Demonstration that Drug-targeted Down-regulation of MYC in Non-Hodgkins Lymphoma Is Directly Mediated through the Promoter G-quadruplex

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Most transcription of the MYC proto-oncogene initiates in the near upstream promoter, within which lies the nuclease hypersensitive element (NHE) III₁ region containing the CT-element. This dynamic stretch of DNA can form at least three different topologies: single-stranded DNA, double-stranded DNA, or higher order secondary structures that silence transcription. In the current report, we identify the ellipticine analog GQC-05 (NSC338258) as a high affinity, potent, and selective stabilizer of the MYC G-quadruplex (G4). In cells, GQC-05 induced cytotoxicity with corresponding decreased transcriptional factors binding to the NHE III₁ region, in agreement with a G₄ stabilizing compound. We further describe a unique feature of the Burkitt’s lymphoma cell line CA46 that allowed us to clearly demonstrate the mechanism and location of action of GQC-05 within this region of DNA and through the G₄. Most importantly, these data present, as far as we are aware, the most direct evidence of intracellular G₄-mediated control of a particular promoter.

The MYC proto-oncogene is a key component of normal cell growth and differentiation, with roles in a multitude of cellular processes. Normally, this gene is subject to tight transcriptional regulation; however, aberrant MYC expression is a common feature in an estimated 80% of all human malignancies (1–3); it is estimated that one-seventh of cancer deaths in the United States are associated with alterations in the MYC gene or its expression (4). Derepression can arise through a variety of mechanisms (5–13), but most often MYC is activated through alterations in cell signaling that lead to increased transcription (14).

Deregulated MYC can lead to transformation (15), often as an early step in the process of multistage cancer development, and one on which all other mutations are based (16, 17). Cancer cells appear to be addicted to a deregulated MYC (18), which can be the “Achilles heel”, offering the potential for a therapeutic window (19, 20). The ability to selectively and potently down-regulate MYC would have considerable potential for both efficacy and safety in a variety of tumor types.

There are several upstream elements within the MYC promoter that can potentially undergo strand separation to form either single-stranded or other non-B-DNA structures (21), which play a critical role in transcriptional control of MYC: the distant Far Upstream Element acts as a cruise control element, Z-DNA found both in the far upstream and the promoter regions, and a GC-rich region within the proximal promoter that acts as an on/off switch (22–28). This near upstream core promoter region, which is responsible for the initiation of 80–90% of MYC transcription (29), contains the GC-rich nuclease hypersensitive element (NHE) III₁ to which double-stranded (Sp1) and single-stranded (CNBP and hnRNP k) transcriptional factors bind. Within the MYC gene’s NHE III₁, a 31-base pair element consisting of five repeats of the sequence (C/T)(C/T)TCCCCA serves as the “on/off switch” for MYC transcription (21). This region has been shown to adopt a G-quadruplex (G₄) on the purine-rich strand in single-stranded DNA, under negative supercoiled conditions, and putatively in cells (30–32). These higher order structures are four-stranded intramolecular foldings of single-stranded DNA, formed by the stacking of two or more tetrads, each comprising four guanines connected by Hoogsteen hydrogen bonds and stabilized with monovalent cations. G₄s are globular in nature, analogous to the tertiary structure of a protein, and they serve as the silencer element for MYC transcription, putatively through sequestration of transcriptional factor binding sites (33–35).

In the present study we identified the ellipticine derivative GQC-05 (NSC338258) as a potent and selective stabilizer of the MYC G₄ structure. We demonstrate that the activity of this compound is dependent on the presence and regulation of a G₄ within the MYC promoter region and that it specifically modulates protein binding to the NHE III₁. We go on to describe a new intracellular test, the “CA46 test” that is of enormous benefit to the study of the MYC NHE III₁ and, in particular, for demonstrating specific MYC G₄ drug targeting within this region. Finally, we discuss our results in relation to cellular targeting of MYC, which, from a therapeutic standpoint, has important implications.

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2 The abbreviations used are: NHE, nuclease hypersensitive element; BL, Burkitt’s lymphoma; SPR, surface plasmon resonance.
MYC Regulation through G4 in Euchromatin

EXPERIMENTAL PROCEDURES

Ex Vivo Compound Characterization—Compounds identified for testing from a pharmacophore query were screened for thermal stabilization using FRET with dual-labeled FAM-TAMRA Pu47, as previously described (36); compounds were obtained from the NCI/DTP Open Chemical Repository (Rockville, MD). GQC-Qi was synthesized in house (supplemental materials). Competition dialysis was performed as reported (37), and allowed to equilibrate for 48 h at 4 °C. CD spectra and thermal stability of MYC Pu47 (5 μM) in the absence and presence of GQC-05 were recorded on a J-810 spectropolarimeter (Jasco, Easton, MD), as previously described (38). Surface plasmon resonance experiments were performed on a Biacore T100 biosensor with immobilized, biotinylated MYC Pu41 on a streptavidin CM5 sensor chip.

Cells, Cellular Viability, and Caspase Activity—Cells were maintained in a 37 °C, 5% CO2 incubator, in exponential growth, for determination of experiment in RPMI-1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cytotoxicity of GQC-05 in RAJI and CA46 cells (ATCC, Manassas, VA) was determined using the MTS assay with compound diluted over a 5–6 log range in 0.5 log steps; IC50s were determined with GraphPad Prism software using non-linear regression modeling. Caspase-3 activation was determined using the ApoAlert Caspase-3 APOPTOSIS kit (Clontech, Mountain View, CA).

qRT-PCR, Western Blotting, and ChIP—RAJI and CA46 were seeded at 1 × 106 cells/ml in at least 4 ml overnight before incubation with compounds at described doses for the ascribed times. Cells were collected, washed with PBS, and RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA) using the Qiacube automated system. Up to 500 ng of cDNA was reverse transcribed with the Quantitect cDNA synthesis kit (Qiagen) prior to quantitative real-time PCR detection on the Bio-Rad MyIQ thermocycler. FAM-labeled TaqMan primers were obtained from Applied Biosystems (Carlsbad, CA) for GAPDH and MYC (exon 1: 01562521_m1; exon 2: 00153408_m1); the ΔΔCt method was used to calculate changes in expression, normalized to the appropriate time-matched DMSO vehicle-treated control cells.

ChIP analyses were carried out using a modified EZ-ChIP kit (Millipore, Billerica, MA) from 5 × 106 cells/IP/treatment. At each time point queried, cells were collected, crosslinked, and sonicated for 45 cycles of 30 s on/30 s off at a maintained temperature of 4 °C until DNA was 300–500 bp (supplemental Fig. S1). ChIP-quality antibodies for each protein of interest were purchased from AbCam (Cambridge, MA) or Millipore; DNA was purified using the QiaQuick PCR purification kit (Qiagen) before 2× dilution and quantitation with qRT-PCR, as described above. Custom TaqMan primers to the NHE III1 region of the MYC promoter and the 3′-UTR of the MYC promoter were designed and purchased from ABI; amplification was detected with the RotorGene Q PCR Detection System (Qiagen).

RESULTS

Identification of the GQC-05 as a MYC G4 Binding Compound

Quindoline derivative i (39) (GQC-Qi) was reported as a MYC G4-stabilizing compound. The compounds NSC176327 and NSC86374 (Fig. 1A) were identified in a high-throughput FRET screen for interaction with, and stabilization of, the MYC G4. They demonstrated ΔTm values of 21 and 17 °C at 1 equivalence, respectively, as measured by circular dichroism (CD). Energy-minimized conformations of the compounds were superimposed to generate a pharmacophore query (Fig. 1B) from which ten additional compounds were identified for further study (Table I). These were screened using the FRET melt assay at concentrations spanning a 2-log range (0.1–10 μM). ΔTm values ranged from 0 to 21 °C, with GQC-05 (Fig. 1C) demonstrating the highest and most robust thermal stabilization of the MYC G4. Consequently, this compound was chosen for further examination.

Validation of GQC-05 as a MYC G4 Binding Compound

CD and Competition Dialysis—To validate the structure stabilized by GQC-05 within the MYC sequence as a G4, CD spectra were recorded in the absence and presence of increasing equivalence of GQC-05 (Fig. 2A). A concentration-dependent stabilization of the positive parallel peak (262 nm) was observed up to 2 equivalents, above which a plateau of molecular ellipticity was noted (Fig. 2A, inset). Thus the ligand:DNA stoichiometry was determined to be 2:1, and thermal stabilization was measured only up to 2 equivalents. GQC-05 stabilized the MYC G4 structure by 3.5 and 7.4 °C at 1 and 2 equivalents, respectively.

Affinity for other promoter G4 sequences, chosen to represent a variety of G4 structures including parallel, mixed, equilibrating, and tandem/more complex varieties (40), and double-stranded (ds) DNA complexes was measured using the competition dialysis assay (Fig. 2B). Concentrations bound per putative binding site ranged from 0.5 to 4.38 μM for all G4 sequences and from 0 to 0.75 μM for all dsDNA. GQC-05 demonstrated the greatest concentration bound to the MYC G4, for which, against its own promoter sequence dsDNA, a 45-fold preference was observed.

Surface Plasmon Resonance (SPR)—SPR was utilized to determine the equilibrium binding constants for GQC-05; GQC-Qi was used as a reference compound. Binding curves for both compounds were fitted to a two-binding-site model, with χ2 values of 0.25 and 2.5, respectively (Fig. 3). GQC-05 exhibits tighter binding with MYC G4 as compared with GQC-Qi with 10-fold lower equilibrium constants (Kd). First site binding constant (Kd1) values were determined to be 0.1 ± 0.01 μM and 1.14 ± 0.25 μM for GQC-05 and GQC-Qi, respectively. For both GQC-05 and GQC-Qi, the second binding sites show ~10-fold lower affinity than the first site, with Kd2 values = 1.43 ± 0.07 μM and 17.8 ± 2.9, respectively. There is a notable
difference in dissociation of GQC-05, as compared with GQC-Qi, in that GQC-05 demonstrates slower dissociation. Overall, there was an observed negative cooperativity between the first and second binding sites for both compounds, with calculated CF values <1 for each compound.

**Cellular Studies in Burkitt’s Lymphoma Cell Lines**

**GQC-05 Shows Higher Cytotoxicity in RAJI, as Compared with CA46, Cells**—A translocation between an immunoglobin chain, in this case the IgH heavy chain that resides on chromosome 14, and the MYC promoter on chromosome 8 defines Burkitt’s lymphoma (BL) and results in aberrant control and up-regulation of MYC expression. For the intracellular characterization of GQC-05, a pair of BL cell lines was used where the translocation leads to the loss of the G4 region in the CA46 cell line, but not in RAJI cell line (Fig. 4A). If GQC-05 were mediating any intracellular effects through stabilization of this structure and modulation of MYC expression, it is expected that the CA46 cells will be considerably less sensitive than the RAJI cells, and indeed that is the case. At 24 h the IC_{50} values of GQC-05 revealed the RAJI cells to be more than 5-fold more sensitive than the CA46 cells (2.6 ± 0.9 \textit{versus} 13 ± 4 \mu M, respectively). For all further experiments, these 24 h IC_{50} concentrations were used to relate any intracellular effects to the inhibitory concentrations.

We further examined stimulation of apoptosis with a caspase-3 activation assay; 50 \mu M doxorubicin served as a positive control. For both RAJI and CA46 cells, doxorubicin and GQC-05 (at the respective 24 h IC_{50} dose) each caused a significant and time-dependent increase in caspase-3 activation (Fig. 4B). There was approximately twice the activation of the apoptosis cascade in the RAJI, \textit{versus} the CA46, cells with GQC-05, despite the concentration used being 5-fold less. In compari-

### TABLE 1

| NSC number | Compound class\(^a\) | Compound | \(\Delta T_m\) (°C) | 10 | 1 | 0.1 |
|------------|----------------------|----------|-------------------|---|---|---|
| 311150     | E                    | GQC-01   | 19                | 1 | 0 |
| 651556     | E                    | GQC-02   | 0                 | 1 | 1 |
| 649091     | E                    | GQC-03   | 7                 | 0 | 1 |
| 79486      | E                    | GQC-04   | 0                 | 1 | 0 |
| 338258     | E                    | GQC-05   | 21                | 5 | 1 |
| 100594     | E                    | GQC-06   | 2                 | 1 | 1 |
| 24037      | NE                   | GQC-07   | 1                 | 1 | 1 |
| 65238      | NE                   | GQC-08   | 0                 | 0 | 0 |
| 118176     | NE                   | GQC-09   | 17                | 1 | 0 |
| 86372      | NE                   | GQC-10   | 12                | 3 | 1 |

\(^a\) E, ellipticine derivative; NE, non-ellipticine derivative.
To localize the action of GQC-05 to the G4-forming region of the MYC promoter in RAJI cells, a Chromatin Immuno-precipitation (ChIP) assay for various transcription factors (the dsDNA binding transcription factor Sp1, the ssDNA binding transcription factors CNBP (G-rich strand) and hnRNP k (C-rich strand)) and regulatory proteins (the G4 resolving protein NM23-H2, and the stabilizing protein nucleolin) was performed over time (Fig. 4D). Since a significant decrease in mRNA was noted within 6 h of treatment with GQC-05 and continued through 48 h, and to observe changes in protein binding to the MYC promoter region that correspond with transcriptional regulation, the ChIP assay was performed 6 and 12 h post-GQC-05.

There was no significant change in RNA Pol II binding to the NHE III1 region of the MYC promoter over time, as compared with the vehicle control-treated cells. In contrast, within 6 h of treatment with GQC-05, hnRNP k was significantly decreased, and CNBP was significantly increased. By 12 h post-GQC-05, the binding of all transcription factors and G4 regulatory proteins was significantly decreased (Fig. 4D).

An Exon-specific Effect of GQC-05 Demonstrates the G4 Is the Direct Molecular Target in CA46—To further characterize the mechanism of action of GQC-05, as it relates to the MYC G4, CA46 cells were re-examined for an “exon-specific” effect due to the translocation event. The BL reciprocal translocation t(8; 14) maintains G4-mediated control of MYC on both chromosomes in RAJI, but only on the non-translocated (NT) chromosome in CA46. Using primers specific to exons 1 and 2, the NT and T mRNA products, respectively, of the CA46 cell line can be examined independently (Fig. 5A). Remarkably, GQC-05 significantly decreased MYC mRNA expression from the NT (exon 1), but not the T (exon 2), allele in the CA46 cells. As a comparison, in the RAJI cells, where both exons remain under G4 control irrespective of translocation status, there are no exon-specific effects noted (Fig. 5B). The previously published quindoline derivative i compound (GQC-Qi) was also evaluated in this CA46 test at 10 μM for 24 h (39), as was 50 μM TMPyP4 and 50 μM doxorubicin. With GQC-Qi, both exon products were significantly decreased from vehicle control ($p < 0.0001$) to 15 ± 3 and 12 ± 4% for the NT and T alleles, respectively, with no apparent exon specificity. TMPyP4 hinted at an early exon-specific effect (64 versus 87% for NT and T alleles, respectively, at 6 h), but through 24 h there was no change in MYC expression with either allele. Doxorubicin has been noted to also decrease MYC expression, but is not ascribed to G4 stabilization; in agreement, there was no significant difference in expression from T and NT alleles 24 h post-treatment with 18 ± 10 and 40 ± 20%, respectively.

GQC-05 Modulates Proteins Binding to the NHE III1 Region of the MYC Promoter in the NT Allele of the CA46 Cells—In the CA46 cells, a ChIP assay for RNA Pol II, Sp1, CNBP, hnRNP k, NM23-H2, and nucleolin was again performed over time (Fig. 5C). Expression of the NT allele was determined first over a time course post-GQC-05, which demonstrated a significant decrease in expression from the NT and T alleles 24 h post-treatment with 18 ± 10 and 40 ± 20%, respectively.

GQC-05 Modulates Proteins Binding to the NHE III1—Compound GQC-05 was identified as ex vivo as interactive with the MYC promoter G4 and demonstrated low micromolar cytotoxicity in BL cell lines in vitro. To tie these two results together, both RAJI and CA46 cells were treated with their respective 24 h IC50 concentrations of GQC-05, and changes in MYC mRNA expression were examined over time (Fig. 4C). In the CA46 cell line, where the activating translocation removes the G4 from the promoter, there is no significant decrease in expression. In comparison, within 6 h there is a robust and significant decrease in MYC expression in the RAJI cells, maintained through 48 h.
A similar pattern of altered protein binding was observed to that seen in the RAJI cells such that no significant change in RNA Pol II binding to the NHE III1 region was found in the CA46 cells promoter over time. Within 6 h of treatment with GQC-05, only hnRNP K was significantly decreased, which remained bound less to the NHE III1 region through 12 h. By this later time point, binding of Sp1, CNBP, NM23-H2, and nucleolin was significantly decreased (Fig. 5). As a negative control, amplification of the 3'-UTR region of the MYC gene was tried for all IPs in both cell lines; no amplification occurred.

GQC-05 Preferential Effect on the MYC Promoter—To determine specificity for the MYC promoter, the regulation of other 'target mRNAs' was monitored over time in CA46 cells, including genes with demonstrated G4s in their promoters (Bcl-2, HIF1α, and hTERT) and with no G4 regulatory element (BAX). CA46 cells were chosen to minimize the effect of MYC regulation itself, since there is no global change in MYC expression in these cells (Fig. 4C). Of those with G4s in their promoters, particular genes were chosen based on the competition dialysis assay in order to represent those with moderately high (Bcl-2), moderate (HIF1α), and low (hTERT) binding of GQC-05, as well as a variation of G4 shapes including mixed, parallel, and tandem. There was no change in mRNA expression, as compared with DMSO control, 6 h post-treatment, versus G4-regulated MYC expression was significantly lowered at this time. There were significant increases in expression noted for both HIF1α (1.4-fold, p < 0.01) and hTERT (1.76-fold, p < 0.05), but not in the G4-containing Bcl-2 or the G4-lacking BAX, at 24 h (supplemental Fig. S2).

Finally, to be assured that changes noted in ChIP experiments were not attributed to altered protein levels, changes in expression of Sp1, CNBP, hnRNP K, Nucleolin, and NM23-H2 were evaluated by Western blot at 6 and 24 h post GQC-05 treatment in CA46 cells (Fig. 5D). The only hint of a modulation in protein expression is a slight, but apparent, decrease in NM23-H2 expression at 24 h. By ChIP, there is no change in NM23-H2 binding to the NHE III1 region at 6 h.

Thus, in both BL cell lines, within a time frame corresponding to altered mRNA expression, significant changes in protein binding, but not expression, to the NHE III1 region of the MYC promoter confirm the location of action of GQC-05 to be within this area and further support the compound’s mechanism of action to be stabilization of the G4 structure. Moreover, there is a demonstrated preferential binding and decreased expression of the MYC gene, over any others examined with G4-regulatory agents.

DISCUSSION

MYC was recently described as the conductor of a symphony of intracellular events with direct or indirect roles in transcription, miRNA regulation, ribosome biogenesis, glycolysis, metabolism, angiogenesis, repression of transcription, pluripotency, and more (41). With MYC’s role in so many hallmarks of
cancer, and growth related events, it is easy to understand why its deregulation is found so frequently as either a causative oncogenic event, or distinctly tied to prognosis and therapy. It is also something to which cancer cells often seem addicted, providing a window of opportunity for new anticancer therapies (15). Specific targeting of protein function has yet to be a successful avenue of drug development. Because the primary method of deregulation is transcriptional overexpression, via various mechanisms (42), targeting transcriptional control is likely to be the most efficacious, with potentially the best selectivity over normal cells due to the increased transcriptional firing in cancer cells. There are many protein regulators of MYC transcription, too many to target collectively, thus the best emerging target is the nascent promoter DNA and its alternate topologies.

**FIGURE 4.** In vitro activity of GQC-05 in BL. A, key reciprocal translocation in the BL cell lines between the IgH heavy chain gene on chromosome 14 and the MYC gene on chromosome 8. CA46 loses the G-quadruplex-mediated control of MYC due to this translocation, while RAJI maintains it. B, 50 μM doxorubicin (DOX) and 2.6 (RAJI) or 13 (CA46) μM GQC-05 cause significant activation of caspase-3 and the apoptotic pathway at 24 h (black bars) and 48 h (white bars). Activation is normalized to DMSO, and one-way ANOVA analyses are between each treatment group and the relevant vehicle control: “Control” for DOX and DMSO for GQC-05. Experiments at each time point are in triplicate, with at least duplicate measurements of caspase-3 activation for each set. †, p < 0.05 as compared with control; ‡, p < 0.05 over time; *, p < 0.05 as compared with DMSO vehicle control. C, MYC mRNA is decreased over time in the RAJI cells, but not the CA46 cells. Data are normalized to DMSO vehicle control (white bars), and no treatment control (black bars) is unaltered in each cell line. GQC-05 (blue bars) is significantly decreased by 6 h, and through 48 h, in the RAJI cell line. Experiments at each time point are in triplicate, with triplicate qRT-PCR measurements. *, p < 0.05, as compared with DMSO vehicle control. D, binding of transcription factors (left) and secondary structure modifying proteins (right) was examined by ChIP and detected by qRT-PCR amplification of the NHE III1 region, over time post-GQC-05 treatment (6 h, black bars; 12 h, white bars). Six hours post-GQC-05, the binding of hnRNP k was significantly decreased and CNBP was significantly increased. Binding of all other proteins, with the exception of RNA Pol II, were significantly decreased by 12 h. Data represent duplicate ChIP experiments, each with technical triplicates of qRT-PCR; *, p < 0.05 as compared with DMSO vehicle controls.
the NHE III1, to which several transcriptional factors bind. This region of DNA also has a propensity to undergo strand separation to form non-B-DNA topologies, through the intermediary of ssDNA, all forms of which play a critical role in transcriptional control of MYC (29). The dynamic interplay between the DNA isomers and regulatory proteins is described in Refs. 15, 29 (Fig. 6).

Targeting MYC through Its G4 Structure—An early study examined the stabilization of the MYC G4 with the cationic porphyrin TMPyP4, which decreased luciferase expression in a wild-type vector. Using the RAMOS and CA46 pair of BL cell lines, we showed that TMPyP4 lowers MYC expression in RAMOS, which maintains the G4, but not in CA46 cells, which have lost the G4-mediated transcription control of MYC in the translocated allele (43). TMPyP4 is a pan-G4-stabilizing cationic porphyrin with generally non-drug-like properties, although it does concentrate in the nucleus (44). Similar effects of small molecule ligands were seen with the quindoline scaftol (39) and Actinomycin D (45) in this same system. Each of these studies was carried out using non-MYC G4-specific agents and at long (i.e. 24–48 h) time points where the effects may be at least partially due to other targets, including dsDNA.

While inferring an action on the region of the promoter lost through the CA46 translocation, and even perhaps within the NHE III1 region, these studies suffered from inherent variabilities between cell lines and deficiencies of the system to make definitive conclusions.

Design and Development of a More Critical System—To address the issue of drug selectivity, we used the MYC G4 as the molecular target in the selection of small molecules through a combined molecular modeling and virtual, as well as cell-free, screening procedure. Through this we identified the 9-dimethylaminoethoxy substituted ellipticine compound NSC338258 (GQC-05) as a potent and selective binder of the MYC G4. This ellipticine derivative demonstrated low micromolar cell kill in the BL RAJI cell line, with 5-fold less activity in the CA46 cells, which was well correlated with a decrease in MYC mRNA, but not in other genes with promoter G4s examined, characteristics similar to previous compounds identified as described above.

The CA46 Cell Line Provides Unique Insight into G4-mediated Control of MYC—One of the most profound aspects of the present study is the ability to monitor independently the two MYC products produced within the CA46 cell line. The translocation event that moves MYC exons near the enhancer ele-
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ment of the IgH heavy chain has been known for more than two and a half decades (46). Within CA46 cells, the major break-
point between chromosomes 8 (MYC gene) and 14 (IgH heavy
chain gene) occurs between exons 1 and 2 of the MYC gene. As
exon 1 is not translated, the protein product resulting from the
translocation is still fully functional (47). While exon 1 is pres-
et in the mRNA of the NT allele, it is absent from the T allele,
and we inferred that monitoring mRNA expression from both
exon 1 and exon 2 could reveal any potential differences in
regulation within the NT and T alleles, respectively. More
importantly, the G4 is retained in the NHE III, region in the NT
allele but lost in the T allele. Therefore, monitoring of the exon-
specific mRNA expression in the presence of a G4-targeted
small molecule should provide critical insight into the targeting
of the MYC G4.

Using this model, we were able to demonstrate that there is,
indeed, an exon-specific down-regulation of MYC expression
in the CA46 cell line produced as a consequence of GQC-05
treatment. Doxorubicin, TMPyP4, and GQC-Qi have all been
noted to decrease MYC expression (39, 48) and were tested for
exon-specific effects as a measure of potential activity on the
G4; however, no compound mirrored the effect of GQC-05.
Previous studies, both from our research group and others,
have focused on the pair of BL cell lines (either RAMOS or RAJ
and CA46) to describe potentially focused action of small mol-
ces (39, 43, 48). However, the newly described CA46 test is
much more robust and direct in demonstrating MYC G4-tar-
geted activity, acting as its own isogenic control. Indeed it is
a distinct advantage to simultaneously monitor the exon-specific
effects in the same cells at the same time to minimize variability
and increase confidence in the conclusions.

Modulation of Protein Binding to the MYC NHE III, with
GQC-05—Many extensive studies have been performed to
describe the binding of transcription factors to the MYC core
promoter, as well as the resolution and stabilization of the MYC
promoter G4 (15, 33, 34, 49). Using GQC-05, we monitored the
modulation of the known transcriptional factors and regulatory
proteins bound to the NHE III, region of MYC and showed that
within six to twelve hours of small molecule administration,
most of these proteins were significantly decreased in both BL
cell lines (Figs. 4D and 5C). RNA Pol II has been found to be
present, but paused, at the promoter and, as shown post-GQC-
05, was not expected to decrease in correlation with mRNA
expression (21). Interestingly, CNBP was significantly in-
creased within 6 h of GQC-05 treatment in only the RAJ cells,
which was not anticipated. While literature supports CNBP as a
single-strand binding transcription factor (50), there is some
new evidence that suggests it may facilitate the formation of a
G4, particularly within the MYC promoter (51). That was dem-
onstrated extracellularly, and may be complicated intracellular-
ly by chaperones, competing proteins, and varying topologi-
cal stresses, which may account for the variation in effect
between the two cell lines.

While ChIP has been used to demonstrate protein binding to
G4 regions, and even changes with nonspecific G4 stabilizers
(52), this study is the first demonstration of a targeted small
molecule stabilizing a specific promoter G4 and disrupting spe-
cific protein binding. This unambiguously confirms the loca-
tion of action of GQC-05 within the G4-forming region of the
NHE III, Together with the exon-specific effect in the CA46
cell line, these data culminate to define the intracellular activity
of GQC-05 on the promoter region through exon 1 by specifi-
cally altering the protein binding within the NHE III, G4-form-
ing region, correlating with a decrease in MYC mRNA expres-
sion and apoptosis. This study is the most direct, and first ever,
demonstration of a small molecule acting to stabilize the MYC
G4 within a cell, silencing transcription, and leading to cell kill.
Not only is this evidence of the molecule’s specific effects, but it
is also the clearest proof of a G4 existing in the intracellular
milieu and in a particular promoter.

The prevalence of putative G4s within critical core promot-
er of many oncogenes makes them an attractive target for
molecularly designed drugs. While many unanswered ques-
tions remain as to the equilibrating dynamics of these unique
structures both extra- and intra-cellularly (53), especially under
different degrees of superhelicity (32), recent studies using
either selective G4 antibodies or telomeric pull-down experi-
ments with molecules (54–56) have shown that indeed these
higher order secondary structures do exist within telomeres
and promoter regions, even in the context of chromatin and
histones. Through the type of cellular studies described here,
which use a combination of selective G4-interactive agents and
existing systems, we hope to encourage the broader scientific
community to look more favorably at small molecule targeting
of G4s.

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