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Turning mirror-image oligonucleotides into drugs: the evolution of Spiegelmer® therapeutics☆

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Spiegelmers are synthetic target-binding oligonucleotides built from non-natural L-nucleotides. Like aptamers, Spiegelmers fold into distinct shapes that bind the targets with high affinity and selectivity. Furthermore, the mirror-image configuration confers plasma stability and immunological passivity. Various Spiegelmers against pharmacologically attractive targets were shown to be efficacious in animal models. Three Spiegelmer candidates: emapticap pegol (NOX-E36; anti-CCL2), olaptesed pegol (NOX-A12; anti-CXCL12) and lexaptepid pegol (NOX-H94; anti-hepcidin), underwent regulatory safety studies, demonstrated good safety profiles in healthy volunteers and were taken into Phase IIa studies in patients. Proof-of-concept for emapticap pegol has recently been demonstrated in diabetic nephropathy patients. Furthermore, promising interim Phase IIa data of olaptesed pegol and lexapteptid pegol also suggest efficacy in the respective patient populations.

Introduction

Single-stranded nucleic acids can adopt 3D structures determined by their nucleotide sequence. This is comparable to proteins where the 3D structure is dependent on their amino acid sequence. Employing *in vitro* selection techniques, functional oligonucleotide sequences with target-binding or even enzymatic activity can be identified from combinatorial oligonucleotide libraries [1–3] and the oligonucleotides are referred to as aptamers or ribozymes, respectively. The screening technology that is employed to identify aptamers has been described as systematic evolution of ligands by exponential enrichment (SELEX). Within the SELEX process, oligonucleotide libraries consisting of random sequence regions and fixed-flanking primer-binding sites are subjected to consecutive rounds of selection and amplification. During the selection step, target-binding oligonucleotides are partitioned from non-binding sequences and the binding sequences are isolated and then enzymatically amplified (e.g. by polymerase chain reaction). Structural motifs commonly found in aptamers comprise not only classical helices with Watson–Crick base pairing but also internal bulges, hairpin loops, G-quartets and pseudo-knots.

Numerous aptamers have been identified against targets as small as nickel (58.7 Da) [4], as large as complement C5 (190 kDa) [5] and even surface molecules on whole cells [6]. Aptamers have been shown to be useful as a chemical alternative to antibodies in a number of applications. These include diagnostics that employ ELISA-type oligonucleotide ligand assays (ELONAs or OLAs) or biosensors, purification processes with aptamer-loaded affinity resins, delivery vehicles for therapeutic siRNAs and therapeutics that can disrupt protein–protein interactions [7].

Chemical modification of aptamers

If RNA- or DNA-based aptamers are intended to be used in a biological environment, and particularly for therapeutic use, they have to be stabilized against cleavage by endo- and exo-nucleases. Because these nucleases are abundant in biological fluids, several modifications of nucleotides were introduced that protect the vulnerable 2′-position of the ribose-phosphate backbone against endonucleases (2′-fluoro, 2′-amino, 2′-*O*-methyl) and the terminal nucleotides against exonucleases. Such modifications have led to increased plasma stability *in vitro* [8]. However, the aptamers usually cannot be completely modified and thereby protected without loss of binding affinity, therefore some degree of biological instability remains.

☆ Spiegelmer is a registered trademark of NOXXON Pharma AG.

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Besides the modifications mentioned above, aptamers that are to be used therapeutically are commonly conjugated to a high molecular weight polyethylene glycol (PEG) moiety (40 kDa), this increases their residence time in the bloodstream by decreasing their elimination from plasma by glomerular filtration [8]. In contrast to the typical random PEGylation of most antibody fragments and several therapeutic proteins, PEG can be conjugated site-specifically to oligonucleotides and aptamers. This is achieved by introducing a linker with an appropriate functional group at the desired position during solid-phase synthesis. As a result, the selective PEGylation of aptamers typically preserves the desired activity in contrast to many proteins that show decreased activity owing to a random reaction at many different sites.

Conventional aptamers in clinical development

The use of aptamers for therapeutic applications became possible when ways of stabilizing nucleic acids against nucleolytic attack in biological fluids were successfully implemented [9]. In 2004, anti-vascular endothelial growth factor (VEGF)-A_{165}-aptamer pegaptanib sodium (Macugen®, Pfizer) for the treatment of the neovascular form of age-related macular degeneration (AMD) became the first, and to-date only, aptamer drug to gain FDA approval [10]. Although Macugen® required injections into the eyeball every six weeks, it was the first treatment for AMD that could effectively slow down progression. It has now been superseded in the market by the broader-acting VEGF-A-targeting therapeutics bevacizumab (Avastin®, Chugai/Genentech/Roche: antibody), ranibizumab (Lucentis®, Genentech/Novartis: Fab fragment) and aflibercept (Eylea®, Bayer/Regeneron: VEGF-trap), which also require intravitreal injections [11].

The original anti-VEGF-A aptamer (t44-OMe) is stabilized by 2′-deoxy-2′-fluoro-pyrimidine and by changing all but two purine nucleotides into 2′-*O*-methylpurines [12]. Additionally, pegaptanib (NX1838, EYE001) carries a 40 kDa Y-shaped (branched) PEG to improve pharmacokinetic (PK) properties further and an inverted dT at its 3′ end as a shield against 3′-exonucleases [13].

Ophthotech is developing E10030, an anti-platelet-derived growth factor (PDGF)-BB aptamer (Fovista®) also known as ARC127 that is currently in Phase III clinical studies for the treatment of neovascular (wet) AMD in combination with ranibizumab (clinicaltrials.gov: NCT01940900). The sequence, a 36mer published in 1996 [14], originated from a DNA library and was only modified with a 3′-3′-linked thymidine nucleotide to reduce exonuclease attacks. Further truncation and post-SELEX modifications (2′-deoxy-2′-fluoro-pyrimidines, 2′-*O*-methyl-purines, internal hexyl-linkers, all wherever possible) to improve nuclease resistance resulted in a 32mer that was later named ARC126 and ARC127 when PEGylated at the 5′ terminus [15]. E10030 is administered by intravitreal injection.

A second therapeutic candidate in Ophthotech's clinical pipeline is the C5-binding aptamer ARC-1905 (Zimura®). The 38mer aptamer sequence was originally identified from a 2′-deoxy-2′-fluoro-pyrimidine-modified library and all but three purines could be exchanged for 2′-*O*-methyl purine nucleotides [5]. For *in vivo* applications, an inverted thymidine and a 40 kDa PEG moiety were added to the 3′ and 5′ end, respectively [16]. ARC1905 has completed Phase I/IIa studies in combination with anti-VEGF treatments.

The only aptamer destined for systemic use and currently active in clinical development is pegnivacogin (RB006, Regado Biosciences), a coagulation factor-IXa-neutralizing aptamer. It was originally identified as a 34-nucleotide-long sequence with 2′-deoxy-2′-fluoro-modified pyrimidines and an inverted T as 3′ cap [17]. For further development it was trimmed to 31 nucleotides, all but one purine was exchanged to the corresponding 2′-*O*-methyl-nucleotides, 2′-deoxy-2′-fluoro-pyrimidines were exchanged for the respective 2′-*O*-methyl nucleotides where possible and a 40 kDa PEG moiety was conjugated to the 5′ end [18]. It is being co-developed with its antidote, anivamersen (RB007), an all-2′-methoxy 15-nucleotide-long reverse complementary oligonucleotide that inactivates the target-binding property of the aptamer by unfolding its active structure and forcing it into a double-helix with the antidote. The two-component anticoagulation system pegnivacogin/anivamersen (Revolixys® kit) has been undergoing Phase III studies in the setting of percutaneous coronary intervention (where it is administered intravenously) and Phase I studies for venous thrombosis (where it is administered via the subcutaneous route).

Mirror-image (L-)aptamers are plasma stable

An alternative way of making therapeutic aptamers is the use of mirror-image RNA or DNA that is not recognized by the ubiquitous plasma nucleases [19]. In a mirror-image aptamer, also known as a Spiegelmer,[†] all chirality centers that are only located in the (deoxy)ribose sugars of the nucleotide, are mirror-inverted [20]. These L-(deoxy)ribose-based nucleotides do not occur in nature and are perfect mirror-images of the corresponding, natural D-nucleotides. By analogy, L-aptamers (Spiegelmers), which are chains of L-nucleotides, are perfect mirror images of their D-aptamer counterparts of the same sequence. Thus, in the only available crystal structure of an L-oligonucleotide, a left-turning helix instead of the typical right-turn helix is observed [21]. Because naturally occurring enzymes are stereoselective, Spiegelmers – unlike aptamers – enjoy a native biostability. As mentioned above, also aptamers can be stabilized against nucleolytic attacks; however, the stabilization process can be cumbersome because vulnerable nucleotides need to be exchanged for modified nucleotides. This is usually not possible for all positions because the overall (target-)binding conformation needs to be maintained.

Generation of Spiegelmers

Ideally, Spiegelmers would be directly selected to bind to a target of choice. However, such a method would require enzymes that recognize mirror-image (oligo)nucleotides and catalyze the respective amplification processes such as PCR, and for RNA also transcription/reverse transcription. Additionally, sequencing of the enriched library after the final round of selection currently depends on cloning in bacteria (*Escherichia coli*) and further polymerase chain reactions, methods that would be difficult to adapt for mirror-image libraries. Therefore, the Spiegelmer identification process relies on the generation of conventional, D-configured aptamers against a synthetic selection target that is the mirror-image of the biological

[†] Derived from the German word Spiegel: mirror, and the Greek word meros: part, which is also used in the words aptamer and polymer.

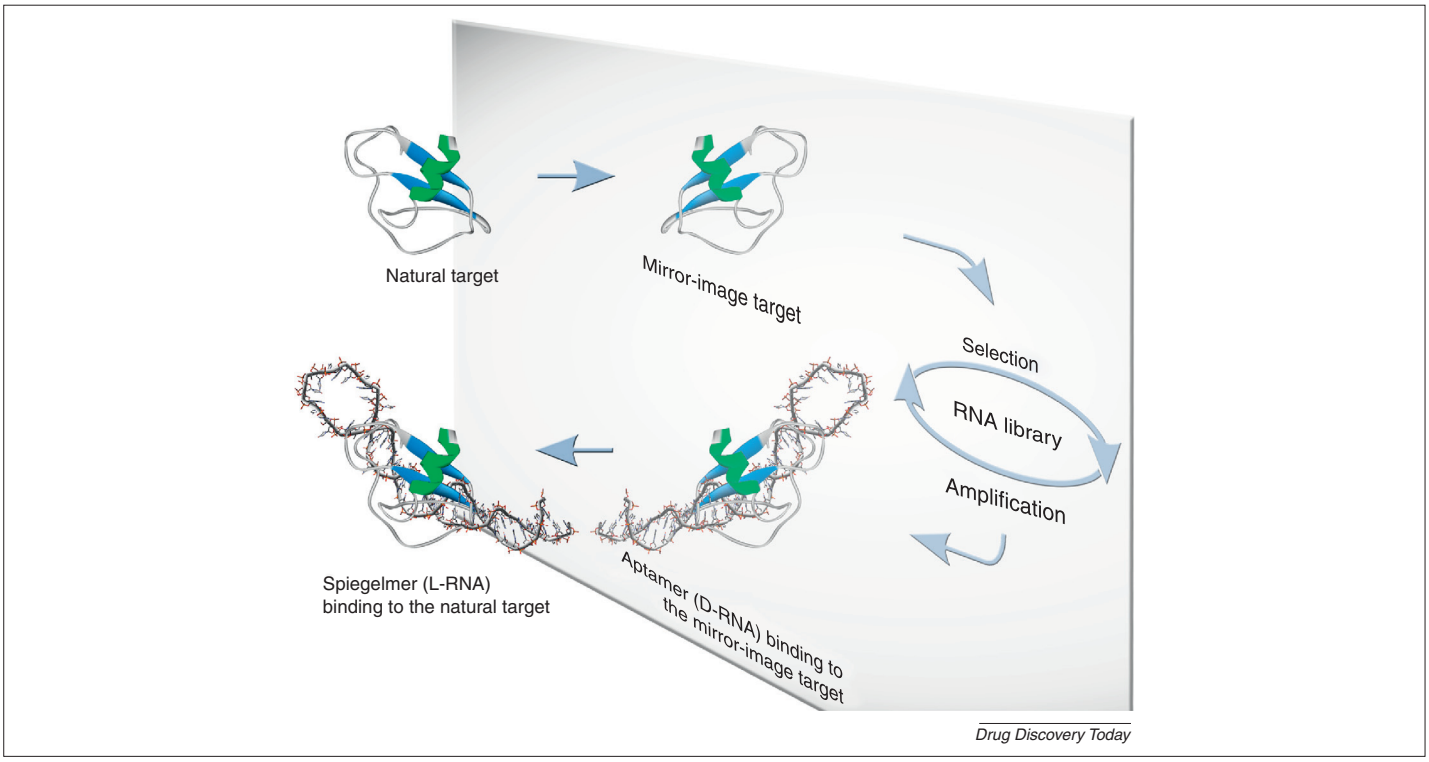

FIGURE 1

Illustration showing the generation of an RNA-Spiegelmer against the chemokine CXCL12. As a first step, a mirror image of the natural target is synthesized. RNA oligonucleotides (aptamers) binding to the mirror-image selection target are then identified by *in vitro* selection from a synthetic oligoribonucleotide library in the natural D-configuration. The natural D-configuration is required because stereoselective enzymes are used for amplification, cloning and sequencing of bound sequences. Identified sequences are finally synthesized using enantiomeric (L-)ribonucleotides. The resulting Spiegelmer binds to the natural target. Artwork by Christian Mihm.

target of interest. The sequence of the identified aptamers is then produced in its respective mirror-image configuration from non-natural L-nucleotides. Following the rules of symmetry, the resulting L-aptamers (Spiegelmers) bind to the natural target with the same affinity as the D-aptamers bind to the mirror-image selection target [20]. The identification of RNA-Spiegelmers is schematically represented in Fig. 1.

The SELEX process generally consists of 10–20 rounds of selection (under physiological conditions, i.e. pH, ions and temperature) and amplification. To separate binding from nonbinding sequences, the target is often biotinylated so that complexes can be captured using streptavidin- or neutravidin-coated beads which can then be separated from the solution by filtration. By adjusting target and oligonucleotide concentrations, wash volumes and competing, unspecific oligonucleotides, an efficient depletion of nonbinding or weakly binding oligonucleotides can be achieved [1,22].

An essential step in developing a commercially viable aptamer is the efficient truncation of the resulting sequences to the minimal binding motif [22]. Each nucleotide that is saved reduces manufacturing costs because of less raw material consumption, increasing yields in solid phase synthesis and more-efficient HPLC purification owing to better separation of full-length product from truncated variants. The length of a final Spiegelmer candidate typically is within the range of 30–50 nucleotides. L-Aptamers against almost 20 targets have been published to date. Although small targets such as adenosine [20] and arginine [68] were used in the initial proof-of-concept studies that supported the general principle of identifying Spiegelmer-based oligonucleotides; later targets with more-important pharmacological relevance have also been pursued and used in subsequent programs (Table 1).

As anticipated, affinity and selectivity of Spiegelmers for their respective targets seem to be comparable to conventional D-configured aptamers. A high selectivity is definitely desirable for *in vivo* applications to reduce unwanted side-effects; however, it can also impose difficulties in animal studies whenever the target of interest is not recognized by the candidate aptamer because of low species homology. This difficulty was first encountered in the anti-monocyte chemoattractant protein (MCP)-1 program [23]. At first, the Spiegelmer NOX-E36 was identified against human MCP-1, but it did not bind to murine MCP-1 that only shares 55% homology to the human protein. As a consequence, an additional drug discovery process was started to identify a rodent-specific anti-MCP-1 Spiegelmer, called mNOX-E36. The rodent anti-MCP-1 served as a surrogate for the preclinical evaluation of Spiegelmer-mediated MCP-1 inhibition in the respective efficacy models but it was also used for GLP toxicity studies in mice. By contrast, the target of NOX-A12, the second Spiegelmer that entered clinical trials, is the well-conserved chemokine stromal-cell-derived factor (SDF)-1 (97% homology between murine and human protein). NOX-A12 is cross-reactive to the murine, rat and cynomolgus monkey protein, thereby enabling preclinical research and development without the need for a surrogate.

The third clinical L-aptamer, the anti-hepcidin Spiegelmer NOX-H94, is selective for the human and cynomolgus monkey target protein but, despite a 75% homology to murine hepcidin,

TABLE 1

Overview of published L-aptamers grouped by development stage.

Target	Designation	K_d [nM]	IC_{50} [nM]	Animal model efficacy	Clinical efficacy
Monocyte chemoattractant peptide MCP-1/CCL2	NOX-E36/ emapticap pegol	1.05 (unpublished)	0.5 [23]	NOX-E36 is human- and cynomolgus-monkey-specific; efficacy studies were done in mouse with mNOX-E36 (see below)	Placebo-controlled Phase I, SAD and four-week MAD completed, PD-effect: reduction of $CCR2^+$ monocytes in peripheral blood [24] Double-blind, placebo-controlled Phase IIa trial in diabetic patients with albuminuria (three months treatment, three months follow-up): reductions in albuminuria and improved glycemic control (HbA1c); renoprotective effect maintained in absence of drug (three months) [25]
Murine MCP-1 (CCL2)	mNOX-E36	0.16 [26]	3 [26]	Lupus nephritis in $MRL^{lpr/lpr}$ mice [26,27] Diabetic nephropathy in uninephrectomized diabetic (db/db) mice [28,29] Liver inflammation and steatohepatitis (CCl_4-, bile-duct ligation or methionine-choline-deficient diet-induced hepatic injury) [30,31] Acceleration of liver fibrosis regression [32] Rodent models of chronic obstructive pulmonary disease (LPS-induced neutrophilia or tobacco-smoke-induced) [33] Pulmonary arterial hypertension in rat (monocrotaline-induced) and mouse (chronic-hypoxia-induced) [34]	
Stromal-cell-derived factor-1 (SDF-1/CXCL12)	NOX-A12/ olaptesed pegol	0.20 [35]	0.20 [35]	Hematopoietic stem cell/WBC mobilization in mice and/or monkeys [35] Reduced MM tumor burden in SCID mice in combination with bortezomib [36] Reduced homing of primary human CLL cells to bone marrow and increased mobilization to peripheral blood of CLL-like cells in mice [37] Rat glioblastoma [38] Pulmonary arterial hypertension (monocrotaline-induced) in rats [39] Diabetic nephropathy in db/db mice (T2DM model) [29,40] Improved pancreatic islet engraftment after transplantation [41]	Phase I, SAD: hematopoietic stem cell and WBC mobilization [35] Phase IIa for chemosensitization in MM (with VD) [42] and CLL (with BR) [43] ongoing
Hepcidin	NOX-H94/ lexaptepid pegol	0.65 [44]	20 [44]	IL-6-induced anemia in cynomolgus monkeys [44]	Phase I: dose-dependent increases in serum iron in healthy volunteers [45] PK/PD study: inhibition of LPS-induced serum iron decrease [46] Phase IIa in anemia of cancer ongoing
TAR (viral RNA)	L-6-4t	100 [47]	–		
Complement C5a	NOX-D19	1.40 [48]	1.90 [48]	Improved vascular integrity and reduced airway remodeling in a murine model for lung transplantation [49]	
	NOX-D20	0.30 [48]	0.28 [48]	Improved survival, organ function, reduced capillary leakage and inflammatory cytokines in a murine model of polymicrobial sepsis [48]	

Glucagon	NOX-G15	3.0 [50]	3.07 [50]	Murine models of type 1 and type 2 diabetes mellitus [50]
Sphingosine-1-phosphate (signaling lipid)	NOX-S93	4.3 [51]	5.5 [51]	Reduction of rhabdomyosarcoma cell seeding to bone marrow (metastasis model) [52]
High mobility group protein A1a/b (HMGA1a/b)	NOX-A50	7.1 [53]	15.4 [53]	Reduction of xenograft tumor growth by Spiegelmer-PEG-polyethyleneimine polyplexes (s.c. implanted pancreatic cancer cell line) [53]
Amylin	NOX-A42		19 [54]	Inhibition of amylinergic responses in the rat brain, for example, treatment of cancer-induced hyperamylinemia [55]
Rat calcitonin gene-related peptide (CGRP)	NOX-C89	32 [54]	4 [54]	Rat migraine model [56]
Ghrelin (acylated)	NOX-B11	35 [57]	5 [57]	Inhibition of growth hormone release induced by exogenous ghrelin in rats [57]
	NOX-B11-2	35 [58]	5 [58]	Amelioration of diet-induced obesity in mice[58]; decrease of calorie utilization; inhibition of body weight recuperation after fasting [59]
	NOX-B11-3	4 [60]	4.5 [61]	
Gonadotropin-releasing hormone (GnRH)	NOX 1257	20 [62]	20 [62]	Inhibition of GnRH-induced luteinizing hormone overexpression in rats following castration [62]
Substance P	SUP-A-004	40 [63]	45 [63]	
Nociceptin	NOX 2149	110 [64]	10 000 [64]	
Staphylococcal enterotoxin B (SEB)	B12b10_65	420 [65]		
Vasopressin	'Williams et al.'	1200 [66]	4000 [66]	
	NOX-F37	1.3 [67]	1 [67]	Aquaretic diuresis (no loss of electrolytes) in rats [67]
Arginine	R16-c	129 000 [68]		
Adenosine	A42d	1800 [20]		

rodent hepcidin is not recognized at all [44]. Preclinical efficacy was demonstrated in a cynomolgus monkey model. In the absence of a second relevant species, the preclinical, GLP-compliant development was only done in monkeys.

In the context of selectivity, an unexpected finding occurred in the C5a program. While selecting oligonucleotides binding to murine C5a, NOX-D19 and its followers NOX-D20 and NOX-D21 were identified as recognizing the intended mouse C5a as well as human C5a but not monkey or rat C5a. The alignment of the involved sequences revealed two out of 74 amino acids that are conserved in the species mouse and human but are different in rat and cynomolgus monkey. Thus, one or both amino acids must be responsible for this unusual specificity phenomenon. The murine target had initially been picked in to enable preclinical research for existing human-C5a-specific Spiegelmers [69].

The basis of every drug discovery approach is the initial preparation of a target molecule. The prerequisite for a Spiegelmer drug discovery process (Fig. 1) is to get access to the non-natural enantiomer of the intended target (in case the target is chiral) thus requiring a synthetic chemical process. Because the most interesting pharmacological targets are polypeptide-based, methods of solid-phase peptide synthesis and ligation techniques are usually employed to prepare mirror-image polypeptides consisting of D-amino acids. Currently, commercial protein synthesis can provide polypeptide chains of approximately 100–150 amino acids in length. However, in academic projects sometimes even considerably longer proteins are prepared chemically; the longest fully synthetic protein reported to date is the 312-residue *E. coli* 4-hydroxy-tetrahydrodipiconilate synthase (EC 4.3.3.7) (DapA) [70]. Taking into consideration that usually only epitopes of polypeptides and proteins are recognized, also smaller domains of larger proteins can serve equally well as selection targets. This approach, already established for the generation of antibodies [71], was also demonstrated for Spiegelmers. Examples include the selection against a 25-amino-acid-long peptide from *Staphylococcus* enterotoxin B (SEB) (239 amino acids) and against a 21-amino-acid-long central DNA-binding domain of the high mobility group protein A1 architectural transcription factors HMGA1a (107 amino acids) and HMGA1b (96 amino acids) [53,65].

Pharmacokinetics

Owing to their low molecular weight, unmodified oligonucleotides including Spiegelmers are rapidly cleared by the kidneys [72]. The renal clearance, however, can be slowed down by attaching moieties such as PEG that increase the hydrodynamic radius of the oligonucleotide. By this means, the half-life of an oligonucleotide can be tuned up to a limit that is set by a combination of biological stability and residual plasma clearance (e.g. uptake by phagocytes and residual renal elimination) [62]. To date, 40 kDa PEG is the only modification for aptamers that has been tested in clinical trials and it was this form of PEG that was approved as part of pegaptanib sodium (Macugen®). PEG of this size is also an integral part of the anti-tumor necrosis factor (TNF)α antibody fragment certolizumab pegol (Cimzia®, UCB), a systemic treatment for rheumatoid arthritis and other autoimmune conditions that has been marketed since 2007. Likewise, all Spiegelmers that have been tested for *in vivo* efficacy are modified with a Y-shaped 40 kDa PEG moiety.

Three clinical Spiegelmer programs revealed nearly dose-linear PK profiles with half-lives in humans of 50 h for NOX-E36, 40 h for NOX-A12 and 23 h for NOX-H94 [24,35,73]. For reasons of safety, the Spiegelmers were generally first administered intravenously as a 15 or 30 min infusion before switching to a slow bolus injection in the majority of the later clinical studies. NOX-E36 and NOX-H94 have also been injected subcutaneously with a reported bioavailability of approximately 50% for NOX-E36 [24].

Efficacy in preclinical disease models

The published models in which Spiegelmers have been tested cover a wide range of chronic diseases (e.g. inflammatory disease, cancer and anemia) as well as acute or subacute diseases. Disease models include stem cell mobilization, glucose tolerance tests in type 1 and 2 diabetes, septic shock as well as chronic studies, such as glioblastoma, tumor xenograft, lupus nephritis and diabetic nephropathy. An overview is presented in Table 1.

Manufacturing

The Spiegelmer oligonucleotides are synthesized by solid-phase chemistry on controlled pore glass (CPG) support employing *tert*-butyl-dimethylsilyl (TBDMS) protected phosphoramidites of L-nucleotides [74]. As L-nucleotides do not exist in nature they have to be synthesized chemically from precursors. The most convenient starting material is L-arabinose, which can be isolated from sugar beet or wood. L-Arabinose is first epimerized catalytically to L-ribose, L-ribose is then per-acylated and, finally, the individual nucleobases are attached using the Vorbrüggen nucleosidation reaction [75]. The resulting L-nucleosides are then converted into fully protected (2′-TBDMS) phosphoramidites following processes that are also well established for the enantiomeric counterparts in the D-configuration. For *in vivo* applications, the mirror-image oligonucleotides are usually PEGylated. For PEGylation, an aminohexyl linker is added to either end of the oligonucleotide and the PEG (NHS-ester) is conjugated to the primary amine of the linker mannerin a site-directed manner [74]. After lyophilization the Spiegelmer drug substance is reconstituted in a formulation solution and then sterile filtered to yield the bulk investigational medicinal product (IMP) for parenteral injection.

Spiegelmer-specific quality control measures include purity (HPLC), mass identity (mass spectrometry) and sequence identity (sequencing) [76] of the unconjugated intermediate Spiegelmer, as well as potency using cell-based assays or, if no reliable cell-based assay can be established, a binding assay. Furthermore, particulate matter, sterility and endotoxin content in the IMP are monitored. Batches of up to 200 g PEGylated Spiegelmer (mass based on the molecular weight of the oligonucleotide part) have been manufactured by contract manufacturing organizations (CMOs) operating under GMP. Scale-up is considered straightforward and will lead to further reductions of cost of goods. The shelf-life of the drug substance and drug product at storage temperatures of −20 °C or 2–8 °C is currently three years (unpublished data) but is likely to increase further as more stability data become available.

Generic names of Spiegelmer drugs

Until recently, no common stem had been established in the international nonproprietary nomenclature for aptamer-based drugs. Precedents were pegaptanib, pegnivacogin or egaptivon

pegol. WHO has now ruled that the stem 'apt' shall be used for aptamers and mirror-image aptamers alike, whereby the stem is to be located as an infix in the middle of the name [77]. Following the new rule, the names olaptesed pegol (NOX-A12), emapticap pegol (NOX-E36) and lexaptepid pegol (NOX-H94) were formed, whereby one can recognize slight but scrambled reference to their targets SDF-1, MCP-1 and liver-expressed antibacterial peptide (another name for hepcidin), respectively.

Proof of mechanism in humans and ongoing clinical trials

NOX-A12: olaptesed pegol

SDF-1 is a major retention factor for hematopoietic cells in the bone marrow. NOX-A12 blocks and detaches SDF-1 from the surface of bone marrow stromal cells (BMSCs), thereby neutralizing the local chemokine gradient in a dual way [78]. As a result, NOX-A12 mobilizes hematopoietic stem cells (CD34-positive cells, colony-forming cells of the different lineages) as well as white blood cells such as monocytes, neutrophils and lymphocytes from the bone marrow into the periphery. This was demonstrated in mice as well as in a proof-of-mechanism Phase I study in healthy volunteers. Here, the dose-linear PK profile of the different doses nicely correlated with a dose-dependent mobilization effect that peaked approximately 12 h after intravenous (i.v.) administration of NOX-A12. In spite of the dose linearity, a saturation of the effect could be observed at the second highest dose [35].

The complex interactions between hematopoietic cells and the local bone marrow microenvironment support growth, development and function, and this can also be attributed to cancer cells. By modulating the microenvironment of the tumor in the bone marrow niche through inhibition of SDF-1 with NOX-A12, tumor cells can be mobilized to peripheral blood where they can be targeted more effectively by chemotherapy and biologics. In addition, SDF-1 itself is a survival factor for some leukemic cells, thus reducing viability if the tumor does not receive SDF-1 and other signals from this niche [79,80]. In this context, NOX-A12 can be differentiated from CXCR4-blocking agents because it neutralizes SDF-1 action on its two receptors: CXCR4 and CXCR7, the latter being responsible for growth, migration and metastasis in some types of cancer [81,82]. Functional proof for effects on cancer cell distribution has been obtained by inhibiting homing of human chronic lymphocytic leukemia (CLL) cells to bone marrow in mice [37]. Further preclinical data also showed that NOX-A12 in combination with the proteasome inhibitor bortezomib significantly reduced tumor load of luciferase-transfected human multiple myeloma cells in mice. Additionally, homing of a multiple myeloma (MM) cell line to bone marrow niches in mouse was reduced in the presence of NOX-A12. Homing to bone marrow is an indicator for bone marrow metastasis, which leads to inhibition of normal hematopoiesis in MM [36].

Currently, NOX-A12 is being tested in two parallel Phase IIa trials. In the first trial NOX-A12 is combined with bendamustine and rituximab (BR) for the treatment of relapsed/refractory CLL patients. In the second trial the combination of NOX-A12 with bortezomib and dexamethasone (VD) in relapsed/refractory MM patients is being investigated. In an analysis of pilot groups that received NOX-A12 alone before the combination with background therapy, the mobilization of malignant cells (CLL and plasma cells, respectively) was maintained over 72 h. After the six months of combination therapy, response rates in both trials compared favorably with historical controls (increased overall response rate and improved quality of responses) with virtual absence of additional toxicity on top of the standard treatment [42,43,83–85].

NOX-E36: emapticap pegol

The MCP-1-neutralizing Spiegelmer NOX-E36 has completed a single ascending dose study (Phase I), a four-week multiple ascending dose study (Phase Ib) including type 2 diabetes patients and a single dose study in renally impaired patients for the investigation of potentially altered elimination kinetics. In all clinical studies, NOX-E36 was safe and well tolerated. The drug showed a dose-linear plasma exposition, and a dose-dependent reduction of peripheral blood monocytes carrying the MCP-1 receptor CCR2 was observed. This demonstrates that the drug effectively antagonizes the CCL2/CCR2 axis in humans [24].

NOX-E36 was recently studied in a double-blind, randomized, placebo-controlled Phase IIa study for the treatment of diabetic nephropathy. Seventy-five type 2 diabetes patients with albuminuria were treated on stable blockade of the renin angiotensin system (RAS-blockade using either an inhibitor of angiotensin-converting enzyme or an angiotensin receptor blocker) and antidiabetic treatment over a period of three months and followed for a further three months [86]. In this study, NOX-E36 reduced urinary albumin excretion, a measure of nephropathy, and also reduced glycosylated hemoglobin (HBA1c), a measure of glycemic control. Importantly, these effects were independent of blood pressure or eGFR, such as those induced by RAS blockade, and the observations were maintained even after cessation of NOX-E36 treatment, suggesting that the Spiegelmer interferes with the underlying pathophysiology of diabetic nephropathy. It is aimed to introduce NOX-E36 as the first agent to modify the course of diabetic nephropathy, to delay end-stage renal disease and ultimately to save patients from the need for renal-replacement therapy (transplantation, hemodialysis), at least for some time [25,87]. Moreover, by improving the kidney function, NOX-E36 could thus reduce the risk of cardiovascular events [88].

NOX-H94: lexaptepid pegol

At first, the hepcidin-neutralizing Spiegelmer NOX-H94 completed a combined single and multiple dose study. The drug was generally safe and well tolerated. PK analyses showed a dose-linear systemic exposure correlated with dose-dependent increases in iron parameters [45]. NOX-H94 was then tested in a randomized, double-blind, placebo-controlled PK/PD study in 24 healthy young men receiving a low-dose lipopolysaccharide (LPS) infusion and a single dose of NOX-H94 or placebo. LPS induced a transient drop in serum iron in the placebo group, probably via interleukin (IL)-6 production, that leads to hepcidin overexpression. Conversely, in the LPS plus NOX-H94 group, where hepcidin is blocked, an increase in serum iron was observed, which was significant at the pre-defined endpoint: 9 h after LPS injection [46]. NOX-H94 is currently being tested in patients with anemia of chronic disease. In a pilot study NOX-H94 has demonstrated efficacy in a subset of anemic cancer patients with functional iron deficiency by positively influencing pharmacodynamic (PD) parameters of iron metabolism and erythropoiesis as well as hemoglobin levels [89]. The Phase IIa studies are being

continued in dialysis patients with erythropoiesis-stimulating agent (ESA)-hyporesponsive anemia [90].

Concluding remarks

The concept of including chiral principles in the evolutionary screening process SELEX has progressed from first proof-of-concept reports to a technology that can deliver highly selective, nonimmunogenic, safe and well-tolerated new chemical entities with high plasma stability and a predictable PK profile. The preparation of the mirror-image target required for the screening process is an important and sometimes challenging step, particularly when targets are chemically or biologically more complex. Whereas smaller peptide targets that are more easily accessible were preferred in the early days of Spiegelmer discovery and development, now encouraging results have been achieved with larger polypeptides, protein fragments or even domains of full-length proteins.

So far, one stabilized D-aptamer (Macugen®) is approved for local treatment of the eye. Three other D-aptamers are currently being evaluated in Phase II and III trials (two of which are also for local ocular treatment). On the Spiegelmer side, three systemically acting drug candidates are in Phase II studies in various indications. So far, Spiegelmers have demonstrated a good safety profile, and all clinical candidates have demonstrated inhibition of their respective target in healthy volunteers as well as patients. Most importantly, the accomplished proof-of-concept for emapticap pegol in a double-blind placebo-controlled Phase IIa study and promising signs of efficacy with olaptesed pegol as well as lexaptepid pegol in patients are encouraging and justify further development.

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