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Synthesis of oligonucleotides carrying nucleic acid derivatives of biomedical and structural interest.

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1. Introduction

The double helix model for the structure of DNA was proposed by Watson and Crick in 1953 [1]. However, it was not until the late 1970s that the crystal structure was resolved from a defined DNA sequence prepared in the laboratory [2]. Since then, advances in the chemical synthesis of oligonucleotides have been crucial for the development of new fields. For example, synthetic DNA was important for the development of DNA recombinant techniques and structural analysis of nucleic acids as well as the characterization of nucleic acid-protein interactions. In addition, optimized strategies for obtaining modified nucleic acids provided good opportunities to use synthetic oligonucleotides in the biomedical field. Starting from the isolation, detection, and analysis of nucleic acid sequences and progressing to the development of the nucleic acid-based drugs, the methodologies used in the preparation of modified oligonucleotides are in constant evolution providing a wide range of versatile tools with application in therapeutics, material science, and molecular computing. The chemical modification of nucleic acids has never been so extensively applied by so many researchers.

This chapter is focused on presenting an overview of the nucleic acids derivatives developed by our group in the last decade as well as highlighting the important progresses made recently in this field. All emerging applications currently exploiting to these valuable and versatile compounds are also referred. Firstly, a complete description of the research findings on oligonucleotides carrying O^6-alkylguanine derivatives involved in DNA repair will be provided. These modified nucleosides may be present as a result of DNA damage, which often occurs transiently but leads to important biological consequences. Secondly, the effects caused by the introduction of synthetic nucleotide derivatives such as conformationally restricted nucleotides, acyclic nucleotides, L-nucleotides and C-nucleotides in the structural properties of DNA will be presented. Finally, the impact of 3ôand 5ômodifications on the gene silencing properties of short interfering RNAs (siRNAs) will be discussed. Excellent reviews have helped compile remarkable advances in the synthesis of nucleic acids derivatives for application in gene silencing therapy [3]: antisense [4], or RNA interference [5], or aptamers [6] and the reader is referred to them. Recent

findings in oligonucleotide labeling [7], DNA structure [8], and DNA nanotechnology [9], have also been duly addressed in literature.

2. Oligonucleotides carrying the DNA lesion O^6-alkylguanine

Nucleobases are responsible for the base-paring properties of nucleic acids. Any modification in the nucleobases may lead to incorrect base pairing, which may induce potential mutations. The reactivity of DNA towards radiation and chemicals changes its molecular structure leading to the formation of DNA lesions. These lesions will be corrected by DNA repair mechanisms to preserve genome integrity [10].

Alkylating agents are a class of chemotherapeutic anticancer drugs that act at multiple sites of the DNA by generating adducts [11]. Of the four bases, the guanine (G) base is the most susceptible to damage, leading to many subproducts [12-13]. A subset of alkylating agents, including nitrosoureas and temozolomide, have a preference for alkylating guanine at the O^6-position, generating the most important lesion in terms of mutagenesis and carcinogenesis [14].

In addition to the other well-known side effects and limitations of chemotherapeutic agents, cyclophosphamide, mechlorethamide, chlorambucil, and nitrosoureas, also present problems of acquired tumor resistance, in part due to the action of DNA repair mechanism. Particularly, the DNA-repair protein O^6-alkylguanine DNA alkyltransferase (hAGT or O^6-methylguanine DNA methyl transferase, MGMT) is responsible for removing alkyl adducts from the O^6 position of guanines (figure 1A). The cytotoxic effects induced by alkyl adducts are blocked playing this correction an important role as a resistance mechanism [15]. It is well established that tumor cells frequently express high levels of hAGT, which is predictive of poor response to chemotherapeutic drugs. This effect has been observed in a large number of cancers [15, 16]. Therefore, pharmacological inhibition of hAGT can enhance the cytotoxicity of a diverse range of anticancer agents [16].

Several methods are available to characterize the activity of hAGT and its inhibition through interaction with small molecules. However, most of the methods commonly used as, for instance, radioactive assays or multiple-step enzymatic reactions [17-20] or *in vivo* hAGT labeling [21-34], require handling hazardous substances or following multiple steps, which discourage their use. For this reason, the development of novel methods based on oligonucleotides sequences for the detection of hAGT activity has been considered an important issue for the measurement of protein activity and for the development of new hAGT inhibitors.

One of the methods developed in our group for the evaluation of hAGT activity was based on the use of a 15-mer oligonucleotide sequence known as thrombin binding aptamer (TBA) [25]. This oligonucleotide forms an intramolecular G-quadruplex with two tetrads and has a strong affinity for Ŭ-thrombin. The introduction of one O^6-methylguanine residue in one of the guanine tetrads prevented quadruplex formation. In this way the introduction of a fluorescent and a quencher molecule at the 5ôand 3ô ends of the oligonucleotide carrying one O^6-methylguanine residue allows to monitor the hAGT activity by following the decrease of the fluorescence induced by the

formation of the quadruplex [26]. As described in figure 1B, the process starts with the methylated TBA that is unfolded. In this conformation the quencher and the fluorophore are beyond the Foster distance. Then, the addition of hAGT removes the methyl group from the mutated guanine residue allowing the TBA to fold back into a chair-like structure. This even produces the approximation of the quencher and the fluorophore and blocks the emission of fluorescence. The hAGT activity can be quantified by the directed decrease in fluorescence.

A more elaborated system was based on the introduction of several TBAs carrying O^6-methylguanine residues in a rectangular DNA origami. The presence of methylated guanine in the TBA prevented its interaction with Ŭ-thrombin. In this way the removal of the methyl group by hAGT could be monitored by the recruitment of Ŭ-thrombin at the O^6-methylguanine repaired sites followed by atomic force microscopy (AFM) [27] (figure 1C). Another version of the use of TBAs carrying O^6-methylguanine residues and Ŭ-thrombin for the measurement of hAGT activity was from the study of the aggregation of gold nanoparticles induced by thrombin [28]. The aggregation induced by thrombin after O^6-methylguanine repair by hAGT was followed by UV-visible spectroscopy, dynamic light scattering (DLS) and magnetic resonance imaging (MRI) measurements [28]. MRI technique was only used in the case of gold-coated iron oxide supermagnetic nanoparticles.

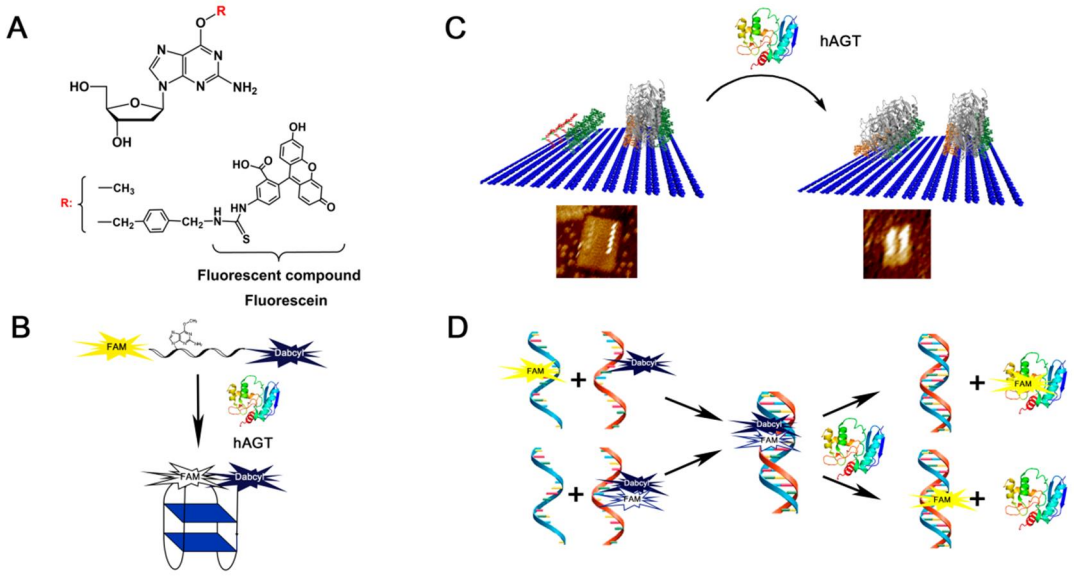

Figure 1: O^6-alkyl guanine derivatives and methods for the evaluation of hAGT activity. A) Chemical structure of O^6-alkyl 2ôdeoxyguaninosine derivatives, B) Fluorescence assay based on the conformational changes of the thrombin-binding aptamer, C) DNA origami as sensor for hAGT activity, D) dsDNA FRET method for the quantification of hAGT activity).

The direct removal of the alkyl group at position 6 of guanine by hAGT inspired alternative methods for the measurement of hAGT activity. In these methods a fluorescent or a quencher tag is covalently linked to the alkyl group, which will be removed by hAGT, and the complementary FRET-pair (quencher or fluorophore) is placed nearby. During the repair process, one of the tags is transferred to the protein. The separation of the fluorophore from the quencher results in fluorescence signal light-up, which will allow the quantification of hAGT repair action [29, 30]. Two methods

have been described recently using this DNA duplex FRET-pair technology with some differences. The fluorescent derivative used and the location of the tag molecules, in the same strand or in an opposite one, are the main differences [29, 30]. These DNA probes may prove valuable in assessing potential resistance arising from cancer treatment with alkylating agents [30] and for the analysis of hAGT inhibitors [29].

3. The effect of chemical modifications in non-canonical DNA structures

In this part we cover some of the synthetic nucleotides designed to modulate the properties observed in non-canonical DNA structures such as triplex, G-quadruplex and i-motif. For a long time, these structures were mainly studied from a structural or theoretical point of view. However, since the demonstration of the presence *in vivo* presence of G-quadruplexes [31] and their importance in several biological processes, the interest in these structures has increased. Synthetic nucleotides may alter the stability and geometry of these non-canonical structures, as well as the interaction between nucleic acids and proteins. There are several reviews describing the impact of modified nucleotides in DNA/RNA structures [32, 33]. Here, we will describe the recent advances that have been focused on the introduction of synthetic nucleotides such as conformationally restricted nucleosides or acyclic nucleotides to enhance some of the properties observed in oligonucleotides forming triplex, G-quadruplex or i-motif DNA structures (figure 2).

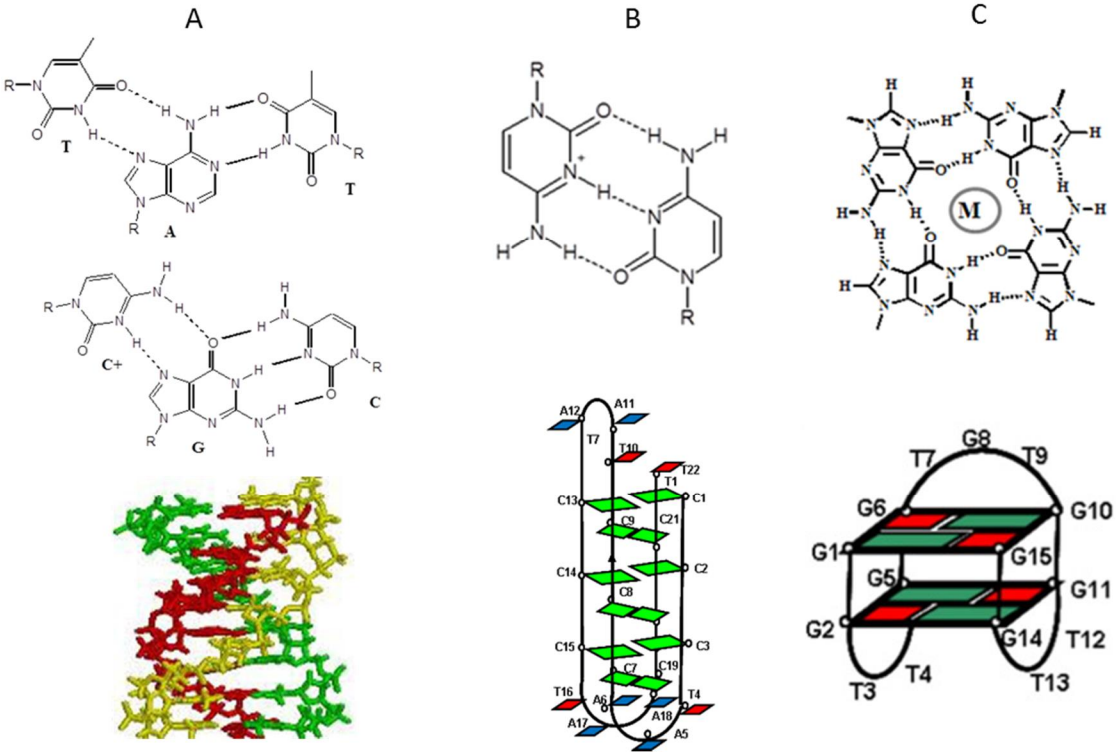

Figure 2. Non-canonical nucleic acids structures. In A is represented the parallel triplex, in B, the i-motif and C, the G-quadruplex. In the top, is shown a scheme of base-pairing units, T.AÅT and C.GÅC+ triads is present in parallel triplex, C:C+ in i-motif and G-tetrads in quadruplex. In the bottom are depicted schemes of the corresponding tridimensional structures.

3.1 Triplex-forming oligonucleotides.

Triplexes are formed when a duplex containing a polypurine/polypyrimidine track interacts with a third strand, by means of specific hydrogen bonds in the major groove of the duplex. The experimental existence of triplexes was demonstrated for the first time in 1957 [34]. Depending on the orientation of the third strand, triplexes are classified into two main categories: parallel triplex, in which a triplex forming pyrimidine strand binds to the purine strand of the duplex in parallel fashion and antiparallel triplex in which a purine-rich strand binds to the purine strand of duplex in an antiparallel orientation. In the first class, the third strand binds through Hoogsteen hydrogen bonds to duplex, forming d(T-AÅT) and protonated d(C-GÅC+) triads (figure 2). This type of triplex is more stable at low pH values. In the antiparallel triplexes, purine-rich strands bind to the duplex using reverse Hoogsteen base-pairing forming d(T-AÅT), d(T-AÅA) and d(C-GÅG) triads [35]. The specificity and selectivity of the third strand to the duplex makes this structure very attractive in molecular biology, diagnostics and therapeutics. Triplex-forming oligonucleotides (TFOôs) may regulate gene expression and induce site-directed recombination as well as site-specific mutations [36].

A number of modifications have been introduced in the backbone, in the bases or in the 3ô or 5ô end of TFOôs to improve selectivity and stability [32, 37, 38]. This includes phosphate modifications, such as phosphorothioate linkages, methylphosphonate or N3ôNP5ô phosphoramidate. The nucleobase modification has mainly focused on the design of cytidine and guanine derivatives to obtain parallel triplexes at neutral pH values [36, 38]. The introduction of psolaren groups to TFOs have also been pursued in order to form adducts and interstrand cross-links to study processes of DNA replication of transcription [36]. In addition, bulge insertions of twisted intercalating nucleic acids (TINAs) have resulted in triplexes and quadruplex with an extraordinarily high thermal stability [39]. Less attention has been paid to the introduction of modifications in the duplex moiety of a triplex structure. However, 8-aminopurines (8-aminoadenine and 8-aminoguanine) are proven to be excellent modifications for parallel- and antiparallel-triplex stabilization [40, 41]. This stabilization has been critical for several recent applications based on triplex-affinity capture [42-44].

Importantly, the sugar backbone modifications have been the most studied modifications in triple strand formation. In that sense, PNAs (peptide nucleic acids) formed by a 2-aminoethylglycine units (figure 3) is an uncharged backbone with a strong affinity to complementary DNA or RNA strands. Moreover, it is able to form stable parallel triplex [45]. In addition, several conformationally restricted ribose modifications have also been considered as good candidates in TFOs. Specifically, LNAs (Locked Nucleic Acids, figure 3) and its derivatives have been extensively studied in triplex. LNA is a 2ô-4ô-bridged nucleic acid locked in an 3ô-endo conformation. The restriction of the sugar-phosphate backbone facilitates a more efficient stacking of the nucleobases. These derivatives improve affinity toward DNA and RNA complements and the stability against nucleases when incorporated into oligonucleotides [46]. Several variants of LNAs have also been reported during the last decade. LNA is a remarkable nucleic acid modification that has been employed in therapeutic strategies both *in vitro* and *in vivo*. Excellent applications have been found in controlling gene expression and in the modulation of the formation of other nucleic acids structures, rather than duplex. It was reported that the incorporation of LNA

derivatives in the homo-pyrimidine strand increased the binding affinity of TFOs in both types of triplexes [47-49]. However, position-dependent stability was observed when they are introduced in the different triads.

3.2. G-quadruplex forming oligonucleotides.

G-rich sequences may form G-quadruplex structures where there is a cyclic arrangement of four guanines (G-tetrads) through Hoogsteen hydrogen bonding (Figure 2). These sequences have been extensively studied as they have been found in telomeric and promoter regions of certain oncogenes. They are very polymorphic and may form intramolecular/intermolecular arrangements. G-quadruplex may be classified according to the orientation of the strands as parallel or antiparallel. Owing to their biological relevance, this structure has been studied in detail and several modifications have been described [50].

Modifications of the nucleobases have been reported for G-quadruplex [51]. Especially the bases that form the tetrads are very important in the formation and the stability of this structure. Importantly, modifications of 8-position of guanine in G-quadruplex have been studied [52]. In the G-quadruplex, the sugar can assume both C2ôand C3ôendo conformations and the guanine can be found either in *syn* or *anti* conformations. The limitation on the conformational equilibrium by the addition of restricted nucleosides affects the stability of this structure [53]. In antiparallel quadruplexes, the conformation of 2ôdeoxyguanosines alternate between *anti* and *syn* conformers. In anti-positions, LNAs are compatible and produce stable G-quadruplex structures [53]. A detailed work of the influence of nucleobase orientation and restricted conformation using methanocarba-(MC)-nucleosides *N*-(*anti*)-MCdG and *S*-(*syn*) MC-dG (figure 3) was accomplished using the TBA [25, 54] as a model compound of antiparallel quadruplex [55]. MC-nucleosides have been studied as substrates for kinases and for their inhibitory effects on cell growth [56]. These compounds are pseudonucleosides in which the sugar conformation is restricted. Both *North*- and *South*-locked derivatives are prepared by shifting the position of the fused cyclopropane ring in these constrained nucleosides [56]. This study shows that having the correct nucleobase orientation is more important for the stability of G-quadruplex than having the correct sugar puckering [55]. In addition, the substitution of thymidines by *North*-MC-dT at the TGT loop of TBA was studied. These substitutions produce a destabilizing effect in the antiparallel quadruplex [57]. In addition, it has been reported that 2ôfluoroarabino nucleic acids-(FANAs)-G and 2ôfluororibo-G (F-RNA) (figure 3) can be used for the manipulation of G-quadruplex polymorphism [58]. 2ôFluoro nucleosides are RNA derivatives that contain a fluorine atom at 2ôposition. 2ôF-RNA derivatives contain a fluorine atom in Ŭ adopting a sugar 3ôendo conformation whereas in 2ôFANA, the fluoride is located in ƀ position adopting a 2ôendo conformation. Both types of derivatives increase oligonucleotide serum stability and binding affinity to their complementary sequence. Similarly, oligonucleotides modified with 2ô fluoronucleosides provide also a sensitive probe for structural study of nucleic acids and their interactions with other biological moieties [59].

Other classes of modifications were introduced in nucleic acids to confer more flexibility in the ribose phosphate backbone. Specifically, a detailed study of the effect of unlocked nucleic acid (UNA, figure 2) derivatives in the stability of TBA G-quadruplex

has been reported [60]. The introduction of these derivatives may control the affinity of the aptamer for the protein in a position-depending manner. UNAs are RNA mimics that lack the bond between the C2′ and C3′ atoms of the ribose ring. This derivative is more flexible than an unmodified RNA monomer. The introduction of UNA monomers may increase or decrease mismatch discrimination against DNA/RNA target strands when it is incorporated in a duplex [61]. Other acyclic nucleoside mimetics studied in G-quadruplex are the acyclic threoninol nucleic acids (aTNA, figure 3) [62]. This mimetic is constituted by threoninol (2-amino-1,3-butanediol) tethered to one of the natural nucleobases A, C, G and T by a carboxymethyl linker [63]. Although the aTNA skeleton is characterized by a more flexible backbone than the natural DNA/RNA, it has been demonstrated that forms a very stable homoduplex with a right-handed structure in an antiparallel orientation [63]. In addition, it has been reported that a fully modified aTNA strand cannot hybridize with the complementary DNA/RNA strand [64]. However, the incorporation of a few aTNA residues in the sequence TG_4T is compatible with the formation of the tetrameric parallel quadruplex [62].

L-nucleosides are opposite enantiomers to D-nucleosides and they have also been introduced in oligonucleotides (figure 3). Their increased nuclease resistance compared to the natural ones has attracted the interest of the groups working in therapeutics. These mirror images have been introduced in aptamers, siRNAs or ribozymes producing long-lasting structures. L-nucleotides have also been incorporated to G-quadruplex. It has been observed that when all D-DNA nucleotides are replaced by L-nucleotides the resulting structure preserves the same biological properties [65]. Recently, L-RNA nucleotides have been also studied. Specifically, it has been demonstrated that L-RNA aptamer is able to bind the natural D-form of HIV-1 trans-activation responsive (TAR) RNA [66]. More recently, a thermodynamic study regarding the characterization of structural motifs formed by β-L-RNA has been reported [67].

3.3 Oligonucleotides forming i-motif structures.

I-motif is another secondary structure formed by a C-C+ base pair, in which two parallel cytosine-rich strands forming duplex are intercalated in antiparallel orientation (figure 2). This structure is found in the complementary regions of G-rich sequences of telomeric regions and protoncogenes [68, 69]. These are more stable at low pH values and may adopt several conformations that are sensitive to pH. Based on these versatile features, i-motif oligonucleotides are attractive compounds in the fabrication of nanodevices or nanostructures [70] and as a potential scaffold for drug delivery [71, 72]. Synthetic nucleic acid analogues that can modulate the stability of the i-motif structures are needed in order to control their properties. In this context, several modified nucleotides have been introduced such as 5-methylcytosines and other epigenetic derivatives [73], PNA [74] or aTNA nucleotides [75]. Recently 2′-*C*-methyl-2′-deoxycytidine has shown to stabilize i-motif (figure 2) [76]. 2′-*C*-methyl-2′-deoxynucleosides are interesting nucleosides because they show differential preferred sugar conformations depending on the absolute configuration at the 2′-carbon. The (2′*S*)-2′-deoxy-2′-*C*-methylnucleosides mainly adopt the C3′-*endo* puckering while the 2′*R* isomers prefer the C2′-*endo* state. These derivatives are nuclease resistance and

have been introduced in therapeutic antisense and DNAzymes oligonucleotides [77]. The limited formation of i-motif at neutral conditions is overcome by the use of 2ô deoxy-2-fluoroarabinose. 2ôF-AraC stabilizes i-motif formation over a wide pH range, including pH 7 [78]. The availability of these oligonucleotides to form i-motif at physiological conditions may find several applications in the study of biological i-motif sequences. In addition, applications in the nanotechnology field based on i-motif conformational changes have been also described [78].

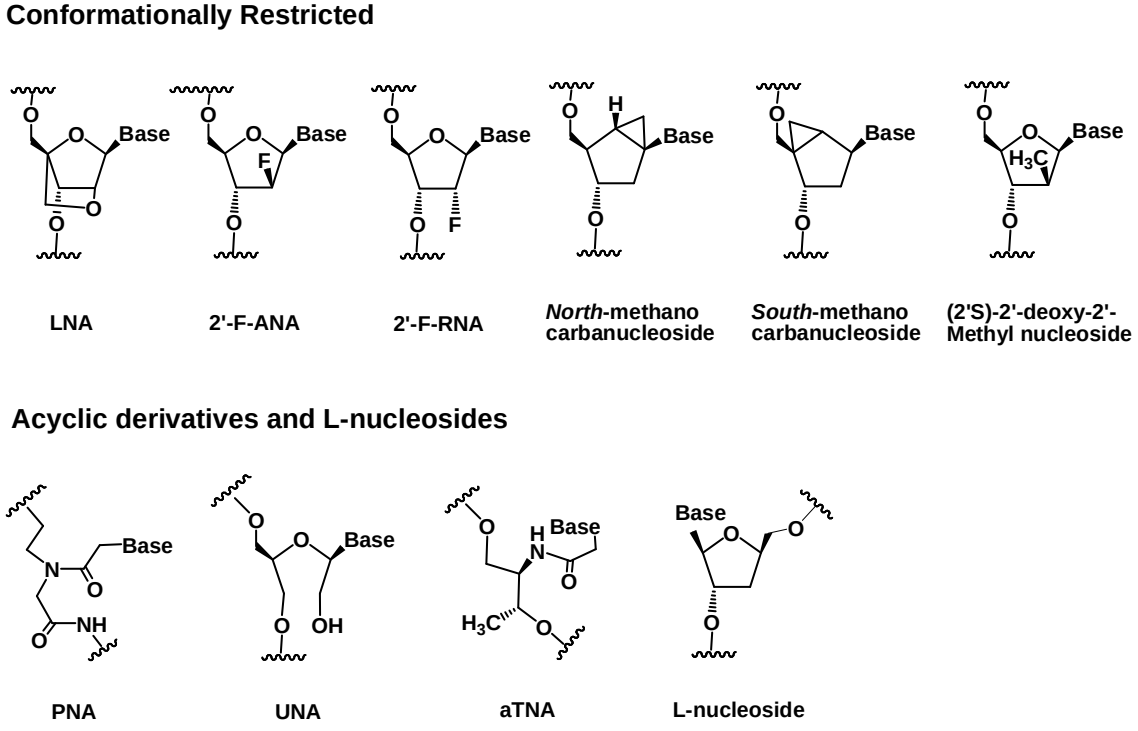

Figure 3. Scheme of modified nucleoside backbones used in the study of non-canonical DNA structures.

4. Modified siRNAs for gene silencing

The discovery of RNA interference and microRNA has triggered an intense research activity in the development of modified RNA as drugs [79]. Short-interfering double-stranded RNAs (siRNA, figure 4) have been demonstrated to be efficient for downregulation of gene expression by targeting a selected mRNA to degradation by the RNA-interfering silencing complex (RISC) [79]. Nowadays, there is no doubt that nucleic acids can be used to interfere with cellular metabolism, in a way that can be developed as novel medicines with potentially more specificity and less toxicity than classical small molecule drugs [80].

However, nucleic acids face multiple obstacles in order to be developed as therapeutic agents. Extensive research effort has been carried out in order to overcome such important barriers [79]. Some of these accomplishments have been carried out by chemically modifying the carbohydrate moiety [81, 82], nucleobases [83],

and/or phosphate backbones [84]. Furthermore, the presence of such chemical modifications and others introduced at specific positions of the siRNA guide strand, such as the two-base overhangs at the 3ôtermini or at the siRNA 5ôtermini [85], are important parameters that govern the efficacy and activity of RNAi [81, 86]. In the following section we will focus on the chemical modifications introduced into the siRNA 3ø5ôoverhangs (PAZ and MID domains). Historically, chemical modifications at the 3ô, 5ô ends have been considered to be the simplest and safest way to enhance nuclease resistance of siRNAs. But recently, it has been proved that, in addition, these modifications can modulate siRNA selectivity and siRNA potency decreasing the amount of potential off-target effects.

4.1 Modifications of the 3ôoverhangs

One of the strategies carried out to enhance siRNA potency and thereby reduce off-target effects relates to modifying the 3ôoverhangs of siRNA oligonucleotides. This has resulted in increasing the strength of the interaction between the siRNA 3ôtermini and the PAZ domain of Ago2 [87]. In this regard, Somoza *et al.* proposed the introduction of L-threoninol derivatives containing several aromatic residues (e.g. pyrene (PYR), anthracene (ANTH), naphthalene (NAPH), trifluoromethylbenzene (TFMB) and fluorobenzene (FB)) at the 3ôtermini of both siRNA strands [88]. The introduction of such modifications of distinct sizes and electronic properties allowed the authors to gain insight into the binding effects among 3ôoverhangs and the PAZ domain. The presence of some aromatic residues at the guide strand was able to stabilize and modulate the interactions with the PAZ pocket. The authors hypothesize that those aromatic modifications capable of promoting stabilization through electrostatic interactions with the PAZ domain, such as PYR and ANTH, resulted in lowest RNAi activities. However, the lower binding affinities of siRNAs modified with TFMB and FB in the PAZ domain resulted in potent RNAi activities comparable to unmodified siRNA at 32 pM [88].

Other aromatic groups like tyramine (TYR), diphenylpropylamine (DPP) and tryptamine (TRYP) have been also introduced in siRNA in order to study the PAZ domain recognition with the modified 3ôoverhangs [89]. Gaglione *et al.* prepared a series of siRNAs containing such aromatic residues at the 3ôtermini in guide and sense strands as well as simultaneously in both strands. The authors found that all single and double modifications were accepted by the RNAi machinery and afforded higher nuclease stabilities in serum than unmodified siRNA. Interestingly, siRNA conjugates only modified with TRYP at the 3ôtermini of the guide strand and double modifications containing either DPP or TRYP at both guide and passenger strands conferred an increased silencing effect according to time-course experiments. The 3ôoverhang interaction of the modified siRNAs with PAZ domain was further corroborated by computational studies.

Similarly, structure activity relationship (SAR) studies carried out by Xu *et al.* unveiled the effect produced by glycerol-based aromatic compounds (figure 4) on the correct strand selection for improving siRNA efficacy [90]. The authors prepared a series of siRNAs in which the first two positions of the guide and the passenger strands were modified at the 3ôtermini with glycerol aromatic derivatives. This strategy allowed the authors to measure the affinity of modified siRNAs with the PAZ domain of Ago2.

Computational and gene silencing studies suggested that modified siRNAs with two-nucleotide 3ô overhangs containing glycerol derivatives, in the guide strand, favored their ability to interact with the PAZ domain. Further computational studies confirmed these findings [91, 92]. Lee *et al.* found different interaction profiles of siRNA duplexes with the PAZ domain of Ago2 depending on the nature of the 3ô nucleotide overhangs of the guide strand [91]. This favored interaction was more pronounced in the case of 3ô UU overhangs than in the case of other nucleotide combinations [91, 92]. Another parameter to be borne in mind toward increasing this strand selection is the pivotal role of the modified 3ô overhangs of the siRNA sense strand, which might promote the recognition of the 5ô termini of the siRNA antisense strand by MID domain and thereby improving the RNAi activity [90].

Other small molecules have been used to modify either guide or passenger strands of siRNA oligonucleotides. Kamiya *et al* replaced the ribose backbone by serinol-based artificial nucleic acids (SNAs) (Figure 4) [93]. SNAs are efficient DNA mimetics containing phosphodiester linkages that have been able to interact with DNA and RNA [63]. The authors prepared a series of siRNA molecules containing preferentially SNA residues at the 3ô termini of passenger and guide strands. These modifications exhibited a greater stability enhancement against exonucleases, when SNAs were introduced at the termini of siRNAs. Furthermore, the presence of the SNAs at the 3ô overhangs of both siRNA strands resulted in an improvement of the antisense selection versus sense strand with optimal RNAi silencing activities.

Alagia *et al* observed a similar behavior with siRNA oligonucleotides 3ô modified with ¨Ţ threoninol backbone (figure 3) [94]. Importantly, this acyclic modification was recognized by the RNAi machinery and also conferred good stabilities in the presence of serum and 3ð5ô exonucleases. As described in other studies containing acyclic modifications, the presence of ¨Ţ threoninol introduced at the 3ô overhang of the antisense strand, as well as the presence of phosphorothioate linkages, promoted potent silencing activities when compared to unmodified siRNAs. These results were hypothesized to be due to the greater affinity of these modifications to the PAZ domain of Ago2, as observed in other studies [95, 96]. Time-course silencing experiments up to five days confirmed that siRNA duplex containing the 3ô overhangs of the passenger and guide strands modified with ¨Ţ threoninol remarkably retained the RNAi activity and therefore inhibited both luciferase production and ApoB mRNA expression.

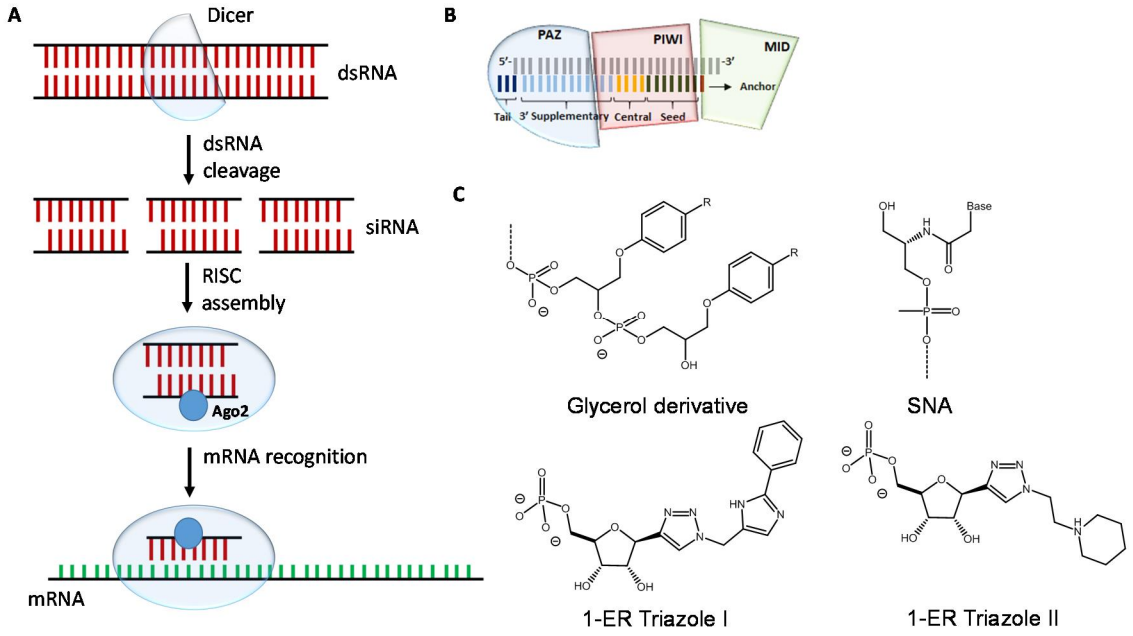

Figure 4. A. Scheme of the RNA interference pathway. B. Scheme of Ago2 and small interfering RNA (siRNA) molecule. Top strand is the passenger or sense and bottom strand is the guide or antisense. C. Chemical structure of some of the modifications described for the 3ô overhang and 5ôend.

4.2 Modifications of the 5ôend

The 5ôends of siRNAs have been also modified with small molecules and the effect on RNAi activity has been evaluated. Great efforts in this field have been mainly carried out by Kubo *et al*. modifying several parameters such as siRNA length and nature of the chemical entities. Lipids and aromatic molecules have been the two major modifications selected by Kubo *et al*. introduced at the 5ôtermini [97, 98]. The synthetic strategy to incorporate such modifications was carried out by introducing covalently a 5ôamino-modifier into the siRNA sense strand. The final 5ôconjugation with the corresponding hydrocarbonated alkyl chains (palmitic acid, cholesterol, lauric acid) [97] and aromatic pendent groups (phenyl, hydroxyphenyl, naphthyl and pyrenyl) were carried out obtaining the corresponding 21-mer siRNA conjugates modified with lipids and aromatic molecules, respectively [98].

In the case of lipid siRNA conjugates, the authors prepared a series of siRNA conjugates targeting *Renilla* luciferase activity and endogenous vascular endothelial growth factor (VEGF) containing 2-nt DNA (dT) as well as the corresponding inverted thymidine (idT) at the 3ôoverhang. This idT residue has proved to be resistant to nuclease degradation [99]. Formulated lipid siRNA conjugates modified with palmitic acid at the 5ôsense strand and the 3ôoverhangs containing idT modifications confirmed long-term gene silencing inhibition activities up to five days and exhibited superior silencing efficiencies than Cholesterol (Chol) derivatives. Despite these interesting results at the nanomolar level, these transfection experiments were tarnished in the absence of cationic lipids in which showed a reduction in VEGF levels

with moderate efficiencies (~25 %) after 48 hours incubation using 1 εM of palmitic siRNA conjugate.

Synthetic dsRNAs of 27-mer in length have revealed to be Dicer-substrates with the ability to activate RNAi process and consequently promote gene silencing with improved efficiencies and longer duration than classical 21-mer siRNAs [100]. These findings inspired Kubo et al. to modify symmetrical 27-mer Dicer-substrate dsRNA either with amino derivatives [101] or palmitic acid at the 5ôtermini of the sense strand [102], according to the same synthetic strategy described previously [97]. The best silencing results were achieved when authors modified 27-mer dsRNA with palmitic acid. The authors showed that Dicer was able to cleave dsRNA to generate the corresponding 21-mer lipid siRNA conjugates exhibiting resistance to nucleases in cell culture medium. Interestingly, 27-mer dsRNA conjugated with palmitic acid afforded better efficiencies and RNAi potencies in the presence of lipofectamine than unmodified siRNAs and 21-mer dsRNA conjugates modified with palmitic acid, Chol and lauric acid at 0.2 nM. Transfection experiments realized without using lipofectamine confirmed the suitability of 27-mer dsRNAs modified with palmitic acid to inhibit luciferase and VEGF production at 600 nM and 5 εM, respectively.

Computational studies have proved to be an important tool in the design of modifications at the 3ôoverhangs to discern conformational changes of Ago2 like the two-state model or lodging/dislodging motion. As previously described, these parameters have been directly involved in RNAi efficacy [89, 96, 103, 104]. In addition to studying 3ôoverhangs of the guide strand, Beal and coworkers performed studies of human-Ago2 (hAgo2) binding modes by computational screenings of purine analogues introduced at the 5ôtermini of the guide strand [105]. This computational analysis allowed the authors to engage a rational design for effective MID and PIWI domain binders to enhance RNAi activity [106]. The authors synthesized three purine derivatives: 7-ethyyl-8-aza-7-deazaadenosine (7-EAA), 7-EAA-triazol and 2-propargylaminopurine triazol derivative (2-AP-triazol) (figure 4) [107]. All of them were introduced at the 5ôtermini of a siRNA guide strand targeting PIK3CB gene [108] and were able to promote gene silencing except for those purine derivatives containing nitrogen substituents or modified Hoogsteen faces. Interestingly, the authors also found new modifications for nucleosides that remarkably improved the silencing effect, when they were introduced at internal positions (either position 12 or 19) of the siRNA guide strand.

The inner positions of the siRNA guide strand have also been the focus of research in order to determine the efficacy and potency of siRNA oligonucleotides. Many groups have modified such positions in order to obtain stable and more effective siRNA molecules against nucleases with longer half-lives. The correct designing of such siRNAs focusing on improving stability and reducing off-target effects has allowed to launch novel modified siRNA conjugates for *in vivo* applications [109]. Such modifications can also involve blunt 19-mer duplexes containing multiple modifications [110]. In particular, Kaufmann and coworkers found that modifying the 2ôposition of the ribose with a 2ô*O*-methyl pendent group greatly increased the melting denaturation of the siRNA duplex and also enhanced its nuclease resistance. Significant contributions in the RNAi activity were revealed depending on the 2ô*O*-methyl ribose position in the siRNA duplex. Thus, while fully modified siRNAs completely lost the siRNA inhibition

activity, alternating 2ô*O*-methyl RNA bases in the guide strand improved the stability in the serum and showed a good tolerance and potency when compared with unmodified siRNAs.

Similarly, other kinds of 2ôRNA nucleobase modifications have been established and evaluated. Prakash *et al*. studied the effect of 2ôF, 2ôOMe and 2ôO-MOE RNA bases introduced at several positions of the siRNA guide strand [111]. While 2ôF RNA bases did not affect the inhibition levels of PTEN mRNA regardless of the strand position, the position of OMe and OMOE substituents remarkably affected the RNAi activity. Thus, the authors found that these bulky modifications actively preferred the 3ôtermini strand than the inner positions of the siRNA guide. Overall, the correct design of siRNAs containing 2ômodifications in the ribose such as F, OMe and/or OMOE groups have proved to be promising siRNA modifications for the validation in animal models [112].

In addition to modifying nucleobases, other chemical modifications have been proposed, which can be introduced either into the guide or the passenger strand without altering the inhibitory properties of natural siRNAs. Alagia *et al*. put their emphasis on positions 9 and 10 of the siRNA guide strand, positions in which are directly involved in the cleavage of the mRNA target by Ago2 [113]. The authors prepared siRNA oligonucleotides containing acyclic nucleic acids based on ¨Ḥ threoninol-thymine and their results were compared to those siRNAs containing a natural uridine at the same scissile phosphate positions in the guide strand. A rational study carried out by the authors allowed them to show that introducing the thymidine modification at position 10 strongly affected the RNAi activity (10 times less) whereas position 11 produced a slight decrease in the siRNA potency when compared with unmodified siRNAs. Further off-target studies involving ¨Ḥthreoninol-thymine building block were conducted by the same authors. The sense/antisense ratio was evaluated by introducing the modification at position 2 of the passenger strand. This produced a total selection of the guide strand versus the passenger strand when ¨Ḥthreoninol-thymine was introduced and therefore produced the expected gene silencing effect with comparable silencing activities to unmodified siRNA.

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