Development of the anti-VEGF aptamer to a therapeutic agent for clinical ophthalmology

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Abstract: Age-related macular degeneration (AMD) is the main cause of loss of sight in the world and is characterized by neovascularization of the macula. The factors producing choroidal vascularization involve various growth factors, including the vascular endothelial growth factor ($VEGF_{165}$). In this context, the systematic evolution of ligands by exponential enrichment (SELEX) became a tool for developing new therapeutic agents for AMD treatment. The SELEX is a combinatorial oligonucleotide library-based *in vitro* selection approach in which DNA or RNA molecules (aptamers) are identified by their ability to bind their targets with high affinity and specificity. Recently, the use of the SELEX technique was extended to isolate oligonucleotide ligands for a wide range of proteins of clinical importance. For instance, Pegaptanib sodium, a 28-nucleotide polyethylene glycol RNA aptamer that selectively binds to $VEGF_{165}$ and inhibits angiogenesis, was approved by the Food and Drug Administration for the treatment of wet AMD, thereby providing significant benefits to a great number of patients with minimal adverse effects.

Keyword: anti-VEGF aptamer, pegaptanib, age-related macular degeneration

Introduction

Molecular sources are becoming gradually more important in the search for compounds that can potentially act as biological agents for *in vivo* and *in vitro* studies and possible drug candidates. According to the increasing amount of new therapeutic targets provided by genome and on-going proteome, the design of specific inhibitors of proteins associated with disease is one of the primary objectives in pharmacological research using the suitability of combinatorial libraries, which assumes that one member of a huge population of different molecules and structures, such as polyamines, carbohydrates, peptides and oligonucleotides, can fit as ligand or inhibitor and modulate target protein activity (Janda 1994; Gold 1995; Ulrich 2006).

The Systematic Evolution of Ligands by EXponential enrichment (SELEX) technique which was introduced in parallel by Gold (Tuerk and Gold 1990) and Szostak (Ellington and Szostak 1990), uses an oligonucleotide-based combinatorial library containing a vast number of different sequences and structural motifs (about 10^{14}) for the *in vitro* selection of DNA or RNA molecules with binding specificity to a desired target. These high-affinity oligonucleotide-target binders are also denominated aptamers. Aptamers can interact with a variety of other selection targets including nucleotides (Sassanfar and Szostak 1993; Meli et al 2002), biologically active peptides, soluble proteins (Jellinek et al 1993; Williams et al 1996; Proske et al 2002), complex targets such as membrane receptors, blood vessels, erythrocyte surfaces (Ulrich et al 1998; Blank et al 2001; Morris et al 2001) and entire cells (Homann and Göringer 1999; Ulrich et al 2002; Guo et al 2006).

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Based on these features, aptamers became ideal candidates for investigation of protein interactions *in vitro*, in animal models and also for developing novel lead compounds for pharmaceutical applications. These features are due to the unique capacity of aptamers to fold into tridimensional structures folding of aptamers based on their nucleic acid sequence. In many cases, aptamers bind with low dissociation constants to their targets in the nanomolar or picomolar range, resulting in larger binding affinities than most of natural ligands or inhibitors used as competitors during the development of the aptamer (Ulrich et al 1998). Moreover, aptamers can interact with functional domains of a protein, most of all due to their smaller size compared to other ligands such as antibodies or other natural ligands, enabling aptamers to easily access their binding sites (Ulrich 2006).

In order to promptly achieve most of the designed properties, several kinds of modifications can be introduced to add specific characteristics to the aptamer such as nuclease resistance and enhancement of stability for *in vivo* applications. These characteristics can be obtained by adding phosphorothioate-based nucleotides to DNA aptamers or 2′-fluoro or 2′-amino-substitution of 2′OH groups of riboses of RNA aptamers (Pestourie et al 2005; Ulrich et al 2006). For improvement of pharmacokinetics and bioavailability high molecular weight or lipophilic moieties are attached to the 5′- or 3′- end of the aptamers. For instance, coupling an aptamer to a polyethylene glycol moiety increases half-lives in plasma to about 9 hours instead of the few minutes observed with unmodified aptamers (Willis et al 1998).

Another benefit of aptamers in *in vivo* experiments or therapeutic applications is the fact that they show low or no immunogenic responses. The proven non-toxicity of these molecules in preclinical and clinical trials, so far, makes aptamers promising candidates for therapeutic use (Eyetech Study Group 2002; Eyetech Study Group 2003). However, serious limitations for some applications of aptamers as diagnostic and/or pharmaceutical drugs are based on their incapacity to passively cross biological membranes, forcing most of researchers in industry to focus on extracellular-acting aptamers (reviewed in Rimmele 2003).

Even though being appropriate for an extensive range of different applications, a valid standardized protocol for aptamer selection for any given target molecule does not exist, as selection of aptamers significantly depends on the properties of a target and its availability in sufficient amount. In countless cases, the accomplishment of aptamer identification with desired properties basically depended on the use of the precise conditions during *in vitro* selection, in which aptamers later exert their function (Jayasena 1999), such as correct concentrations of cations for accurate aptamer folding into secondary and tertiary structures. Additionally, negatively charged target molecules may turn into a problem for DNA and RNA aptamer selection, making it necessary to take these facts into account for the choice of target epitopes and solution conditions used during the SELEX process (Rimmele 2003).

SELEX: the process

The *in vitro* selection of RNA and DNA aptamers against specific targets obeys the same rules as natural selection does. Individual steps of aptamer selection are illustrated in Figure 1 (Ulrich et al 2005). This selection of aptamers consists on the use of a library formed by two constants regions flanking a middle random segment, which can vary from 20 to 75 random-incorporated oligonucleotides. Constant regions should advisably contain restriction enzyme sites to facilitate the insertion of the random regions of selected aptamers into a bacterial vector for sequencing. In addition, a T7-promoter site needs to be included in one of the constant regions in case RNA aptamers shall be selected. For the selection of RNA aptamers the original DNA pool is in vitro transcribed to RNA in the presence of modified nucleotides and T7-RNA polymerase.

Re-iterative *in vitro* selection rounds initiate by presenting the target in standardized concentrations to the pool and separating target-bound molecules from the unbound molecules using several techniques including nitrocellulose filter adsorption (Ulrich et al 1998), affinity chromatography (Trujillo et al 2007), gel-shift separation (Ulrich et al 1998; Tang and Shafer 2006) and magnetic beads (Bruno 1997). Selected RNA molecules are eluted from their target epitope, collected and reverse-transcribed to DNA to be amplified by polymerase chain reaction (PCR). The amplified DNA pool is again *in vitro* transcribed to RNA to originate the second SELEX cycle. As a general statement, the selection stringency in SELEX protocols is usually increased to ensure that only the strongest binders are selected. The originally heterogeneous RNA pool is purified to a homogeneous population of high-affinity target binders to the aimed target. This final RNA pool is reverse transcribed to cDNA, PCR-amplified and used for cloning and DNA sequencing. Consensus motifs located in the previous random sequences are searched within the aptamer clones in order to identify aptamers families with similar sequences and three-dimensional structure. These conserved sequences are often located in stem-loops mediating aptamer-binding specificity (Ulrich et al 2005). Based on observations that these stem-loops by

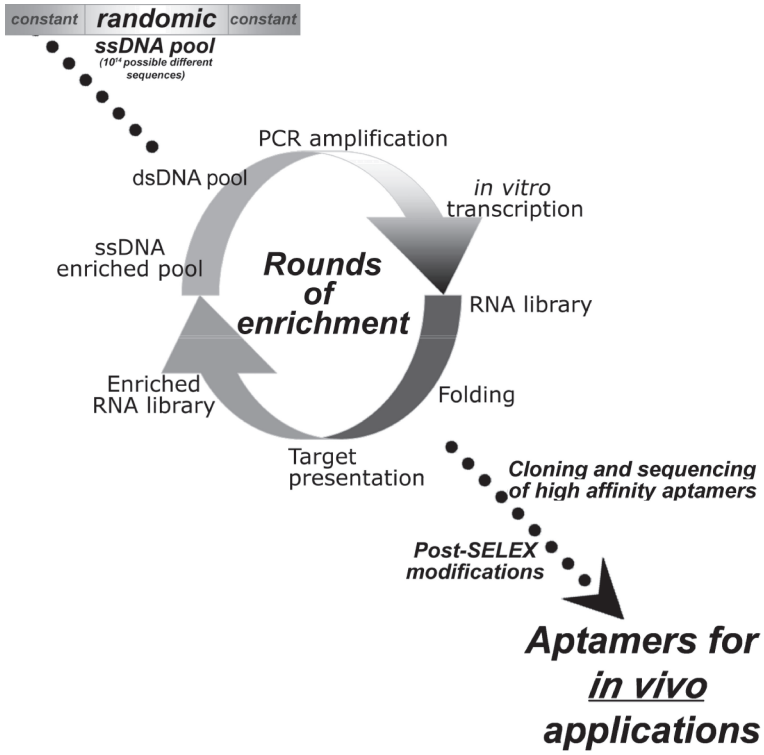

Figure 1 *In vitro* selection of RNA aptamers by using the SELEX (Systematic Evolution of Ligand by EXponential enrichment) technique. A chemically synthesized DNA pool is amplified by polymerase chain reaction (PCR) in the presence of specific primers followed by *in vitro* transcription to the combinatorial RNA library containing 10^{12}–10^{14} different sequences. The presence of 2′F- or 2′-NH_2-modified pyrimidines instead of 2′OH-pyrimidines during the *in vitro* transcription reaction provides nuclease-resistance to the synthesized RNA pool. Secondary structure formation of these RNA molecules is induced by thermal de- and re-naturation. Then the RNA pool containing diverse structural motifs is presented to its selection target (ie, VEGF), followed by collection of target bound RNA molecules which are reverse-transcribed to cDNA followed by PCR reaction. The RNA pool used for the second SELEX cycle is again obtained by *in vitro* transcription. Reiterative SELEX rounds are performed until the diversity of the original combinatorial RNA pool has been narrowed down to a homogeneous population of high-affinity target binders. This final RNA pool is reverse-transcribed to cDNA, amplified by PCR and sequenced for aptamer identification. Using post-SELEX modifications, identified aptamers are optimized for *in vivo* applications regarding nuclease resistance, thermal stability and pharmacokinetics.

themselves may be sufficient for binding specificity and biological activity, aptamers may be truncated to these minimal sequences. As further post-SELEX processes, aptamers can be optimized for *in vivo* applications by introducing specific chemical modifications or attaching reporter groups or hydrophobic linkers to their extremities (reviewed by Ulrich et al 2006).

Age-related macular degeneration

Age-related macular degeneration (AMD) is the most common irreversible cause of vision loss and blindness in the elderly (Klein et al 2004; Michell and Bradley 2006). AMD is defined as the loss of macular function from the degenerative changes of aging by the disruption of the interactions of the retinal pigment epithelium with the neural retina and the underlying choroidal vasculature (reviewed by Rowe-Rendleman and Glickman 2004; Tezel et al 2004).

There are two described forms of AMD: dry (non-exudative) and wet (exudative) (Nowak 2006). The dry AMD represents the form suffered from by more than 80% of the AMD patients and is characterized by the cumulative damage or genetic defects in the retinal pigment epithelium that causes or permits gradual cell loss (Bylsma and Guymer 2005). The wet AMD is described by neovascularization occurring either beneath the retinal pigment epithelium or between the epithelium and the retina, which can result in bleeding or exuding of fluids (Heier 2006). This form of the degeneration is responsible for about only 10%–20% of the total cases of AMD. However, wet AMD causes 90% of cases of severe vision loss in patients with AMD (Berdeaux et al 2005). Its pathogenesis is not well known, although several metabolic, genetic and behavioral risk factors were described for AMD establishment (Churchill et al 2006; Schaumbeg et al 2007; De Angelis et al 2007). For instance, increased plasma levels of MMP-9 metalloprotease (Chau et al 2007), genetic susceptibility (Rivera et al 2005) and behavioral habit of smoking contribute to AMD formation.

The factors initiating and maintaining disease-causing neovascularization in AMD are yet to be identified. However, it is now generally accepted that growth factors, mainly the vascular endothelial growth factor (VEGF), significantly contribute to this process. The VEGF gene family is divided into seven members; snake venom (VEGF-F), VEGF-E, placenta growth factor (PIGF), VEGF-C, VEGF-B, and VEGF-A, which bind with different affinities and specificities to three types of tyrosine kinase receptors (VEGFR1, VEGFR2 and VEGFR3) (Fournier et al 1997).

VEGF-A, a 35–45 kDa homodimeric protein, triggers pharmacological responses after binding to VEGFR1 and VEGFR2 and is also implicated in angiogenesis and neovascular diseases such as AMD and diabetic macular edema, a thickening of the retina occurring as a result of an abnormal accumulation of fluid within the retina (Ng et al 2006). VEGF-A has at least six different isoforms as a result of alternative splicing of a common mRNA. In humans, these are named $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165B}$, $VEGF_{189}$, and $VEGF_{206}$ (Bates et al 2002; Gustafsson et al 2005). $VEGF_{165}$ is the most predominant isoform in the eye and is the target of Pegaptanib for AMD treatment (Figure 2) (McMahon 2000; Giles 2001).

From the RNA library to the pegaptanib

The development of Pegaptanib began in 1994 in order to increase the limited number of available specific ligands for $VEGF_{165}$ for *in vitro* use (Figure 3). These few ligands were represented by a soluble truncated VEGF receptor aimed at the capture of circulating VEGF (Kendall and Thomas 1993) and a monoclonal antibody that inhibited the growth of injected tumor cells in nude mice, but had no effect on the growth rate of tumor cells *in vitro* (Kim et al 1993).

Jellinek et al (1994) used an oligonucleotide-based combinatorial library composed by 30 contiguous randomized positions, flanked by two constant regions for the *in vitro* selection of RNA molecules binding to $VEGF_{165}$. The combinatorial RNA library used in the first selection bound to VEGF with micromolar binding affinity and following 13 SELEX rounds, the affinity of the RNA pool to its selection target improved a hundred times. From this final selected RNA library, 64 sequences were identified that generated 37 consensus regions classified in six structural families. The members of each family had conserved sequences and thus shared a defined secondary conformation in which conserved residues were organized in a singular motif with a respective functional property giving origin to many points of interaction with its target.

Candidates of all six families were screened in nitrocellulose filter binding assays showing their capability to compete with VEGF binding to its receptor; in addition, all RNA ligands competed for the heparin-binding site. Jellinek et al (1993) suggested that many proteins have principal sites for RNA or DNA binding, corroborating the idea of the existence of a common binding site of heparin and VEGF for all aptamers. Using the same binding assay, Jellinek and co-workers used these aptamers to identify two classes of VEGF receptors on human umbilical vein endothelial cells (HUVEC) with different dissociation constants to their ligands. However most interestingly, high-affinity aptamers, denominated as sequences 100, 44, 12, 40, 84 and 126 (family 1–6 respectively) blocked the interaction of $VEGF_{165}$ with its receptor on the cell surface in a dose-dependent manner with dissociation constant in the range of 20–40 nM (Jellinek et al 1994). These observations showed that SELEX was an important tool for the discovery of specific inhibitors for $VEGF_{165}$-induced biological activity for *in vitro* applications and initiated the development of an anti-VEGF aptamer for *in vivo* applications.

The next important step for an anti-VEGF therapeutic aptamer was achieved by Green et al (1995) by introducing chemical modifications granting RNA molecules nuclease resistance and improving their efficacies for use in biological systems (for review see Ulrich et al 2006). The sensibility of RNA is based on 2′-OH groups of riboses which are used by nucleases for cleavage of the adjacent phosphodiester bound (Pieken et al 1991; Cummins et al 1995). For the SELEX procedure, Green et al (1995) incorporated 2′-aminopyrimidines into transcripts by enzymatic synthesis from two random libraries containing 10^{14} different RNA molecules. After 11 reiterative SELEX rounds, affinity of the evolved RNA pool increased a hundred times (dissociation constant ranged from pM to nM), and 79 unique sequences were isolated from the two libraries.

The consensus sequence of 24 conserved nucleotides in the previously randomized region was determined for the highest affinity RNA ligand, named as NX-107 (Green et al 1995). This aptamer with already improved resistance to degradation by nucleases was further modified for increased stabilization and optimization towards therapy by *O*-methylation (2′-OMe) of 2′-OH groups of adenosine in position 12.

Furthermore, poly dT caps with phosphorothioate linkages to the 5′- and 3′-terminals were added without any effect aptamer affinity to $VEGF_{165}$. Further aptamer modifications were tested in order to identify the most effective one: NX-213 was a capped aptamer with 2′-OMe purine modification and

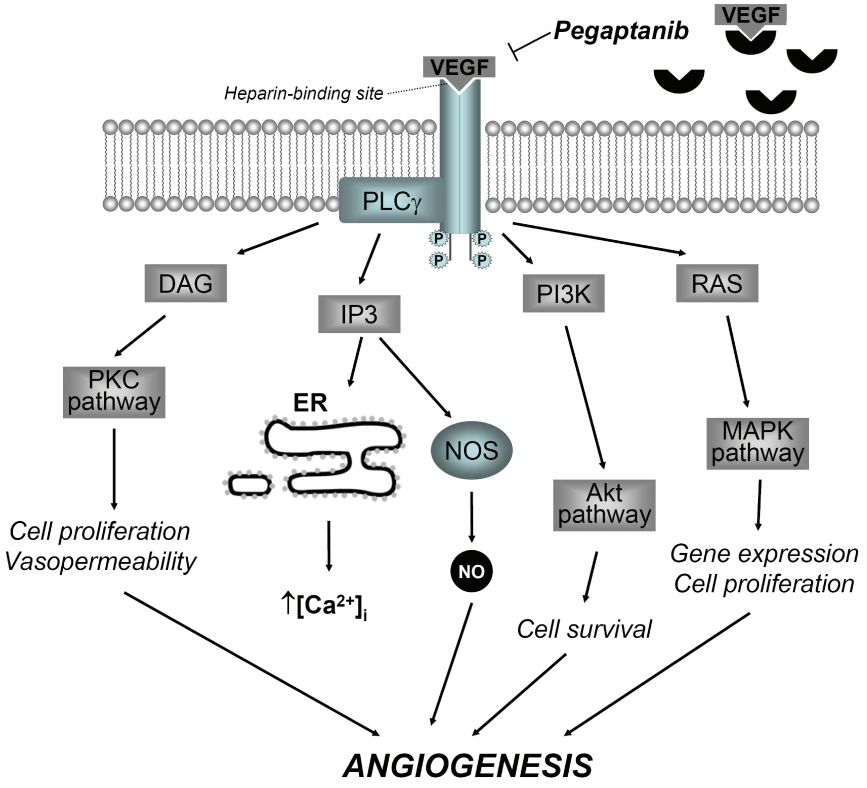

Figure 2 VEGF receptor-induced signaling pathway. VEGF plays a key role in physiological blood vessel formation and pathological angiogenesis such as tumor growth and ischemic diseases. VEGF-dependent cell survival is mediated via phosphatidyl inositol-3-kinase (PI3K)-induced activation of the anti-apoptotic kinase Akt, which inhibits the protein Bad, leading to inhibition of caspase activity and also causes Ca^{2+}-independent activation of nitric oxide synthetase (NOS) through phosphorylation. This pathway is necessary for cellular migration. A major mitogenic signaling mechanism for VEGF involves activation of phospholipase C (PLC-γ) resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate, production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), and subsequent mobilization of intracellular Ca^{2+} and protein kinase C (PKC) activation. PKC mediates activation of extracellular signal regulated kinases (ERK) 1/2 via RAS, Raf-1 and MEK, mediating mitogenesis in association with mitogen-activated protein kinases (MAPK). Binding of the growth factor to its corresponding cell surface receptor (VEGFR) activates complex signal transduction pathways, involving changes in protein phosphorylation, ion fluxes, gene expression, protein synthesis and ultimately a biological response related with angiogenesis. Functional signaling converges at several points, emphasizing how signaling pathways are integrated to form signal transduction networks. For inhibition of this signaling pathway, the Pegaptanib binds to VEGF, blocking the formation of the complex VEGF-receptor and consequently inhibiting signal transduction and angiogenesis.
Abbreviation: ER, endoplasmic reticulum.

NX-178 a capped aptamer without purine modifications. The NX-223 aptamer had an inverted substitution pattern in its nucleotides. Purines of NX-224 were substituted by 2′-OMe-purines, and the NX-191 aptamer was synthesized with 2′-OMe-modifications in all nucleotides. NX-178 and NX-213 with dissociation constants for VEGF binding as low as 0.1 nM, were in the following further characterized for their interaction with heparin-binding proteins and the thermal stabilities of the aptamer's minimal sequence. As a result, a high affinity RNA ligand resistant against degradation was obtained and the 2′-OMe post-SELEX modification in NX-213 significantly improved its half-life in rat urine sufficiently to be a model for the next generation of aptamers as a therapeutic agent in clinical trials (Green et al 1995).

RNA aptamers were selected from a 2′-fluoro-pyrimidine RNA library by Ruckman et al (1998) for further improvement of binding-affinity. 2′-F-pyrimidines are accepted as substrates by T7 RNA polymerase, and this modified oligonucleotide library does not destabilize the duplex conformation of RNA or DNA at physiological pH values in contrast to the 2′-aminopyrimidine substitution, resulting in a greater thermal stability and rigid conformation of secondary structures (Aurup et al 1994; Cummins et al 1995), which is theoretically followed by an increase in affinity (Eaton et al 1995). In addition, Healy et al 2004 verified that this type of modification may improve aptamer tissue residence and plasma half-life. For instance, an aptamer containing 2′-fluoro-pyrimidine and 2′-*O*-methylpurine modifications remained significantly longer in the circulation than did a completely 2′-*O*-methylated composition.

The aptamers against $VEGF_{165}$ were prepared by Ruckman et al (Ruckman et al 1998) using 2′-fluoro modifications RNA libraries containing 30 or 40 random nucleotides selected in 10 reiterative rounds. In comparison to the initial pool, the affinity of the isolated 46 high-affinity RNA to $VEGF_{165}$ increased 1000-fold, resulting in dissociation

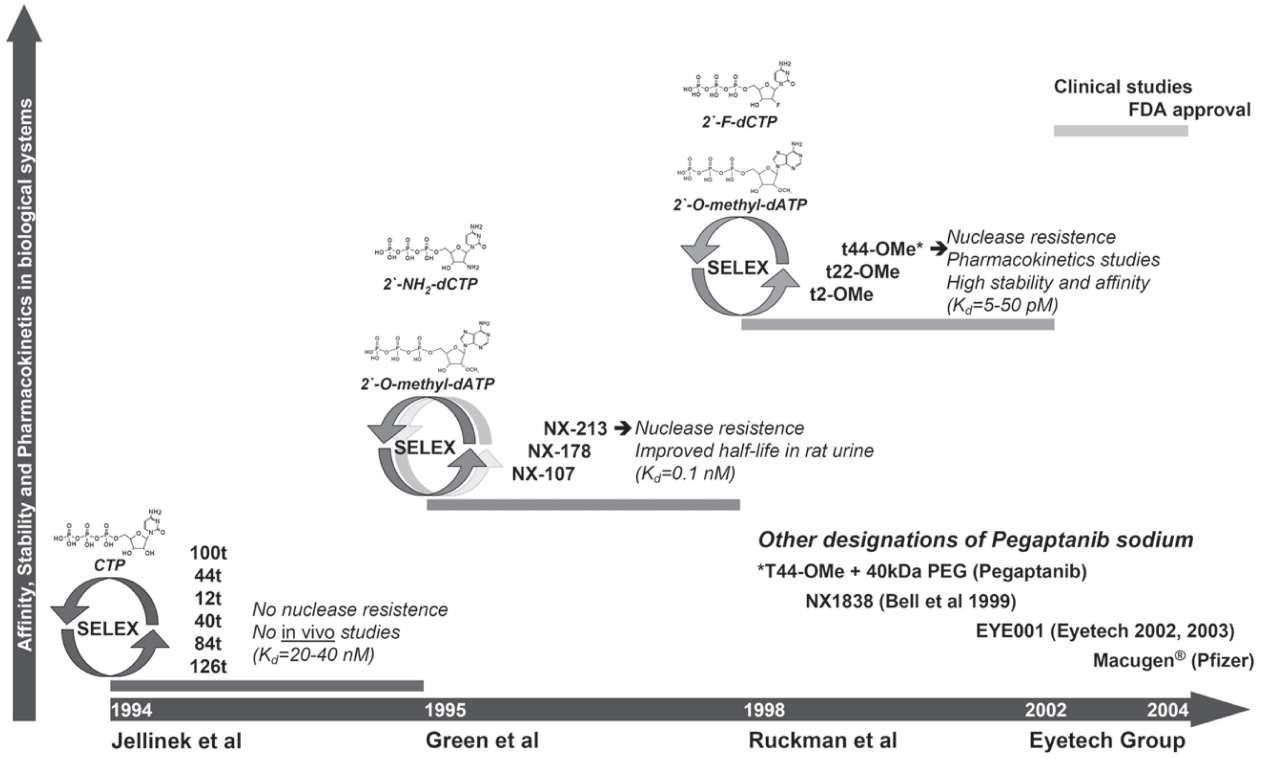

Figure 3 Timeline of the Pegaptanib development towards clinical trials for FDA approval. The first aptamer ligand of VEGF was identified by Jellinek et al (1994) from an unmodified combinatorial RNA library, introducing the idea that a VEGF-binding aptamer could inhibit VEGF-receptor binding. In this work, evolved aptamers were classified in six structural families and one candidate of each family (100t, 44t, 12t, 40t, 84t and 126t) was analyzed in terms of affinity. Observed dissociation constants (K_d) of aptamer-receptor binding were 20–40 nM. The CTP represents the chemical structure of nucleotides for wild type aptamers. Green et al (1995) selected aptamers from two RNA libraries carrying 2′-NH_2 pyrimidine modifications and introduced 2′-OMe purine modifications (exemplified in the figure by 2′-NH_2-dCTP and 2′-O-Methyl-dATP chemical structures, respectively) in already identified aptamers leading to increased nuclease resistance and to improved *in vivo* stability and tissue distribution. Following several experiments in order to determine the best modifications, Green et al (1995) had developed three aptamers named as NX-107, NX-178 and NX-213 with dissociation constants of 0.1 nM. Ruckman et al (1998) repeated the *in vitro* selection of VEGF-binding aptamers. Three modified aptamers with 2′-fluoro pyrimidine and 2′-OMe purine substitutions except for the adenines involved in $VEGF_{165}$ binding (exemplified in the figure by the 2′-F-dCTP and 2′- O-Methyl-dATP chemical structure, respectively) were identified. One of these aptamers was further developed into therapeutics. Following PEGylation T44-OMe was denominated as Pegaptanib. Other names were introduced by Bell et al (1999) (NX1838) and by Eyetech (EYE001) prior to its FDA approval in December, 2004 for therapeutic use in humans.

constants in the 5–50 pM range (Ruckman et al 1998). The evolved RNA aptamers could be grouped in three structural families based on conserved sequence motifs. For each of the aptamer families, minimal sequences sufficient for binding activity were determined. The respective truncated aptamers containing 23–29 nucleotides, denominated as t22.29, t2.31 and t44.29 were selected for 2′-OMe post-SELEX modifications in order to further enhance their nuclease resistance in biological fluids (Beigelman et al 1995).

The 2′-*O*-methylated truncated aptamers (t22-OMe, t2-OMe and t44-OMe) were tested in terms of the divalent cation dependence and thermal stability, the specificity for $VEGF_{165}$ and inhibition of $VEGF_{165}$–induced receptor activation *in vivo* by using the Miles assay (Miles and Miles 1952; Senger et al 1986). With this assay, Ruckman et al (1998) could monitor the effect of aptamers on VEGF-induced increase of leakage of microvessel in guinea pigs. Preincubation with 1 or 0.1 μM of VEGF with the mentioned aptamers (t22-OMe, t2-OMe and t44-OMe) and co-injection both demonstrated that the t44-OMe inhibited the vascular permeability by 58% or 48%, depending on the used VEGF concentrations. Furthermore, improving pharmacokinetics by conjugating the t44-OMe aptamer with a 40 kDa PEG polymer, preventing rapid VEGF elimination from plasma and prolonged systemic exposure facilitating tissue distribution of aptamers (Healy et al 2004), even increased aptamer potency in inhibiting VEGF activity (83% at 0.1 μM of VEGF), although at the same time aptamer binding affinity to VEGF was reduced. Pharmacokinetics and biodistribution of the t44-OMe polyethylene glycol aptamer containing a $VEGF_{165}$-binding sequence of 27 nucleotides plus an additional 3′-terminal deoxythymidine (pegaptanib), now renamed, NX1838 (Tucker et al 1999; Bell et al 1999), were determined in rhesus monkeys. An intravenous administration of Pegaptanib resulted in aptamer half-life of approximately 9 h in circulation whereas unmodified

aptamers were eliminated within minutes (Willis et al 1998) at a clearance rate of 6 ml/h. Subcutaneous administration resulted in almost 80% absorption of aptamers into the plasma compartment, reaching peak concentrations after 8 h (Tucker et al 1999).

Preclinical and clinical studies with NX1838 (now renamed EYE001), were conducted by Eyetech Pharmaceuticals, Inc., for the treatment of wet age-related macular degeneration and diabetic macular retinopathy (Eyetech Study group 2002; Eyetech Study group 2003). In collaboration with Pfizer and following approval by the Food and Drug Administration (FDA), the Pegaptanib sodium is now commercially available under the label Macugen® (www.macugen.com). In view of the breakthrough of Macugen in therapeutic applications, it seems certain that aptamer-based drugs are becoming a novel alternative to conventional drugs in the pharmacological field, both in diagnostic uses and as therapeutics.

In fact, improvements on the aptamer pharmacokinetics made it possible to deposit aptamers in desired tissues under the control of a drug delivery system for a long-term inhibition of VEGF-induced neovascularization. Pegaptanib was encapsulated in poly (lactic-co-glycolic) acid (PLGA) microspheres in order to gradually release the aptamer formulation. The drug was liberated for about 20 days at an average rate of 2 mg/day (Carrasquillo et al 2003). This delivery form represents an attractive alternative to intravitreal injection for AMD treatment.

Pegaptanib as a therapeutic agent

VEGF exterts several physiological and pathological functions such as an increase of vascular permeability causing a fluid leakage in wet AMD. As mentioned above, Pegaptanib selectively interacts with the heparin-binding site of the extracellular $VEGF_{165}$ isoform and prevents $VEGF_{165}$ from stimulating its receptor on the surface of the endothelial cell, thus blocking initiation of the intracellular cascade and consequently inhibiting vascular permeability and retina neovascularization (Figure 2) (Ruckman et al 1998; Eyetech Study Group 2002; Waheed and Miller 2004; Lee et al 2005).

Pegaptanib sodium is completely different from other drugs as the first RNA drug approved by FDA and the first anti-angiogenic agent for AMD treatment (Doggrell 2005). In preclinical trials by using the Miles assay (Miles and Miles 1952; Senger et al 1986), fluid leakage caused by administration of VEFG was completely inhibited by the addition of 100 nM of Pegaptanib. Treatment with Pegaptanib promoted the inhibition of 65% of VEGF-dependent angiogenesis in animal models for corneal angiogenesis (Eyetech Study Group 2002). In another model for retinopathy of prematurity a reduction of 80% of retinal neovascularization was observed in the presence of the aptamer formulation. The phase IA clinical trial (a multi-center, open-label, dose-escalation study) began in 1998 and evaluated the effect of the drug on 15 patients with subfoveal choroidal neovascularization secondary to wet AMD. This test did not reveal any significant safety issues related to intraocular administration of Pegaptanib which could be used safely up to 3 mg/eye (Eyetech Study Group 2002). In phase II trials (multiple-dose safety study), 21 patients were treated with three different doses (0.3, 1 and 3 mg) of the aptamer, and most of the patients (80%) stabilized or improved their vision after 3 months of Pegaptanib treatment (Eyetech Study Group 2003). Although all doses were well tolerated and produced pharmacological effects when compared to sham injection ($p < 0.0001$ for 0.3 mg, $p < 0.0001$ for 1 mg, $p = 0.03$ for 3 mg), dose levels over 3 mg did not result in additional benefits. While the clinical trials demonstrated safety of Pegaptanib in all tested dosages, some undesirable side effects were reported which most medicines reveal. In phase III, most of the undesirable effects were attributed to injection procedures.

Following intravitreous Pegaptanib injection a mild increase of intraocular pressure was observed (Hariprasad et al 2006). Consequently, the FDA recommends tonometry within 30 minutes following injection and biomicroscopy between two and seven days subsequent to the injection. Other undesired adverse effects, which may occur, include endophthalmitis (1.3%), retinal detachment (0.6%), cataracts and possible allergic reactions (Steffensmeier et al 2007). Another obstacle for aptamer-treatment is the cost of Macugen being higher than that of traditional therapies (Zhou and Wang 2006; Smiddy 2007). However, although the cost of treatment with aptamers is elevated, the success of Macugen in effectively treating AMD opens precedents to start more clinical trials for new applications in other ocular diseases, such as diabetic macular edema (DME).

DME is caused by changes in retinal microvasculature and is subdivided in two variants, focal and diffuse. The focal variant is characterized by focal leakage of microaneurysms with lipoprotein accumulation. The diffuse variant causes diffuse leakage from retinal vessels often accompanied by cystoid macular changes (reviewed by Bresnick 1986).

A phase II randomized double-masked trial was carried out to evaluate the safety and efficacy of Pegaptanib in the treatment of DME (Cunningham et al 2005). Three aptamer doses were tested (0.3, 1 and 3 mg) which were well accepted. The patients treated with Pegaptanib showed increments in visual acuity outcomes and additional therapies such as photocoagulation were necessary at a less degree than compared to patients which had not been treated with the aptamer. Although some problems related with the use of Pegaptanib had been reported, more than 50,000 patients were already treated with this aptamer therapy with a notable clinical benefits to a wide range of patients with neovascular AMD.

Moreover, since the aptamer formulation resulted in a reduction of 76% of rhabdomyosarcoma tumor volume, further therapeutic applications for Pegaptanib are foreseen for diseases related to pathological $VEGF_{165}$-induced neovascularization. Dahr et al (2007) published the results of a recent study on Pegaptanib use in 5 patients with von Hippel-Lindau (VHL) disease. The patients were intravitreously injected every 6 weeks with 3 mg doses. The whole treatment contained at least 6 injections. However, no significant reduction in tumor growth was observed, although decrease of exudates indicated decrease in vascular permeability. The ineffective treatment with Pegaptanib in this case is probably due to the fact that missing neoangiogenesis is not sufficient for tumor growth blockade in already vessel-rich environments (Blouw et al 2007). However, aptamer-induced inhibition of neoangiogenesis certainly will be promising for combating tumor growth in other tissue environments.

To prove the efficacy and safety of Pegaptanib, Ng et al (2006) recently show as an unpublished data from VISION (VEGF Inhibition Study in Ocular Neovascularization) that patients having been treated with 0.3 mg Pegaptanib achieved a 45% relative benefit in mean change in vision at the end of two years compared with those having received usual care. In addition to these results, the dosage recommendation could be reviewed to define the lowest effective dose and the longest treatment interval.

Conclusions

The demand for novel therapeutic agents acting on disease-causing or related protein functions has turned the SELEX technique into a promising approach for drug discovery, taking into account that aptamers can be evolved against almost every target. The concept of using aptamers as therapeutic agents is now 17 years old and notable progress has been made in turning this concept into a clinical reality. In this context, Pegaptanib, as the most important clinical aptamer, is the first approved oligonucleotide-based pharmacotherapy for the treatment of age related degeneration.

The development of Pegaptanib was based on considerable technical achievements produced in both academic science and industry. However, due to the instability of RNA and DNA in biological systems, early identified aptamers were not appropriate drug candidates. After significant improvements regarding oligonucleotide modifications many of the stability and pharmacokinetics-related limitations were overcome and stabilized aptamers became available with high prolonged systemic exposure and notable biodistribution within tissues. Chemical modifications of oligonucleotides continue to be developed for further optimization of aptamer efficacy and be tested *in vitro* and in animal models prior to clinical validation. Thus, it seems certain that nucleic acid based drugs will soon become, a standard feature of the pharmacological landscape, both as diagnostics and as therapeutics.

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Note

Considering the quantity of original research articles and reviews available on this topic, we may have not cited all important contributions to the development of anti-VEGF aptamers into therapeutics.

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