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G-quadruplexes in Prague: a Bohemian Rhapsody

Michael O'Hagan, Jean-Louis Mergny, Zoë Ann Ella Waller

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**G-quadruplexes in Prague: a Bohemian Rhapsody****The Sixth International Meeting on Quadruplex Nucleic Acids (31 May – 3 June 2017)****Michael O'Hagan,\*<sup>a</sup> Jean-Louis Mergny,<sup>b,c</sup> Zoë Ann Ella Waller\*<sup>d,e</sup>****a) School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS1 1TS, UK****b) Univ. Bordeaux, ARNA Laboratory, Inserm U1212, CNRS UMR 5320, IECB, F-33600, France****c) Institute of Biophysics, AS CR, v.v.i. Kralovopolska 135, 612 65 Brno, (Czech Republic)****d) School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK****e) Centre for Molecular and Structural Biochemistry, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK****Abstract**

The Sixth International Meeting on Quadruplex Nucleic Acids was held at the Hotel Internationale in Prague, Czech Republic from 31 May – 3 June 2017. A vibrant interdisciplinary community of almost over 300 scientists gathered to share their newest results in this exciting field and exchange ideas for further investigations.

**1. What, where and who?**

The biannual meeting on quadruplex nucleic acids, this year affectionately termed the 'G4thering', is firmly on every quadruplex enthusiast's calendar. Following several successful conferences over the past decade [1-5] this year's meeting, based at the Hotel International in Prague (*Figure 1*), was eagerly anticipated by a record number of delegates travelling from far and wide to join the conversation (*Table 1*).

*Table 1: Details for each G-quadruplex meeting to date*

<b>Year</b>	<b>Venue</b>	<b>Participants</b>
2007	Louisville, KY, USA	104
2009	Louisville, KY, USA	100
2011	Sorrento, Italy	170
2013	Singapore	160
2015	Bordeaux, France	285
2017	Prague, Czech Republic	307

A rich community of chemists, biologists and theoreticians from across the globe, the G-quadruplex field is nothing if not diverse.



Figure 1: The 6th G4thering was held at the Hotel International, Prague.

G-quadruplexes are four-stranded nucleic acid secondary structures formed from guanine-rich sequences (Figure 2) [6-8]. The underlying motif is the G-tetrad, a planar arrangement of four guanine residues stabilized by Hoogsteen hydrogen bonding and coordination to a central cation. Multiple tetrads stack to form the quadruplex, and many different topologies can arise depending on how the primary DNA structure folds into these arrangements. The study of G-quadruplexes is highly interdisciplinary. To start with, it requires a complex suite of spectroscopic methods to elicit their detailed structures. This is complemented by state-of-the-art biochemical approaches, including the development of highly specific probes and reporters, to build evidence that these structures do indeed occur in living systems and understand what role they undertake in health and disease. Fortunately, quadruplex scientists are an ingenious bunch and the literature is rich with work that has truly transformed the field from mere intellectual curiosity to a hot topic that is taking great strides into molecular biology, pharmacology, and even the design of functional nanoscale architectures and molecular machines.

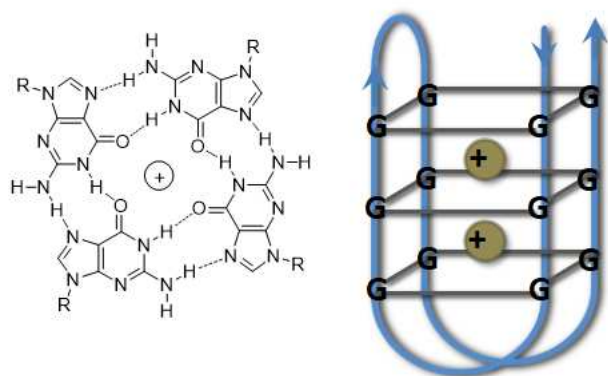


Figure 2: G-quadruplexes are four-stranded nucleic acid structures formed from sequences rich in guanine. These structures are comprised of planar tetrads of guanines, stacked on top of each other and stabilised by Hoogsteen hydrogen bonding and coordination to cationic species.

It was at this cutting-edge of our field that the G4thering was focussed. A busy agenda of almost 50 lectures, over 40 flash presentations and nearly 200 posters showcased a dazzling breadth of top-notch science, building on the fundamental work undertaken over the last few decades to answer ever more complex questions. A key focus of research right now is translating the knowledge of quadruplexes we have amassed through *in vitro* studies towards understanding the precise nature of quadruplex-forming sequences in life. This ranges from studying the effect of crowded cellular conditions on quadruplex structures to investigating their potential roles in complex biological processes such as genome protection, epigenetic regulation and embryonic development.

The quadruplex community is greatly indebted to Lukas Trantirek and the organisational team (including Paula Bates, Nancy Maizels, Michaela Vorlíčková, Anh Tuan Phan, Antonio Randazzo, Jean-Louis Mergny, and Silvie Trantirkova) for their work to bring this year's G4thering to life. As well as the fantastic scientific programme, a varied social schedule proved just as important to the success of the conference. Delegates from all levels career stages and disciplines enjoyed the many opportunities to connect over morning coffee or a late-night beer as well as in the lecture theatre. This meant that G4thering was so much more than just a sequence of excellent talks, and the vibe throughout was that of lively hive of scientists sharing ideas and building friendships in a focussed yet convivial atmosphere.

Our scientific report is structured to mirror the main themes of the meeting agenda which covered quadruplex structure, G4 targets and quadruplex ligands, quadruplex nanotech and biotech, unusual DNA structures, "the i-motif corner" and G4 biology. Owing to the sheer content covered throughout the four days of the G4thering, it has been impossible to cover everything in appropriate depth. Instead, we aim to provide a broad overview of the science discussed, along with citations to the appropriate literature to enable the interested reader to locate further detail.

## 2. Quadruplexes are everywhere!

Due to health reasons, Martin Gellert was unable to attend the G4thering to deliver the opening keynote lecture on the pre-history and impact of the discovery of G-quadruplexes. The attendees sent their well-wishes to Martin in a card which was circulated during the conference. **J.L. Mergny** (Univ. Bordeaux, France) stepped in and expanded his opening

lecture “Quadruplexes Are Everywhere!” with specific reference to Gellert’s seminal work in the field: discovery of the fundamental G-quartet structure by crystallographic methods [9].

Following a general introduction to the G-quadruplex and other associated nucleic acid secondary structures (such as the i-motif, formed by complementary C-rich nucleotide sequences) Jean-Louis gave an overview of the field and how it has progressed throughout the course of thousands of publications, from initial academic curiosity through to work in the clinic. Mergny described the rich occurrence of G-quadruplex forming sequences throughout the genome and the different methods to search for putative G-quadruplex structures *in silico*, such as the Quadparser algorithm [10]. This set the scene to introduce the latest results from their own group, the new and radically different algorithm G4-Hunter [11] which considers G-richness and G-skewness of a given sequence. The algorithm returns a ‘quadruplex propensity score’, rather than a (potentially misleading) yes/no result, since the only way to truly validate a quadruplex structure is through experiment. To that end, Mergny reviewed the evidence for G-quadruplex formation *in vivo* both indirect (such as induced genome instability [12, 13]) and direct (the use of G4-specific antibodies [14] or *in vivo* NMR [15]) as well as highlighting the potential for clinical development for potential quadruplex-related therapeutics [16, 17]. Towards the goal of delivering a quadruplex-based therapeutic strategy to market, AS1411 (a G4-forming oligonucleotide) [18, 19] and CX3543 (a G-quadruplex targeting ligand) [20] are the candidates which have progressed furthest in the drug discovery process to date, but there is still plenty of room for more G4-related clinical development.

New avenues of exploration are opening all the time, with a significant number of new researchers joining the field every month. However, Mergny stressed the need for caution when breaking new ground, such as the need to validate the existence and structure of a proposed quadruplex using a variety of techniques before embarking on application-based studies. Towards this end, we need to work together as an international network to share best practice and provide opportunities for researchers new to the field to take their first steps with confidence. Mergny suggested we can do this by publishing approved standards and guidelines on techniques for quadruplex study, and by maintaining a central online hub for the community to gather and share resources (the excellent quadruplex.org website is much missed!)

Mergny’s take-home message was one of optimism and excitement. The field has strong foundations, but many exciting opportunities to make new discoveries remain. He looked forward to the diverse programme of talks ahead before inviting Ahn-Tuan Phan to chair the first session. With that, the G4thering was underway!

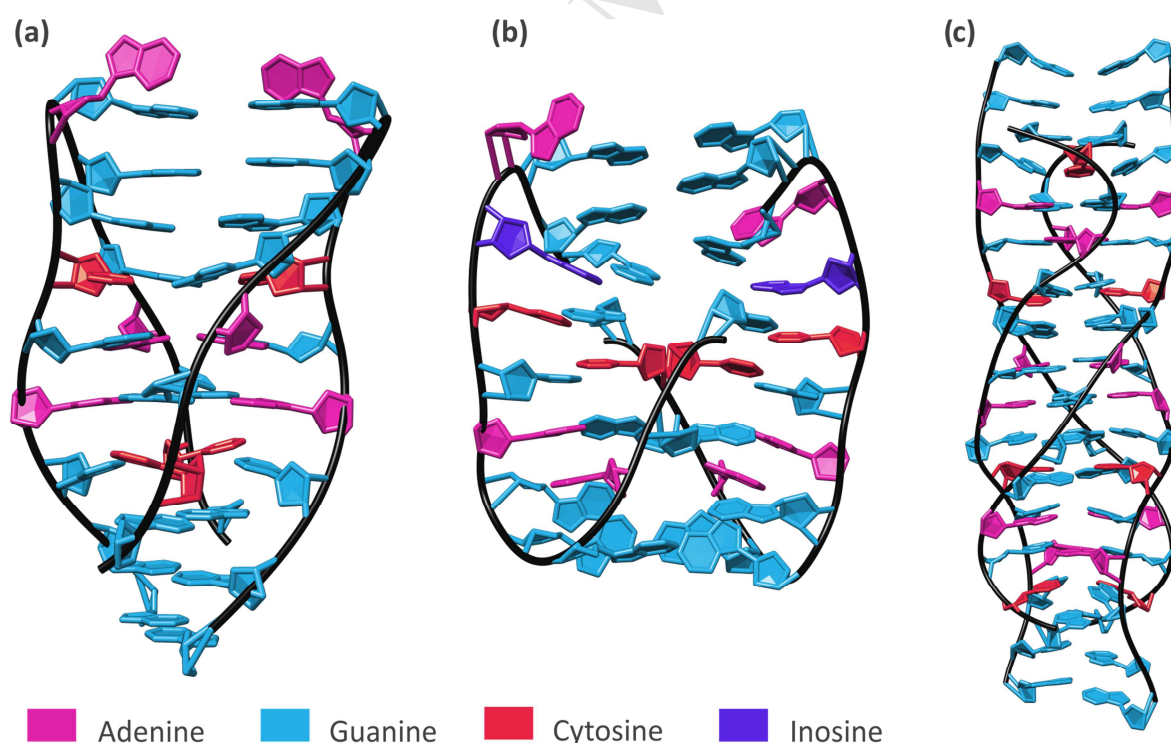
### 3. Getting into the groove: G-quadruplex structure

If there is one thing that sets G-quadruplex DNA apart from the classical Watson-Crick structure of B-DNA, it is the sheer zoo of structures that arises from the myriad ways the nucleic acids strands can twist, turn, loop and bulge to set up the stacking of the core G-tetrads. Furthermore, these sequences are often polymorphic [21, 22], forming different topologies depending on the folding conditions. It’s no surprise that the structural elucidation of these motifs forms a cornerstone of G-quadruplex research, from which



everything else can flow: from the rational design of small molecule ligands, to understanding the interaction of quadruplex DNA with cellular proteins and therefore its biological relevance. A key interest of quadruplex structural biology right now is *how* the different structures fold and unfold under cellular conditions as well as *what* the final folded structure is, which is particularly important when designing approaches that move beyond the test tube to target quadruplexes *in vivo*. Excitingly, researchers are now using their expertise in this field to uncover fascinating new four-stranded structures, beyond the classical G-quadruplex, that may display interesting roles in biology. The work presented in this section reflected many of these themes.

Solution NMR remains an indispensable method in the quadruplex scientist's toolbox, giving unparalleled high-resolution insight into the fascinating topologies adopted by these sequences under biologically-relevant conditions. **J. Plavec** (Nat. Instit. Chemistry, Ljubljana, Slovenia) presented some of the more unusual G-quadruplex structures adopted by the human papilloma virus and a 32-nt fragment of the KRAS promoter (32R-3n) which adopts a very unusual dimeric G-quadruplex structure [23]. Plavec also reported the exciting discovery of novel tetrahelical structures stabilised by G-C, G-A and G-G base pairs, for example the VK1 sequence variant which forms an AGCGA quadruplex (*Figure 3*). In contrast to classical G-quadruplex forming sequences, this sequence does not show as high sensitivity towards the presence of cations or pH [24]. Later in the session, **V. Kocman** (Slovenian NMR Centre, Ljubljana, Slovenia) continued this theme of unusual tetrahelical structures, reporting the observation of GAGA and GCGC quartets formed by AGCGA tracts in the VK34 gene.



*Figure 3: High-resolution structures of representative members of the AGCGA-quadruplex family adopted by d(GCGAGGGAGCGAGGG), VK34, and its inosine analogues [24]. (a) The structure of the VK34 dimer (PDB ID: 5M1L). (b) The structure of the VK34\_I11 (VK34 inosine analog with a G11 to I11 substitution) dimer (PDB ID: 5M4W). (c) The structure of the VK34 tetramer (PDB ID: 5M2L, scaled down approx. two fold compared to structures in panels a and b). Color coding of individual nucleotide residues is indicated.*

Translating promising results obtained *in vitro* to a meaningful effect *in vivo* remains a challenging goal for quadruplex scientists. **C. Sissi** (Univ. Padova, Italy) discussed their group's observation that G4-targeting molecules recognising the c-KIT sequence do not always result in downstream gene silencing *in vivo* [25]. This led to a detailed study of the folding dynamics of individual quadruplex-forming sub-units of the gene. The group elucidated the thermodynamic and kinetic folding profiles of G-quadruplex folding, and identified the process proceeds through several intermediates that may themselves be explored as unique small-molecule receptors. This demonstrated the importance of considering which folding pathway is relevant under cellular conditions to fully exploit the potential of G-quadruplex targeting in therapy.

The theme of G-quadruplex folding pathways continued with **J. Sponer's** talk (Instit. Biophysics, Brno, Czech Republic), which reported molecular dynamics simulation studies on the folding of human telomeric DNA. The folding pathway is described as an extreme case of kinetic partitioning, in which there is competition between well-separated minima separated by large free-energy barriers. This contrasts with the 'funnelled folding' mechanism observed for small proteins, which fold on much shorter timescales. The kinetic partitioning mechanism explains the long folding times of G-quadruplex sequences and the existence of the long-lived sub-states that are observed experimentally [26].

**M. Aznauryan** (Univ. Aarhus, Denmark) continued the theme of G-quadruplex folding dynamics. This talk focused on the use of biophysical tools to examine the folding of human telomeric DNA under physiologically relevant conditions, where the effects of molecular crowding become significant [27]. Single-molecule FRET studies, giving time resolution on conformational dynamics of up to 100ms, showed that interconversion of quadruplex topology occurs through unfolding mechanisms and that molecular crowding agents (such as PEG8000 and Ficoll 70) increase both the rate of folding and the dominance of the folded species in solution. In agreement with Sissi and Sponer, Aznauryan's data highlighted the complexity of G-quadruplex folding pathways, which proceed *via* many different conformational states with different kinetic and thermodynamic stabilities. All of these studies contribute vital knowledge regarding the behaviour of such sequences in media more closely resembling physiological conditions.

The following lecture, by **P. Unrau** (SFU, Canada) described the process of developing a G-quadruplex based fluorophore binding aptamer into a fluorescent switch [28]. RNA-Mango is one of the most red-shifted fluorescent macromolecular probes to date. To better understand the exceptional properties of this probe, the co-crystal structure was determined. A key take-home message was that optimal contrast requires both high affinity for the target but also high fluorescence enhancement. The resultant RNA-based switches make them well-suited to a range of types of nucleic acid detection assays.

Having considered the mechanisms and pathways of quadruplex folding in some of the earlier lectures, **J. Chaires** (Univ. Louisville, Kentucky, USA) provided a complementary story by discussing their work on the *unfolding* of quadruplex DNA by the shelterin complex protein POT1. Though POT1 binds to ss-TTAGGTTAT rapidly, the kinetics of POT1 binding to

the *folded* telomeric sequence are much slower, indicating the limiting factor is the unfolding of the quadruplex. Taken in the context of further experiment, this demonstrates that POT1 acts by conformational selection, in which the unfavourable free-energy barrier to quadruplex unfolding is compensated for by the energy of POT1 binding to the unfolded sequence. This insight may be used to develop inhibitors for the POT1 unfolding pathway.

The final talk of the session was given by **V. Kuryavyi** (Rockefeller Univ., New York, USA) who offered an alternative proposal for the structure of two identical strands of d(GGGAGCGAGGGAGCG). Previously, **J. Plavec** and co-workers had proposed an NMR-based circular DNA model built through the base-pair mediated self-assembly of two composite strands [29]. Kuryavyi and Bracken were curious about the proposed structure and re-analysed and re-visited some of the data, eventually proposing an alternative structure from the experimental data already published.

Following the final lecture of the day was the first **flash presentation session**, part of the student poster competition. Each student who was presenting a poster at the G4thering was asked to prepare a short presentation, summarising the key findings from their poster in 90 seconds. Topics covered a wide range of topics from the formation of G-hairpins, how G-quadruplexes regulate growth in plants, co-evolution of G-quadruplexes in viruses and infected hosts and G4-RNA screener, a motif-independent identification of potential RNA G-quadruplexes.

#### 4. Hitting the target: G-quadruplex ligands

Having spent the previous evening networking and sharing ideas over dinner, wine and posters, the formal proceedings resumed bright and early on Thursday morning, with a diverse line-up of speakers discussing the state-of-the-art in targeting G-quadruplex structures with small molecules. As well as work to identify and validate new G-quadruplex targeting chemotypes, developments in the field place significant focus on using known potent small molecule ligands as tools to give insight into quadruplex biology, whether understanding folding dynamics or visualising the presence of the quadruplexes in cells in real time. Exciting progress has also been made towards realising the hitherto elusive goal of developing G-quadruplex ligands into clinical molecules. Though challenges remain, new opportunities are presenting themselves all the time as our knowledge improves and approaches for identifying promising novel targets become ever more sophisticated.

In remarking that their talk would usually be part G-quadruplex structure section of the conference, **A. T. Phan** (Nanyang Tech. Univ., Singapore) highlighted the holistic nature of the quadruplex field, with the boundaries between work on structure, ligands, targets and biology being fluid rather than existing as tightly-defined divisions. Phan's talk began with a fascinating discussion of the rich variety of quadruplex structures that present exciting opportunities for selective ligand targeting, including left-handed quadruplexes [30] and quadruplex-duplex hybrids [31]. Attention then focussed on considering how structural knowledge informs the design of agents that recognise quadruplex DNA, be they small molecules or proteins. Recent structural work has shown that telomestatin derivatives are able to kinetically trap all-parallel G-quadruplex in potassium solution, and that the RNA helicase RHAU binds specifically to parallel quadruplexes. This is structure, not sequence,



dependent and binding takes place under crowded conditions which favour the formation of parallel G-quadruplex. Phan stressed that the exposed G4 tetrad remains the simplest way of recognising a quadruplex with a small molecule, but presented 'dual specific targeting' as an opportunity distinguish specific quadruplexes, by combining a known tetrad-stacking motif (e.g. Phen-DC3) with a duplex DNA groove-binding motif (e.g. netropsin) to form a termolecular complex [32].

Now that the field of quadruplex ligand design is relatively mature, the development of the next generation of quadruplex-targeting small molecules can build on a wealth of techniques that both predict and measure G-quadruplex binding. **C. Giancola** (Univ. Naples, Italy) made a compelling case for full thermodynamic characterisation of ligand binding using calorimetric methods to more fully understand the nature of G-quadruplex recognition. This is an essential complement to the structural and spectroscopic methods used to characterise quadruplex binding in drug development and lead optimisation. The development of tools to quantify G-quadruplex binding was also the theme of **D. Montesarchio's** (Univ. Naples, Italy) talk, reporting the design of a new high-throughput screening assay based on affinity chromatography for the identification of new G-quadruplex binding chemotypes. Known as G4-OAS (G-quadruplex on oligo affinity support) the assay uses a functionalised resin to bind the quadruplex of interest, which then catches small-molecule binders from combinatorial libraries [33]. Novel chemotypes identified using this method have shown micromolar toxicity in cancer cell lines and binding constants in the order of  $10^7 \text{ M}^{-1}$ .

As well as their potential as therapeutic agents, quadruplex-targeting small molecules are invaluable as probes to visualise the occurrence of G-quadruplexes in live cells. Such tools are vital towards validating G-quadruplex formation as the mechanism of action of candidate drug molecules. Fluorescent probes that light up on quadruplex binding have previously shown promise, though their use has often been hampered by imperfect duplex/quadruplex selectivity giving misleading results. **R. Vilar** (Imperial College, UK) reported a possible solution in the design of DAOTA-M2, a triangulenium molecule whose fluorescence emission lifetime changes significantly on interaction with specific DNA topologies [34]. In measuring emission lifetime rather than emission intensity, the need for perfect topological selectivity is circumvented and this approach has allowed the activity of well-known G-quadruplex ligands, such as BRACO-19 and metal salphens, to be quantified accurately in live cells.

Following lively discussion over morning coffee, **N. Sugimoto** (FIBER, Konan Univ., Japan) began the mid-morning session with a discussion of molecular crowding effects on G-quadruplex formation, noting that this is one of the fundamental differences between *in vitro* and cellular conditions [35]. This inspired Sugimoto's lab to design of PEG-conjugated oligonucleotides containing three G-tracts that could induce quadruplex formation and inhibit gene transcription [36]. The challenge now is to consider how to deliver these oligonucleotides into cells in order to exploit this approach to therapeutic advantage.

A theme of the following two talks was understanding the interaction of G-quadruplex interactions with proteins, and thus obtaining a more complete picture of the role of G-quadruplexes *in vivo*. **H. Balci** (Kent State Univ., Ohio, USA) employed single-molecule FRET

to examine the dynamics of unfolding G-quadruplex DNA by of proteins such as POT1, BLM and RECQL5 [37]. **A. Randazzo** (Univ. Naples, Italy) reported the use of human telomeric DNA to 'fish' for protein interaction partners in nuclear extracts. Interestingly, different topologies of the quadruplex DNA caught different proteins. This approach identified HMGB1, KHSRP and LMNB1 as proteins that recognise human telomeric DNA, suggesting possible roles for these species in G-quadruplex biology [38].

**V. Gabelica** (CNRS, Univ. Bordeaux, France) considered the effect of ligand binding on quadruplex folding. Electrospray ionisation mass spectrometry provides an ideal tool for studying such effects, since it allows the assumption-free elucidation of the cation stoichiometry and therefore indicates how many G-tetrads are present within each species. In conjunction with circular dichroism spectroscopy, time-resolved studies provided experimental evidence for the kinetic partitioning mechanism of folding *via* several 'misfolded' intermediates separated by large free-energy barriers; even c-MYC (typically thought of as an all-parallel structure) was found to form a 2-quartet antiparallel species which exists for several minutes. The effect of well-known ligands could then be quantified, such as PhenDC3's ability to induce transition from a 3-quartet to 2-quartet structure in telomeric quadruplexes [39, 40].

After a delicious lunch in the sun accompanied by (what else?) a cold Czech beer, talks resumed in the afternoon. **S. Neidle** (UCL, London, UK) delivered a comprehensive overview of their lab's work on developing quadruplex-targeting drugs for human cancers. Because many cancers involve the deregulation of several genes, Neidle suggested that true selectivity for a single G-quadruplex (which still remains elusive) may not be a necessary requirement for a therapeutic candidate, as long as the quadruplex/duplex selectivity is sufficiently high. The Neidle group have recently discovered that naphthalene diimide compounds bearing three groove-binding side-chains can be just as active as tetrasubstituted derivatives whilst displaying improved pharmacological properties. Indeed, these compounds display selective nanomolar toxicity towards pancreatic cancer cells and tumour growth arrest in animal models. Critically, the current lead compound has been shown to downregulate several genes, with many of these containing at least one G-quadruplex forming sequence.

The imaging of candidate G-quadruplex targeting molecules in live cells is important towards understanding their mechanism of action. Furthermore, such tools are vital for visualising the presence of G-quadruplexes *in vivo*. **M.-P. Teulade-Fichou** (Instit. Curie, Orsay, France) provided insights from their laboratory following fluorescent tracking studies of PhenDC3 derivatives. This approach required the labelling of the ligand with fluorophores using click chemistry, and different localisation patterns were observed depending on whether the copper-mediated or copper-free click reaction was used: the Cu(I)-alkyne complex being responsible for misleading nucleolar localisation [41] – a key warning for those at the synthesis/biology interface!

**J. Trent** (Univ. Louisville, Kentucky, US) noted the challenges of targeting DNA rather than proteins with small molecules, with selectivity screens being particularly critical in the field of G-quadruplex targeting. Indeed, poor selectivity is a key factor of the high attrition rate of candidate molecules. However, there are key opportunities for targeting novel binding sites

by considering higher-order G-quadruplex structures of up to 192 nucleotides in length, and Trent's lab have successfully identified a novel class of drugs that bind to such higher order quadruplexes. This represents progress within the field to consider much longer, physiologically relevant DNA sequences rather than artificially short strands.

In the final talk of the session, **C. Weldon** (Univ. Leicester, UK), focussed on the role of ligands in regulating the alternative splicing of Bcl-X pre-mRNA. The proximity of the G-tracks to the two alternative 5' splice sites means that stabilisation of this structure can shift Bcl-X splicing in favour of the pro-apoptotic isoform. Using a 7-deaza guanosine footprinting method, it was possible to demonstrate ligand binding to the putative quadruplexes located near to the splicing sites, providing strong evidence for the existence of quadruplexes in long functional RNAs [42].

## 5. From structure to function: G4s in nanotechnology and biotechnology

Targeting G-quadruplexes with small molecules to modulate their function, probe their biology or shut down a disease pathway is only part of the story. Nature's molecules have an impressive track record of inspiring novel technologies: from catalysing enantioselective chemical reactions under conditions far removed from the biological milieu, to use in sensing devices and even as therapeutic agents in their own right. Quadruplexes are no different, and this session looked at ways to harness their functional potential.

**D. Sen** (Simon Fraser Univ., Canada) described how the heme binding properties of G-quadruplexes can be exploited. Ferric heme is able to bind most G4 structures with dissociation constants between 10 nM and 1  $\mu$ M. For example, both the RNA and DNA ALS sequence (GGGGCC) repeats form G-quadruplex structures which can bind heme. Excitingly, the quadruplex-heme complex can then be used to catalyze oxidation reactions [43]. Heme is a useful ligand for G-quadruplexes as it is easily delivered to cells, and its level is controlled tightly within the cellular environment. This work certainly demonstrates that, although heme-quadruplex interactions have been well known for some time, there still significant potential for exploiting this interaction in functional applications.

DNA can be used as a substrate for molecular construction. **H. Sugiyama** (Kyoto Univ., Japan) uses atomic force microscopy to observe G-quadruplex formation at single molecule level and has found that hybrid G-quadruplex can provide a stable scaffold to stabilise T-loop structures. Furthermore, dual-action ligands (which target both G-quadruplex and duplex simultaneously) have been developed. The hybrid molecules employ a porphyrin and a polyamide towards gaining specific binding to a specific quadruplex structure [44]. In collaboration with Hanbin Mao, Sugiyama's laboratory have recently used laser tweezers to measure quadruplex unfolding dynamics under free and caged conditions [45].

**P. Bates** (Univ. Louisville, Kentucky, USA) gave a review of their work on antiproliferative quadruplex-forming oligomers. Others have previously argued against the use of G-rich sequences for such applications due to potential non-specific effects and reduction of effective concentration of the single-stranded species [46]. Bates questioned whether it is fair to assume all antiproliferative oligonucleotides necessarily share a similar mechanism. In particular, they suggested that AS1411 may act as a pro-drug, producing cytotoxic

guanine-based degradation products which may be responsible for the antiproliferative nature of this sequence [47]. In the future, CRISPR-Cas9 screening may help identify resistant cells and contribute further understanding of this fascinating oligonucleotide.

Next up, **S. Dvorkin** (Univ. Ulster, UK) treated the participants to a tutorial on G-quadruplex topologies. Dvorkin actually rejects using terms such as “propeller” or “basket”, claiming they oversimplify the classification of different structures. Using chemical shift indexing, the group aim to be able to distinguish the different possible quadruplex architectures and they propose that a combination of tools could be adopted to allow automated categorisation of quadruplex topologies.

The day concluded with the second **flash poster presentation session**, showcasing a wide range of topics including GC-tetrads, carbohydrate-bearing G-quadruplex ligands with high selectivity [48], *in vivo* NMR and G-quadruplex ligands with *in vivo* activity. The ensuing poster session was accompanied by a fantastic selection of cheese and wine, and it was here that the authors of this meeting report met in real life for the first time after discussing the meeting on Twitter earlier in the day. Social media really is a great way to make new friends at conferences!

Day Three, and despite the festivities taking place the night before, we were woken up with a bang (or should we say a switch?) with **I. Willner's** (Hebrew Univ. Jerusalem, Israel) keynote reviewing their work using both G-quadruplex and i-motifs in the construction of molecular devices and functional materials. Like **D. Sen** the previous day, Willner also documented use of the hemin/G-quadruplex interaction, this time as catalytic labels for optical and sensing platforms [49] as well as driving chemical transformations using a nucleozyme [50], making use of an aptamer to enhance substrate concentration in proximity to the dopamine active site. Willner also explained some of their applications of quadruplexes in material sciences to make hydrogels, where the properties of the hydrogel, such as rigidity, can be controlled by using quadruplex structure: adding crown ether sequesters monovalent potassium and therefore causes G4-unfolding and disruption of the gel structure, with the process being reversed by addition of further potassium ions.

Self-assembly was the theme of **O. Lustgarten's** (Weizmann Inst. Science, Israel) talk, describing an example of a receptor scaffold in which four different strands of DNA are appended with different linkers and with four different fluorophores. These separate strands are able to assemble into a unique quadruplex structure. Binding of a drug induces fluorescence changes. This is drug dependent, and a different fluorescence pattern is observed when analytes are added in different orders, resulting in a fingerprint. A library of 13 strands gives rise to 2560 combinations which, excitingly, could be used to design a molecular-scale security system [51].

As Monty Python used to say: “And now for something completely different!” **M. Fojta** (Inst. Biophys. CAS, Brno, Czech Republic) took the audience on a journey through exploring DNA structure using electrochemical methods. Fojta paid tribute to Emil Palecek who discovered in the 1950's that DNA has intrinsic electroactivity and explained the concept of “structural memory of adsorbed DNA”. The first report of G-quadruplex electrochemistry came from Ana Brett in Portugal [52] and Fojta's work has been looking into the effects of the length of

the oligonucleotide and composition of the nucleotides, including the single-stranded flanking sequence which surrounds the quadruplex. Fojta's work paves the way towards the development of simple electrochemical techniques with applications in label-free monitoring of nucleic acid structural transitions. When the audience had the opportunity to ask questions, a temporary issue with the microphones meant that the chair, Jean-Louis Mergny, had to dart around the audience on foot, with Miroslav Fojta following each time for an up-close-and-personal response!

Computational methods are invaluable towards the study of G-quadruplexes. **A. Tanzer** (Univ. Vienna, Austria) took time to evaluate RNA secondary structure prediction. Using different packages, such as RNAfold and G4-Hunter, the group performed a comparative assessment using a genome-wide screen across different kingdoms of organisms. Only 2% of bacteria and 5% of primate putative sequences were confirmed by RNAfold in the context of the larger sequence. Tanzer also highlighted the importance of considering the sequence context of G-quadruplexes in experimental studies, as well as *in silico* prediction.

## 6. Plenty more fish in the 'C'

It wasn't all about guanine! It would have been remiss to have neglected the G-quadruplex's sister strand and its own stacked structure, the i-motif. **Z. Waller** (UEA, Norwich, UK) discussed the fascinating factors at play in stabilising this structure. The i-motif is generally assumed to require stabilisation under acidic pH, but Waller's lab have demonstrated sequences which are stable under neutral conditions [53]. Work with copper cations [54] has also showed switchable conformations using different redox conditions or chelators. Waller acknowledged that targeting the i-motif with small molecule ligands is much more difficult than G-quadruplex and ligand binding modes are not yet known. Nonetheless, several ligands have been identified [55, 56] and are under further development. The theme of i-motifs at neutral pH was also explored extensively by **C. Gonzales** (CSIC, Madrid, Spain) who used chemical modification strategies to stabilise the structure under a range of experimental conditions. For example, introduction of fluorine atoms at the C2' position of the sugar backbone leads to favourable electrostatic interactions that confer i-motif stability at neutral pH [57]. Gonzales also described the importance of capping interactions for i-motif stability, particularly under physiological-like conditions. They described how GCGC capping tetrads are thought to increase the pKa of the nearby cytosines, affording stability of these structures at higher pH [58].

## 7. That's life! A tour of G-quadruplex biology

Having looked in detail at work to identify quadruplex structure *in vitro* and *in vivo* and the possibilities to target these species selectively with a diverse range of chemical approaches, the meeting was in good shape to turn its attention to using this suite of tools to probe the role of G-quadruplexes in living systems. It was encouraging to see a huge diversity of successful projects underway in this area, which really brings home how the deep understanding of the behaviour of G-quadruplex sequences *in vitro* can be used to interrogate the roles of these structure in a diverse range of physiological circumstances.



**S. Balasubramanian's** (Univ. Cambridge, UK) opening lecture first examined the prevalence of G-quadruplex forming sequence in the human genome, with G4-seq identifying far more G4 sequences than are predicted by bioinformatics methods [59]. Excitingly, G4-seq has now been performed on a number of model organisms studied by DNA biologists and the results are soon to be made available for wider use. It was also noted that stabilising the G-quadruplex with a ligand may do significantly more than simply extending its lifetime, which may have critical implication in ligand-driven experiments, so understanding the G-quadruplex in the context of its wider biological environment is of high importance.

Since guanine is the most readily oxidised DNA base, G-quadruplexes are a potential target of oxidative DNA damage. **C. Burrows** (SLC, Utah, USA) examined this topic, noting that the presence of 8-oxo-G (OG) has a profound effect on reducing G-quadruplex stability. Indeed, presence of OG can lead to a 300% increase in gene expression of G-quadruplex forming sequences [60]. Interestingly, some genes contain inherent protection against this potentially deleterious modification. For example, the vascular endothelial growth factor (VEGF) gene contains a 'spare tyre' G-tract which can be used to repair the quadruplex fold following oxidative damage, triggered by APE1 [61]. This suggests an interesting role for G-quadruplex as sensors of oxidative damage *in vivo*.

What happens at the beginning of life and how are G-quadruplexes involved? **N. Calcaterra** (IBR, Argentina) is exploring the potential roles of G-quadruplex DNA in embryonic development. Using Quadparser, the group have identified 463 developmental genes containing putative quadruplex-forming sequence in their proximal promoter regions. After assessing the quadruplex folding properties of these sequences *in vitro*, the group employed an antisense oligo in zebrafish to inhibit G4 formation and showed this actually reduced gene expression [62].

A key question **T. Bryan** (Univ. Sydney, Australia) is addressing is whether G-quadruplex DNA protects uncapped telomeres. It is already known that G-quadruplexes play a protective role in yeast [63]. POT1 knockdown was shown to result in an increase in G4 localisation at telomeres, suggesting a link between G4 formation and the DNA damage responses that result from loss of POT1. Treatment with a G4-stabilising ligand in the G1 phase of the cell-cycle showed repression of DNA damage responses but, surprisingly, this was accompanied by a decrease in G4 localization at telomeres. This suggests a complex role of quadruplexes in protecting uncapped telomeres in living systems.

Further interesting results were disclosed by **G. Guilbaud** (Univ. Cambridge, UK), who described local epigenetic reprogramming induced by G-quadruplex ligands. Using the BU-locus of chicken cells, they screened for small molecules able to induce G-quadruplex dependent transcriptional reprogramming. The top hit, interestingly, looked to be a weak G-quadruplex ligand, reminiscent of some which were originally used as controls in the early G-quadruplex work. The changes in methylation were also heritable in successive cell divisions [64].

Further work on the role of G4 in replication-dependent genome instability was described by **A. Nicolas** (Institut. Curie, France) [65]. Working in yeast they found that not all G-quadruplex motifs induce such instability, with only those with very short loops (less than 4

nucleotides in length) causing such an effect. It is interesting that such short loops are actually very rare in yeast whereas there are approximately 18,000 sequences with 3 single nucleotide loop in humans, suggesting that evolution may have selected against such sequences.

**G. Capranico** (Univ. Bologna, Italy) described how G-quadruplex ligands can cause modulation of R-loops and therefore induce DNA damage. R-loops are three-stranded structures comprised of both DNA and RNA. Ligands including pyridostatin were shown to induce R-loop formation, using antibodies to visualise the hybrid duplex. The group then demonstrated the associated DNA damage response detected by the formation of  $\gamma$ H2AX foci, a biomarker for DNA double-strand breaks.

The group of **J. Kurreck** (Berlin Univ. Tech., Germany) has investigated the role of G-quadruplexes in the 5'-UTR of mRNAs, following on from work which shows these quadruplexes inhibit gene expression through translation rather than transcription [66]. Since RNA G-quadruplexes are known to be largely unfolded in cells [67], they investigated the effect of knocking-down the Rha helicase. Following knock-down, a proteomic analysis indicated only a small number of proteins are significantly down-regulated by the silencing of Rha. However, G-quadruplex forming sequences in the 5'-UTR of the associated mRNAs are over-represented, occurring approximately three times more frequently (33% of affected genes) than in the full population identified (just 10% of genes). This suggests quadruplexes in the 5'-UTR may play a specific role in regulating the expression of this subset of proteins.

The final trio of talks of the afternoon were centred on the theme of G-quadruplexes in viral DNA. **V. Perumal's** (Indian Instit. Tech., New Delhi, India) group have identified a putative G-quadruplex forming sequence in the promoter region of the preS2/S gene of the hepatitis B virus. In contrast to quadruplexes in other promoter regions (e.g. cMYC) which have been shown to downregulate gene expression, the HBV quadruplex was found to upregulate transcription and the presence of the quadruplex is associated with high levels of hepatitis B virion secretion. Looking next at the HIV-1 virus, **M.-L. Andreola** (Univ. Bordeaux, France) reported the use of small molecule G-quadruplex ligands as antiviral agents. These ligands did not affect the ability of the virus to enter cells but anti-viral activity was correlated with the observed *in vitro* stabilisation of a highly conserved G-rich region in the HIV-1 promoter. G-quadruplex targeting therefore represents a promising antiviral strategy. **I. Frasson** (Univ. Padua, Italy) reported results from the S. Richter group on the analysis of the presence of G-quadruplexes in human viruses. Each class of virus has a differential distribution. For example, retroviruses and herpesviruses are highly enriched in putative G-quadruplex forming sequences whereas double stranded viruses specifically lack putative G-quadruplex forming sequences.

Day three of the G4thering culminated in a splendid gala dinner at Manes Restaurant in central Prague, beautifully positioned on the bank of the Vltava River. Transport to the venue was provided by chartered tram, allowing delegates to arrive in true style.

The final day of the conference opened with a lecture on the role of quadruplexes in transcriptional regulation by **N. Maizels** (Univ. Washington, Seattle, USA). Maizels

highlighted the role of G-quadruplex helicases, such as XPB [68], BLM [69] and WRN [70] in the up- and down-regulation of quadruplex-containing genes. Treating HT1080 cells for 48 hours with PhenDC caused the dysregulation of 1459 genes by at least two-fold, increasing the transcription of 715 genes and decreasing that of 743 others. PhenDC3 upregulates the genes involved in iron homeostasis and heme metabolism, with the heme oxidase HMOX1 upregulated by a factor of 32!

The following talk by **R. v.Schendel** (Univ. Leiden, Netherlands) discussed the use of a *C. elegans* model to study the inhibition of DNA replication by the formation of quadruplexes. Schendel found that G-quadruplex DNA can persist through multiple mitotic divisions without changing conformation, leading to double-strand breaks and deletions of around 120 base pairs following Pol Theta-mediated end joining. The deletions either start at the foot of the quadruplex (for short sequences) or (for longer G-quadruplex motifs) within the rows of the quadruplex. The question remains as to what determines the other end of the mutation [71].

The role of promoter quadruplexes was explored by **D. Yang** (Univ. Purdue, Indiana, USA) who gave a wide-ranging overview covering their structure, function and diversity. Yang noted that a correlation between G-quadruplex formation and transcriptional activity does not necessarily imply causation and that single-nucleotide loops resulting in parallel topologies are very common in promoter quadruplexes.

**M. Fay** (Harvard Medical School, Boston, USA) reported their group's work to interrogate the impact of stress on RNA G-quadruplexes. Fay explained how under such conditions, RNA forms stress granules which stall translation to conserve energy [72]. Using a BG4 antibody, they showed that RNA G-quadruplexes are recruited to stress granules, which is interesting as RNA G-quadruplexes are apparently mostly unfolded under normal cell growth conditions. Furthermore, Fay showed that GGGGCC RNA induces the formation of stress granules *in vitro*. Promotion or disruption of such quadruplexes (with ligands or 7-deaza-G substitution) showed that the quadruplex is necessary for granule formation. The theme of RNA quadruplexes and cellular stress was continued by **P. Ivanov** (Brigham and Women's Hospital, Boston, USA) who discussed functional intermolecular G-quadruplexes derived from tRNA [73]. They showed that cellular stress leads to tRNA in the anticodon loop, creating tRNA fragments which were demonstrated to form folded quadruplexes and inhibit gene transcription *in vivo*, suggesting a key biological role for these structures. The final talk, by **R. Samuel** (Univ. Sherbrooke, Quebec, Canada) looked at the role of RNA quadruplexes in regulating cancer-related microRNAs [74]. Bioinformatics searches were used to find possible 3' untranslated regions with the potential to interact with mRNA containing putative G-quadruplex forming sequences at the binding site. It was shown that the presence of the quadruplex structure greatly affected the ability of the microRNA to bind to its target. This represents an exciting opportunity to regulate gene expression that may complement the approach of targeting G-quadruplexes in gene promoters.

At the closing ceremony, the eagerly-anticipated result of the student poster competition was announced. The standard of the posters presented was of top quality, and with 40 competition participants from 16 different countries the future of our field looks to be in safe hands. The overall winner was **J.-M. Garant** (Univ. Sherbrooke, Quebec, Canada), for

their poster on G4RNA screener, a new bioinformatics tool to identify RNA quadruplexes [75]. The runners up were **C. Baxter** (Univ. Sheffield, UK), presenting on quadruplex-dependent processes in plant species, and **M. Abdelhamid** (UEA, Norwich, UK), who discussed the effects of  $\text{Cu}^+$  on the stability of the i-motif. Congratulations to them all!



Figure 4: Quadruplex self-assembly! Conference delegates get together for a group photo.

## 8. Conclusions and looking forward

Overall it was widely acknowledged that the meeting, the largest of its kind to date, was a great success (Figure 5). A couple of people have already volunteered to host the next G4thering in 2019 – it seems so far away now but time will pass faster than we expect! We have clearly come a long way in recent years, evidenced by the countless tools we have developed which have provided the gateway to much deeper biological understanding of quadruplex DNA. Despite this, ligands which show inter-quadruplex specificity are remain elusive and there is still much more to be learned about the mechanisms of how G-quadruplexes work and function. What is the relationship between G-quadruplexes and disease? Will a G-quadruplex interacting agent make it into the clinic? Can we truly prove these structures exist *in vivo*? Indeed, there remain excellent opportunities within the field to develop research into quadruplexes nucleic acids and though there has been major progress in our field within the last two years, far more is yet to come. We are watching with excitement to see the advances this brings.



Figure 5: A sweet vote of thanks for Lukaz Trantirek and his organising team

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## Highlights

- Meeting report for the Sixth International Meeting on Quadruplex Nucleic Acids, Prague, Czech Republic, 31 May – 3 June 2017
- Over 300 scientists gathered to share their newest results in the Quadruplex field
- The meeting agenda covered quadruplex structure, G4 targets and quadruplex ligands, quadruplex nanotech and biotech, unusual DNA structures, “the i-motif corner” and G4 biology