Small Molecule Stabilization of the *CARD11* G-quadruplex Represses Transcription: Developing a Therapeutic Target for Diffuse Large B Cell Lymphoma

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***Abstract*— Diffuse large B cell lymphoma (DLBCL) involves abnormal B cell growth in the lymphatic system. Abnormalities such as recurrent genetic mutations cause critical components of the BCR signaling pathway to be overactive. This constitutes an oncogenic defect that drives uncontrolled B cell growth. Caspase recruitment domain-containing protein 11 (CARD11) is a critical BCR pathway scaffold protein and its recurrent gain-of-function mutations are frequently found in DLBCL. This study aims to investigate the potential of *CARD11* gene silencing as a therapeutic for DLBCL treatment by targeting the DNA secondary structure- G-quadruplexes (G4s) formed within the gene, as G4s usually act as physical barriers to gene expression. Using circular dichroism (CD), stable G4's were identified within the highly guanine-rich promoter region. Small molecules were screened using a fluorescence-resonance energy transfer (FRET) assay to identify compounds that stabilize G4 structures. To quantify the effects of G4 stabilization on gene expression, qPCR was then used to determine that stabilization of G4s led to repression of transcription and subsequent reduction in mRNA levels of the oncogene *CARD11* and a few others,, which are crucial for DLBCL progression These findings highlight that stabilizing the G-quadruplex structures formed in the *CARD11* promoter region could inhibit DLBCL growth by silencing *CARD11* gene expression and downstream oncogenic signals in the BCR pathway.**

I. Introduction

Diffuse large B cell lymphoma (DLBCL) is a highly aggressive and heterogeneous subtype of non-Hodgkin lymphoma, characterized by the uncontrolled growth of abnormal B cells in the lymphatic system. DLBCL represents the most common form of non-Hodgkin lymphoma, accounting for approximately 30-40% of cases [1,5]. Despite significant advancements in DLBCL treatment, a substantial 60% of DLBCL patients still remain refractory to the standard combinatory treatment R-CHOP (*rituximab*, *cyclophosphamide*, *doxorubicin*, *vincristine*, and *prednisone*), or experience relapse after achieving initial remission [1,4,7].

The B cell receptor (BCR) signaling pathway plays a crucial role in DLBCL development and progression [6]. Upon encountering specific antigens, B cells activate their BCRs, leading to intracellular signaling cascades that promote cell survival and proliferation [1,2,3]. In Activated B cell (ABC) DLBCL, this pathway is constitutively active due to genetic mutations in other key component proteins of the BCR signaling pathway, such as *BCL2* and *MYC*, resulting in enhanced BCR signaling independent of antigen binding [3,6]. Upon activation, BCR sends signals downstream through a multi-protein complex that in turns activates other signaling pathways like the NFκB pathway, which is critical for cell survival and proliferation. The aberrant propagation of survival signals from uncontrolled BCR signaling is a key factor promoting the initiation and progression of DLBCL [1].

The present research focuses on the CARD11 protein, an important component of the multi-protein signaling complex. Since *CARD11* sits at a critical signaling node and its mutations play a vital role in driving lymphoma growth [1,2,4], it represents a promising therapeutic target. Specifically, silencing *CARD11* could overcome a central DLBCL growth mechanism by cutting off key survival signals at their origin - the mutated CARD11 protein itself.

Stabilized G-quadruplex structures (G4s), which are guanine-rich DNA or RNA sequences folded into stacked tetrads, within the *CARD11* gene promoter (Figure 1) likely downregulate *CARD11* and other oncogenes by physically blocking transcriptional machinery and interfering with regulatory elements, leading to reduced gene expression. These double stranded DNA secondary structures are extremely stable and can inhibit gene expression when localized to promoter regions, acting as roadblocks to RNA polymerases and other proteins attempting to bind. By screening a library of small molecules for the ability to selectively bind to and stabilize G4 structures, this innovative approach aims to silence the expression of genes involved in the development of DLBCL, offering a promising avenue for developing novel therapeutic strategies for aggressive DLBCL.

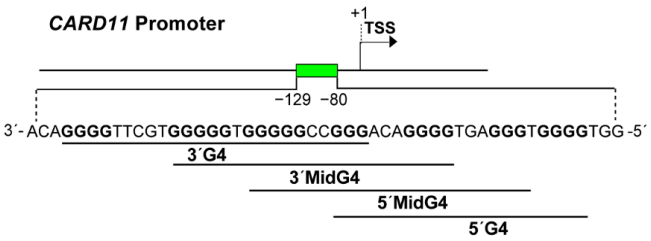

Figure 1. The *CARD11* promoter has potential G4-forming motifs near the transcription start site.

II. Materials and Methods

A. Oligonucleotides

Oligos from IDT and Eurofins were used for circular dichroism (CD) and fluorescence-resonance energy transfer (FRET) experiments. Their concentrations were determined by absorbance measurements at 360 nm using a nanodrop spectrophotometer and calculating the molar absorptivity (ε) via the Beer-Lambert law. Both CD and FRET samples were stored at 4°C.

B. CD Spectroscopy Analysis

We tested G4 formation in *CARD11* oligos using CD with varying KCl concentrations. Previous research has

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revealed that potassium ions stabilize G4 structures, so varying potassium chloride concentrations would allow us to determine the optimal stability conditions of G4 and analyze the G4 folding dependence on potassium levels (Figure 2). Oligos were heated and cooled, and CD spectra data was collected at 263 nm using a Jasco-1100 spectropolarimeter. Data was baseline-corrected, smoothed using the Savitzky-Golay method, and thermal stability was then assessed by increasing the temperature by 1°C/min while recording molar ellipticities, allowing T_m determination within ±1°C using Prism software.

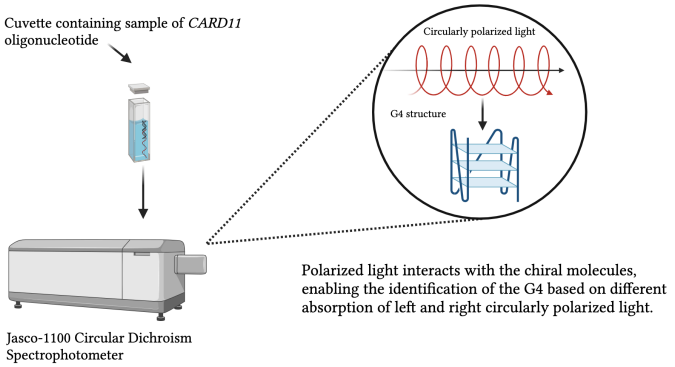


Figure 2. Visual representation of CD methodology to identify G4 structures within the *CARD11* oligonucleotide samples.

C. *Small Molecule Screening*

Custom Fluorescence Resonance Energy Transfer (FRET) probes were created to enable assessment of G4 stability changes induced by small molecules, requiring custom design to target the specific *CARD11* gene sequence. Probes were prepared with 8 small molecules at 40 µM in Dimethyl sulfoxide (DMSO) 16% mixed with FAM/TAMRA-labeled *CARD11* probes in 10mM NaCacodylate and 20mM KCl buffers to provide appropriate salt conditions known to facilitate G-quadruplex formation while enabling testing of the small molecule conditions. The probes underwent gentle centrifugation at 2000 rpm to mix the components while avoiding disruption of structure. Transferal to 96-well plates enabled high-throughput and controlled testing of multiple small molecule conditions simultaneously. The FRET assay was performed at 95°C, slightly below melting temperature, to potently evaluate stability modulations detectable by FRET signal changes. Data normalization on a 0-1 scale enabled standardized comparison across probes and conditions for comprehensive analysis. Curve fitting analysis was utilized to determine the logIC50 values for each small molecule, quantifying the molar concentration required to inhibit 50% of G-quadruplex structure stability. Comparison of the logIC50 values provided key insights into whether the small molecules preferentially disrupt or stabilize G4 structures, based on their binding affinity and potency.

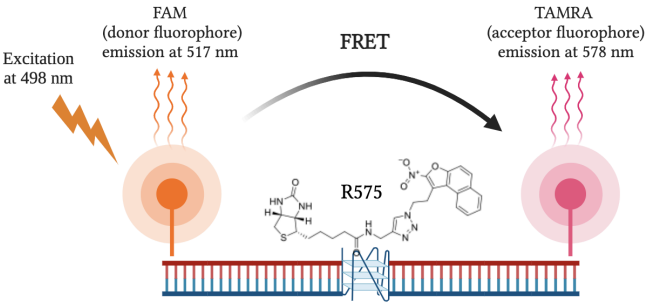


Figure 3. Visual representation of the FRET methodology to test for G4 stabilizing small molecules. R575 used as an example small molecule. In the FRET assay, the G-quadruplex folds upon cooling, bringing the FAM and TAMRA tags on the *CARD11* DNA together, while R575 binding further stabilizes the structure - keeping the tags consistently in close proximity and enabling ongoing energy transfer between them.

D. *Human Cell Lines*

The cell lines RIVA, HBL1, VAL, SUDHL6, and GM16113 from ATCC and DSMZ were stored in RPMI 1640 media with 10% FBS and 1% penicillin-streptomycin. They were grown at 37°C with 5% CO_2. Using these diverse DLBCL cell lines allows the testing of small molecule stabilization of the *CARD11* G4 on malignant cells from varying origins across patients (see Table 1).

Cell line	Cell line origin (biopsy status)
RIVA	Peripheral blood
HBL1	Pleural effusion
VAL	Bone marrow
SUDHL6	Peritoneal effusion
GM16113	Peripheral vein
GM22673	Peripheral vein

Table 1. Cell lines used alongside their biopsy origins.

E. *Gene Expression Measured Using qPCR*

Lymphoma cell lines were treated with small molecules 9037 or R575 to measure relative gene expression. TaqMan probes for genes including *CARD11* were used *in vitro* for this qPCR experiment. Master mixes were prepared with added cDNA for the qPCR. The cycle threshold (Ct) values were normalized to housekeeping gene TBP and compared to untreated controls, yielding ΔΔCt values. The relative gene expression changes were assessed based on these values.

F. *Statistical Analysis*

Data analysis was conducted using GraphPad Prism Software v 9.94. All experiments were performed in triplicate to ensure statistical significance and data

reliability. GraphPad Prism facilitated descriptive statistics, t-tests, ANOVA, and non-linear regression analyses. Visualizations, such as bar graphs and scatter plots, aided in interpreting trends and relationships within the data.

III. Results

The guanine-rich promoter oligonucleotide of *CARD11* displayed distinctive G4 CD peaks at ~260 nm (Figure 4A). Additionally, both these peaks and the oligonucleotides' melting temperatures were affected by the presence of a K+ gradient (Figures 4A-B). Consequently, the *CARD11* exhibits a Guanine rich sequence (3-4 runs of G consecutively), which is consistent with G4 formation (Figure 1). As the concentration of KCl increases, the molar ellipticity decreases, indicating a decrease in the stability of the G4 structures formed by the *CARD11* gene (Figure 4A). The peak at ~ 260 nm with 100mM KCl exhibits the highest molar ellipticity, suggesting that the G4 structure is most stable at this concentration (Figure 4A). As the KCl concentration decreases, the T_m values also decrease, indicating lower stability of the G4 structures, further demonstrating that G4 is most stable at 100mM KCl (Figure 4B).

FRET analysis was performed with the non-interactive small molecule 9037, and we see that 9037 decreased G4 stability in *CARD11*, *BCL2*, and *MYC* genes - confirming its non-stabilizing nature (Figure 4C). To further demonstrate 9037 as a non-interactive small molecule, qPCR was performed to reveal that 9037 did not significantly lower the fold change values in the key gene's expression while in comparison to the vehicle treatment control (VTC) of DMSO at 16% (see Figure 4D). Thus, in subsequent experiments, 9037 was used as a negative control to validate that the screening methodology could differentiate when a small molecule does not stabilize or silence *CARD11* G-quadruplex structures, in contrast to the positive impacts of stabilizing small molecules identified by the screen.

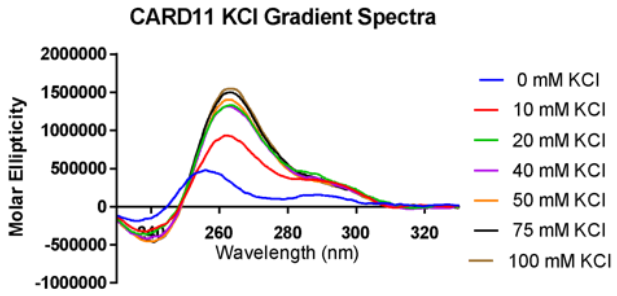


Figure 4A. Upstream G-rich sequence forms a K+-influenced G4 structure, confirmed by circular dichroism peak at ~260 nm.

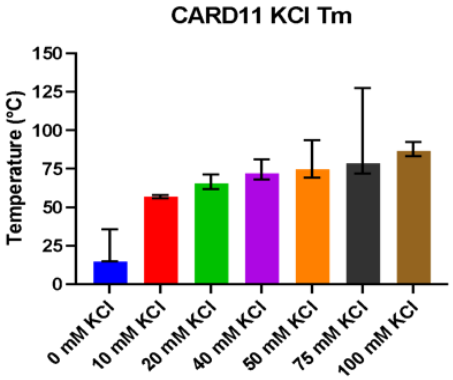
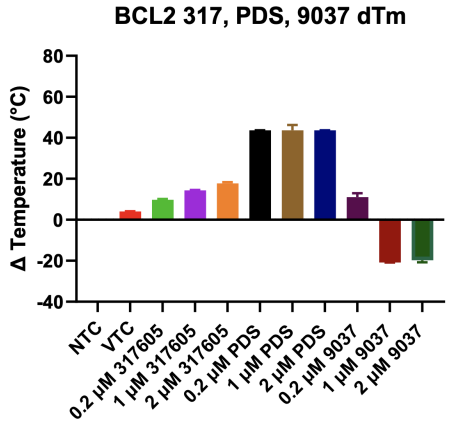
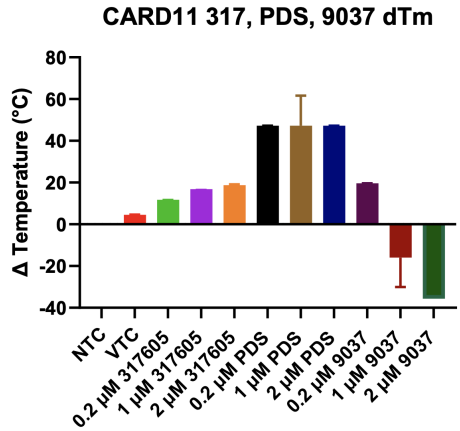


Figure 4B. Corresponding T_m values graphed.





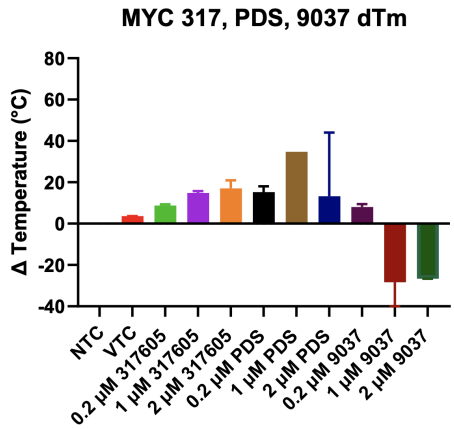


Figure 4C. FRET analysis compared the 9037 impacts (on key oncogenes alongside *CARD11*) to Puromycin Dihydrochloride Salt (PDS, 317605, NTC, and vehicle control: VTC (DMSO 16%).

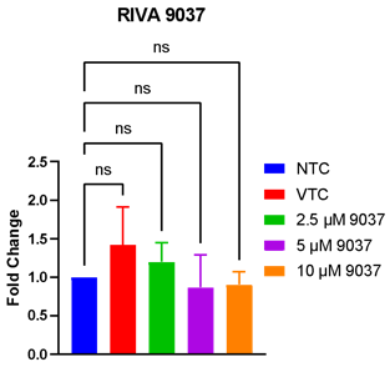


Figure 4D. Fold change *CARD11* mRNA with qPCR data with 9037 tested on RIVA cell line.

FRET assay revealed small molecules R575, 311153, 147481, and 317605 significantly increase G4 Tm, reinforcing structural stability. Small molecules 13248 and 309401 also stabilize G4 but to a lesser extent. Differences arise from diverse chemical structures, binding affinities, and interactions with *CARD11* promoter's G4 motifs. Molecular forces, functional groups, and configurations impact binding.

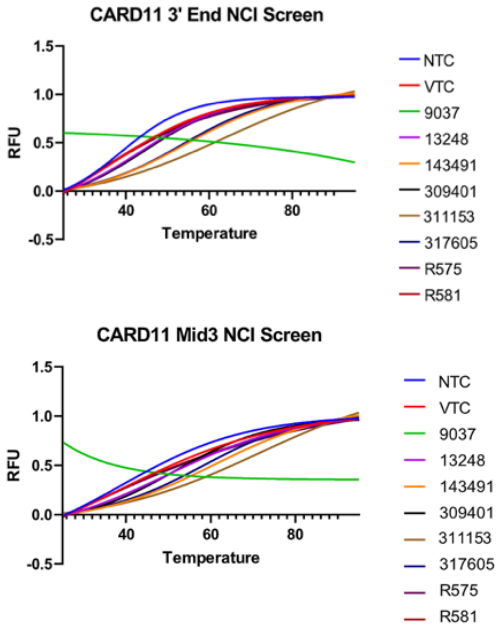
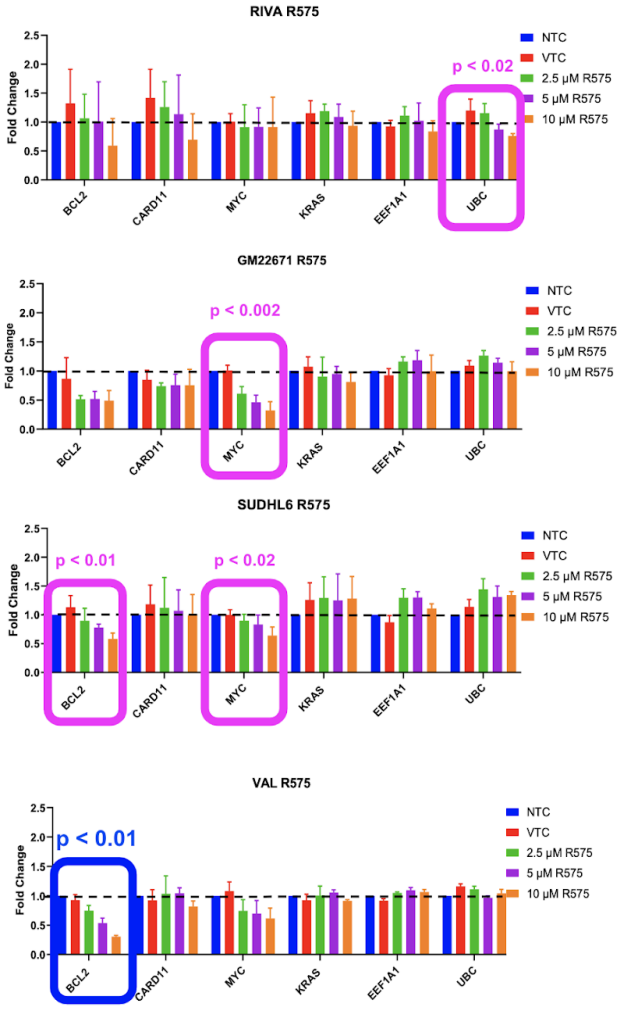


Figure 5. FRET melt curves obtained for small molecules compared to probe + 16% DMSO (vehicle control) at 2.5 µM concentration. Shifts rightwards indicate Tm increase ≥ 4°C, identifying potential candidates. FRET screen used probes in 10 mM NaCac and 20 mM KCl buffer, mimicking the *CARD11* gene's G4-forming conditions.

According to the FRET analysis, small molecule R575 enabled the FAM and TAMRA tagged *CARD11* DNA sequence to retain a consistent 0.7 raw fluorescent unit (RFU) energy transfer plateau from 60°C up to the highest tested temperature of 100°C, without exhibiting the rapid signal decrease that indicates structure melting seen for other small molecules (Figure 5). We thus conclude that R575 allows for stabilized G4 rigidity and continued folding across an expanded temperature range. Small molecule R575 was then investigated for its impact on genes *KRAS* and *TERT* (which contain G4 structures), and *UBC* and *EEF1A1* (which do not contain a G4 structure). In the RIVA cell line, derived from peripheral blood, 10 µM of R575 led to significant downregulation of *BCL2* and *CARD11* genes, indicating effective silencing (top panel, Figure 6). This suggests R575's potential to modulate *CARD11* gene expression, crucial in DLBCL progression. Similarly, the VAL cell line, derived from bone marrow, showed reduced *BCL2* expression with R575 (middle panel, Figure 6). Cell line GM16113, derived from peripheral vein, also experienced silencing effects on *BCL2, CARD11*, and *MYC* genes with R575 (bottom panel, figure 6).



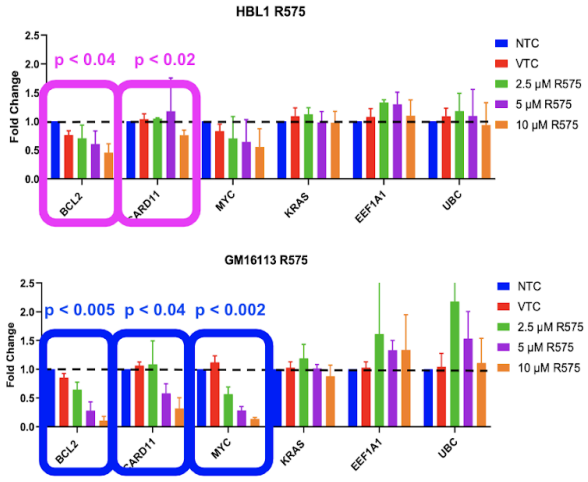


Figure 6. qPCR results for genes *BCL2*, *CARD11*, *MYC*, *MYD88*, *KRAS*, *TERT*, *UBC*, and *EEF1A1* in the RIVA (A), VAL (B), and GM116113 (C) cell lines with varied concentrations of small molecule R575. Boxed experiments show dose-dependent reduced levels of gene expression using ANOVA (purple) or two-sample t-tests (VTS vs. highest dose, pink) statistical analyses. Figures generated by student researcher.

IV. Conclusions

This study demonstrates the potential of targeting G-quadruplex structures within the *CARD11* promoter as a therapeutic strategy for DLBCL. We investigated G-quadruplexes forming in the *CARD11* gene using circular dichroism, identifying stable structures that can act as gene expression barriers. Through screening, the study found a small molecule R575 that substantially stabilizes G4. As stabilized G4 leads to gene silencing, there is no surprise that gene expression analyses revealed that R575 consistently downregulates *CARD11* across DLBCL cell lines, indicating potential for modulating oncogenic BCR signaling. We thus propose G4 in the *CARD11* promoter as a prospective therapeutic target for silencing *CARD11* and disrupting downstream signaling in DLBCL. Small molecule stabilization of these structures shows promises for precise, personalized treatment strategies. Further translational research on G4-targeted therapeutics *in vivo* is warranted to validate and optimize findings toward improved patient outcomes. This innovative approach signifies a potential paradigm shift towards direct DNA/RNA targeting to limit translation of key oncoproteins driving aggressive DLBCL growth.

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References

[1] Swafford K., Acharya, B., Xu, Y.Z., Raney, T., McCrury, M., Saha, D., Frett, B., & Kendrick, S. Targeting a Novel G-Quadruplex in the *CARD11* Oncogene Promoter with Naptho(2,1-b)furan-1-ethanol,2-nitro- Requires the Nitro Group.Genes,13(7),1144 https://doi.org/10.3390/genes13071144 (2022).

[2] Candelaria, M., & Dueñas-Gonzalez, A. Rituximab in combination with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) in diffuse large B-cell lymphoma. *Therapeutic advances in hematology*, *12*, 2040620721989579 (2021). https://doi.org/10.1177/2040620721989579

[3] Kendrick, S., Muranyi, A., Gokhale, V., Hurley, L. H., & Rimsza, L. M. Simultaneous Drug Targeting of the Promoter MYC G-Quadruplex and BCL2 i-Motif in Diffuse Large B-Cell Lymphoma Delays Tumor Growth. *Journal of medicinal chemistry*, *60*(15), 6587–6597. https://doi.org/10.1021/acs.jmedchem.7b00298 (2017).

[4] Davis, R. E. et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature 463, 88-92, doi:10.1038/nature08638 (2010).

[5] Testoni, M., Zucca, E., Young, K. H. & Bertoni, F. Genetic lesions in diffuse large B-cell lymphomas. Ann Oncol 26, 1069-1080, doi:10.1093/annonc/mdv019 (2015).

[6] Davis, R. E. et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature 463, 88-92, doi:10.1038/nature08638 (2010).

[7] De Cian, A., Guittat, L., Kaiser, M., Saccà, B., Amrane, S., Bourdoncle, A., Alberti, P., Teulade-Fichou, M. P., Lacroix, L., & Mergny, J. L. Fluorescence-based melting assays for studying quadruplex ligands. *Methods (San Diego, Calif.)*, *42*(2), 183–195. https://doi.org/10.1016/j.ymeth.2006.10.004 (2007)