



UMEÅ UNIVERSITY

**INVESTIGATING THE BIOLOGY
AND SPECIFIC TARGETING OF
INDIVIDUAL G-QUADRUPLEX
STRUCTURES.**

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Dissertation for PhD

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*“That’s the trouble with science. Always upending itself.
Ruining perfect systems for the little inconvenience of them
being wrong.” – Zahel*

in Rhythm of War
by Brandon Sanderson

Table of Contents

Abstract	ii
Abbreviations	iv
Publication List	vi
Introduction	1
G4 structures – an overview	1
Biological functions of G4s.....	3
Prevalence of G4s in DNA and RNA.....	3
Telomeres	4
Promoters	6
Mitochondrial DNA	8
mtDNA replication	9
G4-dependent switch between transcription and replication	10
mtDNA G4-associated deletions	10
Limitations in G4 research	11
Approaches to selectively target G4s.....	11
G4s as potential drug targets.....	13
Key methods.....	14
MST	14
NMR	14
FRET melting	14
Polymerase-based assays	15
BG4 ChIP-Seq.....	16
Aim of the Thesis	17
Summary of the papers	18
Paper I.....	18
Paper II.....	22
Paper III.....	25
Paper IV.....	28
Conclusion and Future Perspectives	30
References	32

Abstract

G-quadruplex (G4) structures are non-canonical DNA and RNA conformations formed in guanine-rich regions that play roles in gene regulation, genome stability, and RNA processing. However, targeting the approximately 700,000 G4s in the human genome with high specificity remains challenging due to their structural similarities. Despite their biological significance, this inability to selectively study or manipulate individual G4s presents a significant barrier to understanding their distinct roles in human cells and complicates efforts to dissect their contributions to cellular processes.

To address this limitation, we developed a strategy based on click chemistry to covalently link short single-stranded oligonucleotides (Os) to G4 ligands (GLs). This approach combines the stabilising properties of G4 ligands with the sequence specificity of guide oligonucleotides to create G4-ligand-oligonucleotide (GL-O) conjugates. The oligonucleotide forms double-stranded DNA (dsDNA) with the flanking region of the target G4, ensuring selective binding and stabilisation of the desired G4 structure. Through biophysical and biochemical assays, we demonstrated that this approach enables the selective stabilisation of individual target G4s, highlighting its utility for studying specific G4 structures.

In refining the GL-O platform, we systematically evaluated various linker configurations. This work demonstrated that longer and more flexible linkers enhance the adaptability of GL-O conjugates, allowing efficient targeting of G4s with varying distances between the G4-forming region and the complementary oligonucleotide binding sequence. This insight is particularly valuable for addressing steric hindrances and expanding the range of targetable G4 structures.

Additionally, we explored the broader principles of G4 ligand design by focusing on dispersion forces and electrostatic interactions. Synthesising heterocyclic G4 ligands and studying their interactions with G4s showed that dispersion components in arene-arene interactions and electron-deficient electrostatics are central to achieving high-affinity binding and stabilisation. These findings enhance the GL-O approach by providing a framework to fine-tune the stabilisation effect of the GL-Os, potentially reducing off-target effects.

In parallel, we pursued a separate project that examined G4 structures within human mitochondrial DNA (mtDNA), aiming to elucidate their roles in cellular function. Human mtDNA contains regions that have been predicted to form G4 structures *in silico*. We mapped these mtDNA G4s using high-resolution techniques and demonstrated their formation *in vivo*. Stabilisation or replication stalling increases their formation, potentially contributing to mitochondrial dysfunction and genomic instability in disease.

Together, these findings advance our understanding of G4 biology, from selective targeting strategies to the unique dynamics of mitochondrial G4s, offering valuable insights into the biological roles of G4s in maintaining genome stability and regulating cellular processes.

Abbreviations

AFM	Atomic force microscopy
BCL-2	B-cell lymphoma 2
BLM	Bloom syndrome helicase
ChIP	Chromatin immunoprecipitation
c-Myc	MYC Proto-Oncogene
CNBP	Cellular nucleic-acid-binding protein
CSB	Conserved sequence blocks
DHX36	DEAH-Box Helicase 36
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
FRET	Fluorescence resonance energy transfer
G	Guanine
G4	G-quadruplex
GL	G4 ligands
GL-O	G4-ligand-oligonucleotide
GMP	Guanosine monophosphate
K ⁺	Potassium
K _D	Binding affinity
KRAS	Kirsten rat sarcoma virus
KSS	Kearns-Sayre syndrome
LNA	Locked nucleic acids
LSP	Light strand promoter
MAZ	MYC-associated zinc finger protein
MDMD	mtDNA maintenance defects
mRNA	Messenger RNA
MST	Microscale thermophoresis
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting signal
mtSSB	Mitochondrial single-strand binding protein
NHE	Nuclease hypersensitivity element
NM23-H2	Nucleoside diphosphate kinase 2
NMR	Nuclear magnetic resonance
NUMT	Nuclear mitochondrial DNA
O _H	Heavy strand origin of replication
O _L	Light strand origin of replication
OXPHOS	Oxidative phosphorylation

PEO	Progressive external ophthalmoplegia
POLRMT	Mitochondrial RNA polymerase
Poly	DNA polymerase gamma
POT1	Protection of telomeres protein
PQS	Putative quadruplex sequences
Rif1	Replication timing regulatory factor 1
RNA	Ribonucleic acid
RNaseH1	Ribonuclease H1
RPA	Replication protein A
rRNA	Ribosomal RNA
RTel1	Regulator of telomere elongation 1
SP1	Specificity protein 1
SPAC	Strain-promoted alkyne-azide cycloadditions
SPOS	Solid-phase organic synthesis
ssDNA	Single-stranded DNA
TFAM	Mitochondrial transcription factor A
t-loop	Telomere loop
Top3 α	Topoisomerase 3 α
TRF2	Telomeric repeat-binding factor 2
tRNA	Transfer RNA
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WRN	Werner syndrome helicase

Publication List

- I. **"G4-Ligand-Conjugated Oligonucleotides Mediate Selective Binding and Stabilization of Individual G4 DNA Structures."** Berner, A., R. N. Das, N. Bhuma, J. Golebiewska, A. Abrahamsson, M. Andreasson, N. Chaudhari, M. Doimo, P. P. Bose, K. Chand, R. Stromberg, S. Wanrooij and E. Chorell (2024). *J Am Chem Soc* 146(10): 6926-6935.

- II. **"Linker Design Principles for the Precision Targeting of Oncogenic G-quadruplex DNA with G4-Ligand Conjugated Oligonucleotides."** Abrahamsson A., A. Berner, J. Golebiewska, N. Chaudhari, E. Keskitalo, C. Lindgren, M. K. Chmielewski, S. Wanrooij, E. Chorell (2025). *Bioconjugate Chemistry Article ASAP*.

- III. **"Exploring the Dispersion and Electrostatic Components in Arene-Arene Interactions between Ligands and G4 DNA to Develop G4-Ligands."** Andreasson, M., M. Donzel, A. Abrahamsson, A. Berner, M. Doimo, A. Quiroga, A. Eriksson, Y. K. Chao, J. Overman, N. Pemberton, S. Wanrooij and E. Chorell (2024). *J Med Chem* 67(3): 2202-2219.

- IV. **"Enhanced mitochondrial G-quadruplex formation impedes replication fork progression leading to mtDNA loss in human cells."** Doimo, M., N. Chaudhari, S. Abrahamsson, V. L'Hote, T. V. H. Nguyen, A. Berner, M. Ndi, A. Abrahamsson, R. N. Das, K. Aasumets, S. Goffart, J. L. O. Pohjoismaki, M. D. Lopez, E. Chorell and S. Wanrooij (2023). *Nucleic Acids Res* 51(14): 7392-7408.

Publications not included in the thesis

- Kasho, K., *et al.*, A unique arginine cluster in PolDIP2 enhances nucleotide binding and DNA synthesis by PrimPol. *Nucleic Acids Res*, 2021. **49**(4): p. 2179-2191.

- Forslund, J. M. E. *et al.*, The Y951N patient mutation inactivates the intramolecular switch in human mitochondrial DNA POLY. *Proc. Natl. Acad. Sci. U.S.A*

Introduction

Deoxyribonucleic acid (DNA) has been extensively studied since its structure was discovered 70 years ago, leading to groundbreaking insights into its role in storing genetic information, inheritance, and cellular regulation. While its canonical double-helix structure is central to most of its functions, both DNA and its counterpart, ribonucleic acid (RNA), have been shown to form many different secondary structures. Among these structures, G-quadruplex structures (G4s) have been widely studied and are the primary focus of this thesis.

G4 structures – an overview

The first evidence for the self-assembly of guanines was described in the 1910s when it was reported that guanylic acid, now known as guanosine monophosphate (GMP), could form a gel-like substance [1]. Nearly 50 years later, X-ray crystallography revealed that GMP molecules stack upon each other, forming a helical structure stabilised by hydrogen bonds [2]. Research into similar structures expanded in the 1980s, when oligonucleotides derived from immunoglobulin switch regions and telomeric sequences were found to adopt four-stranded structures [3, 4]. These studies also provided the first indications that such structures might have functional significance in these genomic regions [5, 6].

Intensive research has advanced our understanding of G4s and their biological functions in the following decades. These findings highly depend on G4 ligands, which specifically bind to and usually stabilize G4s, but do not bind to regular B-DNA. They commonly consist of a rigid polyaromatic system that stacks on the G4 tetrad by arene-arene interactions. Extensive efforts have led to the design and development of over 4000 such molecules [7]. G4 ligands have allowed some insights into the roles of G4s, especially those involved in telomere maintenance and the regulation of transcription [8-11]. RNA G4s were shown to be involved in alternative splicing [12] and act as modulators of translation [13, 14]. Bioinformatic analyses have revealed widespread yet non-random distribution of G4-forming sequences across genomes, further suggesting their functional significance [15, 16]. These computational approaches were soon validated through experimental approaches [17-19].

In parallel, biophysical experiments have provided crucial insight into the structure of G4s. Revealing that they comprise of at least two planar G-tetrads stacked on each other. Each G-tetrad consists of four guanine (G) bases arranged around a rotation axis and is stabilised by non-canonical Hoogsteen hydrogen bonds between the guanines. This planar structure is further stabilised by a central monovalent cation, such as potassium (K^+), which interacts with the oxygen atoms of the guanines (Figure 1A). When multiple tetrads stack, additional stability is provided by arene-arene (π - π stacking) interactions between the aromatic components of the guanine bases (Figure 1B). Since each tetrad comprises of four guanines, G4s are classified as four-stranded structures. Depending on their formation, G4s can be either intramolecular, where all four strands originate from the same DNA or RNA molecule, or intermolecular, formed by two or more separate DNA/RNA molecules. Further classification is based on the directional orientation of nucleic acid strands. The strands can all run in the same direction (parallel G4s), two strands run in one direction while the other two run in the opposite direction (antiparallel G4s) or one strand runs in the opposite direction compared to the other strands (hybrid G4s) (Figure 1C). [20, 21]

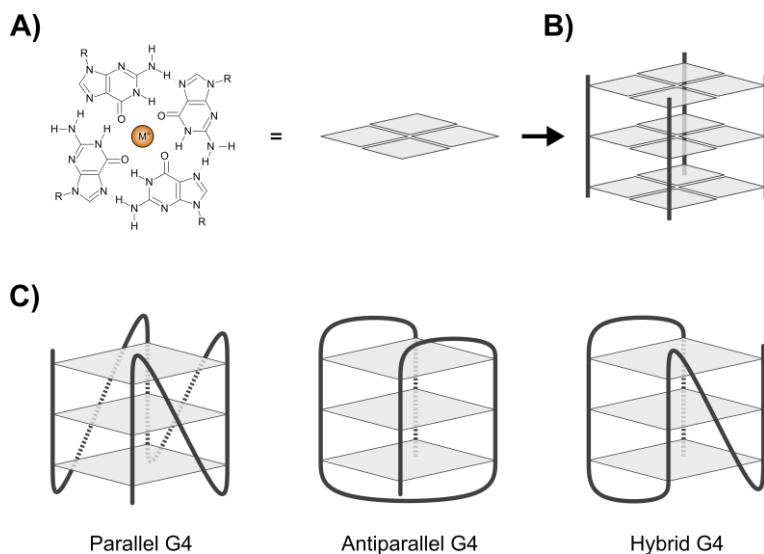


Figure 1 Overview of G4 structures. A) G-tetrad stabilised by a central monovalent metal ion. **B)** Schematic representation of a G4 structure with three tetrads. **C)** Representation of three different conformations of intramolecular G4s.

Biological functions of G4s

G4 structures have been identified across the genomes of all domains of life, as well as in viral species [22-26]. Despite the vast evolutionary diversity among these organisms, G4s exhibit a strikingly similar distribution in their genomes, clustering around promoter regions upstream of transcription starting sites, telomeres, and untranslated regions (UTRs) of messenger RNAs (mRNAs) [16, 27, 28]. Furthermore, there is no evidence suggesting negative selection against G4s during evolution. Instead, they appear to have been conserved and repurposed as an additional regulatory layer influencing multiple biological processes [29].

Prevalence of G4s in DNA and RNA

With the understanding of the basic structure of G4s, researchers began predicting the abundance and distribution of potential G4-forming sequences in genomes. Early computational predictions of G4s in the human genome were based on the assumption that a G4 requires four stretches of at least three consecutive guanines, separated by a short loop sequence [30, 31]. This algorithm identified 375,000 putative quadruplex sequences (PQS) in the human genome. Improved prediction algorithms have increased the number of identified PQS and expanded the tools to predict RNA G4s. These web-based applications now enable screening sequences ranging from short fragments to entire genomes for PQS [15, 31-33]. More recently, computational approaches have been developed to predict the three-dimensional structure of G4s based on the sequence provided [34].

Complementing these *in silico* methods, high-throughput experimental approaches have been developed to identify G4s in cells [17, 19, 35]. Results from these align well with bioinformatic predictions, confirming the accuracy of the algorithms [36]. One thing that stands out in all the results obtained from these methods is the non-random distribution of G4s throughout the genome. Both G4s and PQS are predominantly found in telomeres, promoter regions, and UTRs of RNAs [35, 37]. Some functions of DNA G4s will be discussed in the following sections.

Telomeres

Telomeres are DNA-protein complexes that cap the ends of linear eukaryotic chromosomes. Their primary function is to protect the chromosome ends from being recognised as double-strand breaks by the DNA repair machinery [38-40]. Additionally, telomerase-mediated extension of the telomeres helps resolve the end replication problem, which would otherwise lead to progressive DNA shortening with each cell division [41-43]. In humans, telomeric DNA ranges from 5 to 15 kb in length and consists of 5'-TTAGGG-3' tandem repeats, terminating in a single-stranded 3' overhang of 20 to 400 nucleotides [44-46]. This single-stranded DNA (ssDNA) overhang can loop back and invade the adjacent telomeric region, forming a telomere loop (t-loop). This structure covers the ssDNA, preventing its recognition as DNA damage [47, 48]. Telomeres are also bound by the shelterin complex, which comprises six core proteins with various functions involved in the telomere's correct assembly, protection, and elongation [49-51].

Telomeric G4s

Telomeric sequences were among the first investigated for their ability to form G4s [3-5]. Interestingly, there is no consensus structure for the human telomeric G4. Its conformation depends on the experimental method and salt used to determine the structure [52-57]. When longer stretches (~100nt) of telomeric DNA were analysed using atomic force microscopy (AFM), individual G4s were seen stacking upon each other, forming higher-order structures [58]. In cellular environments, fluorine-19 nuclear magnetic resonance (¹⁹F NMR) studies suggest that telomeric G4s predominantly adopt a hybrid conformation [59]. However, the actual *in vivo* structure of telomeric G4s remains elusive. Further supporting their presence at telomeres, BG4 immunostaining has demonstrated that approximately 25% of BG4 signals are localised to the telomeric regions [60]. Telomeric BG4 foci further increase upon treatment of cells with G4 ligands [61].

Another point to consider is the high conservation of G4-forming sequences in telomeric repeats across species, which implies an essential role for the structures in telomeres [62-65]. A notable exception is *C. elegans*, whose telomeric sequences form hairpin structures rather than G4s [66].

G4s function in telomeres

The precise role of G4 formation in telomere protection remains largely elusive. One of the core proteins of the shelterin complex, the protection of

telomeres protein (POT1), is an ssDNA binding protein with the ability to unwind G4s in the process of binding to telomeric DNA [67]. POT1 has also been shown to stimulate telomerase activity by unfolding G4s, thereby maintaining the DNA in a ssDNA conformation [68]. Another G4 resolving protein, replication protein A (RPA), also localises to the telomeres, further supporting the idea that cells actively resolve G4 structures on telomeres rather than relying on them for specific functions [69]. Additionally, knocking out key G4 helicases (Bloom syndrome helicase (BLM), Werner syndrome helicase (WRN), and regulator of telomere elongation 1 (RTel1)) has shown that their loss leads to fragile telomeres [70-72]. Similarly, treatment of cells with G4 ligands induces telomere fragility, suggesting that unresolved G4s may pose a problem rather than serving a protective role [73, 74]. In contrast, other telomere-associated proteins recognise and bind G4s. For example, replication timing regulatory factor 1 (Rif1) and the shelterin component telomeric repeat-binding factor 2 (TRF2) have been shown to recognise and bind G4s, implying a potential role of G4s beyond being obstacles [75, 76]. It is estimated that most telomeres in human cells form a t-loop at any given time [77]. However, G4s may serve as an additional layer of protection for telomeres that lack t-loops or during S-phase, when t-loops are transiently resolved to facilitate telomere replication.

G4s affect telomerase processivity

Telomerase cannot use most G4s as a substrate and exhibits reduced initiation capacity on parallel G4s compared to ssDNA [61, 78, 79]. Additionally, many G4 ligands have been shown to inhibit telomerase initiation, further strengthening the claim that G4s act as poor substrates for the enzyme [80-82]. However, experiments in different salt conditions suggest that G4 formation during the elongation step may be essential for telomerase translocation along its substrate [83]. It has been proposed that newly synthesised DNA can fold into transient G4 structures upon release from the telomerase. Serving as an anchor to facilitate the realignment of the telomerase RNA template with the DNA substrate [83, 84]. Supporting this, similar experiments with nucleotide analogues that prevent the formation of secondary structures revealed that telomerase could not synthesise DNA beyond four repeats - the length needed to form a G4 [85].

These findings suggest that G4 formation at telomeres is a dynamic process required for proper telomerase processivity [86]. Moreover, this indicates that G4s could have co-evolved with telomerase function, potentially as a regulatory mechanism emerging from the enzyme's activity.

Promoters

The high abundance of G4s in the promoter regions of genes has led to numerous suggestions on their potential role in transcription. G4s have been shown to block RNA polymerase [87, 88] and act as binding sites for transcription repressors [89]. Conversely, some G4s have the opposite effect, enhancing transcription by promoting transcription factor binding [90]. Additionally, G4s can play a role in remodelling the local chromatin structure and forming DNA loops, thereby affecting the expression of the surrounding genes [18, 91].

In this context, oncogenes like vascular endothelial growth factor (VEGF) [92], B-cell lymphoma 2 (BCL-2) [93], and Kirsten rat sarcoma virus gene (KRAS) [94], among many others have been extensively studied [95]. However, the most extensively studied oncogene associated with G4s is MYC Proto-Oncogene (c-Myc) [96, 97]. The G4 sequence of c-MYCs was used as a model in several assays throughout this thesis, and it will be discussed in more detail in the following chapter.

c-Myc G4 Pu27

The c-Myc oncoprotein is a pleiotropic transcription factor that plays a critical role in cell cycle regulation and apoptosis and is associated with many human tumours [98]. Up to 70% of human cancers are estimated to exhibit aberrant c-Myc expression [97, 98]. Since the Myc protein lacks enzymatic activity, efforts to develop direct inhibitors of c-Myc have been largely unsuccessful, almost deeming the protein undruggable [99]. In this context, the promoter region of c-Myc has emerged as a potential drug target for controlling its expression. The regulation of c-Myc expression is complex, involving four different promoters (P_0 , P_1 , P_2 , P_3) and multiple regulatory elements, with P_1 being responsible for most transcripts [100-102]. Upstream of P_1 , a nuclease hypersensitivity element (NHE III1) has been identified as a key regulatory site recognised by transcription factor specificity protein 1 (Sp1) [96, 103]. Interestingly, this 27 bp long GC-rich region (also called Pu27) has been shown to form various intramolecular G4s, making it the key target for G4-dependent transcription regulation of c-Myc [104].

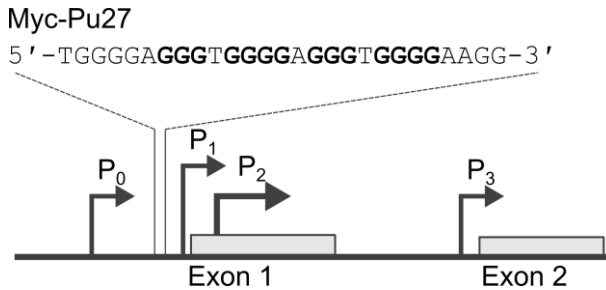


Figure 2 Schematic representation of the c-Myc promoter region. The sequence of the 27-nucleotide G4-forming sequence upstream of the P₁ promoter is shown.

To investigate G4 formation and function, several variations of Pu27 are used in *in vitro* studies. One commonly used variant is Pu22, a shorter 22-base-pair sequence containing two specific mutations (G14T/G23T), favouring the formation of a parallel G4 structure [105]. Another widely studied variant is Pu24T, a 24-base-pair sequence with a slightly truncated and mutated 5' end. In this variant, a guanine in the second G-run is substituted by a thymine (G9T), resulting in a unique yet stable conformation that is predominantly parallel, except for a loop-back of the 3' end into the third G-run [106].

Effects of Pu27 G4 formation on c-Myc transcription

Many proteins involved in transcription regulation have been found to bind to either the folded or unfolded NHE III₁ region. These include Sp1, cellular nucleic-acid-binding protein (CNPB) [107], DEAH-Box Helicase 36 (DHX36) [108], MYC-associated zinc finger protein (MAZ) [109] and nucleoside diphosphate kinase 2 (NM23-H2) [110]. These proteins are associated with a wide array of functions and can have different effects on Myc transcription from P₁. For example, SP1 is believed to bind to the dsDNA and unwind it, allowing G4 formation, while other studies have found SP1 to be a binding protein of the Pu27 G4 itself. In contrast, other proteins such as DHX36 and NM23-H2 exhibit G4 unwinding activity, resolving the G4 structure and thereby enhancing transcription from P₁ [108, 110].

Another G4 binding protein, Nucleolin, interacts with Pu27, blocking accessibility to the DNA for other proteins and ultimately downregulating c-Myc expression [89]. Contrarily, Nucleolin binding to the G4 in the VEGF promoter has the opposite effect, upregulating VEGF expression [111]. This highlights the complex interplay of numerous proteins with opposing roles interacting with Pu27 to regulate c-Myc expression.

Point mutations that decrease the stability of the Pu27 have been found to cause a three-fold decrease in the expression of c-myc, indicating that Pu27 G4 acts as a negative regulator [96]. More strikingly, a recent study found that mutating Pu27 to prevent G4 formation entirely led to a total loss of P₁-driven c-Myc expression, indicating that G4 formation is important for the recruitment of transcription factors and other proteins [112]. Another study further supports this idea, it demonstrated that various transcription factors preferentially bind G4s over the mutated or double-stranded counterparts, suggesting that G4s function not simply as on/off switches but as a binding hub for dynamic regulation of expression [90].

A common approach for studying the effects of G4 formation on c-Myc expression involves using G4 ligands. Hundreds of these G4 ligands have been shown to bind and stabilise Pu27 *in vitro*, with many demonstrating down-regulation of c-Myc expression in cell-based experiments [113, 114]. These findings have contributed to the prevailing belief that G4 formation downregulates gene expression. However, recent research is challenging this view, showing that G4 ligands may compete with proteins for G4 binding and downregulate gene expression by preventing transcription factor access to the G4 rather than by stabilising the G4 itself [90].

Additional factors affecting transcription

Adding to this complexity, G4s have been shown to modulate histone modifications, thereby regulating gene expression at an epigenetic level [115, 116]. Additionally, G4s are associated with phase separation events involving distant DNA strands, altering the local chromatin environment and subsequently affecting transcription [117, 118]. These findings reveal an even more intricate regulatory network than previously thought.

Mitochondrial DNA

Mitochondria originated from endosymbiotic proteobacteria and, through evolution, have specialised in energy production and as a cellular signalling hub [119, 120]. Most of the mitochondrial DNA (mtDNA) migrated to the nucleus through evolution, making most mitochondrial proteins nuclear encoded. In mammals, mtDNA is a circular, multi-copy, double-stranded molecule of 16.6 kb [121]. It is strictly maternally inherited and encodes for two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 13 proteins [122]. These proteins are core components of the oxidative phosphorylation (OXPHOS) complexes [123]. Although these proteins only represent a

minority of the mitochondrial proteome, they are essential, and the loss of mtDNA can lead to severe disease [124]. Hence, the maintenance of mtDNA has been extensively studied; however, further insights into the effects of G4s on mtDNA are needed.

mtDNA replication

Replication and transcription of mtDNA are tightly coupled, and their regulation will be discussed later. Replication is initiated by the mitochondrial RNA polymerase (POLRMT) synthesising an RNA primer at the heavy strand origin of replication (O_H) [125]. This primer is then extended by the replicative DNA polymerase gamma (Poly), which consists of the catalytic subunit PolyA and two accessory subunits PolyB [126, 127]. The helicase Twinkle assists Poly by unwinding the dsDNA [128]. Poly uses the light strand as a template, leaving the original heavy strand as ssDNA, which is bound by mitochondrial single-strand binding protein (mtSSB) [129]. When Twinkle and Poly reach the light strand origin of replication (O_L), this region becomes ssDNA and forms a hairpin structure [130]. POLRMT binds this hairpin and synthesises a short RNA primer that Poly extends to replicate the light strand in the opposite direction of heavy strand replication [131]. After replication, the remaining RNA primers are removed by Ribonuclease H1 (RNaseH1) and Mitochondrial genome maintenance exonuclease 1 (MGME1), and Poly fills the gaps [132-134]. The DNA ends are ligated by DNA ligase III, and the mitochondrial isoform of topoisomerase 3 α (Top3 α) separates the molecules [135, 136].

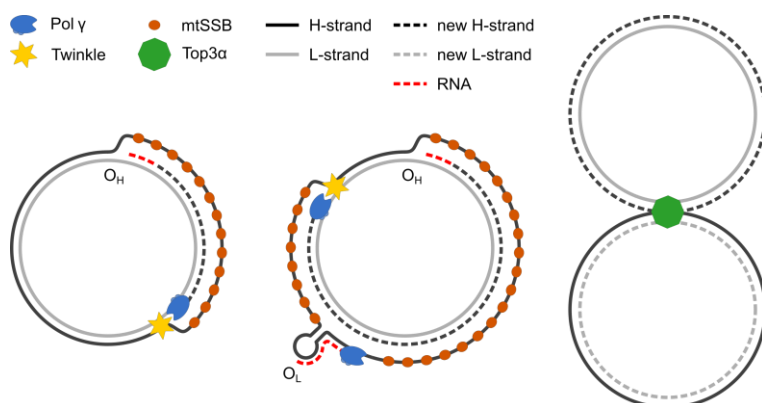


Figure 3 Overview of mtDNA replication. RNA primers (red) are created at O_H and subsequently O_L by POLRMT and elongated by Poly (blue) with the help of Twinkle (yellow). Single-stranded DNA is covered by mtSSB (orange). After replication, RNA is removed by RNaseH1, and the two molecules are separated by Top3 α (green).

G4-dependent switch between transcription and replication

In mitochondria, the transcription from the light strand promoter (LSP) is necessary not only for L-strand gene expression but also for providing the primers for replication at the O_H . The switch from transcription to replication occurs within a region containing three conserved sequence blocks (CSB1-3) [137]. A subset of the transcripts is terminated upon transcription of CSB2, where the newly synthesised RNA forms a hybrid G4 with the non-template mtDNA strand. This G4 structure destabilises the elongation complex, leading to transcription termination [138]. Additionally, G4 formation anchors the RNA to the mtDNA and stabilises the three-stranded structure called the R-loop [139]. The RNA is then processed by RNaseH1, generating a functional primer for mtDNA replication by Poly [140].

mtDNA G4-associated deletions

Beyond their role in regulating the switch between transcription and replication, G4s are also associated with the formation of pathogenic mtDNA deletions [141]. The mechanisms underlying the formation of mtDNA deletions are not yet fully understood. The accumulation of these deletions in post-mitotic tissues can lead to various rare genetic disorders, including progressive external ophthalmoplegia (PEO) and Kearns-Sayre syndrome (KSS) [142, 143]. Moreover, mtDNA deletions have been observed in affected tissues of individuals with Parkinson's disease, a more common neurodegenerative disorder [144]. Demonstrating the pathological consequences of mtDNA deletions and that understanding the mechanisms behind their formation are essential for gaining deeper insights into these diseases.

The two mtDNA strands are highly asymmetric in guanine content, with the heavy strand being enriched in guanines [145]. Due to the slow replication of mtDNA coupled with its replication mechanism, the heavy strand remains single-stranded for an extended period, likely favouring G4 formation [146]. This effect could potentially be further increased by the high concentration of K^+ in mitochondria [147]. Analysis of mtDNA deletion breakpoints has revealed a strong correlation with potential G4-forming sequences, and *in vitro* experiments confirmed the ability of these sequences to form G4s [141, 148]. However, the replicative mitochondrial helicase Twinkle has limited efficiency in unwinding G4s, suggesting the involvement of additional helicases [149]. Among these, the helicase Pif1 is

a potential protein involved in unwinding of mtDNA G4s. Its yeast homolog has demonstrated G4 unwinding activity, and its mutation led to mtDNA instability [150, 151]. Both human and yeast Pif1 localise to the mitochondria; however, there is limited evidence for Pif1 function on mtDNA in human cells [152]. Despite growing interest in mtDNA G4s and their implications in deletion formation, their actual role remains poorly understood. This is mainly due to the lack of tools to study G4s directly in a cellular context. Further research is needed to clarify their impact on mitochondrial genome stability and to investigate potential functions G4s could have.

Limitations in G4 research

As discussed above, the biological functions and the mechanisms underlying G4 formation in a cellular environment remain largely elusive. While BG4 staining and similar visualisation methods demonstrate the presence of G4s in cells, how and when they are formed remains speculative. Furthermore, many techniques used to identify G4s suffer from different biases and might present a skewed picture of G4s formed in cells. For example, several methods rely on the formation of G4 after the DNA isolation, not reflecting the physiological conditions, leading to potentially biased results [17, 19]. This highlights the need for improved methods similar to the approach developed in **Paper IV**.

The second major limitation lies in the use of G4 ligands. These compounds have proven to be a good tool in identifying potential biological functions of G4s but generally lack the selectivity to target individual G4s. Despite this issue, G4-ligands have been used to propose numerous functions for various G4s, often resulting in contradictory findings between studies. Consequently, determining the function of an individual G4 using these ligands is problematic, as off-target effects can cause the observed phenotypes. Hence, while G4 ligands are potent tools in many applications, they cannot determine the functions of specific G4s in a cellular context, and more selective methods are required.

Approaches to selectively target G4s.

Recent advances in G4 research have focused on overcoming the limitations and thus attempting to resolve the contradictions in the field. Precise genome editing tools such as CRISPR/Cas9 allow researchers to selectively modify single G4-forming sequences in the genome. This

enables functional studies of specific G4s by altering the stability of one specific G4 and monitoring the effects this alteration has, limiting the off-target effects [112]. In addition, novel strategies are being developed to block G4 formation. For example, PNAs complementary to specific RNA G4s have been used *in vitro* to block their folding by forming a stable PNA/RNA duplex [153]. While genome editing provides high specificity and *in vivo* relevance, it can be technically complex and time-consuming, whereas G4-blocking strategies like PNA interference offer a more straightforward and reversible approach, though often constrained to *in vitro* settings and could be more susceptible to off-target hybridisation.

Another promising approach is to direct G4 stabilising ligands towards specific G4s, enabling selective stabilisation with minimal off-target effects. This strategy is explored in **Papers I and II** of this thesis, and other researchers have reported similar efforts. For instance, one study used a PNA complementary to the flanking region of a G4 found in the human immunodeficiency virus (HIV), conjugated to a G4 stabilising ligand [154]. This conjugate selectively stabilised the G4 *in vitro*, favouring the formation of the targeted G4 over others in the proximity. Similarly, another method used short oligonucleotides complementary to the flanking sequence of an RNA G4, conjugated to a fluorescent probe that activates upon G4 binding [155]. Increased fluorescence in cells spiked with the RNA G4 of interest, indicating selective detection. Another innovative strategy involved light-activatable ligands, guided by PNAs, to alkylate specific G4s with reported high precision [156]. An alternative method attempts to direct ligands to G4s by attaching DNA groove-binding molecules to G4 ligands, thereby providing some degree of sequence selectivity [157].

Most recently, strategies have been developed that utilise catalytically inactive Cas9 (dCas9) fused to nucleolin or G4 stabilising ligands. Guided by specific gRNAs, these systems allow the precise delivery of G4-binding proteins or ligands to individual G4s within cells [158, 159].

Collectively, these methods mark an important step towards a better understanding of individual G4 functions in cellular processes. However, many of these strategies remain in the early stages of development and face significant challenges, including delivery efficiency and off-target effects, before they can be employed effectively and later translated into clinical applications.

G4s as potential drug targets

G4s are considered promising drug targets for cancer therapy due to their involvement in oncogene regulation and telomere maintenance. Several G4 ligands have demonstrated promising results in selectively killing cancer cells. However, their exact mechanism of action remains poorly understood, as discussed in the previous chapters. Nevertheless, G4 ligands might still hold therapeutic potential. Interestingly, their lack of specific targeting, often seen as a limitation, could be an advantage in a therapeutic context. By binding multiple G4 targets across the genome, these ligands may simultaneously perturb multiple cancer-relevant pathways like telomere maintenance and oncogene expression. Even within the same pathway, G4 ligands might act on multiple levels of regulation. However, this does not necessarily hinder further development of G4 ligands as therapeutics.

Despite this potential, the number of G4-ligands with drug-like properties is limited. Only a few molecules have progressed into clinical trials, and none have advanced beyond phase II [160-162]. Therefore, research is needed to optimise the pharmacological properties of G4 ligands for further clinical development, as in **Paper III**.

Key methods

This section briefly overviews several key methods used in the thesis. It aims to provide a background to help understand interpretations of experiments performed rather than an in-depth review of each method.

MST

Microscale thermophoresis (MST) is a technique used to determine binding affinities (K_D), in this case, between a G4 ligand and G4 DNA. It measures the movement of a fluorescently labelled target, in this case, a folded G4, when a laser applies a microscopic temperature gradient. The binding of a ligand to the fluorescently labelled molecule changes the dynamics of this movement due to changes in size, hydration shell, and charge. This difference can be plotted in relation to ligand concentration, and the binding affinity is determined with titration of the ligand. This method was used in the thesis to determine K_D values of G4 ligands, GL-Os and oligos to a labelled DNA template.

NMR

$^1\text{H-NMR}$ can follow the folding of G4s, more precisely the G-tetrads, by tracking the imino protons of the guanines that arise in the 10-12 ppm range. For instance, a three G4 with three G-tetrads gives rise to 12 sharp imino protons in this range, associated with the 12 guanines involved in its formation. In contrast, imino protons involved in Watson-Crick base pairing can be observed at 13-14 ppm. NMR was used to track interactions of G4 ligands with the G4, leading to a broader linewidth and overlapping peaks because of conformation changes of the G4. Additionally, 13-14 ppm peaks were used to validate dsDNA formation between the template and GL-Os. Furthermore, NMR melting assays were employed to assess G4 stabilisation effects, where the G4 signal was observed at higher temperatures when treated with compounds compared to untreated samples.

FRET melting

Fluorescence resonance energy transfer (FRET) assays were performed to assess G4 stability and the effects G4 ligands have on the stability. In this case, a G4-forming DNA oligo molecule is labelled with two fluorophores on either end. When the G4 is folded, they are in proximity to each other and act as a donor and acceptor pair, and fluorescence is quenched. Increasing the

temperature gradually and continuously monitoring fluorescence can determine the temperature at which the G4 unfolds. The moment the G4 is unfolded, the distance between the two fluorophores increases, and they can't act as a FRET pair anymore, leading to detectable fluorescence from the donor. Adding ligands to the G4 will increase the melting temperature, and differences in the melting temperature can be calculated.

Polymerase-based assays

In this thesis, two different kinds of polymerase-based assays were used. Taq-Polymerase stop assay was used as a complementary approach to NMR and FRET melting experiments to assess the stabilising effects of G4 ligands. For these experiments, short fluorescently labelled DNA primers were annealed to a longer ssDNA template containing a G4-forming sequence, and in experiments with GL-Os, a flanking sequence (Figure 4). Annealing in KCl promotes G4 formation on the template. In the experiment, Taq polymerase extends the primer along the ssDNA template until it encounters a G4. Depending on how stable the G4 is, the polymerase can push through it and reach the end of the ssDNA template. Following extension, reaction products are analysed on a denaturing polyacrylamide gel, allowing the detection of extension products with a single-nucleotide resolution.

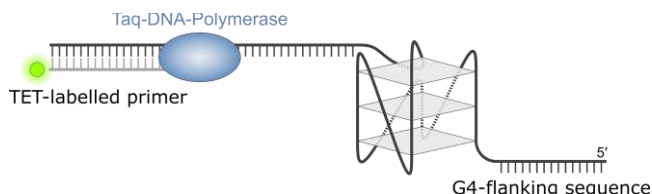


Figure 4 A schematic representation of Taq-Polymerase stop assays shows that a TET-labelled primer (grey) is extended by Taq-DNA-Polymerase (blue) until the polymerase is either stalled at the G4 or pushed through and reaches the 5' end.

The ability of Taq polymerase to overcome the G4 structure serves as a functional readout of G4 stability: the more stable a G4 structure, the less efficiently the polymerase can extend through it. The full-length product can be quantified and compared to control reactions on the gel, allowing a comparison between different G4 ligands.

A second polymerase-based assay was employed to confirm that BG4 does not affect mtDNA replication. A template encoding for one of the identified mtDNA G4s was used. The fluorescent primer was extended by purified Poly, and increasing concentrations of BG4 antibody did not lead to any increase in stalling, in contrast to RHPS4 and PhenDC3.

BG4 ChIP-Seq

ChIP-Seq is a method for analysing protein-DNA interactions. It combines chromatin immunoprecipitation (ChIP) with high-throughput DNA sequencing and can be used to identify DNA-binding sites of proteins. In this thesis, ChIP-seq was used to identify binding sites of the G4-recognizing antibody BG4 on mitochondrial DNA.

Immunoprecipitation was performed on BG4 with a FLAG tag after cross-linking the cells. Cross-linking as a first step ensures that all BG4 DNA interactions occur in living cells and not as an artefact during sample preparation. Cells are lysed, and DNA is sheared. Afterwards, BG4 was pulled down with an anti-FLAG antibody. Samples were further purified for library preparation, and subsequent sequencing was performed. In parallel, samples were prepared for Western blot and qPCR analysis to check for pulled-down mitochondrial proteins and validate sequencing results.

Aim of the Thesis

This doctoral thesis comprises studies investigating the stabilisation of G4 structures and their abundance on mtDNA. One part focuses on developing a novel strategy to specifically target individual G4 structures in nuclear DNA, providing a powerful tool for studying the functions of specific G4s. The second part investigates G4 formation in human mtDNA based on the hypothesis that G4 structures may contribute to mtDNA instability.

The specific aims were:

- I. To establish the GL-O approach as a method for specifically targeting individual G4s with high selectivity and minimal off-target binding and stabilisation.
- II. To further explore the GL-O strategy, focusing on the impact of linker length and flexibility, particularly in relation to the distance between the oligonucleotide binding site and the G4 structure.
- III. To investigate the effect of dispersion and electrostatic components in Arene-Arene interactions of G4-Ligands with G4 structures, aiming to improve the design of G4-ligands.
- IV. To investigate the potential of mtDNA to form G4 structures *in vivo* and determine factors increasing G4 formation, contributing to mtDNA instability.

Achieving these aims allowed us to establish a new methodology for targeting individual G4s by combining a G4 ligand with a guiding oligonucleotide. Additionally, we gained insight into designing future GL-Os by optimising linker composition and selecting appropriate G4-ligands. Furthermore, we gained an understanding of G4 formation on human mtDNA, and our results suggest that unresolved G4 structures can lead to mtDNA instability.

Summary of the papers

Paper I

Research on the biological function of G4s suggests their involvement in various DNA- and RNA-related cellular processes. Additionally, there is evidence that indicates that G4s are linked to diseases such as cancer, making them potential therapeutic targets. However, the detailed understanding of G4s' biological functions and the development of G4-targeting cancer therapies are limited by the lack of tools for precise G4 targeting. Currently, methods to selectively target an individual G4 without affecting other G4s across the genome are limited. G4 ligands are small molecules that can bind and stabilise G4 structures and are widely used in research, with some ligands being used in clinical trials against cancer. However, these G4 ligands lack specificity and cannot discriminate between different G4s, leading to off-target effects. Consequently, none of these compounds have progressed beyond Phase II in clinical trials, and research on the cellular functions of individual G4s remains challenging due to the vast number of G4s within the cell, where targeting one can inadvertently affect many others.

Hence, we developed a novel strategy to selectively target individual G4 structures by combining the sequence specificity of an oligonucleotide with the G4 stabilising properties of a G4-ligand. We conjugated these two components using click chemistry to generate a G4 ligand-conjugated oligo (GL-O). This approach allows us to distinguish between G4 structures based on their flanking regions to which the oligo binds (Figure 5). This GL-O strategy was validated through a series of biophysical and biochemical assays, demonstrating its ability to selectively stabilise specific G4s while minimising off-target interactions.

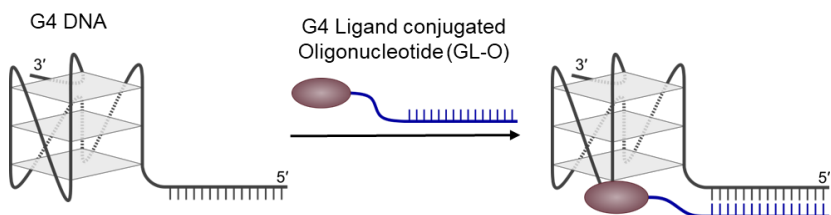


Figure 5 Schematic representation of the GL-O strategy. G4 structure with a 15nt long flanking sequence (left). The G4 Ligand conjugated to a 15-nt oligo complementary to the flanking sequence of the G4 (middle). The oligo guides the G4-ligand to the correct position through double-strand formation between the GL-O and the flanking sequence of the G4 (right).

Synthesis

The basis for synthesising the GL-O was a G4 ligand with drug-like properties that efficiently binds and stabilises DNA G4s [163]. The G4-ligand was functionalised with an azide-substituted piperazine, allowing copper-catalysed click reactions with a terminal alkyne-modified oligodeoxynucleotide. This approach allows for the straightforward synthesis of GL-Os with customisable/different guide sequences.

Validation of the strategy

We first validated that attaching a comparatively large oligo to the small G4-ligand does not negatively affect its G4 stabilisation properties. To assess G4 formation as well as dsDNA formation between the GL-O and the G4-flanking sequence, we performed H^1 NMR. Peaks observed around 11 ppm confirmed that the G4 structure still forms when a flanking sequence is present. These peaks shifted upon the addition of GL-O, indicating an interaction between the G4-ligand and the G4. Furthermore, the appearance of peaks around 13 ppm upon adding GL-O suggested the formation of dsDNA between the GL-O and the flanking sequence. MST was employed to evaluate the binding affinity of the GL-O. The results showed that GL-O binds to G4 with a flanking sequence with significantly higher affinity compared to the individual components, G4 ligand or oligo control. Complementary results from polymerase-stop assays confirmed these findings. The GL-O effectively reduced the full-length product to the same extent as the G4-ligand alone, but at 25-fold lower concentrations. Collectively, these results show that GL-O retains the ability to bind and stabilise G4 structures and does so with considerably higher affinity than the G4-ligand alone.

Selective targeting

To assess specificity, we performed primer extension assays using two different DNA templates in the same reaction, which only differed in their flanking sequence. While the G4-ligand alone showed no discrimination between the two templates, the GL-O exhibited a strong preference for stabilising the G4 in the template with a complementary sequence. Additionally, when comparing G4 stabilisation by the GL-O in the template where it could not form dsDNA to the stabilisation of the G4 ligand alone, we observe a reduced stabilisation effect for GL-O. This suggests that the attached oligo hinders G4 stabilisation in the absence of dsDNA formation. (Figure 6)

To investigate the discrimination abilities of GL-Os, we generated a set of GL-Os with one or two mutations at either the ends or within the central region of the oligonucleotide sequence. Experiments with these mutated GL-Os showed that mutations on the 3' or 5'-ends of the oligo had no noticeable effect on binding or G4 stabilisation. However, two mutations within the oligo's central region were sufficient to significantly reduce binding affinity and G4 stabilisation by GL-O. These results indicate that even minor differences from the target flanking sequences can provide selectivity to GL-Os.

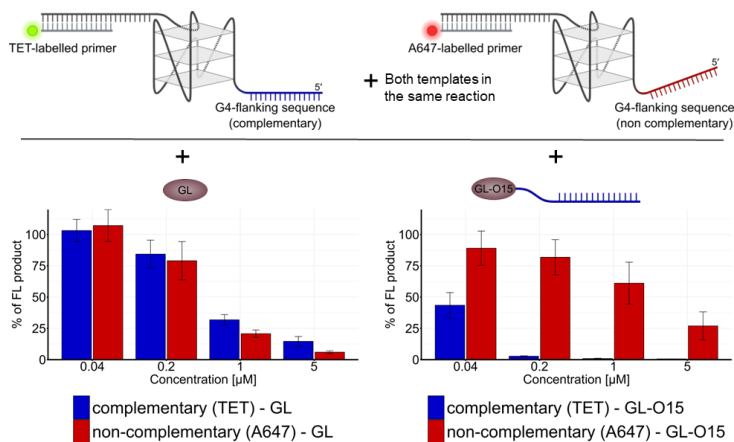


Figure 6 Competition assays performed with GL-O. G4 templates with a complementary flanking sequence (left, labelled with TET-primer) and a non-complementary flanking sequence (right, labelled with an A647 primer) were mixed in the same reaction. Full-length (FL) product quantification is shown relative to untreated samples after addition of G4-ligand alone (bottom left) or GL-O (bottom right). Data represents the mean and standard deviation from three individual experiments.

Insights into strand invasion and binding with LNA

Limited research has been conducted on the nature of the flanking regions of G4s during their formation. Data based on c-Myc's promoter region suggest that approximately 15 nucleotides downstream (3') of the G4 may remain ssDNA [164]. However, if the flanking region is not exposed as ssDNA, GL-O must compete with dsDNA to bind adjacent to the G4. To address this challenge, we synthesised a GL-O modified with locked nucleic acids (LNA) at the three 3' and 5' terminal nucleotides. These LNA modifications potentially allow strand invasion into dsDNA. The LNA modification slightly improved the binding and stabilisation effect of GL-O compared to its unmodified DNA-only counterpart. Notably, in a set-up simulating cellular conditions, where the flanking region may exist as dsDNA, the LNA-modified GL-O was able to invade dsDNA and stabilise the G4 at lower concentrations compared to both GL alone and the unmodified DNA-only GL-O.

Conclusion

In this paper, we developed a novel strategy to target specific G4s by combining the stabilisation properties of a G4-ligand with the sequence selectivity of an oligonucleotide. We show that conjugating the G4-ligand to the oligo allows these components to work synergistically, increasing both binding affinity and stabilisation compared to the individual components while adding high specificity.

This proof-of-concept approach provides a foundation for designing GL-Os capable of stabilising individual G4 structures within cells, offering the field a powerful tool to investigate the specific roles of individual G4 structures while minimising off-target effects. In the long term, this strategy could contribute to the development of therapeutic treatments for cancers driven by G4-regulated oncogenes.

Paper II

In Paper I, we described a novel strategy to selectively target individual G4s by combining the targeting specificity of an oligonucleotide with the stabilisation properties of a G4-ligand. The two components are covalently linked using copper-catalysed click chemistry, creating a triazole linker between the functionalised oligonucleotide and G4-ligand. However, this solution phase method is incompatible when phosphorothioate backbones are used in the GL-O design due to the high affinity of phosphorothioate bonds towards copper [165]. This type of modification increases nuclease resistance and is, therefore, a relevant modification for developing GL-Os for cell-based and future *in vivo* studies [166]. To overcome this problem, we used two different solution-phase click-chemistry strategies compatible with various oligonucleotide modifications. Additionally, we functionalised the G4-ligand into a phosphoramidite, allowing it to be directly incorporated at the 5'-end during solid-phase oligonucleotide synthesis.

This new set of GL-Os, featuring variations in both linker chemistry and length, was evaluated in a series of biophysical and biochemical assays. This analysis provided insights into how these modifications influence G4 binding and stabilisation. We focused on the relationship between linker length and the distance from the oligo binding site to the G4, as this will be crucial for future cell-based experiments.

Synthesis of new GL-Os

Three different conjugation methods were used to generate the new GL-Os. The first conjugation method, strain-promoted alkyne-azide cycloadditions (SPAAC), utilised the same ligand with a terminal azide as in the original GL-O. Two constrained alkyne moieties, differing in carbon chain length, were introduced on the 5'-end of the oligo to enable azide-alkyne cycloaddition reactions with the functionalised G4-ligand (Figure 7 GL-O2 and GL-O3). As a second strategy, we employed amide coupling, synthesising a G4-ligand with a terminal carboxylic acid, which was then reacted with a primary amine (again with two variations differing in carbon chain length) introduced at the 5'-ends of the oligonucleotide (Figure 7 GL-O5 and GL-O6). The third strategy involved directly incorporating the G4-ligand into the oligonucleotide during its synthesis. This was achieved by adding a phosphoramidite into the G4-ligand, allowing it to be integrated at the 5'-end of the oligonucleotide using solid-phase organic synthesis (SPOS) (Figure 7 GL-O4).

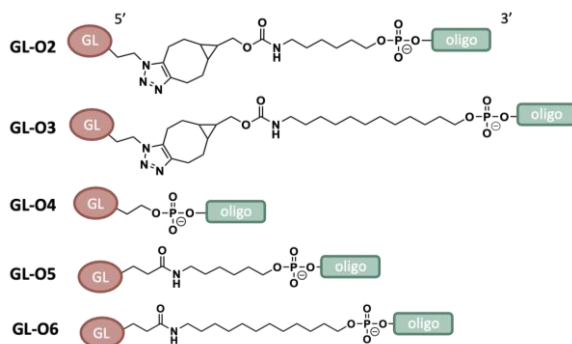


Figure 7. Overview of GL-Os synthesised in this study. GL-O2 and GL-O3 were synthesised using SPAAC with two different linker lengths, with 6 and 12 carbon atoms, respectively. GL-O4 was created using SPOS. GL-O5 and GL-O6 employed amide coupling for conjugation with two different linker lengths, with 6 and 12 carbon atoms, respectively.

Binding and stabilisation of G4 structures

The binding affinity of the new GL-Os was measured using MST with a labelled Pu27 G4 sequence. GL-Os exhibited similar K_D values, ranging from 5 to 40 nM. ^1H NMR analysis on a similar template showed both dsDNA formation and interaction of the G4-ligand with the G4 structure. No distinct differences were observed between the tested GL-Os. These results were confirmed in polymerase-stop assays on comparable DNA templates, which showed no substantial difference. Thus, the variation in linker chemistries and lengths does not significantly impact G4 binding and stabilisation.

Interplay of linker length and binding distance

To further investigate the effects of linker length, we designed a set of G4-containing templates in which the binding site is positioned at varying distances from the G4. This was achieved by either deleting or inserting nucleotides between the G4 structure and the binding sequence, thereby varying their spatial separation. GL-O2, GL-O3, and GL-O4 were selected for these experiments based on their different linker lengths. ^1H NMR experiments and polymerase-stop assays demonstrated that the linker length does not affect binding and stabilisation when the GL-O binds close to the G4, likely due to the flexibility of the template DNA. However, as the distance between the oligonucleotide binding site and G4 increased, GL-O4, which has the shortest linker, lost its ability to stabilise the G4 once the gap extended to 5nt or more. In contrast, GL-O2 and GL-O3, with the longer linkers, were able to stabilise the G4 at a gap length of 9nt, the maximum distance that we tested.

Conclusion

In this study, we expanded on the previously published GL-O strategy by modifying the linker chemistry. We also changed the approach from Cu-mediated click chemistry to two alternative solution-phase click chemistry strategies, and additionally including SPOS as a third approach. These strategies allowed the synthesis of GL-Os with different length linkers ensuring compatibility with phosphorothioate-modified oligonucleotides - a crucial modification for future in-cell studies, required for increasing nuclease resistance of the GL-O compounds.

Adjusting the linker length provides greater flexibility in selecting oligonucleotide recognition sequences positioned further away from the target G4. In G-rich regions capable of forming multiple different G4s, a longer linker enables stabilisation of G4s both near and further away from the binding site. This flexibility ensures that G4s in complex sequence environments can still be targeted. Additionally, when the flanking sequence of the target G4 has high sequence homology to other genomic regions, longer linkers provide the option to bind at a more distant, unique sequence, increasing targeting specificity, which would otherwise not be possible with shorter linkers.

In summary, this study provides additional insights into the design of GL-Os for targeting individual G4 structures in cells and, therefore, studying G4 biology with minimal off-target effects.

Paper III

Most known G4-ligands contain permanently charged species or basic amine residues, leading to poor pharmacokinetic properties of these compounds. This, combined with a limited understanding of interactions between G4-ligands and G4 structures, hinders research on G4s, including their viability as drug targets. Current guidelines agree on a rigid aromatic system as a core component of G4-ligands which allows stacking of the ligand onto the G-tetrad by arene-arene interactions (π - π stacking). The two main components in arene-arene interactions are dispersion and electrostatics. Dispersion is typically the main contributor, increasing with the number of substituents on the arenes, regardless of their electronic properties. Electrostatic effects influence the local dipole and depend on the electronic nature of the arene partner, with electron-rich or electron-deficient components generally enhancing the interaction. Another common element of G4-ligands is cationic groups, which are thought to interact with the negatively charged phosphate backbone of the DNA. However, this contradicts the small amount of binding free energy that salt bridges provide in a solvent-exposed environment.

To investigate how these factors influence G4 binding, we designed and synthesised a series of small molecules based on quinazoline or quinoxaline scaffolds with different substitution patterns. Additionally, we evaluated the effects of the amine side chain by substituting it with a methyl group.

Effects of Dispersion

To assess the effect of dispersion on the interaction of G4 ligands with G4 structures, we designed a set of compounds with an increasing number of substitutes on either the quinazoline or quinoxaline core (Figure 8). The binding and G4 stabilisation properties were evaluated using FRET, MST, and polymerase-stop assays. Results from all experiments consistently demonstrated that increasing the number of substitutes enhances both G4 binding and stabilisation. This confirms that dispersion is an essential component of the arene-arene interaction. Additionally, we observed that components with a quinoxaline core exhibited lower G4 binding and stabilisation compared to their quinazoline counterparts, indicating that already small changes to the aromatic core can have a drastic effect on arene-arene interactions (compounds **8-11**). Nevertheless, increasing the number of substitutes on either core consistently increased the binding affinity of compounds, underlying the importance of dispersion on interactions between G4 ligands and G4 structures.

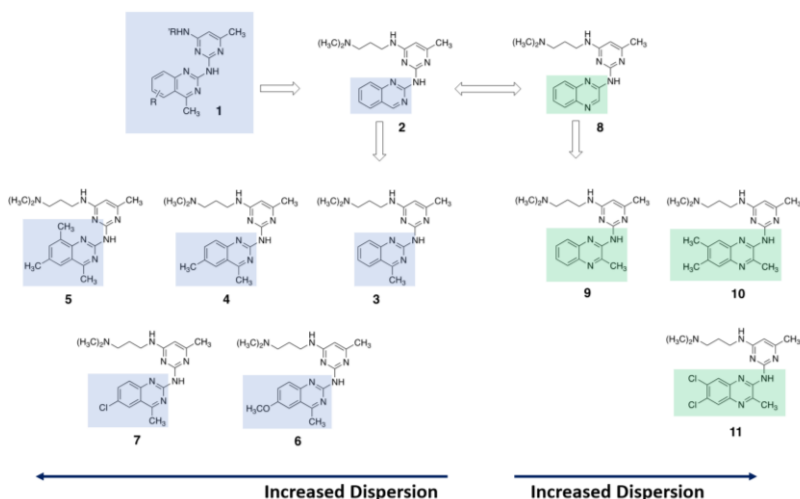


Figure 8 Overview of the compounds used to assess the effect of dispersion. Compounds were designed with a quinazoline (blue, 2-7) or quinoxaline (green, 8-11) core scaffold.

Effects of basic amine side chains

The initial set of compounds had a strong electron deficit because of the aliphatic amine. To investigate the electrostatic component of the arene-arene interactions, we synthesised a new set of compounds based on compounds **2-7** in which the amine was substituted by a methyl group. These new compounds **25-29** (Figure 9) showed decreased G4 binding and stabilisation properties in FRET, MST, and polymerase stop assays. However, consistent with previous results, increasing the number of substitutes increased G4 binding and stabilisation properties, again showing dispersion's importance in arene-arene interactions. Additionally, ITC experiments were conducted, and together with previous studies [167-169] the data showed that the amine's effect is not due to direct electrostatic interactions with the DNA backbone. Instead, it indirectly influences the arene's electrostatic nature, thereby affecting G4 binding and stabilisation.

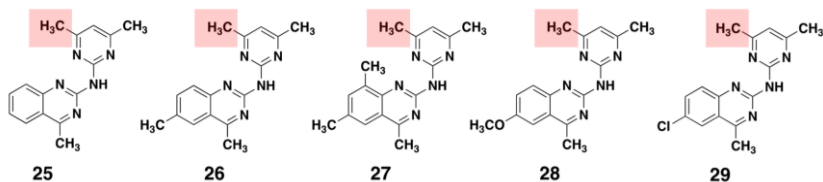


Figure 9 Compounds with replaced amine-side chains. The amine side chain of compounds **3-7** was replaced with a methyl group (red) to generate a new set of compounds (**25-29**).

Physiochemical profiling and G4 stabilisation in cells

To assess the pharmacokinetic properties of the compounds, logD and solubility at pH 7.4 were measured, along with their intrinsic clearance in rat hepatocytes and human microsomes. All compounds exhibited good solubility and low clearance, with only minor differences between the quinazolines (**2-7**, **25-29**) and quinoxalines (**8-11**). The most promising compound (**5**) and its deaminated analogue (**27**) were further evaluated. First, we confirmed that both compounds effectively stabilise various G4 structures while showing no affinity for dsDNA. We assessed their permeability in a Caco-2 assay and their cytotoxicity in three cell lines. To determine whether the compounds stabilise G4s in cultured cells, HeLa cells were treated with the compounds and analysed by BG4 immunostaining, an antibody specific to G4 structures, to visualise and quantify G4 foci. Compound **5** showed a higher increase in detected BG4 foci compared to compound **27** and the untreated control. The observed increase was comparable to that seen in cells treated with PDS, even though PDS was used at a 2.5x higher concentration.

Conclusion

In this paper, we designed a library of G4 ligands to investigate components influencing G4-ligand binding. These compounds allowed us to assess the effect of dispersion and electrostatics on the arene-arene interactions between G4 ligands and G4 structures. We confirm dispersion as a key component in these interactions. Additionally, our data suggests that amine side chains do not interact directly with the DNA backbone but instead induce an electron-deficient arene, increasing the binding strength of the ligand.

Further investigation of the most promising G4-ligand (**5**) and its deaminated counterpart (**27**) revealed good solubility, stability and low cytotoxicity for both compounds. However, the permeability of compound **5** decreased compared to **27** because of the amine group. Nevertheless, compound **5** effectively stabilised G4s in cultured cells to a similar degree to PDS.

In summary, this paper provides insights into the interaction between G4-ligands and their binding targets, paving the way for the development of G-ligands with drug-like properties to target G4s.

Paper IV

Mitochondria are the main provider of cellular ATP and function as a signal hub for many pathways. Unlike other organelles, mitochondria possess their own circular, multi-copy DNA, which encodes the core proteins of the OXPHOS complex. Due to the nature of mtDNA replication, the heavy strand remains single-stranded for an extended period, creating a favourable environment for G4 formation, particularly given its guanine-rich nature. One such G4 (CSBII) has been described as a regulatory switch between transcription and replication. However, the formation of additional G4s and their potential mitochondrial function remain uncertain. It has been hypothesized that persistent mtDNA G4s could slow/stall mtDNA replication and lead to the formation of deletion in the mitochondrial genome. Accumulation of mtDNA deletions in post-mitotic tissues can lead to a variety of late-onset diseases collectively called mtDNA maintenance defects (MDMD).

Due to the limited methods available to directly study mtDNA G4 formation and their effects in cells, we developed a human cell line expressing a mitochondrial-targeted BG4 antibody to identify G4s formed on mtDNA. By performing BG4 pull-down and subsequent sequencing of the captured DNA, we were able to map G4s formed on mtDNA.

BG4 cell line

First, we generated a Flip-In HEK293 cell line engineered to express the BG4 antibody, featuring an N-terminal mitochondrial targeting signal (MTS) from mitochondrial transcription factor A (TFAM) (chosen for its mtDNA nucleoid localization), and a C-terminal FLAG-tag, allowing controlled expression upon doxycycline induction. We performed fractionation and western blot experiments to ensure correct localization of BG4 to the mitochondria and to verify proper folding, which is essential for disulfide bond formation. Immunofluorescence further confirmed the co-localization of MTS-BG4 with nucleoids, confirming the correct targeting to the mitochondrial matrix. Pull-down experiments demonstrated co-immunoprecipitation of various mtDNA-associated proteins, including the mitochondrial DNA polymerase (Poly) and replicative DNA helicase (Twinkle), while no enrichment for other mitochondrial, cytosolic, or nuclear proteins was observed. Additionally, no effects on copy number were observed in a time course experiment expressing MTS-BG4 for up to 24 days. This confirms that MTS-BG4 localizes to mitochondria and the presence of the antibody does not affect mitochondrial DNA stability.

Validating pull-down experiments

DNA Sequencing after BG4 pull-down using the anti-FLAG antibody showed enrichment in multiple regions of the mtDNA, with no nuclear DNA fragments detected. To rule out false positive signals from nuclear DNA regions with high similarity to mtDNA (NUMTs) additional experiments were performed in ρ^0 cells that lack mtDNA. No mitochondrial sequence reads were detected in these samples, confirming that the G4s detected in previous experiments originated exclusively from mtDNA.

Factors affecting G4 formation

To validate the approach further, we treated the MTS-BG4 cells with RHPS4, a recognized G4 stabilizer that has been shown to localize to mitochondria. As expected for a G4-stabilizer ligand, this treatment led to an increase in mtDNA ChIP-seq signals. Interestingly, prolonged RHSP4 exposure led to mtDNA loss, indicating that persistent mtDNA G4s disrupt the mtDNA replication machinery.

Next, we also investigated if mtDNA replication stalling enhances G4 formation on the mitochondrial genome. Cells were treated with ddC, a nucleotide analog selectively incorporated by the mitochondrial DNA polymerase (PolG), leading to chain termination and, therefore, replication stalling. Indeed, ChIP-seq analysis showed increased G4 signals in ddC-treated cells compared to untreated controls. This effect was especially pronounced in the major arc, which remains single-stranded for an extended period during replication, suggesting that slower mtDNA replication enhances G4 formation in these regions.

Conclusion

In this paper, we established a ChIP-seq protocol using a mitochondrial-targeted G4 binding antibody (mito-BG4). A series of *in vitro* and *in vivo* experiments confirmed that mito-BG4 is correctly folded, localized to mitochondrial DNA, and does not interfere with mtDNA replication. Sequencing data revealed that G4s naturally form in mtDNA under normal growth conditions, while treatment with RHPS4 further enhanced G4 signals. Additionally, inducing mitochondrial replication stress increases G4 formation, especially in the major arc. These findings suggest a potential correlation between G4 accumulation during replication stress and mtDNA deletion formation, implicating G4s in mtDNA instability and potentially with the development of mitochondrial disorders.

Conclusion and Future Perspectives

The work presented in this thesis contributes to G4 research in multiple ways. First, we introduce a novel tool to specifically target individual G4s, which can serve as a valuable tool to investigate the biological functions of individual G4s. Second, we developed G4 ligands with improved pharmacological properties, advancing our knowledge of designing potential future therapeutics. Finally, we present the first direct evidence of G4s forming in mtDNA, along with the conditions that favour their formation.

Together, these findings advance our understanding of G4 biology and lay the groundwork for deciphering the functions of individual G4s in cellular processes. With the tools and knowledge generated, future research can develop targeted therapies for a multitude of G4-associated diseases, including cancer, viral infections, and mitochondrial DNA maintenance disorders.

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