApoC-III levels are associated with hypertriglycerideremia, a significant risk for pancreatitis. 300 mg Volanesorsen once weekly are used to lower triglycerides in the treatment of the familial chylomicronemia syndrome and familial partial lipodystrophy [168,169].

A very active area is splice correction in neuromuscular diseases. Nusinersen (Spinraza®) and eteplirsen (Exondys 51®) are two recently approved drugs for the treatment of spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD), respectively. Nusinersen is a second generation 2'-MOE 18mer targeting the SMN2 pre-mRNA upon intrathecal injection [170]. In SMA, a loss-of-function mutation in the SMN1 gene leads to very
low levels of survival motor neuron (SMN) protein deriving from SMN2 gene. Alternative splicing of SMN2 converts it to a SMN1 transcript, resulting in increased levels of SMN protein in the central nervous system (CNS).

DMD arises from mutations in the DMD gene resulting in non-functional dystrophin protein, usually due to a reading frame shift. Removing an exon with an inappropriate number of bases can restore the reading frame down-stream of this exon and allow production of a truncated but functional dystrophin protein. Owing to the genetic heterogeneity of DMD, many different exon-skipping oligonucleotides are required to address the large number of potentially affected exons. Eteplirsen is a 30mer morpholino administered by i.v. infusion inducing splice switching at exon 51 [4,171]. It received accelerated approval from the FDA in late 2016. This was in contrast to drisapersen, a 2'-OMe PS 20mer targeting the same exon 51 of dystrophin pre-mRNA, which has been rejected in early 2016 due to a lack of substantial evidence of effectiveness [4,56].

Table 1. Selected oligonucleotide drugs on the market or in late stage clinical trials. Adapted from [19].

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Indication</th>
<th>Mode of action</th>
<th>Chemistry</th>
<th>Delivery route</th>
<th>Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eye</strong></td>
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<tr>
<td>Fomivirsen Vitravene® ISIS-2922</td>
<td>IE2 gene</td>
<td>CMV retinitis in HIV patients</td>
<td>Translational arrest</td>
<td>DNA PS</td>
<td>Intravitreal injection</td>
<td>Ionis</td>
<td>approved discont.</td>
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<tr>
<td>Pegaptanib Macugen®</td>
<td>VEGF</td>
<td>Neovascular age-related macular degeneration</td>
<td>Aptamer</td>
<td>2'-OMe purines, 2'-F pyrimidines, 5'-pegylated</td>
<td>Intravitreal injection</td>
<td>NeXstar, Pfizer</td>
<td>approved</td>
</tr>
<tr>
<td>Aganirsen GS-101</td>
<td>IRS-1</td>
<td>Ocular neovascularisation</td>
<td>Translational arrest</td>
<td>DNA PS</td>
<td>Eye drops</td>
<td>GeneSignal</td>
<td>Phase III</td>
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<td><strong>Cardiovascular disorders</strong></td>
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<tr>
<td>Mipomersen Kynamro® ISIS-301012</td>
<td>ApoB</td>
<td>Homozygous familial hypercholesterolemia</td>
<td>RNase H</td>
<td>2'-MOE gapmer</td>
<td>s.c.</td>
<td>Ionis</td>
<td>approved</td>
</tr>
<tr>
<td>Volanesorsen ISIS-APOCIIIex</td>
<td>ApoC-III</td>
<td>Familial chylomicronemia syndrome, familial partial lipodystrophy</td>
<td>RNase H</td>
<td>2'-MOE gapmer</td>
<td>s.c.</td>
<td>Ionis</td>
<td>Phase III</td>
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<td>Nusinersen Spinraza®</td>
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<td>SMA</td>
<td>Splice correction</td>
<td>2'-MOE PS</td>
<td>Intrathecal injection</td>
<td>Ionis</td>
<td>approved</td>
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<td>DMD exon 51</td>
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<td>morpholino</td>
<td>i.v. infusion</td>
<td>Sarepta</td>
<td>approved</td>
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<tr>
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<td>DMD exon 51</td>
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<td>s.c.</td>
<td>BioMarin</td>
<td>rejected</td>
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<td>Familial amyloid polynuropathy</td>
<td>siRNA</td>
<td>Lipid nanoparticle</td>
<td>i.v.</td>
<td>Alnylam</td>
<td>Phase III</td>
</tr>
<tr>
<td>IONIS-TTRex</td>
<td>Trans-thyretin</td>
<td>Familial amyloid polynuropathy</td>
<td>RNase H</td>
<td>2'-MOE gapmer</td>
<td>s.c.</td>
<td>Ionis</td>
<td>Phase III</td>
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<tr>
<td><strong>Cancer</strong></td>
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<td>Custirsen OGX-011</td>
<td>Clusterin</td>
<td>Prostate cancer, NSCLC</td>
<td>RNase H</td>
<td>2'-MOE gapmer</td>
<td>i.v.</td>
<td>OncoGeneX</td>
<td>Phase III</td>
</tr>
<tr>
<td>Alicaforsen ISIS-2302</td>
<td>ICAM-1 (CD54)</td>
<td>Pouchitis</td>
<td>RNase H</td>
<td>DNA PS</td>
<td>Enema</td>
<td>Ionis</td>
<td>Phase III</td>
</tr>
<tr>
<td>Mongersen GED-0301</td>
<td>SMAD7</td>
<td>Crohn’s disease</td>
<td>RNase H</td>
<td>DNA PS</td>
<td>Oral</td>
<td>Celgene</td>
<td>Phase III</td>
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<tr>
<td><strong>Inflammatory disorders</strong></td>
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IONS-TTRrx and Patisiran are two oligonucleotide drug candidates in phase III for the treatment of familial amyloid polyneuropathy. Transthyretin (TTR) amyloidosis is a fatal genetic disorder with deposition of misfolded TTR in multiple organs leading to multi-organ failure. Very limited treatment options are available. As a 2′-MOE gapmer, IONS-TTRrx is designed to reduce TTR production by an antisense mechanism [172]. 300 mg are administered as a s.c. injection once weekly. A phase III study was halted when thrombocytopenia was observed as a serious side effect, but continued shortly thereafter under tighter platelet count controls. Patisiran, whose structure is undisclosed, is an siRNA formulated in lipid nanoparticles [173]. It was shown to reduce TTR levels upon i.v. administration as well.

Many drug candidates under investigation for the treatment of cancer or infectious diseases have not yet reached phase III, even though these disease areas continue to benefit from high attention. An exception is Custirsen, a 2′-MOE gapmer in phase III for the treatment of prostate cancer and NSCLC [174]. Miravirsen, an LNA-DNA chimeric 15mer targeting miR-122 to reduce cholesterol levels and the viral load in hepatitis C virus (HCV) infection, is a prominent example in phase II that will be discussed in chapter 2.7.2.1.

Summarizing, local delivery to the eye considerably facilitated early successes of therapeutic oligonucleotides in ophthalmology. To date, first generation chemistry seems to be used only when locally applied. Second generation 2′-MOE gapmers as well as morpholinos have successfully distributed systemically, so did 2′-OMe chemistry but failed to prove efficient in the case of drisapersen. The most productive molecular mechanisms so far are RNase H and splice correction, and most clinical experience is available for the 2′-MOE chemistry whose pharmacokinetics and toxicity have been extensively studied on multiple clinical examples (Tab. 1).

### 1.2.6. Optimization of hybridization-based oligonucleotide drugs

#### 1.2.6.1. In vitro

A key event in drug action is the formation of a high affinity drug-target complex, conventionally characterized by an equilibrium dissociation constant $K_D$. In nucleic acid chemistry, melting temperature ($T_m$) is an established in vitro method to assess target-binding affinity of modified oligonucleotides as a surrogate of $K_D$ [175]. However, recent work suggests that the equilibrium dissociation constant $K_D$ is a poor measure of binding in an open system such as an organism, where drug concentrations temporarily peak before receding, as opposed to a closed in vitro assay system where drug concentrations are artificially maintained. Rather, binding kinetics are proposed to be more informative, especially drug residence time [176-178]. The residence time of a drug-target complex is determined by $k_{off}$, its dissociation rate, and represents a superior in vitro surrogate of a durable pharmacological effect.

Low off-rates may allow the therapeutic effect to persist long after the drug has been cleared from the circulation [176]. In the case of oligonucleotide drugs, off-rates are determined by their sequence length [179]. Thus, $k_{off}$ can simply be modulated by adjusting oligonucleotide length rather than using complex chemical modification. Generally, oligonucleotide length to a large extent also determines binding affinity (which takes into account both $k_{on}$ and $k_{off}$) and selectivity, whereas the former increases with the number of nucleotides, while the latter decreases beyond a certain size [180,181]. Therefore, improving $k_{off}$ of an mRNA targeting oligonucleotide drug is relatively easy. However, the value of doing so is questionable for 3 reasons: First, life-times of most mRNAs, with typical half-lives around 7-10h [182,183], are shorter than residence times of even short (<12 nt) oligonucleotide-RNA duplexes. Second, the mRNAome is very dynamic. Short half-lives of mRNAs indicate that mRNA targets are constantly re-synthesized. In order to cover newly synthesized mRNA target molecules, dissociation from cleaved targets needs to be fast in the case of a catalytic mechanism. In a steric block-type of mechanism like for the SSOs, available drug concentrations at the target site, determining drug-target association, are more critical than $k_{off}$. Third, pharmacokinetic elimination of the oligonucleotide drug from the system is often slower than drug-target
complex dissociation, in which case then it is not the residence time, but the elimination half-life that determines duration of the pharmacological effect [184]. For a drug-target residence time to prolong the binding beyond pharmacokinetic elimination of the drug, the dissociation from its target must be slower than its elimination from the blood, which can heavily vary between oligonucleotide chemistries. Mipomersen for example, a 2'-MOE/DNA PS gapmer, had a half-life of 30 days [3], the 2'-MOE PS 18mer nusinersen had 4-6 months [185], while the half-life of eteplirsen, a 30mer morpholino, was only 3 hours [186]. Thus, in contrast to morpholinos, it is likely that 2'-MOE oligonucleotides persist longer in the circulation than the life-time of their drug-target complexes, indicating that improving $k_{off}$ would not prolong the pharmacological effect.

Optimization of target association rates $k_{on}$, on the other hand, may have a beneficial effect in various scenarios. On-rates are largely independent of oligonucleotide length [179], with the exception of decelerated diffusion upon increasing size of binding partners. Nucleation represents the rate-limiting step in duplex hybridization, meaning the formation of 3 base-pairs, whereupon helix formation, “zipping” of the remaining base-pairs, energetically follows a fast down-hill reaction [179]. Improval of $k_{on}$ is less straight-forward than modulation of $k_{off}$ and needs to be achieved via chemical modification or, alternatively, adjustment of ionic strength or temperature of the medium [179]. Though, optimization of $k_{on}$ is expected to have a beneficial impact in targeting systems where the recycling ability of an oligonucleotide drug is of critical importance as for example with RNaseH-competent gapmers or in RNAi [74]. Furthermore, rapid target rebinding after dissociation might help to prolong the pharmacological effect.

Taken together, even though knowledge of binding kinetics provides a wealth of information and might be considerably more predictive of in vivo behavior than $T_m$, target binding kinetics of oligonucleotide drugs are tedious to perform and are rarely reported.

1.2.6.2. In cells

Cellular testing of candidate molecules is a standard step to be taken prior to in vivo testing. Large investigations on cellular potency of chemically modified oligonucleotides with various mechanisms of action have classified modifications according to their performance in these artificial systems [83,119,120]. However, unassisted cellular uptake is naturally very low and is commonly facilitated by transfection with cationic lipids [187,188]. Use of transfecting reagents introduces an important additional bias as it is commonly recognized that delivery is a key obstacle in successful application of therapeutic oligonucleotides [25,189,190]. Thus, cellular assays depending on transfecting reagents risk to provide a poor estimate of in vivo potency. “Gymnosis”, the concept of delivering naked nucleic acids without the use of transfecting reagents, introduced by Stein et al. [191], has been elaborated and successfully implemented in a dedicated cell line. MHT cells derive from mouse liver cells and kept the ability to take up naked single-stranded oligonucleotides [26]. Though, doses required to produce a detectable effect are considerably higher than with use of transfecting reagents.
1.3. Polyamines

1.3.1. Biosynthesis of endogenous polyamines
The polyamines spermine, spermidine and putrescine are ubiquitous small nitrogenous bases and important modulators of cellular function [192]. They are known to be involved in the regulation of growth, development and mammalian physiology and their levels are tightly controlled by a complex apparatus of enzymes [193]. Putrescine is generated from ornithine by the action of ornithine decarboxylase (ODC), which is considered the rate-limiting step in polyamine synthesis. Spermidine is then formed by spermidine synthase, whereupon spermine synthase can convert spermidine to spermine. Spermidine and spermine synthases are aminopropyltransferases, with decarboxylated S-adenosylmethionine as an aminopropyl donor. Spermine can be back-converted to spermidine by the action of spermine oxidase (SMO), or alternatively via the intermediate of $N^1$-acetylspermine, involving spermine/spermidine-$N^1$-acetyltransferase (SSAT) and acetylpolyamine oxidase (APAO). SSAT and APAO can also restore putrescine from spermidine, via $N^1$-acetylsperrmidine (Fig. 10).

![Polyamine structures and biosynthesis](image.png)

**Figure 10:** Polyamine structures and biosynthesis. Polyamines are shown in blue, enzymes in green. ODC, ornithine decarboxylase; spdS, spermidine synthase; smpS, spermine synthase; SSAT, spermidine/spermine-$N^1$-acetyltransferase; APAO, acetyl-polyamine oxidase; AdoMetDC, S-adenosyl-methionine decarboxylase. Adapted from [193,194].

1.3.2. Discovery and history
Spermine and spermidine were first discovered in human semen. Early descriptions of the colorless crystals from human semen date from 1678 [195] and 1791 [196]. In 1865 Boettcher studied the solubility of the up to 2.2 mm long crystals in different solvents, described their shape and the process of recrystallization [197]. The elemental composition and the name “spermine” were introduced by the Russian physiologist Poehl, who extensively investigated the chemical properties and physiologic functions [198,199]. The scientific history of spermine was taken up by Rosenheim et al., who isolated spermine from various animal tissues and further characterized spermine-phosphate crystals as odorless, optically inactive solid melting between 55 and 60 °C, insoluble in organic solvents but soluble in water [200-202]. They further extracted the free base spermine itself and observed needle-shaped crystals, soluble in water and ethanol, which liquefy upon exposure to air. Besides, they
mentioned the “characteristic odour” and established the chemical formula \( \text{NH}_3(\text{CH}_2)_3\text{NH}(\text{CH}_2)_2\text{NH}(\text{CH}_2)_3\text{NH}_2 \) of spermine [203]. Shortly thereafter spermidine was isolated by the same group [204].

1.3.3. Properties and function

1.3.3.1. Conformation and size in phosphate crystals

The conformation of free spermine has been addressed in a number of x-ray crystallography studies. Studies on spermine phosphate crystals found an “extended zigzag chain configuration” with a 16.23 Å distance between terminal amines [205]. This is in agreement with an NMR study determining the length of “the fully extended spermine to be over 15 Å” [206]. The linear all-trans conformation of the free base was confirmed by both Raman and INS investigations [207-209], showing a predominant all-trans conformation of spermine, spermidine and putrescine in the solid state, randomizing in the liquid state. Furthermore, intermolecular R-HN-H→NH₂-R hydrogen bonds were observed to preferably involve the terminal NH₂ groups of the chain. However, conflicting results were obtained from studies on the crystal structure of spermine tetrahydrochloride, which found secondary C-N bonds to be in gauche conformation, resulting in a twisted molecule, in contrast to spermidine trihydrochloride, for which a planar conformation was found [210].

1.3.3.2. Protonation and RNA binding

Protonation of the primary and secondary amines was observed to strongly depend on temperature, with decreasing \( pK_a \)s upon temperature increase [211]. In an acid-base titration study \( pK_{a1-4} \) of spermine were found to be 8.19, 9.05, 10.21, 10.99 at 25 °C [211], which is in reasonable agreement with values obtained by potentiometric titration (7.95, 8.82, 10.05, 10.86 for spermine, 8.35, 9.83, 10.83 for spermidine) [212]. Thus, due to protonation at physiologic pH, polyamines preferentially interact with negatively charged macromolecules like the nucleic acids. Besides polyamine association with DNA, as it has been described for spermidine and putrescine in phage \( T_4 \) DNA for example [213], spermine, spermidine and putrescine have been found to preferably interact with RNA [214,215]. Spermine was shown to stabilize bacterial RNA against RNase degradation [216] and to play a role in the assembly of ribosomal subunits [217]. Moreover, polyamines influence sedimentation patterns. Multiple studies showed precipitation of RNA by spermine [218,219] or increased sedimentation rates of RNA [220,221], concluding that spermine mediated compaction of the RNA.

Short RNA model sequences such as polyU, polyA, polyC and polyI have been used to investigate sequence dependence of polyamine binding. Gel filtration assays showed that polyI was most susceptible to spermine binding, followed by polyA and polyC, the lowest amount of bound spermine found for polyU [222]. Contrasting results were obtained in another study, with the highest spermine-RNA association for polyU, followed by polyG, and far behind polyC and polyA [223,224]. Among the double-stranded structures, equivalents of bound spermine were consistently higher for poly(A+U) than for poly(G+C) [222-224], in line with previous findings on DNA [225]. Consequently, depending on pH, spermine-triggered precipitation and subsequent centrifugation allowed separation of poly(C+H) and poly(A+U) [214]. Overall, a stronger binding of spermine to double-stranded polynucleotides was observed than to single-stranded polynucleotides, and double-stranded RNA had a higher capacity for spermine binding than double-stranded DNA [223]. Meanwhile, an increase in magnesium or potassium concentration was found to restrict spermine binding [224]. Owing to its phosphate neutralizing capacity, spermine has been used to enhance cellular uptake of oligonucleotides, along with longer non-natural linear polyamines [226].

1.3.3.3. Polyamines increase hybridization affinity of double-stranded oligonucleotides

Polyamine binding to both RNA [219,221,227,228], and DNA [225,229] was found to increase melting temperature. Polyamines were more effective than diamines or magnesium in shifting the transition to higher temperatures, as was shown on the example of polyU [230].
Artificial polyamines such as pentamines, hexamines or tetramines and triamines with varying aliphatic chain length indicated that the more charged an amine, the more efficiently it stabilized RNA-RNA duplexes [231,232]. The same trend was observed with RNA-PODNA and RNA-PSDNA hybrids, while the order of binding affinity with polyamines remained the same as without polyamines. Among the tetramines and triamines tested, spermine and spermidine proved to be most efficient in duplex stabilization and displayed the highest relative binding constant, suggesting that the CH$_2$ chain length of natural polyamines is just ideal for binding to nucleic acids [231]. Spermine was most efficient in stabilizing PSDNA-RNA hybrids, RNA-RNA duplexes being the least susceptible [232]. Furthermore, circular dichroism studies confirmed that polyamine binding did not alter duplex conformation of neither RNA-RNA, PODNA-RNA, PSDNA-RNA, PODNA-PODNA, PSDNA-PSDNA nor PODNA-PSDNA hybrids [232].

1.3.3.4. Mechanistic studies of polyamine-RNA interaction and RNA cleavage

An $^3$H-NMR study investigating the interactions between various amines and single stranded polyA, polyU and polyC found that spermine eliminated the 2'-OH resonance at concentrations of about one spermine per ten phosphates, suggesting it to be rather mobile when bound to RNA [233]. Spermidine behaved in a similar way, whereas for diamines and monoamines a much higher concentration was needed in order to obtain an effect on the 2'-OH resonance, and uncharged amines had no impact at all. A possible explanation was displacement of a bridging water molecule between the 2'-OH and a 3'-phosphate oxygen by the amine, without altering backbone conformation. As a further consequence of hydration changes, elevated polyamine concentrations increased the rate of nonenzymatic RNA hydrolysis in a sequence- and pH-dependent way [234-237]. The pK$_a$s of amines determined their disposition towards catalytic activity in an intramolecular acid-base cooperation enabling the 2'-OH to nucleophilically attack the phosphorus atom [234,235,238].

1.3.4. Polyamine-oligonucleotide conjugates

Conjugation of polyamines to oligonucleotides creates zwitterionic functionality, which in theory can enhance both nucleic acid target hybridization and cellular penetration. Indeed, polyamines introduced at the 5'-terminus, the 2'-O- and the 4'-positions on ribose and at various positions of the nucleobases have been found to improve properties with respect to distinct applications in vitro and/or in cells (Tab. 2-4) [239]. Increased hybridization affinity, nuclease resistance and, in case of terminal oligospermine-conjugation, facilitated cellular uptake were observed. Examples of small melting temperature increases (a few °C per modification) include 2'-aminopropyl- [240], 2'-O-lysylaminohexyl- [241], 2'-aminobutyryl- [242] and 5-aminohexyl-modifications [243], whereas 2'-aminated LNA displayed the highest binding affinity increase to complementary RNA [244]. However, the influence of variables such as polyamine length and shape, the nature of the linker, conjugation site and the position of modifications within the sequence has not been systematically investigated. Certainly, playing on these parameters has the potential to yield polyamine-oligonucleotide conjugates with improved activity and nucleic acid tools with higher sensitivities.

As part of this work, literature on polyamine-oligonucleotide conjugates has been reviewed [239]. A comprehensive overview on conjugate described in literature is given in Tab. 2-4.
Table 2: Conjugation of polyamines to the terminal positions of oligonucleotides. a) mono-spermine conjugates and b) oligo-spermine conjugates (zip nucleic acids, ZNAs). Structure 5 is the phosphoramidite synthon used for sequential addition of terminal spermine-derived residues. Table adapted from [239].

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>Thermo-stability</th>
<th>Nuclease stability</th>
<th>Cellular uptake</th>
<th>Cellular activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>R=</td>
<td>[245]</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[246]</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[247]</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b)</td>
<td>R'=</td>
<td>[248,249]</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[250,251]</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[252]</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[253]</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[252]</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[254]</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Reference Thermo- stability Nuclease stability Cellular uptake Cellular activity
Table 3: Conjugation of amines and polyamines to the ribose units of oligonucleotides. Table adapted from [239].

<table>
<thead>
<tr>
<th>R =</th>
<th>Reference</th>
<th>Thermo-stability</th>
<th>Nuclease stability</th>
<th>Cellular uptake</th>
<th>Cellular activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6) $y_mNH_2$ n = 2</td>
<td>[83]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>(7) $O_{3-4}NH_2$ n = 2 or 3</td>
<td>[255]</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8) $O_{4-6}NH_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(11) $H_{3-4}NH_2$</td>
<td>[241,259,260]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>(12)</td>
<td>[246,261]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(13) $y_mNH_2$ n = 2, 3 or 4</td>
<td>[262]</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>(14) $O_{3-4}NH_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(15) $O_{4-6}NH_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(16) $O_{4-6}NH_2$</td>
<td>[263]</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>(17) $H_{3-4}NH_2$</td>
<td>[263]</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>(18) $H_{3-4}NH_2$</td>
<td>[263]</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>(19) $y_mNH_2$ n = 1, 2, 3</td>
<td>[264]</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>(20) $O_{3-4}NH_2$</td>
<td>[264]</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Conjugation of polyamines to the nucleobases. Table adapted from [239].

<table>
<thead>
<tr>
<th>R&lt;sub&gt;1&lt;/sub&gt; =</th>
<th>Reference</th>
<th>Thermo-stability</th>
<th>Nuclease stability</th>
<th>Cellular uptake</th>
<th>Cellular activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(21) X&lt;sub&gt;N&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[265,266]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>n = 2</td>
<td>[267]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 5</td>
<td>[265,266,268]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 6</td>
<td>[267]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 7</td>
<td>[265,269-271]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(22) X&lt;sub&gt;N&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[266,272]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>n = 2, R&lt;sub&gt;2&lt;/sub&gt; = H</td>
<td>[272]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 6, R&lt;sub&gt;2&lt;/sub&gt; = H</td>
<td>[273,274]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 6, R&lt;sub&gt;2&lt;/sub&gt; = OMe</td>
<td>[279]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(27) X&lt;sub&gt;N&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[279]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>n = 2 or 3</td>
<td>[280-284]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 3</td>
<td>[282,285]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 6</td>
<td>[286]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(28) X&lt;sub&gt;N&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[287]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(29) X&lt;sub&gt;N&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[288]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(30) X&lt;sub&gt;N&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[289]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(31)</td>
<td>[290,291]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(32) X&lt;sub&gt;N&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[292-297]</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>R = Me</td>
<td>[295,296]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R = H</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
1.4. Synthesis of polyamine-conjugated oligonucleotides

Two synthetic strategies towards polyamine-conjugated oligonucleotides are conceivable. The modification can be introduced either pre-synthetically to the monomer or post-synthetically to the crude oligonucleotide, both of which have advantages and disadvantages. The benefit of post-synthetic conjugation is diversification of the oligonucleotide after automated synthesis, meaning that a large number of derivatives can be prepared from a single precursor oligonucleotide. The conjugated group can be introduced either on solid support on the fully protected crude oligonucleotide, or in solution after deprotection of the nucleobases and cleavage from solid support. The solid phase has the important advantage that the crude product can be washed intensely to remove residuals of the conjugation reaction, such as for example copper in copper-catalyzed azide-alkyne cycloaddition (CuAAC). Conjugation in solution allows shortening of the work-up, as it can be done, at least in part, on a purified precursor sequence rather than following the procedures for cleavage from solid support, deprotection, HPLC purification and DMT removal for every single sequence. Thus, post-synthetic conjugation is well suited for high throughput approaches.

On the other hand, pre-synthetic introduction of a modification facilitates oligonucleotide synthesis and work-up, given that the conjugated group is tolerant to conditions of automated oligonucleotide synthesis. Furthermore, CuAAC as the conjugation chemistry used in this project is easier to perform on the monomer than on the crude oligonucleotide and pre-synthetic polyamine introduction would be compatible with a PS backbone. On the other hand, precious conjugate materials are preferably introduced post-synthetically, as the most inefficient reaction in terms of loss of equivalents is the coupling of a (modified) phosphoramidite. Stock solutions of the group to be conjugated in a post-synthetic approach can be recycled. In terms of purity however introduction of the conjugate at the monomer level is safer since the modified nucleoside is purified by column chromatography twice before use on the synthesizer. Besides, removal of copper from the monomer while in organic solvents is more straight forward than from a crude oligonucleotide in aqueous solution.

In this project, both pre- and post-synthetic polyamine-conjugation strategies have been followed. In terms of yield, usually in the nmol range, differences were negligible. Though, due to its compatibility with PS backbones, a major focus was on pre-synthetic conjugation.

1.4.1. Automated oligonucleotide synthesis

Solid-phase oligonucleotide synthesis is a fully automated synthesis procedure of nucleic acids of a defined sequence. In contrast to cellular DNA or RNA synthesis, chemical oligonucleotide synthesis is effected in the 3’ to 5’ direction. To date, the most common method is the repeated coupling of phosphoramidite base-protected nucleoside monomer units onto a solid support in a four-step reaction cycle comprising detrytilation, coupling, capping and oxidation (Figs. 11, 12) [170].

![Nucleoside phosphoramidite building blocks](image-url)

**Figure 11.** Nucleoside phosphoramidite building blocks used in DNA (R=H), RNA (R=OH), 2’-OMe RNA (R=OMe) or 2’-MOE RNA (R=OMOE) synthesis.
Figure 12. Synthetic cycle of automated oligonucleotide synthesis. 1) Detritylation: Removal of the 5'-terminal DMT group under acidic conditions, here dichloroacetic acid in DCM. 2) Coupling of the incoming base to the 5'-terminal OH group upon activation of the phosphoramidite by 5-benzylthio-1H-tetrazole (BTT). 3) Capping: To prevent formation of n-1 sequences, unreacted 5'-OH groups are acetylated in a mixture of acetic anhydride in THF and N-methylimidazole, which acts as a nucleophilic catalyst. 4) Oxidation of the phosphite group to a phosphate triester with iodine in water in the presence of pyridine. Conditions described here are used for RNA/DNA PO synthesis, for modified RNA slightly different reagents and protocols are used.

Upon completion of synthesis, crude oligonucleotides are cleaved from solid support and deprotected under basic conditions. In case of RNA, 2'-O-TBDMS is removed by TEA-3HF treatment. Crude oligonucleotides carrying a 5'-terminal DMT (DMT-on mode) are subjected to HPLC purification. Due to the important shift in retention time by the DMT group, failed sequences are easily removed. The DMT group is then cleaved, the final oligonucleotide is purified by HPLC again and is characterized by LC-MS and quantified by UV.

1.4.2. Polyamine-modification
Conjugation to sugar sites predominantly results in minor groove location of the conjugated moiety. Among the sites accessible to modification and with major groove positioning of the conjugated group are C4 and C5 on pyrimidines as well as C6 of purines, of which C5 of pyrimidines is the only one not implied in Watson-Crick base pairing and pointing away from the base pairing interface [44]. Thus, conjugation to C5 would be expected to not interfere with target hybridization. In addition, pyrimidines are more amenable to modification than purines due to simplified protection schemes and relative ease in handling compared to guanosine and analogs.

The regioselective Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes in the presence of Cu⁺ (CuAAC, commonly called “click chemistry”) is a method to efficiently and selectively modify biomolecules [298]. The resulting triazole function has been shown to have no significant influence on hybridization affinity, unless in consecutive substitutions of neighboring pyrimidines [299]. Thus, CuAAC would be a suitable and relatively straightforward conjugation chemistry for the preparation of polyamine-oligonucleotide conjugates.
1.5. Project outline

The principal objective of this work was the design of high affinity antisense oligonucleotides using polyamine-conjugation to pyrimidine bases, to characterize their binding in biophysical studies and to show their activities in therapeutically relevant biological test systems. Previous studies by Dr. Bettina Wild have shown that major groove location of the polyamine moiety was superior to minor groove location in terms of binding affinity increase. Synthesis of polyamine-conjugated oligonucleotides was rationalized following a pre-synthetic approach, allowing for various sugar and backbone chemistries, and positioning the polyamine fragment in the major groove. Target RNA binding affinities and kinetics of polyamine-conjugated oligonucleotides were investigated in melting and surface plasmon resonance (SPR) studies with the aim to understand binding mechanisms and reasons for major groove preference. For biological testing, four different target systems were chosen:

- Looptomirs targeting pre-let-7a-2 to prevent the oncogenic factor Lin28 from binding and to restore maturation of tumor-suppressive let-7a
- Antimirs targeting miR-122, antagonizing the pro-viral function of miR-122 in HCV
- Gapmers, mipomersen derivatives, targeting ApoB via an RNase H mechanism
- SSOs targeting FECH-C to rescue wild-type splicing and recover functional ferrochelatase in erythropoietic protoporphyria (EPP)

Looptomirs were 2’-OMe PO chemistry, without or with terminal PS, respectively. The looptomir library was used for biophysical investigation of target RNA binding. Antimirs were synthesized using 2’-OMe or 2’-MOE chemistries, with various patterns of phosphorothioation. Gapmers were 2’-MOE/DNA 5-10-5 full PS antisense oligonucleotides and SSOs were full 2’-MOE PS 10mers. Finally, as cellular uptake turned out to be a critical parameter in biological testing of modified oligonucleotides, an uptake assay was developed to count the number of molecules that entered the cells.
2. Results and Discussion

2.1. Selection of the position to be modified

Duplex RNA adopts an A-form helix conformation with a characteristic deep and narrow major groove and a wide and shallow minor groove (Fig. 13) [44]. Location of a conjugated polyamine fragment in both groove was compared by means of two chemically accessible conjugation sites: CS of pyrimidines for major groove, 2'-O for minor groove positioning. Previous work on polyamine-conjugation to oligonucleotides by Dr. Bettina Wild showed that, with respect to melting temperature, triazole-linked polyamine fragments were ideally positioned in the major groove. She introduced polyamine fragments by post-synthetic copper-catalyzed azide-alkyne cycloaddition (CuAAC) on 2'-OMe RNA using TFA-protected spermine azide ([Spm-N3]TFA, Fig. 18C). S-ethyl-S'-DMT-2'-OMe-uridine phosphoramidite was prepared from uridine in 7 steps; 2'-O-propargyl/pentynyl-5'-DMT-adenine phosphoramidite was synthesized from adenine in 4 steps. Alkyne-modified nucleoside phosphoramidites were incorporated into oligonucleotides using standard automated synthesis. (Spm-N3)TFA was prepared following the protocol of Yamada et al. [300] and was introduced onto the fully protected crude 2'-OMe RNA on solid support using CuAAC (Fig. 14).

A library of sequences was synthesized incorporating either major or minor groove modifications (Fig. 15). Hybridization affinity to fully complementary RNA was quantified by melting temperature. When located in the minor groove, a polyamine fragment barely affected melting temperature (Tm) (ΔTm = 0-2 °C). This is in line with literature reports on spermine, spermidine or putrescine conjugated to the 2'-O-positions of PS-DNAs [263], or other 2'-tethers containing an amino group located in the minor groove of duplex RNA [240-242,255,257]. In contrast, conjugated polyamine fragments residing in the major groove drastically increased Tm (ΔTm = 6-12 °C per modification). Likewise, an aminohexyl group in the major groove of a (DNA/2'-OMe)/RNA duplex was reported to increase Tm by 1-5 °C [243,278]. Polyamines tethers positioned in the major groove of duplex DNA however were previously shown to have a much smaller impact on hybridization affinity. CS-conjugated spermine slightly destabilized duplex DNA [273], while C4-conjugation had a stabilizing effect on DNA hybridization in two out of three reports (around 4 °C) [289-291]. To our best knowledge, this is the first report of polyamine-conjugation in the major groove of duplex RNA. The very high hybridization affinity increase in a 2'-OMe/RNA duplex indicates a strong A-form helix or duplex RNA preference of polyamine binding. In fact, free spermine was previously found to have a higher influence on DNA/RNA heteroduplexes than on DNA/DNA duplexes [232], further supporting A-
form helix preference. Respective to favoured major groove location, modelling studies have shown that spermine deeply invades the major groove of duplex RNA. It thereby stabilizes and “locks” the duplex structure by filling up the space and conformationally restricting its mobility [302]. A high electronegative potential in the major groove of an A-form helix was suggested to be a reason for preferred major groove binding of positively charged polyamines [302-304]. Other conceivable explanations could be a higher abundance of H-bonding partners in the major groove, hydration effects or influence on duplex geometry of polyamine-conjugation in favour of more A-form characteristics. Hydration, which is certainly altered upon positioning of a polyamine residue in either groove, may have various influences. Possibly, limitation in major groove hydration may aid base stacking. On the other hand, enthalpic or entropic contributions of hydration changes might also be a source of binding affinity alterations [44]. Or, simply, the shape of the narrow and deep major groove in an A-form helix may be more suitable to accommodate polyamine residues than the wide and shallow minor groove (Fig. 13).

![chemical structures](image)

**Figure 14:** Post-synthetic polyamine introduction onto 2'-OMe RNA. For major groove modifications, 5-ethynyl-2'-OMe-5'-DMT-uridine phosphoramidite was incorporated into a 2'-OMe RNA, the polyamine was introduced by CuAAC while the oligonucleotide was bound on solid support, followed by a washing procedure and conventional work-up.

In an attempt to clarify if duplex geometry was affected upon polyamine conjugation and if there would be a difference between major and minor groove location of the conjugated moiety, circular dichroism (CD) spectra of selected sequences were recorded. CD spectroscopy is a standard technique to investigate helix conformation [305]. A strong positive band at 260 nm, a small local maximum at 225 nm and a strong negative band at 210 nm [305] are characteristic features of A-form helices. Conformational flexibility towards B-form is expected to shift the strong positive band at 260 nm to higher wavelengths, to increase the local maximum at 225 nm to give another strong positive band and to decrease the amplitude of the negative band at 210 nm. Spectra of 12mer 2'-OMe RNA hybridized to fully complementary RNA all displayed strong A-form characteristics (Fig. 15 bottom left). Thus, polyamine-conjugation does not seem to alter A-form duplex geometry. Subtle changes in CD traces as a consequence of differential H-bonding patterns as well as rigidification of the A-form duplex upon polyamine insertion into the major groove are in line with previously reported effects of free spermine on an RNA/RNA duplex [302].

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The triazole moiety resulting from conjugation by CuAAC is unlikely to contribute to enhanced binding, as it was shown to have a negligible impact on DNA/DNA hybridization affinity unless cumulated at consecutive positions [299,306]. Taken together, major groove location of the conjugated polyamine proved much superior in terms of binding affinity over an analogous modification in the minor groove, in line with previous findings for positively charged free spermine identifying the major groove as the preferred binding site on duplex RNA due to its higher electronegative potential [302].
2.2. Synthesis of polyamine-modified oligonucleotides

2.2.1. Synthetic strategies
Conjugates of oligonucleotides can be prepared in a pre-synthetic way, incorporating a phosphoramidite carrying the conjugated moiety, or in a post-synthetic way, introducing the additional residue after automated oligonucleotide synthesis onto the crude oligonucleotide. Both synthetic strategies have advantages and limitations. The benefits of post-synthetic polyamine introduction are 1) easier phosphoramidite preparation and therefore (due to better coupling yield) facilitation of multiple labeling, 2) very low consumption of spermine azide, even for introduction of multiple labels, and 3) the versatility of the approach and the phosphoramidite, allowing compatibility with various other labels thanks to numerous established protocols in the lab including click on solid support, click in solution, reverse click (with azide on the oligo and alkyne on the conjugated group). However, the main shortcomings are its restricted efficiency when combined with PS chemistry as well as a possible copper contamination of resulting oligonucleotides. Even though it is common practice in our lab to introduce functional groups to oligonucleotides by CuAAC and to use these compounds for cellular testing, it has never been shown that final products are free from copper contamination, but eventual copper has never caused a problem in biological testing neither. Though, in many cases PS chemistry is crucial for biological activity of oligonucleotides and therefore a straightforward way to polyamine-introduction to phosphorothioates was highly desirable.

Pre-synthetic polyamine introduction on the other hand turned out to be better compatible with PS chemistry. Oligonucleotide synthesis and work-up take place according to a standard protocol. The limitation of this approach is the phosphoramidite itself. Protection of amine groups needs to fulfill numerous requirements: It has to be compatible with standard oligonucleotide synthesis and work-up, ideally deprotect in basic conditions to allow for compatibility with various ribose chemistries and it should give a stable phosphoramidite. Yamada et al. [300], from whom the protocol for synthesis of the spermine azide was taken, were working with TFA-protection, which was cleaved in basic treatment under standard work-up conditions. However, TFA-protection generated an NMR showing $2^\prime$ compounds, $n$ being the number of TFA protecting groups on secondary amines. Adding to the 2 isomers resulting from phosphitylation, characterization of the phosphoramidite was based on spectra showing 8 compounds. Furthermore, stability of the phosphoramidites was rather limited. After storage times at -20 °C of more than three months phosphorus oxidation or loss of DMT was often observed. Nevertheless, even though a number of other protecting groups have been tried (boc, fmoc, alloc), some of which were working fine to some extent but all having other limitations, TFA was continued to be used for its ease of synthesis and deprotection. The last two steps in the preparation of phosphoramidites, CuAAC and phosphitylation, were performed shortly before use on the synthesizer. An additional drawback of the approach is that the modified amidites did not couple as well as commercially available amidites, and often also more weakly than a 5-ethynyl-uridine phosphoramidite, hypothetically due to water-trapping properties of TFA protection. With boc protection for example, higher coupling yields could be obtained, but boc protection is exclusively compatible with 2'-OMe chemistry due to its requirement for harsh acidic deprotection conditions, which DNA and RNA do not withstand.

Summarizing, yields obtained by pre-synthetic polyamine introduction were usually comparable to yields obtained by post-synthetic modification and in the range of a few nanomoles for a conventional 50 nmol scale synthesis run. Accessibility to both synthetic approaches however allows flexibility with respect to backbone chemistry and further conjugation.
Figure 16: Preparation of 5-ethyl-5′-DMT-2′-OMe-uridine from uridine in 5 steps.

For both pre- and post-synthetic strategies, a precursor nucleoside carrying the 5-ethyl group was prepared (Fig. 16: Here, the example of 2′-OME RNA is shown). 5-ethyl-uridine was synthesized by a Sonogashira cross-coupling reaction of TMS-acetylene on 5-iodo-uridine, followed by desililation and subsequent 5′-tritylation. A TFA-protected spermine azide ((Spm-N₃)TA), synthesized according to a literature protocol [300], was conjugated using CuAAC and the resulting 5-triazole-polyamine-5′-DMT-uridine was converted to the 3′-O-phosphoramidite. To follow the post-synthetic strategy, 5-ethyl-5′-DMT-2′-OMe-uridine was converted to the phosphoramidite and incorporated into an oligonucleotide by automated synthesis. (Spm-N₃)TA was used to introduce the polyamine on solid support, followed by a few additional washing steps and conventional work-up (Fig. 17).

Figure 17: Pre- and post-synthetic polyamine introduction using 5-ethyl-5′-DMT-2′-OMe-uridine as a precursor nucleoside. In the pre-synthetic approach, click chemistry is performed on the nucleoside, which is then turned into the phosphoramidite and incorporated into an oligonucleotide by conventional automated synthesis. For the post-synthetic strategy, 5-ethyl-5′-DMT-2′-OMe-uridine phosphoramidite is used on the synthesizer and click chemistry is performed on the crude oligonucleotide on solid support.

2.2.2. Synthesis of polyamine-modified pyrimidine phosphoramidites
Starting materials were deoxyuridine (dU), 2′-O-methyluridine (mU), and 2′-O-(2-methoxyethyl)uridine (UMOE). dU was commercially available, mU was synthesized in two steps via 2,2′-anhydouridine with subsequent ring opening by methanolate with 66% and 89% yield, respectively, following the procedure of Roy et al. [307], and UMOE was obtained from Dr. Meiling Li.
Figure 18. Preparation of polyamine-modified pyrimidine phosphoramidites. A) Uridine phosphoramidites: a) R=H: I₂, (NH₄)₂Ce(NO₂)₆, AcOH, 80 °C, 20 min, 77%; R=OME: I₂, (NH₄)₂Ce(NO₂)₆, AcOH, 80 °C, 30 min, 90%; R=MOE: I₂, (NH₄)₂Ce(NO₂)₆, AcOH, 80 °C, 20 min, 72%; b) R=H: TMS-acetylene, Pd(PPh₃)₂Cl₂, CuI, Et₃N, DMF, 60 °C, 1h, 52%; R=OME: TMS-acetylene, Pd(PPh₃)₂Cl₂, CuI, Et₃N, DMF, 60 °C, 4h, 73%; c) R=H: DMT-acetylene, Pd(PPh₃)₂Cl₂, CuI, Et₃N, DMF, 60 °C, 3h, 54%; R=OME: DMT-acetylene, Pd(PPh₃)₂Cl₂, CuI, Et₃N, DMF, 60 °C, 2h, 61%; d) R=H: (NH₄)₂Ce(NO₂)₆, acetylene, CuI, Et₃N, DMF, 60 °C, 1h, 29%; R=OME: (NH₄)₂Ce(NO₂)₆, acetylene, CuI, Et₃N, DMF, 60 °C, 2h, 76%.

B) Uridine phosphoramidites: e) R=H: acetylene, N,N-dimethylformamide, TEA, THF, rt, overnight, 75%; f) R=H: (Spm-N₃)TFA, CuSO₄·5H₂O, Na-ascorbate, tBuOH/water, rt, 6h, 86%; g) R=H: (Spm-N₃)TFA, CuSO₄·5H₂O, Na-ascorbate, tBuOH/water, rt, overnight, 76%; h) R=H: 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, TEA, NMI, THF, rt, 6h, 20%; i) R=H: 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, TEA, NMI, THF, rt, 7h, 50%; j) R=H: 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, TEA, NMI, THF, rt, overnight, 29%. B) 2'-MOE-cytidine phosphoramidite: g) TMS-Cl, POCl₃, triazole, TEA, ACN, rt, 5h, 82%; h) aq. NH₃ conc., 1,4-dioxane, rt, overnight, 67%; i) benzoic anhydride, DMF, 0 °C to rt, overnight, 93%; k) (Sp-N₃)TFA, CuSO₄·5H₂O, Na-ascorbate, tBuOH/water, rt, 7h, 53%; l) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, TEA, NMI, THF, rt, overnight, 37%. C) TFA-protected spermine azide (Spm-N₃)TFA used in both pre- and post-synthetic strategies for polyamine conjugation, prepared according to [300].
Iodination at C5 with I₂ and cerium(IV) ammonium nitrate in ACOH was followed by Sonogashira coupling with Pd(PPh₃)₄Cl₂ and CuI in DMF to yield TMS-protected 5-ethynyl uridines. Dimethoxytritylation was achieved using DMT-Cl either with DMAP in pyridine or with 2,6-lutidine in ACN. TMS protection was removed with TBAF in THF to give 5-ethynyl-5'-DMT uridines (Fig. 18A). 5-ethynyl-5'-DMT-2'-deoxyuridine was prepared together with Matteo Fischer.

TFA-protected spermine azide ((Spm-N₃)TFA) was synthesized according to a protocol by Yamada et al. [300] (Fig. 18C). The reactive intermediate trifluoromethanesulfonic azide was generated in situ for azidation of a terminal amine of spermine. In order to facilitate purification, amines were boc-protected using di-tert-butyl-dicarbonate. After purification by flash chromatography to remove bis-azides and unreacted starting material, protecting groups were exchanged by removing boc with TFA in DCM, followed by TFA protection with trifluoroacetic anhydride in pyridine. The availability of spermine azide was a major bottleneck in the synthesis of polyamine-modified uridine phosphoramidites. Due to safety issues it could be synthesized on small scale only. As 1 equivalent was required for Huisgen CuAAC, it resulted in an approximate 1:1 weight ratio with the precursor nucleosides. Post-synthetic click chemistry circumvented this constraint as the mole scale is million-fold lower than in organic synthesis of modified nucleosides (nanomoles vs. millimoles). Automated oligonucleotide synthesis is more inefficient than any other step beforehand, therefore if spermine azide is the limiting step, then the strategy of post-synthetic polyamine introduction should be prioritized over the pre-synthetic approach.

Huisgen CuAAC between 5-ethynyl-5'-DMT-uridines and (Spm-N₃)TFA was achieved with copper sulfate pentahydrate (CuSO₄·5H₂O) and sodium ascorbate in tBuOH/water 1:1. The 3'-phosphoramidites were then synthesized using 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, TEA and N-methylimidazole in THF (Fig. 18A).

Conversion of 5-ethynyl-5'-DMT-2'-MOE-uridine to benzoyl-protected 5-ethynyl-5'-DMT-2'-MOE-cytidine was done by formation and displacement of a 4-triazolid intermediate, following procedures for 5-methyl-2'-MOE-cytidine ([66,308]) and 2',4'-constrained 5-methyl-2'-MOE-cytidine ([66,308]). The 1,2,4-triazole was introduced with phosphorous oxychloride and TMS-Cl in ACN. It was displaced in concentrated aqueous ammonia in 1,4-dioxane. The resulting exocyclic amine function was benzoyl-protected using benzoic anhydride in DMF. Polyamine conjugation and phosphitylation was done analogous to uridine derivatives (Fig. 18B).

### 2.2.3. Oligonucleotide synthesis

Small compound libraries for different “hot targets” of the lab were synthesized (Tab. 5). First, 2'-OMe RNA loop-binding oligonucleotides (looptomirs) targeting the pre-miRNA pre-let-7a-2 were synthesized to establish conjugation chemistry and for biophysical studies. Further on, upon availability of modified 2'-MOE amides, antimiRs targeting miR-122 were prepared with both 2'-OMe and 2'-MOE chemistry and varying phosphodiester-phosphorothioate (PO-PS) patterns. Finally, Apolipoprotein B (ApoB) targeting gapmers (mipomersen derivatives) and splice-switching oligonucleotides against a pathological splice variant of the ferrochelatase (FECH) gene in erythropoietic protoporphyria (EPP) were made.

For synthesis of 2'-OMe, RNA or DNA, standard solid phase automated synthesis conditions were applied, except for prolonged coupling times of 2 x 5 min for modified amides. For sequences containing 2'-MOE phosphoramidites, 4,5-dicyanomimidazole (DCI) was used as an activator, oxidation was done with (15)·(-)-(10-camphorsulfonyl)oxaziridine (CSO) and sulfurization with phenylacetyldisulfide (PADS) [309,310]. This in contrast to 2'-OME, RNA and DNA, where benzylthiotetrazole (BTT) was used as an activator, iodine for oxidation and 3-((N,N-dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-5-thione for sulfurization. 2'-MOE RNA synthesis was extremely sensitive to water. Air humidity was carefully monitored and controlled and reagents were dried on high vacuum overnight.
Two peculiarities of 2′-MOE RNA synthesis were crucial for successful gapmer, antimir and SSO synthesis on the MM192 synthesizer: First, the progress of the synthesis can be visually followed by means of the red color upon DMT removal (deblock) at the beginning of the synthetic cycle that provides an indication on how efficient the previous coupling was. This is an important help to speed up elaboration of optimal conditions, as otherwise after the removal of the synthesis the crude oligo needs to be treated for deprotection and cleavage from solid support prior to evaluation by LC-MS, the total of which takes >24h. On the MM12 machine, deblock color is well visible, on the MM192 however, the plate carrying the columns is not readily visible. Columns were therefore filled with 2 additional filters below the CPG in order to allow visual inspection of the coupling efficiency of the previous amide. Second, deprotection conditions had to be adapted for 2′-MOE RNA. Deprotection and cleavage from solid support was done in 32% aqueous ammonium hydroxide at 55 °C overnight. The reason for this was a change in protecting groups: "(C6H11O)3PPO phosphoramidites from Thermo Fisher carry a benzoyl on the exocyclic amino function rather than acetyl as in RNA, DNA or 2′-OMe RNA. Upon treatment with methylamine, transamination occurs to about 10% instead of hydrolysis, resulting in a N4-methyl-C with a ΔMW +14.

Table 5. Polyamine-modified oligonucleotides. PO, phosphodiester. * = PS; X = mU/prom; X = promU/prom; Y = promC/prom; X = dU/prom; MW in g/mol. â) dimers; °) double negative charge

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2.3. Hybridization affinity to complementary RNA

2.3.1. Melting temperature and thermodynamics

Upon hybridization of complementary oligonucleotides, π-π interactions of stacked bases quench UV absorption at 260 nm. This hypochromic effect is used to determine melting temperature \( T_m \), defined as the temperature at which half of the molecules are hybridized to their complementary target. Melting is recorded at concentrations in the range of 1-5 μM, depending on oligonucleotide length, so as to result in the linear range of absorbance of the spectrophotometer (absorbance around 0.5-1.0). Slow gradients, usually 0.1-0.5 °C/min, are used to ensure thermal equilibrium, which can be assessed by overlaying heating and cooling profiles.

Analysis of thermal melting curves is commonly done in 3 ways. In all cases, an upper \( (A_d(T)) \) and a lower baseline \( (A_u(T)) \) need to be fitted and a plot of absorbance vs. temperature \( Abs(T) \) is turned into an associated fraction plot \( f(T) \), \( f \) being the associated fraction, according to

\[
f(T) = \frac{A_d(T) - Abs(T)}{A_u(T) - A_d(T)} = \frac{[AB]}{[A] + [B]}
\]  

(1)

with \([A]\) and \([B]\) being single strand concentrations, \([AB]\) representing duplex concentration. The associated fraction \( f \) is then fitted to the Boltzmann equation for a sigmoidal function

\[
f(T) = B + \frac{A - B}{1 + \exp\left(\frac{T - C}{D}\right)}
\]  

(2)

where \( A, B, C \) and \( D \) are constants. \( T_m \) can be extracted from either the second derivative, where \( f''(T_m) = 0 \), corresponding to the parameter \( C \), or from \( f(T_m) = 0.5 \). Alternatively, it can be obtained from the van’t Hoff equation

\[
\ln(K_a) = -\frac{\Delta H^\circ}{R} + \frac{\Delta S^\circ}{R}
\]  

(3)

where \( R \) is the ideal gas constant, \( K_a \) is the association constant, \( \Delta H^\circ \) is the enthalpy and \( \Delta S^\circ \) is the entropy of the association reaction. Considering that \( K_a = 2/c_0 \) at \( T_m \), a \( T_m \) can be obtained from the linear fit of \( \ln(K_a) \sim 1/T \).

Thermodynamic parameters \( \Delta H^\circ \) and \( \Delta S^\circ \) can be extracted from the same linear fit of the van’t Hoff representation. Free energy \( \Delta G^\circ \) is determined from \( \Delta H^\circ \) and \( \Delta S^\circ \) or from \( K_a \) according to

\[
\Delta G^\circ = -RT \cdot \ln(K_a) = \Delta H^\circ - T \cdot \Delta S^\circ.
\]  

(4)

However, in order to derive thermodynamics from melting profiles, three prerequisites need to be met [175]: First of all, a two-state equilibrium is required and no intermediate species should be present, thus association and dissociations need to be fast. This is only true for short duplexes up to 12-14mers [311]. Second, the equations presented above are valid for bimolecular reactions. Self-complementary monomolecular hybridizations are independent of concentration and \( K_a = 1 \) at \( T_m \), therefore slightly different equations for \( \Delta H^\circ \) and \( \Delta S^\circ \) follow from van’t Hoff. Third, initial concentrations of the two strands need to be identical. Melting experiments presented here are always bimolecular associations and concentrations of single strands can be considered equal (determined by UV, ±10%). The two-state equilibrium might not be given for some of the longer oligonucleotides in the study.

An Excel template has been created and deposited on the group server to determine \( T_m \) from crude melting profiles in all three presented ways, to extract \( \Delta G^\circ, \Delta H^\circ \) and \( \Delta S^\circ \) from the van’t Hoff equation and to compile \( Abs(T), f(T) \) and hyperchromicity plots as well as graphical representations of the sigmoidal fit and its derivatives.
2.3.2. Binding of polyamine-conjugated 2′-OMe oligonucleotides to complementary RNA

Melting analysis of short polyamine-modified 2′-OMe oligonucleotides binding complementary RNA revealed an important melting temperature increase for a single modification of up to 12 °C, depending on the position of the modification within the sequence. 5′-Terminal positions were most favorable in terms of binding affinity increase, with an additive effect of bis-modification (Fig. 19, Tab. 6).

The higher stabilizing effect of polyamine modifications on the 5′-terminal position suggests that, in line with findings for a spermine on N4 of cytidine by Brzezinska et al. [291], the positively charged chain is oriented towards the 3′-direction from the modification site. The increase in binding affinity is of a fundamentally different nature from other high affinity modifications, e.g. the locked nucleic acids (LNA). For therapeutic application of LNAs, they are preferably interspersed within a DNA sequence, with approximately a third to half of the nucleotides being LNA. With their locked northern sugar puckering they force the duplex structure into an A-form helix, thus influencing sugar conformation of neighboring DNA nucleotides, especially on their 3′ side [312]. Hence, LNAs have the highest stabilizing effect when distributed throughout the sequence, rather than concentrated on the termini [313]. In contrast, polyamine conjugation to terminal positions has a higher stabilizing effect than in internal positions, suggesting that additional interactions between the strands towards the termini contribute to preventing the “zip” from “breathing” [179]. The termini of a duplex are considered the least stable zone and dehybridization is thought to take place from the ends of the duplex towards its center – in analogy to a zip [179]. Therefore, sewing the ends with further interactions may have a beneficial effect on hybridization affinity, more precisely on the dissociation rate (see below).

**Figure 19.** Melting profiles of polyamine-modified 2′-OMe RNA 7mers, 9mers, 10mers and 13mers against complementary RNA. Conditions: 2 μM double strand in 100 mM NaCl, 10 mM phosphate buffer, 0.1 mM Na2EDTA, pH 7.0.
Table 6. Melting data and hybridization thermodynamics of polyamine-conjugated 7mers, 9mers, 10mers and 13mers against complementary RNA. \( T_m \) was obtained from the maxima of the first derivatives of melting curves and is given as average of 3 consecutive melting runs. Thermodynamic parameters \( \Delta H^\circ \), \( \Delta S^\circ \) and \( \Delta G^\circ \) were determined at \( T = 310 \text{ K} \). X = mU\textsuperscript{314}.

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Thermodynamic parameters provide insights into mechanistic aspects of the binding affinity increase upon polyamine conjugation. For example, phosphate neutralization, increased stacking interactions or strengthening of the hydrogen bonding network may indicate enthalpic stabilization. On the other hand, preorganization of single strands or increased hydration may more likely result from entropic stabilization. However, thermodynamics derived from melting analysis have some limitations. Uncertainties in \( \Delta H^\circ \) and \( \Delta S^\circ \) are usually around 10-20% (not shown here), but because these errors correlate and compensate each other, uncertainties in \( \Delta G^\circ \) are typically much smaller and around 2% [314]. In addition, the shorter the oligonucleotides are, the more the prerequisite of the two-state equilibrium is likely to be satisfied. Furthermore, heat capacity changes as a function of temperature (\( \Delta C_p \)) can lead to inaccuracies of thermodynamic parameters determined from melting studies, since these are calculated at \( T_m \) and extrapolated to rt. Thus, the higher the \( T_m \), the higher the risk for inaccuracy due to \( \Delta C_p \). In this light, we can learn more from 7mers, 9mers and eventually 10mers and should deal with 13mers with caution.

Generally, short oligonucleotides with a single polyamine modification underwent entropic stabilization, while bis-modification resulted in enthalpic stabilization (Tab. 6). This suggests that more than one parameter is at play in the mechanism of binding affinity increase. It could not be excluded that the triazole linker has an effect on stacking interactions. Kočalka et al. [299] studied incorporation of 5-triazole-uridine in a DNA duplex and observed a slight \( T_m \) drop for single or bis-modification, while a \( T_m \) increase was found for 4 consecutive modifications. Based on modelling studies, they claimed that stacking of the triazoles in the major groove would support base stacking. In this light, the triazole resulting from CuAAC with spermine azide is expected to have a minor influence on hybridization affinity compared to the polyamine tether. Duplex hydration however may play a certain role for single modifications. Possibly, the polyamine fragment displaces water molecules on the single strand or alters hydration of the duplex. Furthermore, it is conceivable that the single strand may be preorganized in a way that facilitates hybridization to the RNA complement.

\( \Delta G^\circ \) of hybridization for short oligonucleotides correlated well with melting temperature results, as the trends were in line with \( T_m \) for the 7mers, 9mers and 10mers in 8/9 examples (Tab. 6). In the case of longer oligonucleotides (13mers), \( \Delta G^\circ \) did not correlate with \( T_m \), however the two-state equilibrium assumption may be less reliable here and thermodynamics derived from melting profiles have some limitations.
Taken together, polyamine conjugation leads to a very high binding affinity increase for a single modification, while bis-modification has an additive effect. Binding mechanistics however involve multiple parameters.
2.4. Hybridization affinity and kinetics to structured and unstructured RNA

2.4.1. Surface plasmon resonance spectroscopy (SPR)

SPR is a technique to measure interactions between molecules using an optical phenomenon [315]. The underlying principle is a physical process occurring when polarized light hits a gold surface at an incident angle above the critical angle of total internal reflection (TIR). Upon reflection of the light beam at the interface between two media of different refractive index $n$ the photon electrical field can interact with the free electron constellation in the gold film (Fig. 20, left). Photons of the incident light are absorbed and transmit their energy to the outer shell electrons, which convert to plasmons (the particle name of electron density waves), emitting the so-called evanescent wave and dissipating the energy by heat (Fig. 20, right). This energy transfer, called resonance, only takes place if the momentum of the incoming photons equals the momentum of plasmons. The characteristic plasmon momentum is defined by intrinsic properties of the gold film as well as the medium on either side of the film. Thus, surface plasmons are only formed at a specific angle and energy of the incident light. The evanescent wave, on the other hand, is highly dependent on the refractive index of the medium, through which the electric field travels. If the refractive index of the medium changes, the velocity of the plasmons changes and therefore the angle of incident light at which resonance occurs. This can be detected in a very precise manner, either as a resonance angle shift (angular SPR, eliminating the resonance angle from the range of incident angles) or as a wavelength change (spectral SPR), and is displayed as a signal in arbitrary units [RU].

![Figure 20. SPR principle. A) SPR occurs when polarized light hits a metal surface at an incident angle above the critical angle θ, under total internal reflection conditions. B) At a certain angle and a certain wavelength, the electrical field of the incident light interacts with the free electrons of the metal surface, which convert to surface plasmons and dissipate some of the energy as an evanescent wave.](image)

In an SPR experiment the temperature, the nature of the gold film and the angle and wavelength of the incoming light are kept constant, therefore the SPR signal fully depends on the refractive index of the medium. Molecules binding to the surface change the refractive index in the vicinity of the gold film. The mass associated directly correlates with the refractive index, allowing for a conclusion on the number of molecules bound based on the SPR signal (Fig. 21).

The main advantage of SPR over conventional binding assays is that it does not require labeling of the analyte, as a simple mass change in the vicinity of the surface is responsible for a detectable signal. Furthermore, interactions can be monitored in real time and, besides affinities, binding kinetics can be obtained. Thus, SPR is an excellent method for studying interactions of biomolecules such as antibody-antigen interactions, small molecules-protein interactions, DNA, RNA, sugar chains or even lipid membranes. However, in a biological context, SPR is an *in vitro* assay exclusively detecting interaction but not function.
In the context of nucleic acids, SPR is most commonly used to assess nucleic acid–protein interactions as for example RNA binding proteins, transcription factors, interactions involving viral proteins (HIV) or enzymes. Alternatively, SPR is applied to investigate nucleic acid–small molecule interactions such as low molecular weight minor groove binders or intercalators [316]. Nucleic acid hybridization is not commonly the scope of SPR. There are two main limitations of the approach: First, secondary structure considerably influences hybridization affinity of targeting oligonucleotides [317]. While kept at room temperature in a continuous buffer flow, RNA folding is hypothesized to be stable over a couple of days. However, ligand secondary structure on the chip cannot be controlled. Second, hybridization kinetics of nucleic acids may not follow a simple Langmuir 1:1 interaction model. A hybridization reaction consists of two phases, a nucleation step, where the two strands come together and form an initial nucleus of 3 nt, and a zippering step, forming the remaining base pairs [179]. It is unclear if SPR detects nucleation only, considered as the rate-limiting step, or if the monitored kinetics are an overlay of both processes.

What is the motive for putting effort into obtaining kinetic data for a process as complicated as hybridization of complementary nucleic acids? Even though contemporary medicinal chemistry efforts are largely based on equilibrium dissociation constants $K_d$, this artificial affinity constant is valid in a closed system only and may have less value in an ever changing open system such as an in vivo situation (see 1.2.6.1). For small molecule drugs, it has been suggested that the residence time of the drug be more crucial than apparent affinity per se [176,177,318,319]. In an open system, the residence time of the drug, which can be derived from the half-life or from $k_{off}$, may be a better surrogate of in vivo drug efficacy. This might as well apply to oligonucleotide drugs, where in addition separate optimization of $k_{on}$ and $k_{off}$ may allow fine-tuning of oligonucleotide properties for a given application. This is the value of the kinetic characterization of a binding event: It not only facilitates molecular understanding of the binding mechanism, but it may allow for better predictions of pharmacokinetic and pharmacodynamics performance of a given drug in vivo.
2.4.2. Experimental setup

SPR is very sensitive to temperature changes or small variations in experimental conditions. Therefore, a “double-referencing” system is used. First, each analyte injection is run over two surfaces, the active surface carrying the ligand and a reference surface without the ligand, the signal of which is subsequently substracted from the signal on the active surface. Second, buffer injections are interspersed within analyte injections and are also substracted. A resulting example sensorogram of a single injection is shown in Fig. 22. In a typical SPR experiment, five characteristic phases can be distinguished:

1) Pre-injection phase, setting the base-line. Buffer is run over the surfaces.
2) Association phase, starting upon analyte injection. Binding of analyte to ligand is monitored in real time. Calculation of the association rate $k_{on}$ is based on the association phase.
3) Equilibrium phase, when association and dissociation between analyte and ligand are in equilibrium. If the equilibrium is reached, the dissociation constant $K_d$ can be extracted from the steady-state signals of injections of different concentrations.
4) Dissociation phase, post-injection, when the analyte injection is replaced by buffer only. The dissociation rate $k_d$ is determined from this phase.
5) Regeneration phase, to restore the surface after each analyte injection. Remaining analyte is removed by injection of a regenerating agent and the same surface is recovered as prior to any injection.

![Sensorgram diagram](Image)

**Figure 22.** A typical sensorogram showing a single analyte injection. The five characteristic phases are 1) baseline before injection, 2) injection of analyte and association phase, 3) steady-state where association and dissociation are in equilibrium, and 4) dissociation after end of injection. Phase 5) is regeneration of the surface (not shown here).

Multiple immobilization strategies of the ligand on the gold surface are known. A standard approach is the use of biotinylated ligands that are captured by covalently immobilized streptavidin or neutravidin [320]. Chips for amine coupling are covered by a dextran matrix, functionalized with terminal carboxy groups. These are activated by injection of a freshly prepared mixture of 0.4 M $N$-ethyl-$N'$-(dimethylaminopropyl)carbodiimide (EDC) and 0.1 M $N$-hydroxysuccinimide (NHS) and conversion to the NHS ester. Streptavidin is adsorbed to the surface upon injection of a 0.1 mg/ml solution and its free amines react with the NHS ester to form covalent bonds. Subsequent 1 M ethanolamine injection removes non-covalently bound streptavidin from the surface and inactives unreacted NHS esters. As a rule of thumb, for nucleic acid interactions on SPR-2 or MASS-1 machines from Sierra Sensors, a streptavidin immobilization level of 2000 RU is considered optimal. Prior to amine coupling, the surface is prepared by a series of acidic and basic injections, HCl 100 mM and elution buffer (1 M NaCl, 10 mM NaOH) (Fig. 23).
The biotinylated ligand is then immobilized on the streptavidin surface, whereby the intended immobilization level of the ligand is an important issue to consider. For an optimal SPR experiment on SPR-2 or MASS-1, the maximal signal $R_{\text{max}}$ of the analytes is 10-20 RU. This represents a compromise between signal-to-noise ratio and mass transport. The noise on SPR-2 or MASS-1 is typically around 1 RU, therefore $R_{\text{max}}$ should not drop considerably below 10 RU. Mass transport is a phenomenon very often limiting SPR application in the assessment of nucleic acid interactions. Large analytes have a relatively slow diffusion, therefore diffusion to the surface at some point gets slower than association to the ligand. If these rates are in a comparable range, fitting of the association phase needs to consider an additional parameter, a rate constant taking diffusion into account. If association gets much faster than diffusion, the association rate cannot be determined reliably anymore. Diffusion $D$ of molecules in a solution is given by the Stokes-Einstein equation

$$D = \frac{k_B T}{6\pi \eta r}$$

where $k_B$ denotes the Boltzmann’s constant, $T$ the absolute temperature, $\eta$ the viscosity of the solvent and $r$ the radius of the spherical particle. As a rule of thumb, assessment of binding kinetics of oligonucleotides of 9 or more nt in length is mass transport limited which needs to be considered for data fitting. In addition to the size of the analyte, diffusion and therefore mass transport also depends on ligand density on the surface; with faster diffusion for a less densely coated surface. Therefore, the $R_{\text{max}}$ should not greatly exceed the minimal necessary signal. The amount of immobilized ligand needed in order to obtain an $R_{\text{max}}$ around 10-20 RU can be calculated from the ratio of molecular weights of analyte and ligand

$$R_{\text{max}} = \frac{R_{\text{ligand}} \cdot MW_{\text{analyte}}}{MW_{\text{ligand}}}$$

with $R_{\text{ligand}}$ representing the amount of immobilized ligand after ethanolamine injection. In practice, the amount of immobilized ligand should be 20-50 % higher than calculated, as very often surface-immobilized ligands are not fully functional. This can be assessed by a simple analyte injection, whereupon more ligand can be immobilized if necessary. Of note, the ligand is only immobilized on flow cell 2 (FC2), as flow cell 1 (FC1) should remain empty as a reference surface! Therefore, up to ethanolamine, both flow cells have identical treatment, but final ligand immobilization is on flow cell 2 exclusively (Fig. 24). In case of multivalent ligands, a stoichiometry factor needs to be factored into this equation.
2.4.3. Data fitting

Fitting of the association and dissociation phase in a sensorgram yields the association and dissociation rate constants. In order to reliably determine kinetic parameters, sensorgrams should exhibit sufficient curvature. For example, if dissociation is so slow that no signal drop can be observed during the dissociation interval chosen, $k_{off}$ cannot be determined from this dissociation phase. In analogy, if association is so fast that equilibrium is instantaneously reached, $k_{on}$ can hardly be extracted, especially when mass transport comes into play. Many different binding models are conceivable, among them a conformational change model, a model for a heterogeneous surface with multiple binding sites, a model for a decaying surface, a model for a dimerizing analyte or for a multivalent analyte etc., all of which are mathematically challenging. The most simple model which should be applied whenever possible is the Langmuir 1:1 interaction, with or without correction for mass transport, as presented below. For this work, fittings were done according to Langmuir.

Considering the interaction

$$L + A \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} LA$$

the differential equation for the SPR signal change, representing the change in [LA], comes to

$$\frac{d[LA]}{dt} = k_{on} \cdot [L] \cdot [A] - k_{off} [LA]$$

with [L] as the concentration of the free ligand, [A] as the concentration of the free analyte and [LA] as the concentration of the complex. In an SPR experiment, [A] remains constant due to the continuous analyte flow during the injection. It follows the integrate rate equation

$$R = R_{eq}(1 - \exp^{-(k_{on} \cdot C + k_{off})(t-t_0)})$$

where $C$ denotes the free analyte concentration [A] and $R_{eq}$ represents the equilibrium signal

$$R_{eq} = \frac{k_{on} \cdot C}{k_{on} \cdot C + k_{off}} \cdot R_{max}$$

For the dissociation phase, the above equation reduces to

$$R = R_0 \cdot (1 - \exp^{-k_{off}(t-t_0)})$$
with \( R_0 \) as initial signal at \( t = t_0 \). If association and dissociation are so fast that they cannot be fitted, steady-state analysis represents a valid alternative to obtain \( K_D \). Steady-state analysis can only be done if the equilibrium phase is reached, requesting sufficiently long injection times. As a rule of thumb, it requires injections of analyte concentrations in the range of 0.1 \( K_D \) - 10 \( K_D \). Steady-state analysis does not deliver any information about binding kinetics, but is a reliable alternative method to obtain \( K_D \). For small molecules with fast kinetics the equilibrium phase is usually attained and steady-state analysis is therefore a standard approach to determine binding affinities. In contrast, for large molecules with slower dissociation and diffusion, the equilibrium phase is not reached and \( K_D \) is obtained from kinetic fitting. For practical reasons, 10 two-fold serial dilutions of an analyte start concentration around 10 \( K_D \) are injected in duplicates, usually. If an interaction is mass transport limited, meaning that diffusion of the analyte to the surface is slower than the actual association, the event needs to be dissected into 2 phases

\[
A_{\text{bulk}} \xrightarrow{k_m} L + A_{\text{surface}} \xrightarrow{k_m} LA
\]  

(12)

Processing thereof is mathematically more challenging. However, there are a number of software tools available making analysis of sensorgrams accessible to a general public. Zeroing at the start of the injections, aligning them, reference substraction and even mass transport correction are easily performed. For the work presented here, different editions of the Analyzer by SierraSensors were used, as well as Scrubber2 from BioLogic Software.

### 2.4.4. Binding kinetics of polyamine-conjugated oligonucleotides to complementary RNA

The influence of polyamine-conjugation on hybridization kinetics of 2'-OMe oligonucleotides to complementary RNA was investigated by SPR. A biotinylated target RNA was immobilized on the chip surface and 7mers, 9mers and 10mers were analyzed. Sensorgrams are displayed in Fig. 26, kinetic parameters and binding affinities are given in Tab. 7.

**Table 7.** Binding affinities, kinetics and free energy of polyamine-conjugated 2'-OMe RNA 7mers, 9mers and 10mers binding complementary unstructured RNA, determined by SPR at 25 °C. Right, comparison to melting temperature and free energy of hybridization derived from melting experiments (taken from Tab. 6). \( X = \mu \text{ism} \). For explanation of oligonucleotide nomenclature, see Tab. 8.

<table>
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<th>looptomir</th>
<th>sequence</th>
<th>( K_D ) [nM]</th>
<th>( k_m \times 10^3 ) [M·s⁻¹]</th>
<th>( k_m \times 10^3 ) [s⁻¹]</th>
<th>( \Delta G^* ) [kcal/mol]</th>
<th>( \Delta \Delta G^* ) [kcal/mol]</th>
<th>( T_m ) [°C]</th>
<th>( \Delta T_m ) [°C]</th>
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7mer oligonucleotides were not mass transport limited and attained equilibrium, allowing for steady-state analysis. 9mers and 10mers were fitted to a model taking mass transport into account and steady-state was not reached. Polyamine-modification had the highest impact in 7mers. Starting at high nM affinity, a single terminal
polyamine-modification reduced it 165-fold, while an internal modification decreased $K_D$ 55-fold. Bis-modification further increased hybridization affinity, lowering $K_D$ again 10-fold down to pM affinity. The effects of single polyamine-modifications in 9mers and 10mers were similar. For a 5’-terminal modification, $K_D$ dropped 24/-39-fold, respectively, and for internal modifications it was 5/-4-fold. Bis-modification however had a much higher impact in 10mers, where it increased affinity 100-fold, while only 10-fold in 9mers. Overall affinity was highest for the 10mers, in line with melting data. With respects to binding kinetics, $k_{on}$ seems generally to be more affected than $k_{off}$. This was not the case for 7mers, where polyamine-conjugation had an important influence on both $k_{on}$ and $k_{off}$. Thus, binding affinity increases from polyamine-conjugation are primarily $k_{on}$-driven. Of note, terminal polyamine-modification decreases $k_{off}$ a bit more than does internal modification, in line with the suggestion of an end-stabilized zip model.

**Figure 26.** Sensorgrams of polyamine-conjugated 2’-OMe RNA 7mers, 9mers and 10mers binding immobilized unstructured target RNA. Conditions: PBS running buffer containing TWEEN 0.005%, 100 µl injections, flow rate 25 µl/min, regeneration HCl 10 mM and NaCl 1 M 25 µl, 25 °C.

Converting $K_D$s to $\Delta G^\circ$ values represents a linearization and easier quantification of the effect. It clearly shows that polyamine-modification has the highest impact in short oligonucleotides. It further indicates that 5’-terminal
modifications are more efficient than internal ones and that bis-modification has an additive effect, with the exception of the 9mers, even though not quite numerically additive.

2.4.5. Comparison of SPR data to melting data

Binding of polyamine-modified 2′-OMe oligonucleotides to linear target RNA was investigated by SPR as well as in melting experiments (Tab. 7). Both techniques yield free energy of hybridization \( \Delta G^\circ \), allowing for comparison of binding affinities determined by two complementary systems. The differences between these systems were

1) The buffer: PBS with 0.005% TWEEN for SPR (pH 7.4), the melting buffer was very similar in composition but with additional KCl and EDTA (pH 7).
2) 3′-attachment of the target RNA in SPR
3) A moving system, a constant buffer flow in SPR

A critical limitation in \( \Delta G^\circ \) determination from melting data is the assumption that \( \Delta C_P=0 \). If this assumption is not entirely true, then \( \Delta G^\circ \) (at 25 °C) becomes more inaccurate the higher the \( T_M \) is. The loose correlation between \( \Delta G^\circ \) and \( T_M \) at higher \( T_M \)S reflects this problem and indeed indicates that the of \( \Delta C_P=0 \) might not entirely be met (Fig. 25).

\[
\Delta G^\circ = 0.325 \cdot T_M \cdot \left( ^\circ \text{C} \right)^{-1} \text{kcal/mol} + 0.48 \text{kcal/mol} \tag{13}
\]

\[
\Delta G^\circ_{\text{SPR}} = -0.154 \cdot T_M \cdot \left( ^\circ \text{C} \right)^{-1} \text{kcal/mol} - 5.29 \text{kcal/mol} \tag{14}
\]

![Figure 25](image_url). Correlation of \( \Delta G^\circ \) obtained from melting and SPR studies and \( T_M \) of polyamine-conjugated 7mers, 9mers and 10mers. Dashed lines represent the corresponding linear regression.

\( \Delta G^\circ \) as obtained from melting and SPR studies was plotted against \( T_M \) in order to investigate linearity. Overall, we observe lower \( \Delta G^\circ \) in melting experiments, corresponding to higher binding affinity, indicating that SPR conditions might constrain hybridization of a complementary target RNA. 3′-Attachment might restrict association efficiency, while the constant buffer flow potentially favors dissociation. In line with these findings is a comparison of binding affinities of biotin to streptavidin in solution versus surface-bound, which found faster association and slower dissociation for the interaction in solution, resulting in higher binding affinity by several orders of magnitude than on surface [321]. Furthermore, a \( T_M \) increase leads to a higher binding affinity increase in melting experiments than in SPR, i.e. stronger dependence of \( \Delta G^\circ_{\text{melting}} \) on \( T_M \). Linear regression of \( \Delta G^\circ_{\text{melting}} \) and \( \Delta G^\circ_{\text{SPR}} \) against \( T_M \) yields
where the best correlation is seen in the range of 40-60 °C. This is the $T_m$ range where polyamine-conjugation has the highest impact in terms of binding affinity. Compounds with very high binding affinity ($T_m > 70$-80 °C, depending on the application) cannot be optimized further beyond a certain “maximum affinity” as their limitations are not binding affinity but something else [322]. In addition, their binding affinity would be difficult to assess, as a natural limitation of a melting experiment is evaporation of the melting buffer above 90 °C. Supposingly, SPR could go beyond that, however a compound with very high binding affinity already would only marginally benefit from polyamine-conjugation.

In summary, binding affinity assessment by melting experiments resulted in higher impact of polyamine-conjugation than in SPR, in particular for the bis-modifications, binding trends however were consistent across the techniques.

### 2.4.6. Binding kinetics of polyamine-conjugated oligonucleotides to structured RNA

Cellular RNAs are folded into secondary and tertiary structures and often associate with RNA-binding proteins (RBP). Secondary structure plays a critical role as it can compete with binding of antisense oligonucleotides (ASOs) and is often given as a principal reason for ASO failure in vivo [323]. In order to get closer to the in vivo situation with an in vitro assay, SPR was used to assess binding of polyamine-conjugated oligonucleotides to structured RNA. The target sequence of the 7mer, 9mer and 10mer library was taken from the terminal loop region (TLR) of pre-let-7a-2, a tumor-suppressive miRNA involved in the pathogenesis of many human cancers whose processing can be regulated by binding of so-called looptomirs, miRNA-targeting oligonucleotides binding the TLR (Tab. 8, right) [324]. The 3′-biotinylated pre-miRNA was immobilized and 7mer, 9mer, 10mer and 13mer looptomirs were assayed. Equilibrium dissociation constants $K_d$ and association and dissociation rates $k_{on}$ and $k_{off}$ are summarized in Table 8 and sensorgrams are shown in Fig. 27.

#### Table 8. Binding affinities, kinetics and free energy of 7mer, 9mer, 10mer and 13mer looptomirs binding pre-let-7a-2 hairpin, determined by SPR at 25 °C (left); nomenclature of pre-let-7a-2 looptomirs, shown on a truncated pre-let-7a-2 stem-loop structure (right). The conserved Lin28 binding motif in the TLR of pre-let-7a-2 is highlighted in orange. $X = M_{U_{spm}}$. 

<table>
<thead>
<tr>
<th>looptomir</th>
<th>sequence</th>
<th>$K_d$ [nM]</th>
<th>$k_{on} \times 10^3$ [M$^{-1}$s$^{-1}$]</th>
<th>$k_{off} \times 10^{-3}$ [s$^{-1}$]</th>
<th>$\Delta G^*$ [kcal/mol]</th>
<th>$\Delta \Delta G^*$ [kcal/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>L38-7</td>
<td>UAUCUC</td>
<td>8.100</td>
<td>24</td>
<td>197</td>
<td>-6.9</td>
<td></td>
</tr>
<tr>
<td>L38-7-1</td>
<td>XAUCUC</td>
<td>2.71</td>
<td>2610</td>
<td>7.06</td>
<td>-11.7</td>
<td>-4.7</td>
</tr>
<tr>
<td>L38-7-5</td>
<td>UAUCXCC</td>
<td>27.7</td>
<td>986</td>
<td>27.3</td>
<td>-10.3</td>
<td>-3.4</td>
</tr>
<tr>
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<td>10100</td>
<td>0.15</td>
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<td>-6.2</td>
</tr>
<tr>
<td>L36-9</td>
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</tr>
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<tr>
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<td>467</td>
<td>0.082</td>
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<tr>
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<tr>
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<td>-1.2</td>
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<td>0.087</td>
<td>-14.6</td>
<td>-2.6</td>
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<tr>
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<td>L29-13-2</td>
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<td>-1.3</td>
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<td>L29-13-6</td>
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<td>0.029</td>
<td>-13.9</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

\[ X = \text{Starting position of the looptomir} \]
\[ Y = \text{Length of the looptomir} \]
\[ Z = \text{Position of modification} \]
Figure 27. Sensorgrams of 7mer, 9mer, 10mer and 13mer looptomirs binding to immobilized pre-let-7a-2 hairpin. Conditions: PBS running buffer containing TWEEN 0.005%, 130 µl injections, flow rate 25 µl/min, regeneration HCl 10 mM 15 µl, 25 °C.
Generally, binding to structured RNA was weaker than to unstructured complementary RNA. However, the impact of polyamine-conjugation is similar to binding to unstructured RNA. 5’-Terminal conjugation is more effective than at internal positions and bis-conjugation further lowers $K_D$. However, in contrast to targeting of unstructured RNA, here terminal location of the polyamine fragment does not lower $k_{off}$ more than internal location does, it does not seem to consistently “lock the ends of the zip”. In line with findings for binding of unstructured RNA, hybridization affinity increases are predominantly $k_{on}$-driven, except for the 7mers, where $k_{off}$ dramatically decreases upon polyamine-conjugation in both target systems. This is consistent with a literature report on accelerated association of long RNAs in presence of free spermine [325].

In addition to 7mers, 9mers and 10mers, a small library of 13mers was tested. The binding affinity increase mediated by polyamine-conjugation was reduced compared to shorter looptomirs, but trends are in line with 9mers and 10mers. Of note, L29-13 was the looptomir with the highest binding affinity to pre-let-7a-2, pM, emerging from a screen [324]. This suggests that, in analogy to targeting of unstructured RNA, polyamine-conjugation has the highest effect in 7mer looptomirs with intrinsically sub-optimal binding affinity.

Free energy changes upon polyamine-conjugation are much the same in both target systems in case of the 9mers and 10mers, in 7mers Δ$G^*$ was more affected when binding to the pre-miRNA hairpin. Presumably, in line with the highest binding affinity increase in short oligonucleotides such as 7mers, the weaker binding of the unmodified 7mer to the hairpin leaves more room for optimization.

Overall, binding affinity was higher to unstructured RNA than to the folded stem-loop structure. A possible explanation might be that looptomirs are engaged with parts of the terminal loop region (TLR) that are involved in other interactions in a folded hairpin. For example, A(44) of the pre-miRNA hairpin base-pairs with U(22) (Tab. 8, right). As a result, due to involvement in secondary structure of a nt complementary to the terminal site of the 7mer, binding of the 7mer to the hairpin may resemble binding of a 6mer with drastically lower binding affinity. Else, it would have to break up a pre-existing base pair. Alternatively, the TLR might be “constrained” and less predisposed towards oligonucleotide binding than a floppy single strand. In fact, the TLR of pre-let-7a-2 does not seem to be pre-organized in a way to favor accommodation of looptomirs. Association rates $k_{on}$ on the hairpin were slower than on the linear complement. $k_{off}$, on the other hand, did not change a lot when going from the linear complement to structured RNA. Consequently, mass transport was more important for binding to the linear complement, as can be seen from the sensorgrams. It suggests that while compromising annealing rates, conformational restriction did not influence the residence time of the looptomir once it was bound. Furthermore, binding affinity changes between 9mers and 10mers for going from folded RNA to an unstructured target are very striking. Binding affinity of the 10mers against the linear complement was about 50-fold higher than to the hairpin; while only about 5-fold for 9mers. Hybridization affinity to the pre-miRNA hairpin did not change much between 9mers and 10mers, binding to the linear complement however changed about 10-fold. In melting, this corresponds to a difference of about 5 °C between 9mers and 10mers (Tab. 7). Presumably, when binding to structured RNA, nts are not equally important. Some sites might be more easily accessible than others. Possibly, the additional nt of the 10mer compared to the 9mer bound to a kinked complementary nt of the TLR and base-pairing therefore was energetically not favorable.
2.5. Target selectivity: Mismatch studies

The longer an oligonucleotide, the better chance for specificity, however beyond a certain length mismatch tolerance increase and target selectivity decreases again [326]. In practice, a large part of cellular RNA are folded or complexed with proteins, which considerably reduces the number of potential target sites. Hence, selectivity was claimed even for a 7mer ASO [11]. However, high affinity modifications bear the risk of compromising target selectivity. In order to investigate if polyamine-conjugation reduces target selectivity, internal or terminal mismatches were introduced into polyamine-modified 7mers. Melting temperature to complementary target RNA was measured as well as SPR against a folded target RNA, the pre-let-7a-2 stem-loop structure (Fig. 28).

![Figure 28](image.png)

**Figure 28.** Mismatch studies on 7mers. A) Melting profile and temperatures of 7mers with an internal or terminal mismatch (bold red) with and without conjugated polyamine (X), in comparison to fully complementary 7mers (taken from Tab. 6). Melting conditions: 2 μM double strand in 100 mM NaCl, 5 mM phosphate buffer, 0.1 mM Na₂EDTA, pH 7.0. $T_M$ was obtained from the maximum of the first derivative as an average of 3 consecutive melting runs. B) Sensorgrams of mismatched 7mers against pre-let-7a-2. SPR conditions: PBS running buffer containing Tween 0.005%, 130 μl injections, start concentration 400 nM, two-fold serial dilutions, flow rate 25 μl/min, regeneration HCl 10 mM 15 μl, 25 °C.

An internal mismatch in a 7mer fully abolished binding in both melting and SPR, and polyamine-conjugation did not rescue it. No duplex was formed above 10 °C, so at physiological temperature no hybridization would occur. In SPR, measured at 25 °C, no association was seen, therefore $K_D >> 400$ nM. A terminal mismatch, on the other hand, has a reduced impact on binding affinity. Hybridization was possible in the temperature range covered for melting, with a $\Delta T_M$ of -15.8 °C compared to full complementarity. Polyamine-conjugation increased $T_M$ by 9.0 °C, indicating that the mismatched 7mer behaves like a 6mer and affinity-enhancing modifications had a higher impact in the shorter ASOs (Δ$T_M$ = 9.0 °C vs. Δ$T_M$ = 5.2 °C for a fully complementary 7mer with internal modification). The terminal mismatch lowered $T_M$ of the internally modified 7mer by 12.0 °C, in line with what would be expected. In SPR, the terminally mismatched polyamine-modified 7mer started to bind at highest concentrations (400 nM), while the unmodified mismatched 7mer did not. $K_D$ of the terminally mismatched 7mer...
polyamine-modified 7mer is thus expected to be in the range of >400 nM, while for the unmodified mismatched $K_D$ $>>400$ nM.

With respect to target selectivity, this study shows that indeed polyamine-modification decreases selectivity. In 7mers however it would not be a relevant parameter as a single mismatch brings hybridization far below physiological temperature. In short oligonucleotides, a single mismatch has a big impact, thus naturally strengthening target selectivity. In longer oligonucleotides, loss of target selectivity could be unacceptably high.
2.6. Influence of free spermine on hybridization affinity of polyamine-conjugates

Cellular polyamines (spermine, spermidine) are highly regulated and are present at intracellular concentrations in the low mM range [215]. This compares to exogeneous oligonucleotide concentrations, in cellular assays usually in the nM range. In addition, spermine is known to preferably interact with RNA, binding in the major groove with up to 1-4 molecules of spermine per 100 phosphates, and stabilizing duplex structures [232,302,327]. Endogenous polyamines thus might have an impact on the effect of polyamine-conjugation to oligonucleotides. It is conceivable that cellular polyamines reduce or abolish the positive effect on hybridization affinity of conjugation since they occupy the same sites in the grooves of duplex RNA as the conjugated moiety. In order to investigate the influence of free polyamines on hybridization affinity of polyamine-conjugated oligonucleotides, melting studies were performed on modified 7mers in the presence of varying amounts of free spermine, in analogy to literature reports [231,232].

![Figure 29](image)

Figure 29. Melting analysis of polyamine-conjugated 2'-OMe 7mers hybridized to complementary RNA in the presence of free spermine. Conditions: 3 µM double strand in 100 mM NaCl, 5 mM phosphate buffer, 0.1 mM Na<sub>2</sub>EDTA, increasing spermine concentrations, pH 7.0. T<sub>M</sub> was obtained from the maximum of the first derivative as an average of 3 consecutive melting runs.

In line with literature reports, T<sub>M</sub> was higher at increased free spermine concentration [231,232]. T<sub>M</sub> dependence on free spermine concentration of an internally modified 7mer was similar to an unmodified 7mer, while the 5'-terminally modified 7mer showed a reduced T<sub>M</sub> dependence. T<sub>M</sub> of the bis-modified 7mer even negatively correlated with free spermine concentration (Fig. 29). Quantification of linear dependence between T<sub>M</sub> and log([free spermine]) is depicted in Fig. 30. Summarizing, the more a duplex was stabilized by covalent polyamine-conjugation, the less free spermine was able to further increase hybridization affinity. This may indicate that space for spermine accommodation in the major groove of a 7mer duplex is limited and that once the major groove is occupied, additional free spermine has a reduced effect or may even compete with conjugated polyamine fragments and displace them from the groove. The low impact of free spermine on a duplex with terminal location of a conjugated polyamine fragment suggests that 5'-terminal conjugation positions the covalently attached moiety in a way that it optimally fits into the major groove, leaving very little space for additional spermine. Consequently, high intracellular free spermine concentration reduces the impact of polyamine-conjugation on hybridization affinity and thus might play a role when going from <i>in vitro</i> assays to cellular testing. However, even at high endogenous free polyamine levels, polyamine-conjugation is expected to still have an impact on target binding affinity.
Figure 30. Linear regression of $T_M$s of polyamine-modified 7mers at varying free spermine concentrations.

\[ T_M(\text{UAUCUCC}) = (1.29 \cdot \log([\text{free spm}]) + 37.4) ^\circ C \]
\[ T_M(\text{XAUUCC}) = (0.52 \cdot \log([\text{free spm}]) + 46.6) ^\circ C \]
\[ T_M(\text{UAUXCC}) = (1.22 \cdot \log([\text{free spm}]) + 42.6) ^\circ C \]
\[ T_M(\text{XAUXCC}) = (-1.31 \cdot \log([\text{free spm}]) + 56.7) ^\circ C \]
2.7. Cellular testing of polyamine-modified oligonucleotides

Polyamine-conjugated oligonucleotides were tested in a variety of cellular assays. Ideally, the preferred target system would allow for a close relationship between binding affinity and potency of the oligonucleotide. In addition, polyamine-conjugation was expected to benefit from a catalytic type of mechanism such as RNAi or RNase H due to the fact that it causes and increased association rate $k_{on}$ while only marginally affecting the dissociation rate $k_{off}$. Four target systems were chosen for which suitable assays were available. First, pre-let-7a-2 was targeted with looptomirs to prevent degradation of the tumor-suppressive miRNA induced by Lin28 binding. Previous experiments by Dr. Martina Roos pointed to a positive effect of high affinity modifications on looptomir potency. Second, antimirs targeting miR-122, a key miRNA involved in HCV infection and cholesterol homeostasis, were studied. The link between affinity and activity was less obvious here, however the system was considered worth an investigation as a robust assay could be established. Third, mipomersen derivatives were tested, classical gapmer antisense oligonucleotides relying on RNase H, with the aim to reduce ApoB mRNA. Finally, SSOs targeting the transcript from the FECH-C allele were investigated in a splice-switching assay, trying to rescue wild-type splicing of FECH and with the long term goal to recover functional ferrochelatase in erythropoietic protoporphyria (EPP).

2.7.1. Looptomirs targeting pre-let-7a-2

At the time the polyamine-conjugation project was started, the let-7/Lin28 interaction was a very promising target system. It was and still is a highly interesting interaction in the field of miRNAs and cancer and there were indications that increased binding affinity of looptomirs targeting pre-let-7 would enhance their potency.

2.7.1.1. The let-7/Lin28 system

Let-7 was one of the first miRNAs to be discovered in C. elegans [328]. Its absence is associated with a lack of differentiation and ongoing cell division – characteristics of stem cells and cancer. Let-7 orthologs were identified, with conserved sequence in animals from zebrafish to human [329]. In mouse and human, the let-7 family members let-7a to let-7i, sharing the same seed sequence, are located on 13 different loci, along with the related miR-98 and miR-202. Target genes of let-7 are predominantly transcription factors and signaling molecules. Interactions between let-7 and its targets are well conserved among diverse animals [330]. Some important oncogenic let-7 target genes include K-RAS, HMGA2 and C-MYC, suggesting a tumor suppressor role of let-7 (Fig. 31, left). In C. elegans let-7 was found to coordinate developmental timing across tissues and to serve as a differentiation marker. In line with these findings, let-7 was only detected in differentiated tissues in mouse such as the brain, but not in human or mouse embryonic stem cells or pluripotent cells. Thus, regulation of cell differentiation and proliferation was suggested to be a conserved function of let-7 [330].

![Figure 31](image_url)

**Figure 31.** Action and interaction of let-7 and Lin28. **A)** Tumor suppressor pre-let-7 is processed and inhibits oncogenic targets. **B)** The oncogenic factor Lin28 binds to the TLR of pre-let-7, inhibiting maturation of the miRNA.

Levels of mature let-7 were shown to be regulated post-transcriptionally, to a large extent. Conserved nucleotides in the terminal loop regions (TLRs) of let-7 family members are recognized by Lin28, whose expression is
reciprocal to the appearance of mature let-7 (Fig. 31, right). Lin28 is highly expressed in embryonic stem cells and downregulated in most differentiated adult tissues [331]. In human, two isoforms exist, Lin28A and Lin28B, both of which carry a cold-shock domain (CSD) and two zinc-finger motifs (ZFD). The ZFD mediates binding to pre-let-7, of which there are 12 members, recognizing a purin-rich motif in the TLR proximal to the Dicer cleavage site [332]. Lin28, localized in the cytoplasm, depletes mature let-7 in cancer cells. Two mechanisms have been suggested. First, maturation of pre-let-7 was found to be interrupted by inhibition of Drosha and Dicer processing [333]. Alternatively, recruitment of TUTases that polyuridylate the 3’-end of the pre-miRNA was observed upon Lin28 binding [334,335]. Besides pre-miRNAs, mRNA targets of Lin28 have been identified. Lin28 was shown to enhance the translation initiation machinery and to facilitate translation of metabolic enzymes and ribosomal proteins, with the result of an increased global protein synthesis [336]. Example targets are splicing factors, RBPs, cell-cycle regulators and Lin28 itself, suggesting a feed-forward regulatory mechanism [337]. Lin28 overexpression was found in various cancers and was associated with advanced human malignancies and poor prognosis [338]. In contrast to let-7 as a differentiation marker, Lin28 is a pluripotency maintenance factor [172]. Their interactions are manifold, including a variety of feedback- and feed-forward mechanisms (Fig. 32).

2.7.1.2. Looptomirs antagonize Lin28 and enable processing of pre-let-7

In TLRs of pre-miRNAs, single-stranded regions that are accessible to RBPs were reasoned to also be accessible to hybridization with looptomirs. A previous project of the lab aimed at identifying short loop targeting oligonucleotides altering pre-let-7a-2 processing by disturbing the Lin28/pre-let-7 interaction. A 13mer 2’-OMe PO “lead looptomir” had been elaborated which promoted pre-let-7 maturation and efficiently suppressed clonogenic cell growth of cancer cells [324] (Fig. 33, left). Preliminary studies in the process of lead generation had outlined a clear link between binding affinity of looptomirs and their cellular activity, suggesting that polyamine-conjugation would be a promising strategy to enhance potency. Besides, high-affinity modifications would allow for shortened looptomirs with advantageous PK properties (Fig. 33, right).
2.7.1.3. Assessment of mature let-7 levels in a luciferase assay

Cellular activity of polyamine-conjugated looptomirs was to be evaluated in a luciferase assay according to the protocol taken from [324]. Looptomirs at graded concentrations were co-transfected in Huh7 cells with a dual-luciferase vector carrying a fully complementary target site for mature let-7 in the 3'-UTR of the Renilla gene (Fig. 34). However, since we were unable to restore assay conditions as in [324] and could not obtain the same result, a search for new assay conditions was started in order to establish a robust system to assess looptomir performance in cells.

First of all, a cell line with an intact let-7/Lin28 system had to be found. Starting from Huh7 and HEK cells, both of which are cell lines used in [324] and were reported to express both let-7 and Lin28 (mirna.org, www.proteinatlas.org), the dynamic range of the assay was investigated with control reagents as well as a control vector carrying point mutations in the target site (Fig. 35). The results in both Huh7 and HEK cells were comparable. While siRen, an siRNA targeting the Renilla gene and serving as a positive control, was downregulating Renilla on both vectors, pre-let-7a-3 did so only on the vector with a fully complementary target site. The effect on the mutated vector however was different in Huh7 and HEK cells, with a small impact in HEK cells and a contrary effect in Huh7 cells. This may suggest that a feedback loop might be involved here. Unexpectedly, siLin28, an siRNA targeting Lin28, did not affect the fully complementary vector, indicating that Lin28 levels are not in a range where small changes would affect let-7 levels and therefore not suitable for this assay. Presumably, Lin28 expression levels are either too high, with the result that inhibition of translation does...
not have a detectable effect after 48h, or too low and Lin28 is not taking part in the regulation of let-7 levels. Alternatively, a long half-life of Lin28 could drastically reduce the impact of siLin28. In this latter case, intervention with looptomirs could have an effect, while too low Lin28 expression would render looptomirs ineffective. Besides, another potential pitfall could be that endogenous let-7 levels are either too high or too low. In case of high let-7 concentration, far above its binding affinity to the target site, a further increase would not have an impact on an already saturated binding site. On the other hand, if let-7 concentration is far below its binding affinity to the target site, it is conceivable that an eventual increase might still not result in a situation that let-7 concentration is important enough to substantially bind to the target. Considering the non-linear nature of the relationship between concentration and binding, it is easily appreciated how complex this system is. In addition, the simplified view of a single precursor pre-let-7a-2 and a single binding partner Lin28 might not be accurate, as multiple pre-let-7s and both Lin28A and Lin28B might be involved in the feedback loops regulating mature let-7 levels.

![Figure 35](image.png)

Figure 35. Establishment of dual-luciferase assay in Huh7 (A) and HEK cells (B). Two different vectors are used, one with a fully complementary target site for let-7a (nat) and one containing three point mutations within the let-7a target site (mut, plaid). Control reagents at 40 nM were co-transfected with dual-luciferase vectors. After 48h, renilla and firefly luciferase activities were measured. negCON is a scrambled 10mer 2’-OMe PO, siRen is an siRNA targeting Renilla, siLin28 an siRNA against Lin28B, pre-let-7a-3 a synthetic pre-miRNA hairpin. Normalization to negCON and respective vector; error bars, ±1 SD of technical triplicates (a single representative experiment is shown). Relative luminescence, ratio renilla/firefly.

For assay optimization purposes, a number of control looptomirs was synthesized (Tab. 9). L29-13 corresponds to the lead looptomir in [324], L30-14 was the second best looptomir and was used in order to limit sequence-specific effects of L29-13. Concerns about the in cellulo potency of full PO 2’-OMe RNA lead to the synthesis of 2’-OMe looptomirs with terminal PS junctions (L29-13 PS and L30-14 PS), in analogy to [339], where 2’-OMe antimirs with terminal PS were used. Unpublished results of the lab further indicated that in some cases, full PO 2’-OMe would be degraded in cells, while a full PS 2’-OMe looptomir would have too little activity. For the same stability reason a 2’-OMe looptomir with terminal 2’-MOE substitution (L29-13 tMOE) was synthesized. Full 2’-MOE PS looptomirs were obtained from a library synthesized for pre-let-7a-2 loop screening (L29-13 MOE, L30-13 MOE).

After several rounds of transfection optimization, control looptomirs were tested in Huh7 cells and in HEK cells. Fig. 36 shows the best result from several experiments. While siRen and the positive control pre-let-7a-2 were efficiently and reliably downregulating renilla expression, siLin28 as well as the looptomirs had variable influences among replicates. It remained uncertain whether Lin28 levels were in a suitable range for the assay. Notably, variability was drastically increased as soon as an indirect mechanism via Lin28 was involved. This might indicate that the interaction has mechanistically complex consequences, as for example oscillation of levels due to tight regulation by multiple feedback-loops, in accordance to literature [331]. Furthermore, Lin28 levels rise
dramatically according to the proportion of stem cells present in the population [340]. In HEK cells, on the other hand, neither siLin28 nor looptomirs had an effect on renilla expression (not shown) and therefore the cell line was dropped.

Table 9. Pre-let-7a-2 looptomirs. For nomenclature, see Tab. 8. * = PS.

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</tr>
<tr>
<td>L29-13 MOE</td>
<td>MM83</td>
<td>C<em>U</em>C<em>C</em>U<em>G</em>A<em>U</em>G<em>U</em>A</td>
<td>2'-MOE PS</td>
<td>5112.85</td>
<td>5111.91</td>
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<tr>
<td>L29-13 tMOE</td>
<td>MM113</td>
<td>CUCCCUUAGUAGUA</td>
<td>2'-MOE/OMe PO</td>
<td>4322.90</td>
<td>4322.51</td>
</tr>
<tr>
<td>L30-14</td>
<td>Microsynth</td>
<td>AUCUCCCUUGAGU</td>
<td>2'-OMe PO</td>
<td>4541.00</td>
<td>4540.30</td>
</tr>
<tr>
<td>L30-14 PS</td>
<td>MM107</td>
<td>A<em>U</em>CUCCCUUGAU<em>G</em>U</td>
<td>2'-OMe PO/PS</td>
<td>4605.25</td>
<td>4604.07</td>
</tr>
<tr>
<td>L30-13 MOE</td>
<td>MM82</td>
<td>U<em>C</em>U<em>C</em>C<em>U</em>G<em>A</em>U<em>G</em>U</td>
<td>2'-MOE PS</td>
<td>5103.85</td>
<td>5102.30</td>
</tr>
</tbody>
</table>

As low Lin28 expression could possibly be a reason for inefficiency of looptomirs, a cell line engineered for Lin28 overexpression was tested for the luciferase assay. It was a HEK cell line with a stably introduced gene for the expression of a Lin28-GFP fusion protein [341]. The fusion protein has been well characterized and used in a FRET high-throughput screen for small molecule inhibitors of the pre-let-7/Lin28 interaction. Thus, it was known to be able to bind pre-let-7 in vitro. Biological functionality and consequences of pre-let-7 binding of the Lin28-GFP fusion protein were however unknown. Transfection of control reagents indicated that the cell line was behaving in a similar way to conventional HEK cells (Fig. 37). Pre-let-7a-2 as well as siRen were active, while siLin28 again had a contrary effect. The reason for this cannot be elucidated from this data alone, eventually, Lin28-GFP expression levels could help to clarify. Due to its artificial nature, the system was however not investigated further. Model looptomirs were inactive in this cell line (not shown).

Figure 36. Control looptomirs in dual-luciferase assay in Huh7 cells. Reagents at 40 nM were co-transfected with dual-luciferase vectors containing a fully complementary target site for let-7a in the 3'-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. negCON is a scrambled 10mer 2'-OMe PO, siRen is an siRNA targeting Renilla and siLin28 an siRNA against Lin28B. Sequences of looptomirs are given in Tab. 9. "PS" refers to 2 terminal PS linkages in 2'-OMe ribose chemistry, "MOE" is a full 2'-MOE PS sequence. Normalization to negCON; error bars, ±1 SD of technical replicates (a single experiment is shown). Relative luminescence, ratio renilla/firefly.
Figure 37. Dual-luciferase assay in HEK cell line stably overexpressing a Lin28-GFP fusion protein. Two vectors are used, one with a fully complementary target site for let-7a (nat) and one containing three point mutations within the let-7a target site (mut, plaid). Reagents at 40 nM were co-transfected with dual-luciferase vectors. After 48h, renilla and firefly luciferase activities were measured. negCON is a scrambled 10mer 2’-OMe PO, siRen is an siRNA targeting Renilla and siLin28 an siRNA against Lin28B. Normalization to negCON and respective vector; error bars, ±1 SD of technical triplicates (a single representative experiment is shown). Relative luminescence, ratio renilla/firefly.

In search for other cell lines with a let-7/Lin28 system responding to the designed assay, PC3, a prostate cancer cell line, was investigated. It has previously been used to study the biological consequences of Lin28 binding to pre-let-7 [340]. Control reagents and looptomirs were transfected at graded concentrations. SiRen performed well, pre-let-7 substitution however had a reduced effect and siLin28 was inactive. Accordingly, looptomirs were not significantly active (Fig. 38).

Figure 38. Control looptomirs in dual-luciferase assay in PC3 cells. Reagents at graded concentrations (0.6, 2.5, 10, 40 nM) were co-transfected with a dual-luciferase vector containing a fully complementary target site for let-7a in the 3’-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. negCON is a scrambled 10mer 2’-OMe PO, siRen is an siRNA targeting Renilla and siLin28 an siRNA against Lin28B. Normalization to the lowest dose. Looptomirs are given in Tab. 9. Error bars, ±1 SD of technical triplicates (a single representative experiment is shown). Relative luminescence, ratio renilla/firefly.

In order to assess whether the poor activity of siLin28 and looptomirs was due to a detection problem inherent to the luciferase assay, mature let-7 levels were quantified by TaqMan RT-qPCR upon transfection of control reagents in Huh7 and PC3 cells. The result however was inconclusive (not shown). While transfection with
synthetic pre-let-7a-2, the positive control, drastically upregulated mature let-7a levels in both Huh7 and PC3 cells, the effects of siLin28 as well as looptomirs were small, smaller than negCON treatment, and looptomirs behaved the opposite way from siLin28. The experiment would have had to be repeated many times in order to rule out an unspecific effect of the transfection event and to establish whether there is a real effect or not.

A further experiment performed by Matije Lucic addressed mRNA levels of let-7 targets. In different approach of assessing the impact of looptomir treatment on let-7 targets, he quantified mRNA of let-7 targets (Dicer1, HMGA2, CDK6, KRAS) after looptomir transfection in PC3 cells (Fig. 39). Transfection of pre-let-7a-2 downregulated Dicer1, as expected, but did not influence expression of any of the other targets. siLin28, on the other hand, upregulated Dicer1 and CDK6, in contrast to what would be expected. Looptomirs did not significantly affect mRNA levels of let-7 targets. This suggests either a very limited dynamic range in regulation of the let-7/Lin28 system in PC3 cells, or, alternatively, a restricted transfection efficiency.

![Figure 39](image.png)

**Figure 39.** Effect of control looptomirs on let-7 targets. SYBR RT-qPCR on RNA extracted from PC3 cells 48h after transfection at 40 nM concentration. Normalization to β-actin levels and to mock. negCON, scrambled 2'-OMe PO 10mer. Error bars, ±1 SD of technical quadruplicates. Experiment performed by Matije Lucic.

Taken together, under the conditions tested, looptomirs did not have detectable effects on mature let-7 levels. In order to rule out that this was due to a transfection problem of single stranded 2'-OMe RNAs, transfection efficiency was assessed in microscopy experiments on a Cy3-labelled 2'-OMe looptomir in comparison to a labelled pre-miRNA (Tab. 10). Uptake of the pre-miRNA was much better than uptake of the looptomir (Fig. 40). It seemed that transfection conditions were more suitable for double-stranded RNA rather than for single-stranded 2'-OMe PO looptomirs. As a consequence, multiple transfection conditions were tested: different amounts of transfecting reagents (N/P ratio), different transfecting reagents (lipofectamine, oligofectamie, Dharmafect, jetPEI, FuGENE), or transfection on medium with or without serum. The best conditions for looptomir transfection corresponded to the conventional protocol for transfection with lipofectamine, as shown in Fig. 40. However, the limitation of this assay was clearly the fact that the bulky and lipophilic Cy3 substituent would alter significantly the properties of transfected reagents and would have a higher impact in a short single stranded RNA rather than a longer or double stranded RNA.

**Table 10.** Cy3-labelled analytes for uptake studies. $A_{prop}$ and $C_{prop}$ phosphoramidites were incorporated during automated oligonucleotide synthesis. Cy3 was introduced by postsynthetic click reaction on solid support.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Chemistry</th>
<th>MW calc.</th>
<th>MW found</th>
</tr>
</thead>
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<td>Pre-miR-106-1Cy3</td>
<td>5'AAAAGUGCUUACAGUGCGAGGUAAUUGAGA UCUAGUGCAUUGUAGCUCUUCUAC</td>
<td>RNA PO</td>
<td>19450.60</td>
<td>19449.50</td>
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<tr>
<td>L29-13-1Cy3</td>
<td>5'CUCUUUGAUGUA</td>
<td>2'-OMe PO</td>
<td>4806.61</td>
<td>4806.79</td>
</tr>
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</table>
In conclusion, the result of looptomir transfection in a luciferase assay on let-7 as in [324] did not reproduce in our hands and high uncertainty concerning transfection efficiency of short 2'-OMe RNAs in general remained. However, since polyamine-substituents would anyway drastically alter properties of looptomirs, the library of polyamine-conjugated looptomirs was tested in luciferase assays nevertheless, using the best available conditions.

2.7.1.4. Polyamine-conjugated looptomirs in cellular assays

Polyamine-conjugated looptomirs were tested in Huh7 cells using single 40 nM concentrations (Fig. 41). A small effect of siLin28, 10mer and 13mer looptomirs was observed. However, the effect was small and the uncertainty high, thus the result needed to be confirmed in a dose-response experiment. In the following dose-response experiments in Huh7 cells 10mer and 13mer looptomirs were inactive, however (Fig. 42, only 13mers are shown).

Summarizing, the effect of pre-let-7a-2 looptomirs on mature let-7a levels in Huh7 cells, among others, is not very robust. Alternatively, inappropriate transfection conditions for short single stranded 2'-OMe RNAs could have impeded cellular testing of looptomirs. After all, despite extensive optimization attempts and in-depth investigations of assay conditions, the luciferase assay as in [324] could not be used to assess the influence of polyamine-conjugation to looptomirs. Potential pitfalls are manifold: Two years were between the experiments in [324] and the experiments presented here. Cell batches were not the same anymore and common cell culture reagents such as media or FBS or transfecting reagent might have slightly changed in composition. For example, a small alteration of the proportion of stem cells could drastically alter Lin28 levels [340]. The state of the let-7/Lin28 system and its dynamic range in the cell-lines tested remained questionable, as well as transfection efficiency of looptomirs and their stability in cells. Arguably, the let-7/Lin28 interaction is highly regulated by numerous mechanisms and its implications diverse and complex. Disappointingly, the consequence thereof was that the library of polyamine-conjugated looptomirs could never be successfully tested in cells and that the impact of polyamine-conjugation in this steric block-type of mechanism remained unclear.
Figure 41. Polyamine-conjugated looptomirs in a dual-luciferase assay in Huh7 cells (A) and nomenclature of looptomirs (B, taken from Tab. 8). Looptomir sequences are given in Tab. 5. Reagents at 40 nM were co-transfected with a dual-luciferase vector containing a fully complementary target site for let-7a in the 3'-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. negCON is a scrambled 10mer 2’-OMe PO, siRen is an siRNA targeting Renilla and siLin28 an siRNA against Lin28B. Normalization to negCON; error bars, ±1 SD of technical triplicates. Relative luminescence, ratio renilla/firefly.

Figure 42. Polyamine-conjugated 13mer looptomirs in a dose-response dual-luciferase assay in Huh7 cells. Reagents at graded concentrations (0.6, 2.5, 10, 40 nM) were co-transfected with a dual-luciferase vector containing a fully complementary target site for let-7a in the 3'-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. negCON is a scrambled 10mer 2’-OMe PO, negCON-5 is a scrambled 10mer 2’-OMe PO with polyamine-conjugation at position 5, siRen is an siRNA targeting Renilla. Looptomir sequences are given in Tab. 5 and Tab. 9, nomenclature is explained in Fig. 41. Error bars, ±1 SD of technical triplicates. Relative luminescence, ratio renilla/firefly.
2.7.2. Antimirs targeting miR-122

MiR-122 is implicated in two important (patho)physiological events: in lipid metabolism and in HCV infection. Antagonism of miR-122 was found to decrease plasma cholesterol levels in mice by 30-40% [88]. Alongside, miR-122 is known to interact with two binding sites located in the 5'‐UTR of the HC viral genome and thus up‐regulating the viral RNA in infected hepatocytes [342]. MiR-122 is well conserved, highly abundant and expressed almost exclusively in the liver. For all these reasons miR-122 represents an attractive target, especially with phosphorothioates efficiently accumulating in the liver. Thus, miR-122 was the first miRNA inhibited in mice by “antagomirs”, cholesterol-conjugated 2’-OMe PS antimirs [88].

2.7.2.1. Miravirsen, antimir targeting miR-122

Using the very potent LNA-modification, scientists at Santaris identified a rather short miR-122 antimir out of a small library of LNA-modified antimirs, all of which incorporated a few important features [343]: First, for the sake of delivery, the antimirs were supposed to be short. This was achieved by introducing LNA-modifications within a DNA strand, allowing for high melting temperatures. Second, a full PS backbone was chosen and terminal positions were protected with LNA modifications, to favor liver uptake and to promote stability. Third, all antimirs were designed to target the seed region of miR-122, the important site for target recognition, while the very first nt was not covered because of structural studies indicating that it was sitting in a separate binding pocket in Argonaute and would not be accessible for base-pairing [115,116,344]. Using a miR-122 sensor in Huh-7 cells, miR-122 inhibition was found to correlate with $T_m$. The selected lead antimir was SPC3649, later named miravirsen, a DNA-LNA PS 15mer mixmer with a $T_m$ as high as 80 °C and low nanomolar miR-122 inhibitory activity [343]. SPC3649 was shown to efficiently inhibit HCV RNA accumulation in Huh-7 cells harboring the HCV-N replicon [343] and it proved to be a potent inhibitor of miR-122 in mice, superior to previous cholesterol-conjugated antimirs [345]. In non-human primates, SPC3649 drastically lowered cholesterol levels [343], furthermore the viral load was significantly reduced in HCV-infected chimpanzees [346]. Miravirsen was safe in phase I and II clinical trials and showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance [347].

Mechanistically, miravirsen was designed to target mature miR-122 in the RISC. However, Dr. Luca Gebert has shown that the high affinity LNA-modified 15mer was able to invade stem-loop structures of pri-miR-122 and pre-miR-122, additionally inhibiting the biogenesis of miR-122 [348].

2.7.2.2. Design of a lead antimir

Our model system for evaluating antimir activity against miR-122 was a dual-luciferase assay employing a vector with a fully complementary target site for miR-122 in its Renilla 3'-UTR. Antimirs were co-transfected with the miR-122 sensor into Huh-7 cells. After 2 days of incubation, luciferase activities were measured (Fig. 43).

From a previous study of the lab it was known that target binding affinity was not the only determinant of antimir activity [339]. Antimirs targeting miR-122 were conjugated at four different positions with a range of fragments known to associate with RNA. Activities of conjugated antimirs were found to depend on the conjugation position, more than on the nature of the conjugated fragment. It was therefore reasoned that some positions of antimirs would be suitable to carry 2'-substitutions, while some others were not, and that this would be the consequence of how an antimir fits into the miR-122/RISC; more precisely, whether Argonaute proteins are able to accommodate bulky 2'-substituents or not.
Figure 43. Dual-luciferase assay for assessment of antimir activity. A fully complementary target site for miR-122 was cloned into the 3′-UTR of the Renilla gene in a psiCHECK vector. Antimirs and vector are co-transfected into Huh-7 cells. Antimirs are blocking endogenous miR-122, thereby derepressing Renilla expression.

When going from 2′-OMe chemistry to 2′-MOE, 2′-substitution gets bulkier and potentially might be in conflict with Argonaute at some sites. In order to investigate if there are any positions that should not be 2′-MOE substituted, a screen was performed on a library of 16mer 2′-OMe antimirs with two terminal PS, the lead compound of [339], with single 2′-MOE substituents along the seed region of miR-122 (Tab. 11). Activities of antimirs were assessed in a luciferase assay and binding affinities to mature miR-122 were quantified by melting temperature.

Table 11. 2′-OMe antimirs-122 with single 2′-MOE substituent (top section) and 2′-MOE antimirs with two 2′-OMe or DNA substitutions (bottom section). * = PS. Positions 11 and 14 with 2′-OMe and DNA substitution are underlined (bottom section). Synthesis together with Arpad Dunai.

<table>
<thead>
<tr>
<th>Name</th>
<th>Lab book</th>
<th>Sequence</th>
<th>Chemistry</th>
<th>MW calc.</th>
<th>MW found</th>
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<tbody>
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<td>A1-16-6MOE</td>
<td>AD6-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5293.8</td>
<td>5292.7</td>
</tr>
<tr>
<td>A1-16-7MOE</td>
<td>AD7-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5307.8</td>
<td>5308.2</td>
</tr>
<tr>
<td>A1-16-8MOE</td>
<td>AD8-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5307.8</td>
<td>5306.7</td>
</tr>
<tr>
<td>A1-16-9MOE</td>
<td>AD9-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5293.8</td>
<td>5293.1</td>
</tr>
<tr>
<td>A1-16-10MOE</td>
<td>MM127-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5307.8</td>
<td>5306.8</td>
</tr>
<tr>
<td>A1-16-11MOE</td>
<td>MM128-2</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5293.8</td>
<td>5292.9</td>
</tr>
<tr>
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<td>MM129-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5307.8</td>
<td>5308.1</td>
</tr>
<tr>
<td>A1-16-13MOE</td>
<td>MM130-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5307.8</td>
<td>5307.2</td>
</tr>
<tr>
<td>A1-16-14MOE</td>
<td>MM131-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5307.8</td>
<td>5307.4</td>
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<td>A1-16-15MOE</td>
<td>AD10-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5307.8</td>
<td>5307.1</td>
</tr>
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<td>A1-16-16MOE</td>
<td>AD11-1</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5293.8</td>
<td>5293.3</td>
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<td>5619.6</td>
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<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/Ome PO/PS</td>
<td>5780.3</td>
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<tr>
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<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/dna PO/PS</td>
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<td>A2-15-11,14DNA PS MM157</td>
<td>C<em>C</em>AUGUGUCACUCUC<em>C</em>A</td>
<td>2′-MOE/dna PO/PS</td>
<td>5720.4</td>
<td>5720.3</td>
<td></td>
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</tbody>
</table>

Striking differences in activities were obtained. 2′-MOE substitutions at positions 7, 9, 12, 15 and 16 had a very favorable effect. On the other hand, positions 11 and 14 should be avoided, in line with findings for 2′-pentylnyl substitution in [339] (Fig. 44). Comparison to melting temperature reveals that affinity is not the only determinant of cellular activity (Fig. 45). \( T_M \) does not always correlate with activity, especially in the seed region there is limited correlation. 2′-MOE substitution of terminal positions proved to have a stabilizing effect. As expected, \( \Delta T_{M5} \) are rather small, around 2.2 °C. These differences are only just above the noise (around 1 °C) and are not important enough to explain activity differences of 0.5- to 2-fold. In addition, none of the \( T_{M5} \) is below the \( T_M \) of the reference antimir A1-16, while activities for A1-16-11MOE and A1-16-14MOE clearly are.
Figure 44. Cellular testing of 2′-OMe antimirs targeting miR-122 with single 2′-MOE substitutions. Negative control negCON was a randomized DNA PS 15mer, SPC3649 is miravirsen. Antimirs at graded concentrations (0.6, 2.5, 10, 40 nM) were co-transfected with a dual-luciferase vector into Huh7 cells. A single target site for mature miR-122 was present in the 3′-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. Error bars, ±1 SD of biological triplicates. Relative luminescence, ratio renilla/firefly. Activity of the highest dose of the reference antimir A1-16 is shown as dotted line.

Figure 45. Comparison of antimir activity in luciferase assay to melting temperature. Melting conditions: 2 μM double strand in 100 mM NaCl, 5 mM phosphate buffer, 0.1 mM Na2EDTA, pH 7. $T_M$ was obtained from the maximum of the first derivative. The dotted line represents the activity or $T_M$ of the reference antimir A-16.

In order to validate positions 11 and 14 as not being suitable for 2′-MOE substitution, another four lead antimirs were synthesized. Their design was based on the following reasoning:

- Switch to A2-15 format – this is the miravirsen sequence
- Full 2′-MOE chemistry except for positions 11 and 14, as generally 2′-MOE substitutions enhance binding affinity
- Positions 11 and 14 would be 2′-OMe or 2′-deoxy, with the aim to see if even 2′-OMe substitution is too big
• Full PS and PO with terminal PS compounds should be compared, as so far it was not clear which backbone chemistry would be superior

Sequences are shown in Tab. 11. Lead antimirs were tested in a luciferase assay (Fig. 46). Surprisingly, none of the new leads was superior to a full 2’-MOE PS antimir. Both 2’-OMe and DNA modifications at positions 11 and 14 in full PS chemistry drastically reduced antimir activity. The PO analogues had some activity, however considerably lower than full 2’-MOE PS. It seemed that lessons learned from the previous screen of single 2’-MOE substitutions did not directly translate to a new optimized antimir format. However, what can be confirmed from these data is the observation by Davis et al. [119] that, in contrast to full PS substitution, PO backbone chemistry is beneficial with respect to antimir activity.

The lead finding process was stopped here. Lessons learned or confirmed from these experiments were that first, the RISC is sensitive to 2’-substitution and that second, melting temperature is a very weak surrogate for antimir activity. However, optimization attempts of the full 2’-MOE PS antimir were not conclusive. Weaknesses of the study presented here are the lack of a full 2’-MOE PS A1-16 to compare with in the screen of single 2’-MOE substitutions and the switch between A1-16 and A2-15 formats. This is due to the fact that the reference antimir of the lab in [348] and [339] was A1-16, while the miravirsen sequence, the antimir currently in clinical testing, is A2-15.

![Graph showing luciferase activity](image)

**Figure 46.** Cellular testing of 2’-MOE antimirs targeting miR-122 with two 2’-OMe or DNA substitutions at positions 11 and 14. Negative control negCON was a randomized DNA PS 15mer, SPC3649 is miravirsen. Antimirs at graded concentrations (0.6, 2.5, 10, 40 nM) were co-transfected with a dual-luciferase vector into Huh7 cells. A single target site for mature miR-122 was present in the 3’-UTR of the *Renilla* gene. After 48h, renilla and firefly luciferase activities were measured. Error bars, ±1 SD of biological duplicates. Antimir sequences are given in Tab. 11. Relative luminescence, ratio renilla/firefly. Activity of the highest dose of the reference antimir A2-15 MOE PS is shown as dotted line.

### 2.7.2.3. Evaluation of polyamine-modified antimirs

The hypothesis under investigation was whether polyamine-conjugation could enhance antimir activity (Fig. 47). A number of polyamine-modified antimirs were synthesized (Tab. 5) and were tested in a luciferase assay (Fig. 43).

A1-16 PO, a 16mer 2’-OMe full PO antimir, was modified at positions 7 or 13 or both 7 and 13. A1-16 PO and modifications were inactive (Fig. 48, top). In response to that, two terminal PS modifications created an active A1-16 antimir. It was modified at positions 4 or 4 and 13. Modification at position 4 was tolerated, the bis-modified antimir however was inactive (Fig. 48, bottom). Polyamine-conjugation at position 4 reduced antimir potency. Position 13, on the other hand, was not tested as single modification, but might represent the reason for inactivity of the bis-modified antimir, unless uptake problems prevented interaction with the target miRNA.
**Figure 47.** Driving hypothesis: Does polyamine-conjugation enhance antimir potency?

**Figure 48.** Polyamine-conjugated 2'-OMe antimirs with (B) or without (A) terminal PS linkages in a dual-luciferase assay. Negative control negCON was a randomized 2'-OMe 22mer with 3 terminal PS, SPC3649 is miravirsen and Krützfeld is the first antimir-122 as in [88]. Antimirs at graded concentrations (0.6 nM, 2.5 nM, 10 nM, 40 nM) were co-transfected with a dual-luciferase vector into Huh7 cells. A single target site for mature miR-122 was present in the 3'-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. Antimir sequences are given in Tab. 5. Error bars, ±1 SD of technical triplicates. Relative luminescence, ratio renilla/firefly.

In the following, chemistry was switched to 2'-MOE PS, as this was expected to provide a more active unmodified lead. Three different lengths were chosen: 9mers, 12mers and 15mers, all covering the seed region of the mature miRNA. At the site of polyamine-conjugation, sugar chemistry was 2'-OMe, due to limited availability of 2'-MOE amidites. 9mers were too short to have any effect at all (data not shown). While a full LNA tinymer (8mer) was
highly active, full 2’-MOE 9mers were not, and polyamine-conjugation could not rescue activity. The 12mers showed some weak inhibitory activity at the highest dose (Fig. 49, top). However, polyamine-conjugation did not seem to have any effect at all. In the 15mers, the impact of polyamine-conjugation could be assessed (Fig. 49, bottom). The unmodified full 2’-MOE PS A2-15 was probably a little more active than the full LNA 8mer. Polyamine-conjugation at positions 4 or 7 seemed to be tolerated, but only slightly enhanced antimir inhibitory activity. On the other hand, bis-modification at positions 4 and 7 had a negative impact on antimir inhibition. This might indicate a potential transfection issue. It is conceivable that the multiple positive charges interfere with lipofection and therefore uptake of modified oligonucleotides might be less efficient than without polyamine-conjugation.

![Figure 49](image-url)

**Figure 49.** Polyamine-conjugated 2’-MOE antimirs with 2’-OMe substitution at polyamine conjugation site in a dual-luciferase assay. A) 12mers, B) 15mers. Antimirs at graded concentrations (1.5 nM, 6 nM, 25 nM, 100 nM) were co-transfected with a dual-luciferase vector into Huh7 cells. A single target site for mature miR-122 was present in the 3’-UTR of the Renilla gene. After 48, renilla and firefly luciferase activities were measured. Antimir sequences are given in Tab. 5. Error bars, ±1 SD of technical triplicates. Relative luminescence, ratio renilla/firefly.

Finally, 2’-MOE PO 15mers with terminal PS were synthesized and modified at positions 1, 4, 7 and 10. None of the polyamine-conjugated derivatives showed higher antimir activity than the unmodified lead A2-15. Terminal polyamine-conjugation to position 1 was well tolerated, modification at positions 4 and 7 showed reduced activity already, and modification at position 10 abolished antimir activity. Position 10 is the only one among the positions modified here to be within the seed region (Fig. 50).
Figure 50. Polyamine-conjugated full 2′-MOE PO antimirs in a dual-luciferase assay. Antimirs at graded concentrations (0.6 nM, 2.5 nM, 10 nM, 40 nM) were co-transfected with a dual-luciferase vector into Huh7 cells. A single target site for mature miR-122 was present in the 3′-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. Antimir sequences are given in Tab. 5. Error bars, ±1 SD of technical triplicates. Relative luminescence, ratio renilla/firefly.

It can be concluded that in this assay, major groove polyamine-conjugation to antimirs did not enhance antimir potency. It was confirmed, in line with [119], [339] and [122], that binding affinity is not the only determinant of antimir activity. Bulky substituents in both the major and the minor groove can interfere with penetration into the RISC or hybridization with RISC-loaded mature miRNA. Thus, chemical optimization of antimirs should either be restricted to ribose chemistry with minimal 2′-substitution such as in LNA, or activity should be assessed in a case-by-case manner. Alternatively, structural information of Ago proteins could be used to determine optimal 2′-substitution for each nt as well as placement of eventual additional base substituents.

Side-conclusions from these experiments are summarized in Fig. 51. The “antimir map” points out that both 2′-modifications and substituents at position 5 within the seed region are delicate (red, Fig. 51). Modifications outside the seed were usually well tolerated (green, Fig. 51). Exceptions to this simplified rule are a limited number of positions in the seed which accommodate bulky 2′-substituents such as a 2′-MOE very well (positions 9, 12 and 15 of the antimir). Therefore, a case-by-case evaluation is required if the seed region needs to be modified. Of note, positions modified with polyamines shown in red (10, 13) may be tolerated in other cases due to the fact that limited uptake of polyamine-oligonucleotide conjugates might have interfered with activity assessment. The SPC3649 sequence and the modification tolerance pattern identified here do not present any obvious link.
<table>
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<tr>
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<td>C*</td>
<td>A</td>
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<td>C</td>
<td>t</td>
<td>C</td>
<td>C</td>
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</table>

**Figure 51.** Antimir map. Synopsis on tolerance of antimir substitution. Green: modification tolerated; red: modification reduces cellular activity; light colours: smaller amplitude of the effect. 2’mix, 2’-MOE with 2’-OMe at conjugation site; mm, mismatch; tinymer loc., tinymer location.
2.7.3. Mipomersen derivatives targeting ApoB mRNA

2.7.3.1. Gapmers and RNase H

RNase H is an endonuclease cleaving the RNA strand in DNA/RNA hybrids [76,77]. It belongs to a nucleotidyl-transferase superfamily, of which Argonaute 2 is a member, and is well conserved throughout evolution. In human, there are numerous isoforms, the most important being RNase H1 and RNase H2, of which mainly RNase H1 is involved in the mechanism of action of gapmers. Human RNase H1 is 286 amino acids long, 32 kDa, and is ubiquitously expressed in human cells and tissues [350]. It acts in a sequence-nonspecific way and requires divalent cations such as Mg²⁺ and Mn²⁺ in its carboxylate-rich negatively charged active site [351]. Target strand cleavage takes place in an S_{2}2-like manner, where the nucleophile is derived from either water or a 3'-OH of a nucleic acid [351]. Structural studies show that the enzyme extensively interacts with 2'-OH of the target RNA strand [351].

Gapmers are antisense oligonucleotides with a catalytic mode of action containing a central DNA part of 6-10 nt for RNase H recognition and modified nucleosides at terminal positions (“wings”) to enhance target binding affinity and the circulation half-life, and preferably carry a PS backbone [53,54]. The two most often used ribose modifications for the gapmer “wings” are LNA and 2’-MOE. Among the gapmers in clinical testing a large majority are 2’-MOE gapmers, probably due to toxicity concerns with LNA gapmers [64,65,352,353]. Tolerance of ribose modification in gapmers was investigated in a comprehensive study by Stanton et al. [74]. Furthermore, the relation between affinity (T_{M}) and knock-down activity of a cellular target was examined. A moderate association between knock-down and melting temperature up to a certain temperature was found (Fig. 52). With a T_{M} above 80 °C however, knock-down activity dropped drastically. The authors reasoned that this would be due to a loss of catalytic activity, as high affinity binders might not be able to recycle after RNase H cleavage [74].

Polyamine-conjugation to an RNase H-activating DNA PS 15mer ASO was found to be tolerated both in central and distal regions, as assessed in an in vitro RNase H assay [273].

![Figure 52](image.png)

**Figure 52.** Knock-down efficiency of chemically modified gapmers against their melting temperature (Tm). Three different sequences targeting the glucocorticoid receptor and containing most commonly used ribose modifications in their wings of different lengths were transfected into the mouse hepatoma cell line Hepa1-6 at 3 nM concentration. Taken from [74].

2.7.3.2. Mipomersen

Mipomersen (ISIS301012, Kynamro®) is a clinically approved gapmer for the treatment of familial hypercholesterolemia. It is subcutaneously administered once weekly and targets Apolipoprotein B-100 (ApoB-100) mRNA. ApoB is a structural protein on the surface of atherogenic lipoproteins such as LDL or VLDL [354].
There are two isoforms, ApoB-48 and ApoB-100. ApoB-48 is the N-terminal part of ApoB-100, produced by a splice editing process, and is involved in the assembly of chylomicrons in enterocytes [355]. ApoB-100 forms particles with lipids which are intrinsically insoluble, to transport them in blood, and mediates their internalization into hepatocytes via recognition by LDL receptors (LDLRs). Mipomersen only affects ApoB-100 [355].

Cardiovascular diseases such as coronary heart disease, cerebrovascular disease or peripheral artery disease are leading causes of death world-wide. Even though the link between cholesterol levels and cardiovascular disease has been put in question [356], it is a generally accepted strategy to lower plasma cholesterol in order to prevent cardiovascular incidents. State-of-the-art treatment are the statins, HMG-CoA reductase inhibitors, with or without ezetimibe; however, safety issues raise the need for alternatives. Numerous forms of familial hypercholesterolemia (FH) are known, most of which affect the gene encoding the LDLR or the LDLR-binding domain of ApoB-100 with the consequence of reduced cholesterol clearance from the blood. Therefore, mipomersen is a valuable adjunctive therapy in FH or an alternative for statin-intolerant patients.

Mipomersen was developed as a 2nd generation antisense oligonucleotide with PS backbone, 10 internal DNA nts and 5 2’-MOE nts on either side. All cytidines are 5-methylated to prevent unnecessary activation of the innate immune system. Upon s.c. injection of a starting dose of 200 mg/week, mipomersen was shown to reduce ApoB-100 levels in a dose-dependent manner, leading to a remarkably reduced LDL and total cholesterol in a phase 3 study [79]. Thanks to high bioavailability (80-100 %) and slow elimination the effect persisted up to 90 days after treatment [357]. HDL was not affected, and side effects were limited to injection site reactions and few asymptomatic elevations of liver enzymes [357]. Interactions with cytochrome P450 enzymes were neither expected nor observed, thus combination therapy with statins or ezetimibe would be conceivable. In clinical studies, mipomersen was usually administered along with statins [357]. In January 2013 mipomersen was approved by the FDA and obtained orphan drug status.

2.7.3.3. Evaluation of polyamine-conjugated mipomersen derivatives

A number of polyamine-conjugated mipomersen derivatives was synthesized using $^{\text{sp}}$mU$_{\text{MoE}}$, $^{\text{sp}}$mC$_{\text{MoE}}$ and dU$^{\text{sp}}$m phosphoramidites (Tab. 5). Gapmer synthesis was cumbersome. The DNA/2’-MOE transition turned out to be delicate and often, synthesis failed at one of the last five bases. Until now, this problem has not been fully resolved. It is perplexing that these compounds are produced on a very large scale for clinical testing while in our lab we cannot go beyond a yield of a few nanomoles. The reasons for the difficulty in gapmer synthesis on our MM192 have not been fully elucidated. The prevailing hypothesis is that 2’-MOE RNA traps water and thereby lowers coupling efficiency. However, enough material was recovered for cellular testing, except for the bis-modified Mipo-4,14, for which the highest dose had to be omitted. Compounds were transfected into Huh7 cells, which after 24h incubation were harvested, RNA was extracted and ApoB mRNA was quantified by PCR. Biological experiments were carried out by Dr. Helen Lightfoot.

Gapmer experiments allowed for two conclusions: First, polyamine-conjugation is tolerated. Activities at the two highest doses were similar among unmodified mipomersen and single-modified derivatives. Second, polyamine-conjugation does not enhance activity of mipomersen derivatives (Fig. 53). None of the conjugates’ performance was superior to unmodified. This may have various reasons. The most obvious reason could be that a 20mer with a melting temperature of 78 °C simply cannot be improved by additional binding affinity increases. Pedersen et al. [322] suggested that there is an affinity optimum for a given target and a given cell type, and that this is usually between -16 and -22 kcal/mol. Mipomersen has a free energy of hybridization of -30 ± 2 kcal/mol, far beyond the statistically identified “theoretical affinity optimum”. In addition, as was proposed by Stanton et al. [74], a $T_m$ above 80 °C may lead to reduced recycling capacity of gapmers and thereby loss of catalytic cleavage activity. In this light, a more reasonable approach would be to start from shorter gapmers, where binding affinity is a limiting criterion, and try to recover activity by polyamine-conjugation. This would lead to shorter antisense agents with more favorable PK while activity is maintained.
Figure 53. Effect of polyamine-modified mipomersen derivatives on ApoB expression in Huh7 cells. Gapmers were transfected at graded concentrations (0, 7.7, 19.2, 48, 120 nM) into Huh7 cells. After 24h, RNA was isolated and ApoB mRNA levels were quantified by TaqMan RT-qPCR. negCON corresponds to C*C*T*G*T*C*c*a*G*C*T*T*C and negCON-14spm to C*C*T*G*T*C*c*a*G*C*T*T*C, with z = dUspm, randomized 20mer gapmers with and without polyamine-conjugation. A) Normalization to β-actin levels and to lowest dose; B) Normalisation to β-actin levels, lowest dose and negCON. Error bars, ±1 SD of biological triplicates.

Some effects here can hardly be explained. The polyamine-modified negCON-14spm’s highest dose indeed reduces ApoB mRNA levels, even though its sequence is randomized. Does polyamine-conjugation hamper selectivity here and favor off-target binding? The second highest dose has a reproducibility problem as well, with apparent increasing ApoB mRNA levels. The second observation that cannot be explained from this data is the lack of efficacy of the bis-modified mipomersen derivative. While both single modifications were active, the bis-modified was not. This might point to an uptake problem. It is conceivable that bis-conjugation alters transfection efficacy as the overall charge decreases from -19 to -13.

In summary, it can be concluded that, as polyamine-conjugation is tolerated by RNase H, it might be a promising approach to reduce the size of gapmers in order to attain more favorable PK properties. In 20mer gapmers however, polyamine-conjugation does not enhance potency in the above presented cellular assay. Possibly, a reason for this might be limited transfection efficiency of polyamine-conjugated gapmers.
2.7.4. Splice-switching oligonucleotides (SSO) targeting FECH-C

Among the possible mechanisms of action of therapeutic oligonucleotides, splice correction is considered the one with the best correlation between binding affinity and activity and the most robust towards chemical modification [74]. While classical antisense oligonucleotides recruiting RNase H or siRNAs incorporating into RISC depend on interplay with associated proteins, splice correction by oligonucleotides consists in a simple steric block of an RNA binding site, and SSOS do not require any protein co-factors to exert their inhibitory effect. A splice correction system would therefore represent an attractive target to demonstrate enhanced biological activity of polyamine-conjugated oligonucleotides. The model system chosen was a splice correction in the ferrochelatase transcript involved in the pathogenesis of erythropoietic protoporphyria, as this is a prospering project of the lab.

2.7.4.1. Splicing defect in erythropoietic protoporphyria (EPP)

EPP is an inherited disease with multiple genetic variants and varying severity among affected individuals. It manifests as photosensitivity reaction, with pruritus, erythema, swelling upon exposure to daylight, and lichenification, mis-pigmentation and scarring of the skin upon repeated exposure. The disease arises from a deficiency in the enzyme ferrochelatase (FECH), responsible for catalyzing formation of heme by incorporation of iron into protoporphyrin IX. As heme biosynthesis takes place predominantly in erythroblasts, increased levels of protoporphyrin IX in erythrocytes, plasma and the liver are a consequence, predisposing to phototoxic reactions in small dermal vessels and to liver disease. Genetics of the disease are generally assumed to follow a triallelic inheritance model. A genetic pattern very often associated with disease is a mutated, fully dysfunctional allele along with an allele carrying a SNP, a single T>C transition at position IVS3-48 in intron 3 between exons 3 and 4, with the consequence of enhanced use of a cryptic splice site (>95% of patients, Fig. 54). Aberrant splicing leads to a stop codon in the reading frame and to degradation of the transcript by a nonsense-mediated decay mechanism, resulting in a residual FECH activity below 35%, which is the threshold value to develop symptoms.

![Image](image1.png)

**Figure 54.** The T>C transition at position -48 in intron 3 between exons 3 and 4 modulates the splicing efficiency of a cryptic splice site. Taken from [358].

It has been demonstrated that use of the cryptic splice site could be reduced by transfection of LNA-modified antisense oligonucleotides into patient-derived cells, that splicing was successfully redirected toward the physiological acceptor site and that levels of functional FECH mRNA were increased [359]. A current project of the lab is putting effort into optimizing splice correction by functionalized SSOS. The aim of the project is to identify an SSO of optimal sequence and length using 2'-MOE chemistry and to vectorize it in order to facilitate uptake into erythroblasts. Furthermore, it will be tested in a dedicated mouse model. To study splice correction in cells, a minigene system of FECH-C to reproduce alternative splicing in Cos-7 cells was used. Quantification of splicing was achieved after RNA isolation, RT-PCR and separation of correctly spliced and aberrant transcripts on an agarose gel. The splice-switching assay was performed by François Halloy to assess effects of polyamine conjugation to short SSOS.

2.7.4.2. Assessment of splice correction of polyamine conjugated SSO

The model target of candidate SSOS was a mis-spliced ferrochelatase mRNA due to a single T>C point mutation. François Halloy performed a screen of the critical region on the FECH-C mRNA to identify several active SSO of
varying length. In the present study, for the reason of higher impact on binding affinity of polyamine-conjugation to shorter oligonucleotides, a 10mer and a 12mer full 2'-MOE PS SSO were chosen as lead compounds. For a preliminary evaluation of the effect of polyamine conjugation, modifications were introduced into a 2'-MOE PS SSO using a µU supp phosphoramidite. As controls, single 2'-OMe residues were incorporated at the position of polyamine modification (Tab. 12).

Table 12. SSOs targeting FECH-C. X = mU supp; X = suppUMOE; Y = suppCMOE. * = PS. Extract from Tab. 5.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Chemistry</th>
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<tbody>
<tr>
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<td>SSO-10-7sm</td>
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<td>2'-MOE/OMe PS</td>
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<td>2'-MOE/OMe PS</td>
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<td>2'-MOE/OMe PS</td>
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<tr>
<td>SSO-12-1sm</td>
<td>X<em>A</em>G<em>C</em>A<em>G</em>C<em>C</em>T<em>G</em>A*G</td>
<td>2'-MOE/OMe PS</td>
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<td>T<em>A</em>G<em>C</em>A<em>G</em>C<em>C</em>U<em>G</em>A*G</td>
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<td>SSO-12-9sm</td>
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<td>2'-MOE PS</td>
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To quantify splice-switching activity and to assess the effect of polyamine-conjugation thereupon, Cos-7 cells were transfected with a plasmid carrying parts of a mutated gene, a truncated intron 3 and exon 4. They were then treated with SSOs and incubated for 24h or 48h. Emetine, a drug blocking translation and stabilizing RNA against nonsense-mediated decay, was used to stabilize mRNA levels. RNA was extracted and the FECH RNA was amplified by RT-PCR. The correctly spliced and the aberrant transcript were separated on an agarose gel. Splice correction was quantified using the ratio of aberrant/total as determined with Image J software.

Figure 55. Quantification of splice-switching activity of polyamine-conjugated 10mer and 12mer SSOs, first replicate, with different SSO doses. Cos-7 cells were transfected with FECH-C plasmid using Plasmid Xtreme Gene HP DNA transfection reagent and 3h later with SSO 100 nM or 200 nM using Lipofectamine 2000. Emetine 3 µM treatment after another 24h, harvesting after 48h. RNA was extracted and FECH RNA was amplified by RT-PCR. Migration on a 2% agarose gel in TAE buffer for 1h at 85V. Left and right lanes: 100 bp ladder. Bottom line: ratio aberrant/total for quantification of splice correction.

Generally, 10mer SSOs (SSO-10, SSO-10-7sm) had a rather weak effect (Fig. 55 and 56). Polyamine-conjugation slightly increased splice switching activity of the 10mer SSO at 100 nM after 48h incubation (SSO-10-7sm). For 200
nM treatment, the effect was less pronounced (Fig. 55), and for 24h exposure, the effect could not be seen at all (Fig. 56). In 12mer SSOs (SSO-12 and derivatives), polyamine-conjugation did not significantly alter splice-switching activity (Fig. 55 and 56). Melting characterization of SSO-10 and SSO-10-2sm yielded a rather modest binding affinity increase upon incorporation of mUspm of 5.7 °C (Fig. 57). Possibly, ΔT_M upon polyamine-conjugation is lower in 2'-MOE than in 2'-OMe oligonucleotides. Alternatively, as seen in longer oligonucleotides with high T_M, polyamine conjugation may have less an impact in an SSO with very high binding affinity.

Figure 56. Quantification of splice-switching activity of polyamine-modified 10mer and 12mer SSOs, second replicate, with different treatment times. Cos-7 cells were transfected with FECH-C plasmid using Plasmid Xtreme Gene HP DNA transfection reagent and 3h later with SSO 100 nM using Lipofectamine2000. Emetine 3 µM treatment 24h prior to harvesting, harvesting 24h or 48h after transfection of SSOs. RNA was extracted and FECH RNA was amplified by RT-PCR. Migration on a 2% agarose gel in TAE buffer for 1h at 85V. Left and right lanes: 100 bp ladder. Bottom line: ratio aberrant/total for quantification of splice correction.

Figure 57. Melting analysis of polyamine-modified 10mer SSO against complementary RNA. Conditions: 2 µM double strand in 100 mM NaCl, 10 mM phosphate buffer, 0.1 mM Na2EDTA, pH 7.0. T_M (SSO-10) = 69.8 °C; T_M (SSO-10-7sm) = 75.5 °C.

In a second attempt, full 2'-MOE PS 10mer SSOs were synthesized using 6mmU_MOE and 6mmC_MOE phosphoramidites to introduce the polyamine. Positions 2 (C) and 7 (T) were chosen for modification (Tab. 12). After 100 nM treatment, splice correction was not drastically different from unmodified (Fig. 58). Modification at position 2(C) showed an even reduced splice correction effect (SSO-10-2), while modification of position 7 (T) very slightly improved activity (SSO-10-7). The differences however are so small that it is unclear if they are real or if they
would just disappear as noise if the experiment was repeated many times (Fig. 58). A 2'-methoxy residue at the position of polyamine-conjugation did not have any impact, however, as opposed to the previous experiments (Fig. 55 and 56), polyamine-conjugation did not increase splice correction activity of the 10mer SSO.

**Figure 58.** Quantification of splice-switching activity of polyamine-conjugated 10mer SSOs. Cos-7 cells were transfected with FECH-C plasmid using Plasmid Xtreme Gene HP DNA transfection reagent and 3h later with SSO 100 nM using Lipofectamine2000. Emetine 3 µM treatment after another 24h, harvesting after 48h. RNA was extracted and FECH RNA was amplified by RT-PCR. Migration on a 2% agarose gel in TAE buffer for 1h at 85V. Left lane: 100 bp ladder. Bottom line: ratio aberrant/total for quantification of splice correction. negCON sequence: GGACAACGTTCG.

Taken together, from the data presented above it is not clear whether polyamine conjugation to 10mer SSOs improves splice correction (Tab. 13). A small positive effect was observed but experiments would need to be repeated many times to ascertain. It is possible that a higher impact of polyamine-conjugation to SSOs could become evident under alteration of experimental conditions.

**Table 13.** SSOs targeting FECH-C. $X = \text{mU}^{\text{SM}}$; $Y = \text{spmU}^{\text{MOE}}$; $X = \text{spmC}^{\text{MOE}}$. Green, increased potency; red, reduced potency; light colours, reduced effect; grey, indifferent.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<td>SSO-10</td>
<td>G<em>C</em>A<em>G</em>C<em>C</em>T<em>G</em>A*G</td>
<td>2'-MOE PS</td>
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<tr>
<td>SSO-10-7sm</td>
<td>G<em>C</em>A<em>G</em>C<em>C</em>X<em>G</em>A*G</td>
<td>2'-MOE/OMe PS</td>
<td></td>
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<tr>
<td>SSO-12</td>
<td>T<em>A</em>G<em>C</em>A<em>G</em>C<em>C</em>T<em>G</em>A*G</td>
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<td></td>
</tr>
<tr>
<td>SSO-12-1m</td>
<td>U<em>A</em>G<em>C</em>A<em>G</em>C<em>C</em>T<em>G</em>A*G</td>
<td>2'-MOE/OMe PS</td>
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<tr>
<td>SSO-12-1sm</td>
<td>X<em>A</em>G<em>C</em>A<em>G</em>C<em>C</em>T<em>G</em>A*G</td>
<td>2'-MOE/OMe PS</td>
<td></td>
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<tr>
<td>SSO-12-9m</td>
<td>T<em>A</em>G<em>C</em>A<em>G</em>C<em>C</em>U<em>G</em>A*G</td>
<td>2'-MOE/OMe PS</td>
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<tr>
<td>SSO-12-9sm</td>
<td>T<em>A</em>G<em>C</em>A<em>G</em>C<em>C</em>X<em>G</em>A*G</td>
<td>2'-MOE/OMe PS</td>
<td></td>
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<tr>
<td>SSO-10-2</td>
<td>G<em>Y</em>A<em>G</em>C<em>C</em>T<em>G</em>A*G</td>
<td>2'-MOE PS</td>
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<tr>
<td>SSO-10-7</td>
<td>G<em>C</em>A<em>G</em>C<em>C</em>X<em>G</em>A*G</td>
<td>2'-MOE PS</td>
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2.8. Uptake study

As presented in 2.7., polyamine-conjugation to oligonucleotides was generally tolerated in cells, however it failed to enhance potency of antimirs, SSOs or gapmers under conditions used for testing. High binding affinities in vitro did not translate to high potency in cellular assays. One major limitation common to all of these assays was that uptake of modified oligonucleotides was not measured. The finding of severely reduced activity for bis-modified reagents gave an indication that uptake might not be equal among unmodified and conjugated oligonucleotides. All the cellular assays were based on lipofection, meaning complexation with polycationic lipids to facilitate uptake of oligonucleotides into cells. We hypothesized that introduction of positive charges to the oligonucleotide interferes with lipofection. The reduction of the overall negative charge of a conjugate might disfavor complexation with the polycationic lipids used as transfecting reagents. This would imply a reduced cellular uptake of conjugates and consequently a lower intracellular concentration of the modified oligonucleotides. The hypothesis was investigated in uptake assays. Two different approaches were taken to count the molecules of interest inside the cell: chemical ligation qPCR (CL-qPCR) and an uptake assay employing surface plasmon resonance (SPR) for oligonucleotide detection.

2.8.1. Chemical ligation qPCR (CL-qPCR)

Quantification of modified oligonucleotides in cells is rather complex. Reverse transcriptases used in RT-qPCR are thought to tolerate 2'-OMe modifications, but not 2'-MOE substitution [360]. In addition, they require a certain minimal length of the template (17 nt, according to Thermo Fischer) for the stem-loop primers to bind. Among the conjugates used in cellular assays, antimirs A1-16, A1-16-4 and A1-16-4,13 were most suitable as reasonable stocks were available and with their 2'-OMe chemistry and 2 terminal PS linkages they are expected to be sufficiently stable. However, 16mers are too short to be detected with stem-loop primers. An alternative approach is the chemical ligation qPCR (CL-qPCR), developed by a group at Novartis [361] and successfully carried out in our lab by Matije Lucic for modified antimirs of the same format [339] (Fig. 59).

Three different ligator sets were prepared using 5'-biphenylsulfonyl-amidites (BPS-amidites) synthesized by Martina Bigatti [361]. Thereby, the PS linkage was shifted 1 nt to the left and to the right in order to enhance chances to end up with an active pair (Tab. 14). Synthesis was done on a special solid support allowing for mild cleavage conditions (Q-dT-CPG) and PAC-protected purine amidites were used in order to facilitate base deprotection as the 5'-BPS substitution was lost over time under basic conditions.
Table 14. Ligators for CL-qPCR. The G ligator pair includes a 3’-ligator with a 5’-biphenylsulfonyl substitution on a G. A, T in analogy. * = PS

<table>
<thead>
<tr>
<th>Name</th>
<th>Lab book</th>
<th>Sequence</th>
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<td>BPS-GACAATGGAACCAGT</td>
<td>DNA PO</td>
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<td>4826.4</td>
</tr>
<tr>
<td>PS-ligator (G)</td>
<td>Microsynth</td>
<td>TTAACCATTGGAGTG*</td>
<td>DNA PO/PS</td>
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<td>5015.5</td>
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<tr>
<td>BPS-ligator (A)</td>
<td>MM168</td>
<td>BPS-ACATGGAACCAGT</td>
<td>DNA PO</td>
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<td>Microsynth</td>
<td>TTAACCATGGAGTG*</td>
<td>DNA PO/PS</td>
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<tr>
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<td>DNA PO</td>
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<tr>
<td>PS-ligator (T)</td>
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<td>DNA PO/PS</td>
<td>4711.9</td>
<td>4711.4</td>
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</tbody>
</table>

Figure 60. Calibration curves for the three ligator sets (see Tab. 14). Calibration was established from serial dilutions of the template antimir A1-16 (C*C*AUUGUCACUC*C*A, 2’-OMe, terminal PS). A DNA template corresponding to the ligation product was used as positive control for the PCR. NTC qPCR, no template control in the PCR reaction; NTC CL, no template control in the chemical ligation reaction. Ligation conditions: 33 °C for 6h. Error bars, ±1 SD of technical triplicates.
Figure 61. CL-qPCR on antmir on transfection into Huh7 cells. Antimirs were transfected at 40 nM and total RNA was extracted 24h post-transfection. Calibration curves were obtained from serial dilutions of respective antimirs. A1-16, A1-16-4 and A1-16-4,13 are 2’-OMe RNAs with terminal PS substitution (*), as characterized in Tab. 5. Ligation conditions: 33 °C for 6h. A) Three 1:10 dilutions of total RNA were applied in order to target the linear range. B) For analysis of conjugates, only linear range is shown. NTC, no template control. Error bars, ±1 SD of technical triplicates.

Ligation temperature and time were optimized. Best correlations were obtained between 2 µM and 2 nM template antimir after 6h ligation at 33 °C (Fig. 60). However, the reliability of this experiment was insufficient. Quite often, the calibration either yielded a weak slope or failed to deliver any reasonable linear regression, due to a limited ligation efficiency. A representative experiment is shown in Fig. 61. Typically, 1:10 dilutions of total RNA were applied. From these, those within the linear range were selected and were used to quantify antimir
content. Strikingly, the experiment showed that polyamine-conjugation lead to a reduction in transfection efficiency. Only one out of three different cellular extracts contained conjugated A1-16-4, whereas the bis-modified A1-16-4,13 was undetectable in all extracts. The limitation of this approach was that due to the rather weak performance of the chemical ligation, a number of replicates would have been required in order to reasonably evaluate uptake efficiency. qPCR itself was working fine as can be seen from the positive control DNA template in both Fig. 60 and Fig. 61. However, inspite abundant protocol optimization attempts, dependence on template concentration of the chemical ligation step was rather poor, with slopes of -2.11 and -2.12 for the A- and the T-ligator set considerably weaker than in [361]. Extensive replication would have required a lot of material. On one hand, transfections were done in 6-well plates, on the other hand, calibration curves must be established in parallel to total RNA analysis on the same plate in order to quantify the antimir content of a total RNA extract, as discrepancies between individual experiments on different plates were surprisingly high. Taken together, the approach of the CL-qPCR to determine uptake efficiency was not considered safe with the limited amount of material available and an alternative method was sought. However, the limited number of replicates performed strongly suggested that polyamine-conjugation considerably reduced uptake efficiency of short 2'-OMe RNAs into Huh7 cells.

2.8.2. Uptake quantification by SPR
Besides characterizing analyte-ligand interactions, surface plasmon resonance is a highly sensitive method to detect an analyte of interest in a solution of a mixture of diverse entities such as cellular lysates or body fluids [362]. Several studies have shown detection of miRNAs, all of them including a signal amplification step after binding of the analyte to the immobilized target sequence [363-365]. Signal amplification was achieved either by an enzymatic prolongation of the bound miRNA with a polyA tail which was subsequently recognized by polyT sequences coupled to nanoparticles [363], or an antibody recognizing double helices formed upon annealing of the miRNA of interest to the immobilized ligand [364], or a complementary sequence carrying streptavidin [365]. Here, a more simplistic approach was chosen. The signal amplification step was not required due to sufficient amount of a high affinity ligand on the surface allowing for detection of small analytes. 2'-MOE chemistry of the 16mer ligand on the surface allowed for sufficiently high hybridization affinity to detect even very low concentrations of the analyte of interest. Total RNA extracted from Huh7 cells after transfection with antimirs was injected and the signal of a negCON total RNA was substracted. In parallel, calibrations were established with pure antimirs based on the initial binding rate or on the post-injection binding level. The initial binding rate

$$\frac{d[LA]}{dt} = k_{on} \cdot [L][A] - k_{off} \cdot [LA]$$

(15)

can be reasonably assumed to be proportional to $[A]$

$$\frac{d[LA]}{dt} \sim [A]$$

(16)

since $[L] = \text{const.}$ and $[LA] = 0$ at $t_0$. Therefore, a linear regression can be established between analyte concentration and the initial binding rate. Furthermore, post-injection binding levels follow a sigmoidal function and can be fitted to the Boltzmann equation

$$RU([A]) = B + \frac{A - B}{1 + \exp\left(\frac{[A] - C}{D}\right)}$$

(17)

with A, B, C and D being constants. From every signal of an antimir-transfected total RNA was substracted the signal of a total RNA after negCON treatment, at identical concentration as determined by UV. Antimir
concentration was derived from calibrations based both on the initial binding rate as well as the post-injection binding level (Fig. 62). A 2’-MOE PS target RNA was immobilized as a ligand with high binding affinity and maximal nuclease stability.

![Diagram](image)

**Figure 62.** Working principle of the SPR assay to quantify antimir uptake. A) Total RNA extracted from Huh7 cells transfected with antimirs was run over a chip carrying a modified target RNA (2’-MOE PS). B) Antimir concentration was determined using calibration curves based on the initial binding rate (dRU/dt, 1) or the post-injection binding level (RU, 2).

In a first proof-of-principle experiment, a total RNA extract from cells transfected with negCON, containing 3 nM of externally added miraviren (SPC), a 15mer DNA/LNA mixmer serving as a positive control antimir, was injected (Fig. 63, left). After substraction of the signal from total RNA after negCON treatment (Fig. 63, right), 2.99 nM was detected using an SPC calibration established separately on the same chip, based on post-injection binding levels (not shown). In comparison, total RNA extracted from cells transfected with SPC was injected and allowed to quantify the amount of SPC taken up by the cells. In a second spiking experiment with bis-modified A1-16-4,13, 2.97 nM was detected in a total RNA containing 3 nM externally added A1-16-4,13 (not shown). This second experiment was based on a separate calibration curve established using A1-16-4,13. Given that for every analyte a separate calibration is established on the same chip and timewise close to the analysis of total RNA, the setup thus proved highly sensitive to modified oligonucleotides as short as 16mers and suitable to determine antimir content of total RNAs extracted from Huh7 cells after transfection.
Figure 63. Spiking experiment: first proof-of-principle. SPC was externally added to control total RNA and was subsequently detected very precisely by SPR. A) Crude injections, buffer subtraction only; B) subtraction of negCON total RNA. Conditions: PBS running buffer containing TWEEN 0.005%, 100 µl injections, flow rate 15 µl/min, regeneration 50 µl HCl 10 mM and NaCl 1 M, 25 °C.

In a second proof of principle experiment, four serial dilutions of a total RNA after A1-16 transfection were analyzed and respective signals of a total RNA of identical concentration after negCON transfection were subtracted. A1-16 concentration was determined using both the initial rate and the post-injection level (Fig. 64). It was concluded that both mathematical approaches can and should be used and that the sensitivity limit is rather close here. Higher amounts applied would yield more robust results. Thus, depending on the uptake efficiency under investigation, a 5 ng/µl total RNA concentration might be close to the detection limit for a 16mer analyte.

Figure 64. Total RNA dilution: second proof-of-principle. Total RNA extracted from Huh7 cells transfected with A1-16 was serially diluted and referenced with respective total RNA from negCON transfected cells (left). A1-16 concentrations were determined from every concentration using the initial binding rate dRU/dt as well as the post-injection binding level (RU). Conditions: PBS running buffer containing TWEEN 0.005%, 100 µl injections, flow rate 15 µl/min, regeneration 50 µl HCl 10 mM and NaCl 1 M, 25 °C.

Assessment of antimir content in total RNAs extracted from cells after transfection with A1-16, mono-modified A1-16-4 or bis-modified A1-16-4,13 confirmed findings of the CL-qPCR that polyamine-conjugation indeed reduces cellular uptake. Unmodified A1-16 content was significantly higher than content of the conjugates, indicating that polyamine-conjugation might interfere with the process of lipofection (Fig. 65).
Taken together, the two uptake experiments presented above strongly suggest that polyamine-conjugation to short 2'-OMe oligonucleotides severely reduces cellular uptake upon lipofection. Thus, transfection with lipofectamine is an inappropriate protocol for testing polyamine-modified oligonucleotides in cellular assays. An open question remained whether some analytes were selectively lost during RNA extraction, as both CL-qPCR and SPR assays were based on total RNA collected after antimir transfection. Two different extraction procedures were applied, using acidic (pH 4) or basic (pH 8-8.5) phenol/chloroform. However, CL-qPCR was not sensitive enough to detect any of the modified analytes in acidic extracts and technical problems of the SPR impeded comparison of the different extracts on the SPR.

In a more general sense, the state-of-the-art process of evaluating biological activity of modified oligonucleotides in lipofection-based cellular assays should be questioned. It has previously been shown that cationic polymers transfect neutral phosphoramidate morpholinos less efficiently than charged nucleic acids [366]. This suggests that transfection efficiency can be an important confounder when comparing cellular activity of modified oligonucleotides. Transfecting reagents should be avoided in cellular testing of chemically modified oligonucleotides, unless uptake is systematically controlled and shown to be equal to the unmodified parent compound. Otherwise, gymnastic delivery can be considered [191], uptake of “naked” oligonucleotides into special cell lines, requiring however more material. In the present case of polyamine-conjugated 2'-OMe oligonucleotides, synthetic access would first need to be facilitated in order to dispose of sufficient amount of reagents to assess cellular uptake in a transfecting reagent-free setting.

**Figure 65.** Uptake efficiency of polyamine-conjugated 16mer 2'-OMe oligonucleotides into Huh7 cells upon lipofection. Total RNA was extracted from lysates 24h post-transfection and was injected over a surface coated with 2'-MOE PS target RNA. The signal of total RNA after transfection of a negative control (negCON) was substracted. Concentration of oligonucleotides was determined using calibration curves based on the initial binding rate (dRU/dt) or the post-injection binding level (RU) (see Fig. 62 for explanation). Conditions: PBS running buffer containing TWEEN 0.005%, 100 µl injections, flow rate 15 µl/min, 50 µl regeneration HCl 10 mM and NaCl 1 M, 25 °C. Two independent experiments are shown. [a] Uptake measured in amol/ng total RNA. [b] Relative to A1-16. negCON, A*U*CUCCCUUGAU*G*U. 2'-OMe RNA, * = PS.
3. Conclusion and Outlook

3.1. Conclusion

Polyamine-conjugated modified RNA was synthesized, biophysically characterized and was tested in dedicated cellular assays. Besides post-synthetic polyamine introduction by CuAAC on solid support, a pre-synthetic strategy was followed in order to allow for compatibility with PS substitution and the introduction of secondary labels. As a consequence of superior effects of major groove location of the conjugated polyamine on hybridization affinity to complementary RNA, four pyrimidine phosphoramidites carrying a triazole-spermine at position C5 were prepared: mUspm, dUspm, sampMDE and spmCpMOE. These were incorporated into oligonucleotides by standard automated synthesis. Hybridization affinities to target RNA were quantified by UV melting as well as by SPR. Binding to a structured target RNA was compared to unstructured complementary RNA and binding kinetics were assessed in SPR studies. Polyamine-conjugation with major groove location proved highly efficient in duplex stabilization and this effect was mainly attributed to an accelerated association rate.

Small oligonucleotide libraries for four different biological targets were prepared and tested in suitable cellular assays. Among these, looptomirs targeting pre-let-7a-2, antimirs against miR-122, mipomersen-like gapmers targeting ApoB mRNA as well as SSOs to correct FCH splicing in EPP. Due to mediocre effects of polyamine-conjugation on oligonucleotides’ cellular potency, uptake efficiency was investigated. It was shown that tethering of polycationic fragments such as polyamines reduces uptake of modified oligonucleotides, presumably due to interference with the process of lipofection.

Synthetic access to polyamine-conjugated oligonucleotides of various sugar and backbone chemistries was considerably simplified by the pre-synthetic conjugation strategy using the four new phosphoramidites. However, the rate limiting step in the preparation of the polyamine-nucleoside phosphoramidites was access to TFA-protected spermine azide (Spm-N3)TFA. Even though an established literature protocol was available, only small amounts could be synthesized due to explosive potential of the azidation reaction. As only selected positions were to carry the modified pyrimidine, milligram quantities of phosphoramidites were sufficient for the preparation of small libraries of modified oligonucleotides. Upscaling such as it would be necessary for transfecting reagent-free uptake or mouse studies however would require further optimization of the synthetic route in order to obtain reasonable quantities of modified oligonucleotides. Summarizing, synthetic access is granted, but supply of modified phosphoramidites is limited.

Polyamine-conjugation was found to drastically increase hybridization affinity to complementary RNA. For introduction of a single polyamine, a ΔT_M of up to 12 °C was found, while bis-modification had an additive effect. Melting temperature is a standard measure of hybridization affinity, however, correlation with in vivo potency has been shown to be rather poor in some applications [122,339,367]. In a mere steric block type of mechanism a useful alternative in vitro surrogate to estimate in vivo potency is the residence time [176]. A long drug-target binary complex residence time, determined by the dissociation half-life, is highly favorable in terms of duration of a pharmacological effect. On the other hand, in a catalytic mechanism such as cleavage by RNase H or RNAi, a slow complex dissociation has an adverse effect [74]. Since the recycling ability of the oligonucleotide-enzyme complex is a critical determinant of its efficiency, high target binding affinity of RNase H-competent antisense oligonucleotides or siRNAs should be attained by increasing the association rate k_on. Thus, knowledge of hybridization kinetics allows fine-tuning of oligonucleotides’ properties for a given application. In the case of polyamine-conjugation, SPR studies showed that binding affinity increases were predominantly k_on-driven, except for the very short antisense oligonucleotides, where the effect was mediated both by faster k_on and slower k_off. SPR studies further indicated that k_on was typically determined by oligonucleotide length, while k_off was length-independent. These findings are in line with the nucleation-zipping model presented in the pioneering work of Pörschke and Eigen [179]. While k_on and therefore residence time is simply modulated by oligonucleotide length, k_on can be adjusted by polyamine-conjugation. These binding characteristics predispose for use of the
modification in a catalytic system such as in RNase H-competent gapmers or in RNAi, as hybridization to target RNA is favoured while fast dissociation after cleavage of the latter is maintained. Contrariwise, polyamine-conjugation would not be an optimal strategy for the purpose of creation of shorter antisense oligonucleotides, since it does not affect the likeliness of dissociation and therefore cannot keep up the drug-target complex residence time. On that account, mipomersen was the model system chosen, while SSO, antimir and looptomir target systems were tested alongside.

SPR experiments further showed that binding of both polyamine-conjugated and unmodified oligonucleotides to an unstructured target RNA was stronger than to a folded pre-miRNA hairpin and that the observed effect was predominantly due to faster $k_{\text{on}}$ to unstructured RNA. Two possible reasons are conceivable: Nucleation may be hindered by steric constraint of the folded target RNA, or extensive secondary structure might prevent efficient “zipping”, elongation of base pair formation. Besides, comparison of hybridization in melting and in SPR revealed stronger binding affinities in melting, pointing into the same direction of a negative effect of any steric restrictions. With respect to polyamine-conjugation, their affinity ranking and trends in rate constants remain the same, both in melting and in SPR and both against an unstructured and a folded target RNA.

After all, the very high binding affinity did not translate into enhanced potency in cellular assays. Disappointingly, pre-let-7a-2/Lin28, as the most promising target system, could not be used to test the looptomir library due to failure in reproducing the amplitude of the previously reported effect in assays assessing the impact of looptomir treatment on mature let-7 levels. The three other small compound libraries tested showed tolerance of polyamine-conjugation in most cases, however, significantly increased potency could not be detected. Experiments on antimirs against miR-122 lead to an “antimir map”, a tight position-activity relationship and thereby completed pre-existing suggestions from literature that some positions are critical for miRNA-RISC interaction while others can be modified without disturbing RISC architecture and functionality. Essentially, the seed region proved to be more sensitive to conjugation than any positions outside the seed. However, none of the polyamine-conjugated antimirs showed higher potency than an unmodified antimir. In the mipomersen assay established by Dr. Helen Lightfoot none of the polyamine-conjugated gapmers performed better than unmodified mipomersen. Single modifications were shown to be tolerated at all the positions tested, bis-modification however drastically reduced antisense activity. As target binding affinity of unmodified mipomersen is extremely high already, working with a shortened sequence would represent a more promising approach towards revelation of a potency increase upon polyamine-conjugation to gapmers [74]. Indeed, as the 20mer mipomersen derivatives were highly challenging to synthesize, predominantly due to the very delicate DNA/2’-MOE transition significantly reducing synthesis yields, the small amounts of available modified phosphoramidites restricted the choice of a lead compound. Priority was given to full length mipomersen, the approved drug. Among the SSOs, modification of a single specific position resulted in slightly increased potency, which could however not be confirmed in a second replicate. This very weak effect would have to be ascertained in multiple repetitions of the experiment, requiring more material than was available. Though, even if the effect in the splice-switching assay is real, the impact of polyamine-conjugation is probably so small that it does not counterbalance the challenging synthesis.

Reasons for the incongruity between binding affinity and cellular potency could be manifold. For example, a potential pitfall could be unsuccessful delivery of modified oligonucleotides to the cells. In fact, absence of activity of the bis-modified mipomersen derivative while individual modifications were tolerated supported this hypothesis. Uptake studies by CL-qPCR as well as in a dedicated SPR assay indeed showed that polyamine-conjugation hampers uptake of modified oligonucleotides. Since standard transfection procedures such as lipofection are based on complexation of the negatively charged nucleic acid with polycationic lipids or polymers, charge interactions and ratios are critical for successful transfection. It therefore seems likely that polyamine-conjugation, reducing the overall negative charge of an oligonucleotide, interferes with the process of lipofection. An alternative explanation of the same observation could be that polyamine-conjugation lowers solubility and thus precipitates or complexes the conjugates at any time during transfection. For bis-modified short 2’-OMe
oligonucleotides reduced solubility in water was even observed during work-up procedures after synthesis. Precipitation has been reported previously on terminal polyspermine-siRNA conjugates upon a N/P ratio above 4/3 [254]. Possibly, any of the media used in transfection or cell culture might provoke the same effect. The actual mechanism of the reduced transfection efficiency of polyamine-conjugates is not clear from the above presented experiments, however it is fairly clear that limited uptake of polyamine-oligonucleotide conjugates is at least part of the reason why performance in cellular assays is so poor. These results strongly suggest that more attention should be paid to a potential transfection bias when testing modified oligonucleotides in cellular assays and that uptake should systematically be controlled alongside.

3.2. Outlook
Continuation of the project in a transfection reagent-free cellular assay is on-going. Gymnosis is hypothesized to expand the advantage of polyamine-conjugates compared to unmodified analytes over pharmacodynamics, e.g. target binding affinity, to pharmacokinetic aspects. Free uptake is assumed to be facilitated by the presence of a polyamine tether reducing the overall negative charge and thereby exerting a function similar to conventional transfecting reagents. Major challenges to be addressed in this projects are the following:

- First of all, synthetic access to polyamine-conjugated oligonucleotides needs to become more straightforward. Thereby, the potentially explosive azidation reaction of spermine will be a major bottleneck in the upscaling process. Furthermore, yields of automated oligonucleotide synthesis should be addressed. Supposingly, a change in the protection strategy of the amines away from TFA could facilitate automated synthesis due to higher stability of the modified amidite with respect to the PIm, as well as access to dryer starting material. Alternatively, post-synthetic polyamine introduction could be envisaged.

- Second, it seems worthwhile to invest into the elaboration of a suitable lead compound. Ideally, as was learnt from SPR studies, the target structure should be addressed in a catalytic cleavage mechanism, therefore either by RNAi or RNase H-competent gapmers. RNAi requires RISC compatibility, which was shown to not be a given in above presented antimir assays. As the antimir map is not entirely comprehensive covering all possible locations yet, luck would be a major determinant of the outcome. Therefore, gapmers recruiting RNase H such as mipomersen look more promising. An important issue hereby will be the choice in length. As has been outlined above, 20mers are too long for several reasons. In a shorter oligonucleotide polyamine-conjugation would have a better chance to actually make a difference due to a broader optimization potential with respect to binding affinity as well as a more favourable charge ratio. Furthermore, decreasing the size of gapmers will facilitate their synthesis. On the other hand, too short would imply too much of a loss in binding affinity and therefore requirement of higher doses, which might be difficult to provide due to synthetic or uptake limitations, as has been shown in preliminary experiments by François Halloy on SSOs. Taken together, based on the work presented above, an ideal length for a gapmer application would be expected between 14 and 16 nt.

- Moreover, free uptake efficiency will drastically depend on the cell type chosen. A mipomersen assay requires presence of ApoB and therefore has to be done in liver cells. Identification of an ideal cell line for gymnotic delivery might save considerable amounts of compound and allow for a higher dynamic range.

Summarizing, once the synthesis is established, it will become apparent whether there is a reasonable compromise between too short and not binding strongly enough and too long and therefore no impact of polyamine-conjugation. This will finally allow the very interesting evaluation whether the modification has any value in biological applications. Else, there would still be the option for the modification to find use in high affinity primers or probes to optimize detection in biochemical assays, as was suggested from preliminary experiments by Dr. Julian Zagalak on polyamine-conjugated northern blot probes.
4. Experimental

4.1. Synthesis of polyamine-modified phosphoramidites

General experimental details. NMR spectra were recorded on a Bruker Av400 (Faellanden, CH) at a resonance frequency of 400 MHz (1H) and 100 MHz (13C). Solvent signals were used as internal standards. Chemical shifts are given in ppm. 1H-NMR spectra are reported as follows: multiplicity, number of protons (integral), coupling constant J, signal causing proton. For 13C- and 31P-NMR spectra, only chemical shifts of signals are given.

*Spermine azide (Spm-N3)*[^4]. TFA-protected spermine azide was prepared according to a reported protocol [300]. ESI-MS: m/z 515.29 ([M+H]^+), C_{28}H_{30}F_{2}N_{8}O_{6}, calcd 516.37; 1H-NMR and 13C-NMR spectra (DMSO) according to literature.

4.1.1. Pyrimidine modifications

Representative procedure for 5-iodination. Uridine derivatives were dissolved in AcOH. Iodine (0.6 eq.) and cerium ammonium nitrate (CAN, 0.5 eq.) was added and the mixture was stirred at 80 °C until completion of reaction, whereupon it was cooled to rt and solvents were removed under reduced pressure. Work-up was done by crystallization or column chromatography.

5-ido-2'-deoxyuridine. 2'-deoxyuridine (1 g, 4.38 mmol), AcOH (40 ml), CAN (1.18 g, 2.19 mmol) and iodine (674 mg, 2.63 mmol) were mixed and reacted in 20 min as described above. After removal of solvent, the crude was co-evaporated 3x with water and the product was crystallized from EtOH (1.2 g, 77%). ESI-MS: m/z 354.8 ([M+H]^+), C_{11}H_{14}N_{2}O_{3}, calcd 353.97; 1H-NMR (CD_{3}OD) δ 2.21-2.35 (m, 2H, H2'), 3.78-3.86 (m, 2H, H5'), 3.95 (q, J=4 Hz, 1H, H4'), 3.40-3.44 (m, 1H, H3'), 6.24 (t, J=4 Hz, 1H, H1'), 8.55 (s, 1H, H6).

5-ido-2'-OMe-uridine. 2'-OMe-uridine (8 g, 30.9 mmol), synthesized in 2 steps from uridine according to [307]), AcOH (30 ml), CAN (8.47 g, 15.5 mmol) and iodine (4.71 g, 18.5 mmol) were mixed and reacted in 30 min as described above. After removal of solvent, the crude was co-evaporated 3x with water, THF and toluene and the product was crystallized from EtOH (10.68 g, 90%). 1H-NMR (CD_{3}OD) δ 3.44 (s, 3H, OMe), 3.66 (dd, 1H, J=12 Hz, H5'), 3.72-3.74 (m, 1H, H2'), 3.82 (dd, 1H, J=12 Hz, H5'), 3.87-3.90 (m, 1H, H4'), 4.14-4.17 (m, 1H, H3'), 5.80 (d, 1H, J=3 Hz, H1'), 8.59 (s, 1H, H6).

5-ido-2'-MOE-uridine. 2'-MOE-uridine (3.88 g, 12.8 mmol, obtained from Dr. Meiling Li), AcOH (50 ml), CAN (4.22 g, 6.4 mmol) and iodine (1.95 g, 7.7 mmol) were mixed and reacted in 20 min as described above. After removal of solvent, the crude was co-evaporated 2x with EtOH/toluene 1:2, EtOH/water 2:1, then toluene 2x. Purification by column chromatography (solid depot from THF, MeOH/DCM 5%) to afford 5-ido-2'-MOE-uridine (3.93 g, 72%). ESI-MS: m/z 429.0 ([M+H]^+), C_{11}H_{12}N_{2}O_{3}, calcd 428.18; 1H-NMR (CD_{3}OD) δ 3.39 (s, 3H, OMe), 3.59-3.62 (m, 2H, MOE-Et), 3.76-3.94 (m, 4H, MOE-Et, H5'), 4.01-4.04 (m, 1H, H4'), 4.05-4.07 (m, 1H, H2'), 5.91 (d, 1H, J=3 Hz, H1'), 8.67 (s, 1H, H6).

Representative procedure for Sonogashira couplings. 5-ido-pyrimidines were co-evaporated 3x with dry THF or DMF and were dissolved in a mixture of dry DMF or THF and dry TEA (3 eq.). To this was added Cul (0.2 eq.), PdCl_{2}(PPh_{3})_{2} (0.001-0.1 eq.) and, dropwise, TMS-acetylene (3 eq.) and the reaction mixture was refluxed under argon.

5-TMS-acetylène-2'-deoxyuridine. 5-ido-2'-deoxyuridine (10 g, 28.24 mmol), Cul (1.08 g, 5.65 mmol), PdCl_{2}(PPh_{3})_{2} (200 mg, 0.282 mmol) and TMS-acetylene (11.73 ml, 84.72 mmol) were reacted in dry DMF (40 ml) and dry TEA (11.77 ml, 84.72 mmol) as described above. Solvents were removed under reduced pressure upon completion of reaction after 1h. The crude was submitted to column chromatography (solid depot, petrol ether/DCM 1:1 to MeOH/DCM 1:9) to give pure product (4.8 g, 52%). ESI-MS: m/z 325.0 ([M+H]^+), C_{11}H_{20}N_{2}O_{3}Si, calcd 324.11; 1H-NMR (CDCl_{3}) δ 0.00 (s, 9H, Si(CH_{3})_{3}), 2.09-2.24 (m, 2H, H2'), 3.00 (br, 1H, OH-5'), 3.50-3.60 (m, 1H, OH-3'), 3.63 (q, J=8 Hz, 2H, H5'), 3.82 (br, 1H, H4'), 4.32 (br, 1H, H3'), 5.95 (t, J=8 Hz, 1H, H1'), 7.74 (s, 1H, H6), 9.36 (s, 1H, NH-3).
5-TMS-acetylene-2'-OMe-uridine. 5-iodo-2'-OMe-uridine (2 g, 5.21 mmol), Cul (198 mg, 1.04 mmol), PdCl2(PPh3)2 (366 mg, 0.521 mmol) and TMS-acetylene (2.16 ml, 15.62 mmol) were reacted in dry DMF (25 ml) and dry TEA (2.17 ml, 15.62 mmol) as described above. Upon completion of reaction after 2h, the reaction mixture was taken up in EtOAc, was washed with water 3x, then brine and dried over Na2SO4. The crude was filtered over silica gel 1 cm and was submitted to column chromatography (solid depot, MeOH/DCM 3-5%) to give pure product (1.13 g, 61%). 5-TMS-acetylene-2'-MOE-uridine. 5-iodo-2'-MOE-uridine (3.42 g, 8.00 mmol), Cul (305 mg, 1.60 mmol), PdCl2(PPh3)2 (56 mg, 0.08 mmol) and TMS-acetylene (2.36 ml, 23.98 mmol) were reacted in dry THF (20 ml) and dry TEA (3.33 ml, 23.98 mmol) as described above. The reaction was complete after 4h and solvents were removed under reduced pressure. The crude was co-evaporated 3x with THF and subjected to column chromatography (MeOH/DCM 0-3%) to give pure product (2.32 g, 73%). 5-TMS-acetylene-5'-DMT-2'-deoxyuridine. Dry DMT-Cl (6.12 g, 18.06 mmol) dissolved in dry pyridine (25 ml) was added dropwise to a solution of 5-TMS-acetylene-2'-deoxyuridine (4.8 g, 14.79 mmol) and DMAP (108 mg, 0.89 mmol) in dry pyridine (30 ml) and the reaction mixture was stirred for 3.5h at rt under argon. The crude was concentrated and taken up in DCM, washed with water 3x, then brine and dried over Na2SO4. Solvents were removed under reduced pressure and the crude was submitted to column chromatography (petrol ether/DCM 1:1 to MeOH/DCM 1:19) to give pure product (5.07 g, 54%). 5-TMS-acetylene-5'-DMT-2'-OMe-uridine. Dry DMT-Cl (992 mg, 2.93 mmol) dissolved in dry pyridine (3 ml) was added dropwise to a solution of 5-TMS-acetylene-2'-OMe-uridine (848 mg, 2.393 mmol) and DMAP (18 mg, 0.14 mmol) in dry pyridine (5 ml) and the reaction mixture was stirred at rt under argon overnight. The crude was concentrated and taken up in DCM, washed with water 3x, then brine and dried over Na2SO4. Solvents were removed under reduced pressure and the crude was submitted to column chromatography (solid depot from DCM, petrol ether/DCM 1:1 to MeOH/DCM 1:19, all solvents containing 1% TEA) to give pure product (1.16 g, 74%). 5-TMS-acetylene-5'-DMT-2'-OMe-uridine. Dry DMT-Cl (2.32 g, 5.83 mmol) was co-evaporated 3x with dry ACN and was then suspended in dry ACN (50 ml) and cooled to -40 °C. 2,6-lutidine (1.35 ml, 11.66 mmol) and DMT-Cl (2.07 g, 6.12 mmol) was added. The reaction mixture was allowed to go to rt and was stirred under argon for 2h. Solvents were removed under reduced pressure and the crude was co-evaporated 3x with toluene before submission to column chromatography (solid depot from DCM, petrol ether/EtOAc 1:1 to EtOAc 100%) to yield pure product (3.23 g, 75%).
**Representative procedure for desilylation.** Dry 5-TMS-acetylene-5’S-DMT-uridine derivatives were dissolved in dry THF and a 1M TBAF solution in THF (1.1 eq.) was added. The reaction mixture was stirred at rt under argon until completion of reaction. Solvents were removed under reduced pressure.

**5-ethyl-5’S-DMT-2’-deoxyuridine.** 5-TMS-acetylene-5’S-DMT-2’-deoxyuridine (3.14 g, 5.01 mmol) and 1M TBAF in THF (5.51 ml, 5.51 mmol) were reacted in THF (22 ml) for 30 min as described above. The crude was partitioned between EtOAc and saturated aqueous NaHCO3 3x. The organic layer was washed with brine, dried over Na2SO4 and evaporated to dryness to give 5-ethyl-5’S-DMT-2’-deoxyuridine (2.97 g, quant.). 1H-NMR (CDCl3) δ 2.08-2.37 (m, 2H, H2’), 2.68 (s, 1H, C≡CH), 3.12-3.20 (m, 2H, H5’), 3.57 (s, 6H, OMe), 3.89-3.95 (m, 1H, H4’), 4.35-4.36 (m, 1H, H3’), 6.09 (t, 1H, J=4 Hz, H1’), 6.62-6.65 (m, 4H, Ar), 6.98-7.23 (m, 9H, Ar), 7.89 (s, 1H, H6).

**5-ethyl-5’S-DMT-2’-OMe-uridine.** 5-TMS-acetylene-5’S-DMT-2’-OMe-uridine (1.16 g, 1.77 mmol) and 1M TBAF in THF (1.95 ml, 1.95 mmol) were reacted in THF (6 ml) for 2h as described above. The crude was taken up in EtOAc and was washed 3x with water. Aqueous phases were back-extracted with EtOAc, combined organic layers were washed with brine and dried over Na2SO4. Solvents were removed to yield the product (1.00 g, quant.). ESI-MS: m/z 607.2055 [(M+Na)+, C19H16N2O8, calcld 607.2051]; 1H-NMR (CDCl3) δ 2.76 (s, 1H), 3.42-3.53 (m, 2H), 3.64 (s, 3H), 3.79 (s, 6H), 3.92-3.94 (m, 1H), 4.09-4.10 (m, 1H), 4.46-4.49 (m, 1H), 5.94 (d, 1H, J=3 Hz), 6.83-6.86 (m, 4H), 7.21-7.23 (m, 1H), 7.28-7.32 (m, 2H), 7.34-7.37 (m, 4H), 7.43-7.45 (m, 2H), 8.12 (s, 1H). 13C-NMR (CDCl3) δ 55.3, 59.0, 61.8, 68.9, 73.7, 82.0, 83.7, 83.9, 86.9, 87.3, 99.4, 113.3, 126.9, 128.2, 130.0, 130.1, 135.4, 135.6, 143.3, 144.4, 158.55.

**5-ethyl-5’S-DMT-2’-MOE-uridine.** 5-TMS-acetylene-5’S-DMT-2’-MOE-uridine (2.50 g, 3.57 mmol) and 1M TBAF in THF (3.92 ml, 3.92 mmol) were reacted in THF (20 ml) for 1 h as described above. The crude was taken up in EtOAc and was washed 3x with saturated aqueous NaHCO3, then brine and dried over Na2SO4. The residue was evaporated to dryness and purified by column chromatography (solid depot from DCM, petrol ether/EtOAc 1:1 to EtOAc 100 %) to give 5-ethyl-5’S-DMT-2’-MOE-uridine (1.82 g, 81%). 1H-NMR (CDCl3) δ 2.80 (s, 1H, C≡CH), 3.41 (s, 3H, MOE-OMe), 3.44-3.67 (m, 5H, H5’, MOE-Et), 3.81 (s, 6H, DMT-OMe), 4.06-4.11 (m, 1H, MOE-Et), 4.17 (m, 2H, H2’, H4’), 4.45 (q, 1H, J=6 Hz, H3’), 5.99 (d, 1H, J=4 Hz, H1’), 6.86-6.87 (m, 4H, Ar), 7.21-7.24 (m, 1H, Ar), 7.23-7.33 (m, 2H, Ar), 7.36-7.38 (m, 4H, Ar), 7.45-7.46 (m, 2H, Ar), 8.13 (s, 1H, H6), 9.00 (s, 1H, NH-3).

**U to C conversion**

**5-ethyl-4-triazole-5’S-DMT-2’-MOE-uridine.** To a solution of 5-ethyl-5’S-DMT-2’-MOE-uridine (152 mg, 0.24 mmol) in dry ACN (5 ml) and TEA (740 µl, 5.28 mmol) under argon, TMS-Cl (154 µl, 1.2 mmol) was added dropwise. The resulting cloudy mixture was stirred for 15 min, then 1,2,4-triazole (250 mg, 3.63 mmol) was added. After another 10 min the reaction was cooled in an ice bath and POCI3 (66 µl, 0.73 mmol) was added slowly. The reaction mixture was allowed to go to rt and was stirred for 5h. After evaporation to dryness the crude was taken up in EtOAc and was washed 3x with saturated aqueous NaHCO3. Aqueous phases were back-extracted 2x with EtOAc, combined organic layers were washed with brine and dried over Na2SO4. Solvents were removed under reduced pressure and the residue was subjected to column chromatography (MeOH/DCM 0-2%) to yield pure product (134 mg, 82%). 1H-NMR (CDCl3) δ 2.64 (s, 1H, C≡CH), 3.36 (s, 3H, MOE-OMe), 3.41-3.63 (m, 4H, MOE-Et), 3.78 (s, 6H, DMT-OMe), 3.88-3.93 (m, 1H, H5’), 4.03-4.07 (m, 1H, H4’), 4.13-4.18 (m, 1H, H5’), 4.29-4.31 (m, 1H, H2’), 4.40-4.43 (m, 1H, H3’), 5.98 (d, 1H, H1’), 6.82-6.85 (m, 4H, Ar), 7.19-7.23 (m, 1H, Ar), 7.27-7.36 (m, 6H, Ar), 7.42-7.44 (m, 2H, Ar), 8.15 (s, 1H, H6), 8.87 (s, 1H, triazole), 9.24 (s, 1H, triazole).

**5-ethyl-5’S-DMT-2’-MOE-cytidine.** To a solution of 5-ethyl-4-triazole-5’S-DMT-2’-MOE-uridine (134 mg, 0.24 mmol) in 1,4-dioxane (1 ml) was added aqueous ammonia 25 % (1 ml) and the reaction mixture was stirred overnight. Solvents were removed and the crude was partitioned between DCM and water 3x. Organic phases were washed with brine, dried over Na2SO4 and evaporated to dryness, followed by chromatographic purification (MeOH/DCM 0-4 %) to yield product (83 mg, 67 %). ESI-MS: m/z 1277.7 [(2M + Na)+, C33H17N5O8, calcld 627.69]; 1H-NMR (CDCl3) δ 2.93 (s, 1H, C≡CH), 3.39 (s, 3H, MOE-OMe), 3.48-3.55 (m, 2H, MOE-Et), 3.56-3.64 (m, 2H, MOE-Et), 3.65-3.78 (m, 2H, MOE-Et), 3.89-3.93 (m, 1H, C≡CH), 4.04-4.07 (m, 1H, H5’), 4.14-4.18 (m, 1H, H5’), 4.28-4.31 (m, 1H, H2’), 4.40-4.43 (m, 1H, H3’), 5.98 (d, 1H, H1’), 6.82-6.85 (m, 4H, Ar), 7.19-7.23 (m, 1H, Ar), 7.27-7.36 (m, 6H, Ar), 7.42-7.44 (m, 2H, Ar), 8.15 (s, 1H, H6), 8.87 (s, 1H, triazole), 9.24 (s, 1H, triazole).
3.80 (s, 6H, DMT-OMe), 3.88-3.94 (m, 1H, H5'), 4.04-4.05 (m, 1H, H4'), 4.09-4.12 (m, 1H, H2'), 4.22-4.27 (m, 1H, H5'), 4.37-4.43 (m, 1H, H3'), 5.70 (br, 1H), 5.88 (d, 1H, J=1 Hz, H1'), 6.83-6.86 (m, 4H, Ar), 7.19-7.23 (m, 1H, Ar), 7.28-7.32 (m, 2H, Ar), 7.36-7.39 (m, 4H, Ar), 7.46-7.48 (m, 2H, Ar), 8.23 (s, 1H, H6).

5-ethyl-5'-DMT-2'-MOE-ctydine. Benzoic anhydride (33 mg, 0.146 mmol) was added to a solution of 5-ethyl-5'-DMT-2'-MOE-ctydine (83 mg, 0.13 mmol) in DMF (2 ml) cooled in an ice bath. The reaction was allowed to go to rt and was stirred overnight. Another 1.2 eq. benzoic anhydride (4 x 9 mg) was added in 3 portions and the reaction was set to 45 °C and stirred overnight again to reach completion. The crude was partitioned between EtOAc and saturated aqueous NaHCO3. Aqueous phases were back-extracted with EtOAc. Combined organic layers were washed with brine and were dried over Na2SO4. Solvents were removed under reduced pressure and residual DMF was eliminated by co-evaporation with toluene 2x and THF 5x. The residue was purified by column chromatography (MeOH/DCM 0.5-1 %) to yield pure product (90 mg, 93 %). ESI-MS: m/z 732.5 ([M + H]+, C42H32N10O5, calc 731.80); 1H-NMR (CDCl3) δ 2.91 (s, 1H, C=CH), 3.42 (s, 3H, MOE-OMe), 3.46-3.68 (m, 4H, MOE-Et), 3.82 (s, 6H, DMT-OMe), 3.93-3.88 (m, 1H, H5'), 4.13-4.15 (m, 1H, H5'), 4.16-4.18 (m, 2H, H2' and H4'), 4.47 (t, 1H, J=6 Hz, H3'), 6.00 (d, 1H, J=3 Hz, H1'), 6.86-6.89 (m, 4H, Ar), 7.22-7.26 (m, 1H, Ar), 7.31-7.35 (m, 2H, Ar), 7.38-7.41 (m, 4H, Ar), 7.46-7.51 (m, 5H, Ar), 8.26 (d, 2H, J=7 Hz, H2), 8.33 (s, 1H, H6).

Representative procedure for copper-catalyzed azide-alkyne cycloaddition. Dry 5-ethyl-5'-DMT-pyrimidines were dissolved in t-BuOH. TFA-protected spermine azide (Spm-N3)TFA (1 eq.) was dissolved in t-BuOH and was given into the reaction mixture. When fully dissolved, water was added in a 1:1 ratio to t-BuOH. CuSO4•H2O (0.05 eq.) and sodium ascorbate (0.2 eq.) were added and the reaction mixture was stirred at rt. Upon completion, the reaction was concentrated and was taken up in EtOAc. The organic phase was washed with saturated aqueous NaHCO3 3x, then brine and was dried over Na2SO4. The crude was submitted to column chromatography after evaporation to dryness (MeOH/DCM 0-4%).

5-triazospermine-5'-DMT-2'-deoxyuridine. 5-ethyl-5'-DMT-2'-deoxyuridine (200 mg, 0.36 mmol), (Spm-N3)TFA (186 mg, 0.36 mmol), CuSO4•H2O (4.5 mg, 0.004 mmol) and sodium ascorbate (14 mg, 0.017 mmol) were reacted in t-BuOH/water 1:1 (5 ml) for 6h and purified as described above to yield 5-triazospermine-5'-DMT-2'-deoxyuridine (330 mg, 86%). 1H-NMR (CDCl3) δ 1.54 (br, 4H, spm), 1.83-1.89 (m, 2H, spm), 2.24 (br, 2H, spm), 2.46-2.52 (m, 1H, H2'), 2.74-2.80 (m, 1H, H2'), 3.29-3.55 (m, 10H, spm), 3.77 (s, 6H, DMT-OMe), 4.07-4.17 (m, 1H, H4'), 4.25-4.30 (m, 1H, H3'), 4.38 (br, 2H, spm), 5.00 (br, 1H, OH-3'), 6.26-6.33 (m, 1H, H1'), 6.80-6.84 (m, 4H, Ar), 7.16-7.20 (m, 1H, Ar), 7.23-7.28 (m, 2H, Ar), 7.32-7.34 (m, 4H, Ar), 7.41-7.43 (m, 2H, Ar), 8.11-8.20 (m, 1H, H6 or triazole), 8.47-8.60 (m, 1H, H6 or triazole), 9.81-9.98 (m, 1H, NH-3).

5-triazospermine-5'-DMT-2'-OME-uridine. 5-ethyl-5'-DMT-2'-OME-uridine (118 mg, 0.20 mmol), (Spm-N3)TFA (104 mg, 0.20 mmol), CuSO4•H2O (2.5 mg, 0.01 mmol) and sodium ascorbate (8 mg, 0.04 mmol) were reacted in t-BuOH/water 1:1 (2 ml) for 3h and purified as described above to yield 5-triazospermine-5'-DMT-2'-OME-uridine (145 mg, 65%). ESI-MS: m/z 1123.3587 ([M+Na]+), C49H53F9N8O11, calc 1123.3582); 1H-NMR (CDCl3) δ 1.53-1.58 (m, 4H), 1.81-1.91 (m, 2H), 2.17-2.22 (m, 2H), 2.85 (d, 1H, J=8 Hz), 3.30-3.42 (m, 2H), 3.42-3.49 (m, 2H), 3.60 (s, 3H), 3.76 (s, 6H), 3.98-4.03 (m, 1H), 4.07-4.10 (m, 1H), 4.29-4.36 (m, 3H), 6.00 (d, 1H, J=4 Hz), 6.77-6.81 (m, 4H), 7.13-7.16 (m, 1H), 7.22-7.26 (t, 2H, J=8 Hz), 7.32-7.42 (m, 4H), 7.47-7.49 (m, 2H), 7.56-7.62 (m, 1H), 8.06-8.09 (m, 1H), 8.45-8.51 (m, 1H), 9.96-9.98 (m, 1H). 13C-NMR (CDCl3) δ 23.8, 25.6, 26.4, 29.7, 30.9, 36.6, 37.0, 43.9, 44.6, 47.1, 53.5, 55.1, 58.8, 62.7, 69.3, 83.1, 83.5, 86.6, 88.4, 106.2, 113.1, 117.8, 122.5, 126.6, 126.7, 127.8, 128.1, 129.1, 130.1, 135.9, 136.7, 139.0, 144.6, 149.7, 158.4, 158.6.

5-triazospermine-5'-DMT-2'-MOE-uridine. 5-ethyl-5'-DMT-2'-MOE-uridine (300 mg, 0.48 mmol), (Spm-N3)TFA (246 mg, 0.48 mmol), CuSO4•H2O (6 mg, 0.024 mmol) and sodium ascorbate (19 mg, 0.10 mmol) were reacted in t-BuOH/water 1:1 (10 ml) overnight and purified as described above to yield 5-triazospermine-5'-DMT-2'-MOE-uridine (417 mg, 76%). 1H-NMR (CDCl3) δ 1.48 (br, 4H, spm), 1.72-1.82 (m, 2H, spm), 2.06-2.19 (m, 2H, spm), 3.20-4.34 (peak forest, 28 H, sugar-H, OMe, MOE-Et, spm), 5.82-5.92 (m, 1H, H1'), 6.69-6.76 (m, 4H, Ar), 7.05-7.11 (m,
5-triazospermine-N4-bz-5′-DMT-2′-MOE-ctydine. 5-ethynyl-N4-bz-5′-DMT-2′-MOE-ctydine (90 mg, 0.12 mmol), (Spm-N₃)₇FA (64 mg, 0.12 mmol), CuSO₄·5H₂O (3 mg, 0.012 mmol) and sodium ascorbate (10 mg, 0.049 mmol) were reacted in t-BuOH/water 1:1 (2 ml) for 7h and purified as described above to yield 5-triazospermine-N4-bz-5′-DMT-2′-MOE-ctydine (81 mg, 53%). ESI-MS: m/z 1248.5 ([M+H]+, C₃₈H₇₀F₅N₆O₁₉, calc 1248.17); ¹H-NMR (CDCl₃) δ 1.52 (br, 4H, spm), 2.17-2.19 (m, 3H, spm), 2.26 (br, 1H, spm), 3.00-3.62 (br, 19H, spm, MOE-OMe, MOE-Et), 3.76 (s, 6H, DMT-OME), 3.79-3.47 (br, 5H, sugar-H), 6.01 (d, 1H, H1′), 6.60 (d, 1H, 3′-OH), 6.70-6.84 (m, 4H, Ar), 7.16-7.58 (br, 12 H, Ar), 8.04-8.27 (m, 2H, Ar), 8.65-8.77 (m, 1H, H6), 12.22-13.37 (m, 0.5H, NH), 13.52-13.61 (m, 0.5H, NH).

Phosphitylation.

5-triazospermine-5′-DMT-2′-deoxyuridine phosphoramidite. To a solution of dry 5-triazospermine-5′-DMT-2′-deoxyuridine (330 mg, 0.308 mmol), TEA (86 µl, 0.62 mmol) and NMI (2.5 µl, 0.03 mmol) in dry THF (2 ml) was added 2′-cyanoethyl-N,N-dioisopropylphosphoramidite (PCI-reagent, 103 µl, 0.46 mmol) and the reaction mixture was stirred under argon at rt. Completion was reached after 6h upon addition of another 1.5 eq. PCI-reagent (86 µl, 0.62 mmol). The crude was taken up in EtOAc and was washed 3x with saturated aqueous NaHCO₃. Aqueous phases were back-extracted with EtOAc. Combined organic layers were washed with brine and dried over Na₂SO₄. The residue was evaporated to dryness and was submitted to column chromatography (MeOH/DCM 0-2%). The product was precipitated from hexane (78 mg, 20%). ³¹P-NMR (CDCl₃) δ 148.76, 149.07.

5-triazospermine-5′-DMT-2′-OMe-uridine phosphoramidite. To a solution of dry 5-triazospermine-5′-DMT-2′-OMe-uridine (305 mg, 0.28 mmol), TEA (77 µl, 0.55 mmol) and NMI (2.3 µl, 0.03 mmol) in dry THF (2 ml) was added PCI-reagent (93 µl, 0.42 mmol) and the reaction mixture was stirred under argon at rt. Completion was reached after 7h upon addition of another 4 x 0.5 eq. PCI-reagent (132 µl, 0.55 mmol) and TEA (22 µl, 0.55 mmol). The crude was taken up in EtOAc and was washed 3x with saturated aqueous NaHCO₃. Aqueous phases were back-extracted with EtOAc. Combined organic layers were washed with brine and dried over Na₂SO₄. The residue was evaporated to dryness and was submitted to column chromatography (MeOH/DCM 0-2%). The product was precipitated from hexane (182 mg, 50%). ESI-MS: m/z 1323.4662 ([M+Na]+, C₅₈H₇₀F₅N₆O₁₉P, calc 1323.4661); ³¹P-NMR (CDCl₃) δ 150.36, 150.67.

5-triazospermine-5′-DMT-2′-MOE-uridine phosphoramidite. To a solution of dry 5-triazospermine-5′-DMT-2′-MOE-uridine (100 mg, 0.09 mmol), TEA (36 µl, 0.26 mmol) and NMI (1 µl, 0.013 mmol) in dry THF (1 ml) was added PCI-reagent (29 µl, 0.13 mmol) and the reaction mixture was stirred under argon at rt overnight. Completion was reached upon addition of another 2 x 0.75 eq. PCI-reagent (29 µl, 0.13 mmol) and 2 x 3 eq. TEA (72 µl, 0.52 mmol). The crude was taken up in EtOAc and was washed 3x with saturated aqueous NaHCO₃, then brine and dried over Na₂SO₄. The residue was evaporated to dryness and was submitted to column chromatography (solid depot from DCM, EtOAc/hexane 1:1 to EtOAc 100%). The product was precipitated from hexane (34 mg, 29%). ³¹P-NMR (CDCl₃) δ 149.98, 150.05, 150.09, 150.10, 150.14.

5-triazospermine-N4-bz-5′-DMT-2′-MOE-ctydine phosphoramidite. To a solution of dry 5-triazospermine-N4-bz-5′-DMT-2′-MOE-ctydine (81 mg, 0.065 mmol), TEA (27 µl, 0.195 mmol) and NMI (1 µl, 0.007 mmol) in dry THF (2 ml) was added PCI-reagent (22 µl, 0.097 mmol) and the reaction mixture was stirred under argon at rt. Completion was reached upon addition of another 2 x 0.75 eq. PCI-reagent (22 µl, 0.097 mmol) and 2 x 3 eq. TEA (27 µl, 0.19 mmol) and stirring overnight. The crude was taken up in EtOAc and was washed 3x with saturated aqueous NaHCO₃, then brine and dried over Na₂SO₄. The residue was evaporated to dryness and was submitted to column chromatography (solid depot from DCM, EtOAc/hexane 1:1 to 3:1). The product was washed with hexane (35 mg, 37%). ³¹P-NMR (CDCl₃) δ 14.15, 148.75, 149.99, 150.21, 150.62.

5-ethyl-N-5′-DMT-2′-OMe-uridine phosphoramidite. To a solution of dry 5-ethyl-5′-DMT-2′-OMe-uridine (572 mg, 0.98 mmol) and DIPEA (420 µl, 2.46 mmol) in dry THF (2.5 ml) was added PCI-reagent (265 µl, 1.19 mmol) at 0
°C and the reaction mixture was stirred under argon at rt for 2h. An additional 65 µl (0.29 mmol) of the PCl reagent was added and the reaction mixture was stirred for another 30 min. The crude was taken up in EtOAc and was washed 3x with saturated aqueous NaHCO₃, then water phases were back-extracted 3x with EtOAc and combined organic layers were dried over Na₂SO₄. The residue was evaporated to dryness and was precipitated from hexane. The crude product was purified by column chromatography (DCM/MeOH/TEA 97:1.5:1.5) to yield 636 mg (82 %). ESI-MS: m/z 807.3114 ([M+Na]+, C₄₂H₄₉N₄O₉P, calcld 807.3129). ¹H-NMR (CDCl₃) δ 1.00-1.13 (m, 12H), 2.38-2.34, 2.57-2.60 (m, 2H), 2.62, 2.68 (s, 1H), 3.35-3.40 (m, 2H), 3.31-3.60 (m, 2H), 3.49, 3.50 (s, 3H), 3.72, 3.73 (s, 6H), 3.64-3.90 (m, 2H), 3.97-3.99 (m, 1H), 4.10-4.18, 4.20-4.22 (m, 1H), 4.40-4.45, 4.49-4.54 (m, 1H), 5.89 (d, 1H), 6.76-6.79 (m, 4H), 7.10-7.40 (m, 9H), 8.02, 8.11 (s, 1H). ³¹P-NMR (CDCl₃) δ 150.59, 150.61.

4.1.2. Purine modifications

2’-O-pent-4-yn-1-yl-adenosine phosphoramidite. Synthesis according to reported protocol [339]. ESI-MS: m/z 891.32 ([M+H]+, C₄₆H₅₁N₈O₆P, calcld 890.95); ³¹P-NMR (CDCl₃) δ 149.94, 150.67.

2’-O-prop-2-yn-1-yl-adenosine phosphoramidite. Synthesis according to reported protocol [368]. ESI-MS: m/z 965.15 ([M+H]+, C₄₆H₅₅N₈O₆P, calcld 864.95); ³¹P-NMR (CDCl₃) δ 150.28, 150.82.
4.2. Synthesis of polyamine-modified oligonucleotides

Automated oligonucleotide synthesis. Standard RNA, 2'-OMe, DNA and 2'-MOE phosphoramidites were obtained from Thermo Fisher Scientific (Waltham, MA). Oligonucleotides were synthesized on an MM12 (RNA, 2'-OMe or DNA) and an MM192 (2'-MOE) synthesizer from Bio Automation Inc. (Plano, TX). For short sequences (< 30 nt) a 500 Å UnyLinker CPG from ChemGenes (Wilmington, MA) was used, for preparation of 3'-biotinylated pre-miRNA hairpins or single stranded RNAs it was a 1000 Å 3'-biotin TEG CPG from Link Technologies (Lanarkshire, Scotland).

0.08 M phosphoramidite solutions in dry ACN were prepared and coupling times were 2 x 2 min, except for 2'-MOE and modified phosphoramidites, where prolonged coupling times of 2 x 5 min were applied. 3% dichloroacetic acid in dry DCM was applied for 2 x 60s for detritylation, 0.24 M benzylthiotetrazole in dry ACN (RNA, 2'-OMe, DNA) or 1 M 4,5-dicyanoimidazole and 0.1 M N-methylimidazole in dry ACN (2'-MOE) as an activator. Oxidation was done with a 0.02 M iodine solution in THF/pyridine/water 7:2:1 for 6 min (RNA, 2'-OMe, DNA) or 0.5 M (1S)-(−)-(10-camphorsulfonyl)oxaziridine in dry ACN for 2 x 5 min (2'-MOE). Sulfurization was achieved with 0.05 M 3-((N,N-dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-5-thione in pyridine:ACN 3:2 (RNA, 2'-OMe, DNA) or 0.2 M phenylacetyl disulfide in pyridine/ACN 1:1 (2'-MOE) for 2 x 5 min. Capping was done in 60s using 10% acetic anhydride and 10% lutidine in THF as well as 16% N-methylimidazole in THF.

Cleavage from solid support and removal of base protecting groups was achieved with a 1:1 mixture of 25% ammonium hydroxide and 40% aqueous methylamine at 65 °C for 30 min, except for 2'-MOE RNA, which was treated with 32% ammonium hydroxide at 55 °C overnight. Crude RNA was subjected to desilylation before HPLC purification. A freshly prepared mixture of 1-N-methyl-2-pyrrolidone:TEA:HfX3TEA 6:3:4 was added and the crude oligonucleotide was incubated at 70 °C for 90 min. Ethoxytrimethylsilane was added and the crude product was precipitated with diisopropylether.

Purification was done on an Agilent 1200 series preparative HPLC (Agilent Technologies, Santa Clara, CA) on a WatersXBridge OST C-18 column, 10 x 50 mm, 2.5 µm, 5 ml/min, 65 °C using a gradient of MeOH in 0.1 M TEAA buffer over 5-7 min. In a first HPLC run failed sequences were removed, then 5'-terminal DMT was cleaved in 40% AcOH for 30 min at rt, followed by a second HPLC purification step in DMT-off state. Final products were characterized by LC-MS on an Agilent 1200/6130 system (Agilent Technologies, Santa Clara, CA) on a Waters Acquity OST C-18 column, 2.1 x 50 mm, 1.7 µM, 0.3 ml/min, at 65 °C using a gradient of MeOH in a 0.4 M HFIP, 15 mM TEA buffer over 10 min. Pure products were quantified by UV absorbance at 260 nm using a Nanodrop 2000 (Thermo Scientific, Wohlen, CH).

Post-synthetic polyamine or Cy3 introduction. Support-bound crude oligonucleotides containing 2'-propynyl- or 2'-pentynyl-adenosine or 5-ethylidene-2'-OMe-uridine were treated with a mixture of the following solutions: 150 µl PBS, 150 µl MeOH, 40 µl DMF, 20 µl of a 25 mM TBTA solution in DMF, 20 µl of a 50 mM (Spm-N)xTAA solution in DMF or a freshly prepared 50 mM solution of Cy3 azide in DMF, 10 µl of a 50 mM Na-ascorbate solution in water and 10 µl of a 5 mM CuSO4·5H2O solution in water. The reaction mixture was shaken at 45 °C overnight. The CPG was filtered off and washed consecutively with 3 x 200 µl DMF, 3 x 200 µl 0.1 M EDTA in water pH 8, 3 x 200 µl DMF, 3 x 200 µl ACN, 3 x 200 µl chloroform. Oligonucleotides were cleaved from solid support and purified as described above.
4.3. Biophysical characterization

Thermal denaturation studies. Thermal melting was performed on a Cary 300 (Varian, Palo Alto, CA) equipped with a thermocontroller. 2 μM or 3 μM solutions were prepared in phosphate melting buffer (100 mM NaCl, 10 mM phosphate, 0.1 mM Na₂EDTA, pH 7.0). To ensure proper annealing, solutions were heated to 90 °C (5 °C/min), then cooled down to 20 °C and heated again to 90 °C (0.5 °C/min), repeating the melting process 3x. T_M was obtained from the maxima of the first derivatives of melting curves and is given as the average of 3 consecutive melting runs. Thermodynamics were calculated from association constants over a temperature range where the van’t Hoff equation

$$\ln(K_a) = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$$

results in a linear dependence of ln(K_a) vs. 1/T, assuming that ΔC_P=0. ΔH°, ΔS° and ΔG° are given at 25 °C.

Circular dichroism (CD). Spectra were recorded on a J-715 spectropolarimeter (Jasco, Easton, MD) at 25 °C between 320 and 200 nm, at 50 nm/min. 5 μM solutions were prepared in phosphate buffer (100 mM NaCl, 10 mM phosphate, 0.1 mM Na₂EDTA, pH 7.0) and were pre-annealed prior to measurement. 3 scans were averaged.

Surface plasmon resonance (SPR). SPR experiments were performed on a MASS-1 or an SPR-2 (Sierra Sensors, Hamburg, Germany). Neutravidin or streptavidin was covalently immobilized on amine chips (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling at 15 μl/min and 25 °C. 3’-biotinylated target RNA was immobilized by injecting 10-100 μl of a 20-50 nM solution. Binding assays were performed at 25 μl/min, 100 μl injections with a dissociation time of up to 8 min. Analyte solutions were injected at two-fold serial dilutions in PBS buffer containing TWEEN20 0.005%, 8-10 concentrations per analyte in duplicates. Surface regeneration was achieved in 10 μl HCl 10 mM and data analysis was done using Analyzer2 (Sierra Sensors, Hamburg, Germany) and Scrubber2 (BioLogic Software, Campbell, Australia).
4.4. Cellular testing

Cell culture and transfections. Huh-7 cells and HEK293-T cells (ATCC, LGC, Molsheim, France) were cultured as monolayers in DMEM GlutaMAX™-I (31966-021, Gibco®, Life Technologies, Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum, 10270106, ThermoFisher Scientific). Transfections were performed with Lipofectamine 2000 (12252-011, Life Technologies, Carlsbad, CA) according to manufacturer’s protocol and jetPEI (101-10, Polyplus transfections, Illkirch, France) was used for plasmid DNA.

Luciferase assays, general procedure. Cells were seeded in opaque white 96-well plates (136101, Nunc, Roskilde) in 80 µl medium, 10'000 cells/well. 8h after seeding, looptomirs or antimiRs as well as control reagents were transected as described above. After another 16h, reporter plasmids were transfected at 20 ng/well (looptomir assay) or 30 ng/well (antimir assay). Assay read-out was performed with the Dual-Glo® Luciferase Assay System (E2980, Promega, Fitchburg). Cell culture medium was removed and 30 µl of firefly luciferase substrate dissolved in luciferase buffer was added to the wells and was incubated for 10 min. Luminescence was measured on a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad). 15 µl of renilla substrate dissolved in stop&glo buffer was added to the wells and luminescence was measured after 10 min incubation again. Luminescence readings were for 1s per well. Renilla luminescence counts were normalized on firefly luminescence counts. Average and standard deviations were determined from three technical replicates. Eventually, a further normalization was done on a control reagent.

psiCHECK-2 reporter construct cloning. Protocol provided by Dr. Martina Roos. Let-7 vectors were prepared by Dr. Martina Roos, the miR-122 sensor by Dr. Luca Gebert. The target sequences were amplified from synthetic DNA using oligonucleotides from Microsynth (Balgach) and cloned into the NotI and Xhol restriction sites of psiCHECK-2 plasmid (C8021, Promega, Fitchburg). Inserted target sequences were CTCGACTTACCCAGGACTCGAGTTTTT‐CATTTTTGAAGGGCCTCCTGAAGTGGGGGCTACATCAAAAAACTTTAATCTATACAACCTACTACCTAGGGATCTCCCCCTACTCCCTACCTCC TCTAAGGTTGCGCAGGTCACCTGAAGTGCACAGCTAGGCTGGGACCTGTTGCGGCGCTGCTAAGCTCAAGCTAGCTAGTCTCGGC ACCTCGC for the fully complementary let-7 sensor, CTCACCCTCAGCAGCAGCTCGAGTTTTTATTTTAAAGGGG‐GCTCCAGGGATGCGATCCTACCAAAAACTTTAATCTATACACCTACCTACCTAGGGATCTCCCCCTACTCCCTACCTAAGGTTGGGC AGGGTGACCTGAAGTGCACAGCTAGGCTGGGACCTGTTGCGGCGCTGCTAAGCTCAAGCTAGCTAGTCTCGGC for the mutated let-7 sensor and AAACCTGAGCCGAGTGCCTCCCACACAGTGGCGCACAACCAACATGTGACAACCACCCCTCAGCTCTCAGTTTTCAACGGGGCGCAA for the miR-122 sensor (target site in italics). The ligated fragment was transformed into Subcloning Efficiency™ DH5α™ Competent E. coli (18265-017, Life technologies, Carlsbad), plated on LB agar with 100 µg/ml Amp and incubated for at least 16h at 37 °C. The single colonies were picked and resuspended in water. Clones were screened by a Colony PCR for the desired plasmid. Positive clones were used to make glycerol stocks and DNA minipreps and were sequenced by GATC (Cologne). All the PCR purifications were performed following the protocol of MinElute® PCR Purification Kit (28006, Qiagen, Venlo). Miniprep were done according to the protocol of QIAprep® Spin Miniprep Kit (27106, Qiagen, Venlo). Digestions were performed following the guideline of the NEB website (http://www.neb.com) using NotI and Xhol according to the supplied protocol (NEB, Ipswich). The ligations were done with ratios of 1:3, 1:4 (vector to insert) using the dephosphorylated psiCHECK-2 vector with T4 DNA Ligase (M1804, Promega, Fitchburg).

RNA extraction and precipitation. Culture medium was removed from cells and they were washed in PBS 750 µl (for a 12-well plate). 750 µl TriFast reagent (peqGOLD, VWR) was added and cells were lysed by repetitive pipetting. Suspensions were transferred into Eppendorff tubes and incubated for 5 min at rt. 150 µl chloroform was added and suspensions were shaken heavily for 15s, then incubated for another 5 min. They were centrifuged for 5 min at 4 °C and 12’000g. The upper aqueous phase was taken into a new Eppendorff tube. 375 µl isopropanol, 20 µl NaOAc 3 M and 1 µl glycogen was added and samples were stored at -20 °C overnight. The next day they were centrifuged for 20 min at 12’000g. The supernatant was carefully removed and the pellet was
washed twice with cool EtOH 75% 1ml. The pellet was then dried at rt and redissolved in 20 µl water. Concentrations were determined using Nanodrop.

**qRT-PCR.** TaqMan® qRT-PCR was performed using standard reagents from Life Technologies (TaqMan® MicroRNA Assays). RT was done using the TaqMan® primers from MicroRNA Assays and the TaqMan® MicroRNA Reverse Transcription Kit (4366596, Life Technologies, Carlsbad) with 25 ng total RNA. The PCR was performed in a LightCycler 480 instrument (Roche, Penzberg) with GoTaq® Probe qPCR Master mix (A6102, Promega, Fitchburg) according to the manufacturer's protocol. Each reaction was carried out in 4 technical replicates. C_t values were calculated for each replicate and averaged.

**Microscopy experiments.** Cells were cultured in transparent 24-well dishes, 150’000 cells/well. 24h after seeding, reagents were transfected as described above. Medium was removed and cells were washed twice with PBS 200 µl 24h post-transfection. 400 µl fresh medium was added. Microscopy was done on a Leica epifluorescence microscope (DMI6000B), covering an excitation range of 540-590 nm.

**ApoB quantification.** Protocol provided and experiment carried out by Dr. Helen Lightfoot. Huh7 cells (ATCC) were cultured as monolayers in DMEM GlutaMAX™-I (31966-021, ThermoFisher Scientific) supplemented with 10% FBS (fetal bovine serum, 10270106, ThermoFisher Scientific). For gapmer transfections, Huh7 cells were seeded in 96-well plates (50,000 cells/well). GAPmers as well as mock transfections were performed with lipofectamine 2000 (11668019, ThermoFisher Scientific), 24h post-seeding. After another 24h, supernatants were removed and RNA was isolated from the cells. Total RNA was extracted and purified using RNeasy Micro Kit (74004, Qiagen).

10 ng RNA and 500 ng random primer (C1181, Promega) were used for cDNA synthesis with the TaqMan® MicroRNA Reverse Transcription Kit (4366596, ThermoFisher Scientific), according to the manufacturer’s protocol. qPCR reactions were performed using KAPA SYBR® FAST qPCR Master Mix (KK4618, Kapa Biosystems) on a LightCycler 480 Detection System (Roche). Each reaction was carried out in 3 technical replicates. The mean measured levels of ApoB were normalized to the housekeeping gene β-Actin. Data from 3 independent replicates was obtained. Primer pairs utilized are as follows: ApoB F: TGC TAA AGG CAC ATA TGG CCT, R: CTC AGG TTG GAC TCT CCA TTG AG; β-Actin F: CCAACCCGAGAAGATGA, R: CCAGAGGCGTACAGGGATAG. 2^ΔΔCt method was used to calculate the relative transcript abundance.

**Splice-switching assay.** Protocol provided and experiment carried out by François Halloy. COS-7 cells (ATCC) were cultured as monolayers in DMEM GlutaMAX™-I (31966-021, ThermoFisher Scientific) supplemented with 10% FBS (fetal bovine serum, 10270106, ThermoFisher Scientific). For transfections, COS-7 cells were seeded in 24-well plates (135’000 cells/well). FECH-C minigene construct (gift from Prof. D. Schümperli, University of Bern) was transfected 24h post-seeding at 750 ng/well with XtremeGene HP reagent (06366236001, Roche Life Sciences, 2.25 µl/1 µg plasmid DNA). SSO transfection was performed 3h later with Lipofectamine 2000 (11668019, ThermoFisher Scientific; 1.25 µL Lipofectamine/50 nM SSO). Depending on the time course desired, supernatants were removed after 24h or 48h and RNA was isolated from the cells. Emetine (as HCl salt, E2375, Sigma-Aldrich) was used at a final concentration of 3 µM in the cell culture well and was added 24h prior to cell harvest, i.e. immediately after SSO transfection for 24h time points or 24h after SSO transfection for 48h time points. Total RNA was extracted and purified using RNeasy Mini Kit (74106, Qiagen). DNase I treatment was doubled to ensure complete plasmid DNA removal.

1 µg of RNA and 500 pg random primer (C1181, Promega) were used for cDNA synthesis with the TaqMan® MicroRNA Reverse Transcription Kit (4366596, ThermoFisher Scientific), according to the manufacturer’s protocol. For PCR reaction, cDNA solutions were diluted 1:3 prior to mixing: 1 µl cDNA solution, 5 µl DreamTaq 10x Buffer, 1 µl dNTPs (18427, ThermoFischer Scientific), 1 µl FW primer (7.5 µM, 5’-GCTCTCTAACCTGGGTGTGG-3’, Microsynth), 1 µl RV primer (7.5 µM, 5’-GACAATTCAATCCAGCACCTC-3’, Microsynth), 0.25 µl DreamTaq
Polymerase and 40.75 μl RNase-free water. The PCR program was: 1 min, 95 °C; 30 cycles (30s, 95 °C; 30s, 60 °C; 30s, 68 °C); 5 min, 68°C.

PCR samples loaded with 5x GelPilot Loading Dye (239901, Qiagen) were allowed to run for 1h at 85 V in a 2% agarose gel in TAE Buffer and 1/10’000 GelRed Nucleic Acid Stain (41003, Chemie Brunschwig). Gels were revealed in a UV chamber (Universal Hood II, BioRad) and ratios of aberrantly spliced product on (normal+aberrant) were calculated using ImageJ software and background subtraction.

4.5. Uptake study

Cell culture and transfections. Huh-7 cells (ATCC, LGC, Molsheim, France) were cultured as monolayers in DMEM GlutaMAX™-I (31966-021, Gibco®, Life Technologies, Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum, 10270106, ThermoFisher Scientific). 40 nM antimir transfection was performed with Lipofectamine 2000 (12252-011, Life Technologies, Carlsbad, CA) according to manufacturer’s protocol.

RNA extraction and precipitation. 24h post-transfection, culture medium was removed from cells and they were washed twice with ice-cooled PBS 750 μl (for a 6-well plate) and then scraped from wells with a pipette tip (1ml) in 500 μl ice-cooled PBS. Suspensions were transferred to Eppendorfs and were centrifuged for 5 min at 200g and 4 °C. Buffer was removed with a pipette. Cells were lysed in 250 μl NP40 lysis buffer (50 mM HEPES pH 7.5, 150 mM KCl, 0.5% NP40, 0.5 mM DTT, 0.5 mM EDTA, complete protease inhibitor (Roche)) for 15 min on ice. Centrifugation at 14’000g at 4 °C for 15 min. To the aqueous phase was added 250 μl basic or acidic phenol:chloroform (pH 8 or 4) and 25 μl NaOAc 3 M. Mixtures were shaken heavily for >20s, then centrifuged at 12’000g and 4 °C for 10 min. The aqueous phase was isolated and 750 μl EtOH 96% and 1 μl glycolblue (Life Technologies) was added. The RNA was precipitated at -20 °C overnight. Centrifugation at 12’000g and 4 °C for 30 min precipitated the RNA. The supernatant was carefully removed and the pellet was washed 3x with 750 μl cold EtOH 75%, then dried at rt and redissolved in 30 μl water by heavily pipetting up and down, sonicating, vortexing and spinning down again. Concentration was determined using Nanodrop and adjusted by addition of water.

CL-qPCR. Calibration curves were established based on 1:10 antimir dilutions, starting from 2 μM. 3 total RNA 1:10 dilutions were prepared starting from normalized concentrates. To 8 μl of the chemical ligation mix containing 1.0 μl PCR buffer 10X with Mg2+, 1.0 μl polyA 1 μg/μl, 0.1 μl P5-ligator 10 μM, 0.1 μl BPS-ligator 10 μM and 3.8 μl water was added 4 μl of the template (total RNA, antimir dilution or water for a non-template control). The mixtures were incubated at 33 °C for 6h. A qPCR was run in triplicates, composed of 0.15 μl dNTPs 100 mM, 1.0 μl PCR buffer 10X, 0.15 μl forward primer 10 μM, 0.15 μl reverse primer 10 μM, 0.3 μl anti-primer 10 μM, 0.1 μl Hot Start Taq Pol 5 U/μl (Roche, 12032929001) and 2.83 μl of the chemical ligation mix. After activation at 95 °C for 10 min, the reaction was run using the following cycles: 3s, 95 °C; 30s, 48 °C; 10s, 72 °C; 50 cycles. The ramping speed did not exceed 3 °C/s. Template DNA 10 nM was diluted 1:10 for a positive control of the qPCR. Ct values were calculated and averaged and correlated with logarithmic concentration.

Uptake quantification by SPR. SPR experiments were performed on an SPR-2 (Sierra Sensors, Hamburg, Germany). Streptavidin was covalently immobilized on an amine chip (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling at 15 μl/min and 25 °C (3400 RU). 3'-biotinylated target RNA was immobilized by injecting 3x 200 μl of a 100 nM 2'-MOE PS biotinylated target RNA solution (U*G*G*A*G*U*G*U*G*A*C*A*A*U*G*G-Biotin, 683 RU). Binding assays were performed in PBS containing TWEEN20 0.005% at 15 μl/min, 100 μl injections with a dissociation time of 5 min. The surface was regenerated in 50 μl HCl 10 mM and NaCl 1M. For calibration curves, analyte solutions in running buffer were two-fold serially diluted. Total RNA was standardized to 10 ng/μl (experiment 1) and 12 ng/μl (experiment 2) and was injected in duplicates. Analyzer2 (Sierra Sensors, Hamburg, Germany) and Scrubber2 (BioLogic Software, Campbell, Australia) were used to extract post-injection binding
levels as well as initial binding rates. Sigmoidal fitting of binding levels was done in OriginPro (OriginLab). Initial binding rates were determined in the range of 30-60s, based on the rate equation

$$\frac{d[LA]}{dt} = k_{on} [L][A] - k_{off} [LA]$$  \hspace{1cm} (15)$$

where L denotes the ligand, A the analyte and LA the ligand-analyte complex, and on the assumption that [LA]=0 at $t_0$, initial binding rates were simplified to

$$\frac{d[LA]}{dt} = C \cdot [A]$$  \hspace{1cm} (19)$$

with $C$ representing the slope of the linear relation. Uptake was quantified as amount (amol) per ng total RNA.
5. Appendix

5.1. Pre-let-7a-2 loop screen with 2’-MOE 9mers, 11mers and 13mers

5.1.1. Aim of the project
At a time, serious efforts of the lab were focusing on targeting the pre-let-7/Lin28 interaction to enhance levels of the tumor-suppressive let-7 [324,341]. As part of this, the TLR of pre-let-7a-2 was screened for high affinity binding sites for 2’-MOE looptomirs interrupting Lin28 binding while allowing processing of the pre-miRNA (see 2.7.1). A series of 2’-MOE 9mer, 11mer and 13mer looptomirs covering the Lin28 binding site were synthesized, all covering the conserved Lin28 binding motif in the pre-let-7 TLR (Fig. 66). Binding affinities and kinetics to pre-let-7a-2 were investigated by SPR.

![Figure 66. Pre-let-7/Lin28 interaction. Blue, cold shock domain (CSD); green, zinc fingers (CCHC, Cys-Cys-His-Cys-type zinc binding motif); GGAG, conserved binding motif in pre-let-7 TLR. Taken from [369].](image)

5.1.2. Results and Discussion

2’-MOE PS synthesis was established on MM192. A major bottleneck was the adaptation of the deprotection procedure, which was required by a change in base protection of "C MOE. The base protecting group of the "C MOE phosphoramidite was benzoyl, in contrast to conventional acetyl. Acetyl groups can be fully hydrolyzed in MeNH₂, while benzoyl groups only partially hydrolyze and alternatively undergo transamination, resulting in a N₄-Methycytidine. A deprotection protocol avoiding MeNH₂ was implemented, using concentrated ammonium hydroxide at 55-60 °C for 12-16h [370]. Seven pre-let-7a-2 looptomirs of each length (13mer, 11mer and 9mer) were synthesized, all covering the conserved Lin28 binding motif in the pre-let-7 TLR (Tab. 15).

Binding affinities and kinetics to the target pre-let-7a-2 hairpin were investigated by SPR. Sensorgrams are shown in Fig. 68. With respect to mere binding affinities, differences among sequences were not very important, ranging from high picomolar for 13mers to low nanomolar for 9mers (Fig. 67, Tab. 16). The best binding was seen for L30-13. Previous work on 2’-OMe looptomirs by Dr. Martina Roos and Dr. Mario Rebhan identified L29-13 as the strongest binder and far more potent than L30-13 [324]. Thus, ribose chemistry seems to play a critical role in targeting the TLR of a pre-miRNA hairpin. Moreover, rate constants displayed an interesting pattern of position dependence. Looptomirs located close to the 3’-distal region of the TLR would have a slower association rate than looptomirs binding to the central part of the TLR. Likely, central looptomirs would dissociate faster than 3'-distal looptomirs. This finding indicates that, in the present case of pre-let-7a-2, conformational restriction towards the 3’-distal region of the TLR hinders annealing, while it simultaneously slows down dissociation, with a neutral net effect on binding affinity. On the other hand, residence time of the looptomir would be higher for slower kₐff, resulting in a preference for the conformationally restricted target site towards the 3’-distal region of the TLR. L30-13 has an exceptionally slow dissociation rate, which from the present experiment cannot be explained. With
respect to residence time, or half-life of the looptomir-pre-miRNA complex, L30-13 is by far the best looptomir, L29-13 ranked as the second. As expected, dissociation is highly dependent on looptomir length, contrary to association [179].

Table 15. 2’-MOE PS looptomirs against pre-let-7a-2. * = PS; red, conserved binding site.

<table>
<thead>
<tr>
<th>Name</th>
<th>Lab book</th>
<th>Sequence</th>
<th>Chemistry</th>
<th>MW calc.</th>
<th>MW found</th>
</tr>
</thead>
<tbody>
<tr>
<td>L32-13</td>
<td>MM80</td>
<td>U<em>A</em>U<em>C</em>U<em>C</em>G<em>C</em>U<em>G</em>A<em>A</em>U</td>
<td>2’-MOE PS</td>
<td>5087.85</td>
<td>5086.60</td>
</tr>
<tr>
<td>L31-13</td>
<td>MM81</td>
<td>A<em>U</em>C<em>U</em>C<em>U</em>G<em>A</em>U<em>G</em>A</td>
<td>2’-MOE PS</td>
<td>5112.85</td>
<td>5111.41</td>
</tr>
<tr>
<td>L30-13</td>
<td>MM82</td>
<td>U<em>C</em>U<em>C</em>U<em>G</em>A<em>U</em>G<em>A</em>U</td>
<td>2’-MOE PS</td>
<td>5103.85</td>
<td>5102.30</td>
</tr>
<tr>
<td>L29-13</td>
<td>MM83</td>
<td>C<em>U</em>C<em>U</em>G<em>A</em>U<em>G</em>A<em>U</em>A</td>
<td>2’-MOE PS</td>
<td>5112.85</td>
<td>5111.91</td>
</tr>
<tr>
<td>L26-13</td>
<td>MM86</td>
<td>C<em>U</em>C<em>U</em>G<em>A</em>U<em>G</em>A<em>U</em>A*U</td>
<td>2’-MOE PS</td>
<td>5123.84</td>
<td>5123.49</td>
</tr>
<tr>
<td>L34-11</td>
<td>MM87</td>
<td>U<em>A</em>U<em>C</em>U<em>C</em>U<em>C</em>U<em>U</em>G</td>
<td>2’-MOE PS</td>
<td>4290.10</td>
<td>4289.11</td>
</tr>
<tr>
<td>L33-11</td>
<td>MM88</td>
<td>A<em>U</em>C<em>U</em>C<em>U</em>G*A</td>
<td>2’-MOE PS</td>
<td>4299.10</td>
<td>4296.59</td>
</tr>
<tr>
<td>L32-11</td>
<td>MM89</td>
<td>U<em>C</em>U<em>C</em>U<em>G</em>A*U</td>
<td>2’-MOE PS</td>
<td>4290.10</td>
<td>4288.54</td>
</tr>
<tr>
<td>L31-11</td>
<td>MM90</td>
<td>C<em>U</em>C<em>U</em>G<em>A</em>U<em>G</em>A</td>
<td>2’-MOE PS</td>
<td>4315.10</td>
<td>4314.13</td>
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<tr>
<td>L30-11</td>
<td>MM91</td>
<td>U<em>C</em>U<em>G</em>A<em>U</em>G<em>A</em>U</td>
<td>2’-MOE PS</td>
<td>4316.09</td>
<td>4315.25</td>
</tr>
<tr>
<td>L29-11</td>
<td>MM92</td>
<td>C<em>U</em>C<em>U</em>G<em>A</em>U<em>G</em>A*U</td>
<td>2’-MOE PS</td>
<td>4325.10</td>
<td>4325.15</td>
</tr>
<tr>
<td>L28-11</td>
<td>MM93</td>
<td>C<em>U</em>C<em>U</em>G<em>A</em>U<em>G</em>A<em>U</em></td>
<td>2’-MOE PS</td>
<td>4335.09</td>
<td>4334.88</td>
</tr>
<tr>
<td>L36-9</td>
<td>MM94</td>
<td>U<em>A</em>U<em>C</em>U<em>C</em>U<em>C</em>U*</td>
<td>2’-MOE PS</td>
<td>3476.35</td>
<td>3474.50</td>
</tr>
<tr>
<td>L35-9</td>
<td>MM95</td>
<td>A<em>U</em>C<em>U</em>C<em>U</em>G*A</td>
<td>2’-MOE PS</td>
<td>3476.35</td>
<td>3474.60</td>
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<tr>
<td>L34-9</td>
<td>MM96</td>
<td>U<em>C</em>U<em>C</em>U<em>G</em>A*U</td>
<td>2’-MOE PS</td>
<td>3492.35</td>
<td>3491.57</td>
</tr>
<tr>
<td>L33-9</td>
<td>MM97</td>
<td>C<em>U</em>C<em>U</em>G<em>A</em>U<em>G</em>A</td>
<td>2’-MOE PS</td>
<td>3501.36</td>
<td>3500.06</td>
</tr>
<tr>
<td>L32-9</td>
<td>MM98</td>
<td>U<em>C</em>U<em>G</em>A<em>U</em>G<em>A</em>U</td>
<td>2’-MOE PS</td>
<td>3502.35</td>
<td>3503.14</td>
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<tr>
<td>L31-9</td>
<td>MM99</td>
<td>C<em>U</em>G<em>A</em>U<em>G</em>A*U</td>
<td>2’-MOE PS</td>
<td>3527.35</td>
<td>3526.50</td>
</tr>
<tr>
<td>L30-9</td>
<td>MM100</td>
<td>C<em>U</em>G<em>A</em>U<em>G</em>A*U</td>
<td>2’-MOE PS</td>
<td>3528.34</td>
<td>3527.32</td>
</tr>
</tbody>
</table>
Figure 67. Free energies of hybridization $\Delta G^\circ$ (A) and kinetics (B, C) as obtained from SPR experiments on 2'-MOE PS looptomirs binding to an immobilized pre-let-7a-2 hairpin.
Table 16. Binding affinities, kinetics and free energies of 2’-MOE PS looptomirs against pre-let-7a-2, determined by SPR at 25 °C.

<table>
<thead>
<tr>
<th>Name</th>
<th>Lab book</th>
<th>Sequence</th>
<th>$K_D$ [nM]</th>
<th>$k_{on}^{*10^3}$ [M⁻¹s⁻¹]</th>
<th>$k_{off}^{*10^3}$ [s⁻¹]</th>
<th>$\Delta G^\circ$ [kcal/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>L32-13</td>
<td>MM80</td>
<td>U<em>A</em>U<em>C</em>U<em>C</em>C<em>U</em>U<em>G</em>A*U</td>
<td>1.28</td>
<td>34.9</td>
<td>0.045</td>
<td>-13.0</td>
</tr>
<tr>
<td>L31-13</td>
<td>MM81</td>
<td>A<em>U</em>C<em>U</em>C<em>C</em>U<em>U</em>G<em>A</em>U*G</td>
<td>1.33</td>
<td>45.8</td>
<td>0.061</td>
<td>-13.0</td>
</tr>
<tr>
<td>L30-13</td>
<td>MM82</td>
<td>U<em>C</em>U<em>C</em>C<em>U</em>U<em>G</em>A<em>U</em>G+U</td>
<td>0.11</td>
<td>90.1</td>
<td>0.0098</td>
<td>-13.7</td>
</tr>
<tr>
<td>L29-13</td>
<td>MM83</td>
<td>C<em>U</em>C<em>C</em>U<em>U</em>G<em>A</em>U<em>G+U</em>A</td>
<td>0.38</td>
<td>72.9</td>
<td>0.028</td>
<td>-13.1</td>
</tr>
<tr>
<td>L28-13</td>
<td>MM84</td>
<td>U<em>C</em>C<em>C</em>U<em>U</em>G<em>A</em>U<em>G+U</em>A*A</td>
<td>0.99</td>
<td>84.1</td>
<td>0.083</td>
<td>-12.7</td>
</tr>
<tr>
<td>L27-13</td>
<td>MM85</td>
<td>C<em>C</em>C<em>U</em>U<em>G</em>A<em>U</em>G+U*A+A</td>
<td>0.31</td>
<td>145.0</td>
<td>0.045</td>
<td>-13.2</td>
</tr>
<tr>
<td>L26-13</td>
<td>MM86</td>
<td>C<em>C</em>C<em>U</em>U<em>G+A</em>U+G+U<em>A+A</em>U+U</td>
<td>1.00</td>
<td>121.0</td>
<td>0.12</td>
<td>-12.7</td>
</tr>
<tr>
<td>L34-11</td>
<td>MM87</td>
<td>U<em>A</em>U+C<em>U</em>C<em>C</em>U<em>U</em>G</td>
<td>2.24</td>
<td>28.6</td>
<td>0.064</td>
<td>-12.6</td>
</tr>
<tr>
<td>L33-11</td>
<td>MM88</td>
<td>A<em>U</em>C<em>U</em>C<em>C</em>U<em>U</em>G+A</td>
<td>1.04</td>
<td>48.9</td>
<td>0.051</td>
<td>-12.6</td>
</tr>
<tr>
<td>L32-11</td>
<td>MM89</td>
<td>U<em>C</em>U<em>C</em>C<em>U</em>U<em>G</em>A+U</td>
<td>0.64</td>
<td>61.5</td>
<td>0.039</td>
<td>-13.0</td>
</tr>
<tr>
<td>L31-11</td>
<td>MM90</td>
<td>C<em>U</em>C<em>C</em>U<em>U</em>G+A*U+G</td>
<td>0.72</td>
<td>85.4</td>
<td>0.061</td>
<td>-13.1</td>
</tr>
<tr>
<td>L30-11</td>
<td>MM91</td>
<td>U<em>C</em>C<em>C</em>U<em>U</em>G<em>A</em>U+G+U</td>
<td>0.77</td>
<td>84.0</td>
<td>0.065</td>
<td>-13.0</td>
</tr>
<tr>
<td>L29-11</td>
<td>MM92</td>
<td>C<em>C</em>C<em>U</em>U<em>G+A</em>U+G+U+A</td>
<td>0.40</td>
<td>150.0</td>
<td>0.060</td>
<td>-13.2</td>
</tr>
<tr>
<td>L28-11</td>
<td>MM93</td>
<td>C<em>C</em>C<em>U</em>U<em>G+A</em>U+G+U*A</td>
<td>1.19</td>
<td>145.0</td>
<td>0.17</td>
<td>-12.7</td>
</tr>
<tr>
<td>L36-9</td>
<td>MM94</td>
<td>U<em>A</em>U+C<em>U</em>C<em>C</em>U<em>U</em>G</td>
<td>3.62</td>
<td>36.4</td>
<td>0.13</td>
<td>-12.1</td>
</tr>
<tr>
<td>L35-9</td>
<td>MM95</td>
<td>A<em>U</em>C<em>U</em>C<em>C</em>U*U+U</td>
<td>1.49</td>
<td>48.1</td>
<td>0.072</td>
<td>-12.7</td>
</tr>
<tr>
<td>L34-9</td>
<td>MM96</td>
<td>U<em>C</em>U<em>C</em>C<em>U</em>U*G</td>
<td>1.24</td>
<td>55.6</td>
<td>0.069</td>
<td>-12.5</td>
</tr>
<tr>
<td>L33-9</td>
<td>MM97</td>
<td>C<em>U</em>C<em>C</em>U<em>U</em>G+A*U+G</td>
<td>1.53</td>
<td>68.4</td>
<td>0.11</td>
<td>-12.7</td>
</tr>
<tr>
<td>L32-9</td>
<td>MM98</td>
<td>U<em>C</em>C<em>C</em>U<em>U</em>G+A*U+G</td>
<td>2.73</td>
<td>95.6</td>
<td>0.26</td>
<td>-12.3</td>
</tr>
<tr>
<td>L31-9</td>
<td>MM99</td>
<td>C<em>C</em>C<em>U</em>U<em>G+A</em>U+G+U+G</td>
<td>1.44</td>
<td>129.0</td>
<td>0.19</td>
<td>-12.6</td>
</tr>
<tr>
<td>L30-9</td>
<td>MM100</td>
<td>C<em>C</em>C<em>U</em>U<em>G+A</em>U+G+U+G+U</td>
<td>2.83</td>
<td>177.0</td>
<td>0.50</td>
<td>-12.1</td>
</tr>
</tbody>
</table>
5.1.3. Conclusion and Outlook

With respect to the long half-life of the looptomir-pre-miRNA complex, binding sites towards the 3'-distal region were preferred. Association was length-independent and faster the further away from the stem. On the other hand, dissociation was highly dependent on length of the looptomir and there was a trend towards slower dissociation when closer to the stem. Therefore, the more structured the target RNA, the slower the association and the dissociation were. If a lead looptomir had to be selected, L30-13 as the highest affinity looptomir would be chosen. However, affinity distribution for 2'-MOE PS looptomirs was rather flat compared to 2'-OMe PO looptomirs.

The future of this project is uncertain as a basic investigation on the function of pre-let-7a-2 looptomirs was published [324]. Besides, a very attractive small molecule inhibitor of the pre-let-7/Lin28 interaction identified in a high-throughput screening in our lab by far outperforms the potential of RNA-like inhibitors [341].

5.1.4. Experimental

Automated oligonucleotide synthesis. RNA and 2'-MOE phosphoramidites were obtained from Thermo Fisher Scientific (Waltham, MA). Oligonucleotides were synthesized on an MM12 (RNA) and an MM192 (2'-MOE) synthesizer from Bio Automation Inc. (Plano, TX). A 500 Å UnyLinker CPG from ChemGenes (Wilmington, MA) was used for looptomir synthesis, for preparation of 3'-biotinylated pre-miRNA hairpins it was a 1000 Å 3'-biotin TEG CPG from Link Technologies (Lanarkshire, Scotland). 0.08 M phosphoramidite solutions in dry ACN were prepared and coupling times were 2 x 2 min for RNA and 2 x 5 min for 2'-MOE. 3% dichloroacetic acid in dry DCM was applied for 2 x 60 s for detritylation, 0.24 M benzylthietetrazole in dry ACN as an activator. Oxidation was done with a 0.02 M iodine solution in THF/pyridine/water 7:2:1 for 6 min, sulfurization was achieved with 0.05 M 3-((N,N-dimethylamino-methylidene)amino)-3H-1,2,4-dithioazole-5-thione in pyridine:ACN 3:2 for 6 min. Capping was done in 60s using 10% acetic anhydride and 10% lutidine in THF as well as 16% N-methylimidazole in THF.

Figure 68. Sensorgrams of 13mer, 11mer and 9mer 2'-MOE PS looptomirs binding immobilized pre-let-7a-2 hairpin. Sequences of looptomirs are given in Tab. 16. Conditions: PBS running buffer containing TWEEN 0.01%, 130 µl injections, flow rate 25 µl/min, regeneration HCl 10 mM 15 µl, 25 °C.

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Cleavage from solid support and removal of base protecting groups was achieved with a 1:1 mixture of 25% ammonium hydroxide and 40% aqueous methylamine at 65 °C for 30 min in the case of biotinylated RNA, while 2'-MOE was treated with 32% ammonium hydroxide at 55 °C overnight. Crude RNA was subjected to desilylation before HPLC purification. A freshly prepared mixture of 1-N-methyl-2-pyrrolidone:TEA:HFx3TEA 6:3:4 was added and the crude oligonucleotide was incubated at 70 °C for 90 min. Ethoxytrimethylsilane was added and the crude product was precipitated in diisopropylether.

Purification was done on an Agilent 1200 series preparative HPLC (Agilent Technologies, Santa Clara, CA) on a WatersXBridge OST C-18 column, 10 x 50 mm, 2.5 µm, 5 ml/min, 65 °C using a gradient of MeOH in 0.1 M TEA buffer over 5-7 min. In a first HPLC run failed sequences were removed, then 5'-terminal DMT was cleaved in 40% AcOH for 30 min at rt, followed by a second HPLC purification step in DMT-off state. Final products were characterized by LC-MS on an Agilent 1200/6130 system (Agilent Technologies, Santa Clara, CA) on a Waters Acquity OST C-18 column, 2.1 x 50 mm, 1.7 µM, 0.3 ml/min, 65 °C using a gradient of MeOH in a 0.4 M HFIP, 15 mM TEA buffer over 10 min. Pure products were quantified by UV absorbance at 260 nm using a Nanodrop 2000 (Thermo Scientific, Wohlen, CH).

**Surface plasmon resonance (SPR).** SPR experiments were performed on a MASS-1 (Sierra Sensors, Hamburg, Germany). Neutravidin was covalently immobilized on an amine chip (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling at 15 µl/min and 25 °C. 3'-biotinylated target RNA was immobilized by injecting 10-100 µl of a 20-50 nM solution. Binding assays were performed at 25 µl/min, 100 µl injections with a dissociation time of up to 8 min. Analyte solutions were injected as two-fold serial dilutions in PBS buffer containing TWEEN20 0.005%, 8-10 concentrations per analyte in duplicates. Surface regeneration was achieved with 10 µl 0.1 M HCl. Data analysis was done using Analyzer2 (Sierra Sensors, Hamburg, Germany) and Scrubber2 (BioLogic Software, Campbell, Australia).
### 5.2. A small molecule inhibitor of Lin28: Binding studies by SPR

#### 5.2.1. Aim of the project
The Lin28/let-7 interaction has a high relevance in the context of cancer pathobiogenesis [331,333,338,371] and serious efforts have been invested into its mechanistic understanding as well as its targeting [332,372]. In a FRET assay using a GFP-Lin28 donor and a black-hole quencher (BHQ)-labeled pre-let-7 acceptor Dr. Martina Roos and Dr. Ugo Pradère identified a small molecule inhibitor of the pre-let-7/Lin28 interaction out of a library of 16000 drug-like molecules ([Fig. 69](#)). Functional studies showed that let-7 processing was restored in Lin28-expressing cancer cells and differentiation was induced in mouse embryonic stem cells. Mechanics of the inhibition were by the majority unclear and it was to be shown whether the small molecule binds the pre-miRNA hairpin or Lin28. SPR studies on several hit small molecules were run against pre-let-7 or Lin28 as a target, or *vice versa*.

![Diagram of Lin28 and let-7 miRNA processing](image)

**Figure 69.** Small molecule inhibiting the pre-let-7/Lin28 interaction restores processing of pre-let-7 and induces differentiation in mouse embryonic stem cells. Taken from [341].

#### 5.2.2. Results and Discussion
In a first step, biotinylated truncated pre-let-7a-2 was immobilized on a streptavidin-coated chip and small molecules were assayed for binding ([Fig. 70](#)). High immobilization levels had to be attained due to the limiting mass ratio of the small molecules versus the pre-miRNA. This was achieved on a high density amine chip. 5% DMSO in PBS buffer with TWEEN 0.01% was used, requiring DMSO correction of the sensorgrams. Highest concentrations of the small molecules without provoking precipitation in the presence of PBS buffer were 80 µM. At 80 µM, none of the small molecules was binding to pre-let-7a-2. Small signal variations during the injection phase are due to imperfect DMSO correction.

In an inverse experiment, the biotinylated winner hit compound 1632 (cpd 1632) was immobilized on a streptavidin-coated surface and various pre-miRNA hairpins were assayed for binding ([Fig. 71](#)). The ligand was immobilized in 5% DMSO-containing running buffer, while the assay was run in PBS containing TWEEN 0.01%, therefore no DMSO correction was required. No binding of any of the pre-miRNA hairpins to cpd 1632 at 400 nM concentration could be obtained. It cannot be excluded that this hairpin concentration was too low, but higher concentrations were not possible due to the limited amount of material available.

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Figure 70. Sensorgrams showing binding assays of candidate small molecules against immobilized pre-let-7a-2. Running buffer containing DMSO 5%, pre-let-7a-2 immobilization level 1038 RU, expected signal of small molecules >15 RU. Start concentration 80 µM, two-fold serial dilutions, duplicates. Top left, immobilization of biotinylated pre-let-7a-2 in two steps (red, flow cell 2; blue, reference flow cell 1).

Figure 71. Sensorgrams showing binding assays of various pre-miRNAs against immobilized cpd 1632. Running buffer containing DMSO 5% for ligand immobilization, PBS only for binding assay. Immobilization level 495 RU, expected signal of pre-let-7a-2 13662 RU. Start concentration 400 nM, two-fold serial dilutions, duplicates. Top left, immobilization of biotinylated cpd 1632 (red, flow cell 2; blue, reference flow cell 1).
Based on above experiments it was suggested that cpd 1632 binds to a Lin28 binding site rather than to pre-let-7a-2. To investigate this hypothesis, purified Lin28A isolated from HeLa cells overexpressing Lin28A-myc by Dr. Alok Behera was immobilized as a ligand at high levels in order to detect small molecule binding (Fig. 72). In a first experiment, cpd 1632 was found to bind Lin28A with an affinity of 25 µM. None of the other small molecules showed any binding to Lin28A. However, this result has never been reproduced, inspite of intense efforts to restore initial conditions. Therefore, it must be assumed that the protein might have degraded at some point, and it is not clear when. New batches of protein never yielded any positive binding result. It is possible that some other molecules would have shown binding as did cpd 1632 in the very first assay but that the protein was degraded.

![Sensorgrams showing binding assays of candidate small molecules against immobilized Lin28A.](image)

**Figure 72.** Sensorgrams showing binding assays of candidate small molecules against immobilized Lin28A. Running buffer containing DMSO 2% and ZnCl₂ 10 µM, Lin28A immobilization level 1601 RU, expected signal of small molecules 16 RU. Start concentration 200 µM, two-fold serial dilutions, duplicates. Top left, immobilization of biotinylated pre-let-7a-2 in three steps (red, flow cell 2; blue, reference flow cell 1). Lin28A-myc provided by Dr. Alok Behera.

In order to circumvent the potential stability problem of Lin28A, an inverse setup was tried. Biotinylated cpd 1632 was immobilized and Lin28A-myc was used as an analyte (Fig. 73). The difficulty of this approach was the very limited availability of Lin28A and its unknown starting concentration, as determination of concentration would have required considerable amounts of the protein to be sacrificed. With the amount obtained from the overexpression and isolation procedure, an assay was run showing the beginning of a binding event. However, in order to do this experiment properly larger amounts of a recombinant Lin28A would have been required.
As a final control experiment, Lin28A was run over a pre-let-7a-2 surface (Fig. 74). Lin28A did bind pre-let-7a-2, the interaction was however difficult to investigate by SPR due to the big size of the interacting partners. Heavy mass transport limitation of kinetic fitting and failure to attain a steady-state complicated extraction of binding data. Though, the experiment nicely confirmed that the system of the two interactants was intact.

5.2.3. Conclusion and Outlook

The assumed interaction of cdp 1632 with Lin28A was hardly tractable by SPR. The above experiments indicated that most likely cdp 1632 does not bind to pre-let-7a-2 but to Lin28. Furthermore, the pre-let-7/Lin28 interaction was confirmed in the SPR setup. However, as the most interesting experiment could not be reproduced, this work did not result in a figure for the manuscript published in ACS Chemical Biology in August 2016 [341]. The interaction between cdp 1632 and Lin28A was instead shown in a pull-down assay by Dr. Alok Behera.

In contrast to this rather disappointing result, the project brought considerable advances in SPR technology to the lab. The work with small molecules requiring DMSO correction was successfully performed. Thus, assessment of small molecule-RNA interactions by SPR would be easily accessible. On the other hand, work with the zinc finger containing protein Lin28 proved to be tricky. Further progress will have to be made in future projects involving Lin28 or similar zinc finger proteins as one of the interacting partners in SPR. Innovative solutions to improve the stability of the protein in buffers suitable for SPR are desperately needed, as well as a reliable supply of protein at defined concentrations and suitable quality.
5.2.4. Experimental

SPR experiments were performed on a SPR-2 and a MASS-1 (Sierra Sensors, Hamburg, Germany). The biotinylated pre-let-7a-2 was synthesized as described above (see 5.1.4). Synthesis of small molecules and of biotinylated cpd 1632 as well as recovery of Lin28A-myc as described in [341]. Data analysis was done using Analyzer2 (Sierra Sensors, Hamburg, Germany) and Scrubber2 (BioLogic Software, Campbell, Australia).

Small molecules against pre-let-7a-2. Experiments were done on a MASS-1. Neutravidin was covalently immobilized on a high capacity amine chip (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling, injecting a fresh 0.1 mg/ml solution in acetic acid buffer pH 5.5 at 15 µl/min and 37 °C. 3'-biotinylated pre-let-7a-2 was immobilized on flow cell 2 by injecting 60 µl of a 50 nM solution. Binding assays were performed at 25 µl/min, 50 µl injections with a dissociation time of 3 min. Analyte solutions were injected at two-fold serial dilutions starting at 80 µM in PBS buffer containing TWEEN20 0.01% and DMSO 5%, 12 concentrations per analyte in duplicates.

Pre-miRNAs against cpd 1632. Experiments were done on an SPR-2. Neutravidin was covalently immobilized on an amine chip (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling, injecting a fresh 0.1 mg/ml solution in acetic acid buffer pH 6.5 at 15 µl/min and 25 °C. Biotinylated cpd 1632 was immobilized on flow cell 2 by injecting 50 µl of a 50 nM solution in a 5% DMSO-containing running buffer. Binding assays were performed in a running buffer without DMSO at 25 µl/min, 50 µl injections with a dissociation time of 3 min. Analyte solutions were injected as two-fold serial dilutions starting at 400 nM in PBS buffer containing TWEEN20 0.01%, 10 concentrations per analyte in duplicates.

Small molecules against Lin28A. Experiments were done on an SPR-2. Lin28A-myc was covalently immobilized on a high capacity amine chip (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling, injecting a fresh solution in acetic acid buffer pH 6.5 at 15 µl/min and 25 °C on flow cell 2 only. Binding assays were performed in a running buffer containing 2% DMSO at 25 µl/min, 100 µl injections with a dissociation time of 3 min. Analyte solutions were injected as two-fold serial dilutions starting at 200 µM in PBS buffer containing TWEEN20 0.01%, 10 µM ZnCl2 and 2% DMSO, 10 concentrations per analyte in duplicates.

Lin28A against cpd 1632. Experiments were done on an SPR-2. Neutravidin was covalently immobilized on an amine chip (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling, injecting a fresh 0.1 mg/ml solution in acetic acid buffer pH 6.0 at 15 µl/min and 25 °C. Biotinylated cpd 1632 was immobilized on flow cell 2 by injecting 50 µl of a 50 nM solution in a 0.25% DMSO-containing running buffer. Binding assays were performed in a PBS running buffer containing 0.25% DMSO, TWEEN20 0.01%, 25 mM glycine and 12.5 mM Tris at 25 µl/min. Analyte solutions were injected as two-fold serial dilutions, 60 µl injections with a dissociation time of 3 min, 10 concentrations per analyte in duplicates.

Lin28A against pre-let-7a-2. Experiments were done on an SPR-2. Neutravidin was covalently immobilized on an amine chip (Sierra Sensors, Hamburg, Germany) by EDC-NHS-activated coupling, injecting a fresh 0.1 mg/ml solution in acetic acid buffer pH 6.0 at 15 µl/min and 25 °C. Biotinylated pre-let-7a-2 was immobilized on flow cell 2 by injecting 110 µl of a 20 nM solution. Binding assays were performed in a PBS running buffer containing TWEEN20 0.01%. Analyte solutions were injected as two-fold serial dilutions, 100 µl injections with a dissociation time of 3 min, 10 concentrations per analyte in duplicates, starting at a 150 nM approximately.
5.3. Effect of a single stereospecific PS linkage in antimir A1-16

5.3.1. Aim of the project

Synthesis of stereoselective phosphorothioates has been a major challenge in the field of oligonucleotide chemistry. The Wada group developed a strategy for stereopure DNA and RNA synthesis using chiral oxazaphospholidine-containing nucleosides and investigated biophysical properties of stereopure phosphorothioate DNA [373-376]. They found a slightly higher RNA target binding affinity for Rp-PS-DNA and an important destabilization of Sp-PS-DNA:RNA duplexes compared to stereorandom PS-DNA. Dr. Hartmut Jahns elaborated a protocol to introduce a stereoselective bias into RNA synthesis using different activators [31]. Specifically, use of benzylthiotetrazole (BTT) yielded a higher proportion of Rp PS linkages, while tetrazole resulted in a higher fraction of Sp PS linkages. His studies showed a higher target RNA binding affinity for Rp-PS-RNA, in line with above mentioned findings of the Wada group for Rp-PS-DNA, as well as increased knock-down efficiency of Rp-biased PS-siRNAs. In a follow-up project, Dr. Meiling Li prepared stereopure 2’-MOE PS oligonucleotides using chiral bicyclic oxazaphospholidines, as did the Wada group on DNA and RNA [377]. She was thus able to synthesize fully stereopure 2’-MOE PS 12mers, as well as gapmers with stereopure 2’-MOE wings and an internal stereorandom DNA section [377]. Full Rp 12mer splice-switching oligonucleotides designed to restore ferrochelatase splicing in EPP effected slightly more efficient splice correction than their stereorandom counterparts, while Sp analogs had a considerably weaker performance. On the other hand, in mipomersen derivatives, 2’-MOE/DNA gapmers targeting ApoB mRNA, stereochemistry of the 2’-MOE wings did not affect target cleavage efficiency. Consequently, as the chemistry was in place, a little project based on miR-122 antimirs was conceived. Previous studies had shown that structure was highly decisive for RISC compatibility of a given antimir, e.g. variations in 2’-substitution at some positions had drastic effects on antimir activity. The most prominent key position being nt 12 (see 2.1.2.2), an antimir was designed with a single 2’-MOE at position 12 and a single stereospecific PS substitutions within a PO backbone to assess if PS substitution or its stereochemistry would alter antimir activity.

Antimirs with stereospecific PS substitution were synthesized by François Halloy using nucleoside monomers by Dr. Meiling Li. Biological testing was performed by Mirjam Menzi.

5.3.2. Results and Discussion

MiR-122 antimir A1-16 derivatives containing a 2’-MOE substitution at position 12 and a single Rp or Sp PS linkage were synthesized and tested in a luciferase assay (Tab. 17). Of note, all antimirs had a PO backbone with two terminal PS substitutions, therefore the test substances in any case were mixtures of 24 isomers. The goal was to show the effect of a stereospecific PS substitution at position 12.

<table>
<thead>
<tr>
<th>negCON</th>
<th>G<em>T</em>A+G<em>T</em>C+C<em>T</em>A<em>C</em>T*C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC3649</td>
<td>C*+A<em>t</em>+t<em>G</em>T<em>C</em>A<em>C</em>t*C</td>
</tr>
<tr>
<td>A1-16-12MOE</td>
<td>C<em>C+AuUGUCACUC</em>C*A</td>
</tr>
<tr>
<td>A1-16-12MOEsp</td>
<td>C<em>C+AuUGUCACUC</em>UC<em>C</em>A</td>
</tr>
<tr>
<td>A1-16-12MOErp</td>
<td>C<em>C+AuUGUCACUC</em>UC<em>C</em>A</td>
</tr>
<tr>
<td>A1-16-12MOEmix</td>
<td>C<em>C+AuUGUCACUC</em>UC<em>C</em>A</td>
</tr>
</tbody>
</table>

A single PS substitution at position 12 was shown to not affect antimir activity (Fig. 75). Whether it had a PS substitution at position 12 or not and independent of its stereochemistry, antimir activity remained unchanged.
The only slight activity drop was seen for the 10 nM dose of the Sp configuration, however, dose-by-dose comparison did not reveal any statistically significant difference between analytes.

The absence of any effect of a stereorandom PS substitution on antimir activity is not surprising. A single sulfur does not make a big difference in binding affinity to a protein complex such as RISC, since in many cases RNA-binding proteins tolerate PS backbones, or to a target RNA, where a single PS substitution would only very slightly lower melting temperature. Stereospecificity at a single substitution is a very minor change. The stereomix containing 50% of each isomer, statistically, and therefore providing half the dose of the more favorable isomer, while the less favorable isomer would still have some activity, a negligible impact would be expected to arise from introduction of a single stereospecific linkage. Taken together, the difference would be much smaller than what can actually be detected in a luciferase assay.

![Graph](image.png)

**Figure 75.** 2′-MOE antimirs with two terminal stereorandom PS linkages as well as a single stereospecific PS at position 12 in a dual-luciferase assay. Antimirs at graded concentrations (0.6 nM, 2.5 nM, 10 nM, 40 nM) were co-transfected with a dual-luciferase vector into Huh7 cells. A single target site for mature miR-122 was present in the 3′-UTR of the Renilla gene. After 48h, renilla and firefly luciferase activities were measured. Error bars, ±1 SD of biological quadruplicates. Relative luminescence, ratio renilla/firefly.

### 5.3.3. Conclusion and Outlook

A single stereospecific PS linkage at position 12 in A1-16-12MOE does not have an impact on antimir activity in a luciferase assay. PO substitution at this position as well as a stereomixed PS yield the same antimir activity than Rp or Sp isomers.

A potential application of stereospecific PS antimir synthesis could be the rational design of an antimir with an optimized backbone based on modelling studies, such that it geometrically fits into RISC in an optimal way.
5.3.4. Experimental

Antimir synthesis. 2′-MOE phosphoramidites were obtained from Thermo Fisher Scientific (Waltham, MA). Synthesis was done on an MM192 synthesizer from Bio Automation Inc. (Plano, TX). A 500 Å UnyLinker CPG from ChemGenes (Wilmington, MA) was used. 0.08 M phosphoramidite solutions in dry ACN were used and coupling times were 2 x 5 min, except for the oxazaphospholide, of which a 0.11 M solution in dry ACN was prepared and contact time was 3 x 4 min. 3% dichloroacetic acid in dry DCM was applied for 2 x 60s for detritylation, 1.4 M N-phenyl imidazolium triflate in anhydrous ACN as an activator. Oxidation was done with 0.5 M CSO in dry ACN for 2 x 5 min, sulfurization was achieved with 0.05 M 3-((N,N-dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-5-thione in pyridine:ACN 3:2 for 6 min. Capping was done in 60s using 10% acetic anhydride and 10% lutidine in THF as well as 16% N-methylimidazole in THF.

Cleavage from solid support and removal of base protecting groups was achieved with 32% ammonium hydroxide at rt overnight. Purification was done on an Agilent 1200 series preparative HPLC (Agilent Technologies, Santa Clara, CA) on a WatersXBridge OST C-18 column, 10 x 50 mm, 2.5 μm, 5 ml/min, 65 °C using a gradient of MeOH in 0.1 M TEAA buffer over 5-7 min. In a first HPLC run failed sequences were removed, then 5′-terminal DMT was cleaved in 40% AcOH for 30 min at rt, followed by a second HPLC purification step in DMT-off state. Final products were characterized by LC-MS on an Agilent 1200/6130 system (Agilent Technologies, Santa Clara, CA) on a Waters Acquity OST C-18 column, 2.1 x 50 mm, 1.7 μM, 0.3 ml/min, 65 °C using a gradient of MeOH in a 0.4 M HFIP, 15 mM TEA buffer over 10 min. Pure products were quantified by UV absorbance at 260 nm using a Nanodrop 2000 (Thermo Scientific, Wohlen, CH).

Cell culture and transfections. Huh-7 cells (ATCC, LGC, Molsheim, France) were cultured in DMEM GlutaMAX™-I (31966-021, Gibco®, Life Technologies, Carlsbad, CA) supplemented with 10% FBS. Antimir transfections were performed with Lipofectamine 2000 (12252-011, Life Technologies, Carlsbad, CA) according to manufacturer’s protocol and jetPEI (101-10, Polyplus transfections, Illkirch, France) was used for plasmid DNA.

Luciferase assays. Cells were seeded in opaque white 96-well plates (136101, Nunc, Roskilde) in 80 μl medium, 7′500 cells/well. 8h after seeding, antimirs were transfected as described above. After another 16h, reporter plasmids were transfected at 30 ng/well. Assay read-out was performed with the Dual-Glo® Luciferase Assay System (E2980, Promega, Fitchburg). Cell culture medium was removed and 30 μl of firefly luciferase substrate dissolved in luciferase buffer was added to the wells and was incubated for 10 min. Luminescence was measured on a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad). 15 μl of renilla substrate dissolved in stop&glo buffer was added to the wells and luminescence was measured after 10 min repeated incubation. Luminescence readings were for 1s per well. Renilla luminescence counts were normalized on firefly luminescence counts. Average and standard deviations were determined from three technical replicates.
5.4. Site-specific labeling of miRNA precursors: A structure–activity relationship study

Labeled pre-miRNAs are a valuable tool in miRNA research and are playing a pivotal role in the lab [341,368,378,379]. In many cases, the introduction of bulky labels however alters biologic activity of the reagents when transfected into cells (unpublished observation). In order to reduce synthetic efforts every time a labeled pre-miRNAs was needed, a protocol for the design of biologically active labeled pre-miRNAs was therefore highly desirable. The two master students Fabienne Baumann and Matteo Fischer performed a structure-activity relationship study on 4 pre-miRNAs in order to establish general trends that would provide guidance in the design of biologically active pre-miRNA probes. The project was performed in close collaboration with Dr. Ugo Pradère. It lead to the following publication:

**Site-specific labeling of microRNA precursors: A structure-activity relationship study**

Mirjam Menzi, Ugo Pradère, Yuluan Wang, Matteo Fischer, Fabienne Baumann, Martina Bigatti and Jonathan Hall

This work was published in *ChemBioChem* on September 23th 2016 [380].
5.5. New control for RNAi experiments: An siRNA carrying randomized base pairs

Short interfering RNAs (siRNAs) are commonly used for selective downregulation of target gene expression. They are ~19 nt long, double-stranded (ds) RNA with 2 nt overhangs on the 3’-termini of both strands [82]. In analogy to miRNA-mediated gene silencing, the strand with the lower thermodynamic stability at its 5’-end is preferentially loaded into the RISC as a guide strand [381]. The passenger strand is cleaved by Ago2 and expelled [382]. The guide strand (antisense strand) within RISC targets fully complementary RNA, which is cleaved opposite to the central positions by Ago2 [82]. A major complication in post-transcriptional gene silencing by siRNAs are sequence-dependent or -independent off-target effects (OTEs). Although designed to be uniquely complementary to the target mRNA, the guide strand may hybridize to some highly homologous binding sites in other mRNAs. Other sequence-dependent OTEs include immunostimulation, RISC activation of the passenger strand or silencing of mRNAs in a miRNA-like fashion. Sequence-independent OTEs may derive from saturation of the RNAi machinery or cell toxicity. OTEs are considered a major problem in RNAi experiments that can lead to unexpected phenotypes and mis-interpretation of results. A good control in RNAi experiments does not exhibit sequence-dependent effects, while sequence-independent effects are the same as for the effector siRNA.

Here, siRNAs with multiple degenerate positions (siRNDs) assembled from a pool of short RNAs containing randomized bases were shown to form stable duplexes with a \( T_m \) that most closely matched the thermal stability of a perfectly complementary siRNA homolog carrying 1-2 GC base pairs or an siRNA carrying two mismatches. In following studies, siRNDs were shown to be taken up by RISC and, depending on the positions of randomized sites, to form competent gene silencing agents. As such, siRNDs would have highly attractive properties as negative controls in RNAi.

The project was designed and carried out by Dr. Julian Zagalak. Mirjam Menzi performed thermal melting analysis with a range of siRNDs and respective controls. It lead to the following publication:

**Properties of short double-stranded RNAs carrying randomized base pairs: toward better controls for RNAi experiments**

Julian Zagalak, Mirjam Menzi, Fabian Schmich, Hartmut Jahns, Afzal M. Dogar, Florian Wullschleger, Harry Towbin and Jonathan Hall

This work was published in RNA on September 11th 2015 [383].
References


75. Walder JA, Walder RY. Nucleic acid hybridization and amplification method for detection of specific sequences in which a complementary labeled nucleic acid probe is cleaved. (Ed.^(Eds) (Google Patents, 1995)


The text contains multiple references to studies and articles, but the specific content of the document is not legible in its current state. It appears to be a page from a scientific journal or a collection of scientific papers. The text is not legible due to the quality of the image or the resolution at which it is displayed.


