The design of transcription factor-based inhibitors to target Myc: drop the Myc!

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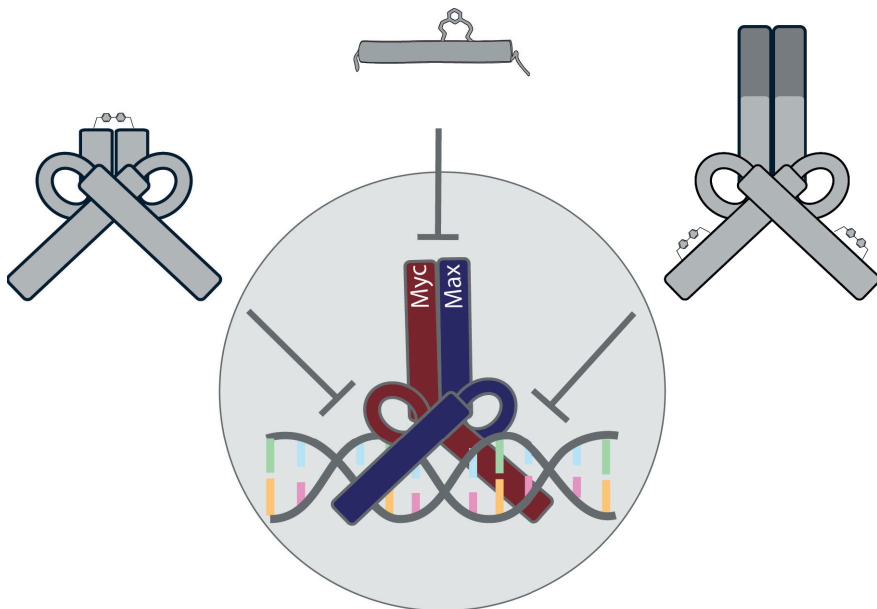
Chapter 1

1

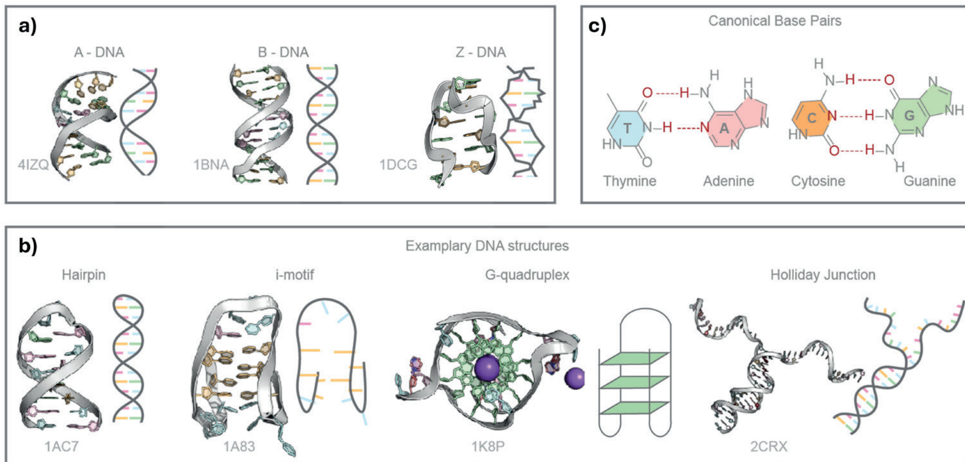
General introduction



Francis Crick described the central dogma of molecular biology as "*the exchange of genetic data, embedded in deoxynucleic acid (DNA), goes in only one direction to ribonucleic acid (RNA) to proteins.*"[1] The first step in this dogma is to read DNA and transcribe this to RNA. This step is performed by the transcription machinery that is recruited towards DNA. This protein complex reads DNA and simultaneously transcribes it to RNA. Subsequently, RNA is translated into the encoded protein.

DNA can adopt multiple conformations that allow DNA binding proteins to recognize DNA sequences. It generally consists as a right-handed double-stranded helical structure, called B-DNA, and was first described by Watson and Crick. [2] Other secondary DNA structures are Z-DNA, G-quadruplexes, i-motifs, hairpins and Holliday Junctions (**Figure 1.1a-b**). [3–7] These secondary structures are the result of canonical base pairing and the chemical composition of DNA. DNA contains a phosphor group, a sugar backbone and four complementary bases: adenine (A) is complementary toward thymine (T) and cytosine (C) is complementary towards guanine (G) (**Figure 1.1c**). The sequence composition combined with secondary elements are important for the flow of genetic data: they allow for specific gene recognition by transcription factors (TFs).

TFs are key proteins involved in the central dogma of molecular biology. They are a group of proteins that can bind a specific DNA sequence and regulate DNA transcription. [8–10] Therefore, these modalities appear to challenge the central dogma



▲**Figure 1.1: Exemplary DNA structures.** a) Possible DNA double helical confirmations. From left to right: A-DNA (PDB = 4IZQ), B-DNA (PDB = 1BNA), Z-DNA (PDB = 1DCG). b) Examples of identified DNA structures. From left to right: Hairpin DNA (PDB = 1AC7), i-loop (PDB = 1A83), G-quadruplex (PDB = 1K8P), Holliday Junction (PDB = 2CRX). For clarification, a schematic representation of each structure has been incorporated next to each structure. c) Canonical base pairing first described by Watson and Crick. Adenosine and Thymine form a couple, Cytosine and Guanine form second couple.

of molecular biology, as they regulate the flow of genetic information from protein to DNA. There are approximately 1600 human TFs that are sequence specific. [10] They often bind small binding sites (binding motifs) that are 6-12 bp in size. Considering that a nine bp sequence occurs ~15.000 times in the human genome and that a sequence becomes unique when it is sixteen bps long, TFs often need to work closely together in order to provide specificity. [11–15] Additionally, DNA methylation,[16] DNA shape[17] and post translational modifications[18–20] can also have an influence on specific DNA binding as well. TFs need to be precise as unspecific binding can result in the development of disease. It is estimated that dysregulation of ~19% of all TFs is involved in a disease, emphasizing their importance as therapeutic targets. [10]

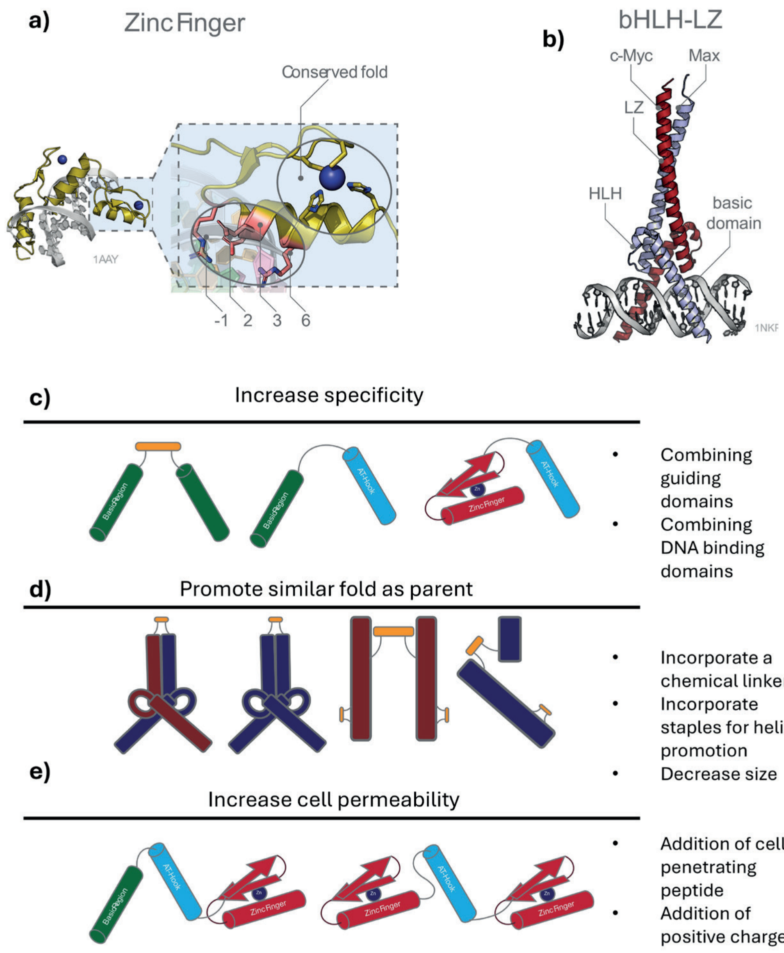
It is suggested that around 22% of the human genome are either intrinsically disordered proteins (IDPs) or have intrinsically disordered regions (IDRs). [21] Without a distinct and stabilized structure, proteins can adopt multiple conformations in solution. Moreover, with no binding pockets, it is difficult to develop small molecules that can recognize and bind this class of proteins.

TFs can contain disordered regions or can be completely disordered. Upon DNA binding, these regions undergo a conformational change and adopt an ordered structure. It is estimated that more than 80% of all TFs contain IDRs. [22] Due to their disordered nature and their location inside the cell, TFs were considered 'undruggable' for a long time. [22] As Johnson and McKnight described in their review in 1989, TFs first need to adopt a tertiary structure before they are able to bind DNA, which is similar for protein-protein interactions (PPI). [23] These structured DNA binding motifs enable another approach to target TFs: designed peptidomimetics and miniproteins based on endogenous TFs. Peptides and miniproteins can bind large surface areas and can recognize DNA or disordered proteins. Often, they are designed to target the DNA sequence of a TF. These artificial TFs therefore block the endogenous TF and thus function as a transcriptional repressor. **Chapter 2** describes an extensive overview of peptides and miniproteins designed to bind to disease-related sequences.

The aim of this thesis is to target the oncogenic TF c-Myc (from now on Myc) with inhibitors based on endogenous TFs. In **Chapter 3** and **4** miniproteins are developed based on a TF, whereas in **Chapter 5** the protein-protein interaction (PPI) between Myc and a binding partner is exploited for inhibitor design. In the following sections the main challenges for artificial TFs and peptide-based inhibitors are specified. Subsequently, Myc is described in detail to provide a better understanding on why we need to target Myc using peptide- and protein-based moieties.

1.1 Difficulties in artificial TF design

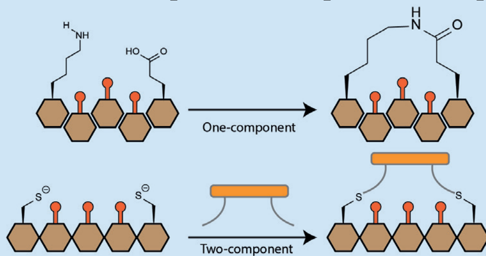
A strategy often observed in literature is to use defined DNA binding motifs of



▲**Figure 1.2: Solutions to overcome challenges in artificial TF design.** a) The crystal structure of Zinc Finger (ZF) 268. A trimeric ZF recognizes DNA inside the major groove. Each ZF contains conserved Histidines and Cysteines that coordinate around a zinc ion. This promotes the formation of one α-helix and a β-hairpin. A single ZF recognizes a triplet of DNA base pairs inside the major groove by residues at position -1, 2, 3 and 6. PDB = 1AAY. b) The crystal structure of c-Myc-Max. This heterodimeric complex adopts a basic Helix-loop-helix (bHLH) fold and utilizes the Leucine Zipper (LZ) domain to dimerize. Once dimerized this complex recognizes DNA. PDB = 1NKP. c) DBD attached via a linker and schematic representation of chimeric TFs developed by the group of Mascareñas. The GCN4 DBD is attached to the AT-Hook,[48] the GAGA ZF is attached to the AT-Hook.[48] d) Promoting similar fold as parent compound. Fully bHLH-LZ either as hetero- or as homodimers covalently attached.[49,50] bHLH-LZ (loop removed) or bZIP TFs covalently attached.[43,51] e) Combining two domains can increase specificity. Chimeric TF that contains the DBD of GCN4 that is attached to a ZF of GAGA by the AT-Hook,[52] Two GAGA ZFs are attached by the AT-Hook.[53] The AT-Hook functions as a cell penetrating peptide.

Info Box I: Stapling techniques

Peptide stapling entails covalently attachment of two residue sidechains to enforce a desired secondary structure within the peptide. This technique is often combined with SPPS. There are two strategies of stapling, one- and two-component stapling.[54] For one-component stapling, two sidechains within the peptide perform an intramolecular reaction to create a staple, whereas for a two-component staple an intermolecular reaction occurs between two sidechains and an external reagent. The latter often utilizes the reactivity of two cysteines.[46] By covalently linking two sidechains a peptide sequence is locked in a favored secondary conformation, depending on the position of the staple. For example, the Grossmann Group demonstrated that a metastasis staple promoted the formation of a β-sheet within their peptidomimetic.[55] For α-helix promotion, the side chain positions relatively towards each other are important. One α-helical turn is approximately four residues long. By incorporating a staple that spans four residues (*i, i+4*) a staple locks a single helical turn.[54] Additionally, a staple that spans two (*i, i*+7) or three (*i, i*+*11*) helical turns is also applied. Besides their ability to lock a peptide into a secondary formation, staples can also promote cell permeability and protease stability.



Info box Figure

Staples can be incorporated via one- or two-component stapling. The one-component strategy entails staple incorporation through an intramolecular reaction, whereas for a two-component staple an intermolecular reaction takes place that often involves cysteines.

endogenous TFs as starting point to inhibit TFs. This includes the use of Zinc Fingers (ZF, **Figure 1.2a**)[24] and motifs that fold upon and get stabilized by DNA binding, such as the Helix-Loop-Helix motif (HLH, **Figure 1.2b**).[25] A difficulty when designing artificial TFs using this strategy is loss of specificity due to the removal of domains from the parent protein. The designed TF needs to fold and act similarly compared to the parent protein. For example, basic Zipper (bZIP) TFs need to act as a dimer and need their basic region for DNA binding.[26] It was therefore envisioned that two basic regions must be linked together to target DNA as the dimerization domain was removed (**Figure 1.2c**).[27,28] To asses this hypothesis, two basic regions of GCN4, a classic bZIP TF, were covalently linked through a disulfide bond.[27] Another study used a light sensitive linker and showed that the *cis* isomer had the highest affinity.[28] Both studies demonstrated that the isolated bZIP domain has low selectivity to DNA and need a DNA guiding domain to coordinate the basic region correctly towards DNA. This was validated through the addition of a metal complex that conjugated two synthetic GCN4 basic regions together.[29,30] The metal complex could coordinate the two monomers differently relative to each other. Other strategies to retrieve the

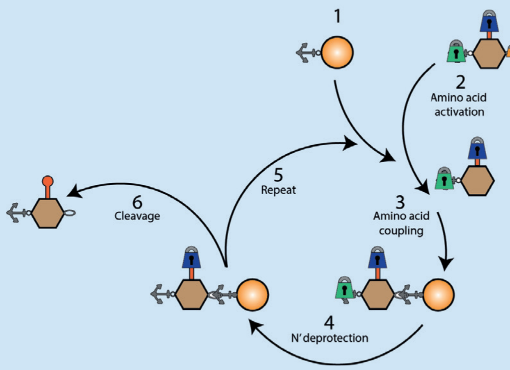
selectivity of the basic region of GCN4 entail the covalent attachment of DNA,[31] pyrrole-imidazole polyamides,[32,33] oligoguanidinium tail[34] or distamycin A[35] to the GCN4 bZIP domain. The latter resulted in a DNA binding hybrid with nanomolar affinity.

The designed peptidomimetic also needs to adopt a similar secondary structure as the parent compound. The correct structure promotes a peptide or miniprotein to recognize and bind specifically to DNA or protein partners. bZIP proteins need to form an α-helix, which normally occurs through dimerization. As a single bZIP domain can adopt multiple conformations in solution, α-helicity can be stabilized by the incorporation of a small peptide scaffold[43–45] or by connecting two residues covalently together (stapling). [41,46] Indeed, a stapled GCN4 DNA binding domain (DBD) was able to bind to DNA and enter RAW264.7 cells. [47] Instead of a two-component method where a staple is covalently attached (**See Info box I**), the incorporation of two histidines provided a non-covalent solution. [48] The addition of palladium resulted in the formation of an α-helix and DNA binding. Moreover, using this technique a reversible staple was created in such a way that DNA binding and cellular uptake could be tuned depending on the palladium concentration. Aside from structure stabilization, the incorporation of a metal also provided the possibility for self-assembly of a chimeric protein system containing a bZIP DBD and minor groove binder. [49,50] Solid phase peptide synthesis (SPPS) allows for the incorporation of unnatural amino acids that can be used for stapling (**See Info box II**). The Moellering group designed an all-carbon stapled artificial TF based on the X-box-binding protein 1 (**Figure 1.2d**). [41] Their miniprotein was able to inhibit hypoxia-dependent gene expression in vivo.

Next to structure, cell permeability is also a major challenge when designing peptide-based inhibitors. Generally, the bigger a compound, the lower the cell permeability. There are multiple techniques to increase permeability such as stapling and decreasing overall size. Additionally, it was observed that increased positive charge can enhance cell permeability. [51] Often cell-penetrating peptides are incorporated into the peptidomimetic design. For example, the AT-hook is a small positively charged AT-rich-minor groove binding motif originally occurring in HMG-I(Y). [52,53] Recent approaches involve the attachment of the AT-hook to the basic domain of GCN4,[36] two GAGA ZFs[38] or between a GCN4 and GAGA domain (**Figure 1.2e**). [37] Alone, the AT-hook has low affinity towards its target and, therefore, two low affinity DNA binders were combined with the AT-hook to increase selectivity and affinity (**Figure 1.2c**). [36] Additionally, it was observed that the AT-hook facilitated cellular penetration (**Figure 1.2e**)[36,37] and promoted selectivity. [37] Thus, the covalent attachment of a linker between DNA binding motifs could improve cellular uptake.

Info Box II: Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) is a technique that enables the straightforward assembly of peptides with virtually any residue sequence. SPPS, first developed by Merrifield in 1963,[61] applies an insoluble resin as starting point (Point 1) from which one by one a single amino acid is coupled until the final peptide or protein sequence is reached. A key aspect within SPPS is the use of orthogonal protection groups (PG) at the functional sidechain of each individual amino acid, compared to the PG at the α-amino group at the N-terminus. A standard coupling cycle is as follows: from the incoming amino acid the carboxyl is converted to an activated ester by an activating agent (e.g. Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium, HATU, Point 2). In the presence of a weak base, the free amine group on the resin-bound amino acid reacts with the activated ester, thereby forming a peptide bond (Point 3). All unreacted products are then drained away and the resin is washed. The resin-bound PG at the N-terminus is removed (Point 4) so that the coupling cycle can start again (point 5) and the peptide can grow on the solid support. Finally, the peptide is cleaved from the resin (point 6). Depending on sequence composition and resin capacity, generally the size of a peptide that can be synthesized using this technique is 30-70 residues.

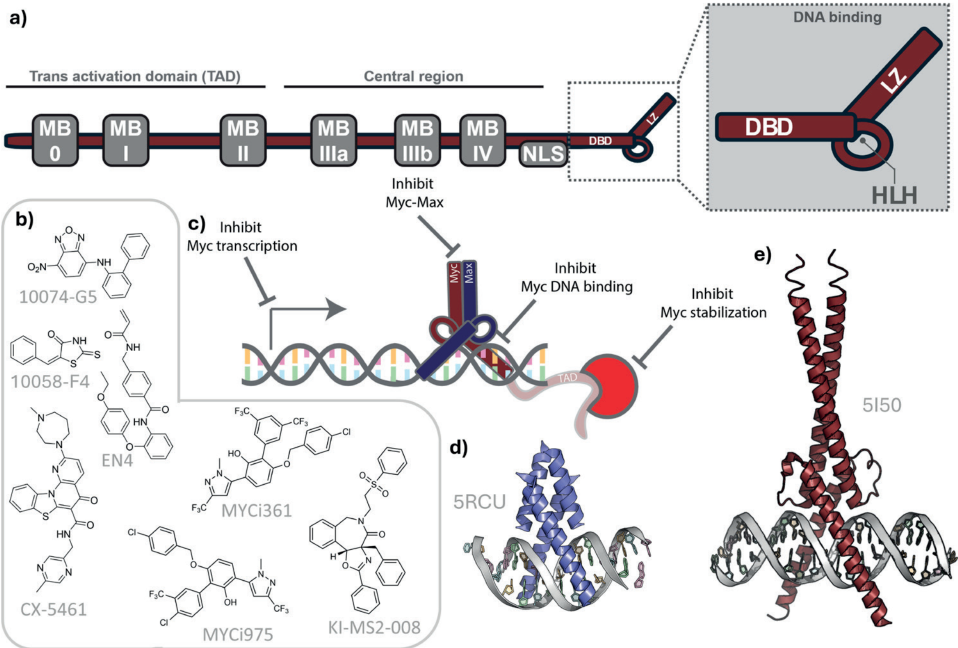


Info box Figure

The general procedure of solid phase peptide synthesis. An insoluble resin (1) is introduced that allows for the pre-activated (2) coupling of an amino acid (3). Next, the orthogonal N' terminal protection group is removed (4) that allows for the next cycle (5) or when finished cleavage of the peptide from the solid support (6).

1.2 Examples of successful ZF-based artificial TF designs

ZFs are a classic example for an artificial TF design and demonstrate the possibilities when a design is successful. They can be engineered to bind a designed sequence and moreover are intrinsic cell permeable. ZFs are small ~30 amino acids long peptides that can bind the major groove of DNA. Generally, a single ZF contains one α-helix and two β-sheets that form a β-hairpin (**Figure 1.2a**). [24] There are multiple classes of ZFs but the most common class is named after its four conserved residues that coordinate around a single zinc ion: Cys2His2. [56] This DNA binding motif contains two conserved histidines inside its α-helix and two cysteines inside its β-hairpin. Within ZFs, the helix is responsible for DNA recognition inside the major groove. Four specific residue positions are responsible for recognizing four bps in the major



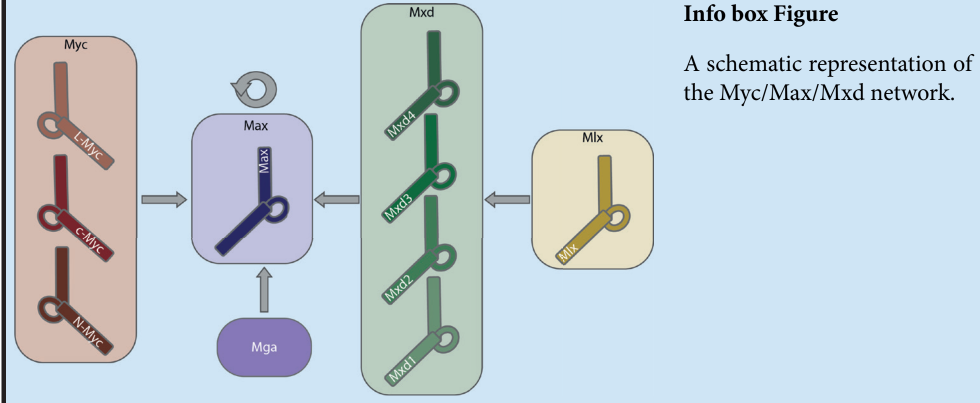
▲**Figure 1.3: Myc is an oncogenic TF that can be targeted by protein modalities or small molecules.** a) Myc is a largely disordered TF that has six conserved Myc Homology boxes (MB) within its Trans activation domain (TAD) and central region. The DBD domain adopts a classic bHLH-LZ fold. b) Examples of small molecules that can inhibit Myc activity. c) There are multiple ways to target Myc. Myc can be inhibited by preventing Myc transcription, stabilization, Myc-Max formation or DNA binding. d) Crystal structure of a Max-Max mimic that recognizes the E-box sequence (PDB = 5RCU). e) Crystal structure of Omomyc. Omomyc recognizes E-box DNA as a dimer (PDB = 5I50).

groove: position -1, 2, 3 and 6 (**Figure 1.2d**). To recognize specific DNA sequences, TFs often harbor multiple ZFs. Based on these findings, there has been research towards the development of artificial TFs based on ZFs that can be engineered to recognize specific sequences[24] and, moreover, can modify DNA.

A pioneer in the field of artificial ZFs was Carlos Barbas III. He envisioned that ZFs could be engineered to target every sequence. As ZFs have specific positions that are important for DNA recognition, he attempted to identify, through phage display, for each possible triplet the optimal α-helix composition. [57–60] This resulted in the identification of a specific α-helix composition for most DNA triplets and the development of Zinc Finger tools; a webserver that allows you to design ZFs based on the DNA sequence input. [62] With the possibility to design ZFs for specific DNA sequences, more complex artificial TFs could be designed. ZFs were designed to function as activators or as repressors, depending on the fused protein domain. [63]

Info box III: The Myc/Max/Mxd network

The Myc family consists of L-Myc, Myc and N-Myc. Myc belongs to the bHLH-LZ TF family and participates in the Myc/Max/Mxd network. Myc is disordered when not in complex with protein partners. It needs to interact with Max to bind DNA. Max can interact with all Mxd1-4 proteins, homodimerize and Mga. The Mxd1-4 proteins also interact with Mlx.



Info box Figure

A schematic representation of the Myc/Max/Mxd network.

Moreover, ZFs proved to be useful tools for the design of artificial TFs that can modify DNA sequences by means of methylation[64–66] or act as nucleases[67,68] or recombinases. [69]

All challenges described above are applicable on peptidomimetics and miniproteins. We needed to overcome these challenges to target the oncogenic TF Myc. Myc is a prime example of an 'undruggable TF' because it is intrinsically disordered without its protein partners and is located inside the nucleus. Moreover, it is highly involved in cancer and therefore an important pharmaceutical target.

1.3 The bHLH-LZ TF Myc

Myc is involved in important cellular processes such as differentiation, growth and death. [70–72] There are three family members in eukaryotes: Myc, N-Myc and L-Myc. Myc is involved in approximately 15% of all gene transcription. [73] Although structurally and functionally similar, these proteins differ in their expression pattern. Myc is widely expressed and the first discovered family member,[74,75] whereas N-Myc and L-Myc are mainly expressed in neural tissues and the lungs, respectively. The general structure of the Myc family is conserved between all three members. All members contain a C-terminal DNA binding and dimerization domain and an N-terminal disordered Trans-activation domain (TAD, **Figure 1.3a**). Within its disordered domain, the Myc family members have Myc homology boxes (MB) that allow other proteins to interact with Myc, regulate DNA binding and facilitate DNA

recognition (**Figure 1.3a**). [76]

Myc belongs to the HLH TF family. This structure contains two α-helices connected by a flexible loop (**Figure 1.2b**). [23,77] Often HLH proteins also contain a basic region (b) that allow them to bind to DNA. Additionally, HLH TFs need to form a dimeric complex to function and bind to DNA. This can be a hetero- or homodimeric complex. Myc has a leucine zipper (LZ) dimerization region. However, it cannot homodimerize and needs to interact with Myc-associated factor X (Max) to bind to DNA. Both Myc and Max are part of the Myc/Max/Mxd network (**See box III**). Other members of this protein network are Max- binding protein (MNT), Max-like protein X (Mlx) and Max gene-associated (Mga). Within this network, Myc can only interact with Max, whereas Max can homodimerize or interact with Mga and Mxd1-4. The Myc-Max complex recognizes Enhancer box (E-box) sequences (CACGTG) and when bound regulate downstream pathways.

Myc contains six MBs which mediate interactions with proteins partners (**Figure 1.3a**). [71] For example, Myc recruits Transactivation/Transformation-Domain Associated Protein (TRRAP) with MBII for histone acetylation and it interacts with Histone Deacetylase 3 (HDAC3) through its MBIIIa domain to promote histone deacetylation. [71,78] These PPI examples highlight why Myc is strictly regulated as it controls through chromatin remodeling a multitude of cellular processes via two contradictory protein partners.

Myc is regulated on two levels: on the mRNA and on the protein level. Both Myc RNA and the protein have short half-life's of approximately 10-20 min and 20 min, respectively. [79] Myc transcription is controlled by secondary DNA structures, including G-quadruplexes and Z-DNA. [70,79] A variety of TFs regulate Myc transcription by binding to Myc enhancer sites. [72,79,80] For example, Bromodomain protein 4 (BRD4) interacts with enhancer regions and stimulates increased Myc expression. [72,80] It was identified that JQ1, a BRD4 inhibitor, led to lower Myc expression levels. [80]

Myc is also regulated through phosphorylation[81] and protein partners. [79] Depending on phosphorylation patterns and cascades, Myc can either be stabilized or it can be marked for ubiquitination and therefore degradation. [81] Other proteins can stabilize Myc by interacting with its MBs, such as Aurora kinase A. [79] Another layer on Myc regulation is through Max. Max is essential for Myc to act as a transcriptional regulator. [70,71,82] By homodimerization or interacting with other members of the Myc/Max/Mxd network, Max monomers are sequestered away from Myc.

A major function of Myc is to sustain cellular proliferation by preventing cell cycle arrest and thereby cellular differentiation. Additionally, Myc is involved in diverse biological processes, such as metabolic reprogramming and angiogenesis. [72] Myc can function as a transcription amplifier that can bind to promotors close

to Transcriptional Start Sites. Contrarily, when interacting with Myc Interacting Zinc Finger 1 (Miz-1), Myc acts a transcriptional repressor, thereby inhibiting cell differentiation and apoptosis.[83,84] It is anticipated that 25-40% of genes repressed by Myc involve Miz-1.[83,85] When bound to Miz-1, Myc can repress genes, such as $p21^{Cip1}$ and $p15^{Ink4b}$, that are involved in cell cycle progression.

Dysregulation of Myc can lead to the development of cancer.[79] Strikingly, Myc engages in more than 50% of all human cancers and moreover, cancer cells become addicted and heavily dependent on Myc activity. Thus, highlighting the importance of Myc inhibition as a pharmaceutical strategy. Under physiological levels, Myc binds to high-affinity promotors guided by PPIs that stabilize Myc-Max-DNA binding by interacting with MBs in the N-terminus of Myc (e.g. WDR5 binds MBIII).[86,87] In Myc driven cancers, Myc is highly overexpressed allowing it to occupy low-affinity promotors as well, which is called promoter invasion.[86] This can eventually lead to uncontrolled cell division. Exemplary cancer types with Myc involvement are lung, breast and brain cancer.[88]

Taken together, it is extremely difficult to inhibit Myc activity. Its complex regulation system, cellular location, involvement in PPIs and its disordered nature make it a challenging pharmaceutical target. This also explains why there is no direct Myc inhibitor on the clinical market while writing this thesis. However, there are attempts to target Myc using small molecules and peptidomimetics.

1.3.1 How to target Myc

Myc was originally deemed 'undruggable'. Improved insight into Myc function facilitated the development of novel approaches to suppress Myc activity. Although Myc is intrinsically disordered, there are small molecules and peptide/protein-based inhibitors developed that can target Myc. Two strategies are applied to inhibit Myc, either direct or indirect inhibition. Additionally, there are also inhibitors dubbed double negative inhibitors as they can inhibit Myc by direct and indirect inhibition. The following section describes examples how Myc is targeted using small molecules and peptide mimetics. The following reviews provide a more extensive overview on Myc inhibitors.[70,71]

1.3.2 Myc inhibition with small molecules

Myc gene transcription is regulated through secondary DNA elements that can be targeted. Within its promotor, Myc contains a G-quadruplex that functions as a gene silencer.[92,93] The G-quadruplex is untwined to activate Myc transcription. A Myc inhibition strategy is to stabilize the G-quadruplex in the Myc promotor and thereby prevent Myc transcription.[70] Small molecules have been identified that can stabilize the Myc G-quadruplex (**Figure 1.3b-c**).[92–96] One of these compounds, CX - 5461

(**Figure 1.3b**), is currently in clinical trials for BRCA-deficient breast cancer. [97] Additionally, peptides have been identified to stabilize the Myc G-quadruplex. [98,99] Other strategies entail the inhibition of upstream proteins of Myc, such as BRD4,[80] the interaction with Myc stabilizing proteins, such as Aurora A,[100] or the development of Proteolysis Targeting Chimeras (PROTACs). [101–104]

Small molecules have also been identified that target the Myc-Max interaction (**Figure 1.3c**). [71] Nomura *et al.* recently developed EN4 (**Figure 1.3b**). [105] EN4 is a covalent ligand that can target cysteine171 in Myc, whereas it is suggested that it is unable to interact with N- or L-Myc as this cysteine is not conserved among Myc family members. It was suggested that EN4 destabilizes Myc, which renders it unable to interact with Max. Han *et al.* developed small molecules that could inhibit Myc in cells by Myc-Max inhibition, hampering Myc expression and promoting Myc degradation through phosphorylation. [106] Moreover, their lead compound, MYCi361 and its improved version MYCi975 (**Figure 1.3b**), could suppress tumor growth in mice. Struntz *et al.* identified KI-MS2-008 that could stabilize the Max-Max homodimer, while simultaneously reducing both expression levels of Myc and proteins regulated by Myc (**Figure 1.3b**). [107] Other examples of small molecules that can prevent Myc-Max formation are 10058-F4 and 10074-G5 (**Figure 1.3b**). [108,109] These compound could bind Myc while not properly folded and prevented it from refolding to a bHLH-LZ fold with Max.

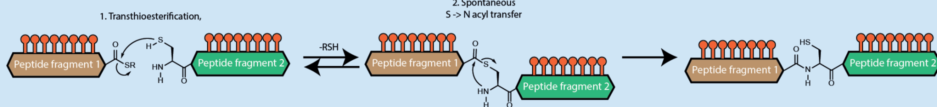
1.3.3 Myc inhibition with peptido- or protein mimetics

Transcription repressors have been designed to inhibit Myc activity based on endogenous Myc inhibitors (**Figure 1.3c-e**). Recently, Speltz *et al.* developed a transcription repressor based on Max that displayed Myc inhibition (**Figure 1.2d, 1.3d**). [42] The flexible loop was removed from the Max sequence and the Zipper domain was decreased in size. The DBD and the remaining part of the Zipper region were covalently linked with a maleimide linker, thereby replacing the flexible loop. Additionally, the miniprotein ME47[110,111] and recombinant Mad1,[112] were reported to inhibit Myc activity as well. ME47 is a chimeric protein that contains the DBD of Max fused to the dimerization domain of E47, whereas Mad1 contains the first 147 residues of Mxd1. Other strategies entail peptides that are based on the bHLH-LZ domain of Max,[113] the LZ domain of Max,[114] covalently linked Myc-Max or Max DBDs[115,116] or covalently linked bHLH-LZ region of Myc-Max and Max-Max dimers (**Figure 1.2b**)[39,40]

One of the first attempts to use bHLH-LZ proteins as a template was based on the Myc-Max heterodimer by the Kent group in 1995. [117] Using Native Chemical Ligation (NCL, see **Info Box IV**), Myc and Max monomers were covalently attached, thereby forming the heterodimer. Firstly, through thioester formation the C-terminal HLH-LZ region was attached to the N-terminal basic region to generate the monomers.

Info Box IV: Native chemical ligation

As proteins often contain more than 70 amino acids that cannot be synthesized with SPPS, a different approach has been developed: native chemical ligation (NCL). [91] Here, the C-terminal thioester of fragment 1 reacts with the N-terminal cysteine of fragment 2 (point 1), which is followed by a spontaneous intramolecular S-to-N acyl shift (point 2). This entails that the α-amino group of the cysteine attacks the thioester, forming an amide bond and releasing the thiol group. After its development, this technique has been applied to the synthesis of proteins including for example Max and allows for the incorporation of post translational modification that can be studied in an in vitro context. [92,93]



Info box Figure

Native chemical ligation (NCL) is often applied for protein synthesis. First, a transthioesterfication occurs (1) followed by a spontaneous S->N acyl transfer shift (2) leading to the release of the thiol group, thereby retrieving the original cysteine.

Secondly, an oxime reaction provided the covalent attachment of both monomers that resulted in the heterodimer. Additionally, a one-pot thioester and oxime formation yielded in the formation of a Max-Max homodimer. Krylov *et al.*,[118] proposed a different strategy. This included the removal of the TAD and exchange of basic regions that would prevent correct DNA binding. Overexpression could then inhibit Myc-Max activity. A similar strategy was applied by Soucek *et al.* to develop Omomyc. [119]

Omomyc is based on Myc with four point mutations in its LZ domain that enables homodimerization (**Figure 1.3e**). [119] Omomyc can interact with Myc, Max and Miz-1, but is unable to interact with Mxd proteins. [120] The crystal structure of Omomyc displayed a DNA recognition triad consisting of a His12, Glu16 and Arg20. [86] Strategies to analyze Omomyc entail Omomyc expression in mammalian cells,[119] recombinant expression and purification,[86] flow chemistry[39,40] and NCL. [121] Strikingly, Omomyc has the strongest affinity for the E-box sequence as a homodimer and is unable to bind to E-box DNA when bound to Myc.[39] It is able to inhibit Myc activity when expressed inside cells[86,122] and it is claimed that it has cell penetrating properties. [123] There are, however, also reports showing that Omomyc is unable to enter cells and that a cell penetrating peptide is beneficial to assist cell permeability. [124] Jung *et al.* proposed that Omomyc mainly competes with Myc-Max for low-affinity promotors as the removal of the N-terminus renders it unable to interact with stabilizing PPIs. [86] By competing with Myc at low-affinity promotors, Omomyc counteracts aberrant expression behavior of Myc. Currently, Omomyc-103 is the first direct Myc inhibitor in clinical trials. [125]

Info Box V: AlphaFold

New and powerful AI methods have been developed to predict how proteins fold. This led to the development of AlphaFold2 by Deepmind.[127] AlphaFold is a deep-learning method that predicts how proteins fold based on their primary sequence. AlphaFold is built around a deep neural network that is trained on evolutionary, physical and geometric data of protein structures.[127] It contains two main stages. The first stage includes Evoformer, which is a novel architecture that operates on two datatypes: multiple sequence alignments (MSA) and pair representations. After multiple rounds of Evoformer processing, the obtained data is interpreted by the second stage that translates the Evoformer data into a 3D structure. The output of AlphaFold2 is an end-to-end structure prediction with additional prediction scores for each individual residue in the protein. AlphaFold has been applied to the whole human genome,[129] including IDPs through fragmentation[130] and PPIs.[131]

Myc is intrinsically disordered which hinders its characterization and crystallization. Additionally, IDPs and IDRs do not adopt defined binding pockets, which impedes the use of small molecules. Therefore, another approach is pursued to target disordered proteins: the use of deep-learning tools such as RFdiffusion[126] and AlphaFold-Multimer.[127] For example, recently the Baker lab used RFdiffusion to design IDR binding proteins.[128] In the absence of a crystal structure we used AlphaFold-Multimer[127] to predict how Myc and Miz-1 interact. This deep-learning tool predicts how proteins fold based on their primary sequence without the need for a crystal structure (**See Info Box V**). Based on the prediction we could design Miz-1 based Myc inhibitors.

1.4 Aims and outline of this thesis

Despite being involved in more than 50% of human cancers, there are no direct Myc inhibitors on the clinical market. Through substantial amount of process over the past decades we got a better understanding of Myc activity and Myc involvement in cancer. Because Myc is both an IDP and TF it becomes more difficult to inhibit. An increase in knowledge enabled scientists to develop inhibitors towards Myc via multiple strategies. Additionally, the development of AlphaFold2 revolutionized the field in protein structures. This thesis explores the field TF-based inhibitors via SPPS and protein expression. Additionally, it utilizes AlphaFold2 to design novel peptidomimetics based on a more than decade old unresolved PPI between Myc and Miz-1. The aim of this thesis is to design TF-based peptide modalities that can target Myc and validate these compounds through biochemical assays.

Myc is not the only disease related TF. Therefore, in **Chapter 2** a comprehensive overview is provided that entails synthetic peptide modalities able to tackle disease related DNA or RNA. **Chapter 3** describes the development of DuoMYC. Here, we

performed a structure-based study based on the Omomyc sequence that led to the design of a smaller bHLH miniprotein that functioned as an artificial transcriptional repressor and could inhibit Myc activity in vitro. In **Chapter 4** we explored the possibilities of expressed protein stapling to improve stability and cell permeability of Omomyc. A protocol was developed to express proteins that could be stapled, while remaining active. In **Chapter 5** a different approach was pursued to inhibit Myc. Whereas previous chapters explore the ability to inhibit Myc through artificial transcriptional repressors, in this chapter we use AlphaFold2 to predict how Myc and Miz-1 interact and used this prediction to develop peptidomimetics that can inhibit Myc-Max complex formation. **Chapter 6** combines all the findings of this thesis and describes the most important conclusions and future perspectives.

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