Aptamers: future pharmaceutical drugs

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ABSTRACT:

In vitro selection strategies are powerful tools for the identification of nucleic acids with unsuspected activities. Their successful application during last two decades has positioned these technologies among those most promising for the development of therapeutic and diagnostic agents. Aptamers are DNA or RNA molecules with special conformational features, able to efficiently bind to a target molecule. They are obtained by SELEX, a particular *in vitro* selection strategy, and have found utility during last years as a novel class of pharmaceuticals compounds. This review summarizes the progress made in the use of aptamers as therapeutic agents, paying special attention to those examples that have concluded in pharmacological formulations currently included in preclinical and clinical trials.

1. INTRODUCTION:

The structural flexibility of nucleic acids, particularly RNA, results in their great functional versatility. Besides their well known function as key elements in the storage and transmission of the genetic information, nowadays it is assumed the participation of the RNA in a variety of biological functions that position nucleic acids in the centre of the essential cellular processes. This structural diversity has been successfully exploited for the *in vitro* identification and development of RNA and DNA molecules exhibiting, *a priori*, unsuspected functions. For this purpose, technologies known as *in vitro* selection and evolution strategies have been developed and applied during the last 20 years (reviewed in (Breaker, 1997; Burke and Berzal-Herranz, 1993; Gold *et al*, 1995; Marton *et al*, 2010; Romero-López *et al*, 2007; Wilson and Szostak, 1999)). Although all the strategies respond to the common general scheme represented in Figure 1a, they are designed on-demand according to the needs or interests of the researcher to end isolating RNA or DNA molecules exhibiting a desired phenotype from a large pool of variants.

Specific and efficient binding of nucleic acids to a target molecule is a phenotype that has attracted much attention, most likely due to the further potential blocking effect on the target function. RNA or DNA molecules that efficiently interact with a specific molecule are named aptamers. The isolation of aptamers is performed by the application of a particular *in vitro* selection methodology, known as SELEX (Systematic Evolution of Ligands by Exponential enrichment) (Tuerk and Gold, 1990). This process consists of iterative series of synthesis, binding, positive selection and amplification steps over a randomized oligonucleotides pool to yield a population enriched in those molecules able to bind to the desired target molecule (Figure 1b). During this selection procedure, the recovered molecules can also be counter-selected against a non-desired target in order to deplete the non-specific binders. The exploitation of this strategy has allowed for the identification of RNA or DNA molecules with potential clinical applications for a great variety of diseases (Ni *et al*, 2011). From basic research to preclinical and clinical studies, aptamers have shown to be promising candidates to develop pharmaceutical compounds. This review will summarize the current state of the art in this field, focusing on the ongoing clinical trials.

2. APTAMER STABILIZATION:

From a wide point of view, nucleic acid-based therapeutics, particularly RNA-based treatments, have important challenges to overcome, such as specific cell targeting, delivery and stabilization. The latter is mostly relevant in the case of inhibitor RNA molecules, mainly due to the highly abundant cytoplasmic and serum ribonucleases. This hurdle has been successfully solved by the development of modified nucleotides, which can be incorporated at precise positions during the chemical synthesis with minimal interference in the desired activity. Besides preventing degradation by exo- and endonucleases, chemical modifications may improve pharmacokinetic and pharmacodynamic properties of the nucleic acid, while reducing the

immunogenicity (reviewed in (Wang *et al*, 2011)). Thus, most of the aptamers with potential clinical application have been subjected to these chemical substitutions (see section 4).

One of the main challenges for the practical use of modified aptamers has been the implementation of the chemical synthesis to get modified nucleotides susceptible to be inserted into the aptamers. Advances during last years have increased the efficiency and reliability of the manufacturing process, thus reducing the production costs. Theoretically, incorporation of modified nucleotides follows two well-defined strategies:

1) During the SELEX step, by their inclusion during the initial RNA library synthesis step. This approach allows for the performance of the selection procedure in the presence of the chemical substitution, thus minimizing potential steric hindrance and favoring the acquisition of the functional three dimensional conformation by the aptamer. Modifications include changes in the backbone and/or the sugar moiety, and can be added during the transcription step by conventional or specifically modified T7 RNA polymerases (Burmeister *et al*, 2005; Chelliserrykattil and Ellington, 2004; Green *et al*, 1995; Jellinek *et al*, 1995; Kato *et al*, 2005; Lin *et al*, 1996; Ruckman *et al*, 1998).

First generation of modified nucleotides included the replacement of the non-bridging oxygen atoms in the phosphate linkage by sulfur atoms (Figure 2A). This substitution has been widely employed in antisense oligonucleotides-based therapeutics, and to a lesser extent, in aptamers (King *et al*, 2002; Tam *et al*, 1999). The main disadvantage of this strategy is the marked decrease in the inhibitor specificity. Further advances performed on oligonucleotide chemistry prompted the development of second generation molecules containing chemical modifications affecting to the ribose moiety at the 2' hydroxyl group: 2'-O-methyl, 2'-O-fluoro and 2'-O-methoxyethyl sugar substitutions (Figure 2a). They overcome many of the drawbacks detected for the phosphorothioate compounds and often provide increased aptamer efficiency and even a higher thermal stability of the duplex (Botkjaer *et al*, 2012; Burmeister *et al*, 2005; Darfeuille *et al*, 2002; Rhie *et al*, 2003; White *et al*, 2008). These features have also been subsequently improved with the development of chemical analogs of nucleic acids. Among them, the so-called LNA (locked nucleic acid) are of particular interest. LNA nucleosides contain a methylene linkage between the 2'-O and the 4'-C of the ribose molecule, which locks the sugar moiety into the energy-favorable C3'-*endo* conformation (Singh *et al*, 1998). LNA-modified oligonucleotides show high thermal stability when complexed with their DNA/RNA target, with increasing melting temperatures up to 10 ºC compared to unmodified duplexes (for a review, see (Barciszewski *et al*, 2009)). These chemical and biophysical properties also confer to LNAs high affinity and specificity for their targets. Hence, LNA-aptamers can be considered as the third generation of RNA-derivative drugs.

In contrast to all these chemical substitutions, spiegelmers are obtained by SELEX where the target is the chiral mirror of the natural form. Then, the isolated D-RNA molecules can be converted to the L-RNA form to assess the recognition of the wild-type protein. The resulting aptamers are enantiomers of natural RNAs with increased nuclease resistance due to the stereo-selectivity of natural ribonucleases (Wilson and Keefe, 2006).

2) Post-selection editing process by site-specific engineering. This approach is preferably used for the incorporation of chemical substitutions that cannot be added during *in vitro* RNA synthesis, such as fluorophores, peptides, lipids and polysaccharides conjugates (Figure 2b). The addition reaction is usually performed at the 3' and/or 5' termini of the nucleic acid molecule, thus minimizing potential disruptions of the aptamer folding. These kind of conjugates have already been successfully used for the development of aptamer-based optical sensors detecting specific molecules (drugs as cocaine, cellular or viral protein factors, nucleotides,…) (Citartan *et al*, 2012) and for the engineering of cell and tissue specific oligonucleotide drugs delivery (for a review, see (Zhou and Rossi, 2011)).

It is especially interesting the conjugation with polyetilenglicol moieties (PEG). The covalent linking of PEG chains, named PEGylation, has become a leading drug modification approach during last two decades. The addition of PEG provides important advantages to the carrier therapeutic compound, such as longer *in vivo* half-life, reduction of aggregates formation and low immunogenicity (Healy *et al*, 2004). Firstly tested in proteins, the PEGylation technology arrived to the aptamer field, yielding effective formulations that are currently being tested in clinical trials (see section 4) (Boomer *et al*, 2005).

3. DEVELOPING APTAMERS FOR THEIR THERAPEUTIC USE:

The potential use of aptamers as therapeutic agents was discerned since their discovery at 1990 (Tuerk and Gold, 1990). Unlike other RNA tools, such as antisense oligonucleotides, ribozymes and siRNAs, aptamers work by the recognition of both the primary structure and the three dimensional conformation of the target, thus providing higher specificity than their counterparts (Darfeuille *et al*, 2006). This feature is also companied by low immunogenicity and toxicity, thus giving further advantages to aptamers over other therapeutic agents, as antibodies. Furthermore, the *in vitro* obtaining procedure greatly simplifies the efficiency and manufacturing process. This section will summarize the main recent advances in the preclinical studies performed with aptamers and current aptamer-based drugs research.

3.1. Antiviral aptamers:
Targeting viral factors has become one of the most popular aptamer therapeutic uses, since they can interfere at any stage of the viral cycle, including entry, translation, replication, packaging and budding. The first viral target chosen for the aptamers isolation was the viral polymerase, largely due to its innate ability for interacting with nucleic acids. The pioneer study performed by Tuerk *et al.* (Tuerk and Gold, 1990) not only rendered efficient aptamers for the T4 phage DNA polymerase, but also informed about the nature and composition of the protein domain involved in the initiation of replication. This work set the basis for further investigations about the potential of human virus proteins as efficient targets. During last years, this has been a leading proposal for the development of novel combinatorial therapeutic strategies based on drugs cocktails containing conventional viral replication inhibitors and nucleic acid-based inhibitors. This would greatly improve the efficacy of current antiviral treatments and would

diminish the appearance of escape viral mutants. Many efforts have been made to target protein factors involved in different steps of the viral cycle (reviewed in (Binning *et al*, 2012)):

1) Virus entry: HIV targets helper T-cells by glycoprotein 120 (gp120). The use of gp120 as protein target for aptamers allows, not only for the inhibition of viral entry, but also for the specific delivery of other antiviral compounds (Dey *et al*, 2005; Khati *et al*, 2003; Mufhandu *et al*, 2012; Neff *et al*, 2011; Sayer *et al*, 2002; Zhou *et al*, 2008; Zhou *et al*, 2011a; Zhou *et al*, 2011b; Zhou *et al*, 2009).

2) Replication: viral RNA-dependent RNA polymerases have been a common target for the identification of aptamers. These proteins ususaly share classical reverse transcriptase features with unique conformational and functional properties. Hence, the identification of aptamers against them has been a major goal, not only from a clinical point of view, but also for the investigation of their exceptional functioning. Aptamers targeting HCV NS5B protein effectively inhibited its function *in vitro* and in infected cells, demonstrating the feasibility of this approach for the design of novel anti-HCV drugs (Bellecave *et al*, 2008; Biroccio *et al*, 2002; Jones *et al*, 2006). To date, however, information about the replication mechanism is still scant. In the case of HIV, aptamers against the reverse transcriptase (RT) have also been extensively reported. The application of *in vitro* selection strategies demonstrated that HIV-1 RT is an effective antiviral target, even in those viral strains that are resistant to conventional non-nucleoside inhibitors (Chaloin *et al*, 2002; Chen and Gold, 1994; DeStefano and Cristofaro, 2006; DeStefano and Nair, 2008; Joshi and Prasad, 2002; Li *et al*, 2008; Mosing *et al*, 2005; Schneider *et al*, 1995; Tuerk *et al*, 1992). Some of these aptamers were chemically modified by the inclusion of phosphorothioate nucleotides to yield efficient inhibitor RNA molecules *in vitro* (DeStefano and Nair, 2008).

3) Protein synthesis and maturation: many viruses have developed alternative translational mechanisms to that employed by the cellular cap-mRNAs, thus rendering a candidate target for virus inhibition. For example, HCV genome is a single stranded, plus polarity RNA molecule containing in its 5' end an internal ribosome entry site (IRES), which directs protein synthesis by a cap-independent pathway. This region exhibits a high sequence and structure conservation rate that has been widely exploited as potential therapeutic target (for a review, see (Romero-Lopez *et al*, 2006)). Aptamers against isolated functional domains of the IRES have been shown to act as efficient inhibitors of viral translation, both *in vitro* and in cell culture (Kikuchi *et al*, 2003; Kikuchi *et al*, 2005; Kikuchi *et al*, 2009; Tallet-Lopez *et al*, 2003). These studies demonstrated the efficacy of targeting functional genomic domains in RNA virus and served as starting point for the development of novel strategies combining two or more inhibitor RNA molecules targeting the whole IRES (Romero-López *et al*, 2005; Romero-López *et al*, 2012; Romero-López *et al*, 2009; Romero-López *et al*, 2007). These compounds interfered with HCV translation in the range of nanomolar, providing the proof of concept for the engineering of new chimeric nucleic acid-based inhibitors.

After their synthesis, protein products may need further processing and maturation. This has been also considered an interesting hot point for the control of the viral infection. In the case of HCV, the NS3 protein displays protease and helicase activities in two well-defined regions, playing key functions for the viral polyprotein maturation and viral RNA synthesis (Tanji *et al*, 1994; Wardell *et al*, 1999). In the past, efforts were focused on the development of selection strategies for the identification of aptamers targeting different functional domains (Fukuda *et al*, 2004; Fukuda *et al*, 2000; Nishikawa *et al*, 2004; Nishikawa *et al*, 2003). These findings prompted an initial attempt of engineering dual-aptamers, generated by the combination of two independently isolated aptamers with different target sites, linked by a non-related sequence (Umehara *et al*, 2005). The chimeric molecules retained both aptamer specificities and acted as efficient inhibitors of protease activity and virus replication in cell culture.

4) Encapsidation: the isolation of aptamers targeting viral core proteins is an interesting strategy for the investigation of the packaging process and the signals that govern it. For example, RNA aptamers against the nucleocapsid protein were isolated by different groups (Berglund *et al*, 1997; Kim and Jeong, 2003; Kim and Jeong, 2004) and proved efficient inhibition of the HIV genomic RNA packaging stage in cell culture.

3.2. Aptamers in oncology:

Cancer has been classically considered the result of genetic alterations affecting to essential signaling pathways, thus conferring proliferative and invasive properties to the carrier cell (Hanahan and Weinberg, 2000). These particular features exhibited by the malignant cell may be exploited by aptamers to specifically target and inhibit tumor progression. Aptamers have been developed against a variety of cancer targets, including extracellular ligands, cell surface proteins and intracellular factors.

The isolation of cell-type specific aptamers, mostly directed against cell surface receptors, achieves the direct action over the desired target cell (Cerchia and de Franciscis, 2007). It also provides specific delivery for a wide variety of drugs and reagents and its active accumulation when combined with a cell-specific aptamer, thus improving the efficacy of the treatment. To date, numerous aptamers directed against a specific cell type or tissue have been isolated by conventional SELEX methodologies or through innovative cell or tissue-SELEX processes. The latter are becoming emerging methods for the identification of aptamers targeting specific and likely unknown, cell surface molecules (Phillips *et al*, 2008). Their main advantage is that selected molecules recognize the native state of the ligand and even could likely differentiate not only cancer cells from non-tumoral tissues, but also between different types of cancer. Nevertheless, the high complexity and diversity of the cell and tissue surface conditions the selection pressure to avoid high diversity in the isolated aptamer pools. Hence, despite of the potential of the method, care should be taken in the subsequent analysis and validation of the selected aptamers.

The receptors for tyrosine kinase (RTK) are candidate targets since they appear constitutively activated in a variety of cancers, such as multiple endocrine neoplasia type 2A and 2B

syndromes and in familiar medullary thyroid carcinoma (Putzer and Drosten, 2004). The development of the live cell-based selection techniques (Cerchia *et al*, 2005) allowed for the isolation of the efficient inhibitor D4, a modified 2'-fluoropyrimidine RNA aptamer able to interact with the extracellular protein domain and interfere with the subsequent signaling pathway, preserving its activity in tissue-like microenvironments (Vento *et al*, 2008).

A recent application of aptamers is their use as drug carriers. Since Lupold *et al*. (Lupold *et al*, 2002) identified two RNA aptamers against the extracellular portion of the prostate-specific membrane antigen (PSMA), great efforts have been made to engineer aptamer-based therapeutic reagents that specifically target PSMA-positive malignant cells. The combination of these aptamers with small interfering RNAs (siRNAs) (Chu *et al*, 2006) targeting prostate cancer-specific prosurvival genes involved in the tumor progression, such as *bcl*2 and *plk*1, showed several advantages (Chu *et al*, 2006; Kim *et al*, 2010; McNamara *et al*, 2006). These constructs promote the efficient cellular uptake, simplify the compound formulation and present low toxicity compared to other delivery systems as cationic lipids, peptides or even viral vectors. In addition, they provide a feasible tool for the clinical use of siRNAs.

3.3. Anticoagulant aptamers:

Anticoagulants are a major class of pharmaceutical agents that can be used to prevent clotting events during certain clinical situations or for the treatment of cardiovascular diseases. The most commonly used reagent, heparin, may unleash serious secondary effects, such as hemorrhages, decrease in the platelet number and even allergies. Thus, the discovery of new anticoagulant drugs was a major goal during many years. In this context, aptamers appeared as a good alternative to classical drugs.

Thrombin protein is a preferred target for the development of anticoagulant compounds. This factor is a serine protease with a key role in hemostasis by the activation of procoagulant factors, which greatly amplify the coagulation reaction. The use of aptamers able to control this cascade was glimpsed in the early 1990's, with the isolation of a DNA aptamer that interfered with the proteolytic activity of thrombin (Bock *et al*, 1992). The great potential of this compound as anticoagulant prompted further pharmacokinetic and pharmacodynamic tests in different animal models (Griffin *et al*, 1993). These initial trials showed a proper dose-response relation between the aptamer administration and the anticoagulation activity. Importantly, maximum therapeutic effect was assessed at ten minutes after infusion, thus reflecting a short half-life. This feature could be very useful for the aptamer application to extracorporeal circuits. The results derived from the subsequent Phase I clinical trials were unsuccessful (Kim *et al*, 2008), but they opened a wide field for the development and engineering of novel efficient anticoagulant aptamers. The isolation of nuclease resistant, modified RNA-based aptamers against thrombin by other groups has confirmed the suitability of this strategy (Jeter *et al*, 2004; Kubik *et al*, 1994; White *et al*, 2001). Furthermore, these investigations pointed to the heparin binding site within thrombin as the optimal target region for the efficient aptamer interaction and

the consequent inhibitory activity. All together, these findings may promote the rational design and engineering of novel aptamer-based antithrombin drugs.

One of the main causes for morbidity during the anticoagulant treatments is bleeding. Thus, the idea of using aptamer compounds as anticoagulants has become even more attractive by the incorporation of RNA aptamer-based antidotes, which could provide the safest mechanism for the control of the drug activity (see section 4.3.1) (Rusconi *et al*, 2002). More recently, the development and engineering of light-inducible anti-thrombin aptamers is a promising strategy for the improvement of pre-existing compounds (Buff *et al*, 2010).

4. PRECLINICAL AND CLINICAL TRIALS:

The above mentioned reports provided the proof of concept to accomplish further validation for the use of aptamers as therapeutic reagents. Despite numerous obstacles, more than ten aptamer-based therapies have reached the clinical trial step. This section will summarize the main advances in the field of aptamer therapeutics for the treatment of cellular diseases (Table 1).

4.1. Anti-angiogenic aptamers:

Numerous pathological processes, such as tumorigenesis, ocular neovascular diseases or inflammatory events are intrinsically linked to the angiogenesis phenomenon. Hence, the control of the neovascularization events in these patients is a good alternative to interfere with the progress of the disease.

4.1.1. Therapy against VEGF: Pegaptanib.

VEGF is a pro-angiogenic factor, which can be produced as four principal isoforms, being VEGF165 the most abundant in serum (Ferrara, 2004).

Three different SELEX process were carried out by different groups of the NeXstar Pharmaceuticals Company to identify aptamers targeting VEGF. First of them, reported on 1994, described the isolation of aptamers blocking VEGF activity *in vitro*, confirming the potential suitability of the strategy (Jellinek *et al*, 1994). Subsequent approaches made use of amino-modified aptamers to increase their stability and resistance to nuclease attack (Green et al, 1995). Finally, the use of fluoro-oligonucleotides set the basis for the further development of Pegaptanib.

Pegaptanib (commercially available as Macugen, OSI Pharmaceuticals/Pfizer) is the first nucleic acid that was approved for its use in clinical applications. This compound proceeds from a 2'-fluoropyrimidine RNA aptamer identified in 1998 by Ruckman *et al.* (Ruckman et al, 1998) from a conventional SELEX procedure using as target VEGF165. Three different aptamer families were isolated with binding affinities to VEGF in the range of low picomolar. Representative molecules from each group were truncated to get the minimal region able to bind to VEGF and fully modified with 2'-O-methyl purines and a 3'-3' linked deoxythimidine cap, with the aim of

increasing their intracellular stability. One of these aptamers, t44-OMe, appeared as the most efficient inhibitor of vascular leakage from dermal microvessels following injection into guinea pigs. It was further modified by the addition of 40 kDa PEG at the 5' end to get the current Pegaptanib compound. Though it was observed an evident reduction in the binding affinity to VEGF after all these modifications, the resulting compound was even a more effective inhibitor than its unmodified counterparts. This is likely due to a longer tissue residence and serum half-life. Subsequent works investigating the pharmacodynamic properties of Pegaptanib in monkeys demonstrated that the biologically active aptamer could be detected in the vitreous humour of the eye even 28 days after administration, without detecting significative secondary symptoms (Drolet *et al*, 2000; Tucker *et al*, 1999). These studies prompted the initiation of the clinical trials, which finally led to the approval of Pegaptanib in December 2004 for its therapeutic use in patients with age-related macular degeneration (AMD).

4.1.2. Other antiangiogenic targets:

1) Aptamers against the complement system: ARC1905.

The complement system is a part of the innate immune system, which is stimulated during sepsis to generate a series of activation products that finally unleash the cleavage of the C5 molecule, generating the anaphylatoxin C5a and C5b. This determines the recruitment of inflammatory cells and the liberation of pro-angiogenic factors, such as VEGF (for a review, see (Ward, 2010)). This mechanism plays a key role in the progression of numerous pathophysiologic disorders. Interfering with C5 activity has been largely proposed as a good strategy for the treatment of pathologies related to the vascularization and inflammatory processes. Different methods for blocking this cascade have been reported, including the use of neutralizing antibodies and pharmacological inhibitors (reviewed in (Woodruff *et al*, 2011)).

The well-known advantages of aptamers as therapeutic compounds, and the previous successful use of Pegaptanib as antiangiogenic drug, promoted the development of anti-C5 RNA-based inhibitors. The application of a SELEX methodology from a starting pool of 2'-fluoropyrimidine-modified oligonucleotides yielded seven aptamers able to interact with C5 with a Kd in the range of nanomolar (Biesecker *et al*, 1999). These molecules effectively interfered with the C5 hemolytic activity in serum. Furthermore, they efficiently blocked downstream steps in the complement cascade governed by C5. A truncated version of one of these aptamers was further optimized by the application of a second selection procedure. This experiment yielded the 2'-fluoroaptamer ARC1905, with increased affinity by C5. Subsequent conjugation with PEG at the 5' end and 3' capping achieved a stable molecule, which is currently being tested in clinical trials (Phase I) for the treatment of AMD by Ophthotech.

2) Targeting of platelet-derived growth factor (PDGF): E10030.

Platelet-derived growth factor (PDGF) is an essential, ubiquitous mitogen and chemotactic factor required for the normal development of the heart, ear, central nervous system (CNS), kidney, eye and brain. In adults, it contributes to the normal maintenance of kidney, pancreas and CNS, and also regulates the blood vessels formation. Its overproduction in the absence of

exogenous stimulation leads to several diseases, such as atherosclerosis, glomerulonephritis, renal failure, glioblastoma, medulloblastoma and fibrosis (reviewed in (Reigstad *et al*, 2005)).

The critical function of PDGF during neovascularization events promoted its selection as potential target to interfere with the angiogenesis process. In 1996, Green *et al.* identified a group of DNA molecules able to interact with PDGF-AB with subnanomolar affinity (Green *et al*, 1996). One of these aptamers, E10030, was reduced to the minimal active region, and subsequently modified by 5'-PEG addition and 3'-capping to improve the exonuclease resistance. It is currently being assayed in AMD patients, in a Phase II clinical trial, by Ophthotech.

4.2. Aptamers as antiproliferative agents:

4.2.1. <u>AS1411</u>:

The antiproliferative DNA aptamer AS1411 was identified from a cell-based screening of guanosine-rich oligonucleotides that interfered with cellular propagation (Bates *et al*, 2009b). The initial pool of oligonucleotides was shown to form homoquadruplex stabilized by G-quartets, the essential requirement for their interaction with the nucleolin protein on the cell plasma membrane (Bates *et al*, 2009a). Nucleolin is a multifunctional protein that is highly expressed by cancer cells, both intracellularly and on the cell surface, and plays a critical role in the activation of the transcriptional factor NF- B (Girvan *et al*, 2006). AS1411 has proved its efficiency in many different animals and preclinical cancer models, and is currently being tested in Phase II clinical trials by Antisoma Research.

4.2.2. <u>Spiegelmers for cancer therapies</u>:
Many attempts have been accomplished for the development of spiegelmers targeting malignant cells. Two compounds, NOX-A12 and NOX-E36, are currently being tested in Phase I clinical trial for their potential as antiproliferative drugs by Noxxon Pharma AG. They bind chemokines ligand 12 and 2, respectively, which are mainly involved in tumor metastasis and inflammation (Sayyed *et al*, 2009). Both of them are conjugated to a 3'-PEG molecule. NOX-A12 is being assayed for the treatment of lymphoma, multiple myeloma and hematopoietic stem cell transplantation, whereas NOX-E36 is being administered to Type 2 Diabetes mellitus patients that suffer glomerulosclerosis for the treatment of tissue injury and inflammation.

4.3. Anticoagulant aptamers in clinical trials:

4.3.1. <u>Aptamers targeting Factor IXa: REG1</u>.

In 2002, Rusconi *et al.* (Rusconi et al, 2002) described the identification of the 2'-fluorpyrimidine aptamer RB006 targeting factor IXa, a key partner in the blood clotting formation. A chimeric molecule was further engineered by the addition of a 2'-methoxy-RNA oligonucleotide complementary to a portion of the RB006, thus achieving a regulatory sustainable system for

the anticoagulant activity. Conjugation of this construct with polyetilenglicol (PEG) or cholesterol (Rusconi *et al*, 2004; Rusconi *et al*, 2002) significantly increased its serum half-life in different animal models. The resulting construct, named REG1, was further tested by Regado Biosciences Inc. in clinical trials. Recently, in a Phase IIa assay, REG1 showed well tolerability in patients suffering percutaneous coronary intervention, with no angiographic thrombotic complications and good reversible response mediated by the antidote domain (Becker and Chan, 2009; Cohen *et al*, 2010). These promising results have prompted the following Phase IIb study, currently ongoing.

4.3.2. Inactivation of the von Willebrand factor for the control of the coagulation cascade: ARC1779.

Platelet adhesion and aggregation in sites of vascular injury, stenosis and endothelial denudation is dependent on the von Willebrand factor (vWF). As well as its immediate potential as anticoagulant target, it has great potential in combined therapies for the treatment of acute coronary syndromes.

ARC1779 is a chimeric DNA/RNA aptamer that binds to vWF with high affinity. This compound contains 13 unmodified 2'-deoxynucleotides and 26 2'-O-methylnucleotides. It is capped in its 5' end by a PEG moiety of 20 kDA and by a deoxythimidine nucleotide placed at the 3' termini. All these modifications contribute to increase the aptamer serum stability (Gilbert *et al*, 2007). Pharmacodynamic properties of ARC1779 were firstly assayed in a Phase I clinical trial by Archemix Corporation, showing dose dependence with respect to the area distribution and low toxicity. These results promoted the initiation of the subsequent Phase II clinical tests (Spiel *et al*, 2009). Injection of ARC1779 demonstrated to be an effective approach for the inhibition of vWF and vWF-dependent platelet function in patients with acute myocardial infarction.

4.3.3. Clinical trials with anti-thrombin aptamers: NU172.

Nu172 is a DNA aptamer selected to bind and interfere with the thrombin function (Waters *et al*, 2009). Te application of a conventional SELEX method yielded a pool of parental, potentially inhibitory aptamers. One of them was subsequently truncated to the minimal core active aptamer. This compound, without further modifications, is currently being tested in Phase II clinical trials for anticoagulation in heart disease therapies by ARCA Biopharma. Unfortunately, preliminary results have shown a limited efficacy of NU172.

4.4. Procoagulant aptamers:

Hemophilia A and B are bleeding disorders characterized by deficiencies in coagulation factor VIII (FVIII) and factor IX (FIX), respectively, resulting in abnormally prolonged bleeding. Factor replacement has been the most widely used treatment for these patients. However, it has been described that around 15% of the patients may develop a rejection reaction by the production of antibodies able to neutralize the replacing proteins (Srivastava and Group, 2005). Hence, the combination of these therapies with bypassing agents is a major goal. It is well-known that in

hemophilic patients the extrinsic coagulation pathway, negatively regulated by the tissue factor pathway inhibitor (TFPI) is intact (Crawley and Lane, 2008). The inhibition of TFPI would be, therefore, a suitable strategy to improve the blood clotting propagation.

Waters *et al.* described in 2011 (Waters *et al*, 2011) the isolation of a modified RNA-aptamer targeting TFPI by using a conventional SELEX method. The starting library was composed by a randomized mix of oligonucleotides containing deoxycytidine and 2'-O-methyl-guanosine, -adenine and -thymidine. The parent aptamer was subsequently truncated to define the minimal region able to interact with TFPI, and modified by the addition of a 3'-deoxythymidine cap and a 5'-hexylamine linker with a 20 kDa PEG moiety, to finally generate ARC19499. This molecule efficiently binds to TFPI, promoting the thrombin generation in hemophilia plasma. In monkeys treated with neutralizing antibodies, it corrected clotting and bleeding times. These results demonstrate the suitability of this strategy for its use with hemophilic patients in a combined therapy. Archemix Corp. is currently tested its activity in humans in a Phase II clinical trial.

5. PERSPECTIVES:

The emergence of the *in vitro* selection methodologies and particularly the isolation of aptamers, as novel useful tools for the development of therapeutics and diagnostics has moved to several companies for the exploitation of these technologies. The most significant companies holding key patents and/or license for the therapeutic application of the aptamer technology are OSI Pharmaceuticals, Noxxon Pharma AG and Archemix Corp.

Despite of the initial promising results offered by the use of Pegaptanib in AMD patients, it was replaced by a cheaper product, just two years after its approval. The new compound, though more effective than the aptamer drug, promoted important side-effects and was only tolerated by a low percentage of the affected individuals. New clinical trials were accomplished to further study the long-term pharmacodynamic features of Pegaptanib and its therapeutic potential for other clinical optic pathologies.

The great advances carried out in the field of aptamers production and their clinical applications have demonstrated that interfering with therapeutic targets function may be achieved by small molecules. This may be an useful strategy to complement alternative therapies, such as the use of neutralizing antibodies. These approaches are currently being extensively investigated by several companies with the aim of improving the therapeutic response and the long-term effects, as well as minimizing the toxicity and secondary effects.

In summary, aptamers provide a promising and unique tool for therapeutics. Given the fast progresses made in the field, it seems likely that in the next decade aptamers may be a common instrument for the clinical practice.

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Table 1. Aptamers currently being tested in clinical trials.

Pathway	Target	Aptamer	Pathology	Corporation	Clinical Trial	References
Coagulation (inhibition)	Thrombin	NU172	Heart disease	ARCA Biopharma	Phase II	(Waters et al, 2009)
	vWF	ARC1779	von Willebrand Disease	Archemix Corp.	Phase II	(Gilbert et al, 2007; Spiel et al, 2009).
	Factor IXa	REG1	Cardiovascular diseases	National Heart, Lung, and Blood Institute (NHLBI)	Phase I	(Rusconi et al, 2004; Rusconi et al, 2002) (Becker and Chan, 2009; Cohen *et al*, 2010).
Coagulation (induction)	TFPI	ARC19499	Haemophilia	Archemix Corp.	Phase II	(Waters et al, 2011)
Malignant cell proliferation	Chemokine ligand 12	NOX-A12	- Lymphoma - Multiple myeloma - Hematopoietic stem cell transplantation	Noxxon Pharma AG	Phase I	(Sayyed et al, 2009)
	Nucleolin	AS1411	Primary Refractory or Relapsed AML	Antisoma Research	Phase II	(Bates *et al*, 2009b) (Bates *et al*, 2009a) (Girvan et al, 2006).
Inflammation	Chemokine ligand 2	NOX-E36	Type 2 Diabetes Mellitus Systemic Lupus Erythematosus	Noxxon Pharma AG	Phase I	(Sayyed et al, 2009)
Angiogenesis	PDGF	E10030	AMD	Ophthotech Corporation	Phase II	(Green et al, 1996)
	VEGF	EYE001	AMD	OSI Pharmaceuticals	Phase II/III	(Ruckman et al, 1998) (Drolet et al, 2000; Tucker et al, 1999)
Complement system	C5	ARC1905	AMD	Ophthotech Corporation	Phase I	(Biesecker et al, 1999)

AMD: Age-related macular degeneration; AML: Acute Myeloid Leukemia; PEG: Polyetileglicol; TFPI: tissue factor pathway inhibitor; VEGF: vascular endothelial growth factor; vWF: von Willebrand factor.
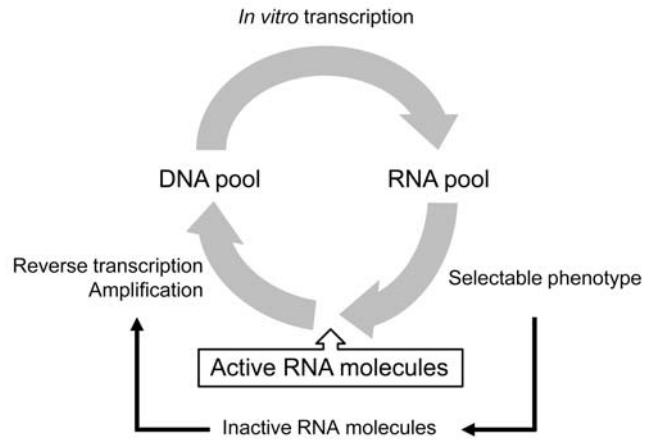
Figure legends:

Figure 1. *In vitro* **selection methods.** A) General scheme of an *in vitro* selection procedure involving an RNA library. An RNA population containing active and inactive variants is subjected to a selective pressure. Active RNA molecules are selectively recovered and reverse transcribed. Amplification of the cDNA templates generates a DNA pool, which is transcribed to yield a new RNA population that can be sampled and subjected to further selection rounds if required. B) SELEX process. Identification of RNA aptamers targeting a specific ligand by the application of an *in vitro* selection method. Figure adapted from (Romero-López et al, 2007). Suppression of transcription and reverse transcription steps allows for the isolation of active DNA oligonucleotides.

Figure 2. Chemical modifications. Diagram shows the most common chemical substitutions incorporated into synthetic oligonucleotides aimed to improve their pharmacodynamic properties. Pre-SELEX (A) and post-SELEX (B) modifications mentioned throughout the text are included. PEG, polyethilenglycol.
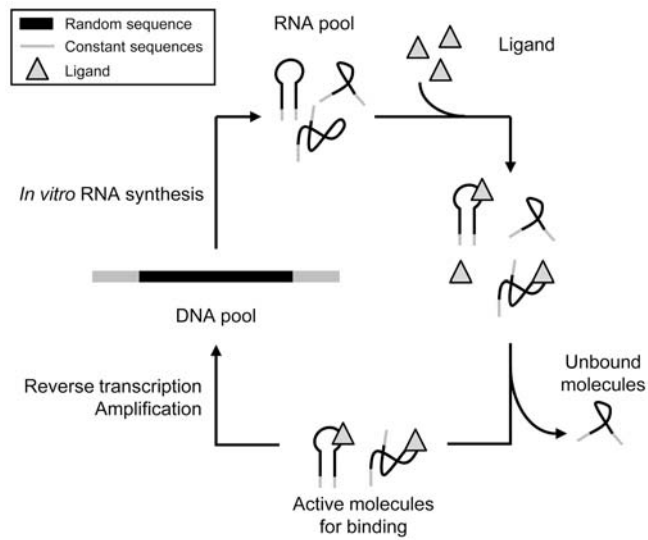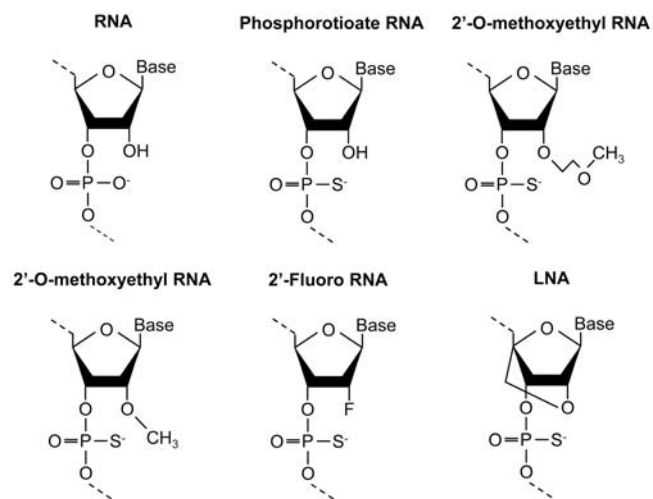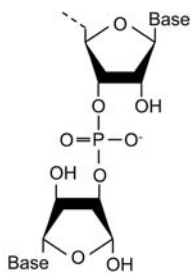
Figure 1

A)

B)

Figure 2

A)

B)