Mini review

Drug Testing and Analysis

Received: 4 May 2011 Revised: 13 June 2011 Accepted: 24 June 2011 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/dta.330

RNA interference for performance enhancement and detection in doping control

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ABSTRACT: RNA interference represents a comparably new route of regulating and manipulating specific gene expression. Promising results were obtained in experimental therapies aim at the treatment of different kinds of diseases including cancer, *diabetes mellitus* or Dychenne muscular dystrophy. While studies on down-regulation efficiency are often performed by analyzing the regulated protein, the direct detection of small, interfering RNA molecules and antisense oligonucleotides is of great interest for the investigation of the metabolism and degradation and also for the detection of a putative misuse of these molecules in sports. Myostatin down-regulation was shown to result in increased performance and muscle growth and the regulation of several other proteins could be relevant for performance enhancement. This mini-review summarizes current approaches for the mass spectrometric analysis of siRNA and antisense oligonucleotides from biological matrices and the available data on biodistribution, metabolism, and half-life of relevant substances are discussed. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: doping; gene doping; siRNA; antisense oligonucleotide; miRNA; mass spectrometry; sport; clinical study

Introduction

The Prohibited List of the World Anti-Doping Agency (WADA) includes 'The transfer of nucleic acids or nucleic acid sequences' in category M3 and 'gene doping' which was specified in the 2011 list.[1] This also covers antisense oligonucleotides and small, interfering ribonucleic acid (siRNA) molecules that could be used to down-regulate a gene to achieve performance enhancement. While antisense oligonucleotides, as forerunner of siRNAs, act unspecifically by blocking the translation of mRNA to the corresponding protein in the nucleus or cytoplasma, siRNA utilizes the endogenous micro RNA (miRNA) pathway which was shown to result in higher efficiency.[2] Research on antisense nucleotides pioneered this therapeutic arena and, consequently, the drug candidates of this category have now reached more advanced phases of clinical studies; it is, however, expected that the alternative of siRNA will be the more efficient and controllable molecule option for gene knock-down. So-called antagomirs have recently been reported as a new complementary strategy that could also become relevant to doping controls. Antagomirs block the endogenous miRNA action and therefore increase the expression of certain target genes. They are still in an early research state[3] and will only be mentioned briefly in this paper. Primarily, this review discusses RNA interference in the context of performance enhancement and doping control and therefore focuses on upcoming pharmaceuticals, research on metabolism and excretion, and analytical approaches for detection.

Structure, mechanism, and modification

Antisense oligonucleotides are usually composed of single-stranded deoxyribonucleic acid (DNA) molecules with 15–20 bases,[4] but few experiments with single-stranded ribonucleic acid (RNA) molecules were also performed.[5] These molecules are designed to block the translation of mRNA by binding to the mRNA and inducing unspecific degradation of the duplex by for example, RNase H which cleaves DNA-RNA duplexes. Antisense oligonucleotides can also target pre-mRNA to induce exon skipping, i.e. the antisense oligonucleotide specifically covers exonic splicing enhancer motifs of the pre-mRNA and thus excludes certain exons from the transcript.[6]

siRNA molecules are double-stranded and most commonly 21 base pairs long. Nineteen of the 21 base pairs form an alpha helix, while the 3′ end has a two-base overhang on each side. These two bases are usually deoxy-nucleotides. The activity and efficiency of the compounds depends on the base composition.[7] Different positions are important for binding of the RISC (RNA-induced silencing complex), the attachment to the mRNA, and the cleavage of the mRNA-antisense duplex, and numerous studies were performed to probe for the impact of different bases in varying positions.[8]

Additionally, the half-life of antisense oligonucleotides and siRNA molecules in the circulation was found to be significantly increased by chemical modification of bases, which protect the molecules from nuclease digestion.[4,9,10] The first type of modification used in antisense nucleotides was the exchange of phosphate residues by phosphothioate moieties, which have been employed in siRNAs as well. In the meantime, two complementary generations of modifications were investigated, namely variation of the 2′ position of the ribose residue and several more complex modifications such as locked nucleic acids (LNA) or internally segmented siRNAs or the modification of the nucleotide bases.[11] The introduction of alterations must

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be conducted with utmost precision to preserve or even improve the efficiency of the siRNA molecule. In the context of the analysis of modified molecules, these chemical modifications add an enormous amount of variability to the structure, which complicates the detection or prediction of the composition of a pharmaceutical or illegally manufactured and distributed product. On the other hand, the prolongation of half-life is crucial for efficient detection and artificial modifications support and facilitates the identification of xenobiotics.

Transfer to cells

In addition to the improvement of the half-life and efficiency by chemical modification, the antisense oligonucleotides and siRNAs need to have an adequate formulation to allow for the aimed cell transfer. There are several different possibilities to overcome biological barriers[2,12] that can be divided into non-directed (e.g. coupling to cholesterol or polyethyleneimine, transport in liposomes or other nanoparticles) and directed transport (e.g. coupling to antibodies, receptor ligands or aptamers).[13] Depending on the purpose and use of such formulations, the analytical process is also influenced. Covalent coupling to cholesterol or for example, a receptor ligand, results in a permanent change of the drug molecule's structure and its detection will have to consider the modified analyte. In contrast, liposomes or the coupling to aptamers, which are cleaved and removed after cell entrance by the dicer enzyme, would not alter the structure of the released therapeutic agent.

Relevant molecules in research and clinical studies

Antisense oligonucleotides for blockage of translation

The list of antisense oligonucleotides and siRNA molecules in clinical studies on the clinicaltrials.gov homepage shows 51 studies for antisense oligonucleotides and 20 studies for siRNA[14]. Two products based on antisense oligonucleotide therapeutics have already been approved on the European market. Fomivirsen (Vitravene™) was available since 1998 to treat cytomegalovirus retinitis (CMV) in immunocompromised patients.[15] It consists of a 21mer oligonucleotide with phosphorothioate modification and the sequence 5′-GCGTTTGCTCTTCTTCTTGCG-3′ but was discontinued. Pegaptanib (Macugen™) is a pegylated 2′-fluor-pyrimidine und 2′-O-methyl-purine substituted 27mer (5′-[40kDPEG]-[HN-$(CH_2)_5$O]CGGArArUCAGUGAAUGCUUAUACAUCCG3′-dT, r = ribonucleotides)) to treat macular degeneration by down-regulating the vascular endothelial growth factor (VEGF) 165.[16,17]

Most of the ongoing clinical studies investigate medications for different types of cancer such as neoplasms, leukemia, skin melanoma, or lung, bladder, prostate, or breast cancer. Further studies include oligonucleotides designed to treat diabetes mellitus I and II, Crohn's disease, asthma, or Dychenne muscular dystrophy (DMD). Among these, DMD, diabetes mellitus I and asthma are conditions that might possess potential for (mis)use in sports for performance enhancement as DMD treatment compensates for muscle wasting; insulin has admittedly been used by athletes;[18–20] and the incidence of asthmatics in sports has been shown to be significantly higher than in the average population.[21] Regarding the phase I/II clinical study for the phosphorothioate oligodeoxynucleotide ASM8 against mild asthma, the idea is to counteract the allergen-induced eosinophilic response by down-regulation of receptors for eotaxin (CCR3) and the granulocyte-macrophage colony-stimulating factor (common beta chain).[22] This may not be relevant for sports drug testing in its current setting but could replace the use of, for example, beta-2-agonists or corticosteroids that were shown to have performance-enhancing properties after systemic but not inhaled application.[23] Also, if other ways of treatment are developed that counteract the bronchoconstruction, those may well play a role as doping agents.

Concerning diabetes, the clinical study includes the *ex vivo* treatment of autologous, monocyte-derived dendritic cells targeting the primary transcripts of CD40, CD80, and CD86 co-stimulatory molecules to suppress the autoimmune reaction. As for the asthma study, this application may not be relevant for doping purposes but, for example, a controlled increase in endogenously produced insulin could be of use for athletes if its elevated secretion is adequately regulated.

DMD is a muscle-wasting disease with a dramatic and fatal course. In this case, the idea of the use of antisense oligonucleotides is different from those reported above as DMD patients miss intact dystrophin due to different kinds of mutations within the gene. These mutations are supposed to be excluded by binding of an antisense oligonucleotide to the pre-mRNA which induces exon skipping. Consequently, a protein is synthesized that is missing selected sequences but exhibits greater activity than the mutated form and aims to attenuate the disease or lead to the milder form referred to as Becker muscular dystrophy. [6,24,25] This strategy was used also in a study with myostatin (a natural muscle growth inhibitor) where the down-regulation of the corresponding gene is aimed to be applied in muscle-wasting conditions such as DMD. Deficiency of myostatin or false expression due to mutation leads to considerably increased muscle growth and enhanced performance as shown in dog races.[26,27] The approach of exon-skipping that was successfully used for treating DMD is used here to produce a non-functional protein and the muscle mass of mice was significantly increased *in vivo*.[28] Furthermore, experiments with a classical antisense approach showed muscle growth in mice after injection and oral application.[5] The confirmation of the oral availability is a very important factor concerning the easy self-application as therapeutic agent in a clinical setting as well as for cheating athletes.

Antisense oligonucleotides for modification of endogenous miRNA

Another use of antisense oligonucleotides and ribo-oligonucleotides is the regulation of endogenous miRNA. miRNA molecules influence gene expression in a similar way as exogenous siRNA and blockage of miRNA function also changes the expression of certain proteins. It was shown that miRNAs have a wide range of functions and are involved in insulin secretion; heart, skeletal muscle, and brain development; and lipid metabolism.[3,29,30] The molecules used to regulate miRNA can be DNA[30] or RNA oligomers[3] with at least 19 bases as shown by Krützfeld *et al.*[29] The compounds bear modifications similar to those of antisense oligonucleotides and siRNA molecules, which results in similar analytical strategies. The mechanism of inhibiting miRNA action has not yet been fully elucidated but it was shown that the so-called 'antagomirs' are found exceptionally in the cytoplasma after injection into mice. Interestingly, the regulation of miRNA persists for many days or even weeks, which indicates some kind

of 'recycling' of the molecules. The research of this particular route of modifying gene expression is even younger than that of classical RNA interference but as it allows the up-regulation of many different proteins – wherever miRNAs are involved – this technique is at least as important for sports drug testing.

siRNA

Clinical studies involving siRNA are as yet generally less evolved than those including antisense oligonucleotides but it is expected that siRNA will be the more important agent in future applications due to its increased efficiency of silencing and the more controlled mechanism as siRNA uses an endogenous pathway.[2] Many molecules are produced for local use (e.g. intraocular administration), and are not for systemic use which circumvents stability and biodistribution issues.

Besides siRNA-based drugs allow the treatment of different eye diseases, drug candidates aiming to cure selected forms of cancer, renal failure, or delayed graft function after kidney transplantation are being developed. Substances that are relevant for doping controls could involve a great variety of molecules and pathways. For instance, the regulation of fat and glucose metabolism could be useful for various sport disciplines. Further, the suppression of inflammation or other (sport-related) health issues and disease states might possibly be corrected by RNA interference instead of conventional pain killers. Moreover, the improvement of general wound healing would be a conceivable application.

Among all of these targets for RNA interference, the myostatin gene currently possesses the greatest potential for misuse in sport due to the extensive research conducted and the advanced drug development programme. Hence, it represents an excellent example to establish detection assays that could be adopted for other upcoming compounds. As the targets are expected to be very variable in their structure and extent of modification, the method of choice should be as flexible as possible and will have to be optimized for the emerging therapeutic agent.

Biodistribution, stability, and half-life

Half-life in the circulation, excretion rates into urine, and also tissue distribution are important factors for the analysis and window of detection of the analytes and their corresponding metabolites. In Table 1, studies on the stability of nucleotide-based drug candidates considering different modifications and different matrices are summarized. The half-life of a 'naked' antisense or siRNA molecule in the circulation is very limited while different modifications significantly delay the degradation process. Additionally, different kinds of modifications and formulations for transport into cells influence the biodistribution and may therefore be varied according to the target tissue.[31] Gao *et al.*[31] (Table 1, 1) compared different modifications and their degradation and biodistribution after injection into mice. Following the administration of an unmodified siRNA, no intact remainders were detected in the circulation after 5 min. In contrast, the same molecule altered to comprise a few locked nucleic acid modifications and a covalently bound cholesterol residue reached a half-life of 30 minutes. It was shown that the modification, as well as the dose, influences biodistribution. The kidney was found to be the tissue of highest siRNA levels 30 min post-application for most drug candidates bearing derivatized nucleic acids, and the detection window for the administered compound was longest in the lung were siRNA was detectable for 24 h when linked to cholesterol or extensively modified with locked nucleic acid residues. Different kinds of nanoparticles were also tested and tissue distribution investigated. Soutschek *et al.*[32] (Table 1, 3 and 4) incubated modified siRNA molecules in plasma and found 50% of the cholesterol-linked molecule and 5% of the unconjugated siRNA after 60 min. They further described a study where the half life of a cholesterol-linked molecule was 95 min after intravenous injection to rats and 6 min for the unconjugated pendant. In terms of drug detection, a valuable study was reported by Yu *et al.*[33] (Table 1, 5). Here, a modified antisense oligonucleotide was injected to different animals and humans and the half-life in humans was 1.26 h for a 200 mg intravenous injection. A study in rats showed that 20% of the intravenously injected dose was excreted in urine after 200 h and 25% were excreted after approximately 325 h, which provides an estimate for the detection window in urine, which is obviously much longer than the short half-life in blood and could therefore be the more important matrix for doping control analysis purposes. Miyao *et al.*[34] (Table 1, 2) investigated the stability of a biotinylated deca-thymidine oligonucleotide and found 30% of the dose that was intravenously applied in mice excreted in urine after 60 min.

Zhang *et al.*[35] (Table 1, 6) tested different sample preparation steps and the possible degradation of an antisense oligonucleotide. Freeze and thaw cycles, 8 h at room temperature or 24 h in the autosampler after extraction, did not result in significant degradation. Additionally they confirmed that evaporation to dryness is a critical factor for degradation and that samples should not be completely dried during sample preparation.[35,36]

The half-life and biodistribution of RNA-based drug molecules is considerably depending on the structure, the modification, and transport vehicle such as nanoparticles. A 'naked' antisense oligonucleotide or siRNA molecule is degraded quickly and the detection within that period of time seems improbable; however, various clinically relevant modifications were shown to result in a half-life required for efficient medicinal activity, which in turn extends the window of opportunity to detect such molecules after application.

Analytical strategies

The detection of antisense oligonucleotides and siRNA by mass spectrometric techniques[37] is still in an early development stage. For clinical studies and general research on RNA interference, most commonly the target protein or mRNA molecules were analyzed. The direct analysis of the active drug molecules from biofluids has only been subject of a few studies where mass spectrometric assays were applied (Table 2).[38,39] Mass spectrometry of oligonucleotides remains a challenging task and is by far not as sensitive as peptide or protein analysis, which is most probably due to inefficient ionization of basic and acidic groups, adduct ion formation, and a wide charge distribution.[40,41] Nevertheless, different chromatographic and mass spectrometric strategies were tested and oligonucleotides and siRNA measured from blood or urine. On the chromatographic side, ion exchange chromatography seems to be the method of choice to separate different compounds and avoid cation adducts that are readily formed but disadvantageous to mass spectrometric analyses.[42] Regarding mass spectrometry, mostly negative ionization is used and high resolution/high accuracy instruments

Table 1. Stability of antisense oligonucleotides in different matrices or solvents.

Type of molecule	Structure	Matrix	Stability	Ref
1) siRNA Underlined letters indicate the position of the LNA nucleotides. Lower case indicates phosphorothioate linkage.	1) Unmodified siRNA: sense 5′-GACGUAAACGGCCACAAGUUC-3′ Antisense 5′-ACUUGUGGCCGUUUACGUCGC-3′	Blood (intravenous injection into mice, 400 μg/kg)	Very low levels after 1 min, < LOD at 5 min	[31]
	2) Internally segmented siRNA Sense: 5′-GACGUAAACG-3′ 5′-GCCACAAGUTC-3′ antisense 5′-ACUUGUGGCCGUUUACGUCGC-3′		>unmodified siRNA; < cholesterol-coupled siRNA; similar to 6)	
	3) Locked nucleic acid modified siRNA sense 5′-GACGUAAACGGCCACAAGUTC-3′ antisense 5′-ACTUGTGGCCGUUTACGTCGCU-3′		>unmodified siRNA; < cholesterol-coupled siRNA; similar to 6)	
	4) Phosphorothioate modified siRNA sense 5′-gacguAAACGGCCACaaguuC-3′ antisense 5′-ACUUGUGGCCGUUUACGUCGC-3′		>unmodified siRNA; < cholesterol-coupled siRNA; slightly less stable than locked nucleic acid modified or internally segmented siRNA	
	5) Cholesterol-coupled siRNA sense 5′-GACGUAAACGGCCACAAGUTC–Cholesterol-3′ antisense 5′-ACUUGUGGCCGUUUACGUCGC-3′		20% decline after 5 min 50% decline after 30 min	
	6) Locked nucleic acid modified siRNA sense 5′-GACGUAAACGGCCACAAGUTC-3′ antisense 5′-ACUUGUGGCCGUUUACGUCGC-3′		60-80% decline after 5 min	
2) Antisense oligonucleotide	5′-T-pR1-TTTTTTTT-pR2-T-3′ R1: biotinylation R2: methoxyethylamine	Blood/urine (intravenous injection into mice)	95% degraded within 2 min 30% of the dose excreted in urine within 60 min	[34]
3) siRNA	sense 50-GUCAUCACACUGAAUACCAA*U-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAc*a*C-3′; cholapoB-1-siRNA: sense 5′-GUCAUCACACUGAAUACCAAU*chol-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAc*a*C-3′; The lower-case letters represent 2′-O-methyl-modified nucleotides; asterisks represent phosphorothioate linkages.	Human serum, in vitro incubation (5 μM, 37 °C)	50% of cholesterol conjugated duplex 5% of unconjugated duplex after 60 min	[32]
4) siRNA	Unknown	Rat plasma (intravenous injection of 50 mg/kg)	t ½ of 95 min cholesterol conjugated t ½ of 6 min unconjugated siRNAs	[32]
5) Antisense oligonucleotide, ISIS 301012, Targeting human apolipoprotein B-100	5′-GC^{M}C^{M}TC^{M}AGTC^{M}TGC^{M}TTC^{M}GC^{M}AC^{M}C^{M}-3′ Fully thioated ^{M} = 5-methyl cytosine Underlined: 2′-O-(2-methoxyethyl) modified ribose	Human blood (comparison to mouse, rat and monkey), 200 mg *i.v.* injection	t ½α of 1,26 h t ½β of 31 days (determined following s.c. injection)	[33]
		Rat urine, 5-mg/kg *i.v.* bolus injection in rats.	20% excreted after 200 hours 25% excreted after 325 hours	
6) Antisense Oligonucleotide	5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′ Phosphorothioate backbone	Rat plasma/after extraction (15 or 150 ng/ml)	No degradation after 2 freeze/thaw cycles No degradation after 8 hours at room temperature Little degradation after 24 hours in an autosampler	[35]

Table 2. Mass spectrometric assays for detection of antisense oligonucleotides and siRNA and their metabolites

Type of molecule	Structure	Matrix analyzed	Extraction method and analysis	Sensitivity/ amounts used/ dose	Molecule analyzed	metabolites	Ref.
1) siRNA (HBV263 targeting hepatitis B virus; siRNA Therapeutics)	Sense: 5′-BGGAfCfUfUfCfUfCfUf-CAAfUfUfUfUfCfUTTB-3′ Antisense 5′-rArGrAmAmAmAfUfUm-GmAmGmAmGmAmAm-GfUfCcCmUmU-3′ F = 2′-fluoro m = 2′-O-methyl r = RNA base B = inverted abasic residue	Spiked Rat/human plasma Human/rat liver microsomes	LLE/SPE with ion pair agents; Ion-pair LC-MS	1 nmol/250 μL plasma for metabolite identification	Single strands and their metabolites	Antisense strand preferentially degraded from the 3′-end Sense strand preferentially degraded from the 3′-end	[39]
2) siRNA (model molecule)	Sense: 5′-BUGUGCACUUCGCUU-CACCUTTB-3′ Antisense 5′-AGGUGAAGCGAAGU-GCACATsT-3′ Uridine and cytosine bases: 2′-flouro groups Antisense strand: all guanine and adenine bases 2′-O-methyl groups S = phosphorothioate linkage T = DNA base thymine B = inverted abasic residue	Spiked urine Rabbit vitreous humor (intravitreal injection into female New Zealand White rabbits)	Direct injection Phase-lock light gel tubes; Ion-pair LC-MS	5 μM (concentration in urine) 15 μg in 30 μL	Duplex and single strands	Sense strand degraded faster starting at 5′-end Only antisense strand degraded	[43]
3) siRNA (model molecules complementary to myostatin mRNA)	siRNA 1: sense: 5′- GCUGGUCCAGUGGAUCUA-AdTdT −3′ Antisense: 5′- UUAUAGC-CACUGGACCAGdTdT-3′ siRNA 2: sense: 5′-CAACUUmAmGG-CAUUGAAAUAdTdT-3′ Antisense: 5′-UAUUUCAsAsUGCCUAAGUU-GdTdT-3′ Internal standard: 5′- GCAGGUCAAGUGGAUC-GADdTdT-3′	Spiked human plasma	miRNA isolation kit; cation exchange resin; flow-injection to MS	siRNA 1: LOD: 250 pmol/ml siRNA2: LOD: 1 nmol/ml	Intact single strands	n.a.	[44]
4) Antisense oligonucleotide	5′-TCGTCGTTTTGTCGTTTT-GTCGTT-3′ Phosphorothioate backbone Internal standard: 5′-TCGTGGTTAGCCGGCC-GTTCGGC-3′	Spiked rat plasma	LLE/SPE with ion pair agents; Ion-pair LC-MS	Validation in range of 5–2000 ng/ml	Intact molecule and 2 metabolites by MRM	5′N-5/3′-N-5 5′N-4/3′N-4	[35]
5) Antisense oligonucleotide, ISIS 301012, Targeting human apolipoprotein B-100	5′-$\underline{GC^{M}C^{M}TC^{M}}$AGTC^{M}TGC^{M}TTC^{M}-$\underline{GC^{M}AC^{M}C^{M}}$-3′ Fully thioated ^{M} = 5-methyl cytosine Underlined: 2′-O-(2-methoxyethyl) modified ribose	Human (and monkey) urine	two-step solid phase extraction (strong anion exchange, RP C_{18}); Ion-pair LC-MS	200/400 mg/day; 3 i.v. injections within 3 days, 3 s.c. injections within 22 days.	Oligonucleotide and metabolites	GTCTGCTTCGCACC TCTGCTTCGCACC TGCTTCGCACC GCTTCGCACC GCTTCGCACC GCCTCAGTCT GCCTCAGTC GCCTCAGT TTCGCACC GCCTCAG	[33]

are crucial for identification of intact molecules, fragment ions, and for sequence determination.[39,43,44] In Table 2, recent studies on the mass spectrometric analysis of antisense oligonucleotides and siRNA from matrices by *in vitro* incubation of molecules or after application to mouse or man are summarized. Zou *et al.*[39] (Table 2, 1) used a highly modified siRNA molecule targeting hepatitis b virus for *in vitro* studies for metabolite identification in blood and liver microsomes. Molecules were purified by liquid-liquid extraction (LLE) followed by solid-phase extraction (SPE) using buffers that allow ion exchange purification. Separation of target analytes before mass spectrometry was performed by ion-pair chromatography using triethyl amine (TEA) and hexafluoroisopropanol (HFIP). Significant differences were found between degradation in serum and human liver microsomal preparations. While degradation of the antisense strand preferentially occurred in serum, liver microsomes predominantly metabolized the siRNA molecule starting at the sense strand. It remains to be shown whether this difference is observed also after application of molecules (i.e. *in vivo* following administration). In a study by Beverly *et al.*[43] (Table 2, 2), the *in vitro* metabolism of a modified siRNA molecule and the *in vivo* degradation of the same molecule in rabbit ocular vitreous humor was investigated. The sample preparation for the *in vivo* experiments employed phase-lock light gel tubes, and spiked urine samples were directly subjected to ion-pair LC-MS after 1:1 dilution. Also here, the metabolite pattern looked different with the sense strand being degraded completely and the antisense strand being nearly fully intact after 6 days in urine but the siRNA in ocular vitreous humor being preferentially metabolized in the antisense strand. In both studies, the employed modifications prevented the molecules from fast degradation. The metabolite pattern fits well the knowledge on modifications; however, the set of nucleases is obviously different in the studied fluids and metabolites will have to be identified for each drug candidate depending on the structure and application route as well as the target tissue and the matrix analyzed. A third study on the analysis of siRNA from blood was performed by Kohler *et al.*[44] (Table 2, 3) who employed an miRNA purification kit for isolation of the siRNA molecules from human plasma. The mass spectrometric analysis was accomplished by flow-injection without prior LC separation after treatment of the analytes with a cation exchange resin. This method was validated including the use of an internal standard and a detection limit of 250 nmol-1pmol/ml of plasma was achieved for differently modified siRNA molecules. This method requires the differently modified molecules to bind to the purification kit. Single- as well as double-stranded RNA oligonucleotides were bound but, for example, oligonucleotides with high phosphorothioate modification or DNA oligonucleotides were lost during purification (data not shown).

For antisense oligonucleotides, Zhang *et al.*[35] (Table 2, 4) developed a comparably sensitive method for detection of a fully thioated antisense oligonucleotides that was validated in a range from 5–2000 ng/ml of plasma. As for the siRNA approach,[44] an internal standard was used and the analysis were done after addition of the oligonucleotide to rat plasma. By using a similar sample preparation as described above from Zou *et al.*[39] and employing multiple reaction monitoring (MRM) for analysis of the intact molecule as well as two metabolites, a method with good validation parameters was established. A study by Yu *et al.*[33] (Table 2, 5) used samples from a clinical study to elucidate the renal elimination of the drug candidate. After intravenous or subcutaneous injection of a highly modified antisense oligonucleotide urine samples were collected and analytes isolated by a two-step SPE. Ion-pair LC-MS was applied and the intact molecule as well as 3′- and 5′- degraded metabolites were detected giving a first idea of the metabolism of such molecules and their traceability in urine.

Further improvements and optimization of these promising approaches and more detailed analysis of the metabolism will provide powerful tools for efficient detection of siRNAs as well as antisense oligonucleotides in blood or urine. Furthermore, a general improvement of sensitivity would increase the power of the methods tremendously although the sensitivities of the developed assays are sufficient for amounts currently used in clinical studies.

Other agents based on short oligonucleotides

Alternatively to transferring a directly interfering molecule into an organism as described above, short hairpin RNA (shRNA) can be used for similar purposes and is transferred as a shRNA expression cassette in viral vectors. The detection of this kind of RNA interference is more commonly used in the field of molecular biology (also regarding possible detection strategies) and is therefore not described in more detail.[2]

Conclusion

While research on the interference of endogenous miRNA is still in a basic research state, siRNA is used in clinical studies and few antisense oligonucleotides are found on the pharmaceutical market for topical use. As siRNA was shown to be more efficient than antisense oligonucleotides, siRNA molecules might replace the first generation RNA interference drugs in clinical studies and approved therapeutics in the future. RNA interference is highly relevant for doping controls, and antisense oligonucleotides inhibiting the expression of myostatin were already shown to be efficient for muscle growth and performance. Analytical strategies are being developed and promising milestones were accomplished. Additionally, the knowledge on degradation and biodistribution is growing constantly and will help to optimize methods for the mass spectrometric detection of therapeutics aiming RNA interference.

Acknowledgments

The presented work was conducted with support of the Federal Ministry of the Interior of the Federal Republic of Germany, Antidoping Switzerland (Berne, Switzerland), and the Manfred-Donike-Institute for Doping Analysis (Cologne, Germany).

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