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Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides☆

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ABSTRACT

Pharmacokinetic properties of oligonucleotides are largely driven by chemistry of the backbone and thus are sequence independent within a chemical class. Tissue bioavailability (% of administered dose) is assisted by plasma protein binding that limits glomerular filtration and ultimate urinary excretion of oligonucleotides. The substitution of one non-bridging oxygen with the more hydrophobic sulfur atom (phosphorothioate) increases both plasma stability and plasma protein binding and thus, ultimately, tissue bioavailability. Additional modifications of the sugar at the 2′ position, increase RNA binding affinity and significantly increase potency, tissue half-life and prolong RNA inhibitory activity. Oligonucleotides modified in this manner consistently exhibit the highest tissue bioavailability (>90%). Systemic biodistribution is broad, and organs typically with highest concentrations are liver and kidney followed by bone marrow, adipocytes, and lymph nodes. Cell uptake is predominantly mediated by endocytosis. Both size and charge for most oligonucleotides prevents distribution across the blood brain barrier. However, modified single-strand oligonucleotides administered by intrathecal injection into the CSF distribute broadly in the CNS. The majority of intracellular oligonucleotide distribution following systemic or local administration occurs rapidly in just a few hours following administration and is facilitated by rapid endocytotic uptake mechanisms. Further understanding of the intracellular trafficking of oligonucleotides may provide further enhancements in design and ultimate potency of antisense oligonucleotides in the future.

Contents

1. Introduction

There has been significant growth and maturity in the number and type of oligonucleotide therapeutics being studied in clinical trials today. The pharmacokinetics and biodistribution of oligonucleotides has been predominantly published for antisense single-stranded DNA/RNA oligonucleotides (ASOs) and, to a lesser extent, double-stranded siRNA. ASOs are synthetic DNA/RNA like oligonucleotides, typically comprised of 16–21 nucleotides, which bind to RNA through sequence-specific Watson-Crick base pair interactions [1] and are highly water soluble (>50 mM), stable for years under refrigeration and administered in simple saline solutions. Two general chemical classes of ASOs are commonly used today: (1) single-stranded ASO that modulate RNA function by several mechanisms including degradation of the target RNA by the enzyme, RNase H, or modulate RNA intermediate

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metabolism such as splicing and (2) double-stranded synthetic oligonucleotides that work through an RNA-induced silencing complex (RISC) to promote degradation of the target RNA [2]. Importantly, ASOs target RNA in the nucleus (preRNA, mRNA, non-coding RNA, toxic nuclear localized RNA, etc.) and/or cytoplasm and thus must cross a biological membrane to exert the desired pharmacology. Once bound to the target RNA, ASOs or the antisense strand of the siRNA duplex can affect the metabolism of the target RNA by a number of mechanisms, including degradation via the recruitment of endogenous RNase H1 recognition of the RNA/DNA duplex, or in the case of siRNA the RISC complex [3,4]. ASOs have been developed in numerous chemical classes, most of which contain phosphorothioate backbones plus one or more 2′-ribose sugar modifications (2′-MOE, cEt, LNA, and 2′-OMe) or with sugar-phosphate modifications (e.g., morpholino and PNA).

For the purpose of this review, the primary publications available and used for summarizing the pharmacokinetic properties are for single-stranded phosphorothioate modified chimeric (DNA/2′-modified) ASOs (Fig. 1). Where possible, published data from other classes of compounds will be compared or contrasted with this class. Oligonucleotides are distinguished from other classes of pharmaceutical agents by both the mechanism of action and physical–chemical or pharmaceutical properties. These distinguishing features provide an opportunity to pursue a large spectrum of first-in-class or best-in-class therapeutic agents for molecular targets that are unreachable by other classes of agents, e.g., small molecules and antibodies, which predominantly target proteins.

2. Pharmacokinetic properties of antisense oligonucleotides

The primary route of administration for oligonucleotides for systemic applications is by parenteral injection, either intravenous (IV) infusion or subcutaneous (SC) injection. Following systemic administration, phosphorothioate-modified single-stranded ASOs rapidly transfer from blood into tissues (minutes to hours). Pharmacokinetic properties of ASOs are similar across species and gender (5–9). Rapid transmission into cells is predominantly facilitated by endocytotic uptake. Once intracellular, ASOs exhibit long half-lives (2–4 weeks) and prolonged activity in suppressing or altering expression of their target RNA.

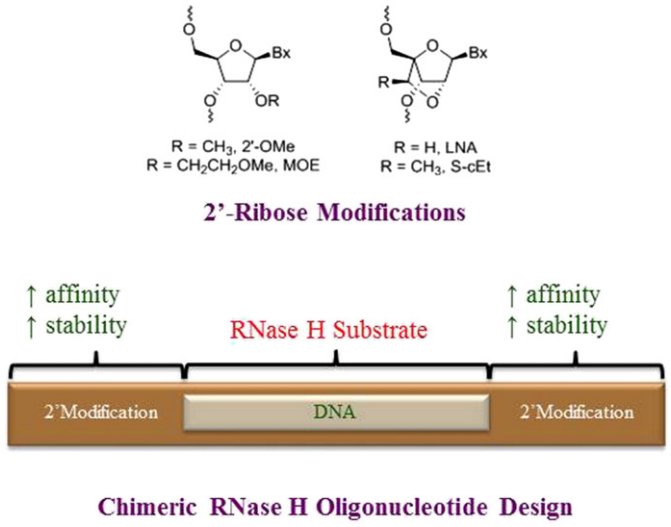

Fig. 1. Chemistry and structure of 2′-modified chimeric rnaseh oligonucleotides. The chimeric RNaseH oligonucleotide is generally designed with a phosphorothioate backbone and two regions of 2′-modified ribose residues on either end of the molecule which flank a central DNA region (gap) that supports RNase H activity upon hybridization to a complementary RNA. This design provides enhanced stability and potency, favorably attenuates non-hybridization-based toxicities, while supporting sequence-specific reduction of the target mRNA. 2′-OMe denotes 2′-O-methyl; MOE, 2′-O-methoxy ethyl; LNA, locked nucleic acid; S-cEt, (*S*)-constrained-2′-O-ethyl.

2.1. Absorption and distribution following IV/SC administration

Following SC administration, ASOs are rapidly absorbed from the injection site into the circulation with peak plasma concentrations consistently reached within 3 to 4 h [5–9]. Nearly complete absolute bioavailability has been observed for multiple ASOs after SC administration in monkeys [5,6]. Following either IV or SC administration, plasma concentrations rapidly decline from peak concentrations in a multi-exponential fashion—characterized by a dominant initial rapid distribution phase wherein drug transfers from circulation to tissues in minutes or a few hours, followed by a much slower terminal elimination phase (half-life of up to several weeks). The apparent terminal elimination rate observed in plasma is consistent with the slow elimination of ASOs from tissues, indicating equilibrium between post-distribution phase plasma concentrations and tissue concentrations (Fig. 2) [6]. The partition ratios between liver and post-distribution plasma concentrations are similar across animal species (approximately 5000:1 for 2′-MOE modified ASOs), and therefore post-distribution plasma concentrations are also expected to provide a surrogate for tissue exposure in humans [6].

ASOs that contain a phosphorothioate backbone are extensively bound to plasma proteins (≥85%) across all species [5,9], with albumin being the protein that appears to bind the greatest amount of the drug in most species including man. Protein binding to albumin is of relatively low affinity thus prevents loss of drug to renal filtration and yet still facilitates uptake in tissues (K_d approximately 150 μM) [5]. Plasma protein binding is not saturated at clinically relevant doses due to the large capacity of binding to proteins in plasma.

In contrast, oligonucleotides that lack charge or are less extensively or more weakly bound to plasma proteins (peptide nucleic acids (PNAs), morpholinos, and unmodified and unformulated siRNA) exhibit more rapid clearance from blood primarily due to either metabolism in blood or excretion in urine [10–12]. These compounds and their metabolites are readily filtered and excreted, resulting in much lower or negligible tissue uptake. For these reasons, the kidney (the organ with highest concentrations of these oligonucleotides) is the primary organ of distribution but with still very low concentrations [13]. Thus, appropriate and balanced plasma protein binding is required for broad and significant delivery to tissues and cells systemically. Either too tightly bound or not bound enough result in poor distribution properties.

For all animal species evaluated, ASOs distribute broadly into most tissues with the exception of the central nervous system after systemic administration, as is the case with all second-generation ASOs (Fig. 3) [5,15]. The major systemic tissues of distribution include liver, kidney, bone marrow, adipocytes (cell body but not lipid fraction), and lymph nodes [5–7,14–18].

2.2. PK/PD: improved potency of ASOs drives antisense activity beyond the liver and kidney

The broad distribution of antisense drugs can be exploited to provide activity in numerous tissue targets outside of liver and kidney. It has been well understood that where the antisense oligonucleotide accumulates in highest concentrations (liver and kidney), good antisense activity is routinely observed [13–17]. Nevertheless, antisense activity has been shown in all tissues of distribution for antisense molecules (Table 1) [18]. For example, while much less ASO is distributed to adipose tissue, with potent second-generation chemistry, antisense activity can be quite robust [19–21].

3. This property has been exploited for the development of cancer therapies based upon chemical modifications that increase binding affinity and thus potency of the antisense drug [22–24]

More recently, it has been demonstrated that antisense drugs can efficiently target muscle tissues, which has served as the basis for developing therapies for muscular dystrophies such as Duchenne muscular

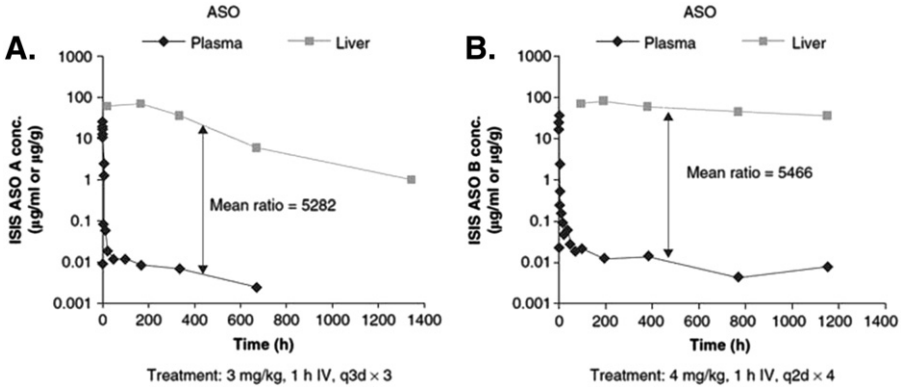

Fig. 2. Two different sequences in the same 2′-MOE chimeric chemical class exhibit different liver and terminal plasma half-lives but similar liver: plasma concentration ratios over time, consistent with an equilibrium established between tissue and post-distributional plasma drug levels.

dystrophy and myotonic dystrophy [25–27]. Important in this endeavor is identification of potent molecules, effective at nM or low μM concentrations, with broad and optimal biodistribution properties. One can speculate that the reasons we have seen less than robust pharmacology in muscle targeted ASOs can be traced to poor tissue bioavailability coupled with insufficient potency of the constructs utilized [21,28]. Recently, animal studies have demonstrated that potent, well-distributed ASOs can produce robust and durable pharmacology (as measured by specific and nearly complete knockdown of RNA levels) in muscle [29,30]. Clinical confirmation of these observations is needed to confirm therapeutic application.

4. Biodistribution following intrathecal administration

Antisense drugs do not cross the intact blood-brain barrier. However, delivery of the antisense drugs in the cerebral spinal fluid (CSF) surrounding the spinal cord and brain results in broad distribution into spinal cord and brain tissue [31–34]. These preclinical findings have now been translated to the clinic. Investigators first demonstrated safe and well-tolerated intrathecal administration of a 2′MOE modified second-generation ASO in SOD1 familial ALS patients [35]. The second-generation 2′-MOE ASO targeting SOD1 was administered by intrathecal infusion over a 12-h period. Maximum concentrations were measured in CSF and plasma at the end of the infusion. CSF concentrations were well-predicted directly from preclinical animal PK data. The exposure of the drug in blood after direct administration into CSF was multiple orders of magnitude lower than exposures observed with direct administration by SC or IV administration reflective of the lower total dose administered and the partial transfer from the CNS to the systemic circulation [36,37].

Preclinical models have been used to further assess direct administration of modified ASOs into cerebrospinal fluid (CSF). It has been demonstrated that bolus injection into the CSF results in better distribution in the CNS than slow infusion [34]. This paper reports broad distribution throughout the central nervous system, including all concentrations of the spinal cord and brain structures in mice and nonhuman primates. Active uptake into neurons broadly, including motor neurons with robust antisense activity, has been demonstrated. Distribution kinetics from the CSF into CNS tissues is rapid with a distribution half-life of less than 1 h that is likely due to the combination of uptake into CNS tissues and transfer to systemic circulation. Similar to plasma following systemic dosing, the CSF concentrations of drug exhibit multiphasic kinetics with a long terminal half-life at very low concentrations in CSF that appear to be in equilibrium with central nervous system tissues (Fig. 4). Once again, there appears to be uptake of oligonucleotides broadly in multiple cell types in the CNS. Combined with a good clinical safety profile observed to date, the likely application in neurodegenerative disease alone are broad and has the opportunity to address directly disease causation in patients that have extremely high unmet need [34,35,38].

5. Mechanism of cellular uptake of ASOs

Understanding how ASOs traffic into and within cells has the potential to improve ASO design and ultimately lead to more potent ASO drugs. In vivo experiments in mice [39] demonstrated that slowly

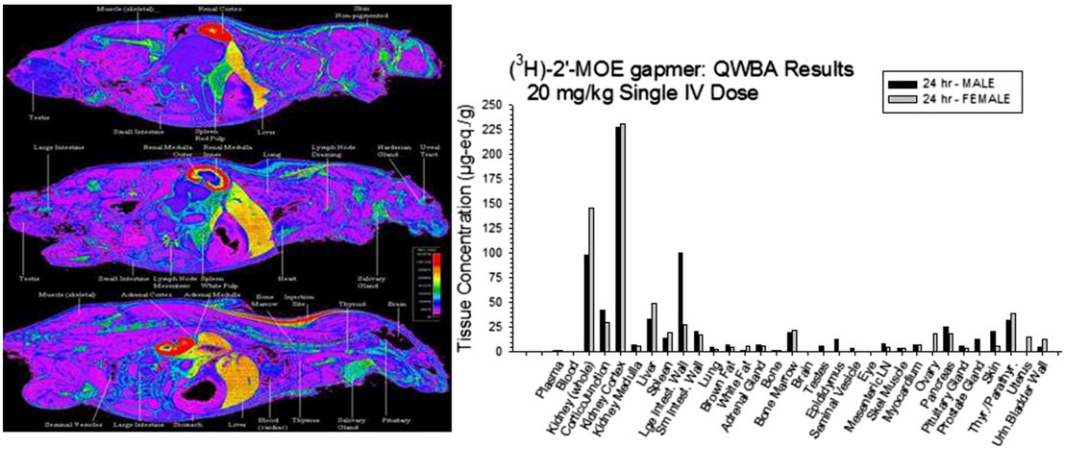

Fig. 3. ASOs administered systemically distribute broadly to various tissues as demonstrated by quantitative whole body autoradiography (left) and tissue distribution (right) at 48 h after a single subcutaneous injection of a [^{35}S]-labeled 2′-MOE modified chimeric antisense oligonucleotide at a target dose of 25 mg/kg to a male rat.

Table 1
MALAT1 mRNA knock down (% control) in multiple tissues and organ systems (mouse) following SC administration of a MALAT1 2′ gapmer cEt (Gen 2.5) antisense oligonucleotide–quantitative RT-PCR.

Tissues	Target K_d (%)
Liver	−95
Kidney	−97
Adipose	−75
Heart	−65
Mes LN	−61
Diaphragm	−50
Brain	0
Duodenum	−64
Stomach	−55
Lung	−86
Adrenal	−90
Gall bladder	−95
Bladder	−51
Prostate	−79

infused drug systemically in vivo resulted in substantially greater uptake in the liver compared with bolus administration, implicating a saturable uptake process. The lower plasma ASO concentrations presented to the liver over a longer period of time (via infusion) allowed a larger amount to be taken into cells. However, there was little to no ASO activity associated with the higher concentrations in liver suggesting that a nonproductive uptake pathways exists in cells that endocytose ASOs presented at slow and low concentrations [39]. Thus, better productive uptake in vivo (liver in this case) was facilitated by bolus injection of ASOs that we speculate may overcome the saturable nonproductive pathway. Competition of the nonproductive pathways with nonsense ASOs resulted in less bulk uptake in liver with paradoxically greater antisense activity as measured by target RNA silencing [39] with lower total exposure. In composite, this work suggests at least two pathways of uptake in cells in vitro and in vivo, one leading to productive antisense activity and the other resulting sequestration (nonproductive uptake) of the ASO (Fig. 5). Understanding early endosome uptake and sorting pathways may unlock the mechanism of these observations. Further, intracellular protein binding may be associated with localization or trafficking of phosphorothioate ASOs [40].

Ex vivo primary hepatocytes readily and productively take up ASOs and exhibit robust RNA inhibition in culture without transfection but rapidly lose this ability likely due to loss of transporter expression. To better understand the mechanism of transfection-free uptake into

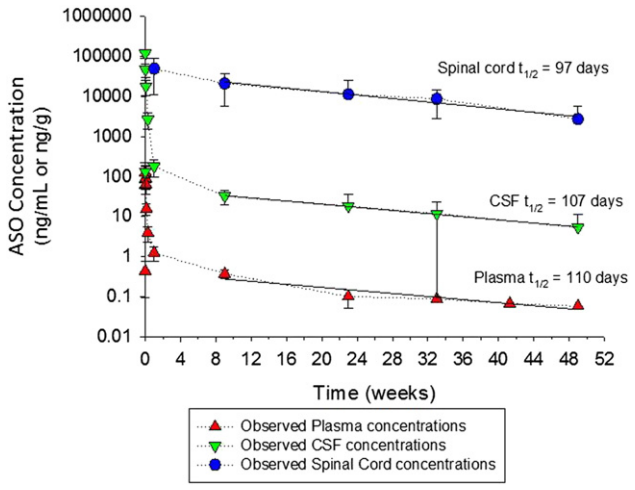

Fig. 4. ASOs with 2′-MOE modifications administered intrathecally by lumbar puncture bolus injection to the CSF of monkeys distribute rapidly to CNS spinal cord tissues with subsequent transfer to systemic circulation and exhibit long half-lives of multiple months in CSF, plasma, and CNS tissues requiring infrequent dose administration.

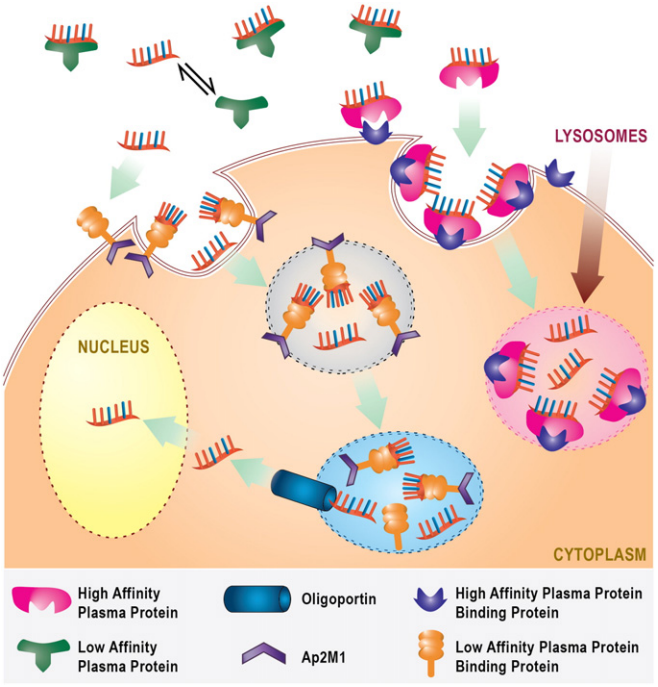

Fig. 5. Diagram depicts the theoretical dual cell uptake mechanisms for antisense oligonucleotides that result in productive and nonproductive intracellular distribution (figure adapted from reference [41]).

cells [41], we identified a stable mouse hepatocyte cell line (MHT) that take up single-stranded ASOs productively by free uptake in simple aqueous buffer solutions without transfection agents [41]. The study of ASO uptake in these cells implicates endocytosis as the dominant uptake mechanism requiring binding to surface proteins. Further, the importance of DNA in the productive uptake of oligonucleotides was confirmed, where both the base and the deoxy sugar were required for optimal uptake. Nanomolar concentrations of ASOs taken up by endocytosis were shown to reduce target RNAs within hours with maximal knockdown apparent by 24 h, which are similar to in vivo observations. Microscopy imaging demonstrated that the bulk ASO found in the cells is immediately and predominately associated with intracellular vesicular structures consistent with lysosomes.

Additional work in the MHT cells and in vivo has identified AP2M1 as an important clathrin-associated adaptor protein for active or productive free uptake of ASOs [41]. Paradoxically, the knockdown of clathrin did not alter productive uptake of ASOs, thus suggesting the active pathway is an as-yet uncharacterized clathrin-independent but AP2M1-dependent pathway. As we better understand the pathways that result in efficient delivery to intracellular active compartments and perhaps even more clearly understand the vesicular processing pathways in the cell, we have an opportunity to better design ASOs to highest potential potency. Further work to understand endosomal vesicular sorting and ASO trafficking is ongoing.

5.1. Optimizing cellular biodistribution through receptor targeting

Recent progress in identification and utilization of ligand conjugation strategies confirm that potency can be substantially improved through more efficient uptake in the target cell. Triantennary GalNAc conjugates of siRNA or single-stranded antisense molecules have greatly enhanced the targeted distribution of these molecules to hepatocytes and resulted in significant improvements in potency [42,43]. In general, liver (and specifically hepatocyte) distribution and activity is enhanced approximately 10-fold with concomitant reductions in distribution to other organs and cell types (Fig. 6). Increased potency of these molecules, as manifested by reduction of target mRNA at much lower doses

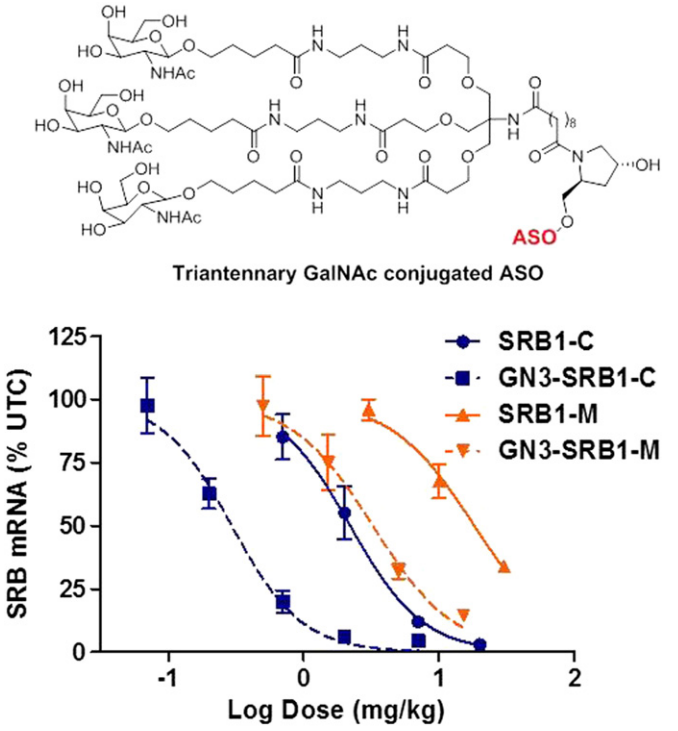

Fig. 6. GalNAc conjugation to single-stranded chimeric ASOs produces orders of magnitude greater potency in vivo (mouse) for reducing hepatocyte mRNA targets (example for SRB1 mRNA) with up to a 100-fold decrease in the mg/kg dose of ASO needed to effect a 50% reduction in mRNA compared to SRB1-C (2′MOE ASO). SRB1-C-1′MOE chimeric ASO; SRB1-M-cEt chimeric ASO; GN3-GalNAc conjugate.

in mice, is directly correlated with the increase in exposure to the hepatocyte. While this specific ligand appears limited to hepatocyte targeting, it nevertheless points the way to potential optimization for other cell types through cell-specific endocytotic pathways.

6. Conclusion

Pharmacokinetic properties and biodistribution of oligonucleotides are largely driven by chemistry of the backbone and thus are sequence independent within a chemical class. In particular, chemical modifications in sugar chemistry (2′MOE, 2′OMe, LNA or cEt) or base chemistry have much less effect on the pharmacokinetic properties than does the overall charge at the phosphate bridge in the backbone (PS vs PO vs morpholino). Tissue bioavailability (% of administered dose) is assisted by plasma protein binding that limits glomerular filtration and ultimate urinary excretion of oligonucleotides. The substitution of one non-bridging oxygen with sulfur (phosphorothioate) increases both plasma stability and plasma protein binding. Additional modifications of sugars at the 2′ position increase RNA binding affinity and significantly increase tissue half-life and prolong RNA inhibitory activity and ultimately can modulate protein binding. Oligonucleotides modified in this manner consistently exhibit the highest tissue bioavailability (>90%). Plasma protein binding of 2′-MOE and PS modified oligonucleotides has been shown to be of relatively low affinity (μM), prevents renal filtration, while enhancing distribution to tissues and into cells. Systemic biodistribution is broad, and organs typically with highest concentrations are clearance organs, liver, and kidney followed by bone marrow, adipocytes, and lymph nodes. Both size and charge for most oligonucleotides prevents distribution across the blood brain barrier. However, modified single-strand oligonucleotides administered by intrathecal injection into the CSF distribute broadly in the CNS. The great majority of intracellular oligonucleotide distribution systemically or in the CNS occurs rapidly in just a few hours following administration and is facilitated by rapid endocytotic uptake mechanisms. Both systemically and within the CNS tissues of distribution, cells differentially take up oligonucleotides. The conjugation of triantennary GalNAc ligands that direct ASOs or siRNA to cell-specific endocytotic receptors on hepatocytes has been shown to alter distribution and enhance potency for targets in hepatocytes. Further understanding of vesicular sorting and the intracellular trafficking of oligonucleotides may provide substantial enhancements in design and ultimate potency of antisense oligonucleotides in the future.

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