Targeted Knockdown of *MYC* in AML Cells Using G-quadruplex Interacting Small Molecules

by

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ABSTRACT

Acute Myeloid Leukemia (AML) is a disease that occurs when genomic changes alter expression of key genes in myeloid blood cells. These changes cause them to resume an undifferentiated state, proliferate, and maintain growth throughout the body. AML is commonly treated with chemotherapy, but recent efforts to reduce therapy toxicity have focused on drugs that specifically target and inhibit protein products of the cancer's aberrantly expressed genes. This method has proved difficult for some proteins because of structural challenges or mutations that confer resistance to therapy. One potential method of targeted therapy that circumvents these issues is the use of small molecules that stabilize DNA secondary structures called G-quadruplexes. G-quadruplexes are present in the promoter region of many potential oncogenes and have regulatory roles in their transcription. This study analyzes the therapeutic potential of the compound GQC-05 in AML. This compound was shown *in vitro* to bind and stabilize the regulatory G-quadruplex in the *MYC* oncogene, which is commonly misregulated in AML. Through qPCR and western blot analysis, a GQC-05 mediated downregulation of *MYC* mRNA and protein was observed in AML cell lines with high *MYC* expression. In addition, GQC-05 is able to reduce cell viability through induction of apoptosis in sensitive AML cell lines. Concurrent treatment of AML cell lines with GQC-05 and the *MYC* inhibitor (+)JQ1 showed an antagonistic effect, indicating potential competition in the silencing of *MYC*. However, GQC-05 is not able to reduce *MYC* expression significantly enough to induce apoptosis in less sensitive AML cell lines. This resistance may be due to the cells' lack of dependence on other potential GQC-05 targets that may help upregulate *MYC* or stabilize its protein product. Three such genes identified by RNA-seq analysis of GQC-05 treated cells are *NOTCH1*, *PIM1*, and *RHOU*. These results indicate that the use of small molecules to target the *MYC* promoter G-quadruplex is a viable potential therapy for AML. They also support a novel mechanism for targeting other potentially key genetic drivers in AML and lay the groundwork for advances in treatment of other cancers driven by G-quadruplex regulated oncogenes.

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Chapter 1: Introduction

1.1 Acute Myeloid Leukemia

1.1.1 Overview

Acute leukemia is a malignancy that results from genetic damage and aberrant gene expression in blood cells, and it is the most common type of cancer in children (Tarlock and Cooper, 2016). A subset of this disease, Acute Myeloid Leukemia (AML), forms when blood cell precursors of myeloid lineage are unable to differentiate and begin to proliferate in excess. The cancer cells expand in the bone marrow and displace the normal blood cell progenitors. This causes disease through inhibition of blood cell production, as well as eventual metastasis and displacement of other essential organs.

1.1.2 Current Treatments

AML is currently treated by administering chemotherapy agents, such as cytarabine and anthracyclines (Tarlock and Cooper, 2016). Cytarabine is a cytosine analog with an arabinose sugar in place of deoxyribose. Presence of cytarabine in a cell brings DNA synthesis to a halt when DNA Polymerase attempts to integrate it into the growing DNA strand and is stalled by the difference in structure of the nucleoside's sugar (Galmarini et al., 2001). This can be effective against rapidly dividing cancer cells, but will also be detrimental to other rapidly dividing cells, such as blood cells, hair follicles, and the cells lining the gastrointestinal tract. Loss of these normal tissues can give patients nausea, anemia, bleeding, and alopecia (Tarlock and Cooper, 2016). Anthracyclines are a class of compound that damage the DNA through multiple mechanisms including intercalation into the DNA that leads to topoisomerase II-mediated double stranded breaks and the production of free radicals that damage DNA structure (Hortobágyi, 1997). The DNA damage is used to target replicating cells by clogging up DNA replication machinery. In addition to the previously listed side effects of targeting cell replication, anthracyclines can also induce heart failure because of the free radicals they produce (Hortobágyi, 1997). Both compounds are harsh on patients but effective in killing leukemia cells. However, because both compounds inflict heavy damage on the patients' DNA, it is possible that secondary cancer can occur. Furthermore, approximately 30% of patients experience relapse

after treatment, usually returning to the clinic with a poor prognosis (Tarlock and Cooper, 2016). The high rate of relapse in AML patients and toxicity of commonly used therapies highlight a strong need for improved AML therapies that will combat drug resistance and minimize side effects.

1.2 Targeting the *MYC* Oncogene

1.2.1 *MYC* Upregulation in AML

One method of treating patients with cancer that minimizes side effects is the use of compounds that inhibit the gene product or products that are misregulated in the cancer being treated. AML is associated with a number of genetic drivers that could serve as targets. One gene that is being investigated as a novel target in AML therapies is the transcription factor c-Myc. This protein regulates many hallmarks of cancer such as cell growth, proliferation, cell-cycle, apoptosis, and cell differentiation (Vita and Henriksson, 2006). Its involvement in many pathways makes it a potent oncogene when misregulated. Many of the gene fusions common in AML (e.g. *FLT3-ITD* (Gilliland and Griffin, 2002), *AML1-ETO*, *PML/RARa*, and *PLZF/RARa* (Müller-Tidow et al., 2004)) have been correlated with an increase in c-Myc expression. Similar increases in expression have been observed in chemotherapy-resistant AML as well (Pan et al., 2014), indicating that these cancers may rely on c-Myc upregulation to boost growth and cause the patient to relapse. In addition to its role in the previously mentioned pathways, c-Myc is also an important factor in the maturation of blood cells, and its expression is turned off to allow myeloid precursors differentiate. In fact, when myeloid progenitor cells are forced to express c-Myc, their ability to differentiate into more specialized cells is lost (Selvakumaran et al., 1996). By expressing c-Myc at high levels, AML cells are able to retain their undifferentiated, highly proliferative states. The many roles c-Myc has in cancer growth and survival make it an attractive target for AML therapy. In fact, c-Myc inhibition has been correlated with a decrease in AML cell proliferation and prolonged survival of mice transplanted with AML (Brondfield et al., 2015). Reliance on c-Myc overexpression is a common mechanism for many other tumor types as well, and an inhibitor of this transcription factor would be useful for treatment of a wide variety of cancers (Vita and Henriksson, 2006).

1.2.2 Obstacles to c-MYC Inhibition

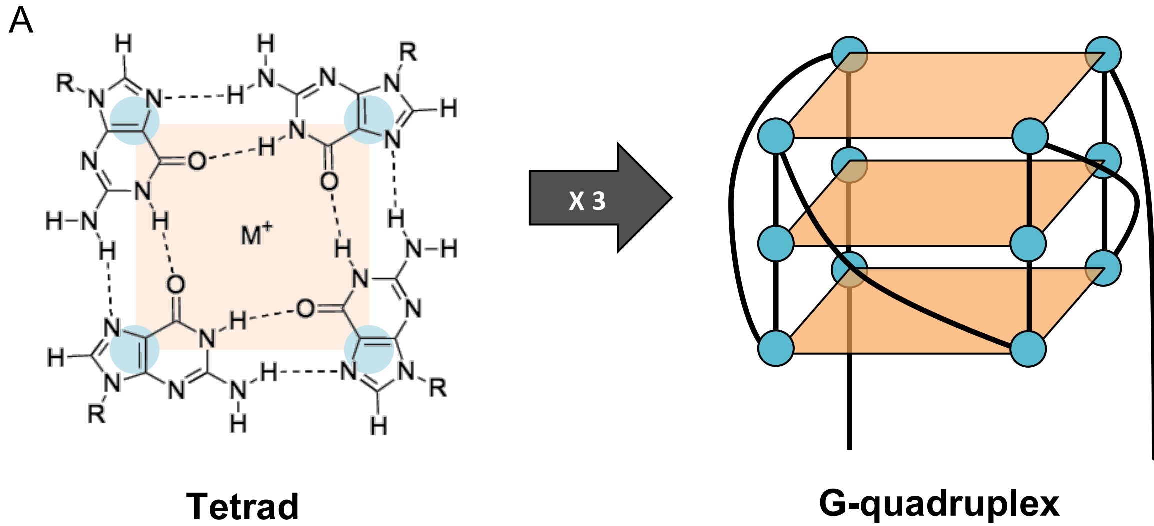
Although it is an attractive target, c-Myc has been notoriously difficult to suppress with small molecule inhibitors. Neither its interaction with co-factor Max nor its binding to the target DNA sequence are easily interrupted with small molecule binding. A few notable exceptions to this predicament have been described. The compound 10058-F4 is able to disrupt c-Myc/Max heterodimerization and induce apoptosis in AML cells (Huang et al., 2006). However, the Max binding surface on c-Myc is flat, large, and lacks distinctive features to target. Due to these setbacks, 10058-F4 does not have the specificity needed to be useful in the clinic (Prochownik and Vogt, 2010). Cells also gain resistance to protein-targeted compounds by strategically mutating the compound's target site so that it can no longer bind to the protein it was meant to inhibit. One way to circumvent these issues is to target the oncogene's transcription by altering its promoter. With this approach, the target is less likely to mutate because promoter alterations are more likely to be deleterious to transcription of the entire gene. The compound (+)JQ1 targets the transcription of *MYC* by inhibiting the BET family of proteins (Filippakopoulos and Knapp, 2014). BET proteins dock onto acetylated lysines on histone tails and rearrange the chromatin around genes to promote their transcription. (+)JQ1 binds these BET proteins and prevents them from docking onto histones. This effectively inhibits *MYC* expression, but it also affects a number of other genes regulated by BET proteins. The wide reach of BET inhibition could potentially result in significant side effects if (+)JQ1 is used as a therapy for AML.

1.2.3 Inhibition of *MYC* through Targeted Stabilization of G-quadruplex DNA structures

Another method to target *MYC* transcription is the use of small molecules to stabilize DNA secondary structures called G-quadruplexes. These structures are formed when stress from negative superhelicity is imposed on guanine-rich stretches of DNA, causing the DNA to form tetrads of Hoogsteen base-paired guanine nucleotides to relieve the tension (Bochman et al., 2012). A few of these tetrads will stack on each other to form a 3D structure called a G-quadruplex (Fig. 1A). Guanine-rich sequences with the potential to form G-quadruplexes are enriched in the promoter regions of many genes involved in cell replication, proliferation, apoptosis, and other highly regulated processes with the potential for oncogenesis (Eddy and

Maizels, 2006). Often, the presence of a G-quadruplex in a promoter inhibits transcription of its associated gene, as is the case for *MYC* (Brooks and Hurley, 2010) (Fig. 1B). Since the G-quadruplex structure is in dynamic equilibrium with the promoter's transcriptionally active single stranded and double stranded forms, there exists the opportunity to repress *MYC* transcription by stabilizing the G-quadruplex. This has been achieved previously *in vitro* using the porphyrin molecule TMPyP4 in Burkitt's Lymphoma cells (Siddiqui-Jain et al., 2002).

Not only is the G-quadruplex an effective target for gene regulation, but its variable structure also creates the opportunity for gene-specific regulation. G-quadruplex structures can vary in the length and composition of the loops connecting the tetrads, the number of stacked tetrads in the structure, and the orientation of the DNA strands (Brooks et al., 2010). If small molecule inhibitors are designed to interact with G-quadruplexes in the variable loops they can target specific structures, and consequently specific genes.



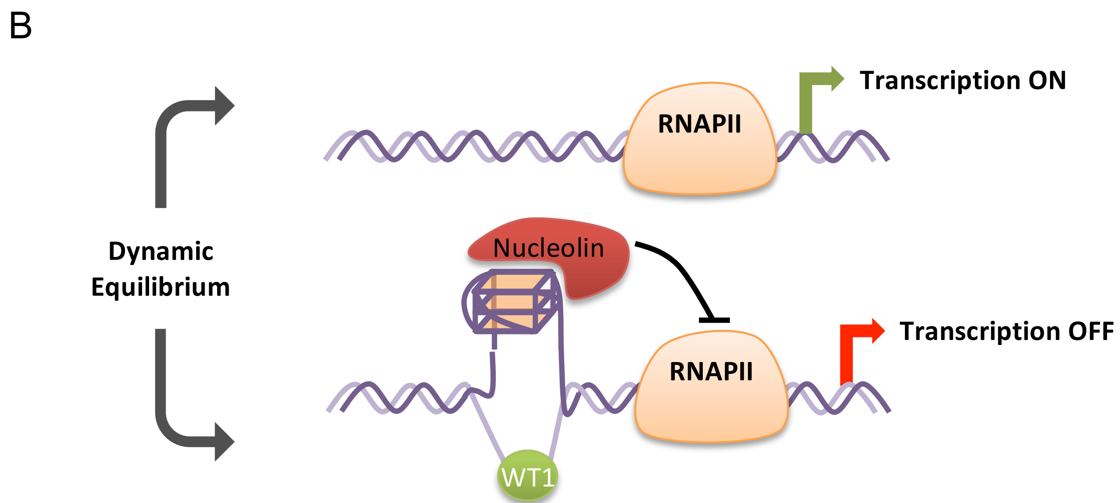


Figure 1. G-quadruplex Structure and Transcription Regulation Dynamics (A) Example of a G-quadruplex structure. Tetrads are formed from Hoogsteen base pairing of four guanine nucleotides. Two or more tetrads stack to form a G-quadruplex. (B) Diagram of the G-quadruplex mediated transcription regulation in the *MYC* gene. The transcriptionally active dsDNA form of the gene is in dynamic equilibrium with the transcriptionally inactive G-quadruplex form of the gene. When the G-quadruplex is formed, it recruits the protein Nucleolin to repress gene transcription.

Finally, G-quadruplexes make excellent targets for oncogene repression because they are enriched in the target cancer cells. G-quadruplexes form to relieve stress imposed on the DNA by transcriptional unwinding (Sun and Hurley, 2009). When a cancer cell upregulates expression of an oncogene, it also increases the amount of time the promoter of that oncogene spends in its G-quadruplex form. Therefore, a small molecule inhibitor will be much more likely to find its target in a cancer cell than in a normal cell with less transcription of the target gene. This will reduce the off-target effects of G-quadruplex targeting cancer therapies.

1.3 Research Hypothesis

This study's intent is to assess the effect of the G-quadruplex interacting compound GQC-05 on AML. This compound has previously been shown to interact with the G-quadruplex in the NHE III region of the *MYC* p2 promoter and repress the gene's transcription in Burkitt's lymphoma (Brown et al., 2011). Here, the goal is to determine GQC-05's phenotypic effect in different types of AML, as well as its mechanism of action when inducing cell death. Given that GQC-05 has other known G-quadruplex targets, another goal is to cross-examine the genome for other GQC-05 regulated genes that could represent important targets for AML therapies. The hypothesis is that GQC-05 will knock down expression of *MYC* and induce cell death in AML cells with *MYC* overexpression. In addition, there are likely other genes targeted by GQC-05 that contribute to its cytotoxicity in AML, which could possibly serve as targets for future AML therapy development.

Chapter 2: Materials and Methods

2.1 Cell Culture

All cell lines were authenticated using Short Tandem Repeat (STR) analysis by the University of Arizona genomics core. The CMY, CMK, and CMS cell lines were a generous gift from Dr. Jeffrey W. Taub, Wayne State University. The KG-1a, HL-60, and MV-4-11 cell lines were grown in IMDM media (Corning) supplemented with 20% Fetal Bovine Serum (FBS; Atlas Biologicals), 1% L-Glutamine (Caisson Labs), and 1% penicillin/streptomycin (Gibco). The UT-7epo cells were grown in similar IMDM media that was supplemented with 1 U/mL recombinant erythropoietin (rhEPO; R&D Systems). The Molm-13, Kasumi-1, CMY, NB4, TF-1, M-07e, CMK, HEL, THP-1, U937, AML-193, and CMS cells were grown in RPMI 1640 (Corning) with 10% FBS and 1% penicillin/streptomycin and L-glutamine. The RPMI growth media for TF-1 and M-07e was supplemented with 2 ng/mL granulocyte macrophage colony-stimulating factor (GMCSF; R&D systems), and the media for AML-193 contained 2 ng/mL GMCSF as well as 5 µg/mL Insulin Transferrin Selenium A (ITS; Gibco). PBMCs were isolated from whole blood by density centrifugation using Ficoll (GE Life Sciences) and grown in RPMI (10% FBS) supplemented with 10 ng/mL IL-2 (R&D Systems). All cells were grown at 37°C with 5% CO_2. For 6 well plate assays, cells were plated at 500,000 cells/mL (KG-1a and TF-1) or 250,000 cells/mL (CMK) with 4 mL in each well. Cells were allowed to grow overnight before treatment.

2.2 Antibodies, Primers, and Compounds

Primary antibodies for c-Myc (Rabbit mAb #5605), Bcl-2 (Rabbit mAb #2870, Mouse mAb #15071), and PARP (Rabbit mAb #9532) were purchased from Cell Signaling Technology. The GAPDH (Mouse mAb sc-166545) primary antibody was purchased from Santa Cruz Biotechnology. Secondary Rabbit and Mouse antibodies were obtained from Jackson Immunoresearch.

Gene specific qPCR primers for c-Myc (Forward: 5’-GCCCACCACCAGCAGCGACTC-3’, Reverse: 5’-GCACCTCTTGAGGACCAGTGG-3’), Bcl-2 (Fwd: 5’-AGTACCTGAACCGGCACCTGC-3’, Rvs: 5’-ACTTGTGGCCCAGATAGGCAC-3’), YWHAZ (Fwd: 5’-AGAGAAAGCCTGCTCTCTTGC-3’, Rvs: 5’-CGTCTCCTTGGGTATCCGATG-3’), and GAPDH

(Fwd: 5’-TGGACCTGACCTGCCGTCTA-3’, Rvs: 5’-AGGAGTGGGTGTCGCTGTTG-3’) were obtained from Life Technologies.

The G-quadruplex interacting compounds GQC-05 and GSA 1103 were kindly provided by Dr. Laurence Hurley, School of Pharmacy, University of Arizona. (+)JQ1 and Etoposide were obtained from Selleckchem.

2.3 Drug Dose Response Viability Assays

For the assays used to determine IC50 values, AML cells (2000 cells/well) or PBMCs (10,000 cells/well) were plated on 384 well plates. At 24 hours, cells were treated with various drug doses in quadruplicate wells for 72 hours. For GQC-05, treatments were 1:3 serial dilutions ranging from 60 μM to 3.05 nM. 5-Azacytidine treatments were previously performed at 1:3 dilutions ranging from 100 μM to 5.08 nM. For the GQC-05/(+)JQ1 competition assays, GQC-05 treatments were 1:3 dilutions from 30 μM to 1.52 nM, with (+)JQ1 treatments of 1:4 dilutions ranging from 20 μM to 4.9 nM being added as well. After 72 hours of treatment, Cell Titer-Glo® (Promega) was added to the cells, and luminescence was read using the PerkinElmer EnVision Multilabel Plate Reader. Samples were normalized to untreated controls and dose response curves and IC50 values were determined using GraphPad Prism.

2.4 Western Blots

Cells were plated at 2,000,000 cells/well in 6 well plates and allowed to grow for 6 or 24 hours after treatment. Treated cells were washed in PBS (Paradigm) and lysed using RIPA lysis buffer (Thermo Fisher) supplemented with 1x Halt Protease & Phosphatase inhibitor cocktail (Thermo Fisher). Gel electrophoresis was performed on 15 or 10-well NuPAGE 4-12% Bis-Tris gels from Thermo Fisher. Samples were then transferred to Invitrolon PVDF transfer membranes (Invitrogen) and blocked overnight in blocking buffer consisting of 1x TBS (Quality Biological) with 0.1% Tween® 20 (Thermo Fisher) and 5% dehydrated milk. Primary antibodies were used at concentrations of 1:1000 except the control gene GAPDH, which was 1:20,000. The secondary antibody concentration used was 1:20,000. Blots were imaged after treatment with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher) using the Bio-Rad ChemiDoc™ Imaging System. Bands were quantified using the Bio-Rad Image Lab™ software.

2.5 Quantitative Polymerase Chain Reaction

Cells were plated at a concentration of 2,000,000 cells/well in 6 well plates and treated for 6 hours. Total RNA was isolated from treated cells using the Qiagen RNeasy® Plus Mini Kit as per manufacturer instructions. RNA samples (1 μg) were reverse transcribed using the iScript Select cDNA synthesis kit (Bio-Rad). qPCR reactions were prepared in triplicate using iQ SYBR GreenSupermix (Bio-Rad) and were performed using a CFX96 Real-Time qPCR Detection System (Bio-Rad). Analysis of fold change gene expression was performed on Excel (Microsoft) spreadsheets using the Comparative C_T method (Schmittgen and Livak, 2008).

2.6 Luminescence-based Caspase 3/7 Activity Analysis

Cells were plated in 6 well plates as described above. Aliquots (4 x 25 μl) were taken for apoptosis assays and transferred in quadruplicate to 384 well plates. Caspase-Glo® 3/7 (Promega) was used for the apoptosis assay to determine the activity of caspases 3 and 7, two enzymes used by cells during apoptosis. Caspase-Glo® 3/7 contains a substrate that, when cleaved by caspase 3 or 7, produces ATP. A thermostable luciferase ATPase also present in the solution emits luminescence proportional to the amount of ATP in the sample. Luminescence was measured using the EnVision Multilabel Plate Reader (PerkinElmer).

2.7 Flow Cytometry

Cells were plated and treated in 6 well plates as described above. After 24 hours of treatment, 500,000 cell aliquots of each sample were taken, including 4 aliquots of the 10 μM Etoposide positive control and 1 aliquot of each other treatment. Cells were washed twice with cell staining buffer (FBS diluted 1:20 in PBS with 1% Sodium Azide), and then resuspended in 50 μL 5X Annexin binding buffer (Invitrogen) with 5 μM Sytox® Red dead cell stain (life technologies) and 1.25 μg/mL Brilliant Violet 421™ Annexin V (Biolegend). Instrument controls included Sytox® only, Annexin V only, or unstained sample. Samples were then incubated in the dark at room temperature for 15 minutes and diluted 1:10 in PBS prior to analysis. Flow cytometric analysis was performed by Dr. Mrinalini Kala of the University of Arizona Translational Flow Cytometry Laboratory on a FACSCanto flow cytometry system (BD Biosciences).

2.8 Fluorescence Microscopy

Cells were plated and treated in 6 well plates as described above. After 24 hours of treatment, 50 µL cell aliquots of each sample were transferred to wells of a black clear-bottom 96 well plate. 50 µL of a dye mixture containing 2.5 µg/mL Brilliant Violet 421™ Annexin V and 10 µM Vybrant™ DyeCycle™ Ruby stain (Invitrogen) in 5x Annexin Binding buffer was added to each well. The samples were then allowed to incubate in the dark at room temperature for 20 minutes before they were imaged using the ImageXpress® Micro XLS Widefield High-Content Analysis System (Molecular Devices). Images were then analyzed using Molecular Devices' MetaXpress™ High Content Image Processing Software.

2.9 RNA-seq Analysis

Cells were split to 400,000 cells/mL in a T75 culture flask. After 48 hours of growth, they were plated in 6 well plates (500,000 cells/mL) and allowed to grow overnight before 6 hours of treatment. Total RNA was isolated using the Qiagen RNeasy® Plus Mini Kit. RNA samples were then sent to the DNASU sequencing core at Arizona State University for library preparation, NextGen sequencing using the Illumina NextSeq500 platform, read alignment to the human genome build 38 (GRCh38) toplevel assemble using STAR v2.5.1b, and normalized TPM value generation using Cufflinks v2.2.1. TPM values were converted to $\log_2$ TPM values for ease of handling. Genomics Suite 6.6 (Partek) was used to perform analysis of variance (ANOVA) and identify differentially expressed genes with p values < 0.05 and a ≥2 fold difference in gene expression between DMSO and GQC-05 treated samples.

2.10 siRNA Gene Knockdown

For gene silencing studies, 2 µL of 20 µM siRNA stocks were added to four wells of a six well plate. The four siRNA stocks included All Stars Negative Control siRNA (Qiagen), All Stars Hs Cell Death Control siRNA (Qiagen), and the c-Myc siRNAs *MYC*_5 (Qiagen #S100300902) and *MYC*_7 (Qiagen #S102662611). For KG-1a cell assays, 1 mL of diluted transfection reagent containing 50 µL Lipofectamine® RNAiMAX transfection reagent (Invitrogen) in 5 mL Opti-MEM™ media (Gibco) was added to the four wells with siRNA and one of the two empty wells. 1 mL Opti-MEM™ was added to the final well. For TF-1 cell assays the diluted transfection reagent

contained 25 µL RNAiMAX in 5 mL Opti-MEM™. The plates were incubated at room temperature for 30 minutes before addition of 500,000 cells in 1 ml of media to each well. The media used was RPMI with 1% L-Glutamine for TF-1 cells and IMDM with 1% L-Glutamine for KG-1a cells. After addition of cells, the plates were placed in the incubator at 37°C, 5% CO_2. After 6 hours, 2 ng/µL GMCSF was added to wells of the TF-1 plates. 24 hours after cell plating, 250 µL of growth media with FBS was added to each well. IMDM with 20% FBS and 1% L-Glutamine was used for KG-1a cells, and RPMI with 10% FBS and 1% L-Glutamine was used for TF-1 cells. After 48 hours, aliquots of cells were taken for viability analysis using Cell Titer-Glo® (Promega) and caspase activity analysis using Caspase 3/7-Glo® (Promega). The remaining cells were harvested for RNA extraction.

Chapter 3: Results

3.1 Phenotypic Effect of GQC-05 on a Panel of AML Cell Lines

3.1.1 AML Cell Lines have Variable Sensitivities to GQC-05

In order to examine the activity of G-quadruplex interacting drug GQC-05 in AML, drug dose response assays were performed on a panel of 16 AML cell lines derived from pediatric and adult patients, as well as PBMCs, which were used as a normal cell control. Viability of the cells was measured after 72 hours of treatment with a range of concentrations for either GQC-05 or 5-Azacytidine, a common therapeutic agent used in AML. Cell line response to treatment was varied (Fig. 2A). GQC-05 was generally more potent, with IC50s ranging from 53 nM to 422 nM while 5-Azacytidine IC50s ranged from 199 nM to 4.431 μM (Table 1).

Cell Line	GQC-05 IC50 (nM)	5-Azacytidine IC50 (nM)
MV-4-11	53	2005
Molm-13	54	199
Kasumi-1	57	1547
UT-7epo	76	2361
KG-1a	92	4431
CMY	105	1527
NB4	153	401
TF-1	165	1783
M0-7e	190	879
CMK	202	739
HEL	204	1105
THP-1	224	3497
U937	315	381
AML-193	351	2855
CMS	353	2542
HL-60	422	3205
PBMC	191	-

Table 1. IC50 Values of the AML Cell Line Panel Treated with GQC-05 and 5-Azacytidine

3.1.2 c-Myc and Bcl-2 Protein Expression is Variable in AML Cell Lines

Protein expression of the two potential GQC-05 targets, c-Myc and Bcl-2, in the panel of 16 AML cell lines was analyzed by western blot. Expression of both c-Myc and Bcl-2 protein was widely varied between cell lines with 9 cell lines showing expression of c-Myc protein and 11 cell lines expressing Bcl-2 protein (Fig. 2B). Interestingly only 6 AML cell lines showed expression of both proteins.

3.1.3 AML Sensitivity to GQC-05 is Correlated to Base c-Myc Protein Expression

Quantification of c-Myc and Bcl-2 protein expression in each AML cell line and its comparison to the IC50 values of those cells reveals a correlation between high c-Myc protein expression and sensitivity to GQC-05 (Fig. 2C). Cancers with high *MYC* expression are often dependent on maintenance of this high level of expression to survive. This correlation indicates that c-Myc downregulation may be a mechanism used by GQC-05 to induce cell death in AML cells, though the outliers suggest it is likely there is also a secondary mechanism. Bcl-2 protein expression appears to have no correlation to GQC-05 sensitivity.

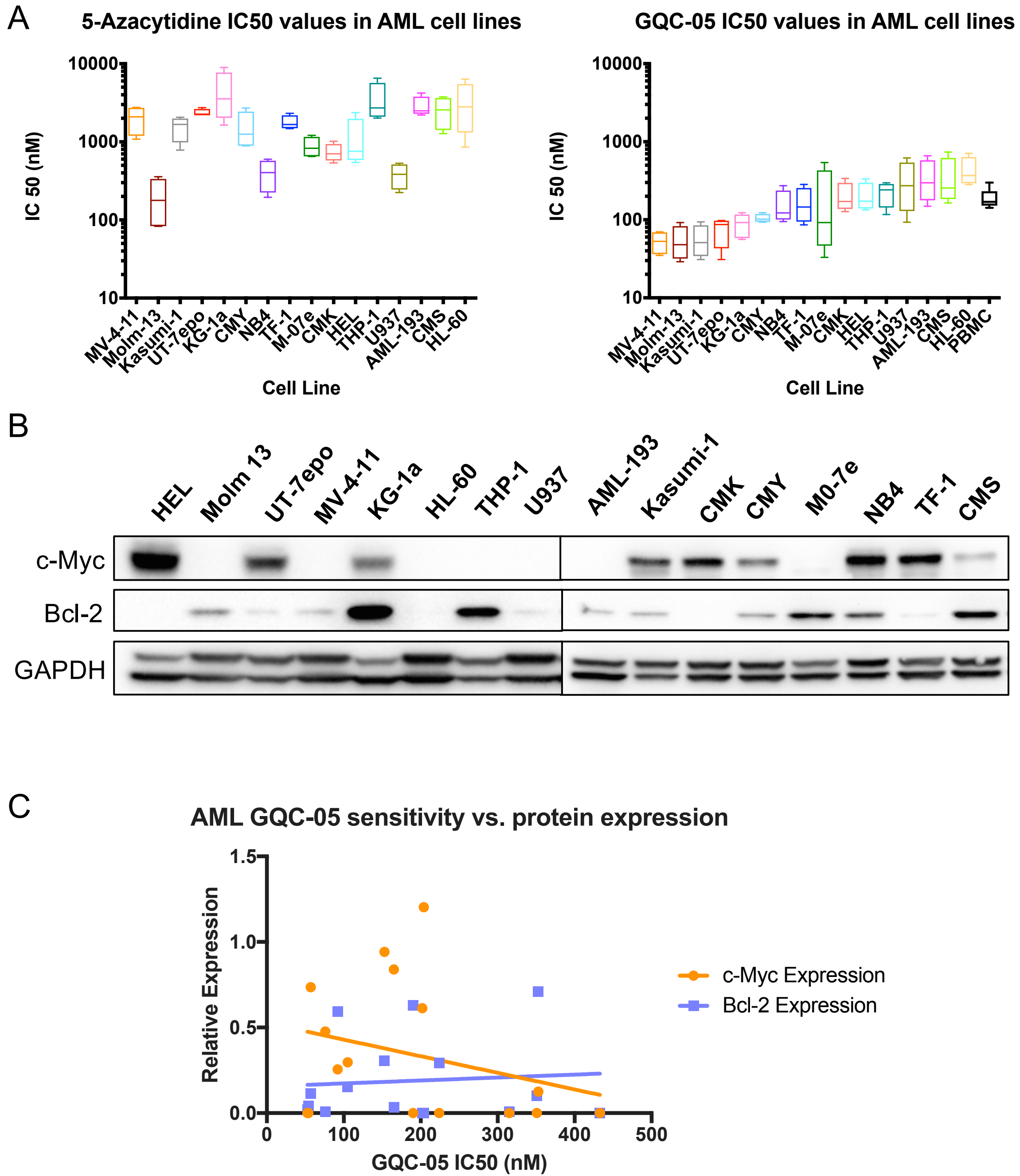


Figure 2. Phenotypic Effect of GQC-05 on a Panel of AML Cell Lines and Correlation to Protein Expression (A) Range of IC50 values in a panel of 16 AML cell lines and a PBMC sample control after 72 hr treatment with GQC-05 (right) or a current AML therapy 5-Azacytidine (left). Viability was measured using a luminescence assay. Results are an average of four experimental replicates. (B) Western blot indicating base c-Myc and Bcl-2 protein expression in the same panel of AML lines. (C) Graph indicating a correlation between base c-Myc expression and sensitivity to GQC-05 in AML cell lines. No correlation was seen with Bcl-2 expression. Protein expression was

quantified by measuring western blot c-Myc band intensity and normalizing it to the GAPDH band intensity.

3.2 GQC-05 Downregulates *MYC* Expression in AML Cell Lines with High Base *MYC* Expression

3.2.1 c-Myc Protein is Reduced in Four *MYC*-expressing AML Lines after GQC-05 Treatment

GQC-05 was able to knock down c-Myc protein expression after 6 hours in four cell lines that have high c-Myc protein expression: CMK, KG-1a, UT-7epo, and TF-1 (Fig. 3A). Knockdown was more significant for GQC-05 treatment than for similar treatment with two other *MYC* inhibitors, the bromodomain inhibitor (+)JQ1 and a second G-quadruplex interacting compound GSA 1103. This data shows that GQC-05 is able to knock down c-Myc protein expression in AML cells, and further supports c-Myc downregulation as an important factor in the cytotoxicity of GQC-05.

3.2.2 GQC-05 Reduces *MYC* mRNA and Protein in KG-1a, CMK, and TF-1 Cell Lines

Expression of *MYC* mRNA in the *MYC* expressing cell lines KG-1a, CMK, and TF-1 was analyzed after 6 hours of GQC-05 treatment. All three cell lines show a decrease in *MYC* mRNA expression after treatment with GQC-05, though KG-1a showed the strongest response (Fig. 3B). When the same set of cell samples were analyzed via western blot, a downregulation of c-Myc protein was observed as well (Fig. 3C). GQC-05's ability to knock down both c-Myc protein and *MYC* mRNA suggests that it may be inhibiting transcription of the gene rather than translation.

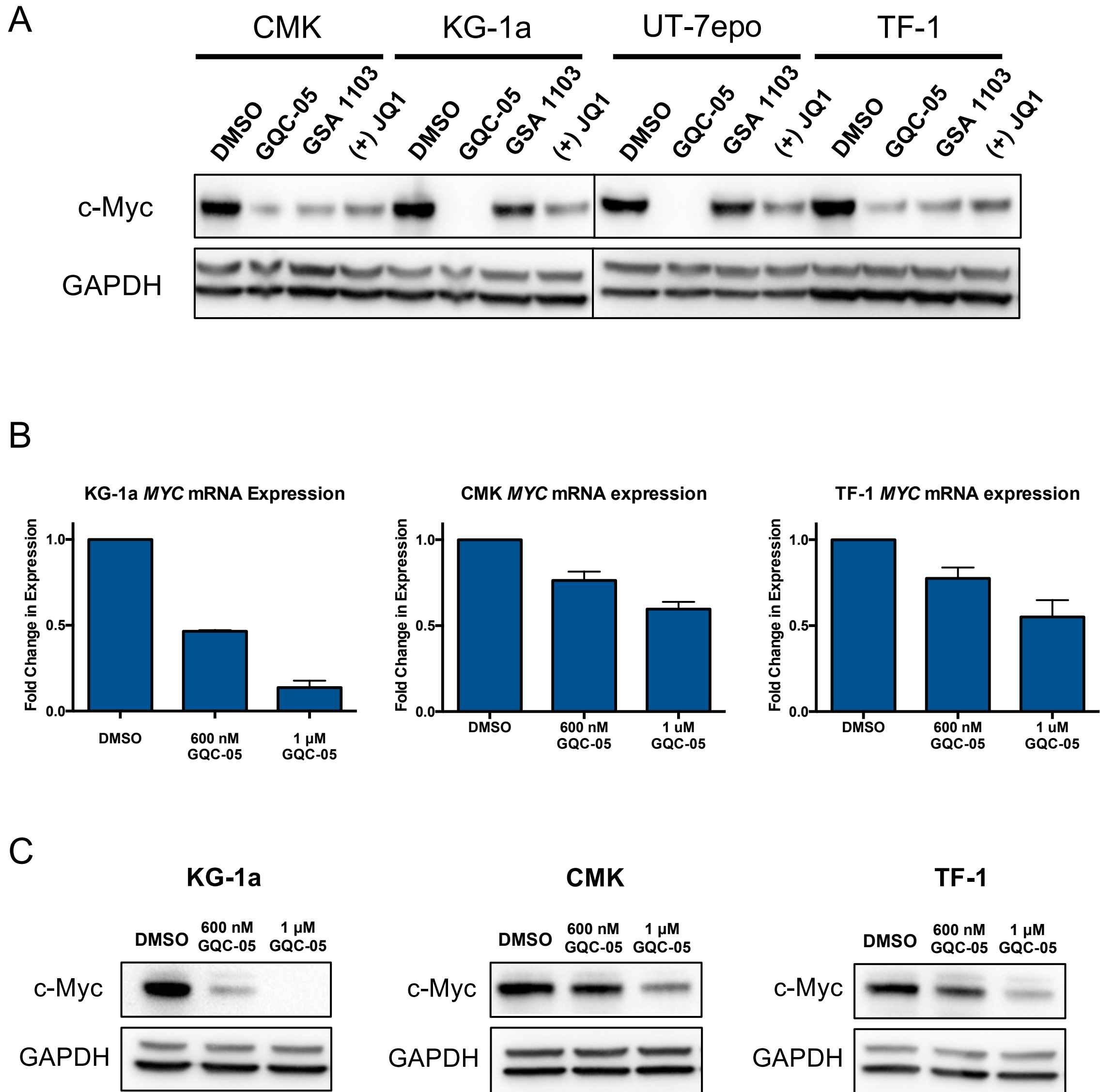


Figure 3. GQC-05 Induces Loss of c-Myc in AML Lines with High *MYC* Expression (A) Western blot showing loss of c-Myc protein in AML cell lines after 6 hr treatment with the *MYC* inhibitors GQC-05, GSA 1103, and (+)JQ1. GQC-05 and (+)JQ1 were at 1 μM while GSA 1103 was at 3 μM. (B) qPCR analysis of AML cells treated 6 hr with GQC-05 shows loss of *MYC* mRNA after treatment. KG-1a and CMK results are an average of three experimental replicates, and TF-1 results are an average of two. (C) Western blot showing loss of c-Myc protein in AML cells after 6 hr treatment with GQC-05. qPCR and western blot analysis of c-Myc loss were done on the same set of samples.

3.3 siRNA Mediated Knockdown of *MYC* Reduces Viability and Induces Apoptosis in Sensitive AML Cell Lines to a Lesser Degree than GQC-05

KG-1a and TF-1 cells were transfected with two *MYC* specific siRNAs as well as positive and negative control siRNAs for 48 hours. Only *MYC* specific siRNA *MYC*_5 was able to reduce cell viability, with a viability loss of only 13% in the GQC-05 sensitive cell line KG-1a (Fig. 4A). However, both *MYC* specific siRNA *MYC*_5 and *MYC*_7 were able to knock down expression of *MYC* mRNA in both KG-1a and TF-1 (Fig. 4B). Furthermore, apoptosis was induced by *MYC* knockdown in KG-1a cells as evidenced by the increase in caspase 3/7 activity with *MYC* specific siRNA treatment. No such induction was seen with TF-1 cells (Fig. 4C).

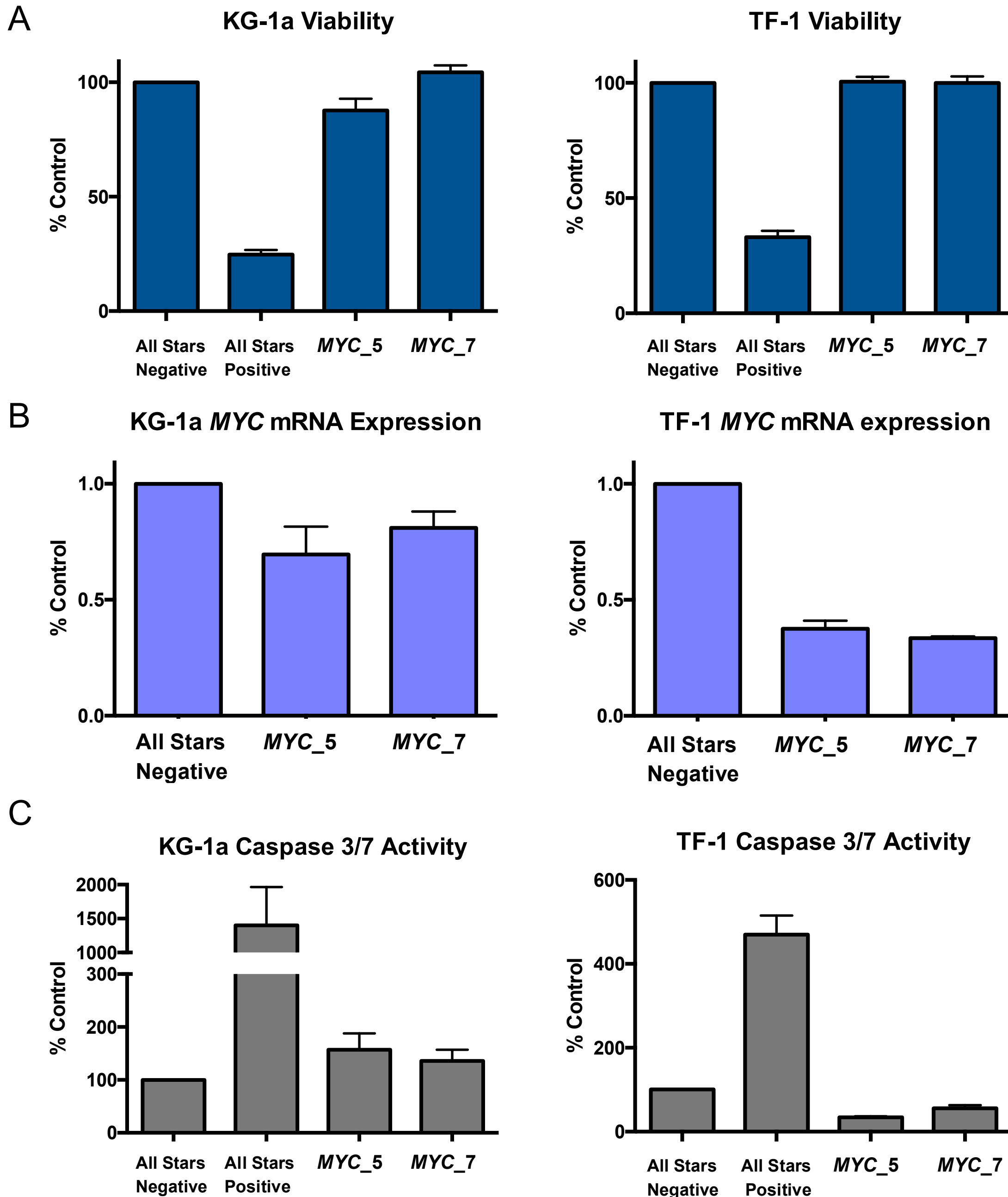
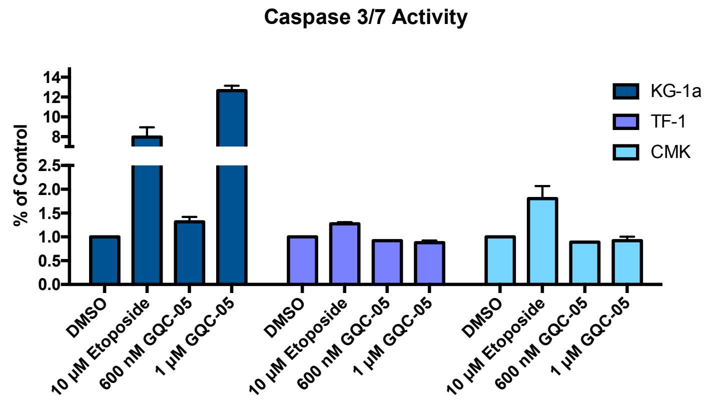


Figure 4. siRNA Knockdown of *MYC* in AML Cells Reduces Viability and Induces Apoptosis in GQC-05 Sensitive AML Cells (A) Viability data after 48 hour siRNA treatment in KG-1a and TF-1 indicates a 13% loss of viability in KG-1a cells treated with *MYC* specific siRNA *MYC*_5. (B) qPCR analysis shows siRNA mediated knockdown of *MYC* by both *MYC* specific siRNAs *MYC*_5 and *MYC*_7 in KG-1a and TF-1 after 48 hours. (C) Caspase 3/7 activity is increased in KG-1a cells treated 48 hours with *MYC* specific siRNA, but not in TF-1 cells. All results for Figure 4 include at least two experimental replicates.

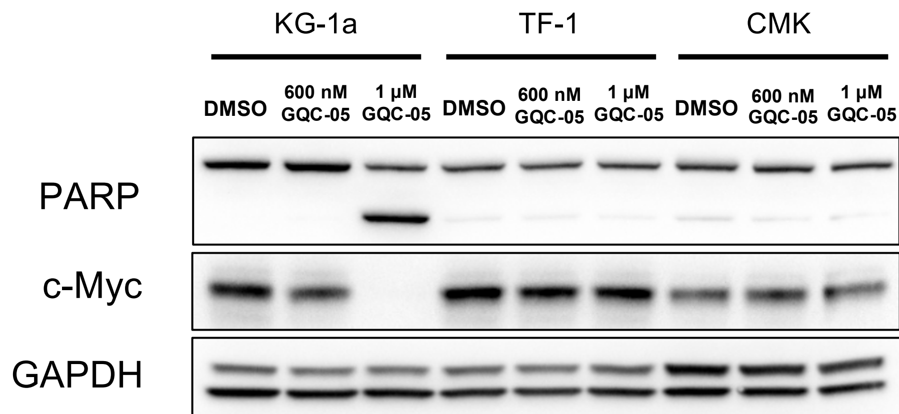
3.4 GQC-05 Induces Apoptosis in Sensitive AML Cell Lines

KG-1a, CMK, and TF-1 cells were analyzed for induction of apoptosis after 24 hours of treatment with GQC-05. Etoposide, a chemotherapy clinically used to treat AML, was used as an apoptosis positive control. Apoptosis was induced in KG-1a cells treated with GQC-05, as evidenced by the increase in caspase 3/7 activity (Fig. 5A), PARP cleavage (Fig. 5B), and Annexin V staining (Fig. 5C+D). It is also apparent that c-Myc downregulation is induced in conjunction with PARP cleavage in KG-1a cells. However, both of the less sensitive cell lines, CMK and TF-1, show that c-Myc protein expression is no longer downregulated by GQC-05 at 24 hours, and there is little increase in caspase 3/7 activity, PARP cleavage, or Annexin V staining after GQC-05 treatment. This suggests that CMK and TF-1 have some mechanism of resistance to GQC-05 mediated knockdown of c-Myc and induction of apoptosis.

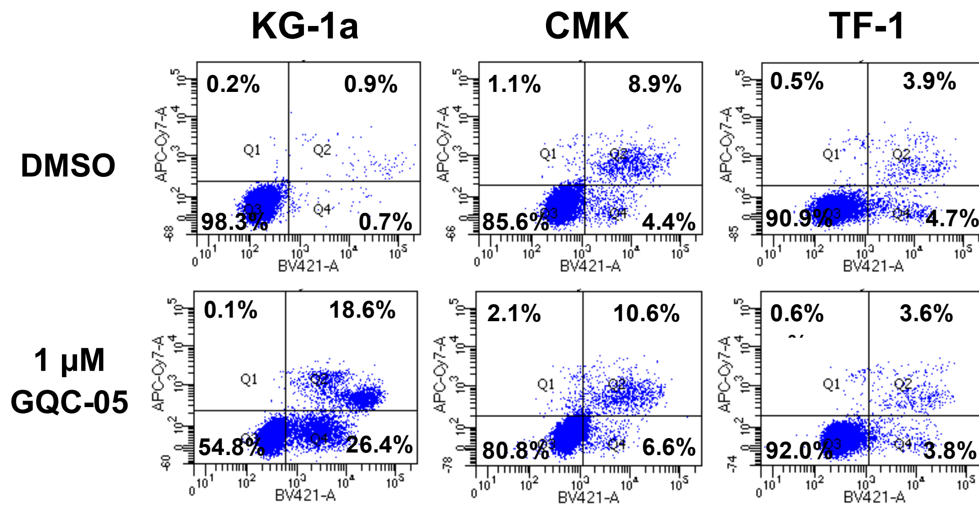
A



B



C



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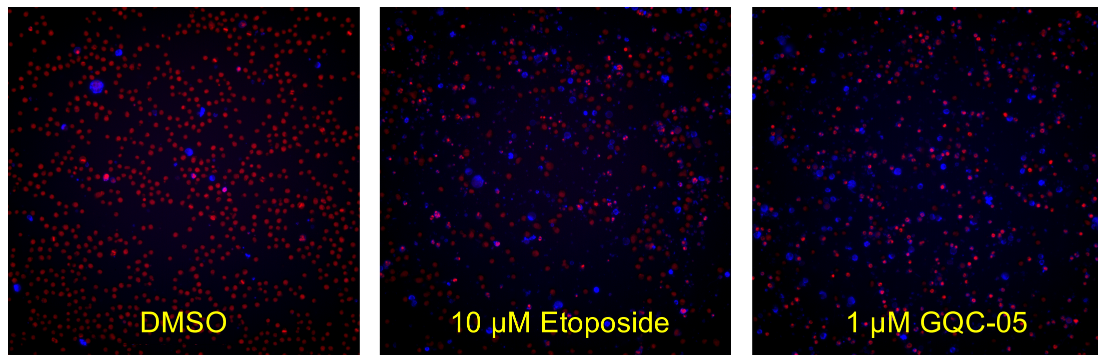
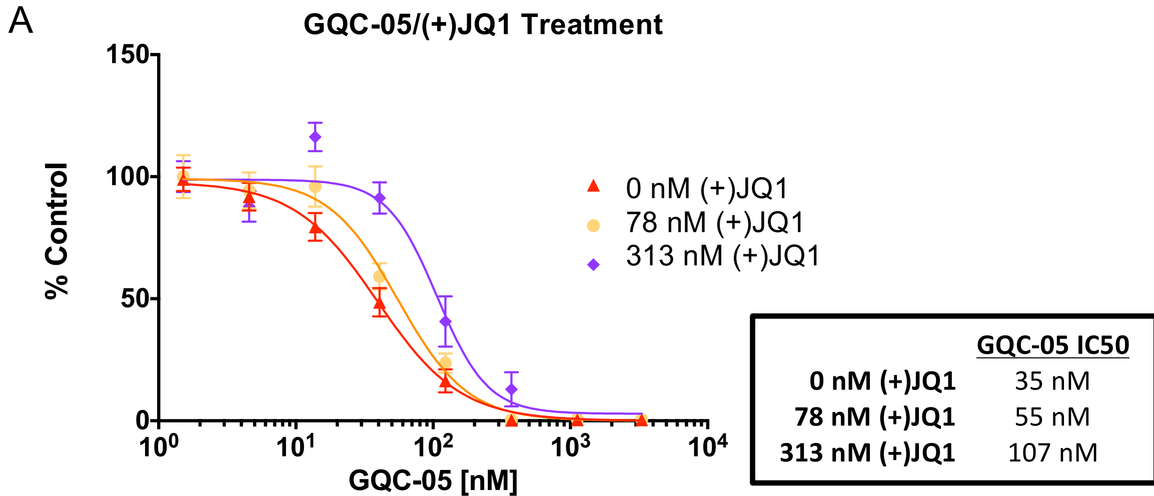


Figure 5. GQC-05 Induces Apoptosis in Sensitive AML Cell Lines. (A) Caspase 3/7 activity in KG-1a, TF-1, and CMK cells after 24 hour treatment. Apoptosis was induced in the GQC-05 sensitive KG-1a cells, but it was not significantly induced in the more resistant CMK and TF-1 cells. Etoposide was used as a positive apoptosis control. KG-1a results are an average of three experimental replicates. All TF-1 and CMK results but the 600 nM GQC-05 treatment are an average of two experimental replicates. (B) The same treated cells were analyzed for PARP cleavage and c-Myc protein expression by western blot analysis. PARP cleavage and c-Myc protein loss were only significantly induced in the KG-1a cells. (C) Annexin V/ Dead Cell Apoptosis Assay of KG1-a cells treated with GQC-05. Analysis was done by flow cytometry using Brilliant Violet Annexin V (x-axis) and Sytox Red (y-axis) staining in the same treated cells. Annexin staining and cell death increased with GQC-05 treatment in KG-1a cells, but not significantly in CMK or TF-1 cells. (D) Fluorescence microscopy analyzing Annexin V (blue) staining in KG-1a cells. The red Vybrant DyeCycle Ruby stain is a cell permeable nucleus marker indicating the location of all cells. These images highlight the increase of Annexin V binding to KG-1a cells after treatment with GQC-05.

3.5 Combined Treatment of AML with GQC-05 and (+)JQ1 Reveals an Antagonistic Effect

In order to examine the effect of a GQC-05 and (+)JQ1 combination treatment on AML cells, KG-1a cells were treated with a range of GQC-05 concentrations in combination with a range of (+)JQ1 concentrations. After 72 hours, viability was measured to determine the compound combination's effect on cell viability. Dose response curves revealed that addition of (+)JQ1 to the cells increased the IC50 of GQC-05 (Fig. 6A). When comparing the loss of viability after treatment with either individual compound to that of the combination treatment, it becomes apparent that the two compounds are antagonistic (Fig. 6B).



	GQC-05 IC50
0 nM (+)JQ1	35 nM
78 nM (+)JQ1	55 nM
313 nM (+)JQ1	107 nM

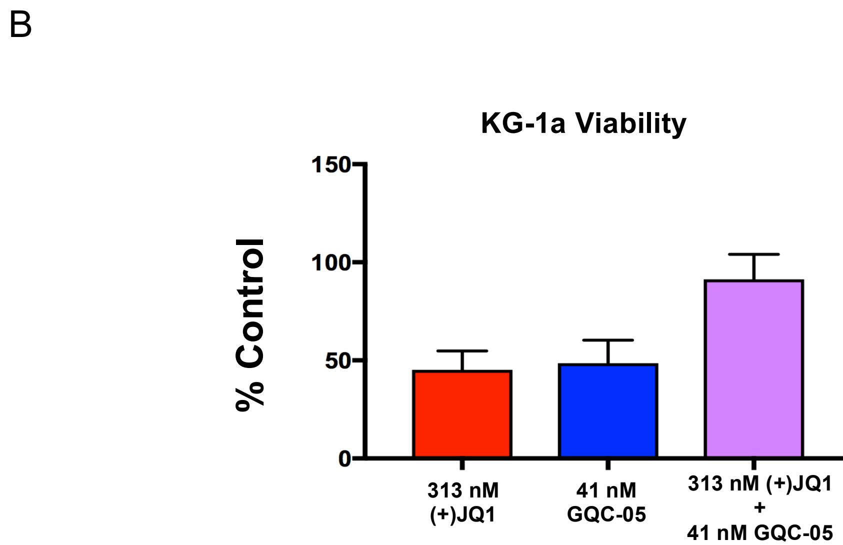
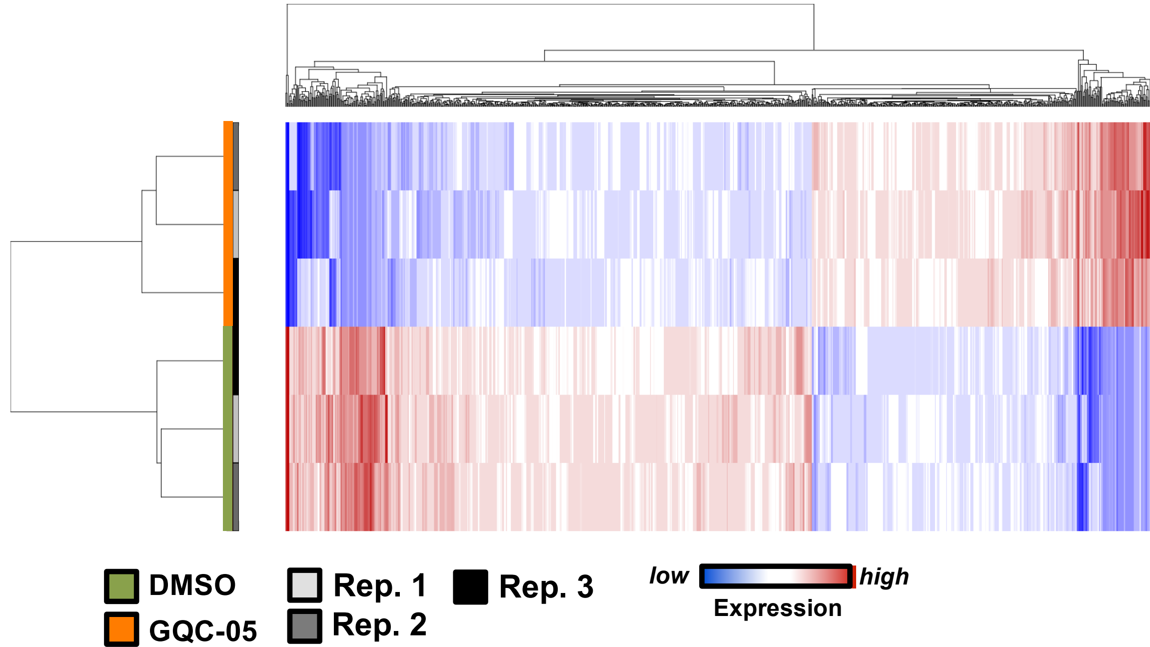


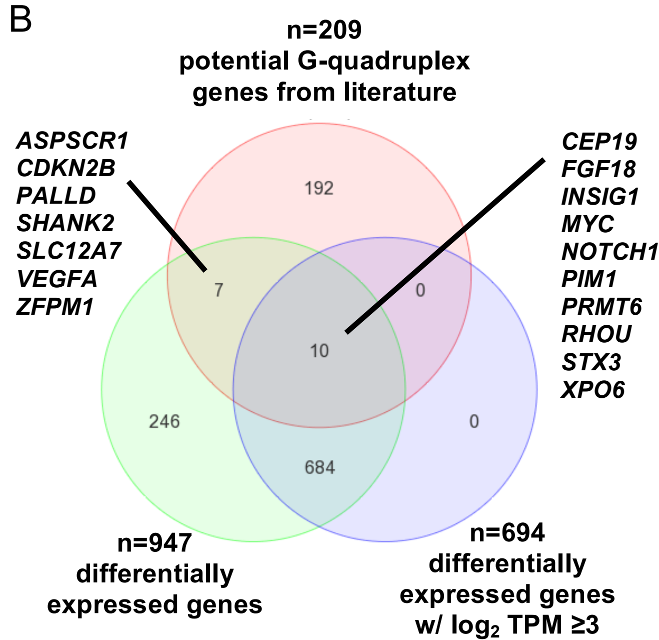
Figure 6. GQC-05 and (+)JQ1 Antagonism (A) 72 hr treatment of KG-1a cells with combinations of various doses of GQC-05 and (+)JQ1 show a decrease in cytotoxicity of GQC-05 when (+)JQ1 is also added. The IC50 values of GQC-05 increase with the (+)JQ1 dose. Viability was measured using a luminescence assay. Two replicates of this experiment produced similar results. (B) Viability in cells treated with either GQC-05, (+)JQ1, or a combination of the two was normalized to viability of untreated cells. This analysis highlights a much larger drop in viability in the two single treatments compared to the dual treatment.

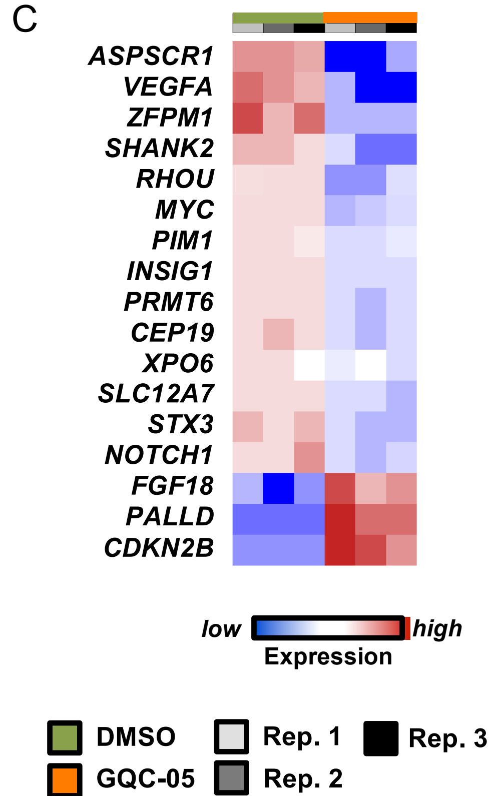
3.6 RNA-seq Reveals Additional Potential Targets of GQC-05

3.6.1 Expression of 17 Potentially G-quadruplex Regulated Genes are Modulated by GQC-05

RNA-seq was performed to better understand the global effect of GQC-05 treatment on AML cells. After 6 hours of treatment with either DMSO (vehicle control) or GQC-05, total mRNA was isolated from the samples. RNA-seq was performed on each sample, and log_2 TPM values were generated for 19,873 protein-coding genes. Of these genes, 947 had a fold change in expression of ≥2 between the DMSO and GQC-05 treated samples, and were considered to be significantly modulated by GQC-05. The three DMSO samples clustered separately from the GQC-05 samples when hierarchical clustering analysis was applied based on the expression of the 947 genes, indicating a high experimental reproducibility (Fig. 7A). To separate genes that may be amplified in the cell or have a greater impact on cellular processes, a list of 694 more highly expressed genes with at least one log_2 TPM value of ≥3 was generated. Genes on either list were cross-referenced with a list of 209 genes known to be associated with high G-quadruplex forming potential (Eddy and Maizels, 2006; Lam et al., 2013) (Fig. 7B). The 17 genes that were modulated by GQC-05 and known to have G-quadruplex forming potential were considered to be potential targets of GQC-05. Most of the 17 genes were downregulated by GQC-05, but three genes were upregulated (Fig. 7C). The significance of the gene expression modulation is highlighted by the relatively constant expression of the housekeeping genes *YWHAZ* and *GAPDH* (Fig. 7D).


A
Hierarchical clustering of 6 samples using 947 genes
DMSO
GQC-05
Rep. 1
Rep. 2
Rep. 3
low
high
Expression


B
n=209
potential G-quadruplex
genes from literature
ASPSCR1
CDKN2B
PALLD
SHANK2
SLC12A7
VEGFA
ZFPM1
CEP19
FGF18
INSIG1
MYC
NOTCH1
PIM1
PRMT6
RHOU
STX3
XPO6
192
7
0
10
246
0
684
n=947
differentially
expressed genes
n=694
differentially
expressed genes
w/ log2 TPM ≥3


C
ASPSCR1
VEGFA
ZFPM1
SHANK2
RHOU
MYC
PIM1
INSIG1
PRMT6
CEP19
XPO6
SLC12A7
STX3
NOTCH1
FGF18
PALLD
CDKN2B
low
high
Expression
DMSO
GQC-05
Rep. 1
Rep. 2
Rep. 3

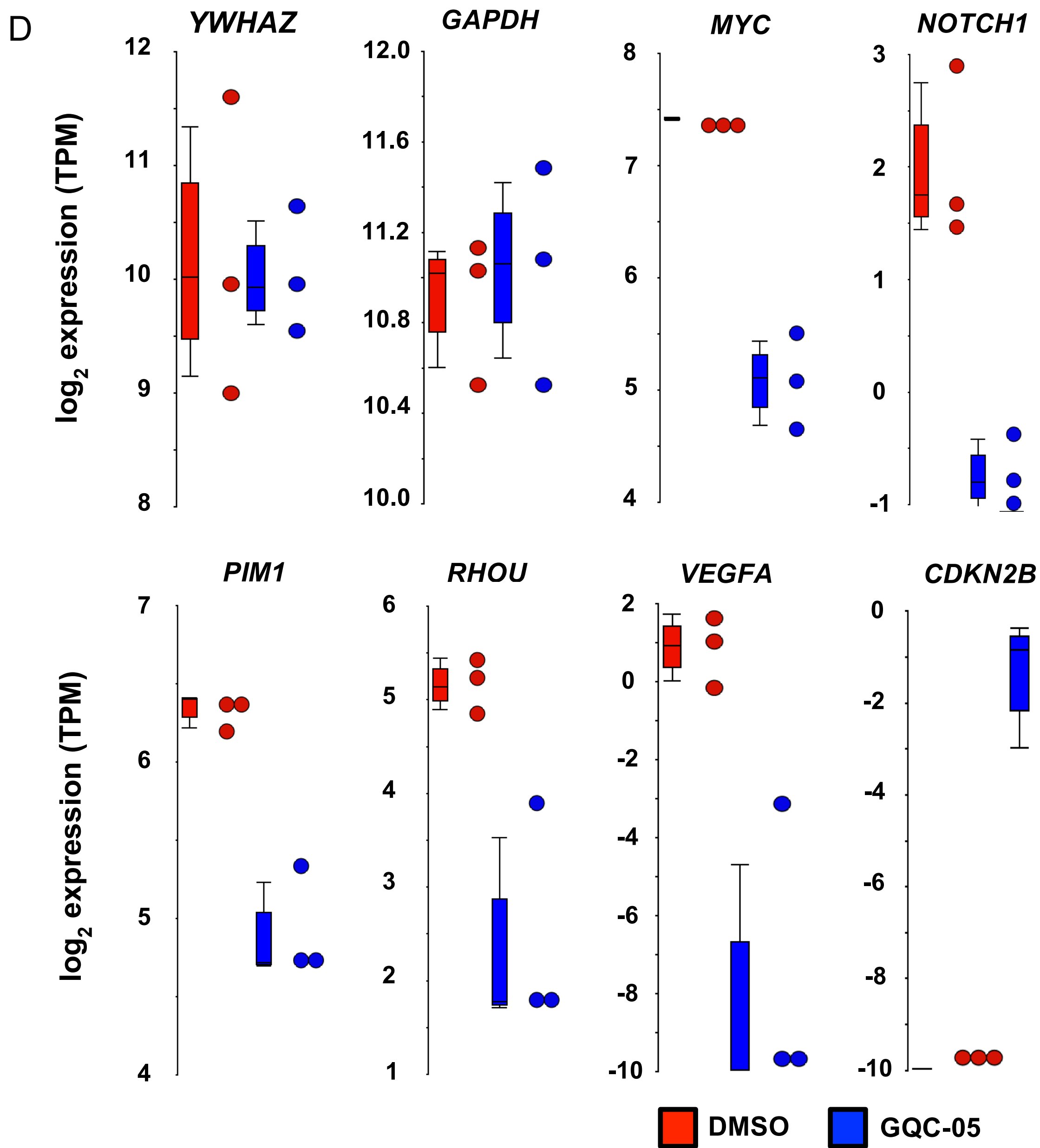


Figure 7. RNA-seq Analysis of KG-1a Treated with GQC-05 Introduces 17 Potential Targets of GQC-05. (A) Hierarchical clustering analysis of the 947 genes determined to have a ≥2 fold difference in expression between the DMSO (vehicle) and GQC-05 treated samples. (B) Diagram demonstrating the overlap between three sets of genes: 209 genes known in the literature to have high G-quadruplex forming potential (red), the 947 genes differentially expressed between DMSO and GQC-05 treated RNA-seq samples (green), and 694 genes from the previous set with one log_2 TPM ≥3 (blue). (C) Relative expression of the 17 genes of interest in each of the RNA-seq samples highlights which genes are upregulated and which are downregulated by GQC-05.

(D) Graphs of the $\log_2$ TPM values from each RNA-seq sample for 6 genes of interest known to have cancer involvement and the 2 housekeeping genes *YWHAZ* and *GAPDH*.

3.6.2 Four Oncogenes with Significant Expression in KG-1a Cells are Downregulated by GQC-05

8 of the 17 genes modulated by GQC-05 are known to be oncogenic (Eddy and Maizels, 2006; Freier et al., 2006; Heimann et al., 2001; Marcucci et al., 2008; Pogue-Geile et al., 2006; Stein et al., 2012; Vega and Ridley, 2008; Wei et al., 2013), and 6 of them were downregulated. Of those 6 genes, 4 had a TPM of ≥3 in at least one sample: *MYC*, *NOTCH1*, *PIM1*, and *RHOU* (Table 2). The International Cancer Genome Consortium (ICGC) Data Portal was used to gather pathway involvement data for each of the 17 genes.

Gene	TPM ≥3	Δ Expression	Cancer Involvement	Pathway Involvement
ASPSCR1	No	Down	Oncogene (fusion: *TFE3*)	Membrane trafficking/ Glucose metabolism
CDKN2B	No	Up	Tumor suppressor	Cell cycle
PALLD	No	Up	Oncogene	Cytoskeleton/ Cell mobility
SHANK2	No	Down	Upregulated	Protein-protein interactions at synapses
SLC12A7	No	Down	-	Ion transport
VEGFA	No	Down	Oncogene	Endothelial growth signaling
ZFPM1	No	Down	Upregulated	Erythroid/ megakaryocytic cell differentiation
CEP19	Yes	Down	-	Centrosome protein
FGF18	Yes	Up	Oncogene	Cell growth/ invasion
INSIG1	Yes	Down	-	Lipid metabolism
MYC	Yes	Down	Oncogene	Transcription (multiple pathways)
NOTCH1	Yes	Down	Oncogene	Cell differentiation/ Development
PIM1	Yes	Down	Oncogene	Cell proliferation/ Survival
PRMT6	Yes	Down	Oncogene	Chromatin rearrangement/ Cell growth
RHOU	Yes	Down	Oncogene	Cytoskeleton/ Cell migration
STX3	Yes	Down	-	Membrane trafficking
XPO6	Yes	Down	-	Nuclear transport

Table 2. Expression and Gene Function of Each of the 17 GQC-05 Modulated Genes.

Chapter 4: Discussion

4.1 Selective Cytotoxicity of GQC-05 on a Subset of AML Cell Lines

Though many cases of AML have been linked to *MYC* overexpression and dependency, a varied cellular response to the known *MYC* inhibitor GQC-05 was expected due to the genetic heterogeneity of AML as a disease (Li et al., 2016). In line with this reasoning, the IC50 values of GQC-05 observed from the panel of 16 AML cell lines ranged from 199 nM to 4.431 μM. These differential IC50s indicated that GQC-05 may be an effective treatment for a subset of AML cell types. To determine if this subset is sensitive because of its expression of a gene targeted by GQC-05, the base expression of both *BCL2* and *MYC* were analyzed. These two genes were chosen because GQC-05 is known to bind the G-quadruplexes in their promoters *in vitro* (Brown et al., 2011). *BCL2* expression did not correlate with GQC-05 sensitivity, but the slight correlation of *MYC* expression to GQC-05 sensitivity suggests that *MYC* inhibition may play a role in the cytotoxicity of GQC-05. However, the correlation is not strong enough to suggest that *MYC* inhibition is GQC-05's only mechanism of action, and there is a possibility that it may be targeting other G-quadruplex regulated genes.

4.2 Mechanism of Action of GQC-05

4.2.1 *MYC* Expression Knockdown

GQC-05 was shown to knock down *MYC* expression more completely in the sensitive AML lines KG-1a and UT-7epo than in the more resistant cell lines CMK and TF-1. GQC-05 also outperformed *MYC* inhibitors GSA 1103 and (+)JQ1 in *MYC* inhibition in the more sensitive cell lines, while all three inhibitors showed similar inhibition in CMK and TF-1. This discrepancy may be due to the expression of another GQC-05 target in the sensitive cells. When this alternative target is inhibited, *MYC* downregulation could be strengthened. This theory is further supported by the inability of *MYC* specific siRNA to reduce cell viability or induce apoptosis as significantly as GQC-05. While this may be due to the incomplete knockdown of *MYC* by the siRNA, the reduction in KG-1a cell viability is only 13% while c-Myc mRNA levels are knocked down 30%. This suggests that incomplete knockdown may not entirely explain the reduced potency, and another gene target needs to be downregulated for the full effect.

4.2.2 Induction of Apoptosis

GQC-05 stimulates an apoptotic pathway to kill sensitive AML cell lines, as evidenced by a significant increase in caspase 3/7 activity, PARP cleavage, and Annexin V staining after treatment. However, apoptosis is not induced in the less sensitive AML cell lines CMK and TF-1, and expression of c-Myc protein in these cell lines at 24 hours has rebounded. This suggests that, because GQC-05 is not able to knock down *MYC* expression as significantly in some AML cell lines, *MYC* expression is able to rebound before apoptosis can be induced. Again, this could be due to GQC-05 mediated inhibition of a secondary target that is partially responsible for *MYC* upregulation in sensitive cell lines.

4.2.3 Antagonism with *MYC* Inhibitor (+)JQ1

The cytotoxicity of GQC-05 in KG-1a cells is reduced when the *MYC* inhibitor (+)JQ1 is added into the mix. Antagonism between these two *MYC* inhibitors offers insight into the molecular mechanism of GQC-05. (+)JQ1 is a bromodomain inhibitor that represses *MYC* by inhibiting the protein responsible for rearranging the chromatin near *MYC* into a transcriptionally favorable form (Filippakopoulos and Knapp, 2014). GQC-05, on the other hand, inhibits *MYC* through G-quadruplex stabilization, and the *MYC*-repressive G-quadruplex forms from single stranded DNA. It is possible that (+)JQ1 and GQC-05 are competing for double stranded and single stranded forms of the same stretch of nucleotides, which leads to an antagonistic combinatory effect. Another explanation for this antagonism could be a secondary GQC-05 target that, when repressed, interferes with the pathway (+)JQ1 targets to repress *MYC* expression.

4.3 Potential Targets of GQC-05

RNA-seq analysis of GQC-05 treated cells identified several genes that were downregulated by the compound. The three non-*MYC* potential targets of GQC-05 with oncogenic significance include the transmembrane receptor gene *NOTCH1*, the signal transduction kinase gene *PIM1*, and the rho GTPase gene *RHOU*.

4.3.1 *NOTCH1*

The *NOTCH1* gene codes for a transmembrane receptor protein involved in development and T-cell differentiation. When Notch-1 proteins receive a signal, an intracellular fragment of the

protein called the ICN detaches, translocates to the nucleus, and directly activates the transcription of *MYC* (Fig. 8), making it a potent oncogene when misregulated (Weng et al., 2006). Notch-1 activating mutations that prolong protein half-life or enable ligand-independent cleavage of the ICN segment are commonly identified in T-ALL, but are also present in some instances of AML (Tohda, 2014). Expression of *NOTCH1* has been shown to vary in AML samples and cell lines, with some having high expression while others have little (Kanamori et al., 2012). A dual inhibition of both *NOTCH1* and *MYC* in *MYC*-dependent AML cell lines that express both genes could have a much stronger *MYC* expression knockdown effect than direct *MYC* inhibition alone. *NOTCH1* is a promising target for many blood cancers, and G-quadruplex mediated inhibition of this gene would be a novel and effective way to treat these diseases.

4.3.2 *PIM1*

Pim-1 is a Ser/Thr protein kinase encoded by the *PIM1* gene. This kinase has been shown to phosphorylate the c-Myc protein, leading to an increase in the stability and amount of c-Myc in the cell (Zhang et al., 2008) (Fig. 8). *PIM1* has been shown to be upregulated and oncogenic in some AML cases, particularly those with the FLT3-ITD fusion (Kim et al., 2005). Inhibition of *PIM1* expression by GQC-05 could explain the high sensitivity of FLT3-ITD positive cell lines MV-4-11 and Molm 13 to GQC-05 despite their low expression of c-Myc protein. This gene is also an important target because the FLT3-ITD mutation in AML has been detected in around 35% of AML cases, and is generally an indicator of poor prognosis (Kindler et al., 2010). An effective treatment for this subset of AML is sorely needed.

4.3.3 *RHOU*

The protein product of *RHOU* is a Rho GTPase capable of inducing reentry into the cell cycle. RhoU, also known as Wrch-1, is capable of activating the protein kinase PAK-1 (Tao et al., 2001). The PAK-1 protein is an important target in AML, and its inhibition leads to apoptosis, differentiation, reduction in AML progression, and downregulation of *MYC* (Pandolfi et al., 2015) (Fig. 8). GQC-05 mediated transcriptional inhibition of the PAK-1 activator RhoU could potentially decrease the amount of active PAK-1 in the cell enough to induce apoptosis and *MYC* downregulation. This indirect inhibition of PAK-1 could be useful for treatment of other cancer

types as well, including breast cancer (Shrestha et al., 2012), bladder cancer (Redelman-Sidi et al., 2013), and non-small cell lung cancer (Mortazavi et al., 2015).

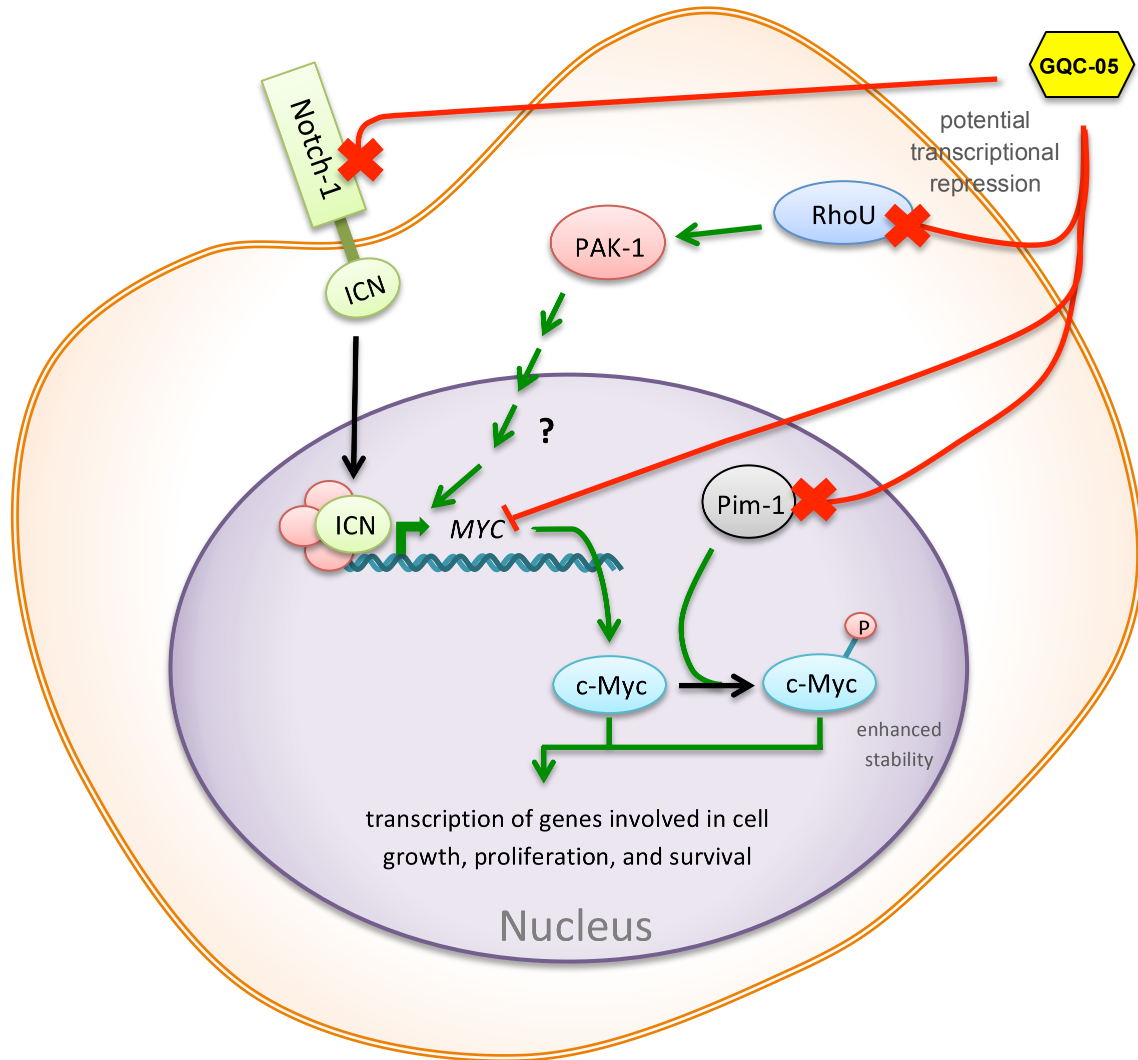


Figure 8. c-Myc Linked Pathways Potentially Targeted by GQC-05: Pathway connections of the three genes potentially downregulated by GQC-05 (*NOTCH1*, *PIM1*, and *RHOU*) to *MYC* regulation and c-Myc stability. After a signal is received, the ICN segment of the Notch-1 protein is cleaved and translocates to the nucleus, where it directly binds *MYC* and facilitates its transcription. RhoU protein activates the PAK-1 kinase, which through an unknown mechanism upregulates *MYC* expression. Pim-1 is capable of phosphorylating and stabilizing the c-Myc protein.

4.4 Conclusions and Future Experiments

MYC inhibition by GQC-05 is integral to the compound's cytotoxicity and ability to induce apoptosis in AML. Though direct inhibition of *MYC* through stabilization of the gene's NHEIII G-quadruplex is certainly likely, it is also probable that downregulation of *NOTCH1*, *PIM1*, *RHOU*, or a combination of the three by GQC-05 may be needed to knock down *MYC* significantly enough to induce apoptosis. Functional validation of the role of these genes in AML will need to be performed. Future testing of AML cell lines for sensitivity to single gene or combination knockdown of these four genes by RNAi or CRISPR could determine which genes are vital for growth and survival of AML cells. After identification of the most important GQC-05 targets, compound libraries could be screened to identify new compounds with structural similarity to GQC-05 that are more specific for the genes of interest. Identification of these compounds is crucial for identification of a compound with reduced toxicity that can be progressed to animal models and potentially clinical trials.

Another area of interest for future studies would be the characterization of any regulatory G-quadruplexes that form in the promoter regions of *NOTCH1*, *PIM1*, or *RHOU*. A better understanding of the regulation of these genes could prove beneficial for more advanced design of gene specific inhibitors that could benefit patients diagnosed with one of the myriad of diseases driven by these oncogenes.

From the studies outlined in this thesis, it can be concluded that *MYC* inhibition through GQC-05 mediated G-quadruplex stabilization is a promising avenue for the development of therapies for AML and potentially other cancers.

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