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Citation
Ellenbroek, B. D., Kahler, J. P., Evers, S. R., & Pomplun, S. J. (2024). Synthetic peptides: promising modalities for the targeting of disease-related nucleic acids. *Angewandte Chemie (International Edition)*, *63*(20). doi:10.1002/anie.202401704

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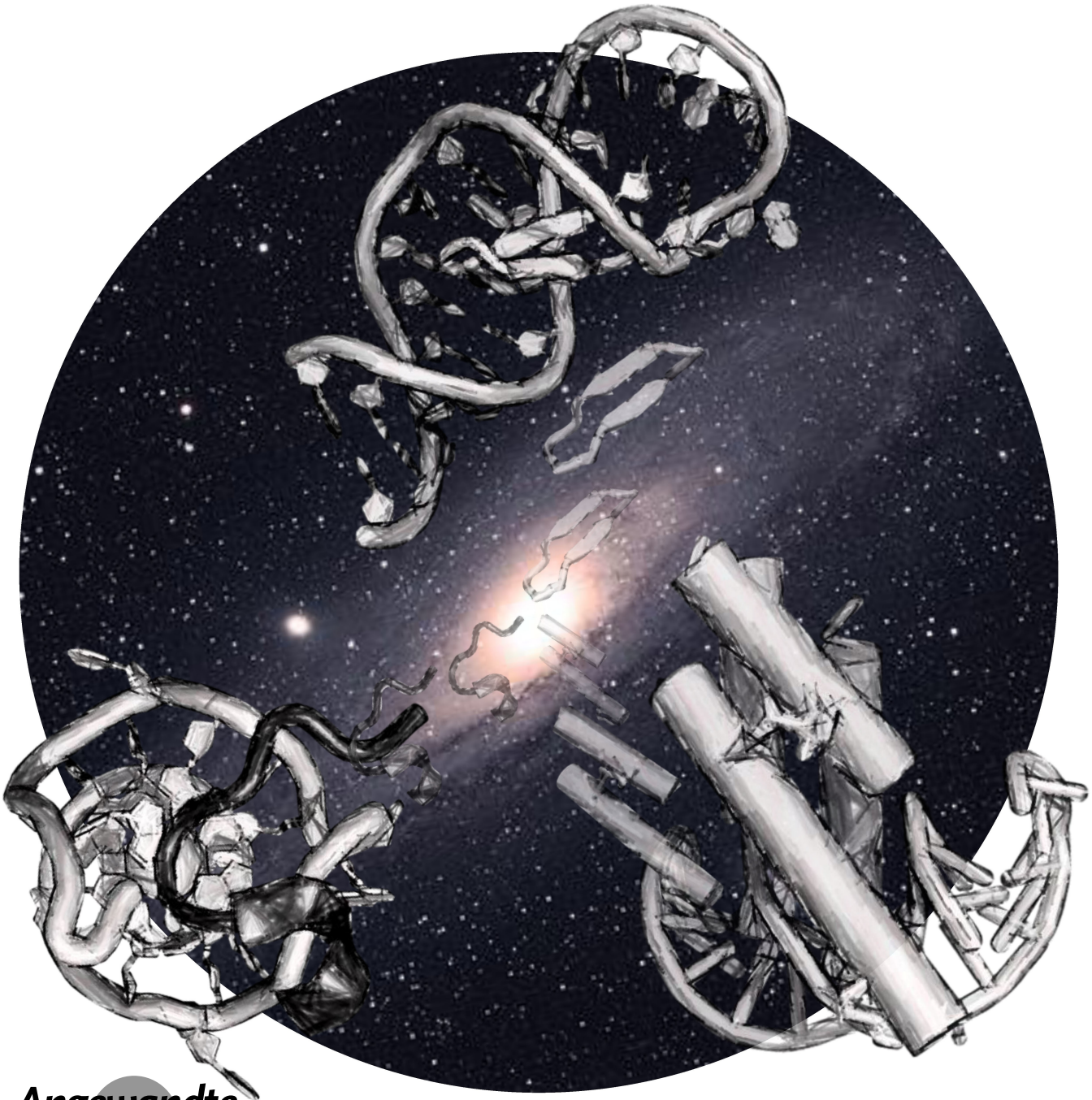
Note: To cite this publication please use the final published version (if applicable).

How to cite: *Angew. Chem. Int. Ed.* **2024**, e202401704
doi.org/10.1002/anie.202401704

Medicinal Chemistry

Synthetic Peptides: Promising Modalities for the Targeting of Disease-Related Nucleic Acids

Brecht D. Ellenbroek[+], *Jan Pascal Kahler*[+], *Sophie R. Evers, and Sebastian J. Pomplun**



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Abstract: DNA and RNA play pivotal roles in life processes by storing and transferring genetic information, modulating gene expression, and contributing to essential cellular machinery such as ribosomes. Dysregulation and mutations in nucleic acid-related processes are implicated in numerous diseases. Despite the critical impact on health of nucleic acid mutations or dysregulation, therapeutic compounds addressing these biomolecules remain limited. Peptides have emerged as a promising class of molecules for biomedical research, offering potential solutions for challenging drug targets. This review focuses on the use of synthetic peptides to target disease-related nucleic acids. We discuss examples of peptides targeting double-stranded DNA, including the clinical candidate Omomyc, and compounds designed for regulatory G-quadruplexes. Further, we provide insights into both library-based screenings and the rational design of peptides to target regulatory human RNA scaffolds and viral RNAs, emphasizing the potential of peptides in addressing nucleic acid-related diseases.

1. Introduction

Nucleic acids (NAs) play fundamental roles in all processes of life. Ribonucleic acids (RNA) have even been postulated as the potential linchpin in the origin of life itself.[1] Deoxyribonucleic acids (DNA) serve as repositories for the genetic blueprints inherent to every living organism and cell. As first described by Watson and Crick, the DNA houses the A, C, G, and T sequence in a double-helix arrangement,[2] delineating the unique code of each gene. Polymerases transcribe genes into RNA, which in the case of messenger RNA (mRNA) subsequently can undergo ribosomal translation, dictating the amino acid sequence of a final protein product. This unidirectional genetic flow, from DNA to RNA and from RNA to protein, has historically been termed the ′central dogma of molecular biology′.[3] Discoveries from the last decades, encompassing regulatory DNA elements (e.g., G-quadruplexes or G4s,[4] depicted in Figure 3), a variety of non-coding RNAs[5] (ncRNAs, such as micro RNAs (miRNA),[6] as depicted in Figure 5), and the intricate role of transcription factor (TF) proteins,[7] collectively illuminate the intricate and multi-faceted nature of nucleic acids′ functions and the genetic information cascade, far surpassing the unidirectionality of the traditional ′central dogma′.

Despite their fundamental role in health and disease, only few therapeutic compounds targeting specific disease related NAs have been developed and approved for use in humans. Classical small molecule drugs usually target proteins, and specifically proteins that have well-defined binding pockets, such as receptors or enzymes. DNA in its double helix does not present ‘druggable’ binding pockets for small molecules. DNA-targeted small molecules, indeed, usually have sequence agnostic unspecific affects,[8] ultimately leading to DNA damage and cell death.[9] An exceptional class of small molecules, the pyrrole-imidazole polyamides, mainly discovered and investigated by Dervan et al., can target specific DNA sequences, via minor groove binding and base pair recognition.[10] However, the advancement towards therapeutic use has remained limited.[11] RNA can fold to several more or less defined three-dimensional structures.[12] Compared to proteins these motifs are less defined and structurally more flexible, but, nevertheless, targeting these motifs with small molecules has recently shown intriguing potential.[13,14]

Chemical modalities beyond small molecules can target specific NA sequences. Antisense oligonucleotides (ASOs) can be designed to hybridize with complementary RNA sequences, thereby modulating gene expression through different mechanisms.[15] However, challenges associated with poor pharmacokinetics, limited cellular uptake, and inefficient delivery to target tissues have constrained the translation of ASOs into successful therapies. Nevertheless, to date, nine ASO-based drugs have secured regulatory approval for clinical use.[16] Other approaches include the use of engineered DNA-binding proteins, such as zinc finger (ZF) proteins[17] and transcription activator-like effector nucleases (TALENs).[18] While these proteins can be engineered to bind specific DNA sequences, obstacles like suboptimal cellular entry and issues related to specificity have impeded their widespread implementation in clinical settings. The revolutionary CRISPR-Cas system presents another avenue for NA manipulation.[19] This technology holds immense potential for precise genome editing, but hurdles related to efficient delivery of CRISPR components into target cells remain a substantial challenge. Developing effective delivery strategies that ensure the transport of Cas proteins and guide RNAs into the desired cells while minimizing off-target effects is a critical area of ongoing research.[20]

Peptides have emerged as a compelling class of molecules with the potential to tackle challenging drug targets in biomedical research.[21] Peptides offer a unique balance of specificity, affinity, and structural diversity. Their larger size and capacity for intricate three-dimensional structures enable precise interactions with target sites that may be beyond the reach of conventional small molecules.[22] At the same time, their size is significantly reduced compared to

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other biomolecular drugs, making their production and handling more straightforward. While there are several challenges related to peptides' pharmacokinetic properties, these modalities hold potential to tackle intracellular targets. For the discovery and the development of bioactive peptides there are a variety of robust strategies: when structural insights are available, the rational design of short active peptides can be pursued, often stemming from larger protein structures.[23] Alternatively, powerful affinity selection approaches, such as phage display,[24] or affinity selection mass spectrometry,[25] can be leveraged to screen libraries of billions of sequences for high-affinity hits, all in one experiment. A plethora of non-canonical amino acid building blocks can be used to improve the affinity and overall properties of peptide hits. Generally speaking, peptides present a versatile platform for addressing a wide range of therapeutic challenges, from targeting protein-protein interactions to modulating complex NA structures.

In this review we highlight the utilization of synthetic peptides to target disease related NAs. In a first section we will focus on proteomimetic peptides able to specifically target E-Box DNA and inhibit the oncogenic transcription factor Myc. Notably, one peptidic Myc inhibitor, a variant of the miniprotein Omomyc,[26] is currently in human clinical trials, underlining the promise of this targeting strategy. Several synthetic analogs have been developed and show intriguing potential.[27–33] Further, we will describe peptides targeting DNA G-quadruplex structures, modulating gene transcription.[34–37] In a second section we will describe the development and discovery of peptides targeting diverse RNA structures involved in disease. Specifically, we will focus on compounds targeting viral RNAs and non-coding microRNA (miRNA) involved in cancer. For these targets we will present proteomimetic strategies, library selection platforms and unnatural modifications such as nucleobase amino acids and peptide stapling, leading to intriguing bioactive candidates.

2. Targeting of DNA with Synthetic Peptides

DNA stores genetic information and can be called the code of life. Replication of cellular DNA is necessary for cell division and proliferation. Amongst other functions, DNA encodes for proteins which are synthesized by first transcription of DNA into RNA followed by translation of RNA into the respective protein. DNA transcription is regulated by many different proteins such as transcription factors which can have an effect on the level of transcription of a certain protein and thus indirectly on the amount of protein eventually synthesized.

DNA sequences can form secondary structures and the biologically most prevalent one is the canonical double-helix, so-called B-DNA, proposed by Watson and Crick in

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Sophie Evers earned both her Bachelor Chemistry and Master Drug Discovery in Amsterdam (University of Amsterdam and VU Amsterdam), especially fascinated by biochemistry. During her internship in the Strømgaard group (University of Copenhagen), she discovered her ideal blend of chemistry in biological systems through synthetic peptides. To explore another facet of peptides, she joined the Pomplun lab to explore the field of peptides targeting nucleic acids. Currently, she is undertaking her PhD in chemistry under the guidance of Dr. Nina Hartrampf at the University of Zurich. There, her research focuses on automated fast-flow peptide synthesis of a phosphorylated protein domain.

Brecht Ellenbroek received his B.Sc in Chemistry in 2018 at the University of Amsterdam/Vrije Universiteit of Amsterdam and his M.Sc. in Drug Discovery in 2021 at Vrije Universiteit of Amsterdam. He is currently a PhD candidate in the research group of Dr. Sebastian Pomplun at the University of Leiden. His research focuses on the development of miniproteins that can target disease related nucleic acids.

Jan Pascal Kahler studied Chemistry at TU Darmstadt, Germany, where he obtained both his B. Sc. and M. Sc. He then pursued his PhD in the field of activity-based protein profiling in the laboratory of Prof. Dr. Steven Verhelst in Leuven, Belgium, focusing on quenched activity-based probes for serine proteases. Currently he is a postdoctoral researcher in the group of Dr. Sebastian Pomplun at Leiden University, the Netherlands, where he works on the design and evaluation of artificial transcription factors.

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1953.[2] Here, two complementary single strands of DNA running in opposite directions (ssDNA) form double-stranded DNA (dsDNA) through canonical pairing of the individual bases which constitute the DNA building blocks: adenine pairs with thymine and guanine pairs with cytosine (Figure 1c).[38] In this right-handed double helix, the base pairs lie in one plane approximately vertically to the helix axis with their aromatic rings stacking on top of each other, stabilizing the helix (Figure 1a, middle). As the backbone carbons of both strands are not positioned directly opposite each other on the axis of the helix two grooves of different size, major and minor groove, are running around the B-DNA.[39] The wider major groove is larger and provides more sequence information than the minor groove. It is thus more easily accessible to and is where most DNA binding proteins bind.[38]

Further, dsDNA can adopt an A- and Z-form as well as other motifs such as G-quadruplexes (G4s, see 3.1). The A-form of DNA, too, is a right-handed double helix which adopts a wider helix with a less accessible major groove (Figure 1a, left). Z-DNA on the other hand, is a left-handed helix with almost equally-sized grooves (Figure 1a, right).[38]

As DNA transcription and its regulation directly impact the eventual protein levels, focusing on targeting DNA represents a strategic approach to influencing disease-related proteins at an earlier level than directly targeting the proteins themselves.

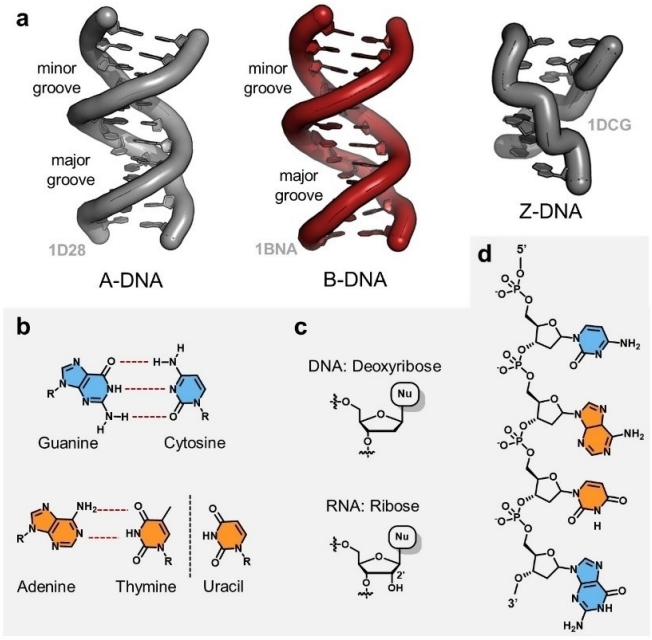

Figure 1. The structure and components of nucleic acids. (**a**) DNA secondary structures with B-DNA being the most common one (PDB: A-DNA 1D28; B-DNA 1BNA; Z-DNA 1DCG, Structures generated using PyMol). (**b**) Canonical base pairing. Adenine pairs with thymine through two H-bonds and guanine with cytosine through three H-bonds. In RNA thymine is replaced by uracil. R denotes the connection with the sugar-phosphate backbone (**c**) Structures of deoxyribose and ribose that form part of the backbone of DNA and RNA, respectively. (**d**) The structure of DNA with the ribose-phosphate backbone.

2.1. Targeting dsDNA with Synthetic Peptides

Many strategies have been developed to target dsDNA and various dsDNA targeting small molecules such as cisplatin, doxorubicin and etoposide are widely used in cancer therapy. However, these small molecules do not recognize specific sequences and act as general cytotoxic agents. Conversely, proteins can distinguish between different DNA sequences. Nevertheless, entire proteins are not cell permeable and therefore of limited use when it comes to developing therapeutics for intracellular targets. Peptides are positioned between small molecules and proteins and might offer intriguing possibilities to develop and advance DNA-targeted therapeutics. Early on, Dervan et al. developed DNA binding molecules based on the structures of natural products Netropsin and Distamycin A, resulting in DNA-binding peptide-like compounds.[40]

A generally used method to obtain peptides that can target DNA is to modify existing natural TFs.[41] Most of these studies were aimed at a broader understanding of TFs and their binding to DNA. For instance, Mascareñas et al. introduced two His mutations in the basic region of yeast TF GCN4 which acted as a metal-dependent staple inducing formation of an α-helix and allowing for specific binding to dsDNA and internalization.[42,43] In a different study, stapling of the same peptide by various cysteine crosslinkers afforded peptides binding to dsDNA with enhanced cell penetration and proteolytic stability.[44] All known DNA targeting peptides were developed based on natural TFs or by screening large peptide libraries. Designing sequence specific peptides *de novo* has not been achieved to date.

2.1.1. Targeting dsDNA with Synthetic Peptides Derived from Myc and Max

The c-Myc TF plays a role in a multitude of cellular processes such as cell proliferation and survival, differentiation, apoptosis, metabolism and DNA repair.[45] It belongs to the basic helix-loop-helix leucine zipper (bHLHLZ) family of TFs. The full length protein consists of an N-terminal transactivation domain followed by a central domain with a nuclear localization signal and the C-terminal bHLHLZ domain. While monomeric Myc is unable to bind to DNA, it heterodimerizes with its partner Myc-associated protein X (Max) through coiling of their zipper domains. This enables the Myc/Max heterodimer to bind to a DNA sequence called enhancer box (E-box) with the canonical sequence CACGTG (Figure 2a, left).[45] Activation of gene transcription is achieved by recruitment of different cofactors such as histone acetyltransferase complexes (TTRAP, GCN5, TIP60, TIP48) eventually leading to histone acetylation and allowing RNA polymerase II binding.[32,46–48]

Acting within the Myc/Max/Mxd network, Myc can only heterodimerize with Max whereas Max itself can homodimerize as well as heterodimerize with other bHLHLZ proteins in the network, such as Mxd1-4 and others. These interactions sequester Max, effectively competing with Myc for Max binding. The Max/Max homodimer can also bind to

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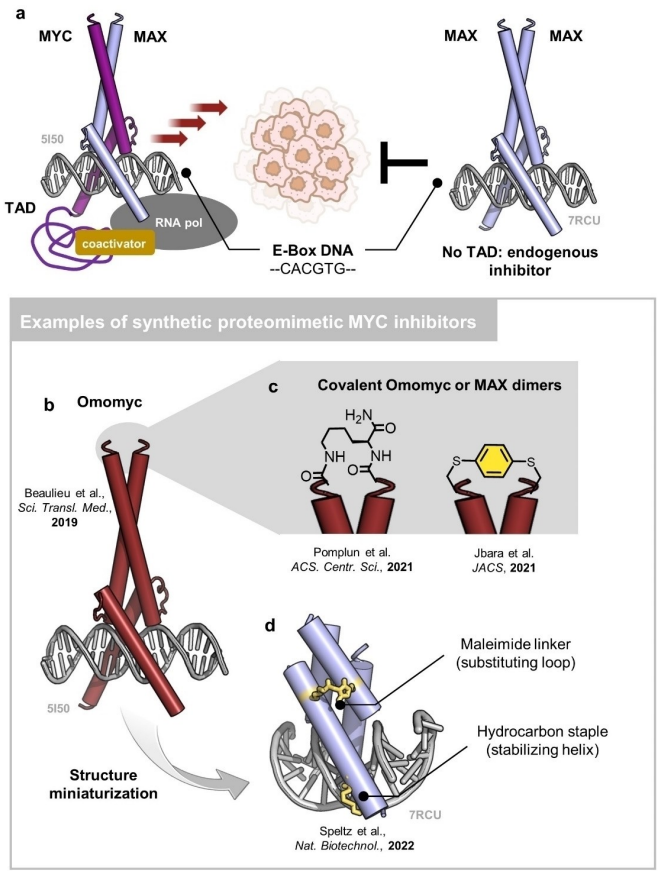

Figure 2. The Myc/Max system in gene transcription and synthetic transcription factors that inhibit Myc. (**a**) Myc heterodimerizes with Max to bind to the E-box DNA sequence. The transactivation domain (TAD) acts as binding site for further cofactors eventually allowing RNA polymerase to bind, leading to gene transcription and subsequently cell proliferation. Conversely, Max can homodimerize and bind the E-box sequence (PDB 7RCU). As it is lacking a TAD, no gene transcription and cell proliferation is initiated. The Max homodimer essentially acts as an endogenous inhibitor. (**b**) Omomyc is based upon the structure of Myc but possesses four mutations, allowing it to homodimerize and bind to the E-box sequence (PDB 5I50). Importantly, cell permeability of Omomyc has been shown by microscopy, cell-based assays and in an animal model.[26] (**c**) Omomyc made by fast-flow SPPS has been synthetically dimerized and the dimers used to show DNA binding. Also the synthetically dimerized Omomyc displays cell permeability as shown by fluorescence microscopy and cell-based assays.[27,28] (**d**) The Max basic helix together with a minimal zipper helix has been linked with a maleimide linker and stapled using hydrocarbon stapling in an attempt to minimize the structure of synthetic transcription factors and improve cell penetration (PDB 7RCU). Cell penetration has been shown by fluoroscence microscopy and in cell-based assays.[70] Representations of crystal structures were generated using PyMol.

E-box sites and block Myc/Max from binding (Figure 2a, right).[32]

The Myc TF modulates about 15 % of the entire genome and it is highly abundant in proliferating cells. Under normal circumstances the level of Myc is tightly controlled by several mechanisms on the genetic, mRNA and protein level. Aberrant Myc control and Myc overexpression is observed in over 50 % of human cancers. When abnormal Myc levels occur in combination with defective regulation of stress response checkpoints, non-canonical E-box sites become occupied by Myc resulting in activation of otherwise silent genes. This eventually leads to Myc involvement in all hallmarks of cancer such as proliferation angiogenesis and invasion.[32,46,49,50] Targeting Myc and Myc-controlled expression thus constitutes a promising pathway in cancer therapy and given its universal role, might make it an especially interesting target in cancers with poor prognosis and limited treatment options.

However, Myc is an intrinsically disordered protein (IDP) and by itself lacks features such as defined pockets which would be paramount for the development of classical small molecule drugs. Therefore, only a handful of small molecule drugs have been developed. These include the Max homodimer stabilizer KI-MS2-008,[51] EN4 which reduces Myc stability by covalently targeting C171[52] as well as Myci361 and Myci975 which disrupt Myc/Max dimerization and destabilize the Myc protein.[53] An interesting new approach is a recently developed bivalent proteolysis targeting chimera (PROTAC) aimed at Myc which combines a TNA aptamer with an E-box DNA motif to achieve selectivity.[54]

2.1.2. Omomyc

Developed by Soucek et al. in the late 90s, Omomyc (Figure 2b) is a Myc-derived protein which, in contrast to its parent protein, is able to homodimerize. However, as it does not contain a transactivation domain, it is unable to activate gene transcription. Omomyc's ability for homodimerization is achieved by four distinct mutations in its leucine zipper region which prevents homodimerization of canonical MYC: Two argininines lead to unfavorable equally charged contacts in a Myc homodimer and were replaced by the AA found in Max at their respective positions (R77Q and R78N). The same was done mutating E70I. Finally, E63 was replaced, not with asparagine, as found in Max but with a threonine as this lead to a better complementarity of the two monomers.[55] Omomyc has been found to form homodimers as well as heterodimers with Myc and Max. Whereas Omomyc homodimers and Omomyc/Max-heterodimers can bind DNA at the E-box motif thereby preventing Myc gene regulation, Omomyc/Myc heterodimers do not bind DNA. Omomyc thus competes with Myc for DNA binding while at the same time sequestering Myc away from DNA.[26,56]

When conditionally expressed in cells, Omomyc has been found to bind to the E-box sequence thereby blocking Myc binding and thus transactivation of genes. It does, however, have little effect on or even potentiates Myc gene repression.[57] It has been suggested that this could be by Omomyc interaction with Myc interacting zinc finger 1 (Miz-1).[58] Yet, blocking of repression has been found as well.[56]

Omomyc displays a Myc-dependent apoptotic effect on cells.[57] In mouse skin, Omomyc was able to restore normal tissue homeostasis and counteract the Myc-induced onco-

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genic activity.[59] When expressed in transgenic mice inducible by doxycycline, Omomyc lead to regression and even eradication of lung carcinoma, whereas the effects on normal tissue are reversible and mostly found in highly proliferating cells.[60,61] It also leads to tumor regression in transgenic mice with an SV40-driven pancreatic islet tumor, suggesting that endogenous Myc is not only necessary to maintain the tumor environment in Myc-driven tumors but also in tumors driven by other oncogenic processes.[62] Similar effects have been found with glioma.[63] The reason for the remarkable effects on tumor growth while simultaneously exhibiting little side effects has been suggested to be rooted in Omomyc blunting Myc promoter invasion. Omomyc is believed to mainly affect low affinity Myc-binding sites that are only occupied at high Myc levels whereas Myc-binding to high affinity binding sites also occupied at physiological Myc-levels are hardly affected.[56]

Due to the need to conditionally express Omomyc in these studies, its use was mainly limited to being a proof of principle and research tool instead of being used as a potential cancer treatment. More than 20 years after its emergence, however, Soucek et al. found that Omomyc possesses cell penetrating properties, most likely conferred by the basic region and its generally high arginine content.[26] Omomyc is considered to mainly enter the cell by clathrin-mediated endocytosis and macropinocytosis. Treatment of a mouse NSCLC xenograft with Omomyc slowed tumor progression and in combination with paclitaxel almost led to tumor abrogation.[26] Although another study in which Omomyc is fused to a cell-penetrating peptide (CPP) indicates less favorable cell penetrating properties for Omomyc when not combined with the CPP,[64] cell penetration of Omomyc has been corroborated by Demma et al. in an extensive study in which they also further examined the Omomyc binding to E-box sites.[65]

Several solid phase peptide synthesis (SPPS) approaches have been used to produce Omomyc[27–29,31] (as well as Myc and Max).[30,66–68] Brown et al. synthesized the whole protein using either microwave or infrared irradiation during SPPS and also explored native chemical ligation (NCL) to be able to make Omomyc from two fragments. Furthermore, they developed a high-throughput microbial expression workflow to introduce mutations and improve Omomyc binding affinity. They were able to significantly improve the K_D towards a fluorescently-labelled E-box DNA and found that the coiled-coil dimerization domain could be almost entirely removed and replaced with a disulfide bond.[69]

Omomyc, Max and the DNA binding domain of Myc have been synthesized by fast-flow solid-phase peptide synthesis. In a first approach all six combinations of homo- and heterodimers were obtained using an S-arylation approach with palladium oxidative addition complexes. All dimers except Myc/Myc and Omomyc/Myc do bind DNA in electrophoretic mobility shift assays (EMSAs), confirming earlier findings (Figure 2c, right).[27] In a second approach, Pomplun et al. obtained homodimers of Max and Omomyc as well as Myc/Max and Myc/Omomyc heterodimers by fast-flow synthesis using a special linker to the resin allowing their parallel (homodimers) or sequential (heterodimers) synthesis (Figure 2c, left).[28] The dimers display binding in EMSAs and inhibit cell proliferation. Penetration into the nucleus is shown using a fluorescently-tagged analog and can be improved by adding a nuclear penetration sequence to the protein. Remarkably, the size of these synthetic cell penetrating modalities ranges from 160 to 230 amino acid residues. In both studies RNA sequencing and gene set enrichment analysis (GSEA) were performed and show significant modulation of Myc-target genes.

In an attempt to find new stapling and S-alkylation strategies, a Myc-derived 38mer was used in Zn^{2+}-promoted stapling reactions. This method was then expanded to S-palmitylate full length Omomyc which might increase cell permeability and serum stability as the palmityl-group promotes albumin binding.[71]

2.1.3. Max-Derived Mini Proteins

A miniprotein derived from a fusion of Max and E47, the miniprotein ME47, has been developed in an attempt to inhibit Myc-binding to DNA using a "smaller protein with a simplified structure".[72] The chimeric ME47 comprises the Max basic region in conjunction with the HLH of TF E47. The strongly homodimerizing HLH leads to stable ME47 dimers which bind DNA with affinity in the low nanomolar range but, due to the differences between its HLH and the HLHzip of Myc and Max, no interaction with other proteins of the Myc/Max system is possible.[33] The crystal structure has been solved to a resolution of 1.7 Å and shows that ME47 usually exists as a dimer in solution, although about 17% of it forms tetramers. Interestingly, no monomeric ME47 is found, further highlighting that no interaction with Myc or Max is to be expected. As is to be expected with intrinsically disordered regions, when crystallized in the presence of dsDNA, alpha-helicity increases.[72] ME47 reduces cell proliferation when ectopically expressed in TNBCs MDA-MB-231 and delays tumor growth in mouse xenografts. Using FLAG-tagged ME47 in a ChIP-qPCR experiment, it is shown to bind and compete with Myc for binding with E-box as Myc-binding is reduced in a competitive ChIP-qPCR experiment.[73]

Using phage-assisted continuous evolution (PACE), Inamoto et al. further evolved ME47. First they mutated R12A and C29A and eventually added the LZ domain of their previously developed FosW protein to stabilize dimerization and correct folding of the protein which shows a tendency to misfold. This yields MEF which they term a 'franken-protein' as it is composed of domains from different classes of TFs, preventing heterodimerization. MEF displays slightly increased E-box binding and strong cooperative binding.[74]

In recent work Moellering and co-workers used the basic helix along with the minimal zipper helix from Max to target E-Box DNA with high affinity. The individual helices harbor olefin containing amino acids allowing for side-chain stapling by ring-closure-metathesis and appropriate amino acids for ligation with a maleimide linker. This resulted in a synthetic TR composed of stapled basic and minimal zipper

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helices connected by a maleimide linker termed 'cross-dimer' (Figure 2d). Their synthetic TFs exhibit an effect on Myc-regulated genes, are cell permeable and, owing to stapling, are more resistant to proteolytic cleavage. Most interestingly, they could further expand their design to make synthetic TFs targeting different DNA sequences by sequence alignment.[70]

Similar to Omomyc, O'Neil and co-workers at MSD developed Mad, an artificial transcriptional repressor based on Mxd1, a Max binder and Myc antagonist. Mad is composed of the first 146 AAs of Mxd1 including an S146A mutation to prevent phosphorylation, ubiquitination and subsequent degradation along with a domain for interaction with mSin3, a transcriptional repressor. Mad exhibits better properties than Omomyc in cell proliferation assays and penetrates the cell where it can be found in the nucleus. It further interacts with Max but not Myc as seen in coimmunoprecipitation experiments and displays stronger downregulation of Myc-upregulated genes than Omomyc. Lastly, a ChIP assay confirms that Mad binds to E-box motifs in Myc-regulated genes and actually competes with Myc for binding.[75]

2.2. Targeting G-Quadruplexes with Synthetic Peptides

2.2.1. Structure and Biological Relevance of G-Quadruplexes

Guanine-rich sequences of ssDNA (e.g. unwound dsDNA) as well as RNA are prone to form guanine-quadruplex (G4) secondary structures (Figure 3). In these G4s four guanines assemble into a planar quartet formed by Hoogsteen-type base-pairing and the G4 is then established by more than two tetrads stacking on top of each other making use of π–π-interactions. The tetrads are further stabilized by binding of positively-charged ions such as Na^+ and K^+, with the latter being the most stabilizing one.[76,77] Depending on the direction of the four strands forming the edges of the G4, different topologies of them can form.[78] Moreover, G4s can be intra- and intermolecular. The cytosine-rich ssDNA complementary to G4s has been found to forge secondary structures termed i-motif[79] but whether these form under the same conditions as G4s or are mutually exclusive has not been conclusively proven thus far.

The consensus sequence for G4s to assemble is $G_{3\text{-}5}N_{1\text{-}7}G_{3\text{-}5}N_{1\text{-}7}G_{3\text{-}5}N_{1\text{-}7}G_{3\text{-}5}$ with the N-termed nucleotides functioning as linkers between the columns of guanines.

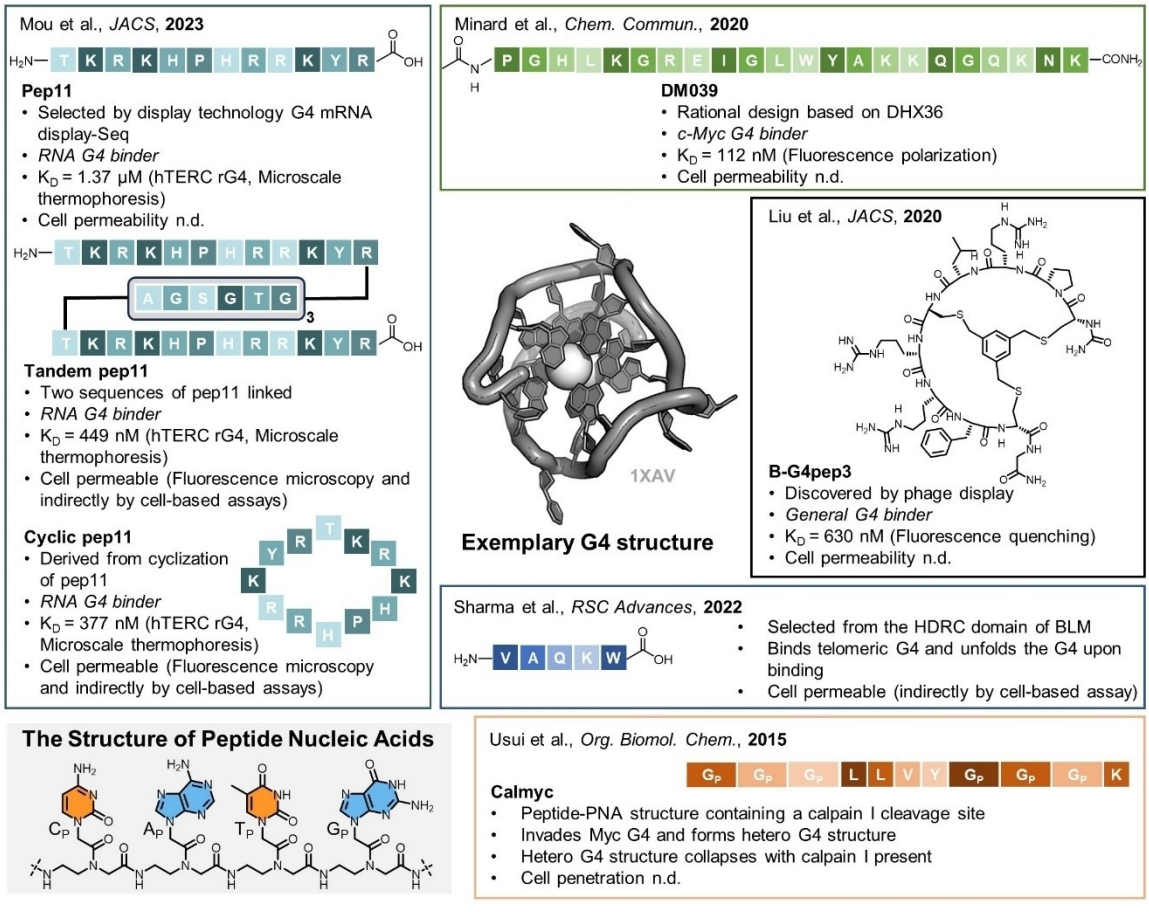

Figure 3. Peptides targeting G-quadruplexes. General G4 structure (PDB 1XAV, middle) and examples of G4-targeting peptides. Lower left panel shows the structure of Peptide Nucleic Acids (PNAs) and lower right panel shows a peptide-PNA structure. Subscript P denotes PNA. Representation of crystal structure was generated using PyMol. n.d. = not determined. Top left: Mou et al.;[97] Top right: Minard et al.;[36] Top middle right: Liu et al.;[94] Lower middle right: Sharma et al.;[98] Bottom right: Usui et al.[34]

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Simply scanning the human genome for this sequence suggested that there may be about 300,000 potential G4s.[80] However, two guanines from within a column can be separated by other nucleotides forming a bulge between the guanidine stretch thereby leading to non-consensus G4 sequences.[81] Thus, using Illumina sequencing in a method called g4-seq a total of 716,310 potential G4-forming sequences were identified.[82]

G4s are more abundant at telomeres, promoter regions, 5' and 3' untranslated regions (UTRs) along with ribosome binding sites (RBSs) suggesting a biological function and thereby making them potential therapeutic targets.[37,83] Generally, G4s might play a role in cellular functions such as transcription, replication, genomic stability and others.[78] For instance, in telomeres, G4s might prevent normal function of telomerases by segregating the 3' end and hampering its extension.[84] Stabilizing telomeric G4s is thus a valuable strategy to target telomerase activity in cancer. Nevertheless, it has also been found that telomerase can unwind telomeric G4s and might play a role in their recruitment making it crucial to establish when stabilization of G4s is beneficent.[78,85]

G4 enrichment in oncogenic promoter regions such as the Myc oncogene[86] suggests a role in regulating transcription. The Myc oncogene promoter entails a G4 and treatment with G4 stabilizing porphyrin TMPyP4 reduces MYC expression, [87] hinting on G4s functioning as negative regulators of gene expression. Similarly, stabilizing a G4 in the KRAS promoter with TMPyP4 results in reduced expression[88] and small molecules binding to the c-kit G4 reduced c-kit expression in MCF7 and GIST882 cells[89,90] exemplifying the value of G4 binding molecules as pharmacological compounds. However, many small molecules make use of π-stacking interactions with the surface of G4s which is a good strategy for gaining selectivity over dsDNA but does hardly allow to differentiate between different G4s . Peptides offer a more complex array of possible interactions and are thus a valuable tool to target and differentiate between different G4s.[36]

2.2.2. Targeting G4s with Synthetic Peptides

Many proteins bind and either stabilize, unwind or degrade G4s.[37] For instance, nucleolin, a protein binding to histones and playing a role in chromatin decondensation has been found to stabilize and fold G4s. It is involved in formation of the G4 in the Myc promoter region thereby reducing promoter activity and acting as tumor suppressor.[91] Furthermore, topoisomerases promote G4 formation and helicases unwind G4s catalyzing ssDNA formation.[37] The multitude of G4 binding proteins thus provide a logical and promising starting point for the design and development of G4 targeting peptides and numerous peptides have been developed. Therefore, this chapter will only highlight some recently developed peptides (for a comprehensive review see Sharma, Saxena and Kaur[37].)

Generally, peptides binding G4s can be classified according to different categories, one of which being their binding mode. As such they can intercalate into the planes of the G4 and they can bind to the loops and bulges between guanines. Additionally, not directly binding to the G4s, they can also bind at the dsDNA-ssDNA junction and bind the complementary cytosine-rich ssDNA.[37]

Making use of the crystal structure of bovine helicase DHX36 bound to the G4 found in the Myc oncogene promoter region,[92] Minard et al.[36] designed a peptide targeting this G4. The 22 AA peptide termed DM039 spanning residues 57–78 as the minimal Myc-binding domain of DHX36 bound the Myc G4 selectively not only over dsDNA and non G4 ssDNA but also over other G4s such as the c-kit1 and c-kit2 G4s. However, when they introduced either an i, i+4 or an i, i+7 staple the peptide bound with reduced affinity to the Myc G4 and the selectivity over other G4s was lost. This suggests that a certain amount of flexibility is required for binding and thus an induced-fit binding mechanism.[36]

The group of Shankar Balasubramian[35,93] used phage display from a library of 4x4 and 3x3 bicyclic peptides to screen for G4 binders. They cyclized the linear peptides the phages displayed using TBMB and screened on biotinylated G4s. They found a bicycle with nanomolar affinity towards the c-kit2 G4 and by computational modeling demonstrate the affinity is derived from molecular preorganization.[94]

Exploring an interesting concept Usui et al.[34] employed peptide NAs (PNAs, Figure 3 lower left panel) for the formation of switchable heteromolecular G4s in the Myc promoter region (see Brodyagin et al.[95] for a review of PNA and their application). They designed calmyc, a guanine-rich PNA construct comprising a calpain I protease cleavage site. Calmyc is able to invade the Myc G4 and form two 3-tetrad PNA-DNA hybrid G4s with ssDNA from the Myc promoter. These hybrid G4s displayed higher stability than the DNA G4 but collapsed upon calpain I addition allowing for control over the DNA secondary structure and stability.[34] They later employed calmyc to break G-wires.[96]

Saxena and co-workers demonstrated that not only stabilization but also unwinding of G4s can have beneficial effects, highlighting the complicated roles G4s might play. They employed a peptide library based upon the bloom syndrome protein helicase domain (BLM) to target telomeric G4. BLM unfolds telomeric G4s enabling normal telomer replication which is defective in Bloom's syndrome leading to telomere shortening and genomic instability.[99] The 5-mer BLM-peptide, VAQKW, derived from the library binds to the G4 loop with the C-terminal tryptophan intercalating into the G4-planes. This intercalation destabilizes the stacking interactions and leads to unwinding of the G4. Interestingly, the peptide displays an antiproliferative effect on MDA-MB-231 breast cancer cells.[98]

In a different project, they developed a 10-mer peptide, QW10 (QQWQQQQWQQ), consisting of glutamine for hydrogen-bond interaction with guanine bases and tryptophane for intercalation into the G4 planes. QW10 stabilizes telomeric[100] as well as the c-Myc[101] G4s in the presence of potassium. Conversely, another human telomeric G4 is destabilized by this QW10.[102] Moreover, they then shorten QW10 to a 5-mer peptide (QQWQQ) which destabilizes

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and unfolds human telomeric G4s by tryptophan intercalation.[103]

Recently, an RNA display platform termed G4-mRNA display-seq has been developed for screening of peptide binders to RNA G4s. They found a peptide binder, pep11, that bound specifically to RNA G4s. Furthermore, binding could be increased by cyclization of the peptide, showing that sequences found by this technique can be valuable as starting points for rationally designing peptide binders to G4s.[97] Although this method was developed using RNA G4s, it should be amenable to DNA G4s.

3. Targeting of RNA with Synthetic Peptides

In the process of transcription, RNA polymerases, assisted by various enzymes, transcribe genetic DNA into RNA. A key molecular distinction between RNA and DNA oligonucleotides lies in the presence of a 2' hydroxyl group in RNA and the use of the uracil nucleobase (RNA) instead of thymidine (DNA). RNA serves diverse functions within cells. mRNA undergoes translation to synthesize amino acid sequences, ultimately forming proteins. A substantial portion of transcribed RNA is classified as ncRNA. ncRNA can fold into various (semi)stable secondary and tertiary structures (Figure 4), and performs a multitude of functions.[104,105] Prominent examples of ncRNA include transfer RNA (tRNA) and ribosomal RNA, both crucial for the translation process. Another noteworthy category of ncRNA comprises microRNAs (miRNAs), serving as crucial regulatory elements in gene expression (elaborated upon in the subsequent chapter). Numerous long and short ncRNAs harbor yet unknown functions.[106,107]

Typical RNA motifs include double stranded RNA (dsRNA), hairpin RNA, stemloops, bulges and internal loops.[107,108] Longer ncRNAs can also fold into tertiary structures.[109] RNA structures are less rigid and defined than protein structures, posing challenges in the discovery of selective high-affinity ligands. Nevertheless, there is a growing interest to identify modalities that specifically target RNA.[13,14] Disney et al. have been pioneers in developing strategies for discovering small molecules that target specific RNA motifs.[110] Given the expansive surface area of RNA scaffolds and their relatively low structural diversity, larger modalities like peptides and peptidomimetics emerge as intriguing options for achieving high-affinity and specific RNA targeting. This chapter delves into the exploration of peptidic modalities designed to target disease-related RNAs, such as pre-miRNAs and viral RNAs. For an extensive overview on cyclic peptides that target RNA, we refer to a recent review by Pal and 't Hart.[111]

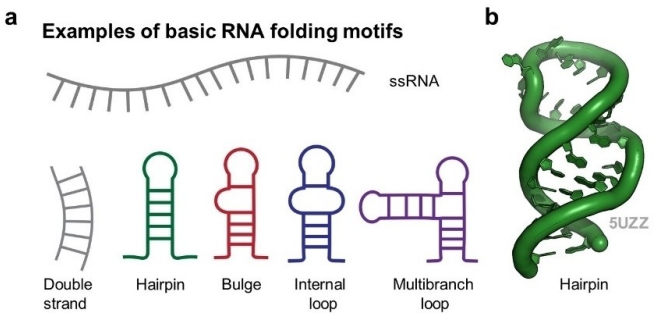

Figure 4. RNA secondary structures (**a**) graphic representation of common secondary RNA structures. (**b**) Crystal structure of an RNA hairpin. (PDB 5UZZ, structure created using PyMol).

3.1. MiRNA

MiRNAs play pivotal roles in gene regulation and many miRNAs have been implicated in disease.[112–117] These small, approximately 22-nucleotide-long, single-stranded oligonucleotides primarily modulate the translation of specific mRNAs, often by inhibiting protein synthesis.[112,115,118] It is estimated that miRNAs influence over 60% of all genes. Elevated levels of specific miRNAs have been linked to diseases such as cancer, metabolic disorders, vascular diseases, neurological conditions, and Alzheimer's disease, making them attractive targets for therapeutic interventions.[115,116,119,120] Antisense strategies have been proposed to target miRNAs, exemplified by the clinical candidate Miravirsen, blocking the hepatitis C virus miRNA-122 interaction.[121–123]

Two key precursors of mature miRNAs, namely primary miRNA (pri-miRNA) and precursor miRNA (pre-miRNA) hairpins, represent preferential targets for inhibiting miRNA activity.[112] Pri-miRNAs possess a large stem-loop structure that undergoes capping, polyadenylation, and cleavage by the RNAse Drosha in the final step, generating hairpin-shaped pre-miRNA in the nucleus. Subsequently, pre-miRNA is translocated from the nucleus to the cytoplasm where it undergoes cleavage by the RNAse Dicer resulting in the formation of mature miRNA duplexes. The guiding strand of these miRNA duplexes can interact with the RNA-induced silencing complex (RISC), forming the miRNA-RISC complex, which can bind to mRNA and either block or degrade targeted genes (Figure 5). The relatively defined structures of these miRNA precursors offer feasible targets for ligand development. Inhibiting the interaction between Drosha or Dicer and the RNA scaffolds blocks production of mature miRNA and with that miRNA activity. In this chapter we describe rational design and library-based strategies for the discovery of peptides targeting disease related pri- and pre-miRNAs (Figure 6a).

3.1.1. MiR-21

miRNA-21 (miR-21) is widely implicated in cancer development, and therefore also referred to as an oncomir.[124] It blocks genes that are involved in apoptosis. Inhibition of miR-21 is therefore an interesting pharmaceutical approach. In the following sections we describe both rational design and combinatorial library approaches that led to peptidic miR-21 inhibitors.

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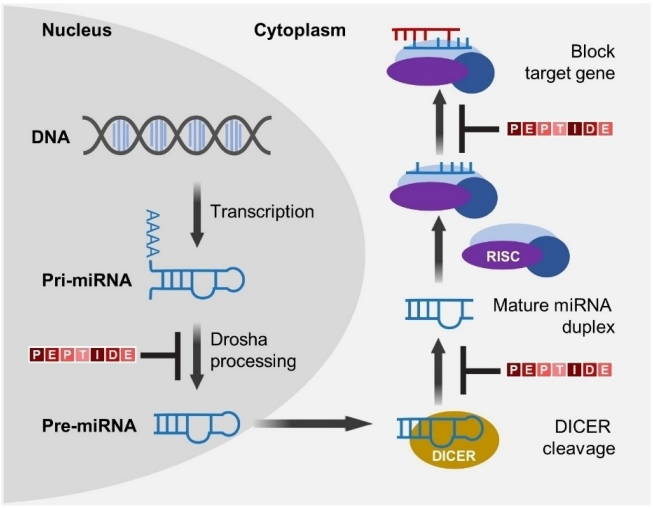

Figure 5. The biogenesis of mature miRNA. Pri-miRNA is formed by RNA polymerase II transcription in the nucleus and processed by Drosha resulting in pre-miRNA. After being transported to the cytoplasm, pre-miRNA is cleaved by DICER, generating mature miRNA which is subsequently incorporated into the RISC complex to degrade or block targeted genes. Maturation steps that can be inhibited with peptides are highlighted by a red peptide representation.

Chirayil et al. performed a screening with a combinatorial peptoid library containing 7680 members to identify binders for the apical loop of pri-miR-21, the binding site of Drosha.[125,126] They identified a peptoid with an affinity of 1.9 μM against pri-miR-21 (Figure 6b, left).[126] However, in the presence of magnesium concentrations needed for microprocessor activity, which is necessary for RNA cleavage, no binding was observed. Building on these findings, Diaz and collaborators identified a novel peptoid through a library screen, utilizing the peptoid identified by Chirayil et al. as the library scaffold.[125] The screen resulted in a compact peptoid with an affinity of 12 μM (Figure 6b, left).

Bose et al. utilized phage display to discover high affinity peptides binding to pre-miR-21.[113] The best hit, a 12-mer peptide with the sequence ALWPPNLHAWVP demonstrated a binding affinity of 12.7 nM (Figure 6b, upper middle). The bulges in the pre-miR-21 secondary structure are necessary for peptide recognition. Removal of the bulge near the terminal loop resulted in weak binding of the peptide, thereby demonstrating that the secondary structure is important for RNA recognition. The peptide was able to inhibit Dicer processing of pre-miR-21 to mature miR-21, as demonstrated by Dicer cleavage assays. Additionally, the TUNEL assays showed an increase of 30% in apoptosis caused by their hit compound.

Given the advantageous properties of macrocycles, such as stability and cell permeability,[127] the Varani group has focused on the design of macrocyclic peptides that can target RNA, including pre-miR-21.[128–130] They developed β-hairpin mimetics with a D-Pro/L-Pro template that are on average 12 amino acids in size.[128–130] This specific shape allows the peptides to mimic the orientation of the sidechains in an α-helix.[131] This strategy was used to identify peptides that can target pre-miR-21,[129] pre-miR-20b,[130] transactivation response (TAR) RNA and Rev Response Element (RRE) RNA.[131–138]

Dicer binds pre-miRNA at the apical loop. A library, which was originally designed for the bovine immunodeficiency virus (BIV) TAT RNA binding domain (RBD)[134] was screened against pre-miR-21 by Shortridge et al.[129] They identified a peptidomimetic that could bind the minor groove of pre-miR-21 with an affinity of 200 nM (Figure 6b, lower middle). L50 could also inhibit Dicer cleavage and prevent miR-21 maturation.

To identify a mirror image peptide that is able to inhibit miR-21, Sakamoto et al. screened a phage display library against L-pre-miR-21.[139] The obtained hit was resynthesized using D-amino acids and used in a Dicer assay (Figure 6b, lower right) that showed an IC_{50} of 540 nM and Dicer inhibition.

In 2020 Pomplun et al. designed a combinatorial library platform to target miRNA with biohybrid peptidomimetics displaying amino acid side chains and nuceobase functionalities.[140] Combining 12 nucleobase building blocks with 18 natural amino acids provided a combinatorial 100 million-membered library of 10-mer nucleobase peptides. The authors demonstrated how the nucleobase side chains increase overall affinity for nucleic targets, compared to using natural peptides. A hit compound with an affinity of 9 nM towards pre-miR-21 was identified from the selection. The biohybrid hit compound contains 4 nucleobases and 6 natural side chains. (Figure 6b, upper right) Overall this work shows that the RNA binders can be challenging as only one hit was selected from a library with 100 million compounds. At the same time the study points out the importance of ultralarge libraries and smart library design.

Structure-based approaches have been performed to design double-stranded RNA binders.[144,145] Kuepper et al. performed a structure-based approach to target mature miR-21 and thereby prevent miRNA-RISC interaction.[146] As template they used the *tomato aspermy virus* 2b (TAV2B) protein that can bind double-stranded RNA non-specifically and acts as a gene silencer.[146] Trimming this protein resulted in a peptide sequence that consists of 33 amino acids displaying an affinity of 1.19 μM towards palyndromic RNA (pal-RNA). Kuepper et al. showed that their peptidomimetic could bind miR-21 as well. Remarkably, the implementation of two hydrocarbon staples significantly improved affinity to 18 nM against double stranded miR-21, 75 nM against pre-miR-21 and 70 nM against a pal-RNA duplex. Additionally, the peptide was able to inhibit Dicer cleavage of pre-miR-21. In another study the Grossmann group coupled the unstapled TAV2B peptidomimetics to complementary DNA to generate peptide-oligonucleotide conjugates that were able to form DNA/RNA/peptide complexes with single stranded RNA and make them more sequence specific.[147]

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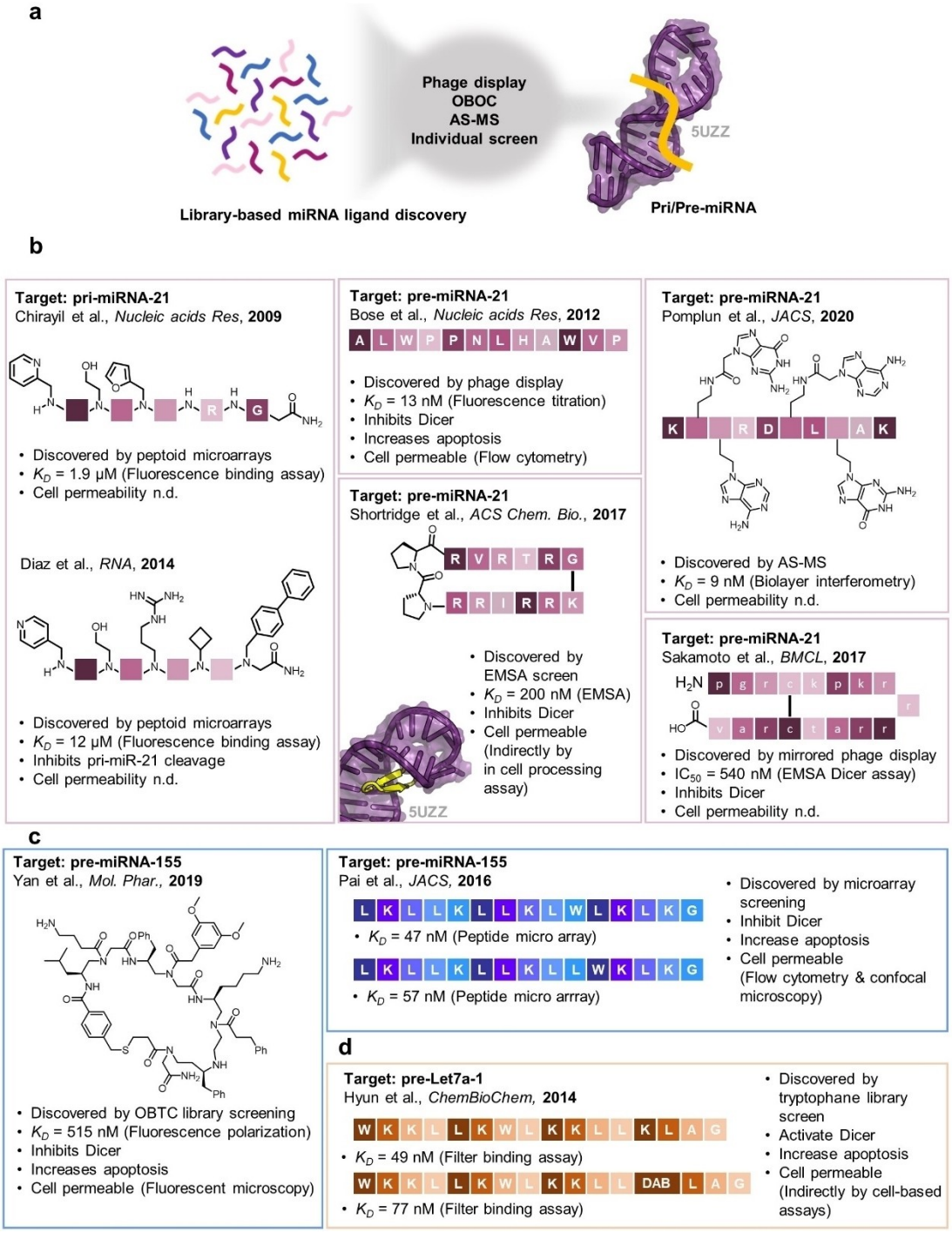

Figure 6. Peptide sequences that target disease-related miRNAs. (**a**) Different methods can be used for the screening of peptide-libraries ultimately resulting in pre/pri-miRNA binders (PDB 5UZZ). (**b–d**) Hit peptides identified in screenings against pri/pre-miR with their corresponding affinities and downstream effects. b right: Chirayil et al. and Diaz et al.;[125,126] upper middle: Bose et al.;[113] lower middle: Shortridge et al.,[129] PDB 5UZZ; upper right: Pomplun et al.;[140] lower right: Sakamoto et al.;[139] c right: Yan et al.[141] ; upper right: Pai et al.;[142] lower right: Hyun et al..[143] Representations of crystal structures were made using PyMol. Dab = L-2,4- diaminobutyric acid. n.d. = not determined.

3.1.2. MiR-155

Another miRNA with important implications in cancer is miR-155.[148] miR-155 is involved in apoptotic and it has been shown that the inhibition of miR-155 prevents cancer development in mice.[148]

Several strategies for the targeting of pre-miR-155 using library screening techniques have been described. It has been shown that amphiphilic peptides have increased

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affinity towards hairpin RNA.[143,149,150] As such, Pai et al. developed a 185 membered amphiphilic peptide library based on the sequence LKKLLKLLKKLLKLKG, a peptide originally designed to bind calmodulin, [142] and screened it in a micro array format against fluorescent pre-miR-155.[114] This first screening round identified twelve peptides that displayed affinity towards pre-miR-155. A Dicer inhibition assay identified two peptides showing IC_{50}s of 0.77 μM and 1.6 μM and K_Ds of 47 nM and 57 nM, respectively (Figure 6c, right). These two hits promoted apoptosis through the caspase pathway.

Yan et al. designed a cyclic γ-substituted-N-acylated-N-aminoethylamino acids (γ-AA) library using the one-bead-two-compounds (OBTC) strategy.[141,151] They screened a 320,000 membered library and identified a cyclic peptidomimetic able to bind pre-miR-155 and inhibit Dicer activity in vitro and in vivo with an IC_{50} of 22 μM (Figure 6c, left).

3.1.3. MiRNA Let7a-1 and MiRNA-23b

Upregulation of miRNAs does not necessarily directly contribute to cancer development in all cases. Let7a-1 is an example of a miRNA that promotes caspase dependent apoptosis, thereby preventing uncontrolled cell growth.[152,153] Decreased let7a-1 maturation could lead to the development of cancer. Similar to the library designed by Pai et al.,[114] an amphiphatic library was designed, in which in the sequence LKKLLKLLKKLLKLAG each leucine was replaced by a tryptophane.[143] Multiple scanning rounds resulted in the identification of two peptides binding pre-let7a-1 with affinities of 49 and 77 nM (Figure 6d). More importantly, one of these peptides increased Dicer induced pre-let7a-1 cleavage resulting in reduced activation of target genes in cells. The researchers suggest that binding of the peptidomimetic causes a conformational change in pre-Let7a-1, thereby making the Dicer cleavage site more accessible. This research demonstrates that peptidomimetics can also be utilized to promote Dicer activity.

Another miRNA involved in cancer development is miR-23b. An interesting design was developed by Krishnamurthy et al.[154] They developed helix-threading peptides (HTPs) using ring-closing metathesis. HTPs are heterocyclic peptides that can intercalate into RNA structures. RNA footprinting showed that two HTPs were able to selectively bind and protect hairpin RNA from nuclease activity. One peptide was able to bind pre-miR-23b with an affinity of 2.33 μM. However, as this peptide could bind multiple RNA sequences, more optimization is necessary for a selective miR-23b binder.

Various techniques exist for identifying peptidomimetics with selectivity towards miRNA. Library screening has proven to be a robust method uncovering multiple peptidomimetics capable of disrupting miRNA maturation at different stages of its biogenesis. While many strategies focus on the apical loop to inhibit Dicer or Drosha activity, peptidomimetics can also be employed to upregulate Dicer activity, influencing downstream processes. The growing accessibility of diverse unnatural amino acids further broadens the chemical space available for miRNA targeting.

3.2. Viral RNA

Viral RNAs, especially those of the Human Immunodeficiency Virus type-1 (HIV-1), have been investigated as compelling targets for therapeutic intervention. Following the entry of HIV-1 into the host cell, its genomic RNA undergoes transcription into complementary DNA (cDNA) which is then transported to the nucleus and integrated into the host DNA.[155] RNA polymerase II (pol II) is then recruited to the HIV-1 promoter in the viral 5′ Long Terminal Repeat (LTR), allowing the transcription of the viral genome back into genomic RNA.[155] Two essential regulatory proteins, TAT and Rev, play a pivotal role in the growth and replication of HIV-1.[156] The effectiveness of both proteins relies on their binding to specific RNA elements; Tat binds to TAR RNA, and Rev binds to RRE RNA. Consequently, peptides that disrupt the TAT:TAR or Rev:RRE interaction have been explored as a potential strategy to address HIV-1 infection.[157]

3.2.1. Peptidomimetics Targeting TAR

TAT, a cell penetrating transcriptional transactivator protein, plays a central role in the early development of HIV-1.[158] The target of TAT is TAR, which is an RNA hairpin located at the 5' end of viral transcripts.[159] TAT interacts with the major groove of TAR through an arginine-rich motive (ARM). Upon binding TAR, TAT recruits the superelongation complex (SEC), which contains the positive transcription elongation factor b (P-TEFb). P-TEFb consist of Cdk9 and Cyclin T1 that can phosphorylate RNA pol II.[156,159] As such, when TAT binds the conserved TAR hairpin, Pol II is activated and the HIV-1 transcripts are formed. Whereas TAT can only bind to TAR, P-TEFb is involved in gene activation for multiple genes of the host. Therefore, TAT:TAR is an interesting pharmaceutical target.

As TAT:TAR binding is critical for the formation of HIV-1 mRNA in the early stages, much effort has been performed to block this interaction using peptidomimetics. Early publications focused on the analogous BIV-derived TAR stem-loop interaction with TAT because of limited structural information.[160,161] Later, linear peptides[149,150,162,163] or peptoids[164] were developed to block TAT:TAR or Rev:RRE. However, because of their high flexibility SARs remained limited. As previously mentioned, Varani and co-workers designed a D-Pro/L-Pro framework to target RNA using cyclic peptidomimetics. They were also successful in targeting RRE and TAR RNA using a similar cyclic framework, initially based on the BIV TAT peptide,[131–138] or a γ-AA framework.[163] To obtain TAT mimicking peptides, as starting point the BIV TAT was used and multiple optimization rounds were performed to design cyclic peptides with high affinity towards HIV TAR, with

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their most recent peptide targeting HIV-1 TAR within the picomolar range (Figure 7, lower left).[132] Nevertheless, cellular data showed only limited inhibition of binding between TAR RNA and P-TEFb. The researchers suggest that targeting the TAR loop alone is not sufficient to block the formation of the SEC.

High affinity binders towards RNA can be obtained using an RNA binding protein as a blueprint. Crawford et al. used laboratory evolution to alter the RNA Recognition Motif (RRM) of U1A to find a selective binder of the TAR hairpin.[165] They identified a selective TAR-Binding-Protein (TBP) with low nanomolar affinity that could interfere with TAT:TAR binding (Figure 7, upper panel). The Wedekind lab crystallized the most promising candidate, TBP6.7, in complex with the TAR hairpin which revealed an arginine trio located in a β2-β3 loop (Figure 7, upper panel).[166] Remarkably, constrained isolated β2-β3 loop peptides could also bind TAR RNA with a K_D of 1.8 μM and inhibit TAT binding (Figure 7, upper panel).[166] Further research using variants of TBP6.7 revealed the importance of the spacing and position of the arginines located in the 'arginine fork'.[167] Moreover, cyclization of the β2-β3 loop of TBP6.9 resulted in an affinity of 3.6 μM against TAR RNA (Figure 7, middle). A follow-up cyclization study showed that napthalene and methylene linkers improved affinity.[168] Whereas the napthalene linker showed increased affinity, the methylene-linked peptides showed higher antiviral activity in cells and lower toxicity. One noteworthy observation was that introducing an Arg-to-Lys mutation in the cyclic peptide led to a tenfold reduction in TAR binding. This finding suggests that TBP-derived cyclic peptides employ an arginine-fork motif to recognize the TAR major groove. Moreover, these cyclic peptides demonstrated the ability to block the TAR binding by the TAT ARM and release the TAT ARM peptide from its bound state. This suggests a potential overlap between the site of cyclic peptide binding to TAR and TAT ARM-RNA binding site.

SPPS gives straightforward access to a vast chemical space and a variety of scaffolds beyond linear peptides. For example, branched peptides are able to have multivalent interactions with RNA that can provide high selectivity and affinity. Bryson et al. developed an on-bead library screen containing 4096 branched peptides, screened them against labeled TAR RNA and recovered hits using de novo sequencing by MALDI/MS resulting in the identification of a selective binder towards TAR RNA (Figure 7, lower right).[169] Most hits contained N-terminal arginines. The linear counterpart of the strongest binder showed a decrease in affinity, thereby illustrating the influence of branching. Later research demonstrated that these branched peptides are cell permeable.[170]

3.2.2. Peptidomimetics Targeting RRE

Rev is involved in a later stage of HIV-1 development. Rev facilitates the transport of unspliced and single-spliced

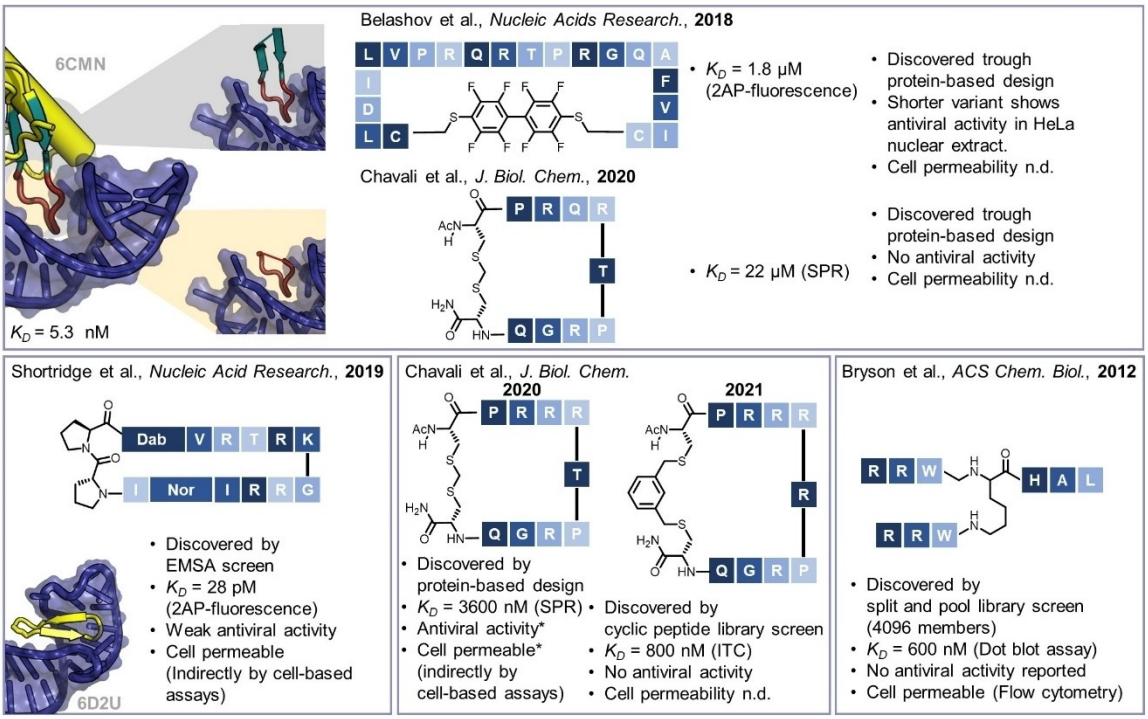

Figure 7. Peptides targeting TAR RNA with their corresponding affinities and downstream effects. Upper panel Belashov et al.[166] and Chavali et al.,[167] PDB 6CMN; lower panel left Shortridge et al.,[132] PDB 6D2 U; middle Chavali et al.;[167,168] right Bryson et al.[169] Representations of crystal structures were generated using PyMol. Dab = L-2,4- diaminobutyric acid. Nor = norarginine. n.d. = not determined. *as reported by Chavali et al., *J. Biol. Chem.* **2021**.

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mRNAs from the nucleus to the cytoplasm where they encode structural viral proteins.[171,172] Similar to TAT, Rev also contains an ARM that provides cell permeability and RRE binding. A difference compared to TAT is that Rev can bind RNA multivalently.[172] The ARM of Rev ($Rev_{34\text{-}50}$) is in total 17 amino acids long and folds into an alpha helix in solution (Figure 8, upper left).[173,174] $Rev_{34\text{-}50}$ can bind the stem-loop IIB of RRE in its major groove using a specific asparagine, threonine and four arginines.

Multiple studies have effectively induced alpha helicity in RRE binding proteins. The group of Frankel designed a combinatorial library against RRE[175,176] and showed that it binds partly as an alpha helix when bound to RNA.[177,178] One of their hit-compounds had a 15-fold increase in specificity towards RRE compared to $Rev_{34\text{-}50}$.[175] Remarkably, this peptide contained a proline in the middle of the helix but was still able to partly form an α-helix, whereas the parent peptide adopted a random coil. Another combinatorial library using an ARM as framework identified the specific RRE binding sequence R_6QR_7.[179] Optimization of this sequence using a macrolactam staple resulted in an IC_{50} of 150 nM.[180] Harrison et al. also introduced a lactam bridge in their peptidomimetic design.[181] They designed Rev analogs by appending the small lactam constrained peptide, c-(1,5-*cyclo*)-[KARAD]-NH_2 known for inducing alpha helicity,[182] to the $Rev_{34\text{-}50}$ peptide. To avoid steric clashes between the cyclic peptide and RNA, various linkers between the C- and N-terminus of $Rev_{34\text{-}50}$ were modeled, resulting in a linker consisting of three alanines (Figure 8, lower left). Additionally, the lactam bridge was also introduced at non-essential positions inside the $Rev_{34\text{-}50}$ sequence yielding eight peptides in total. The most promising peptide featured the N-terminal c-(1,5-*cyclo*)-[KARAD]-NH_2 sequence with a three residue linker. These works demonstrate that the introduction of a staple, such as a lactam bridge, can increase the affinity of peptidomimetics. However, the specific positioning of these staples is critical, as they can also interfere with binding.

In nature, alpha helicity can involve the use of metal ions. Zinc fingers, for example, fold around a zinc ion through the interaction of two conserved histidines and two conserved cysteines.[183] This DNA recognition motif adopts a structural configuration consisting of an α-helix and two β-sheets, with the α-helix specifically interacting with the major groove of the DNA. McColl et al. utilized this zinc finger design by introducing $Rev_{34\text{-}50}$ in the alpha helix of the well-studied zinc finger 268, whereas the β-sheets remained intact.[184] This peptidomimetic was able to recognize RRE in vitro and in vivo. Cowan and co-workers reported metal chelate Rev conjugates with tenfold higher binding affinities for the RRE compared to Rev.[185] These chelate-Rev conjugates formed coordination complexes with metals, and the arginine-rich $Rev_{34\text{-}50}$ facilitated localization to the RRE stem loop-IIB site. Choi et al. engineered self-assembling cyclic peptide gold nanoparticles, incorporating the Rev ARM for RNA recognition and a cysteine-rich domain for gold binding (Figure 8, lower right).[186] These elements were strategically separated by a flexible linker. The cyclic and linear peptides showed similar affinities for RRE RNA in EMSA assays. However, the cyclic variant displayed enhanced selectivity and the capacity for multivalent binding to RRE RNA. Additionally, the cyclic peptide could recognize RRE RNA in both the cytoplasm and the nucleus, whereas its linear counterpart only recognized nuclear RRE

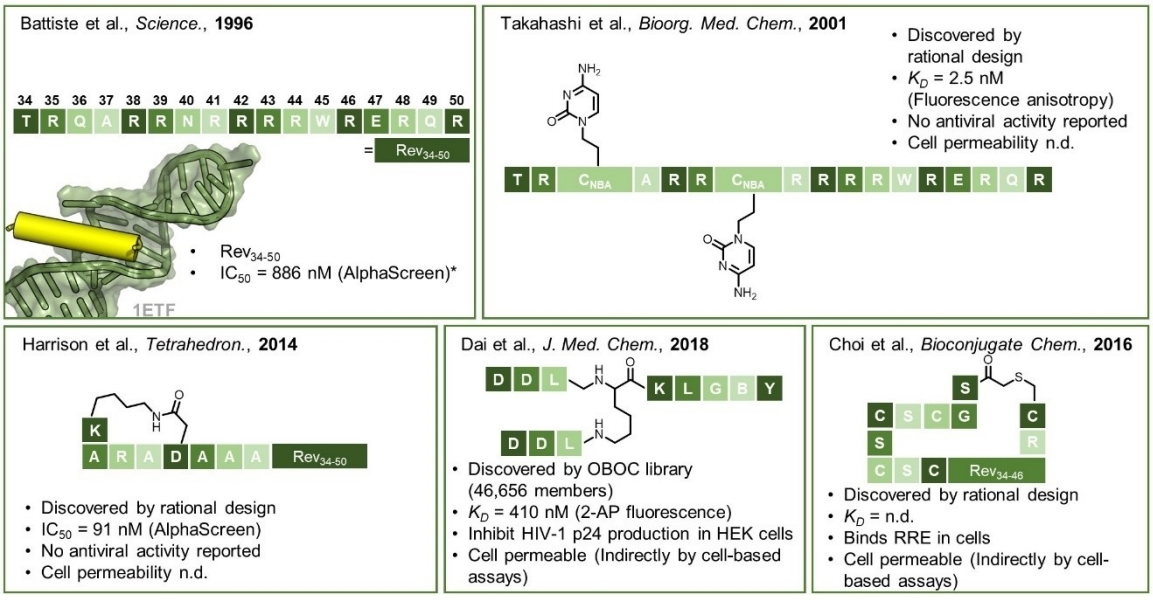

Figure 8. Peptides binding RRE RNA with their corresponding affinities and downstream effects. Upper left panel Battiste et al.,[174] PDB 1ETF; Upper right panel Takahashi et al.;[195] Lower left panel Harrison et al.;[181] lower middle panel Dai et al.;[189] lower right panel choi et al.[186] Representations of crystal structures were generated using PyMol. B = lysine boronic acid. n.d. = not determined. *as reported by Harrison et al., *Tetrahedron.*, **2014**.[181]

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RNA. These studies show the influence that metal ions can have on affinity and that they can be used as an alternative way to promote alpha helicity.

Branched peptide libraries were devised targeting RRE, incorporating boronic acid to facilitate reversible interaction with the 2'-hydroxyl of RNA. This led to the development of selective branched peptides with binding affinities in the micromolar range.[187–192] To further optimize binding, a branched peptide library was designed that introduced both boronic acid and acridine.[187] The latter was introduced because of its intercalating properties. Additionally, a library was designed that contained merely unnatural amino acids.[189] To determine RRE binding, the formation of p24, a HIV-1 capsid protein, was monitored resulting in seven hits in the micromolar range. The strongest binder had an affinity of 410 nM (Figure 8, lower middle).[189] Additionally, it was shown that this peptide binds not only to the stem-loop II regions of RRE, but also in the bulge region of stem loop I, competing for Rev protein primary and secondary binding site.[188] In conclusion, branched peptides exhibit promising results with regard to RRE binding. Their ability to perform multivalent and long-range interactions with RNA make them interesting scaffolds for the development of RNA binding therapeutics.

The Mihara group implemented NBA into Rev_{34-50}.,[193–197] resulting in peptidomimetics that exhibited increased α-helicity in comparison to their PNA counterparts.[197] From a small library, hit compounds were designed that have affinity towards RRE in the nanomolar range and showed higher affinity towards RRE compared to the native Rev_{34-50}. Introduction of PNA moieties in Rev_{34-50} resulted in lower affinity towards RRE compared to Rev_{34-50} but still in the nanomolar range (Figure 8, upper right).[193,194] Overall, incorporating both PNA and NBA proved to be a compelling strategy for targeting RNA, potentially leading to the development of highly effective RNA binders.

3.2.3. Hepatitic C Virus

Another virus that is considered a pharmaceutical target is Hepatitic C (HCV). HCV interacts with ribosomes through their internal ribosome entry sites (IRES).[198] In order to promote viral RNA translation, it is essential for HCV to also interact with the human La protein using their IRESs.[199] The human La protein is characterized as an initiator factor and can bind HCV RNA with its three RRM sites.[198] Pudi et al. isolated a 24-mer from the second RRM that was still able to interact with RNA (Figure 9). Moreover, this peptide could compete with the natural La protein and prevent HCV translation when fused to TAT. The peptide was trimmed down by Mondal et al. to its core sequence only consisting of seven amino acids eventually.[200] As the heptamer adopts a β-turn, Manna et al. prepared a cyclized variant (Figure 9).[201] To facilitate cell permeability, both the linear and cyclized heptamer were fused to hexa-arginine and administered to Huh7 cells. The cyclized peptide displayed an increase in affinity and bigger decrease in HCV RNA. Similar to the Wedekind lab this protein guided approach yielded a peptide that could interact and interfere with viral RNA maturation and proves that proteins can be utilized as a blueprint towards RNA binding peptidomimetics.

4. Summary and Outlook

In this review article we have summarized recent developments of peptides and peptidomimetics as modalities that can target different types of NAs involved in disease. With their molecular weight and size in between small molecules and large biologics, peptides represent an intriguing option for recognizing DNA and RNA sequences or structures with specificity, while maintaining the potential to reach intracellular targets.

The two main discovery strategies for NA binding peptides are proteomimetic design and screening of large combinatorial libraries. In the proteomimetic design strategy the DNA or RNA binding domains of natural proteins are excised to produce minimalistic peptide scaffolds. When isolated from their complete protein context these peptides often loose the secondary structure necessary for NA binding. Chemical modifications, such as stapling or cyclization, are often used to stabilize such structural features in

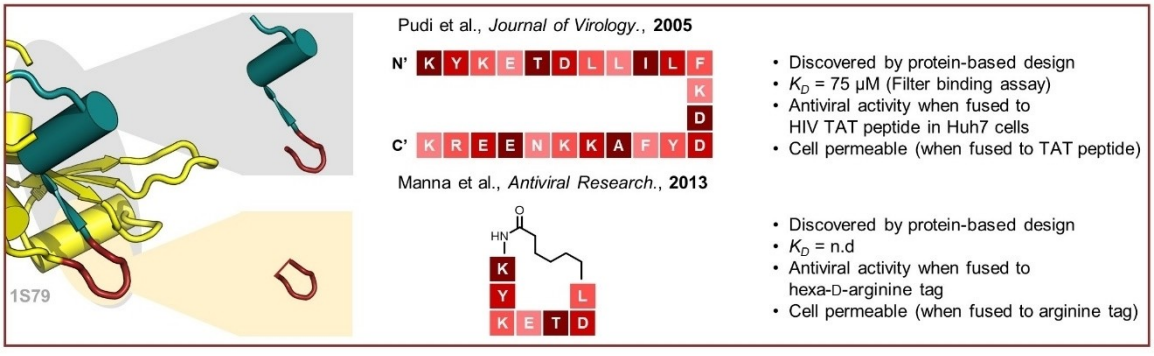

Figure 9. Peptide binders of HCV IRES RNA, based on the human La protein with their corresponding affinities and downstream effects developed by Pudi et al.[199] and Manna et al.,[201] PDB 1S79. Representation of crystal structure was made using PyMol. n.d. = not determined.

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the short peptides. These modifications often also help to improve certain properties of the peptides, such as cell penetration and proteolytic stability.

Several types of combinatorial libraries and screening or affinity selection platforms have been used to discover DNA and RNA binding peptides and peptidomimetics. These platforms include combinatorial one-bead-one-compound (or one-bead-two-compound) libraries, magnetic bead based affinity selections, phage display and microarrays. Interestingly, powerful platforms such as mRNA display or DNA encoded libraries, are almost never used in this context. The exposed NA tag, emblematic for these types of libraries, would make screenings for NA binding compounds rather complicated. However, with careful experimental design, DNA or mRNA encoded libraries can be used in specific cases.[202]

Selectivity is a major challenge for NA targeting modalities. Interestingly, in several studies, described in this review and elsewhere, peptides originally developed for one specific NA target, are used to address more or less unrelated sequences. Examples include: the miR-21 binder discovered by Pomplun et al., binds to miR-155 with similar affinity;[140] the Grossmann group utilized peptides derived from the viral suppressor of RNA silencing, TAV2b, to target human miR-21;[146] the Arora group used DNA binding domains of TAV2b and the transcriptional repressor MAX to design RNA binding modalities.[144] Based on these and other reports and on our own experience with NA binding modalities we would like to call for caution when it comes to making or interpreting claims about selectivity. Also natural TFs and other NA binding proteins usually have a certain degree of promiscuous generic affinity for any NA. However, in the complex cellular environment they ultimately recognize preferentially their specific target sequences. Assays the aim to showcase selectivity should be carefully designed and include biological evaluation in a cellular context.

Cell penetration is a general limitation for peptidic drug modalities. Many of the compounds reported in this review have not been tested for cell penetration or cellular activity. It is likely that these compounds might require extensive further development, in order to achieve bioactivity. On the other side a common trait of many NA binding peptides is a high Arginine content which leads to polycationic sequences with intrinsic cell penetration. Remarkably, even very large polypeptides, such as Omomyc (~90 amino acids or ~180 amino acid residues for the dimer), display intrinsic cell penetrating activity triggered by their polycationic DNA binding domains.[26–28] A similar observation had been made by Carlos Barbas III, who found multivalent zinc finger proteins to be, to a certain extent, intrinsically cell penetrating.[203,204] However, in order to achieve potent activity inside cells improved cell penetration of peptide-based modalities is necessary and will require further research and development.

Recent developments in engineering NA binding peptides have shown the potential of these modalities. A particularly prominent example is the development of Omomyc derivatives to treat MYC-driven cancers. An analog called Omomyc-103 is currently in clinical trials.[205] To date there are no approved drugs based on peptidic modalities targeting NA. Omomyc showcases the feasibility of the approach and the potential of peptidic modalities to address targets generally considered undruggable. With continuous developments in the field of peptide engineering and improved understanding on how to improve their pharmacokinetic properties,[127] we believe that peptide-based modalities can play an important role in identifying NA targeted therapies.

Acknowledgements

BDE, JPK and SJP acknowledge funding from the ERC StG (SynTra–101039354). We gratefully acknowledge Miguel Mata for his help in preparing graphic elements for the Figures.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Manuscript received: January 24, 2024
Accepted manuscript online: March 8, 2024
Version of record online: ■■, ■■

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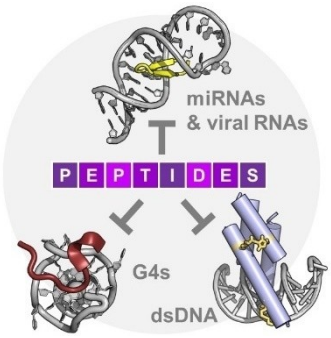
Reviews

Medicinal Chemistry



Nucleic acids play pivotal roles in life processes. Their dysregulation is implicated in numerous diseases while therapeutic compounds targeting them remain limited. In this review we focus on synthetic peptides which offer potential solutions for targeting double-stranded DNA, regulatory G-quadruplexes and non-coding RNAs.

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