Development of an aptamer-based therapy for neovascular retinal diseases

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Resumo

Em estágios avançados de doenças da retina, como retinopatia diabética, degeneração macular associada à idade e oclusões da retina, ocorre neovascularização devido à sobre expressão de fatores, como o fator de crescimento endotelial vascular (VEGF) e a nucleolina (NCL) em resposta à hipoxia. Os tratamentos atuais incluem corticosteroides e anticorpos específicos, mas têm eficácia limitada a longo prazo e efeitos colaterais. Os aptâmeros surgiram como uma alternativa promissora por serem moléculas que se ligam a alvos com alta especificidade, além de serem mais fáceis de sintetizar e menos imunogénicos. O AT11 é um aptâmero derivado do AS1411 que, devido à capacidade de adotar estruturas em G-quadruplex, pode ligar-se à NCL, uma proteína relacionada com vários fatores de crescimento, nomeadamente o VEGF. A NCL pode atuar como co-receptor do VEGF, participando na sua via de sinalização, afetando os processos de angiogénese. Deste modo, este estudo visou testar se a afinidade do AT11 para a NCL se traduziria depois numa afinidade também para o VEGF, inibindo assim as suas vias de sinalização, e consequentemente, diminuindo a neovascularização característica das doenças da retina. Além disso, o AT11 poderia ser utilizado em conjunto com um corticosteroide, como a dexametasona, tornando o tratamento mais eficaz. Foram realizados estudos biofísicos, como o dicroísmo circular e ensaios de fluorescência, seguidos de testes em células endoteliais da veia umbilical humana (HUVEC), para avaliar a interferência do AT11 e da dexametasona, bem como da combinação de ambos, na proliferação celular. O ensaio antiangiogénico revelou que o aptâmero inibiu com sucesso a angiogénese, atingindo valores bastante inferiores a 50%, e também contribuiu para uma maior eficácia da dexametasona, atuando de modo sinérgico na inibição da angiogénese aquando da combinação de ambos como agentes terapêuticos.

Keywords

Aptâmeros em G-quadruplex; retinopatias; VEGF; angiogénese; neovascularização.

Resumo Alargado

O olho é um órgão vital e delicado, responsável por capturar imagens do mundo ao nosso redor, enviando-as para o cérebro para a sua interpretação. Apesar das camadas de proteção que o cercam, o olho pode ser afetado por diversas doenças que prejudicam gravemente a qualidade de vida, podendo resultar na perda de visão. Um tipo comum dessas doenças afeta a retina através de problemas vasculares, causando complicações sérias. A retina, que converte imagens em sinais elétricos para o cérebro, pode ter a sua função comprometida devido à angiogénese e aos processos inflamatórios associados. Estas doenças são uma das principais causas de cegueira em países desenvolvidos, e espera-se que se tornem um problema significativo globalmente devido ao aumento da prevalência da diabetes, um fator de risco para essas condições.

Uma das principais causas as das doenças vasculares da retina são episódios de hipoxia, frequentemente desencadeados por condições como diabetes ou hipertensão, que prejudicam o fluxo sanguíneo normal. Esses episódios levam a uma superprodução de VEGF, desencadeando processos que resultam em inflamação e crescimento anormal de novos vasos sanguíneos. Uma proteína que é bastante importante nesta cascata de inflamação é a NCL, que durante esses episódios de superprodução de VEGF, migra para a superfície celular, onde atua como recetor para fatores de crescimento e intervém no processo pró inflamatório e angiogénico.

O AS1411 é um aptâmero, que, devido à formação de uma estrutura de G-quadruplex (G4) bem definida, pode ligar-se especificamente à NCL e, portanto, tem potencial para ser usado no tratamento de patologias neovasculares. No entanto, a eficácia do AS1411 pode ser limitada devido ao facto de poder apresentar várias estruturas G4, dificultando o isolamento da estrutura com maior atividade biológica Deste modo, este estudo propõe utilizar um aptâmero derivado do AS1411, o AT11, também com elevada especificidade para a NCL, para não só superar as dificuldades de alterações na conformação que o AS1411 apresenta, mas também para auxiliar o tratamento destas doenças neovasculares, através da sinergia com um corticosteroide já usado neste âmbito, a dexametasona.

De modo a avaliar a efetividade da sinergia entre os dois compostos, estes foram testados isolados e combinados em células HUVEC, avaliando assim a sua capacidade angiogénica.

Os resultados mostram que o AT11 forma uma estrutura molecular bem definida quando associado à dexametasona, e não interferiram de modo algum na proliferação celular. Os ensaios de angiogénese demonstraram que a dexametasona tem, de facto, uma

capacidade anti-angiogénica bastante elevada, algo que também se pode constatar no AT11, ainda que em menor grau. A utilização da combinação de ambos elevou ainda mais a capacidade anti-angiogénica da dexametasona, tendo resultado na total inibição da angiogénese. Estes resultados sugerem que o AT11 incrementa as capacidades da dexametasona e pode ser utilizado como um complemento ao tratamento com este fármaco para aumentar a eficácia do mesmo.

Abstract

In advanced stages of retinal diseases such as diabetic retinopathy, age-related macular degeneration, and retinal occlusions, neovascularization occurs due to overexpression of factors such as vascular endothelial growth factor (VEGF) and nucleolin (NCL) in response to hypoxia. Current treatments include corticosteroids and specific antibodies, but they have limited long-term efficacy and side effects. Aptamers have emerged as a promising alternative because they are molecules that bind to targets with high specificity, are easier to synthesize, and are less immunogenic. AT11 is an aptamer derived from AS1411 that, due to its ability to adopt G-quadruplex structures, can bind to NCL, a protein related to various growth factors, including VEGF. NCL can act as a co-receptor of VEGF, participating in the signaling pathway, affecting angiogenesis processes. Thus, this study aimed to test whether the affinity of AT11 for NCL would also translate into affinity for VEGF, thereby inhibiting the signaling pathways and consequently reducing the characteristic retinal neovascularization. Additionally, AT11 could be used in conjunction with a corticosteroid such as dexamethasone, making the treatment more effective. Biophysical studies, such as circular dichroism and fluorescence assays, were conducted, followed by tests on human umbilical vein endothelial cells (HUVEC) to evaluate the interference of AT11 and dexamethasone, as well as the combination of both, in cell proliferation. The anti-angiogenic assay revealed that the aptamer successfully inhibited angiogenesis, reaching values below 50%, and contributed to increased efficacy of dexamethasone, acting synergistically in angiogenesis inhibition when both were combined as therapeutic agents.

Keywords

G-quadruplex aptamers; retinopathies; VEGF; angiogenesis; neovascularization.

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List of Abbreviatures

AGE – Advanced glycation endproducts
ALR2 – Aldose reductase gene
AMD – Age-related macular degeneration
Anti-VEGF – Anti-vascular endothelial growth factor
Ang-1 – Angiopoietin 1
Ang-2 – Angiopoietin 2
BRVO – Branch retinal vein occlusion
CD – Circular Dichroism
CA - Carbonic anhydrase
DME – Diabetic macular edema
DR – Diabetic retinopathy
FRET – Fluorescence resonance energy transfer
FT – Fluorescence titrations
G4 – G-quadruplex
GH-IGF - growth factor–insulin growth factor
K_D – Apparent equilibrium constant
KPi - Potassium phosphate buffer
NCL - Nucleolin
NMR – Nuclear Magnetic Resonance
PDR - proliferative diabetic retinopathy
PKC - Protein kinase C
RAS - Renin-angiotensin system
RAGE - Advanced glycation endproducts receptor
RD - Retinal detachment
TDS – Therma Difference Spectra
*T*m – Melting temperature
VEGF – Vascular endothelial growth factor
VH - Vitreous hemorrhage

1. Introduction

There is one Chinese proverb that says: “Present to the eye, present to the mind”, emphasizing the importance of this complex organ that plays a crucial role in perceiving our surroundings and providing information for understanding what is happening. Essentially, eyes are the door for our brain to process and assign meaning to everything we see, being equally important in human relationships, since “one look is more valuable than a thousand words”.

Given the complexity and vital role of our eyes in enhancing our quality of life, it becomes imperative to take good care of them. However, several complications that can arise impacting our life, and these situations are appearing more frequently due to the population aging, and surprisingly, the most affected areas are developed countries, despite the information available and health care access.

1.1. Anatomy and physiology of the human eye

The eye has three layers, compartments, and fluids (Figure 1). These multiple features help to protect the eye from all the environment aggressions.

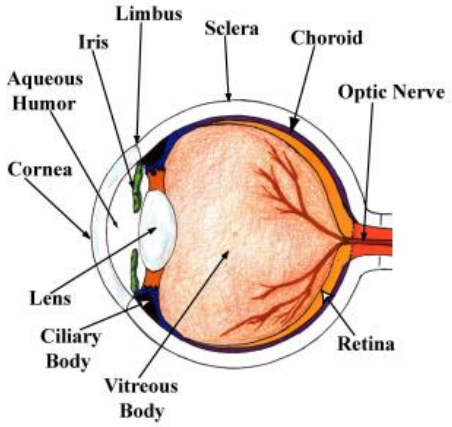

Figure 1 – Sagittal section of the eye (adapted from [1]).

Those three layers of the eye start with an outer fibrous layer (cornea, sclera, and lamina cribrosa), then move to a middle vascular layer (iris, ciliary body, and choroids), and finally we have the inner nervous layer (pigment epithelium of the retina, retinal photoreceptors and retinal neurons) [1], [2].

For further comprehension of these layers’ structures, starting at the outer fibrous layer, there are two parts, the anterior and the posterior. The anterior is formed by the cornea,

that is transparent, and the posterior is formed by the sclera and lamina, which is white, and these two parts are known as limbus, and they provide protection to the retina and other ocular tissues [2].

Afterwards there is the middle layer, that is composed by a structure called uvea, having the iris as the anterior part, being the posterior part called choroid [2].

The inner layer is the neurosensory layer, that forms the retina and has an anterior part that is composed by the ciliary body and iris. This layer is extremely important because it has photoreceptor cells on the retina [2].

To understand the function of these layers, it is important to know that the main function of the eye is to transmit a clear image of objects to the brain through the optic nerve, which starts with the refraction of the light through the cornea, being converted to electrical energy on the retina, allowing the transmission of the image to the brain. This main function of the retina is enhanced by the choroid, that provides oxygen and nutrients for the development of this function, and the outer layer that provide protection to the ocular tissues.

1.2. Retinal diseases

1.2.1. Neovascular retinal diseases

Neovascular retinal diseases involve the abnormal growth of new blood vessels (neovascularization) in the retina [3]. This is often associated with conditions that affect the blood vessels in the eye, leading to the growth of fragile and leaky vessels. Two primary neovascular retinal diseases are diabetic retinopathy (DR) and age-related macular degeneration (AMD) [3].

Treatment options for neovascular retinal diseases may include: Intravitreal Injections of anti-vascular endothelial growth factor (anti-VEGF) drugs to inhibit the growth of abnormal blood vessels [4]; Laser Therapy that may be used in some cases to seal or destroy abnormal blood vessels; and Photodynamic Therapy involving a combination of a light-activated drug and laser treatment to target abnormal vessels.

1.2.1.1. Diabetic retinopathy

Diabetic retinopathy (DR) is the leading cause of blindness in working age adults, affecting nearly 90% of the people with type 1 diabetes and nearly 80% of the people with

type 2 diabetes in a period of 10 years [5]. The major cause for this occurrence is the high levels of glucose and metabolic syndrome associated with diabetes (Figure 2). This disease begins to manifest by the development of ischemic zones within the retina, the formation of capillary microaneurysms and retinal exudation caused by capillary leakage. There are a variety of pathways from which the disease can appear [6], for example the carbonic anhydrase (CA) pathway, in which the vascular permeability is increased by the increase of pH, and this enzyme is overexpressed in diabetic retinopathy, being the reduction of CA a major factor for hypoxia reduction as it was shown in animal studies [7]. Another important pathway in this disease is the Growth Hormone and Insuline Growth Factor (GH-IGF) pathway, in which this hormone acts as a modulator for the retinal precursor cells, and this triggers angiogenesis as a response to hypoxia [6]. It is important to know that hyperglycemia, shown in Figure 2, is in the beginning of all these processes, by activating PKC, inducing oxidative stress, inflammation and renin-angiotensin system (RAS) pathway activation [6], [8]. Several studies have shown that genetic factors can increase an individual's susceptibility to developing DR [9]. These genetic factors may impact various aspects of the disease, including its severity, progression, and response to treatment [10], [11], [12]. One of the key genes implicated in DR is the gene called VEGF (vascular endothelial growth factor) responsible for encoding this growth factor, that plays a crucial role in the development of new blood vessels and is involved in the abnormal blood vessel growth observed in DR [13], [14].

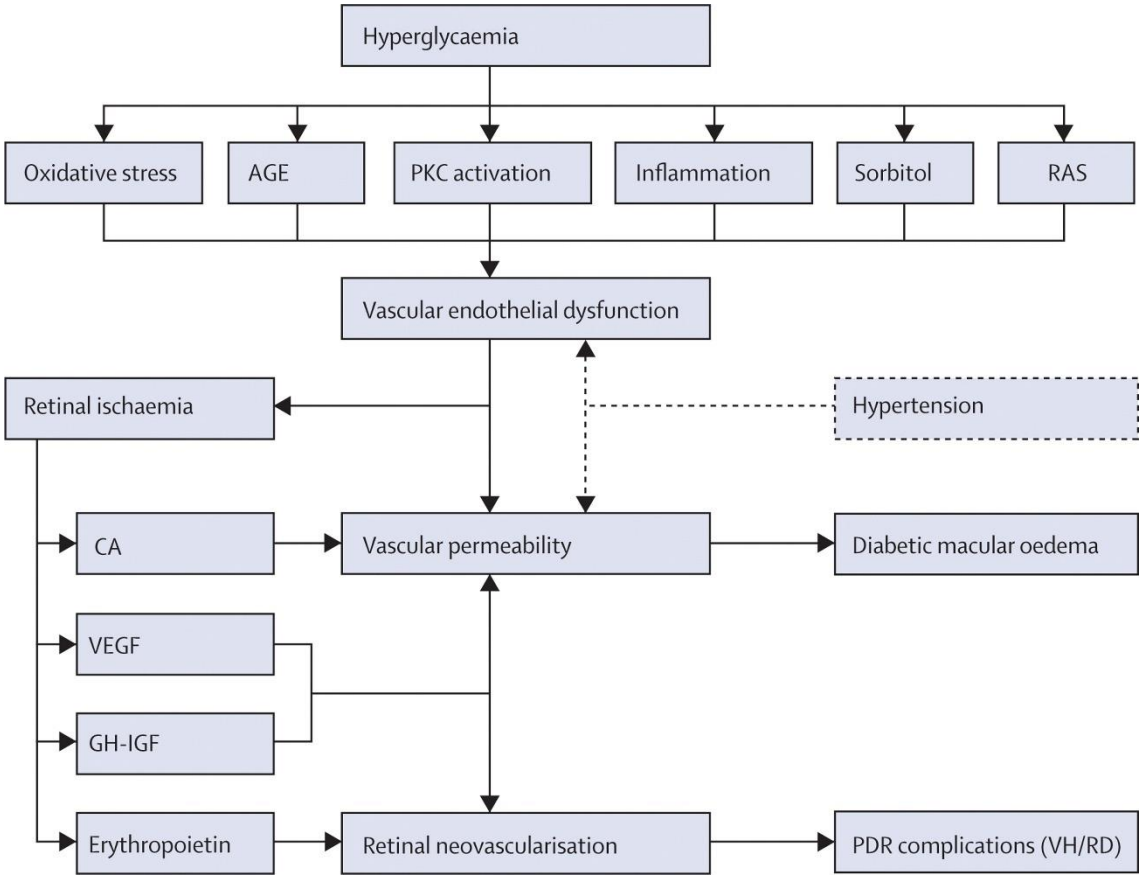

Figure 2 – Pathways in the development of DR (adapted from [6]).

Certain variations or mutations in the VEGF gene have been associated with an increased risk of developing DR and its progression to a more severe stage. Variations in other genes, such as the aldose reductase gene (ALR2) and the receptor for advanced glycation end products (RAGE) gene, have also been linked to DR [6].

However, it is important to note that genetic factors alone do not determine the development of DR. The interplay between genetic predisposition and environmental factors, such as blood sugar control, blood pressure, and duration of diabetes, also contribute to the development and progression of the disease [15].

Further research is still needed to fully understand the genetic components of DR and how they interact with various environmental factors. Such knowledge may help identify individuals at high risk for developing the condition and enable targeted preventive measures and treatments. Diabetic macular edema (DME) is the most advanced and severe state of DR, and in this cases, anti-VEGF injections have been used as a part of the treatment. Another treatment involves focal laser photocoagulation, which is expected to reduce moderate visual loss by 50% [16]. Nowadays, it was discovered that the inhibition of VEGF in the eye can offer an innovative approach instead of the intravitreal

steroids, which led to the approval of ranibizumab and aflibercept, by the FDA, to the treatment of patients with DR and DME [17].

1.2.1.2. Branch retinal vein occlusion (BRVO)

Branch retinal vein occlusion (BRVO) is a vascular disorder that occurs when a vein that carries blood away from the retina becomes blocked or occluded, leading to decreased blood flow and subsequent damage to the affected area of the retina [18]. BRVO can be classified into two types based on the location of the blockage. Branch Retinal Vein Occlusion – Macular Edema (BRVO-ME) occurs when the occlusion affects a smaller branch vein closer to the macula [19], [20] resulting in the accumulation of fluid or swelling in this area. Macular edema can lead to central vision loss or blurry vision. In Branch Retinal Vein Occlusion – Non-Macular Edema (BRVO-NME) the occlusion occurs further away from the macula, resulting in less severe visual symptoms [20], [21] However, some patients may still experience visual disturbances, peripheral vision loss, or distorted vision. The exact cause of BRVO is not always clear, but there are several risk factors that can contribute to its development, including atherosclerosis, hypertension and glaucoma [21], [22] . In atherosclerosis, hen fatty deposits (plaques) build up in the blood vessels, they can narrow the vessel and increase the risk of occlusion. Hypertension (high blood pressure) can strain the blood vessels, making them more prone to blockages [21], [22]. Glaucoma is a risk factor since result from increased intraocular pressure that may affect blood flow and contribute to BRVO [23]. DR can damage blood vessels in the retina and increase the risk of vein occlusion. BRVO is more common in older individuals. The severity and duration of symptoms may vary depending on the extent and location of the occlusion [21]. The treatment approach for BRVO aims to manage complications, improve vision, and prevent further progression. It may include Anti-VEGF injections of ranibizumab, aflibercept, or bevacizumab to reduce macular edema by blocking VEGF [4], [24], thus preventing abnormal blood vessel growth and fluid leakage. In some cases, laser therapy can be performed to seal leaking blood vessels and reduce swelling in the macula. Also, intravitreal injections of corticosteroids may be considered to reduce inflammation and macular edema. The visual outcomes of BRVO vary depending on factors such as the extent of the occlusion, location, and presence of macular edema. Some individuals may experience spontaneous improvement in vision over time, while others may have persistent visual impairments. Regular monitoring by an eye care professional is crucial to manage any long-term complications and adjust the treatment plan as needed [25].

1.2.1.3. Age-related macular degeneration

Age-related macular degeneration (AMD) is a condition that results in significant central visual impairment. It is an acquired degeneration that works through a combination of non-neovascular and neovascular derangement. AMD mainly affects adults older than 65 years, and it is estimated to impact around 170 million people worldwide [26].

Dry AMD (Non-neovascular) is the more common form of AMD, accounting for about 90% of cases. It is characterized by the presence of drusen, which are small yellow deposits that form under the retina. Dry AMD progresses slowly and may lead to a gradual loss of vision.

Wet AMD (Neovascular) is more severe. It occurs when abnormal blood vessels grow beneath the retina and leak fluid and blood, causing rapid and severe damage to the macula. Wet AMD can lead to a sudden and significant loss of central vision [27].

Blurred or distorted central vision and difficulty reading or recognizing faces are the most common symptoms, and the treatment for wet AMD may involve anti-VEGF medications injected into the eye to inhibit the growth of abnormal blood vessels.

Clinical cases classify AMD as “vision threatening AMD” and “non-vision threatening AMD”, being late stage and early stage respectively. The late stage includes geographic atrophy and exudative AMD, being known as wet AMD, which can be an evolution of geographic atrophy AMD, once it leads to the death of photoreceptors, and where new blood vessels are formed [28], [29], [30].

1.3. Targets and treatments for retinal vascular diseases

1.3.1. Targets for vascular diseases

Targeting specific genes in vascular diseases is crucial, primarily because it enables us to gain insights into the disease mechanisms. This understanding is pivotal for developing more precise and personalized treatments, as diseases manifest differently in individuals. Additionally, targeting specific genes is essential for early disease detection, making it easier to predict and intervene. This, in turn, facilitates drug development by providing a clearer understanding of the disease and its underlying mechanisms.

1.3.1.1. Angiopoietin-Tie pathway

The angiopoietin-Tie pathway is a signaling system involved in the regulation of angiogenesis, the process of new blood vessel formation [31]. This pathway consists of angiopoietin (Ang) ligands (Ang-1, Ang-2, Ang-3, and Ang-4) and their receptor, Tie-2 and is particularly relevant in the context of retinal vascular diseases, because it plays a crucial role in the development and maintenance of blood vessels, including those in the retina [32].

Ang-1 is a key ligand that activates the Tie-2 receptor. It is produced by pericytes, smooth muscle cells, and endothelial cells. Ang-1 promotes vessel stability, maturation, and integrity. It helps to maintain the quiescent state of blood vessels and inhibits leakage [32]. Ang-2 (Angiopoietin-2), on the other hand, is often considered to be destabilizing. It is produced by endothelial cells and can act as both an agonist and antagonist of Tie-2. In the context of angiogenesis, Ang-2 is involved in vessel destabilization and promotes angiogenesis [33].

Tie-2 Receptor is expressed on endothelial cells. When Ang-1 binds to Tie-2, it activates signaling pathways that promote vessel stability, maturation, and quiescence. This activation is essential for maintaining the integrity of existing blood vessels.

Ang-2/Tie-2 Interplay is crucial since Ang-2 can act as a context-dependent agonist or antagonist of Tie-2 [32], [33]. In situations where angiogenesis is needed, such as during tissue repair or in pathological conditions, like retinal vascular diseases, the Ang-2/Tie-2 interaction promotes vessel sprouting and growth.

Modulating the angiopoietin-Tie pathway has been explored as a potential therapeutic strategy for retinal vascular diseases [31], [33]. The goal is to restore the balance between Ang-1 and Ang-2, promoting vessel stabilization and inhibiting abnormal angiogenesis. There is already a successful antibody designed for intravitreal use, and it can simultaneously bind to and neutralize Ang-2 and VEGF-A, controlling pathological angiogenesis in retinal diseases, the name is Faricimab [34]. This pathway has another study associated to it, but not for the same disease, however, the target remains the same, and there are inhibitors that were in clinical trial phase by 2021, like vanucizumab and Tavi6 [35].

1.3.1.2. Complement System

The complement system is a part of the immune system that plays a role in the inflammatory response and immune surveillance (Figure 3). In the context of retinal vascular diseases, the complement system has been implicated in various pathological processes, including inflammation, oxidative stress, and damage to blood vessels [36], [37]. Targeting the complement system in the treatment of retinal vascular diseases is an area of active research and several potential strategies have been explored.

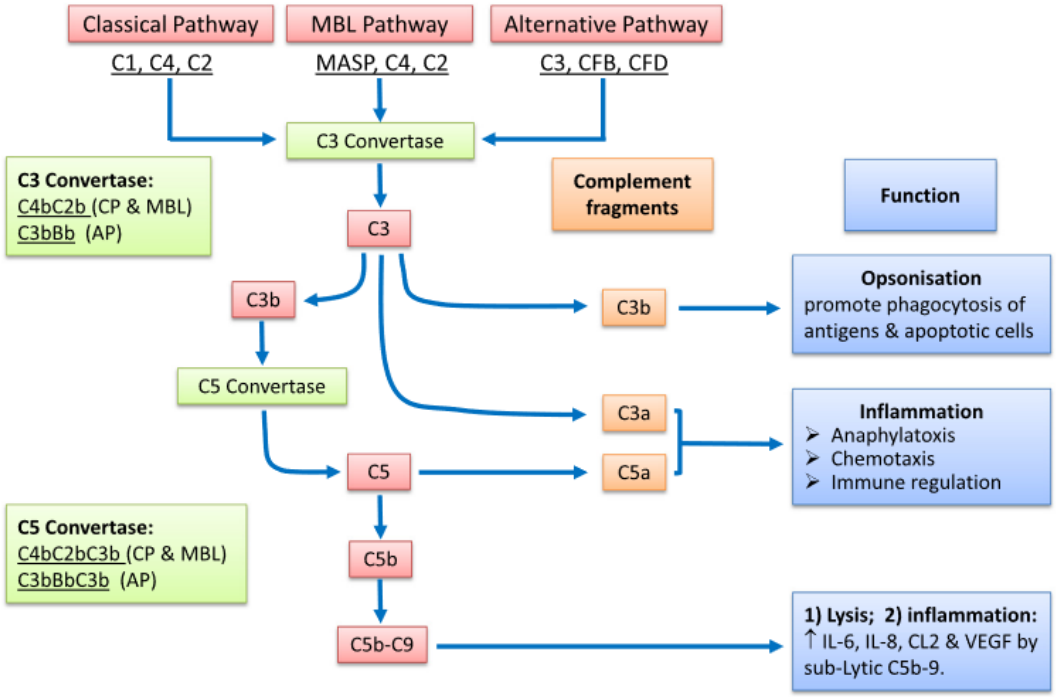

Figure 3 – Complement system activation and immune regulation (adapted from [36]).

Drugs that specifically target components of the complement system can be developed to inhibit its activity. For example, inhibitors of complement proteins, such as C3 and C5, have been studied [37], [38], [39]. Eculizumab, a monoclonal antibody that targets C5, has been used in certain disorders associated with complement dysregulation.

Blocking the receptors that mediate the effects of complement activation can also be a therapeutic strategy. This approach may prevent the downstream inflammatory responses associated with complement activation.

In addition to directly targeting complement components, drugs that inhibit downstream inflammatory cytokines produced due to complement activation may be beneficial to mitigate inflammation and reduce damage to retinal blood vessels [36].

Introducing factors that regulate the activity of the complement system can also help to maintain its balance. These regulators may prevent excessive activation and subsequent tissue damage. For example, factors like CD55 and CD59 are natural regulators of the complement system [37], [39].

Gene therapy techniques can be explored to modulate the expression or activity of specific complement components addressing the underlying genetic factors contributing to complement dysregulation in retinal vascular diseases [36], [40]

Given the complexity of pathways and multiple factors involved in retinal vascular diseases, combination therapies targeting both complement and other relevant pathways may be more effective in managing the diseases.

In Table 1 we can see the checkpoint of inhibitors for this cascade that were approved or in clinical trial phase by 2023.

Drug	Target
Ravulizumab	C5 inhibitor
Avacopan (CCX168)	C5a receptor blocker
Cemdisiran	Small interfering mRNA inhibitor of synthesis of C5
Pegetacoplan (APL-2)	C3 inhibitor
Iptacopan (LNP023)	Complement fB inhibitor
IONIS FB-LRx	Complement fB inhibitor
Narsoplimab	MASP-2 inhibitor
LNP023	Factor D inhibitor

Table 1 – Inhibitors for complement system approved or in clinical trial by 2023 (adapted from [41]).

1.3.1.3. VEGF

VEGF is a protein that plays a crucial role in angiogenesis, the formation of new blood vessels from existing ones. VEGF is essential for the normal development and maintenance of blood vessels in the body.

In the context of DR, the role of VEGF becomes particularly significant. Persistent high blood sugar levels associated with diabetes can result in damage of the small blood vessels that supply the retina [24]. In response to this damage, the body increases VEGF production as a compensatory mechanism to promote the growth of new blood vessels and improve blood supply to the retina.

However, in DR, the production of VEGF can become excessive and uncontrolled which leads to the growth of abnormal blood vessels, a characteristic feature of the disease. The abnormal blood vessels are fragile, leaky, and prone to bleeding, causing further damage to the retina and potentially leading to vision loss if left untreated [24]. Given its role in abnormal angiogenesis, VEGF has become a target for treatment in DR and other retinal diseases. Anti-VEGF drugs, such as ranibizumab, aflibercept, and bevacizumab, are commonly used to inhibit the effects of VEGF and reduce the growth of abnormal blood

vessels [4]. These drugs are often injected into the eye to directly target the retina and help manage the progression of DR [42].

It's worth noting that while anti-VEGF therapy has shown significant benefits in many patients, there can be variations in individual responses and treatment outcomes. Factors such as the severity and stage of the disease, patient characteristics, and adherence to treatment play significant roles in determining the effectiveness of anti-VEGF therapy for DR [42].

1.3.2. Aptamers

The word aptamer is derivative from the Latin *apus* to fit, and the Greek *meros*, part of a region, and this is clearly suggestive for the meaning and significate of aptamer, that refers to oligonucleotides that have the capacity to bind to a target with high affinity and specificity. These oligonucleotides are small sequence-specific nucleic acids (DNA or RNA) with 20-100 nucleotides, and often compared to antibodies. In fact, despite yielding the same effect in terms of target interaction, aptamers have the advantage of efficient synthesis, because of the absence of cell or animal culture requirements during the production process [43], [44].

The method used to obtain aptamers is SELEX (*Systematic Evolution of Ligands by Exponential Enrichment*). This technique consists of using oligonucleotides of DNA or RNA as primers and exposing them to the target molecule. Unbound sequences are excluded, and those that bind are subsequently amplified through PCR, for further optimization of those binds, as it is shown in figure 4.

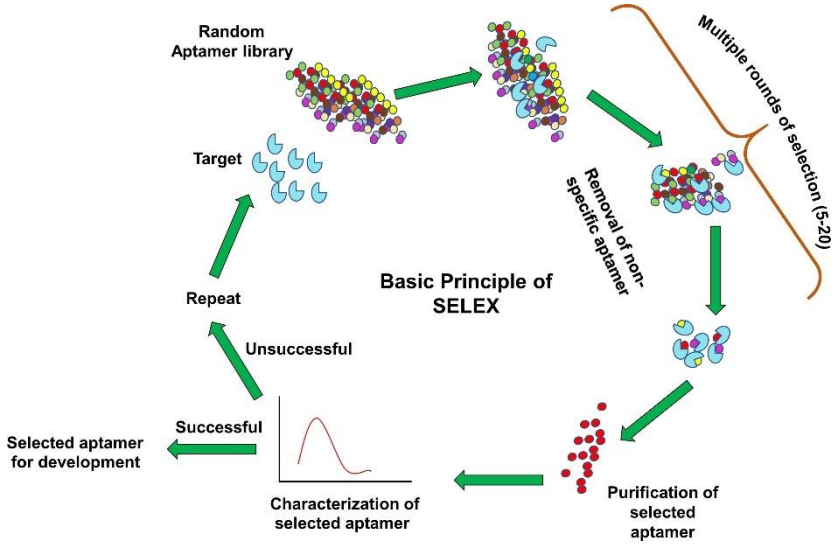

Figure 4 – SELEX method (adapted from [45]).

Aptamers take advantage of an important feature called G-quadruplex (G4) structure (Figure 5), that have G-quartets as motifs. Each G-quartet is formed by four guanines stabilized and linked by Hoogsteen bonds in a co-planar square, and their accumulation forms the G4 structure, that is stabilized by cations, such K^+ or Na^+, at the center of the G-quartet. G4 can adopt three conformations: parallel, anti-parallel and hybrid [46], resulting from different polarities [46].

G4s not only are important for regulatory roles and biological processes, but also for carcinogenic processes like the growth and development of tumors and neurological disorders, therefore, the pathway of therapeutic methods using G4 is being considered [47], [48], [49].

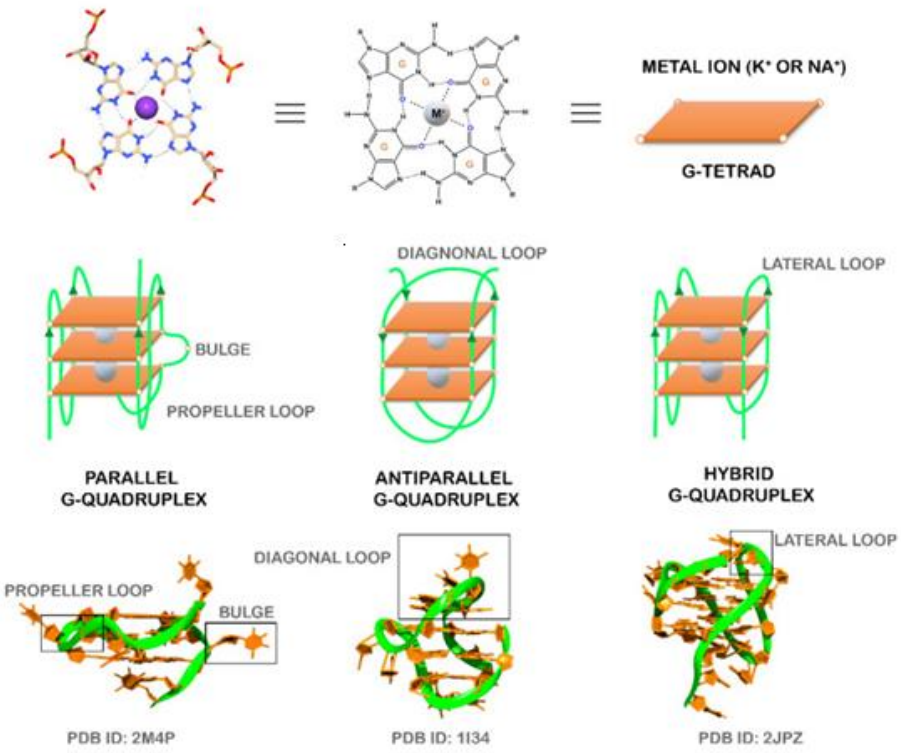

Figure 5 – Structure of a G-quartet and the different topologies of G4 (adapted from ([50]).

Aptamers can have often be used in innumerous processes in medicine (Figure 6). One example of an aptamer that is well known by the scientific community for having a high anti-cancer potential is the AS1411, which binds directly to nucleolin (NCL) NCL, that is an integral part of the ribosomal biogenesis. NCL, a widespread protein, that plays diverse roles in biological processes. Predominantly expressed in the cell nucleus, it regulates ribosome genesis and ribosomal RNA synthesis. Additionally, in the cytoplasm NCL functions as a shuttling protein between ribosomes and the nucleus. Notably, NCL

exhibits high expression on the cell surface of proliferating cells, influencing the regulation of growth, adhesion, and angiogenesis.

This aptamer is also implicated in antiviral activity against HIV [51], [52]. Another aptamer that has already been studied and it is even commercialized is Pegaptanib, also known by Macugen (brand name). Pegaptanib is an anti-VEGF agent, which means that it helps to reduce the growth of abnormal blood vessels, and it slows the progression of AMD. This aptamer is administered via intraocular injection, and it is an RNA aptamer chemically modified with polyethylene glycol (PEG) [53], [54]. The pegylation of aptamers helps to improve its stability and pharmacokinetics, making it more suitable and efficient for therapeutic use. Pegaptanib has been approved in 2000, being one of the first approaches to these diseases, which subsequently led to the development of another anti-VEGF agents like Ranibizumab [55] and Aflibercept [56].

Despite the lack of studies regarding the use of aptamers in retinal diseases, the high affinity, for example, of the AS1411 for NCL [57] suggest its potential success in inhibiting it. This marks a promising development towards retinal diseases, since NCL is overexpressed in these conditions.

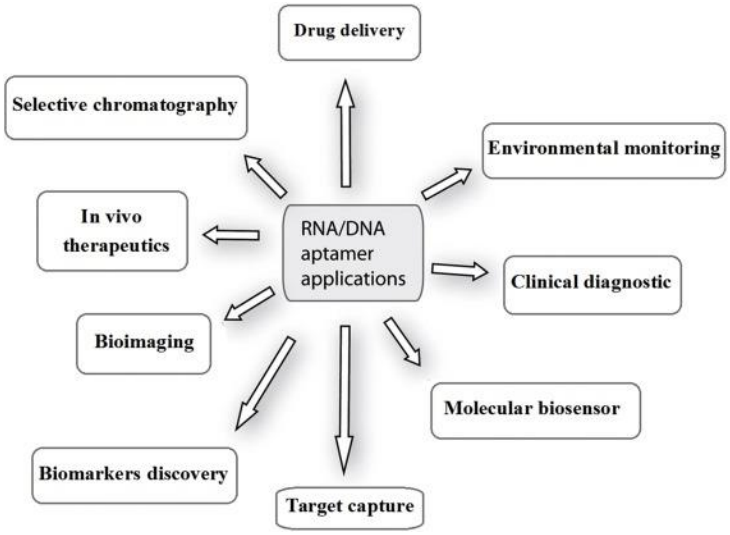

Figure 6 – Various application fields of aptamers (adapted from [58]).

1.3.2.1. Structural characterization of aptamers using biophysical techniques

For further comprehension and analysis of aptamers, several biophysical studies are used to characterize both conformational and interaction properties, such as nuclear

magnetic resonance (NMR), fluorescence titrations (FT), circular dichroism (CD) and thermal difference spectra (TDS).

NMR can be used to study structural, dynamics of G4 and ligands complexes and kinetic properties. The principle of NMR consists of every nucleus being electrically charged and many of them have spin, which means that if we apply an external magnetic field, there is a possibility that an energy transfer occurs. This transfer is going to have a certain wavelength in which we will be able to identify the corresponding radio frequency. Ideally, the conditions that are used for this method are set to mimic cellular media, but as the technique got optimized, it started to work under conditions closer to the ones that we can see in living cells, or even directly on them, such as oocytes [59]. As said before, the most important signals to analyze in G4 and G4/ligand complexes are the ones resulting from Hoogsteen pairing. This signal is expected to appear near the region of 10-12 ppm, indicating the formation of the G4 [50], [57]. NMR has valuable advantages, but it has some disadvantages too, such as the time required to perform the technique and the concentration of aptamer and ligands [50], [57].

To determine the binding affinity between the G4 aptamer and the target or ligand, we can employ fluorescence titration, in which the aptamer or the ligand must have a fluorophore capable of emitting fluorescence upon reaching a specific level of excitation [60], [61]. This is important because the fluorophore concentration will have impact on the emitted fluorescence along with factors like the temperature and the intensity of the excitation source. Another experiment based on the fluorescence domain that can be conducted is fluorescence resonance energy transfer (FRET). This method assists in gathering information about stability of a G4 conformation and potential interactions with the ligand. FRET is a straightforward technique based on an energy transfer between a previously excited donor and an acceptor.

FRET- melting analyzes the folding and unfolding states, with the maximum fluorescence, indicating the completely unfolded state of the molecule [62]. Unlike NMR, FRET requires a low quantity of sample, but on the other hand, it requires a proper labeled aptamer and the information that provides is limited.

CD (Figure 7) is another method to study chiral molecules by providing information about the conformation or secondary structure of proteins. This is particularly valuable as protein structures are sensitive to environmental, temperature or pH changes. The utilization of this method can provide structural, kinetic, and thermodynamic information about macromolecules such proteins or nucleic acids. CD measures a range of wavelength at the visible and ultra-violet region of the electromagnetic spectrum, and it is based on the differential absorbance by a substance of right and left-handed circularly polarized light (Figure 7).

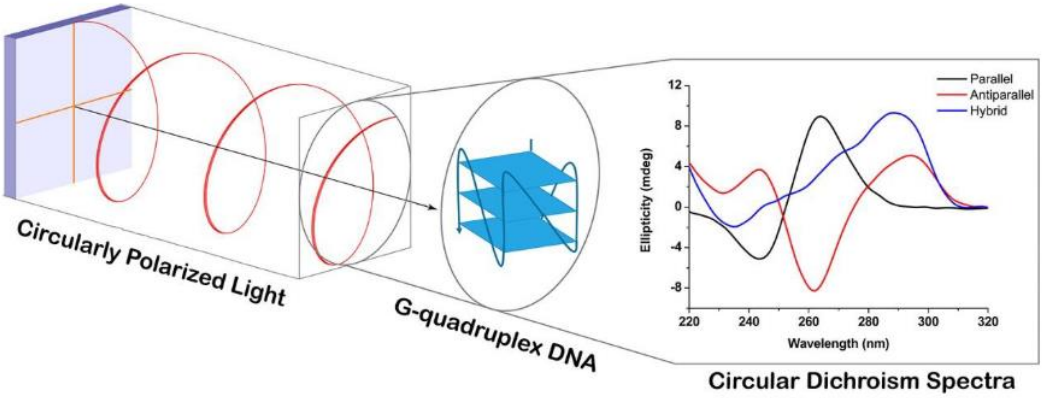

Figure 7 – Schematic representation of CD operation mode and the different spectra topologies for G4 (adapted from [63]).

This technique requires a small amount of sample and offers valuable information about the G4, since it can show the type of conformation obtained, as different conformations exhibit different graphical patterns. For example, antiparallel G4 has two positive peaks, one at about 295 nm and the other at about 240 nm, but it also has a negative peak located at 260 nm. Parallel G4 has only one positive peak at about 260 nm and a negative peak located at about 240nm, while hybrid G4 has his two positive peaks located at 295 nm and 260 nm and its negative peak located at 240 nm [50], [64]. A drawback of CD is its low resolution, rendering it incapable of detecting multiple conformations of the same molecule [65], [66].

1.3.2.2. Aptamers in retinal diseases

Aptamers exhibit remarkable potential for the inhibition of pathological pathways associated with retina angiogenesis, which led to different aptamer-based developments as highlighted in table 2. To this investigation, we focused on the VEGF target, where we identified two aptamers that already have been developed for it [67], [68]

Aptamer	Target	Disease	Application
ky2	Kanamycin B	Bacterial keratitis	Bind with kanamycin B and construct a drug carrier system
neo5	Neomycin B	Bacterial keratitis	Bind with neomycin B and construct a drug carrier system
S58	TGF-β receptor II	Glaucoma	Anti-fibrosis after filtration surgery
NX1838	VEGF	Glaucoma	Anti-fibrosis after filtration surgery
		Neovascular glaucoma	Anti-angiogenesis
		AMD	Anti-angiogenesis
F1	Brain-derived neurotrophic factor	Glaucoma	Constructed biolayer Interferometry-based 3D aptasensor for early diagnosis
ARC126/E10030	Platelet-derived growth factor	AMD	Anti-angiogenesis
APT-F2P/RBM-007	Fibroblast growth factors 2	AMD	Anti-angiogenesis and anti-fibrosis
Avacincaptad pegol	Complement component 5	AMD	Reduce geographic atrophy growth
AS1411	Nucleolin	AMD	Reduced choroidal neovascularization and attenuated infiltration of macrophages
		Retinoblastoma	Inhibit tumor growth
Apt-3	C promoter binding factor 1	AMD	Anti-angiogenesis
CD44 aptamer	CD44	AMD	Delivery of drugs for retinal pigment epithelial
V7t1	VEGF	AMD	Constructed target-induced dissociation assay for detecting VEGF
#4s aptamer	Advanced glycation end products	DR	Inhibit early development of disease
APT1	Connective tissue growth factor	DR	Constructed BLI-based enzyme-linked aptamer sandwich assay for early diagnosis
APT12TM	Lipocalin 1	DR	Early diagnosis
XQ-2d	CD71	Uveal melanoma	Inhibit tumor growth
HMGAap	High mobility group A	Retinoblastoma	Inhibit tumor growth

Table 2 - Aptamers applied in ophthalmology (adapted from [54]).

Aptamers can be designed to bind specifically to biomarkers associated with retinal diseases [67]. When labeled with imaging agents, aptamers can be used for diagnostic imaging techniques, such as optical coherence tomography (OCT) or molecular imaging, to detect and visualize specific molecular signatures indicative of retinal diseases.

Also, by conjugating aptamers to therapeutic agents, drugs can be delivered specifically to the affected retinal cells, minimizing off-target effects, and improving the efficiency of treatment [68]. This targeted approach may enhance the therapeutic efficacy while reducing potential side effects.

In diseases like DR and AMD, abnormal angiogenesis can contribute to vision impairment. Aptamers can be designed to target and inhibit specific factors involved in angiogenesis, thereby helping to control the pathological growth of blood vessels in the retina.

Aptamers can be engineered to target molecules associated with neurodegenerative processes in the retina. By binding to specific factors involved in neurodegeneration, aptamers may have a neuroprotective effect, potentially slowing down or preventing the progression of diseases affecting the neural tissues of the retina [68].

Due to the role that inflammation plays in various retinal diseases, aptamers can be developed to modulate the immune response by targeting specific inflammatory mediators. This approach may help in controlling the inflammatory processes associated with retinal diseases [69], [70].

Aptamers can also be valuable tools in research settings for studying the molecular mechanisms underlying retinal diseases. They can be used to selectively isolate, and study specific proteins or other biomolecules associated with pathological processes in the retina.

2.Aims of work

This work aimed to verify the angiogenesis inhibition capacity of AT11 conjugated with dexamethasone, by inhibiting the VEGF pathway.

For this purpose, we defined the following list of objectives:

1. To characterize AT11 as G4 aptamer for targeting VEGF;
2. To evaluate AT11 interaction with dexamethasone and VEGF;
3. To evaluate in vitro cell viability and angiogenesis inhibition of AT11 conjugated with dexamethasone in a HUVEC model.

3.Materials and Methods

3.1. Materials and reagents

For the preparation of the solutions used in this work, ultrapure-grade water obtained from a Mili-Q system from Millipore (Burlington, MA, USA) was utilized.

AT11 (5′-TGG-TGG-TGG-TTG-TTG-TGG-TGG-TGG-TGG-T-3′) was purchased from Eurogentec (Seraing, Belgium) with HPLC-grade purification and a purity of 98%, while 5'-Cy5- TGG-TGG-TGG-TTG-TTG-TGG-TGG-TGG-TGG-T-3′ was acquired from STAB-VIDA (Caparica, Portugal) with a purity of 98 % too. Stock solutions were prepared using Milli-Q water and stored at -20 °C until use.

To determine the concentration of the oligonucleotide samples, the absorbance was measured at 260 nm with a UV-Vis spectrophotometer (Thermo Scientific™ Evolution 220) using the molar extinction coefficient (ε) provided by the manufacturer.

Normal Primary Human Umbilical Vein Endothelial Cells (HUVEC) (PCS-100-010), Vascular Basal Cell Medium (PCS-100-030), and Endothelial Cell Growth Kit-VEGF (PCS-100-041) were acquired from ATCC (Manassas, Virginia, USA). 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was acquired from Thermo Fisher Scientific (Waltham, MA, USA, CAS: 298-93-1).

Angiogenesis Assay Kit (*In vitro*) (ab204726) was acquired from Abcam (Cambridge, UK).

Dexamethasone was acquired from Tokyo chemical industry (Japan, CAS: 50-02-2; purity ≥ 99 %), and stocked on a 10 mM DMSO solution (Thermo Fisher Scientific, Waltham, MA, USA, CAS: 67-68-5, purity ≥ 99,5 %). The oligonucleotide was annealed in 20 mM potassium phosphate buffer (KPi) containing 65 mM KCl (Labkem, Barcelona, Spain, CAS: 7447-49-7, purity ≥ 99%) (working buffer) before each experiment by heating it for 10 min at 95 °C followed by an ice cooldown of 10 min. To prepare KPi (pH 6.9), 1.945 g of KH_2PO_4 (Chemlab, Zedelgen, Belgium, CAS: 7778-77-0, purity ≥ 98%) and 0.990 g of K_2HPO_4 (Honeywell, USA, CAS: 7758-11-4, purity ≥ 99%) were mixed and solubilized on water until perform 1 L.

3.2. Methods

3.2.1. Characterization of G4 structure from AT11

3.2.1.1. CD titration and CD-melting

The equipment used for CD experiments was a Jasco J-815 CD spectropolarimeter with a Peltier-type temperature controller (model CDF-426S/15). For the reading's achievement, quartz cuvettes with 1 mm path-length at 20 °C were utilized. The spectral width was set to 220-340 nm, with 1 nm bandwidth, 1 s integration time over 3 averaged accumulations and a speed of 200 nm/min. AT11 was annealed as previously described before the CD spectra measurement.

In order to determine the melting temperature (T_m) of the G4 structure with and without dexamethasone, it was performed a CD-melting, on the Jasco J-815 CD spectropolarimeter. AT11 was annealed the same way as described, being dexamethasone directly added to the quartz cell to perform 0, 1 and 2 molar equivalents from a 10 mM stock. The wavelength of maximum ellipticity (260 nm) was monitored at temperatures ranging from 20 °C to 100 °C with a heating rate of 2 °C/min.

Data were converted into fraction folded (f) plots according to Eq. (1) and fitted to a Boltzmann distribution using OriginPro2021:

$$f = \frac{CD - CD_{\lambda}^{min}}{CD_{\lambda}^{max} - CD_{\lambda}^{min}} \qquad \text{Eq. (1)}$$

A two-state transition model was used to determine the T_m, where CD is the ellipticity at each temperature and CD^{max} and CD^{min} are the highest and lowest ellipticities, respectively. T_m have been calculated based on the corresponding temperature value to half of the total reached ellipticity. Data has been normalized and fitted to Boltzmann distribution using OriginPro2021.

3.2.1.2. Fluorescence titration

Dexamethasone as a ligand was also tested using fluorescence titrations. These experiments were conducted on a Horiba FluoroMax 4 fluorometer (Japan) equipped with a Peltier-type temperature control system at 20 °C.

Samples were analyzed in a high-precision quartz suprasil cuvette (light path 10 mm × 4 mm) with a 700 µL optimal volume. Before the titrations were performed, a solution of 5´-Cy5-AT11 at 1 µM concentration has been prepared and annealed as previously described on the working buffer. Excitation wavelength was 647 nm, the same as the maximum absorbance of Cy5 fluorophore. Fluorescence spectra have been acquired between 660 and 800 nm with 0.5 s integration time, an emission slit fixed at 2 nm, an excitation slit fixed at 1 nm, and a step size of 1 nm, averaged over 3 scans. To calculate the AT11/ligand affinity, the change in fluorescence adding increasing concentrations of ligands was measured by fluorescence titrations. After each addition, 10 min were necessary for the solution equilibration, and then the spectrum has been acquired. Data was converted into a fraction of bound ligand (α) plots using the following equation (Eq. 2):

$$\alpha = \frac{I - I_{\lambda}^{free}}{I_{\lambda}^{bound} - I_{\lambda}^{free}} \qquad \text{Eq. (2)}$$

here, I is the fluorescence intensity at 667 nm at each AT11/ligand ratio and I_{bound} and I_{free} are the fluorescence intensity of the fully bound and free AT11 respectively. Data points were fitted afterwards according to the most suitable model (Hill) using Origin Pro 2021.

We moved on using the same procedure to determine the affinity of AT11 to dexamethasone and AT11 and AT11/dexamethasone complex to VEGF. For AT11 titration with VEGF, AT11 concentration was set at 1 µM, while adding VEGF with concentrations in the range of 0 to 20 nM and measuring in every 10 minutes after the addition. AT11/dexamethasone complex had the same 1 µM value for the concentration, as well as dexamethasone, varying once again the concentration of VEGF after 10 min of equilibration (from 0 to 20 nM), being the spectra recorded 10 minutes after each protein addition. AT11 was titrated with dexamethasone too, at concentrations of 0 to 50 nM.

3.2.2. In vitro assays

3.2.2.1. Cell line

In vitro studies were performed with HUVEC (PCS-100-010) acquired from ATCC (USA). These cells proliferate in a Vascular Basal Cell Medium (ATCC, PCS-100-030, USA), and they are adherent cells that can have VEGF added or not to the media. The appearance of these cells is cobblestone with large dark nuclei, and they present a high

mitotic index, being small and evenly sized during proliferation. The main cause for the selection of these cells was the fact that this line is the model which is typically used for anti-angiogenic effects of any substance evaluation. After the addition of an adequate extracellular matrix the angiogenic process is stimulated leading to the formation of tubes by these cells.

3.2.2.2. MTT assay

The cellular viability when exposed to both dexamethasone and AT11, was evaluated by MTT assay. HUVEC were harvested when 80-90% confluency was reached and seeded on 96-wells using 100 µL of Vascular Basal Cell Medium supplemented with Endothelial Cell Growth Kit-VEGF from ATCC at a density of 5×10^3 cells per well. The medium was removed 24 h after, and new medium containing AT11, dexamethasone or a combination of both, was added to the wells. For the new medium that was added after 24h, different concentrations and proportions of compounds were used (10 µM of dexamethasone and 100 µM of aptamer and for the combination of both AT11 and dexamethasone, proportions of 1:1 and 1:2, respectively, were used). Once again, medium with the tested stimuli was removed, after 72 h, and 100 µL of MTT was added at a concentration of 1 mg/mL. After 4 h, MTT was removed, and DMSO was used to dissolve formazan crystals. Once the crystals were dissolved, absorbance was read on a plate reader (Bio-rad xMark spectrophotometer from Bio-rad, USA) at 570 nm.

3.2.2.3. Angiogenesis assay

To test the anti-angiogenic properties of the compounds used, an Angiogenesis Assay Kit (*In vitro*) was used. This kit was acquired from Abcam, and the manufacturer's guidelines were followed. Like in the MTT assay, HUVEC were harvested when reaching 80-90% confluency and seeded on 96-wells using 50 µL of extracellular matrix provided with the kit, with a density of 5×10^3 cells per well, being the negative controls seeded without the matrix. The stock compounds were prepared at concentrations of 10 µM for dexamethasone and 100 µM for the aptamer. In the experimental wells, isolated dexamethasone and isolated AT11 were tested at concentrations of 0.5, 1, and 2 µM. Additionally, a combination condition was tested with a 1:2 ratio of AT11 to dexamethasone, where the concentration of AT11 remained the same as in the isolated AT11 tests.The incubation proceeded for 18 h, being after incubated for 30 min with the staining dye provided with the kit and were carefully washed to proceed to the

observation on a microscope (AxioObserver Z1) using light and fluorescence. Results were analyzed using ImageJ software.

3.2.2.4. Statistical analysis

All data was analyzed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla California USA). Statistical significance of these studies was evaluated with resource to one-way ANOVA and p-values ≤ 0.05 were considered as significant.

4.Results and discussion

The main goal of this chapter is to evaluate the potential of the AT11/ligand complexes to inhibit angiogenesis in neovascular diseases.

4.1. Characterization of G4 structure in AT11

When performing CD titrations, it is important to vary experimental conditions such as pH, temperature, or ligand concentration to observe changes in the circular dichroism spectra, and by that, acquire the maximum information in relation to any change that can happen to our object of interest.

In this work, KPi was used as buffer, because it offers many advantages such as maintaining constant pH (some biomolecules often have pH dependent conformation and stabilities), buffering capacity, (minimizes pH fluctuations during the titration and ensures the stability of the environment), compatibility with biomolecules (these buffers are generally considered biologically compatible hence are less likely to interfere with the structural integrity of proteins and nucleic acids), and adjusting ionic strength (buffers ionic strength can be optimized to mimic physiological conditions or specific experimental requirements) [71], [72].

KCl in the buffer solution when working with G-quadruplex-forming aptamers is needed to optimize the conditions for G4 stability, since the concentration of KCl may be adjusted based on the specific requirements of the experiment; hence, the use of KCl is crucial for maintaining the G4 and ensuring that the aptamer adopts the desired conformation for its functional role, such as binding to a target molecule with high specificity.

The CD profile of AT11 is described in literature as a right-handed parallel-stranded G4 [73], [74], showing one minimum at 240 nm and a positive peak at 260 nm at the CD spectra [74] which is the expected profile when running the experiment for the characterization.

4.1.1. CD titration

CD titration of AT11 was performed by adding increasing concentrations of dexamethasone between 5 and 20 μM, and the spectra obtained are present in Figure 8.

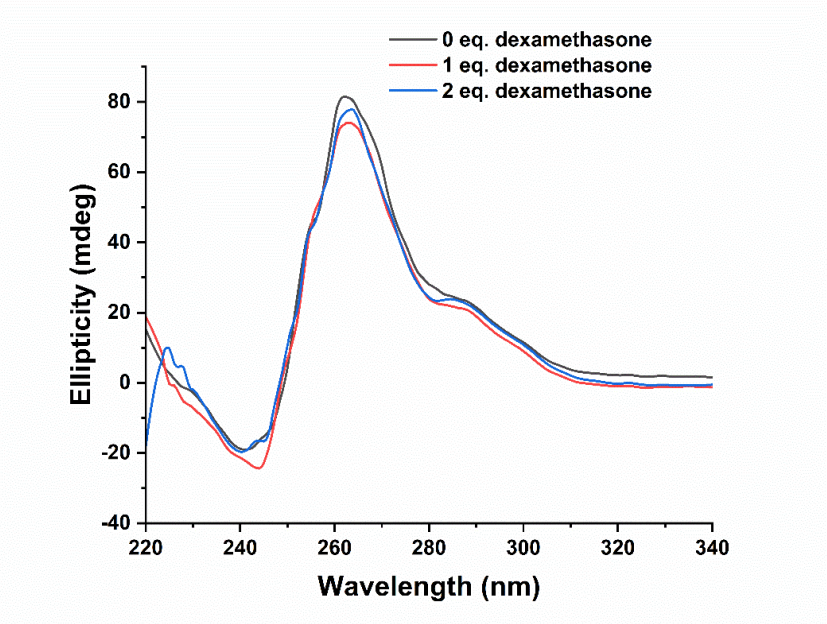

Figure 8 – CD spectra of AT11 with concentrations of dexamethasone from 5 μM to 20 μM.

The spectra showed a typical parallel G4 structure, as we can see at Figure 8, by the positive peak at 260 nm and the negative one at 240 nm, that are specific for this kind of structure [74]. The purpose of this experiment was to observe the pattern of AT11 by adding dexamethasone to it, seeing if the conformation changes or not. As we can see, the spectra indicates that there was no alteration in the conformation of the oligonucleotide, suggesting that from equimolar to doubled equivalents of dexamethasone, AT11 maintains a well-defined G4 structure with a parallel topology, confirming the AT11 stability in presence of dexamethasone across a range of concentrations.

4.2. Interaction of AT11 with ligands

4.2.1. CD melting

To evaluate ΔT_m and the grade of stabilization of the G4 structure for dexamethasone, it was needed to perform CD-melting assays, to acquire more precise data. As shown in Figure 9, we can observe that the melting temperature without the ligand is 41.8 °C. When equivalents of dexamethasone were added, the curves are almost overlapped and the *T*m does not change, indicating that dexamethasone did not promote a thermal stabilization of the G4 structures presented in Figure 9.

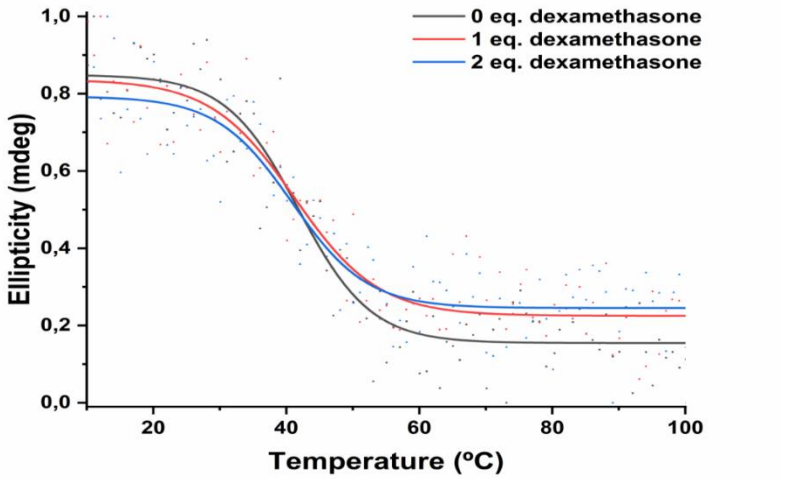

Figure 9 – CD-melting curves from 0 to 2 equivalents of dexamethasone.

Similar findings were also obtained with the AT11-L0 aptamer and dexamethasone, wherein no thermal stabilization was evident across various concentrations of the ligand. In contrast, only C8 and PhenDC3 induced a concentration-dependent thermal stabilization of the structure [68].

4.2.2. Fluorescence titrations

For the determination of the apparent equilibrium constants (K_D) of dexamethasone and VEGF, we carried out fluorometric titrations, observing a decrease in the intensity once these ligands were added to the pre-folded 5'Cy5-AT11.

Spectra were acquired between 660-800 nm and by plotting the maximum fluorescence intensity recorded at 667 nm K_D was determined. Fitting was made using Hill saturation binding model for the three cases and we analyzed K_D and Hill Coefficient (n).

In Figures 10 and 11 we can observe the fluorescence titrations between AT11 and dexamethasone and between AT11 and VEGF, respectively.

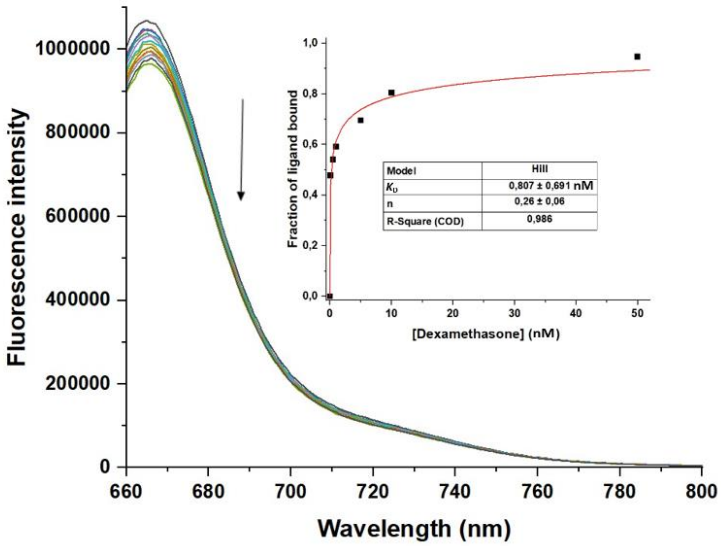

Figure 10 – Fluorescence intensity spectra and K_D determination of AT11 (1 μM) titrated with dexamethasone.

Dexamethasone showed a low K_D, in the nanomolar order, meaning that dexamethasone have a slightly high affinity for AT11 (K_D = 0.807 ± 0.691 nM). The fluorescence intensity and wavelength remain with nearly the same pattern over concentration variations, supporting that dexamethasone and AT11 can be used as a synergy option for VEGF targeting. However, despite the partially positive results of K_D, we obtained n = 0.26 ± 0.06, which is an indicator of negative cooperative binding, meaning that the binding of subsequent substrate to other active binding sites is discouraged after the first bind being achieved. These results show that despite the high affinity that AT11 has for

dexamethasone, saturation can be achieved faster, since n < 1. With this information, we next had to confirm the affinity of AT11 to VEGF.

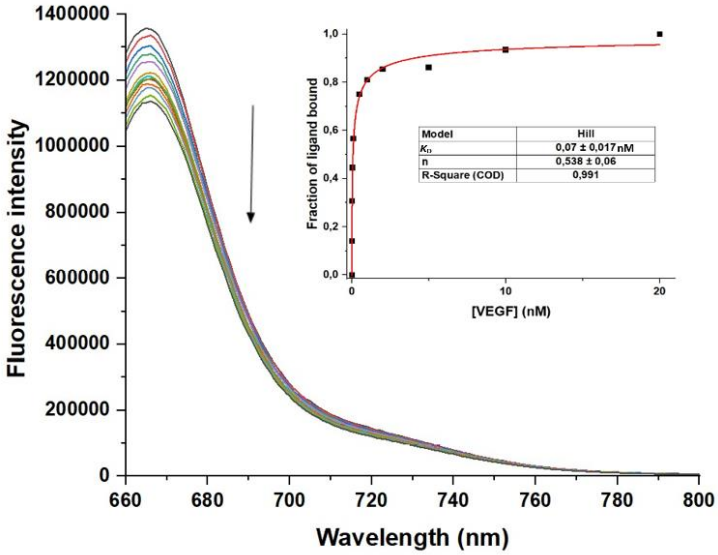

Figure 11 – Fluorescence intensity spectra and K_D determination of AT11 (1 μM) titrated with VEGF.

In Fig. 11 we acquired the spectra of fluorescence intensity of AT11 titrated with VEGF, and we obtained K_D = 0.07 ± 0.017 nM, being in the nanomolar order, indicating a high affinity of AT11 to VEGF, providing the information that AT11 can be used to target this growth factor. However, a similar issue to the one described in the dexamethasone titration was observed, with n = 0.538 ± 0.06. Despite this value is higher than the one showed for dexamethasone, it remains < 1, which indicates that when a molecule of VEGF binds to AT11, it prevents the subsequent binding of other VEGF molecules, suggesting that the saturation can be rapidly achieved, as we can see for the graphic curve in Figure 11.

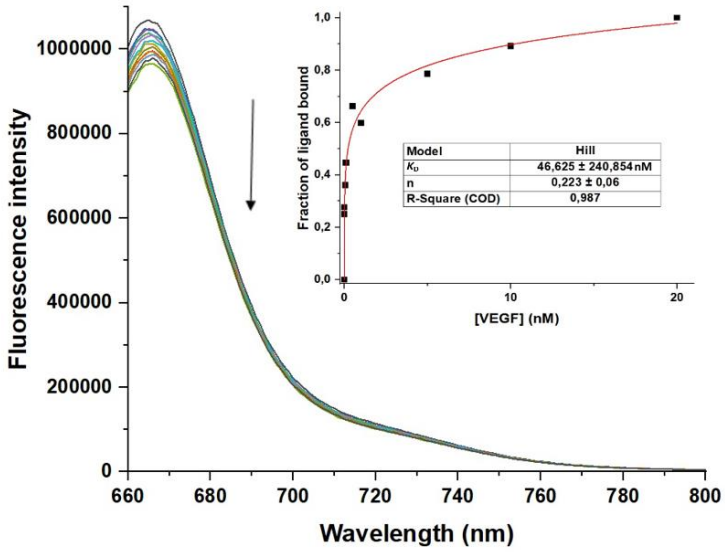

Figure 12 - Fluorescence intensity spectra and K_D determination of AT11 (1 μM) and dexamethasone (1 μM) titrated with VEGF.

Once the high affinity of the G4 structure for dexamethasone and VEGF was confirmed, the next step was to evaluate whether AT11 and dexamethasone together exhibited high affinity for VEGF. Upon adding VEGF to the pre-folded 5'Cy5-AT11, a slight decrease in the fluorescence intensity was observed. Figure 12 shows the fluorescence titration between AT11/dexamethasone when titrated with VEGF. For this experiment, Hill saturation binding model was used. As observed, the obtained K_D is considerably higher in the nanomolar order (46.625 ± 240.854 nM), suggesting a lower affinity. Additionally, we obtained n = 0.223 ± 0.06, which is < 1, indicating once again that the binding of one first substrate will discourage the subsequent binding of another one.

The results from the evaluation of the affinity between AT11 and dexamethasone for VEGF showed indicate a potential opportunity to exploit their synergy to inhibit angiogenesis by targeting VEGF. Given that both AT11, as illustrated in Figure 11, and dexamethasone, as supported by various studies, have demonstrated isolated affinity for VEGF, we sought to examine the behavior of this complex in vitro. The objective was to investigate whether AT11 could enhance the inhibition of angiogenesis when administered to cells with VEGF overexpression.

4.2.3. MTT assay

The viability of HUVEC cells was evaluated when exposed to different concentrations of dexamethasone and AT11 aptamer, being the results obtained depicted in Figure 13 A and B, respectively.

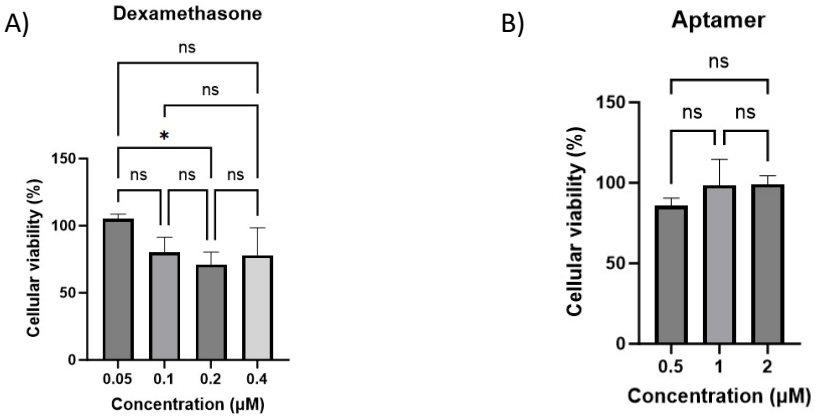

Figure 13 - Percentage of viable HUVEC after 72 h incubation with A) dexamethasone at a concentration of 0.05 to 0.4 µM and B) AT11 aptamer at a concentration of 0.5 to 2 µM. ns-non significant differences.

As shown in Figure 13, AT11 reduces de viability but not significantly, which suggests that AT11 is not a significant inhibitor of cell proliferation reducer and does not induce any major cytotoxic effect. The same observation applies to dexamethasone, since cellular viability did not decrease below 50% at any of the tested conditions.

Given that the main purpose of this work was to reduce angiogenesis without compromising cell viability, it has noteworthy that both compounds when administered individually, do not exhibit any cytotoxic effect., Consequently, they are suitable be tested in angiogenesis assays. However, the combined effects of these two compounds had to be tested, since their synergistic interaction could potentially yield more robust outcomes in angiogenesis control.

In Figure 14 is shown the results from the test of AT11 and dexamethasone at the HUVEC viability, at respective proportions of 1:1 (Figure 14 A) and 1:2 (Figure 14 B).

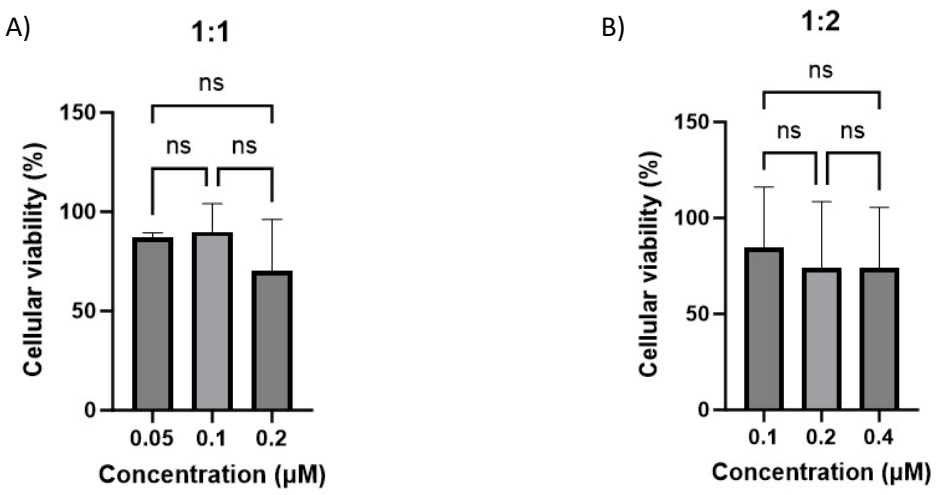

Figure 14 - Percentage of viable HUVEC after 72 h incubation with A) dexamethasone at a proportion of 1:1 for AT11 aptamer and B) dexamethasone at a proportion of 2:1 for AT11 aptamer.

As expected, AT11 and dexamethasone at 1:1 and 1:2 proportions, respectively, did not affect the viability of HUVEC, making these concentrations suitable for angiogenesis assay as well, since we can evaluate this parameter without disturbing normal cell proliferation.

4.2.4. Angiogenesis

In this experiment, as previously described, we considered essential the study of how dexamethasone and AT11 can affect angiogenesis, that is a main characteristic of vascular diseases of the retina. The assay was carried out to determine the effects of activators or inhibitors of neovascularization over time.

To determine the angiogenic activities of dexamethasone, a concentration of 10 µM was employed. Additionally, it was combined with AT11 at a concentration of 100 µM, which was also individually tested to evaluate its angiogenic activity. As we can see in Figure 15 A, HUVEC cells organized themselves on a capillary-like structures, when seeded on a Matrigel, with these formations being clearly visible in the untreated control group. Moreover, the combination of dexamethasone and AT11, at ratios of 1:1 and 2:1, respectively, as well as when administered separately, exhibited a significant impact on tube formation.

Moreira et. al tested the angiogenesis inhibition of AT11-L0, also a derivative of AS1411, but didn't obtain any significant results regarding to the angiogenesis inhibition,

however, it was explained that it could be due to the concentration of the aptamer being too low, since AS1411 presented great results [68]. AT11 however, exhibit an inhibition more accordingly to AS1411, maybe due to the overexpression of NCL in VEGF-dependent cells. These results are exposed in Figure 15.

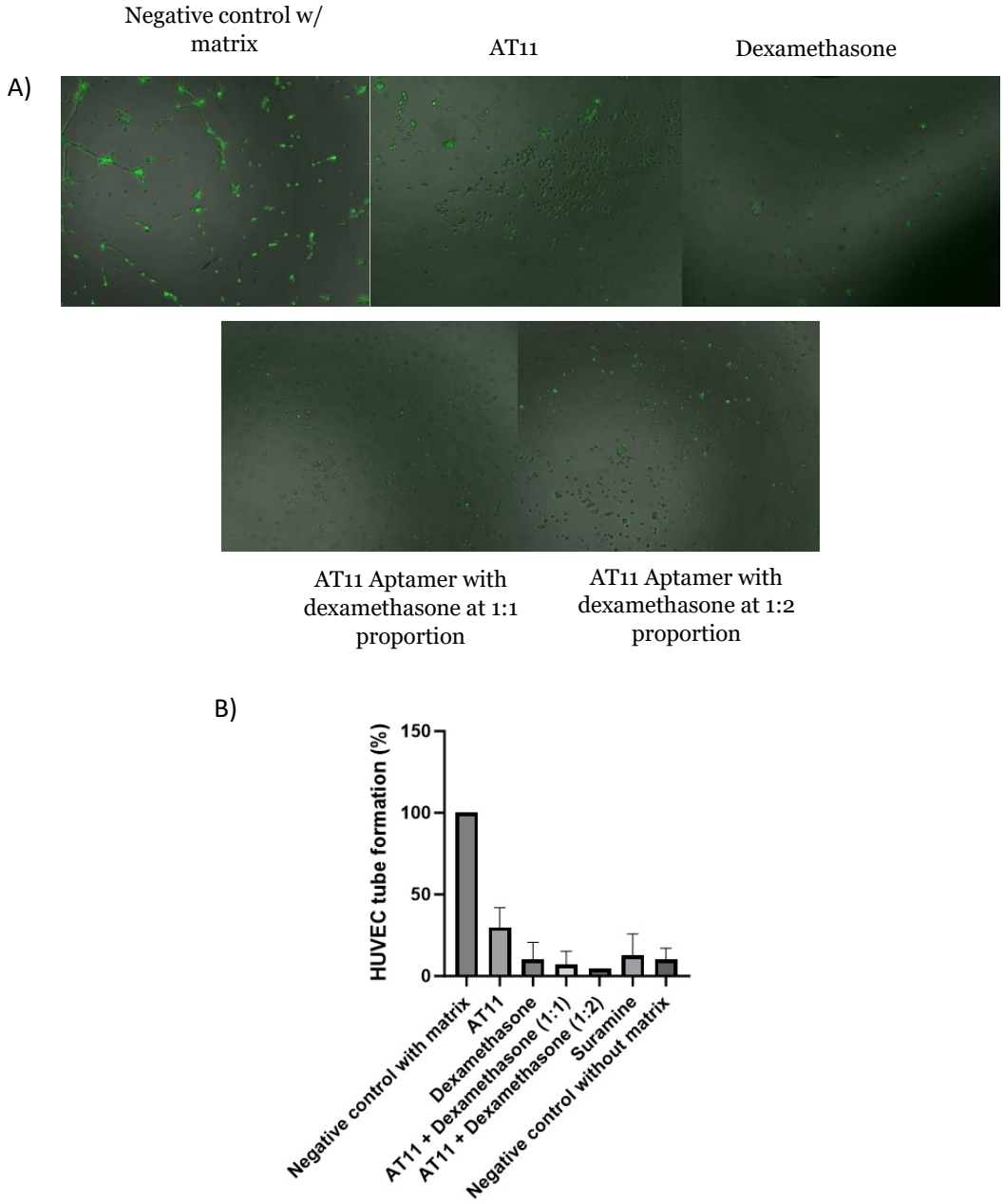

Figure 15 – A) Representative image of tube formation on HUVEC. B) HUVEC tube formation.

At the level of angiogenic properties, AT11 has demonstrated promising results, as evident from the obtained data. Despite its slightly lower inhibitory capacity, it still

significantly reduced angiogenesis by more than half. Dexamethasone is already known for its antiangiogenic properties, as demonstrated by Langendorf et al. [75]. Additionally, since it is already used in the treatment of retinal vascular diseases [76], [77], combining this compound with AT11 could be a viable approach to expedite the antiangiogenic process and enhance treatment efficiency.

This potential synergy arises from AT11's ability to target VEGF. Given that HUVEC cells are VEGF-dependent and exhibit VEGF overexpression, AT11 can potentially assist dexamethasone in specifically targeting cells with this VEGF overexpression condition. This targeted approach could effectively inhibit the cells that initiate the angiogenic process, providing a means to improve the overall efficacy of the treatment.

5. Conclusion and future perspectives

This work aimed to use AT11 aptamer conjugated with dexamethasone, to effectively inhibit angiogenesis in retinal diseases. AT11 is an aptamer derivative from AS1411 that targets NCL, but once NCL is deeply related to VEGF, it could have promising effects when targeting this growth factor.

Through CD spectroscopy, it was observed that AT11 formed a majority parallel-stranded G4 structure with a well-defined profile, using a 65 mM KCl buffer. In contrast, AS1411 has a drawback by adopting multiple G4 structures, a limitation effectively overcome by AT11.

The primary objective was to assess whether AT11, in conjunction with dexamethasone, could effectively target VEGF. Affinity tests for VEGF revealed that AT11 alone could target VEGF with high affinity, as evidenced by its low KD value, despite the low n value. Subsequently, affinity testing of AT11 for dexamethasone showed similar results to those obtained for AT11's affinity for VEGF. Although AT11 combined with dexamethasone showed lower affinity for VEGF, with a slightly higher KD value and low n value, they still suggest that the compound formed exhibits affinity for VEGF. When tested *in vitro*, both AT11 and dexamethasone successfully inhibited angiogenesis, although dexamethasone had slightly superior results. When AT11 and dexamethasone were combined, demonstrated that their synergy enhanced angiogenesis inhibition, particularly at 1:1 and 1:2 proportions, respectively, without exhibiting cytotoxicity, as observed in MTT assays.

Despite their diverse applications, G-rich aptamers encounter hurdles such as susceptibility to nuclease degradation and renal excretion [78]. Hence, further exploration, particularly at preclinical and clinical stages, is imperative to fully harness their potential in therapeutic and diagnostic realms. It's noteworthy that current research predominantly focuses on DNA G-rich aptamers, yet RNA G-rich aptamers play crucial roles in numerous biological processes [78]. Following significant advancements in oligonucleotide modifications, many of the stability and pharmacokinetics-related challenges have been addressed, leading to the availability of stabilized aptamers with prolonged systemic exposure and enhanced biodistribution in tissues [79]. But as described before, once again, ongoing developments in chemical modifications of oligonucleotides are still aiming to further optimize aptamer efficacy, with rigorous testing in vitro and in animal models preceding clinical validation. Consequently, it appears inevitable that nucleic acid-based drugs will soon become commonplace in the

pharmacological realm, serving as both diagnostics and therapeutics. Thus, directing attention towards RNA counterparts could offer compelling alternatives to existing biomedical approaches.

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