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Meeting report:
Seventh International Meeting on Quadruplex Nucleic Acids
(Changchun, P.R. China, September 6-9, 2019)

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Abstract:

DNA is prone to structural polymorphism: beyond the iconic Watson-Crick double helix, nucleic acids can adopt a number of unusual motifs, at least *in vitro*. Scientists around the world gather every two years to discuss two of these oddities: G-quadruplexes and i-DNA. The seventh international meeting on G-quadruplex Nucleic Acids was held in Changchun, in Jilin province of the P.R. of China, approx. 1000 km North-east of Beijing. Nearly 320 participants gathered from Asia, Europe, North America and Oceania. More than 80 talks and as many posters summarized our current knowledge of these unusual DNA and RNA structures. During this meeting, the creation of the *G4 society* was **announced**, in order to coordinate efforts and share tools and knowledge in our field (<https://www.g4-society.org>).

Keywords:

G-quadruplex; G-quartet; i-DNA; self-assembly; Drug-DNA recognition; antiproliferative agents

Glossary:

- a *DNAzyme* is a DNA-based enzyme. In the case of G-quadruplexes, the **hemin-G4** complex has interesting peroxidase activity.
- a *G-quadruplex* (G4): an unusual nucleic acid structure formed by G-rich DNA and RNA sequences and based on the formation of G-quartets.
- a *G-quartet* (also called *G-tetrad*) results from the coplanar association of four guanine bases.
- a *G4-ligand* is a compound able to recognize G-quadruplexes.
- i-DNA is an unusual DNA structure formed by cytosine-rich sequences based on the formation of C.C⁺ hemiprotonated base pairs. Stability is optimal under mild acidic conditions.

Foreword:

The International quadruplex meeting is now a well-established “routine” held every odd year. The first two editions (2007, 2009) were held in Louisville, KY, USA followed by Sorento (Italy) in 2011, Singapore in 2013, Bordeaux, France in 2015 and Prague, Czech Republic in 2017. People attending these conferences have different **backgrounds**, from modellers and chemists to cell biologists (**Figure 1** presents a group picture of all participants). The topics covered here reflect this diversity. I apologize in advance for not covering all talks, as parallel sessions prevented me from attending all lectures - I had to **rely** on friends, spies and informal chats to cover some of the missing talks!

1. Introduction:

Jonathan B. Chaires (U. Louisville, KY, USA) provided an overview and historical perspective of the quadruplex field in his inaugural lecture. As of August 2019, the quadruplex field gathers 8,467 articles and 253,174 citations in the Science Citation Index. Over 200 G4 structures are available in the PDB. Among institutions, The Chinese Academy of Sciences (CAS) provided the largest share of articles (620), followed by CNRS (426) and Inserm (313), two institutions in France. CAS leadership in the field illustrated how appropriate was the choice of P.R. China to hold this meeting. As Chaires mentioned “*G4 may be events not things*”, illustrating their dynamic behavior. Brad also introduced the initiative to create a G4 society, to coordinate efforts and share tools and knowledge in our field (<https://www.g4-society.org>). Following discussions with Bruce Armitage, the location of the next meeting (2021) was proposed to be Pittsburg, PA, USA, and this proposal met unanimous approval.

2. A paradise for structuralists!

Anh Tuan Phan (NTU, Singapore) gave a fascinating lecture on our understanding of quadruplex folding and recognition. He first demonstrated that there is an actual “zoo” of G4 structures, which can be formed by classical G4-forming sequences, as well as those challenging the classical definition of G4 and escaping the classical consensus, with bulges, missing guanines, long loops or stem loops. In particular, he described the folding of multiple variants of the well-known anti-proliferative DNA sequence AS1411. Each of these variants differs from the original sequence mainly by only one base substitution, yet they fold into very different topologies.^{1 2 3} Some robust motifs have emerged, including a 12-nt left-handed G4-forming motif^{4 5} and a compact 7-nt knot-like G-quadruplex peripheral motif involving a G.T.T triad and a G.T base-pair. He concluded that a small change in sequence can lead to large changes in structure (from right- to left-handed, for example). But the reverse is also true: a large change in sequence does not always change the fold. He then analyzed the interaction of quadruplexes with ligands, including small molecules (exemplified by a macrocyclic hexaoxazole and Phen-DC3) and proteins. Recognition is based on common principles: docking on the hydrophobic surface of a G-quartet and electrostatics, with the former defining the specificity and the latter enhancing the affinity. He illustrated that the quartet is the simplest solution for specific recognition. In the case of the Rhau protein (a helicase also known as DHX36), Rhau prefers parallel over antiparallel structures as the loops in the latter topology are hindering recognition. In that case, preferential binding results from the level of accessibility of the terminal tetrad which is recognized, rather than from the parallel topology itself.⁶

Janez Plavec (Ljubjana, Slovenia) also provided beautiful examples of quadruplex folds. The final topology is not always straightforward to predict. For example, the GGGTAGGGAGCGGGAGAGGG sequence derived from the RankL promoter, does not form a G4 structure with three quartets, but rather adopts a fold with two quartets and two base-triads. He demonstrated that the fifth base (A5; underlined above) is required for this unexpected strand slippage.⁷ He then analyzed the folding of GGGGCC repeats, showing that two similar but distinct antiparallel topologies may be observed. Folding is slow (several weeks) and reannealing (heating) is necessary to observe conversion between these two topologies. Finally, he presented data on G4 folding with sequences of four repeats of human telomeric repeat containing 8 oxo-G oxidation products at different positions.⁸

As noted by **Marta Szachniuk** (Poznan University of Technology, Poznan, Poland), the complexity of the G-quadruplex motif can be intimidating, especially for newcomers. She proposed an automated, computational procedure to annotate G4s in nucleic acid 3D structures and describe their features. Tetrads are assigned to one of the three so-called ONZ (Oval-Needle-Zigzag) classes.

3. Understanding folding:

Understanding the structural dynamics of G-quadruplexes under physiological conditions is of fundamental importance for the biology of these structures.

Harald Schwalbe (Johann Wolfgang Goethe-Universität, Frankfurt/M., Germany) illustrated how time-resolved NMR can be used to follow quadruplex folding.^{9,10} The reaction is triggered in the NMR tube itself with a rapid mixing device or by light from a laser. Potassium-induced G4 DNA folding is slow and biphasic, with the initial formation of the kinetic product. The final thermodynamically equilibrated state contains a mixture of folded G4 species and a partial population of unfolded state at shorter time points (≈ 90 minutes). Long lived intermediate states point to a kinetic partitioning folding mechanism. On the other hand, RNA folding is faster and monophasic. This is likely due to the absence of *syn* guanines in RNA strands, as shown by 2'F-ANA substitutions in DNA, which forces the nucleotides into an *anti* conformation and leads to acceleration of folding kinetics. He analyzed non canonical polymorphisms with sequences such as c-myc, in which runs of guanines are of unequal length (GGG and GGGG), creating stable substates with different G-registers. Transient conformational selection can be performed with a photocage of the imino proton side involved in Hoogsteen bond formation to trap a pure conformation: a photo-labile protection can be released with light, allowing one to follow G-register exchange mechanism, which proceeds by strand slippage.

Klaus Weisz (U. Greifswald, Germany) showed that the deliberate incorporation of guanosine analogs into G-quadruplexes allows for well defined refoldings into new G4 topologies and conformations. On the other hand, analysis of NMR high-resolution structures of the novel folds reveals interactions operative in G-quadruplex folding like unfavorable steric interactions of sugar substituents within the narrow groove or the formation of non-conventional CHF or CHO hydrogen bonds.¹¹

For **Naoki Sugimoto** (Konan U., Kobe, Japan), the “Watson-Crick world is not enough!”. Naoki illustrated how molecular crowding can destabilize duplexes but stabilize G4. He determined the thermodynamic parameters for duplex formation under crowding.¹² He presented results analyzing how replication can be affected by the resolving of the non

canonical structure, which is the rate determining step. Efficiency depends on stability but also on the type of structure formed: a hairpin can be opened in a stepwise fashion, while this is hardly the case for a G-quadruplex.¹³ Thanks to the microinjection of a fluorescent G4-forming oligonucleotide, he analyzed crowding in various organelles and concluded that the nucleolus behaves differently from the rest of nucleus.¹⁴

Understanding quadruplex formation is also important for RNA structure. **Jiang Zhou** (Peking University, Beijing, China) investigated the formation and structure characteristics of the G-quadruplex formed by a microRNA, miR-92a, using ESI-MS and CD. miR-92a, which is a potential biomarker for colorectal cancer, has a G-rich primary sequence. The residues involved in G4 formation were determined by systematic mutations.

Finally, a number of contributions analyzed the effect of loop sequence on quadruplex stability. For example, **Mingpan Cheng** (Nanjing U., China) demonstrated that loop permutations affect the topology and stability of G-quadruplexes¹⁵ as well as i-DNA.

4. Methods:

Gary N. Parkinson (University College London, UK) reported on the development of a new long wavelength synchrotron beam line resource (I23) at Diamond, UK, designed for the routine collection of SAD data on previously unavailable anomalous scattering atoms, such as the potassium ions bound in the central G-tetrad core. Tuning the beam line to these intrinsic scattering atoms has enabled Dr Parkinson, in collaboration with I23 staff, to validate the use of the new automated pipeline and determine the de novo structures of unusual DNA and RNA G-quadruplex topologies. It is now hoped that challenging crystallographic G4 structures with complex topologies can be routinely determined independent of previous anticipated topologies.¹⁶ **Alessandro Paciaroni** (Università di Perugia, Italy) illustrated how elastic neutron scattering experiments of G-quadruplex hydrated powders can provide information on hydration water surrounding nucleic acid structures.

Hanbin Mao (Kent State U., OH, USA) uses optical tweezers to evaluate G-quadruplexes from mechanical perspectives at the single molecule level. He was able to measure the mechanical stability of DNA G-quadruplexes at different angles, and found that the properties of DNA G-quadruplexes were anisotropic and could be altered by G4-ligands such as a telomestatin derivative.¹⁷

Shuntaro Takahashi (Konan U., Kobe, Japan) illustrated how pressure can affect the stability and recognition of G-quadruplexes. The stability of G-quadruplex is largely decreased by pressure (as compared to other DNA structures such as duplexes) indicating water release upon folding. Increases in pressure also affect G4 recognition by small ligands such as thiazole orange.¹⁸ **Xinhua Guo** (Jilin U., Changchun, China) reported how surface-enhanced Raman spectroscopy (SERS) can be used for obtaining secondary structural information of DNA quadruplexes, including i-DNA¹⁹ and G-quadruplexes.²⁰ Sensitive SERS signatures featuring the structures of G-quadruplexes and i-motifs may be obtained. **Guoqing Jia**, from Can Li's lab (Chinese Academy of Sciences, Dalian, China) showed that Raman Optical Activity spectroscopy, a new chiroptical method, can probe G-quadruplex DNA at the vibrational level, which provides information on topology (antiparallel or parallel) and allows one to distinguish local structural differences within similar antiparallel conformations. **Frédéric Rosu** (IECB, Pessac, France) presented an interesting model for the CD spectra of G-quadruplexes based on time-dependent density functional theory (TDDFT) calculations of

the stacking between two proximal G-quartets. The CD effect is due to the bases only, and there is no contribution from the solvent or the sugar-phosphate backbone to the signal. They were able to reproduce reference spectra provided that all 8 guanines are included in the calculations. The presence of a central cation was necessary to reproduce experimental CD for some topologies.

5. Applications to nano- and biotechnology:

G-quadruplexes and i-DNA are likely to be relevant for biology (see below), but may also be used for non biological applications in nanotech and biotech, thanks to their self-assembly properties²¹. The self-assembly of guanosine derivatives has been known for over a century, and GMP structure was established more than 50 years ago. Jeff Davis (Maryland, USA) described how supramolecular G-quadruplexes, assembled from 5'-modified guanosines are (i) scaffolds that catalyze photochemical reactions in solution and (ii) templates for writable-erasable hydrogels. Davis first highlighted a catalytic [2+2] photocycloaddition, triggered by G-quartet formation, that gave a single cyclobutane stereoisomer in high yield. He also described a 5'-hydroxamate G that forms G-quartet hydrogels with KCl. This G-quartet polymer is a supramolecular siderophore, forming red complexes by binding Fe³⁺. The hydrogel's surface could be patterned with a solution of FeCl₃, either by hand or with a 3D-printer. This rewritable hydrogel, combining self-assembled motifs from Nature (G₄•K⁺ quartet and siderophore•Fe³⁺ complex), is strong, transparent and moldable.²² Xiaodong Shi (University of South Florida, Tampa, FL, USA) developed a new class of guanosine derivatives that allows the formation of a discrete G8-octamer.²³

Itamar Willner (The Hebrew University of Jerusalem, Israel) presented a number of biotech- and nanotech- applications based on quadruplex formation, explaining how these structures can be used as functional units for sensing, catalysis and materials science.^{24,25} Hemin/G-quadruplexes synthesized by different DNA machineries can act as DNAzyme reporters for sensing. DNAzymes can be immobilized on electrodes, attached to quantum dots (allowing chemiluminescence resonance energy transfer, or CRET), to perform the multiplexed analysis of genes. Stimuli-responsive DNA-based hydrogels were designed with shape memory properties. Unusual DNA can be of interest in material sciences, thanks to its self-healing functions, drug uptake and release and mechanical functions. Finally, G4 can be combined with DNA origami for biocatalysis in confined nanoenvironments. Quadruplexes can also be used to design logic devices, as shown by Shuo Shi (Tongji University, Shanghai, China). Jinglin Fu (Rutgers University, Camden, USA) illustrated how DNA-mediated Proximity Assembly Circuits can be used for Actuating Biochemical Reactions. Proof of concept for DNA-mediated proximity assembly of an enzyme/cofactor pair was presented, based on a DNA hairpin-locked cofactor.²⁶

Huangxian Ju (Nanjing University, China) provided some examples of the involvement of quadruplexes in analytic sciences while Jun Zhou (Nanjing U., China) optimized DNAzyme activity based on primary sequence of the G4-prone DNA sequence. John D. Brennan (McMaster, Hamilton, Canada) illustrated an application of DNAzymes to develop a Colorimetric Paper Sensor for *Helicobacter pylori*. This system lays a foundation for developing DNAzyme-based simple devices as point-of-care diagnostics for monitoring pathogens in complex samples. Yingfu Li (McMaster U., Hamilton, Canada) showed that *in vitro* selection experiments often lead to the discovery of G-rich DNA molecules, which are prone to G-quadruplex formation. He recently discovered a G-rich DNA molecule capable of adopting a folded structure in 7 M urea. This molecule adopts an intramolecular all-parallel

guanine-quadruplex structure with 5 quartets²⁷ and can be programmed into a molecular switch for the detection of single nucleotide polymorphism in long DNA sequences in 7 M urea.²⁸

6. i-DNA formation and recognition:

Most of the talks during this meeting were dedicated to G-quadruplexes. Still, some presentations illustrated our **increased** understanding of the folding and recognition of the C-rich “other strand”. The i-motif is becoming fashionable again now that its formation has been demonstrated in cells by *in vivo* NMR (see next §) and by specific antibodies.²⁹ **Daniel Renciu**k (IBP, Brno, Czech Republic) is interested in the sequence determinants of i-DNA stability.³⁰ He presented an analysis of the complex melting of C-rich sequences containing long runs (5 or more) of cytosines. **Jun Zhou** (U. Nanjing, China) analyzed the stability of a number of different i-DNA sequences in order to understand the rules governing i-motif formation. **Byeang Hyeon Kim** (Pohang University of Science and Technology, Korea) discussed fluorescent probes for monitoring the i-motif transition. Finally, evidence for i-DNA formation in cells will be discussed in the next **section**.

7. Evidence for quadruplex (G4 and/or i-motif) formation in cells:

Lukas Trantirek (CEITEC, Brno, Czech Republic) presented results showing that exogenous DNA fragments (< 50 nt) can be directly probed by in-cell NMR after electroporation into human cells. Endogenous nucleic acids do not contribute to the in-cell NMR spectrum, as genomic DNA is too big (and thus invisible in solution NMR), small RNA are not abundant enough (they are below NMR detection limit) and exchangeable protons are not visible in isolated nucleotides. Strand concentrations of 10-15 μ M can be reached, and DNA quickly accumulates in the nucleus. Interestingly, he could follow the thermal denaturation and renaturation of i-DNA in cells³¹ (Imino protons are visible at 20°C, disappear when the cells are heated to 40°C and become visible again when the solution is cooled down). In parallel, NMR measurements in crude cellular homogenates can be made. This allowed **Dr. Trantirek** to conclude that the human telomeric G-quadruplex does not adopt a parallel fold in cell-like setting, and that polyethylene glycol (PEG200), one of the most commonly used mimics of cellular molecular crowding, does reliably emulate molecular crowding in cells. Notably, not only isolated DNA can be studied, but **drug-DNA** complexes can be electroporated into cells, allowing to study the impact of the intracellular environment on the integrity of the complexes.³²

Prince Kumar Lat from Dipankar Sen’s lab **at** Simon Fraser University (Barnaby, Canada) presented his latest data on RNA and DNA G4 auto-biotinylation pulldown strategy (involving bound heme and biotin-tyramide), in trial *in vitro* experiments as well as from living cells. He showed that the method works very well (sensitively as well as specifically) *in vitro*, and he presented his early *in vivo* data. Using a biotin blot (like a Northern blot), he has now figured out the cell and tissue culture conditions optimal for this approach, both with mammalian cell lines and freshly dissected *Drosophila* salivary glands. He is on the verge of obtaining (hopefully G4) DNA and RNA sequences that he has already pulled down from live cells using this strategy. Meanwhile, preliminary microscopy of *Drosophila* salivary glands shows strong G4 signals **s** in nuclei and especially nucleoli.

Zheng Tan (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) argued that some researchers are still not fully convinced of the presence of G4 in living cells. To probe

G4 formation, his group designed an engineered 11kDa G4-binding protein (patent pending). This protein selectively binds *in vitro* to all kinds of G4, with Kd values varying from 0.3 to 3.1 nM. He then performed G4-ChIP analysis in human A549 cells as well as mouse and chicken cells. Reads were found to be enriched near TSS; the protein was found to bind to non canonical G4 containing bulges, G-vacancies or long loops. The main conclusions were that (i) G4 form in the large majority of genes; (ii) G4 are associated with ssDNA; (iii) G4 formation is correlated with negative supercoiling; (iv) G4 forms in open chromatin; (v) G4 formation is anti-correlated with cytosine methylation in A549 and HeLa cells and (vi) correlated with histone modification in HeLa cells.

8. AS1411 and other G-rich oligonucleotides

Dihua Shangguan (Institute of Chemistry, Chinese Academy of Sciences, Beijing, China) addressed the mechanism of the anti-proliferation effect of G-rich oligonucleotides (GRO). The toxicity of GROs was mainly contributed by the selective anti-proliferation effect of guanine-based degradation products, such as dGMP, deoxyguanosine and guanine. He revealed that guanine deaminase (GDA) was a major factor in determining the cell-type sensitivity to the guanine-based molecules. The GDA-deficient cells could not efficiently remove the excess guanine-based molecules in cells, resulting in metabolic disturbance of other types of nucleotides, subsequently resulting in DNA synthesis inhibition and cell apoptosis/death. DNA synthesis could be restarted by post addition of adenine- and cytosine-based nucleosides, subsequently reduced the anti-proliferation effect of guanine-based molecules.³³

9. G-quadruplex ligands:

Over the years, a number of G4 ligands have been developed. It was therefore important to report the latest progress made in this field. Some of the compounds described during this conference are presented in **Figure 2**. **Valérie Gabelica** (IECB, Pessac, France) revisited thermal denaturation experiments by monitoring with mass spectrometry the evolution of the stoichiometry of K⁺ and ligand complexes as a function of temperature. We learned that slipped-strand structures are entropically favored compared to perfectly aligned quadruplexes. Mass spectrometry also revealed counterintuitive phenomena, such as the appearance of new stoichiometries at high temperature. The binding of selective G4 ligands (PhenDC3, TrisQ) is also entropically favored, and this raises the point that relying on thermal denaturation for a first ligand screening may have selected entropy-favored binding modes.³⁴ The presentation clearly illustrated the complementarity of mass spectrometry with other thermodynamic and structural approaches.

Hiroshi Sugiyama (Kyoto University, Japan) developed cyclic imidazole/lysine polyamides, cIKP, as a new class of G4 ligands. They designed hybrid molecules that were constructed with dual DNA-binding components, a cyclic imidazole/lysine polyamide (cIKP) and a hairpin pyrrole/imidazole polyamide (hPIP), with the aim of specific quadruplex targeting by reading out the local duplex DNA sequence adjacent to designated quadruplexes in the genome.³⁵ **Peter Scott** (U. Warwick, Coventry, UK) introduced optically pure peptide-mimetic metallohelices as G4 ligands. These self-assembling multimetallic coordination compounds exhibit antimicrobial properties. **Xiaogang Qu's** group showed several posters about G4 ligands, especially chiral metal triplex metallohelices. They found that the Δ-enantiomer binds to D-DNA while the Λ-enantiomer binds to L-DNA G-quadruplexes, and exhibit nearly identical stabilization effects and binding affinities.³⁶ Furthermore, they found

that one enantiomer of chiral metallohelices, NiP, rather than NiM, has the ability to predominantly reduce cell viability in breast CSCs compared to the bulk cancer cells.³⁷

Chunyang Cao (Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China) presented two ligands interacting with the G-quadruplex found in the oncogene RET. One ligand is a natural product, COL, which has a different scaffold, and the other is the commonly used berberine molecule. NMR analysis revealed some interesting characteristics. For example, COL was found to interact with a specific residue of the RET G-quadruplex, while keeping the G4 melting temperatures nearly unchanged.

Two communications illustrated the use of fluorescence lifetime imaging microscopy (FLIM) to visualize G4 ligands in cells. **Peter A. Summers** (Imperial College, London, UK) focussed his efforts on the design of fluorescence lifetime-based probes which are concentration independent and can be used for FLIM in live cells. G4 specific binding is not a prerequisite for imaging, as the probe can be measured in both G4 and dsDNA environments. This allows for the displacement of the probes (from G4 to dsDNA) by a strong G4 ligand such as pyridostatin to be monitored in real time. **Ta-Chau Chang** (Academia Sinica, Taipei, Taiwan) presented time-gated FLIM experiments with a G-quadruplex (G4) fluorescent probe, 3,6-bis(1-methyl-2-vinylpyridinium) carbazole diiodide (o-BMVC). This provides an objective method to screen human cancer cells based on a large contrast in the number of o-BMVC foci between cancer cells and normal cells.³⁸ Although o-BMVC foci were only tested in 6 cancer and 3 normal cell lines together with 4 clinical human cancers of head and neck, thyroid, pleural effusion, and colon cancers, these preliminary results lay the foundation for the hypothesis of o-BMVC foci as a common signature of cancer cells. To examine whether the o-BMVC foci are the G4 foci in cells, Prof. Chang will attempt to isolate o-BMVC foci by using laser micro-dissection. Further studies for verifying the presence of G4 structure by NMR, determining the DNA sequence by gene sequencing, and identifying the surrounding proteins by mass analysis from isolated o-BMVC foci will be conducted for better understanding G4 formation and biological function. In addition, a high-throughput method was developed for the initial screening of G4 binding ligands in cells.³⁹

Quadruplex imaging in live cells was the topic of a number of talks. **Zhou Nie** (Hunan University, Changsha, China) reported a new benzothiazole-based G4-targeted fluorescence light-up probe for the direct visualization of the native RNA genome of HCV in living host cells.⁴⁰ **Fangwei Shao** (Zhejiang University, China) designed a light-up probe to visualize RNA G-quadruplexes in live cells, based on Pt(bis(1,10-phenanthroline-2-yl)amine) complexes. Interesting differences in both the quantity and lifetime were found between normal and cancer cells. **Hongxia Sun** (from Yalin Tang's lab in the Chinese Academy of Sciences, Beijing, China) designed and synthesized a series of fluorescent probes for G-quadruplex recognition and imaging in live cells,^{41, 42} leading evaluation method for intracellular quadruplex-targeting ligands.^{43,44}

Danzhou Yang (Purdue University) found that a medicinal natural product epiberberine specifically recognizes the hybrid-2 telomeric G4 and converts other telomeric G4 forms to hybrid-2.⁴⁵ She determined the structure of the EPI-hTelG4 complex in K⁺ solution by NMR showing an extensive four-layer binding pocket of EPI specific to the hybrid-2 telomeric G4. She also determined the solution structures of the 1:1 and dimeric 4:2 complexes of a Pt-tripod with the hybrid-1 human telomeric G-quadruplex, in collaboration with **Zong-Wan Mao** (Sun Yat-sen Univ, Guangzhou, China). The unprecedented dimeric 4:2 structure is interlocked by a non-canonical A:A pair at the 3'-end. The specific and strong binding of the

Pt-tripod is achieved by the non-planar tertiary amine conformation and three properly sized cationic platinum arms which interact with most of the loops and flankings. These studies provide important insights into understanding the dynamic binding of small molecules to G-quadruplexes and a structural basis for future rational anticancer drug design of non-planar platinum compounds targeting G-quadruplexes.⁴⁶ In addition, D. Yang uncovers that clinically-tested anticancer indenoisoquinolines strongly bind and stabilize the MYC promoter G-quadruplex and lower MYC expression levels in cancer cells, and suggests that dual targeting of MYC G4 and topoisomerase I may serve as a novel strategy for anticancer drug development.⁴⁷

Claudia Sissi (University of Padova, Italy) is interested in targeting a different oncogene promoter, the Epidermal Growth Factor Receptor (EGFR). The sequence located ≈ 272 bases upstream the transcription start site is highly polymorphic. The G-rich strand folds into two main G-quadruplexes whereas the C-strand can either form a hairpin or an i-DNA structure depending on pH.^{48,49} Selective targeting of this region is therefore challenging. However all these arrangements have a conserved small stem-loop element in one loop formed at the same site on the two strands. Beside representing a potential enucleation site for the structural rearrangement of the double helix, small molecules were found to be able to target it. **Subhrangsu Chatterjee** (Bose institute, Kolkata, India) is interested in two evolutionary consensus G-quadruplex motifs found in tandem upstream of the Mitogen-activated protein kinase (MAPK12) promoter, and synthesized naphthelenediimide derivatives that bind to the corresponding quadruplexes. These compounds induce apoptotic death in cancer cells via the RAS-MAPK pathway.

Marie-Paule Teulade-Fichou (Institut Curie, Orsay, France) is interested in how G4-ligands may affect protein-G4 interactions. She showed that the PhenDC3 compound decreased the binding of the nucleolin protein to G-quadruplexes, while other compounds (PDS, Pt-tpy, RHPS4) had no effect. She also investigated the interaction between G4 RNA and the EBNA1 protein from Epstein-Barr virus, and screened for molecules which would disrupt this interaction: novel PDC analogs from the PhenDH series were found to be active.⁵⁰

Zhishu Huang's group (Sun Yat-sen Univ, Guangzhou, China) has developed a number of different scaffolds for G4 ligands, including quindolines, berberine and acridines. An emphasis was put on imidazoles and isaindigotones. In particular, she showed that (i) one can visualize DNA G4 in cells with ISCH-1, which binds to rDNA; (ii) one can recognize telomeric multimeric G4 with IZNP-1;⁵¹ (iii) one can track RNA G4 with the QUMA-1 compound⁵² with similar accuracy as an antibody and that (iii) the IZCZ-3 G4 ligand is specific for the c-myc quadruplex.⁵³ This compound does not inhibit other genes (kit, Hras, PDGFA) and is effective in mice.

Mojgan Dajvaheri-Mergny (CRC, Paris, France), using a wide set of analysis methods including transcriptomic and proteomic analyses, cell imaging and biochemical studies, reported that the triarylpyridine G4 ligand 20A promotes senescence in cancer cells through Ataxia Telangiectasia Mutated (ATM) and autophagy activation. Inhibition of both pathways directs cells to apoptosis, positioning ATM/autophagy axis as a linchpin between senescence and apoptosis.⁵⁴ From a therapeutic point of view, inhibiting the ATM/autophagy pathway impairs senescence and drives cells to apoptotic cell death, which may offer a promising strategy to optimize the potential therapeutic efficacy of G4 ligands in cancers.

Hong Xu (UBC, Vancouver, Canada) demonstrated that the CX-5461 compound selectively kills cells with are BRCA deficient, and can be used to targets BRCA-deficient cancers.⁵⁵ An increase in telomere defects and an induction of DNA damage is observed upon treatment. DNA damage is replication dependent, and repaired by the BRCA pathway. CX-5461 was found to be more effective than a PARP inhibitor in a number of breast cell lines. This molecule is active in a mouse PDX model and kills BRCA-deficient cells. Deficiency in NHEJ increased sensitivity to this ligand. Phase I clinical trials were recently initiated with this molecule; a total of 20 patients were enrolled. This molecule appeared well tolerated, with preventable photosensitivity; one patient obtained a partial remission with a 67% reduction of the tumor burden.

10. Quadruplex-binding proteins:

Both **Marie-Paule Teulade-Fichou** (Institut Curie, Orsay, France) and **Luying Chen** from the Danzhou Yang group (Purdue, USA) analyzed binding of nucleolin to G4, for which contradictory results have been reported. They both observed that a long central loop was necessary for optimal binding, and little or no binding was found when all loops were short. The Teulade-Fichou group used crosslinking coupled with LC MS/MS analysis to conclude that the RBD2 domain of nucleolin was necessary for binding.

Yinsheng Wang (University of California Riverside, USA) employed a quantitative proteomic workflow together with biotin affinity pull-down for discovering novel G4-binding proteins. Three different quadruplexes were tested (derived from human telomere and promoters of *cMYC* and *cKIT* genes), and only a few proteins bind to all three G4 sequences. One of them is YY1, a well-studied transcription factor, which has K_d values for G4 structures of 21 nM (for *cKIT* promoter sequence) and 81 nM (for telomere sequence). Binding is mediated via its zinc finger motif, and PDS and TMPyP4 both disrupt the interaction of this protein with the G4. YY1ChIP-Seq peaks partially overlap with BG4 ChIP peaks (40%). As YY1 is known to form dimers, the authors performed a proximity ligation assay to show that YY1 can also promote ligation between two distant G4, and between G4 and double-stranded DNA with a YY1-binding motif, where the YY1-G4 DNA interaction is also involved with DNA looping. The authors also uncovered another function of the SRA stem-loop-interacting RNA-binding protein (SLIRP), which binds to G4 structure and interacts with the majority of the human helicase proteome; the latter facilitates 2'-O-methylation rRNA and promotes protein translation.⁵⁶⁵⁷

Alessio De Magis (Bonn, Germany), from the Paeschke lab, described how G4-interactive protein screening allowed the identification of Zuo1, a multimeric protein with cytosolic and nuclear functions. Zuo1 is recruited to G4 sites shown by ChIP-seq. By ChIP with the BG4 antibody, he observed a decrease in G4 content in cells when Zuo1 is knocked-out. Interestingly, a specific G4 ligand, Phen-DC3, was able to rescue zuo1delta growth defects. As Zuo1Δ cells are sensitive to DNA damage agents, he proposed a model in which Zuo1 supports the NER pathway at G4 sites.

G4-binding proteins are not only relevant for DNA, but also for RNA. **Jean-Pierre Perreault** (U. Sherbrooke, QC, Canada) is interested in quadruplex motifs in RNA molecules. He presented results on the RNA G-quadruplex formed at the 5'-end of the 5'-UTR of the BCL2-associated athanogene 1 (BAG-1) mRNA. The BAG-1 protein is overexpressed in colorectal cancer; its regulation is complex and translation of the mRNA can be both cap-dependent and independent. He showed that G4 formation can contribute to the regulation of different

translation mechanisms: the RNA quadruplex acts as a steric block which controls the initiation of translation at each start codon of the transcript (at least three are present) and maintains the global 5'UTR secondary structure required for IRES-dependent translation.⁵⁸

Daisuke Miyoshi (Konan U., Kobe, Japan) found that G4-RNA – protein interactions are critical for liquid-liquid phase separation, a phenomenon essential in biology. Stress granules containing G4 RNAs and ribonucleoproteins were formed in MCF-7 cells after photodynamic therapy treatment.⁵⁹

11. Helicases, repetitive motifs and genomic stability:

Danzhou Yang (Purdue University) discovered that DDX5 DEAD-box RNA helicase proficiently unfolds MycG4-DNA and activates MYC transcription in cancer cells. Her results show that DDX5 is a highly active DNA and RNA G4-resolvase that does not require a single-stranded overhang in an ATP-independent manner.⁶⁰

Ximiao Hou (Northwest A&F University, China) used single molecule FRET (smFRET) to show that Pif1 unfolds G4 structures in the sequential and repetitive manner. Her team systematically addressed Pif1-mediated unwinding of various G4s and found that Pif1 is sensitive to G4 stability and topology, as Pif1 preferentially unfolds antiparallel over parallel quadruplexes and cannot unfold G4 with short (single-nt) loops such as (GGGT) repeats.⁶¹

Alain Nicolas (Institut Curie, Paris, France) presented ChIP-seq results showing that G4-dependent genome instability of the human minisatellite CEB1 in yeast is associated with the extensive accumulation of ssDNA (RP-A ChIP) during replication of the leading strand template. **He next** reported an extensive analysis of the occurrence of thermodynamically stable G4 motifs with short loops (G4L1) across 495 species genomes. Remarkably, the G4-L1 loop composition exhibits distinctive trends, leading to the preferential accumulation of the least stable G4-L1A in vertebrates but G4-L1G in other eukaryotes. It suggests negative selection against the most stable G4 motifs, thus neutralizing their detrimental effects on genome stability while preserving the less stable G4 motifs to be associated with biological regulatory roles.⁶²

12. Quadruplexes and telomerase:

Telomerase has long been the target of choice for G-quadruplex ligands. The rationale behind this strategy was that if the ligand could lock the telomerase substrate (the G-rich telomeric overhang) into a G-quadruplex conformation, it would no longer be extended by this enzyme. Hundreds of compounds were developed to that aim, but no single telomerase inhibitor (G4-based or otherwise) has been FDA-approved so far. In addition, the popular TRAP assay is inappropriate for G4-ligands and cannot be used directly to gauge inhibition.

The laboratory of **Tracy M. Bryan** (Children's Medical Research Institute, Australia) has demonstrated that, while antiparallel & hybrid G4 cannot be extended by telomerase, parallel topologies (intra- or inter-molecular) are good substrates for both ciliate and human telomerase. Now, in collaboration with Antoine van Oijen (University of Wollongong, Australia) they have analyzed parallel G4 interaction with telomerase using single-molecule FRET, to explore the mechanism by which telomerase binds and unfolds G4. Unwinding requires telomerase activity and the RNA template region of the telomerase component is also

important for this process. Furthermore, G4-stabilizing ligands do not always inhibit this process.

13. Quadruplexes in different species:

Chun Kit Kwok (City Univ. of Hong Kong, China) discussed the formation of RNA G-quadruplexes in plants, using SHALiPE probing⁶³ of RNA G4. This method allows probing of G4 that really forms *in vivo* in the whole plant. He found out that (i) hundreds of folded rG4 exist in plants; (ii) rG4 can be enhanced by cold and (iii) rG4 regulate plant development.

Ilaria Frasson from the Richter's lab (University of Padua, Italy) analyzed G-quadruplex propensity in human viruses. They found that the occurrence and location of potential quadruplex-forming sequences were characteristic features of each human virus class and family. They also provided evidence of the existence of quadruplex interacting proteins and of the possibility to target quadruplexes with small molecules in HIV-1 and HSV-1 viruses.⁶⁴

Mateo Nadai, also from the Richter's lab, is focussing his efforts on targeting the G4-prone LTR region of the HIV-1 virus with tetrasubstituted naphthalene diimide derivatives. These compounds exhibit good antiviral activity with IC₅₀ values in the nanomolar range. **Kyeong Kyu Kim** (Sungkyunkwan University School of Medicine, Suwon, Republic of Korea) is specifically interested in quadruplexes present in the gene promoter regions of the human cytomegalovirus genome.⁶⁵ In this study, 36 putative G4-forming sequences were identified in the promoter regions of 20 genes in the 235-kb genome. However, only 9 out of 20 genes were affected by G4 formation in their expression. In addition, promoter regulation by G4 was confirmed in UL35 in the context of virus infection.

In bacteria, **Akhilesh Mishra** from the Jayaram lab (Indian Institute of Technology Delhi, India) analyzed the possible role of G-quadruplexes in antigenic variation in *Neisseria gonorrhoeae*. They analyzed $\approx 1,100$ promoters and found a potential G4-forming motif just upstream of the LTA synthase gene, an important gene for growth of this pathogenic bacteria.

14. G-quadruplexes and R-loops:

R-loop formation results from RNA invasion of a DNA duplex, leading to the formation of a DNA/RNA hybrid. R-loops and G4 are compatible in terms of sequence – both being favored by GC skewness – and G4 formation is possible in the displaced DNA strand of an R-loop. Cooperation between these two structure has been proposed in yeasts and prokaryotes.

Giovanni Capranico (U. Bologna, Italy) not only demonstrated that G-quadruplex ligands stabilize G4s but also that they simultaneously increase R-loop levels in human cancer cells.⁶⁶ This increase is observed within minutes; G4 ligands cause a spreading of R-loops towards G4-prone adjacent regions. The finding that the effects of G4-ligands is mediated at least in part by R-loop formation is an important observation, since R-loop induction was found with three different G4 ligands (Pyridostatin, Braco-19 and FG) belonging to different chemical families. Interestingly, differences were found in the biological outcomes of R-loop induction between these compounds. In addition, R-loop induction mediated DNA damage induced by these compounds. Overall, these findings emphasize the interplay between R-loops and G-quadruplexes, and establishes a model of action of G4 ligands in cancer cells.

15. Awards:

The meeting awards were sponsored by the Royal Society of Chemistry and Journal of Materials Chemistry B. Besides 10 poster awards, four researchers received a prize for their oral presentations. **Jun Zhou** (Nanjing University, China) described position effects in G-quadruplex and i-DNA structures, and how these effects can modulate G4 functions such as DNAzyme.⁶⁷ **Sunny Y. Yang** (The University of British Columbia, Canada) from the Wong lab developed an approach called G4RP-seq to identify native transiently folded G4-RNA on a transcriptome-wide scale from living human cells.⁶⁸ G4RP-Seq is based on G4-RNA-specific precipitation using a biotinylated version of a G4-specific ligand developed by David Monchaud's lab.⁶⁹ **Fuan Wang** (Wuhan University, China) described self-catabolic DNAzyme nanosponges used for smart anticancer drug delivery. **Li Zhou** (Guangzhou University, China) presented a new concept of supramolecular co-assembly between guanosine monophosphate (GMP) and an azobenzene intercalator.⁷⁰ The resulting fluorescent crystals can be photoreversibly disassembled/reassembled upon UV/blue irradiation.

16. Closing remarks:

Jean-Louis Mergny (Institut Curie, Orsay, France) pointed out that quadruplexes are still full of surprises, and much remains to be done, both *in vitro* and in cells. He insisted on the need to have the proper tools for quadruplex studies available to all. This includes ligands (several high affinity selective compounds are commercially available), antibodies (both against G4 and i-DNA), databases (for example for G4 ligands or RNA G4) and prediction algorithms such as G4-Hunter.⁷¹ He noticed that the human telomeric motif and AS1411 sequences are among the most studied DNA G4 structures: from a structural point of view, they are also the most complicated. **AGGGA repeats, which have been recently reported to be involved in a genetic disease, also form quadruplexes.** For cellular studies, he promoted the use of “G4-poor” model organisms such as *Dictyostelium discoideum*⁷² or *Plasmodium falciparum*. The lower density of quadruplex-prone motifs should facilitate the deconvolution of biological effects.⁷³ Regarding G4 ligands, he argued that “more is not always better”⁷⁴: high affinity is not always compatible with high selectivity! In addition, G4 ligands should not be considered as simple transcriptional inhibitors, as a number of genes can be upregulated in the presence of such compounds.

17. Conclusions:

Regular meetings between researchers interested in these DNA and RNA oddities are always welcome. A number of burning questions remain on the roles and effects of quadruplexes both for biotech/nanotech applications and in biology. All participants embraced the perspective of the eighth meeting being held in Pittsburgh, PA, in 2021 with great enthusiasm. We hope to see there a few friends and colleagues who could not make it **to this meeting** (some flights were cancelled between Beijing and Changchun). In the meantime, information will be shared in the G4-society website (<https://www.g4-society.org>), and initiatives for local G4 gatherings are more than welcome. **Maybe** now is the time to rejuvenate the initiative to write guidelines!

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Figure Legends:



Figure 1: The official picture of all participants (courtesy of Kai Dong) taken in front of the Changchun Institute of Applied Chemistry of the Chinese Academy of Sciences.

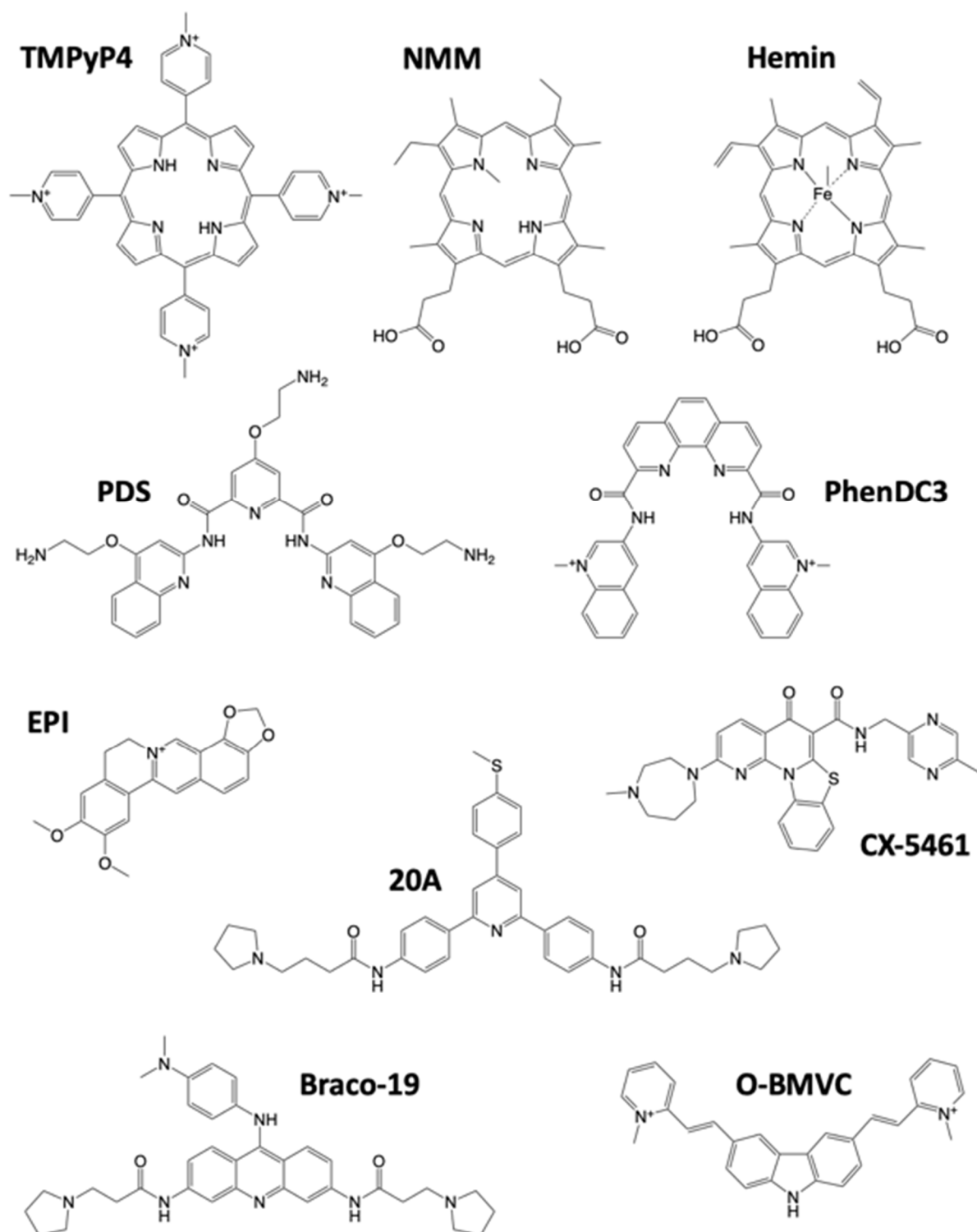


Figure 2: The usual suspects: some of the G-quadruplex ligands discussed during this conference. TMPyP4 is a tetracationic porphyrin with high affinity but limited specificity for G-quadruplexes. NMM stands for N-methyl mesoporphyrin IX. Hemin, as an iron-containing porphyrin endogenously produced in the human body, is a natural G4 ligand. PDS corresponds to Pyridostatin. CX-5461 is in phase I clinical trial for patients with BRCA1/2 deficient tumours (Canadian trial NCT02719977). EPI is epiberberine while 20A is based on a triphenylpyridine scaffold. Braco-19 is a trisubstituted acridine derivative. O-BMVC is a G-quadruplex fluorescent probe. Many of these compounds are commercially available.



Figure 3: The Changchun organisation team.

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