Targeting the Process of c-MYC Stabilization in Chronic Myelogenous Leukemia

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Abstract

Currently there is no curative therapy for Chronic Myelogenous Leukemia (CML), and patients must remain on the current prescribed treatment, tyrosine kinase inhibitors (TKI), indefinitely. Although many patients can survive in the chronic phase of the disease under TKI treatment, some patients do progress to the terminal blast crisis phase of the disease. Patients in this terminal phase do not respond to TKI treatment. We evaluated the therapeutic benefit of targeting the oncogene c-MYC in CML, using the CML cell line K562. This was achieved by inhibiting the enzyme O-linked β-N-acetylglucosamine Transferase (OGT), using two indirect inhibitors 2-deoxyglucose and Azaserine, and the direct inhibitor ST078925. Treatment with these inhibitors resulted in decreased half-life of c-MYC protein in K562, reduced c-MYC protein in K562 cells, and reduced K562 cell growth. Together these results suggest that targeting c-MYC through OGT may be a potential therapeutic option for patients with CML.

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List of Abbreviations

2-DG: 2-Deoxyglucose
BC: Blast crisis phase of Chronic Myelogenous Leukemia
ChIP: Chromatin immunoprecipitation
CP: Chronic phase of Chronic Myelogenous Leukemia
CLP: Common lymphoid progenitor
CML: Chronic Myelogenous Leukemia
CMP: Common myeloid progenitor
Co-IP: Co-immunoprecipitation
DMEM: Dulbeco's Modiifed Eagle's Medium
GFAT: Glutamine-fructose-6-phosphate-transaminase
GMP: Granulocyte-macrophage progenitor
H3K4me3: Histone H3 lysine 4 trimethylation
H3K27ac: Histone H3 Lysine 27 acetylation
HAT: Histone acetyl transferase
HBP: Hexosamine Biosynthesis Pathway
HSC: Hematopoietic Stem Cell
IP: Immunoprecipitation
iPSC: Induced pluripotent stem cell
UDP-GlcNAc: Uridine diphosphate-N-acetylglucosamine
MEP: Megakaryocyte-erythroid progenitor
MPP: Multipotent progenitors
O-GlcNAc: O-linked β-N-acetylglucosamine
OGA: O-GlcNAcase
OGT: O-GlcNAc transferase
PAGE: Polyacrylamide gel electrophoresis
p-TEFb: Pause-release factor
PTM: Post translational modification
RNA pol II: RNA polymerase II
SDS: Sodium Dodecyl Sulfate
Ser62: Serine residue 62 of c-MYC
TBP: TATA binding protein
TFIIHp89: Transcription factor II H protein 89
THR58: Threonine residue 58 of c-MYC
TKI: Tyrosine Kinase inhibitor

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Introduction

The Transcription Factor c-MYC

Life is very complex, and relies on the careful control and regulation of the immense number of processes and chemical reactions that occur at each and every moment within every cell of the human body. Transcription factors are one of the many ways that cells can regulate which genes are expressed and which are not. Transcription factors can bind DNA and recruit the core transcriptional machinery to initiate transcription, the first step of gene expression (Coulon, Chow, Singer, & Larson, 2013). Many cancers are driven by the aberrant expression or activity of transcription factors, and may present new therapeutic targets.

c-MYC is a proto-oncogene that encodes the transcription factor c-MYC, which in humans is widely expressed during embryogenesis and in highly proliferative tissues. The MYC gene was originally discovered in studies of chicken tumours caused by oncogenic retroviruses (Vennstrom, Sheiness, Zabielski, & Bishop, 1982). This led to the identification of v-MYC. Similar human retroviruses were not discovered. However, the human MYC gene was found to be consistently altered by chromosomal translocations in Burkitt's lymphoma, and it was this discovery that identified c-MYC as an oncogene (Dalla-Favera et al., 1982). Recently, elevated c-MYC protein levels have been identified in various human cancers including myeloid leukemias. Moreover, its deregulated expression is associated with more aggressive tumors (Pelengaris, Khan, & Evan, 2002).

The protein c-MYC belongs to the MYC family of basic helix-loop-helix-leucine zipper transcription factors including n-MYC, and l-MYC (Chi V. Dang, 2012). The role of l-MYC has not

been studied as extensively, however n-MYC can replace c-MYC and rescue defects in mouse embryonic development and impairment of cellular differentiation, and allow mice to survive into adulthood following deletion of c-MYC (Malynn et al., 2000). The 439 amino acid protein c-MYC has two important regions: an N-terminal transcriptional regulatory domain; and, a c-terminal DNA binding domain. A schematic of c-MYC is depicted in Figure 1.1. The N-terminus, is largely unstructured and comprises the transcriptional regulatory region of c-MYC. Within this segment are the highly conserved MYC boxes I and II (MBI and MBII). The proline, glutamatic acid, threonine, and proline rich segment (PEST) is found in the middle of c-MYC and also contains the highly conserved MYC boxes III and IV (MBIII and MBIV). This segment also contains a nuclear localization sequence. Finally, at the C-terminus of c-MYC is the DNA binding and basic-helix-loop-helix leucine zipper (bHLHZ) domain (Conacci-Sorrell, McFerrin, & Eisenman, 2014). In order to bind DNA, c-MYC forms a heterodimer with the small protein MAX, and together they preferentially bind DNA at promoter proximal E-box motifs (5'-CACGTG-3') where they can stimulate gene transcription (Chi V. Dang, 2012). MBII of c-MYC is an important region of c-MYC transcriptional activation domain. It can bind histone acetyl transferase (HAT) complex members such as TRRAP-GCN5, Tip60, and Tip48, which promote histone acetylation, a mark associated with gene expression (Conacci-Sorrell et al., 2014).

As a transcription factor, c-MYC regulates the expression of genes related to cell proliferation, metabolism, apoptosis, and many biosynthetic pathways leading to increased biomass accumulation and cell growth (Kress, Sabò, & Amati, 2015). An illustrative list of the types of genes that c-MYC regulates are summarized in Table 1.1. Genome wide studies of c-MYC binding have revealed that c-MYC preferentially binds E-box motifs (5'-CACGTG-3') in CpG

islands within gene promoter regions (Fernandez et al., 2003). Furthermore, c-MYC bound promoters are associated with the positive epigenetic marks of transcription: Histone H3 lysine 4 trimethylation (H3K4me3); and, Histone H3 Lysine 27 acetylation (H3K27ac). In addition, the promoters are bound by the basal transcriptional machinery and are found within non-condensed or “open” regions of chromatin, as they are within regions sensitive to DNAse I digestion. All these findings demonstrate that c-MYC binds genes which are permissive to transcription (Kress et al., 2015). In addition, induced pluripotent stem cell (iPSC) studies suggested that c-MYC cannot act as a pioneer transcription factor binding condensed chromatin on its own, rather it requires other factors to open chromatin first (Soufi et al., 2015).

Table 1.1. An illustrative summary of the diverse c-MYC regulated genes involved in cell growth, metabolism, regulation of cell cycle, apoptosis, DNA replication, and global transcription. Genes that are activated by c-MYC (↑) or genes that are repressed by c-MYC (↓) are listed. Table is adapted from (Kress et al., 2015).

Categories		**Genes (↑ (activated) or ↓ (repressed) by c-MYC**
Cell Growth	Ribosome Biogenesis	↑ rRNA, Ribosomal proteins
	Protein Synthesis	↑ tRNA ↑ eIF2A and EIF4E
Cell Cycle	Cell Cycle Inhibitors	↓ p15, p21, p27
	Cyclins	↑ Cyclin D, Cyclin E
Apoptosis	Pro-apoptotic genes	↑ BAX, NOXA, BIM
	Anti-apoptotic genes	BCL-2, BCL-X (↓ in primary cells, ↑ in cancer cells)
Metabolism	Glycolysis	↑ HK2, LDHA
	Nucleotide Biosynthesis	↑ RRM2, PPAT, GART, CAD, SHMT
	Amino acid synthesis	↑ SHMT
	Lipid Synthesis	↑ FASN, SCD
DNA replication	Pre-replicative complex	↑ CDT1, MCM5, MCM7, MCM2
	Initiation of DNA replication	↑ CDC7
RNA Related	Global transcription	↓ NELF ↑ GTF2H1, GT2H4

The ability of DNA binding transcription factors to recruit core transcriptional inititiation complexes and RNA polymerase II (RNA pol II) to specific DNA sequences is widely seen as one the principle ways in which transcription factors exert their control of gene expression (Hochheimer & Tjian, 2003). In line with this, c-MYC has been shown to form complexes with the transformation/ transcription domain associated protein (TRRAP) and the histone acetyltransferase GCN5 in order to acetylate histones to promote transcription. In addition c-

MYC interacts with TATA binding protein (TBP), a key member of the pre-initiation complex (McEwan, Dahlman-Wright, Ford, & Wright, 1996). As mentioned previously, c-MYC binds regions of DNA which are already acetylated, which suggests a role for c-MYC to maintain transcriptionally active chromatin. In addition to these findings, recent studies have identified that c-MYC can influence gene expression by releasing promoter-proximal RNA pol II pausing through the recruitment of the pause-release factor (P-TEFb) to actively transcribed genes (Rahl et al., 2010).

Recent studies have examined the genome wide binding of c-MYC under different levels of c-MYC protein expression. This was important to understand how c-MYC can play a role in the development and progression of so many different cancers where aberrantly high levels of c-MYC are observed. In instances of relatively low amounts of c-MYC, it primarily occupies promoter regions marked with H3K4me3 and RNA polymerase II (RNA pol II). As c-MYC levels rise, the amount of c-MYC binding these regions increases, and it begins to occupy other promoter regions at more distal sites marked with active histone marks (H3K4me3, H3K27ac). As the levels of c-MYC continue to rise, it occupies almost all active promoters, including lower affinity E-box motifs. At very high levels of c-MYC, it was found to bind sequences non specifically (Fernandez et al., 2003; Lin et al., 2012). According to this model, in cancers with elevated c-MYC expression the role of c-MYC is to amplify the current gene expression program by accumulating at the promoters of actively transcribed genes across the genome. As previously mentioned, c-MYC can recruit p-TEFb and allow release of paused pol II at many transcriptionally active genes, and it is this function of c-MYC which might underlie its ability to act as an amplifier of gene expression. This may also explain why c-MYC is implicated in many

different cancers across many different tissues. While an initial gene mutation can initiate a change in cell growth or proliferation, the ability of c-MYC to amplify those signals transcriptionally might be the key to realize the drastic proliferative and growth changes observed in tumours (Lin et al., 2012).

c-MYC is a relatively unstable protein with an estimated half-life of 20-30 minutes in healthy cells (Hann, Thompson, & Eisenman, 1985). The levels of c-MYC are regulated at virtually every level of control, and this is all to ensure that c-MYC protein levels are present at the appropriate time during the cell cycle. c-MYC expression is regulated at the transcriptional level by the regulation of RNA Pol II initiation and elongation. It is regulated after transcription by controlling the export of c-MYC mRNA from the nucleus to the cytoplasm, and as well at the post-translational level by various post translation modifications (PTMs) (Tansey, 2014). One important PTM of c-MYC is ubiquitination, as c-MYC protein is rapidly degraded through the ubiquitin/26S proteasome pathway following the addition of this mark (Salghetti, Young Kim, & Tansey, 1999). c-MYC stability has been demonstrated to be regulated by two N-terminal phosphorylation sites within MYC box I (MBI) of c-MYC, namely Threonine residue number 58 (Thr58), and Serine residue number 62 (Ser62). Ser62 phosphorylation by ERK which precedes Thr58 phosphorylation stabilizes c-MYC protein levels. In contrast, Thr58 phosphorylation by the glycogen synthase kinase, GSK-3, promotes c-MYC degradation through the ubiquitin proteasome pathway (Sears et al., 2000). Following phosphorylation at Thr58, Fbw7 a component of an E3 ubiquitin ligase complex, binds c-MYC and directly promotes its ubiquitination, which leads to rapid degradation of c-MYC (Welcker et al., 2004). Other studies have shown that Thr58 is a mutational hot spot in Burkitt's lymphoma, and that these

mutations prevent phosphorylation at Thr58 and stabilize c-MYC protein in these cells (Bahram, von der Lehr, Cetinkaya, & Larsson, 2000; Gregory & Hann, 2000). Finally, recombinant c-MYC that has its Thr58 mutated to an Alanine, has an increased half life in vivo compared to WT c-MYC (Sears et al., 2000). These observations suggest that Thr58 mutations can increase the stability of c-MYC and highlight the importance of Thr58 in the regulation of c-MYC protein stability.

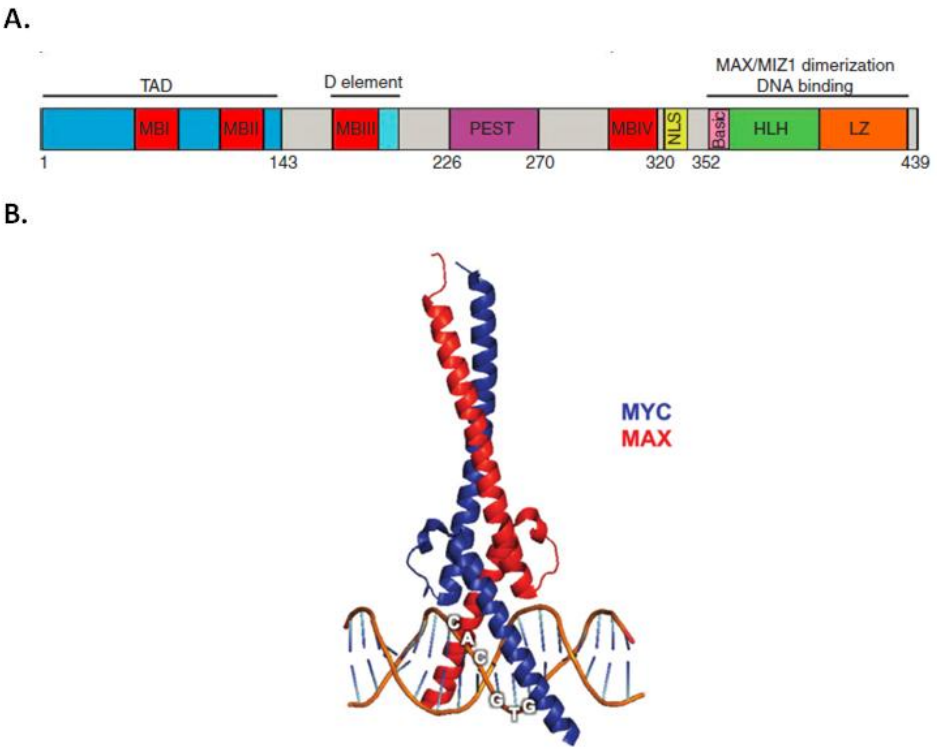

Figure 1.1. (A) Structure of c-MYC. Schematic of the c-MYC protein structure including important elements such as the conserved MYC boxes,I, II, III, IV (MBI, MBII, MBIII, MBIV), and the basic helix-loop-helix/ leucine zipper domain (bHLH-LZ). Figure adapted from (Farrell & Sears, 2014). (B) Schematic depicting c-MYC heterodimerization with the protein MAX, and binding of the heterodimer to DNA at (5'-CACGTG-3') E-box motifs. Figure adapted from (Conacci-Sorrell et al., 2014).

Protein Glycosylation

Protein glycosylation is an important post translational modification of proteins, and plays many diverse roles in the cell, from cell surface signalling to regulating nuclear functions. There are three main types of protein glycosylation: N-linked glycosylation; O-linked glycosylation; and, C-mannosylation (Moremen, Tiemeyer, & Nairn, 2012). O-linked glycosylation, or O-GlcNAcylation, involves the addition of a single O-linked β-N-acetylglucosamine (O-GlcNAc) moiety to the hydroxyl group of Serine (Ser) or Threonine (Thr) residues of proteins. O-GlcNAc, unlike N-linked glycosylation, does not expand to form complex linkages of sugars, but rather remains as a single modified sugar group (Butkinaree, Park, & Hart, 2010). The cycling of O-GlcNAc is mediated by the opposing actions of the enzyme O-GlcNAc transferase (OGT) which catalyses the addition of O-GlcNAc, and the enzyme O-GlcNAcase (OGA) which catalyses the removal of O-GlcNAc (Singh, Zhang, Wu, & Yang, 2015). The addition of O-GlcNAc to a Thr or Ser residue is shown in Figure 1.2.

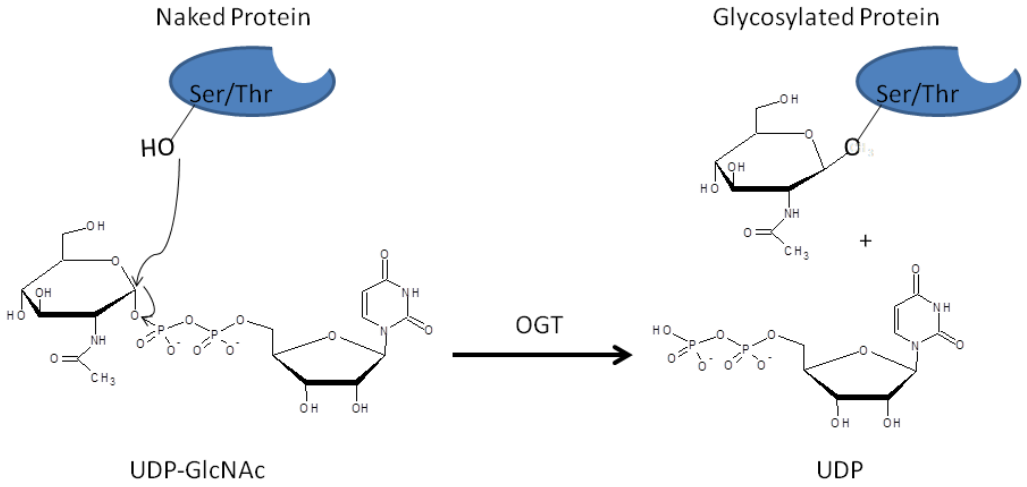

Figure 1.2. Glycosylation of a target protein. Addition of O-GlcNAc to an unmodified Serine (Ser) or Threonine (Thr) residue on a target protein by OGT, from the donor molecule UDP-GlcNAc. Figure adapted from (Lazarus et al., 2012).

O-GlcNAc is found ubiquitously in animals, and is essential to mammalian life. So far, only one gene containing the catalytic domain of OGT has been found in mammals and insects. In addition, the genes encoding human and mouse OGT are highly conserved, both in protein sequence and structure (Hanover et al., 2003; Kreppel, Blomberg, & Hart, 1997). With respect to OGT's importance in the cell, deletion of OGT in mice was found to be embryonic lethal, further supporting the importance of O-GlcNAc (Shafi et al., 2000). In agreement with these findings, O-GlcNAcylated proteins are found both in the nucleus, and cytoplasm, and span a wide array of functional classes of proteins from cytoskeletal elements such as actin, to transcription factors such as the oncogenic transcription factor c-MYC. Furthermore, it is estimated that approximately 25% of O-GlcNAcylated proteins are involved in transcription or translation, highlighting the importance of this mark on the regulation of gene expression (Zachara & Hart, 2006).

The substrate of OGT is the activated sugar molecule Uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is the end product of the nutrient sensing hexosamine biosynthesis pathway (HBP). The HBP is summarized is Figure1.3. ATP is required for the first reaction, however beyond the first step the reactions are irreversible and no longer require ATP input (Bouché, Serdy, Kahn, & Goldfine, 2004). It is estimated that 2-5% of intracellelular glucose is processed by the HBP, and converted to glucosamine-6-phosphate by the rate limiting enzyme glutamine-fructose-6-phosphate-transaminase (GFAT) (Marshall, Bacote, & Traxinger, 1991). As such, the production of UDP-GlcNAc is sensitive to glucose and glutamine availability, as well as the inhibition of HBP enzymes, especially GFAT (Butkinaree et al., 2010). In addition, cells with lower levels of available UDP-GlcNAc have lower levels of protein O-GlcNAcylation (Boehmelt et al., 2000). Together this suggests that protein O-GlcNAcylation can be reduced by limiting glucose availability, inhibiting HBP enzymes, or directly inhibiting OGT itself.

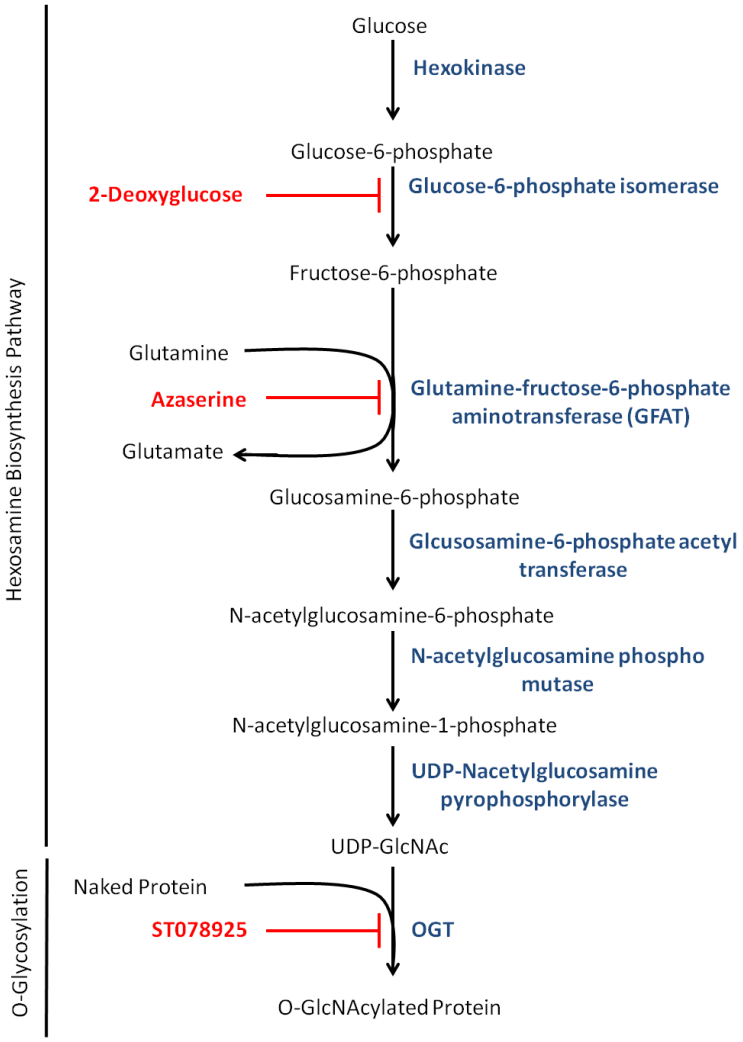

Figure 1.3. Diagram of the Hexosamine Biosynthesis Pathway, depicting key intermediates, enzymes, and inhibitors. 2-deoxyglucose is an inhibitor of Glucose-6-phosphate isomerase (Ralser et al., 2008), Azaserine is an inhibitor of GFAT (Ngoh, Facundo, Zafir, & Jones, 2010), and ST078925 is a direct inhibitor of OGT (Capotosti et al., 2011). Figure adapted from (Butkinaree et al., 2010).

Previously, several studies have used the inhibitor of glucose-6-phosphate isomerare, 2-deoxyglucose (Ralser et al., 2008), and the inhibitor of glutamine-fructose-6-phosphate aminotransferase (GFAT), Azaserine (Ngoh et al., 2010), to reduce the pool of available UDP-GlcNAc, and thereby reducing global protein glycosylation. In addition, high throughput screens

have been employed to discover specific OGT inhibitors (Capotosti et al., 2011; Gross, Kraybill, & Walker, 2005). There is a commercially available analog (ST078925, TIMTEC) of one of the validated OGT inhibitors from this study.

It is believed that OGT plays the role of an important nutrient sensor in the cell. Its substrate, UDP-GlcNAc, relies on inputs from many metabolic pathways including glucose, amino acids, fatty acids, and nucleotides. As such, OGT can link cellular metabolism to signal transduction and transcription. This link between metabolism and O-GlcNAcylation may help to explain why aberrant O-GlcNAcylation is seen in diseases with altered metabolism, such as diabetes and cancer (Ma & Hart, 2014).

Chronic Myelogenous Leukemia

Blood is primarily composed of mature terminally differentiated cells, and like other tissues, it has tissue specific stem cells that are responsible for maintaining it. In the case of blood, these are hematopoietic stem cells (HSCs) which are capable of self-renewal or differentiation to yield more mature daughter cells on the path to ultimately yield terminally differentiated blood cells such as red blood cells, T-cells, macrophages, etc. (Bryder, Rossi, & Weissman, 2006). The earliest work in the field of hematopoiesis was done by Till and McCulloch who demonstrated that some bone marrow cells were capable of forming colonies in the injected spleens of lethally irradiated mice. These colonies contained cells of multiple blood lineages (Till & McCulloch, 1961). This work paved the road for how the hematopoietic system is viewed today; as a pyramidal hierarchy that begins with a multipotent HSC at the top, differentiating into the two blood lineages and mature cells at the base (Doulatov, Notta,

Laurenti, & Dick, 2012). A simplified hematopoietic hierarchy is depicted in Figure 1.4. Briefly, a hematopoietic stem cell (HSC) is capable of undergoing self-renewal, or can differentiate to yield multipotent progenitors (MPP). These MPPs can differentiate to yield the oligolineage committed progenitors: the common myeloid progenitor (CMP); or, the common lymphoid progenitor (CLP). CLP cells can differentiate to yield mature lymphoid cells. The CMP can further differentiate to give rise to a granulocyte-macrophage progenitor (GMP), or a megakaryocyte-erythroid progenitor (MEP), which terminally differentiate to mature myeloid blood cells. The hematopoietic system is highly tuned in that signals that promote proliferation and differentiation are highly regulated to ensure that HSCs are not depleted, and mature cells are not over produced. These regulatory mechanisms that maintain the balance of self-renewal and proliferation are extremely important. If they are lost, bone marrow failure or leukemias can develop (Blank & Karlsson, 2015).

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder resulting from the malignant transformation of a hematopoietic stem cell (HSC) by the BCR-ABL oncogene. BCR-ABL is a constititutively active non-receptor tyrosine kinase that is a product of the reciprocal translocation event between chromosomes 9 and 22. The result of the translocation is the fusion of the Abelson murine Leukemia (ABL) gene from chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22 to generate the BCR-ABL fusion oncogene. This aberrant kinase can then promote cellular growth by activating downstream signalling molecules such as RAS, PI3K, AKT, JNK, and SRC family kinases (Ren, 2005). CML has an incidence of 2 in 100,000 adults, and represents 15% of all newly diagnosed cases of leukemia (Jemal, Siegel, Xu, & Ward, 2010). Patients will typically present in the chronic

phase of the disease with anemia, splenomegaly, and fatigue (Jabbour & Kanterjian, 2014). CML is characterized by an initial chronic phase (CP) which progresses to an accelerated phase (AP) , and finally to a terminal blast crisis phase (BC), where blast cells make up 20% or more of peripheral blood (Radich, 2007; Sawyers, 1999). The current therapies include tyrosine kinase inhibitors (TKI), which target the kinase activity of BCR-ABL, and are effective at inducing remissions and increasing survival of CP CML patients (Eiring, Khorashad, Morley, & Deininger, 2011). Most patients in the chronic phase respond well to TKI treatment, however the survival rates drop from 80% in the chronic phase to 50% in the accelerated phase to 20% in the blast crisis phase (Radich, 2007). Although tyrosine kinase inhibitors are successful at eliminating the bulk of CP CML cells, there is evidence of the persistence of primitive quiescent leukemia stem cells (LSC) in the bone marrow of patients receiving long term conventional treatment (Chu et al., 2011). In agreement with this observation, relapse is often seen after patients discontinue TKI treatments, even in those who have undetectable levels of BCR-ABL. Indeed, in the Stop Imatinib (STIM) study 61% of patients relapsed after cessation of Imatinib treatment. While all the patients who relapsed responded when TKI therapy was reintroduced, this presents several problems (Mahon et al., 2010). Firstly, there are many incidences of patients developing point mutations in the kinase domain of BCR-ABL, rendering the oncogene resistant to first generation TKIs. This has led to the development of second generation TKIs such as dasatinib or nilotinib, and while patients resistant to imatinib do respond to these new treatments, it remains unclear if patients can also develop resistance to these newer drugs (Eiring et al., 2011). Secondly, there are many adverse effects reported with the usage of Imatinib. This is thought to contribute to the lack of compliance and the necessity to reduce dosages in some

patients, which can further contribute to drug resistance. These problems are further compounded by the fact that patients need to remain on TKI indefinitely in order to avoid relapse (Eiring et al., 2011; Mughal & Schrieber, 2010). Therefore, a "cure" for CML patients is unlikely to be achieved through TKI treatment, and a new approach to target CML cells is needed.

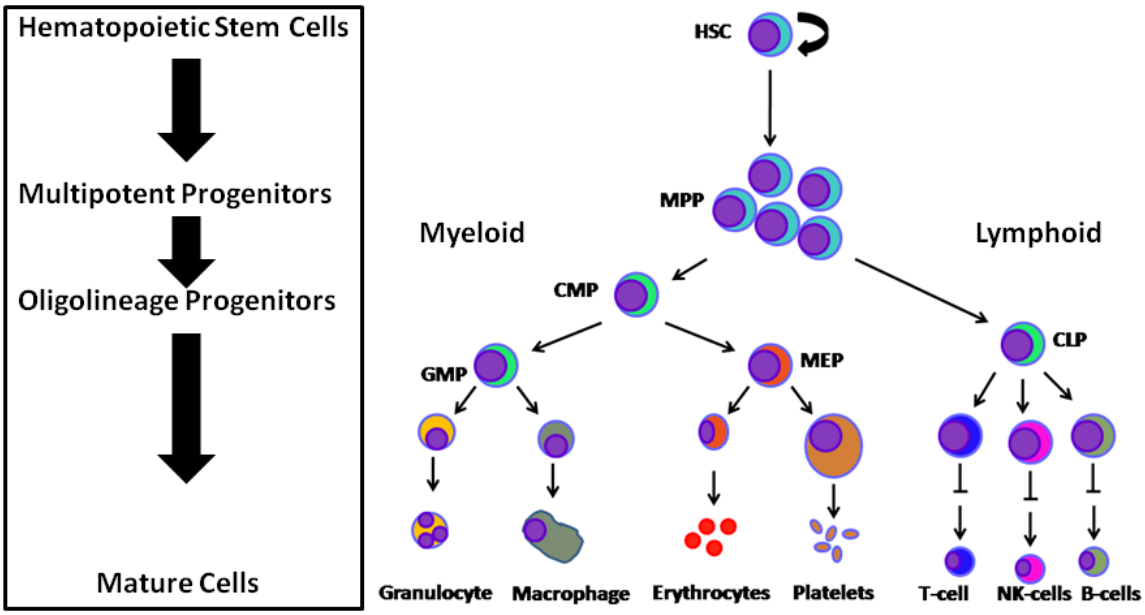

Figure 1.4. Hematopoietic hierarchy pyramid. A hematopoietic stem cell (HSC), is capable of undergoing self-renewal, or can differentiate to yield multipotent progenitors (MPP). These MPPs can differentiate to yield the oligolineage committed progenitors, the common myeloid progenitor (CMP), or the common lymphoid progenitor (CLP). CLP cells can differentiate to yield mature lymphoid cells. The CMP can further differentiate to give rise to a granulocyte-macrophage progenitor (GMP), or a megakaryocyte-erythroid progenitor (MEP) which terminally differentiate to mature myeloid blood cells. Figure adapted from (Blank & Karlsson, 2015).

c-MYC and Chronic Myelogenous Leukemia

The protein c-MYC has been shown to play an important role in many cancers, and it is estimated that c-MYC is involved at least in part in up to 40% of cancers (CV Dang, Le, & Gao, 2009). Indeed in Chronic Myelogenous Leukemia (CML), the causative oncogenic kinase BCR-ABL was shown to be able to induce c-MYC mRNA expression through the activity of the kinase Jak2. BCR-ABL phosphorylates and activates Jak2 at Tyrosine 1007, and Jak2's kinase activity is required for c-MYC mRNA induction (Xie, Lin, Sun, & Arlinghaus, 2002). Transgenic experiments using mouse models of CML have revealed the critical importance of c-MYC protein for the maintenance of leukemia. Mouse models with inducible BCR-ABL expression can initiate a CML like myeloproliferative disease in mice. Mouse models with inducible knockout of the c-MYC gene in hematopoietic cells have also been generated, and these cells can initiate CML if BCR-ABL is also expressed. Once c-MYC knockout is induced, these cells can no longer initiate CML, highlighting the necessity of c-MYC for BCR-ABL to induce oncogenic transformation in these cells. Furthermore, when c-MYC expression is withdrawn from already established tumors, the tumor regresses, and there is reduced proliferation of the CML cells (Arvanitis & Felsher, 2006; Reavie et al., 2013). This suggests that c-MYC plays an important role in initiating and maintaining CML, and that established tumors require c-MC expression in order to survive. In addition, c-MYC was also shown to prevent the differentiation of K562 CML cells induced by imatinib treatment (Gómez-Casares et al., 2013). Finally, it was recently shown that OGT and glucose availability was necessary to maintain c-MYC protein levels and oncogeneicity in a murine model of T-cell leukemia (Swamy et al., 2016).

c-MYC transcript and protein levels are overexpressed in CML cells compared to normal hematopoietic cells, and these levels are further increased in the transition from CP to BC (Reavie et al., 2013). In addition, c-MYC expression was found to be higher in untreated CML patients, as well as in those not responding to imatinib treatment. Moreover, c-MYC mRNA levels at diagnosis could predict response to imatinib treatment, with higher levels leading to a lower probability of response (Llorca, Mauleon, & Vaqu, 2011).

The means by which c-MYC proteins levels are elevated in CML is unclear (Malempati et al., 2006). These results taken together suggest a central role of c-MYC in CML initiation and maintenance. Furthermore, decreasing the levels of c-MYC protein pharmacologically, thereby reducing its transcription activation activity in CML cells, may prove to be an attractive therapy in combination with TKIs to eradicate resistant CML cells. This is supported by recent findings in murine T-cells, where c-MYC protein levels were down regulated following either OGT deletion or inhibition. In this context, OGT was found to be crucial for self-renewal and malignant transformation of murine T-cell progenitors, and it was suggested that it was perhaps mediated through c-MYC (Swamy et al., 2016).

c-MYC and OGT

While c-MYC transcript levels are increased in both CP and BC CML compared to healthy cells, c-MYC protein levels are much more elevated. These results are demonstrated in Figure 1.5. (Reavie et al., 2013). However, the mechanism whereby c-MYC protein levels are stabilized in these cells remains unknown.

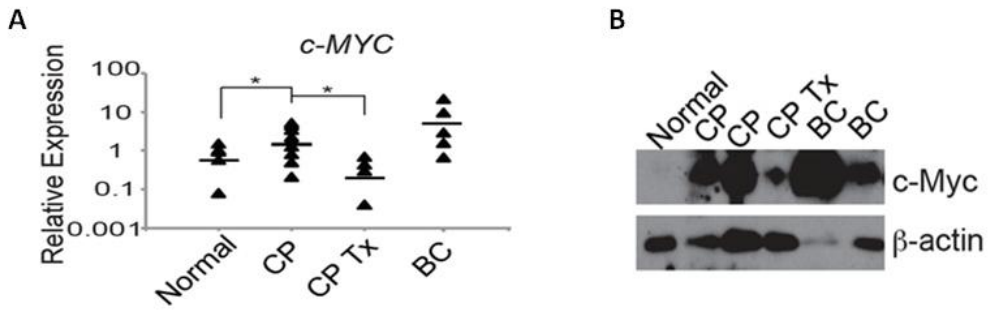

Figure 1.5. c-MYC protein levels are much more elevated than mRNA levels in CML patients compared to healthy persons. (A) Relative c-MYC mRNA levels from normal persons, chronic phase CML patients (CP), chronic phase CML patients undergoing TKI treatment (CP Tx), and blast crisis phase CML patients (BC). (B) c-MYC protein levels from cells of normal persons, chronic phase CML patients (CP), chronic phase CML patients undergoing TKI treatment (CP Tx), and blast crisis phase CML patients (BC). Figure adapted from (Reavie et al., 2013).

In addition to phosphorylation, c-MYC is modified post-translationally by the enzyme O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT) at Thr58 (Chou, Hart, & Dang, 1995; Kamemura, Hayes, Comer, & Hart, 2002). OGT glycosylates c-MYC at Thr58 and this can compete with phosphorylation at this residue thereby increasing the stability of c-MYC. Interestingly, OGT inhibition leads to a decrease in c-MYC protein levels but not a decrease in c-MYC mRNA levels in prostate cancer. Furthermore, the expression of c-MYC was positively correlated with that of OGT in prostate cancer (Itkonen et al., 2013). Finally, it was also found that MYC driven breast cancer cells were sensitive to OGT inhibition and exhibited reduced cellular viability and increase apoptotic markers compared to non c-MYC driven cancer cells (Sodi et al., 2015). It is still unknown whether CML cells are sensitive to OGT inhibition or whether OGT inhibition can reduce c-MYC protein levels in these cells.

Current treatments for CML target BCR-ABL and are successful at eliminating the bulk of CML cells but fail to eradicate leukemia stem cells that drive relapse (Chu et al., 2011). Moreover, no curative pharmacologic therapy exists for CML. Since c-MYC is necessary for initiation of CML, and c-MYC protein levels are aberrantly stable in CML, this leads to the question of whether CML cells can be targeted by reduction of c-MYC protein. This could be achieved by chemically reducing the activity of OGT which is capable of glycosylating c-MYC at Thr58. This would destabilize c-MYC and lead to its degradation by the ubiquitin proteasome pathway (Figure 1.6.). Since CML tumors appear to require c-MYC expression in order to establish and survive, the CML cells would likely die as a result of OGT inhibition.

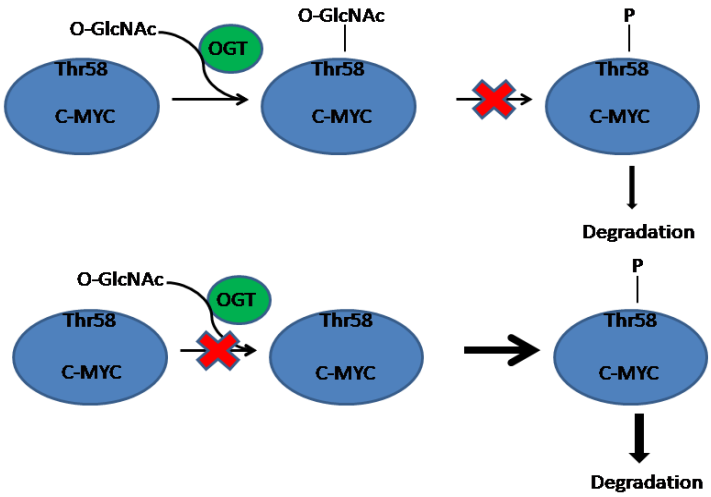

Figure 1.6. OGT can glycosylate c-MYC thereby stabilizing it. Therefore, by inhibiting OGT it may be possible to destabilize the network and disrupt its leukemogenic program.

Rationale:

Currently there is no curative therapy for CML. Patients must remain on the current prescribed treatment, TKI (tyrosine kinase inhibitors), indefinitely. Although many patients can survive in the chronic phase of the disease under TKI treatment, some patients do progress to the terminal blast crisis phase of the disease. Patients in this terminal phase do not respond to TKI treatment. Previous findings and our results suggest that c-MYC is a potential therapeutic target in CML through the inhibition of OGT. Therefore, it is important to explore the possibility of using drugs that can reduce c-MYC protein levels by inhibiting OGT in CML cells, and evaluate their ability to eradicate chronic phase and blast crisis phase CML cells.

Hypothesis:

In chronic myelogenous leukemia (CML), c-MYC forms a complex with OGT that induces the glycosylation of c-MYC at Thr58. This leads to aberrant stabilization and increased levels of c-MYC protein which drives leukemogenesis which then leads to cellular reprogramming of CML cells, such that they lose dependence on the original oncogene BCR-ABL for tumor maintenance and become dependent on c-MYC for survival. By inhibiting OGT and thereby reducing c-MYC levels, the resistance of leukemia stem cells to conventional therapy can be overcome, and the leukemia stem cells can be eradicated.

Specific Aims:

Aim I: Therapeutic analysis of OGT inhibition on c-MYC protein levels, cell survival, and proliferation in the CML Cell line K562.

Aim II: Elucidate the mechanism whereby OGT inhibition affects c-MYC protein stability.

Aim III: Study the c-MYC-OGT complex using immunoprecipitation. Analysis of c-MYC and OGT chromatin binding, using ChIP in the CML cell line K562. Evaluate the effect of OGT inhibition on c-MYC and OGT binding, and the expression of c-MYC target genes

Material and Methods

Antibodies

The c-MYC antibody for Western blotting, Chromatin Immunoprecipitation and, Immunoprecipitation experiments was anti c-MYC (ab32072), purchased from Abcam. The OGT antibody for Western blotting and immunoprecipitation was anti OGT (11576-2-AP), purchased from Proteintech. The TFIIHp89 antibody used for Western blotting was anti TFIIHp89 (SC-293), purchased from Santa Cruz Biotechnology. The MAX antibody used for Western blotting was anti MAX (SC-197), purchased from Santa Cruz Biotechnology.

Cell Culture

K562 cells were grown in Dulbeco's Modiifed Eagle's Medium (DMEM), with 10% fetal bovine serum, and 1% Pennicilin/streptomycin at 37°C. Cells were maintained at a density of 0.5 x 10^6 cells/mL.

Subcellular fractionation and Nuclear Extraction

Cells were concentrated by centrifugation and washed with PBS. The cells were then lysed in swelling buffer (25mM Hepes k pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40) on ice for 30 minutes. The supernatant was kept as the cytoplasmic fraction. Nuclei were pelleted by centrifugation, and then washed 2 times with cold PBS. Nuclei were then resuspended in Buffer C (20mM HEPES pH7.9, 1.5mM $MgCl_2$, 0.6mM KCl, glycerol(25% v/v), 0.5mM DTT, 1X protease inhibitor cocktail), and vortexed on ice every 15 minutes for 1 hour. An equal volume of buffer D (20mM HEPES pH 7.9, 5mM $MgCl_2$, 0.6mM KCl, glycerol (20% v/v) 0.5mM DTT, 1X PIC) was added, and then tubes were centrifuged to pellet debris, and the supernatant was kept as the

nuclear extraction. Samples not used immediately were frozen in liquid nitrogen and kept at -80°C.

Co-Immunoprecipitation

Dynabeads Magnetic beads were chemically cross-linked using dimethyl pimelimidate (DMP) to the specified antibody. Nuclear extraction was prepared from K562 cells. Protein concentration of the nuclear extract was determined by Bradford Assay. The nuclear extract was then pre-cleared with protein A Dynabeads for 1hour at 4°C, and then added to antibody cross-linked beads overnight at 4°C. The beads were washed with IP buffer 150mM. Proteins were eluted from the beads using 1X SDS gel loading buffer (5% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM DTT).

Protein half-life determination

K562 cells were transferred to fresh DMEM medium. Then cells were treated with either 100µg/mL of cycloheximide alone or in combination with 10µM MG132. Whole cell extracts were taken at the indicated time points by resuspending the cells in SDS-PAGE loading dye (200mM Tris ph 6.8, 40% Glycerol, 8% SDS, 0.4% bromophenol blue, 200mM DTT), and subsequently boiling the mixture at 95°C for 5 minutes while vortexing. The same volume of lysate for each time point was then resolved by 10% SDS-PAGE. The relative amount of c-MYC protein compared to the internal loading control was determined through densitometric analysis. The time needed for a 50% reduction of c-MYC protein from the original amount was then calculated.

Chromatin Immunoprecipitation

K562 cells were grown in Dulbeco's Modiifed Eagle's Medium (DMEM). 100 million cells per condition were collected by centrifugation, and then proteins were cross-linked to DNA with 1% formaldehyde in DMEM for 30 mintues at room temperature. The cross-linking reaction was then quenched by adding 100mM glycine, and inverting on a waver for 5 minutes. The cross-linked cells were collected by centrifugation. The pellet was then washed with 1X PBS and the pellet was then frozen at -80°C. The pellet was thawed on ice, and then incubated with 10X packed cell volume of swelling buffer (25mM HEPES ph7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.1% v/v NP-40, 1mM DTT, 1X protease inhibitor cocktail (PIC)) for 15 minutes with vortexing. The cells were then dounced 40X with a Type B pestle. The lysed cells were collected by centrifugation at 4°C. The pellet was then resuspended in 400µL of 1% SDS sonication buffer (50mM HEPES pH7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% w/v Na-deoxycholate, 1% w/v SDS, 1X PIC). The tube was then sonicated in a Bioruptor at 4°C for 25 cycles at high power setting (30 seconds ON, 60 seconds OFF per cycle). The solution was then diluted with sonication buffer without SDS (50mM HEPES pH7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% w/v Na-deoxycholate, 1X PIC) to have a final SDS concentration of 0.1% SDS. Tubes were then spun at 14000 rpm for 15 minutes at 4°C, supernatants were collected, and DNA concentration was measured. 50µL was kept as input for later qPCR. 0.5µg/µL of λ DNA was added as well as 1µg/µL of ovalbumin to the chromatin, which was then pre-cleared on magnetic beads for 2 hours at 4°C. 1000µg of the pre-cleared chromatin was then added to the beads with the indicated antibody, and were rotated over night at 4°C.

Results

c-MYC and OGT are located in K562 nuclei and interact in nuclear extract

We elected to use the human CML cell line K562 for our studies, as it is a Philadelphia Chromosome positive cell line derived from a 32 year old woman in Blast Crisis phase of CML (C. Lozzio & Lozzio, 1975). Firstly, we wanted to examine the subcellular localization of c-MYC and OGT in K562 cells. While c-MYC is a transcription factor and expected to be found within the nucleus, OGT is responsible for glycosylating both cytoplasmic and nuclear proteins, and has been found in both cellular compartments (Smith et al., 2004; Vella et al., 2013). We separated nuclear and cytoplasmic proteins from K562 cells, loaded equal amounts of total protein, and separated them by 10% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE). Probing of the membrane with antibodies against c-MYC and OGT revealed that c-MYC protein was located exclusively in the nuclear fraction, while OGT was located primarily in the nuclear fraction. The proteins TFIIHp89 and TATA Binding protein (TBP) were used as nuclear fraction controls as they are both transcription factors and expected to be located in the nucleus. Tubulin, a cytoplasmic protein was used as a cytoplasmic fraction control (Fig. 2). This demonstrated that K562 nuclear proteins could successfully be isolated, and that the nuclear extract contained both c-MYC and OGT. Next, K562 nuclear extract was incubated with magnetic beads cross linked with either c-MYC antibody or OGT antibody (Fig. 3A,B) in a reciprocal co-immunoprecipitation (co-IP) experiment. The c-MYC IP was successfully able to pull down OGT, and the OGT IP was able to pull down c-MYC. The reciprocal co-IP confirms that c-MYC and OGT can interact in K562 nuclear extract. A pan protein glycosylation antibody (RL2)

was used in an RL2 IP of K562 nuclear extract. The RL2 IP was able to pull down c-MYC protein, and this suggests that c-MYC protein is glycosylated in K562 nuclear extract.

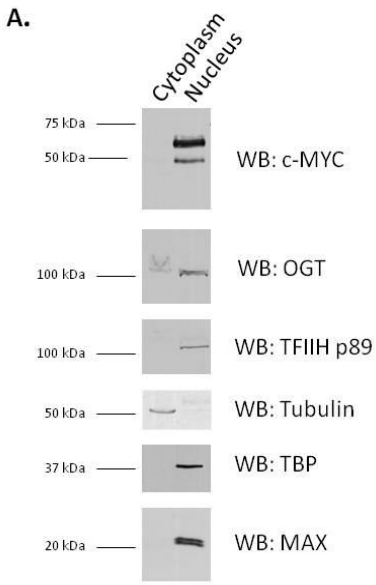

Figure 2. Nucleus and cytoplasmic protein fractionation of K562 cells and resolution by 10% SDS-PAGE. c-MYC, OGT, and MAX are found in the nucleus fraction. TFIIH p89 and TBP (TATA binding protein) were used as loading controls for the nuclear fraction. Tubulin was used as a loading control for the cytoplasmic fraction.

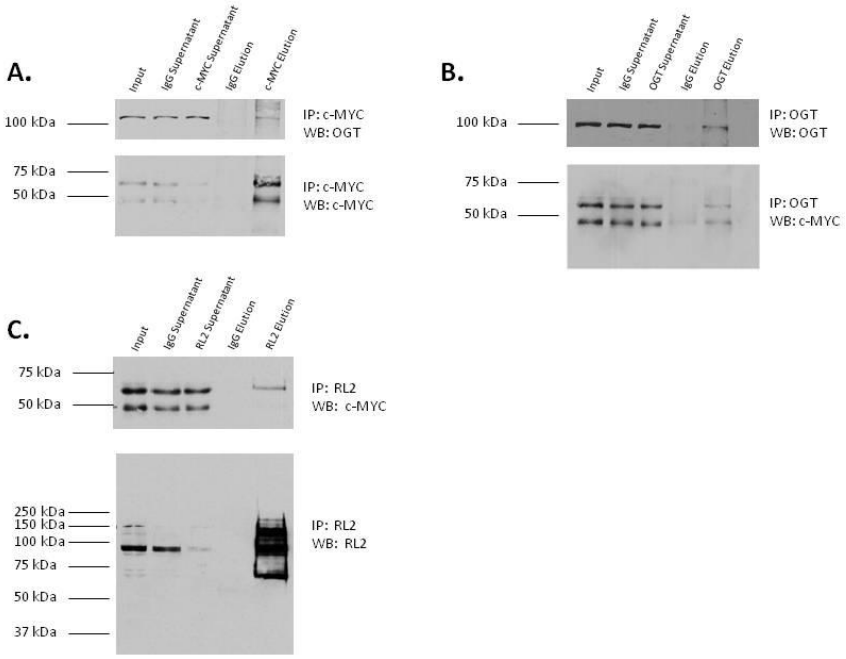

Figure 3. (A.,B) Endogenous c-MYC and OGT interact in K562 cells. Reciprocal co-immunoprecipitation experiments of c-MYC and OGT, from K562 nuclear extract. (C) The pan protein glycosylation antibody RL2 can immunoprecipitate c-MYC. Large scale K562 nuclear was used for the immunoprecipitation experiments.

c-MYC protein levels in K562 are dependent on cell culture time

Initially we wanted to examine the stability of c-MYC protein levels during a period of cell culture time. K562 cells were grown for up to 96h, and nuclear extracts were prepared every 24h (Figure 4.). c-MYC protein levels decreased steadily over the 96h, compared to the loading controls TFIIHp89 or TATA binding protein (TBP). OGT protein levels decreased slightly after 48h, as well as MAX protein levels.

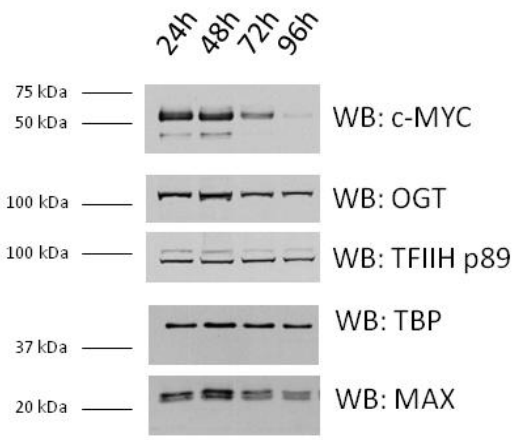

Figure 4. c-MYC protein levels decrease steadily over a 96h culture period. K562 nuclear extracts were prepared at the indicated times, and subjected to 10% SDS-PAGE.

c-MYC protein levels in K562 are dependent on cell culture media glucose levels

In order to eliminate cell culture variables, we demonstrated that glucose levels alone affect c-MYC protein stability. K562 cells were grown in the DMEM media until exponential phase of growth was reached, at which time the cells were transferred to MEM glucose-free media and cultured with increasing concentration of glucose over 48h (Fig. 5). C-MYC protein levels rose in a dose dependent manner with increasing glucose levels (Fig. 5A). In addition, K562 cell growth increased with increasing concentration of glucose (Fig. 5B). These findings suggest that c-MYC protein level and K562 cell growth is dependent on cell culture media glucose concentration.

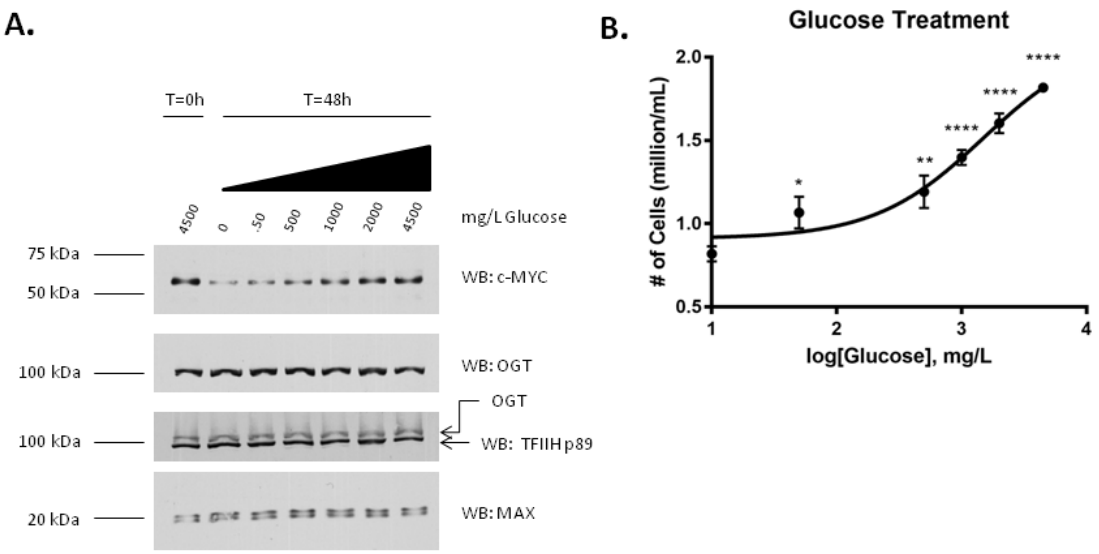

Figure 5. (A) Glucose is required to maintain c-MYC protein levels in K562 cells. Nuclear extract was prepared from K562 cells growing in DMEM medium (T=0). K562 cells were subsequently washed and transferred into minimum essential medium (MEM) without glucose. Different concentrations of glucose were supplemented into the medium, and the cells were allowed to grow for 48h. (B) Cell growth of K562. The cell growth of K562 is greater as glucose concentration increases; statistical analysis was performed by one-way ANOVA (Mean, and SEM, *p<0.05, **p<0.01, ****p<0.0001, n=3).

Inhibitors of OGT reduce c-MYC protein and cell growth in K562

In order to assess the therapeutic benefit of OGT inhibitors, K562 cells grown in DMEM were treated with various OGT inhibitors. 2-Deoxyglucose (2-DG) is a non-metabolizable glucose. It is metabolized by hexokinase to form 2-Deoxyglucose-6-phosphate, but cannot be fully processed into UDP-GlcNAc, the substrate of OGT (Pelicano, Martin, Xu, & Huang, 2006). 2-deoxyglucose should therefore inhibit the ability of OGT to glycosylate proteins, by limiting the pool of UDP-GlcNAc in the cell. Treating K562 cells with increasing concentration of 2-DG resulted in a dose dependent decrease of c-MYC protein (Fig.6A). K562 cell growth also decreased in a dose dependent manner at 48h (Fig.6B).

Azaserine is an inhibitor of GFAT, the rate limiting enzyme of the hexosamine biosynthesis pathway. Inhibiting GFAT reduces the level of UDP-GlcNAc, thereby inhibiting OGT (Olivier-Van Stichelen et al., 2012). For this reason, we decided to treat K562 cells with Azaserine. Treating K562 cells with increasing concentration of Azaserine resulted in a dose dependent decrease of c-MYC protein (Fig. 7A). K562 cell growth also decreased in a dose dependent manner (Fig. 7B).

ST078925 is a specific inhibitor of OGT. Treating K562 cells with increasing concentration of ST07895 resulted in a dose dependent decrease of c-MYC protein (Fig. 8A). K562 cell growth also decreased in a dose dependent manner (Fig. 8B).

From the densitometric analysis of the western blots, the concentration of drug necessary to achieve a 50% reduction of c-MYC protein (IC50) was calculated, and determined to be 1174.9mg/L of 2-deoxyglucose, 5.2µM of Azaserine, and 107.15µM of ST078925 (Table 2). The concentration of drug necessary to achieve a 50% reduction of cell growth (IG50) was also calculated and determined to be 1614mg/L of 2-deoxyglucose, 3.3 µM of Azaserine, and 92 µM of ST078925 (Table 3). Together these results indicate that inhibitors of OGT reduce c-MYC protein and inhibit K562 cell growth in a dose dependent manner.

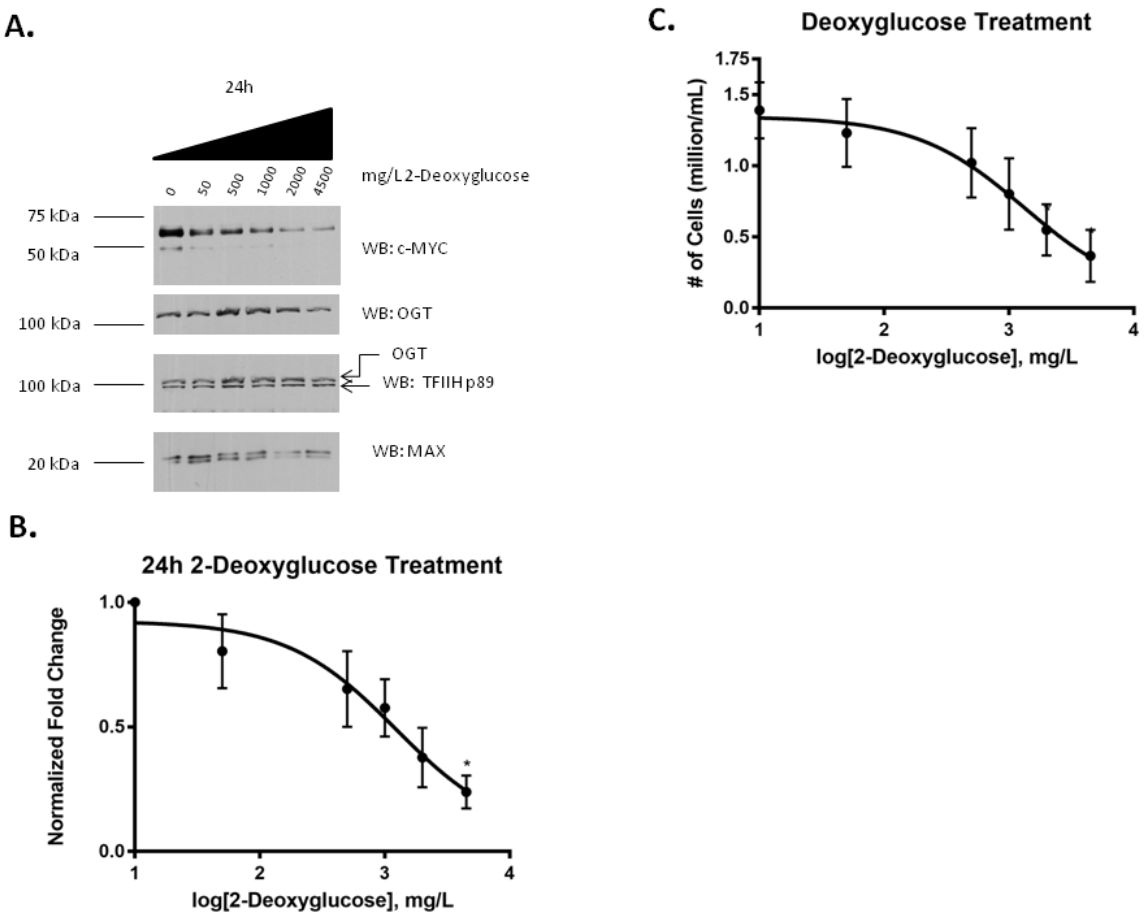

Figure 6. 2-Deoxyglucose (2-DG) reduces c-MYC protein in a dose dependent manner. (A) A representative Western blot of K562 nuclear extract, from 3 independent experiments. K562 cells grown in DMEM medium are treated with a concentration gradient of 2-DG. (B) Densitometric quantification analysis of 3 independent western blot experiments. c-MYC protein levels decrease as the concentration of 2-DG increases; statistical analysis was performed by one-way ANOVA (Mean, and SEM, *$p<0.05$, n=3). (C) K562 cell growth is also reduced in a dose dependent manner upon 2-DG treatment at 48h; statistical analysis was performed by one-way ANOVA (Mean, and SEM, *$p<0.05$, n=3).

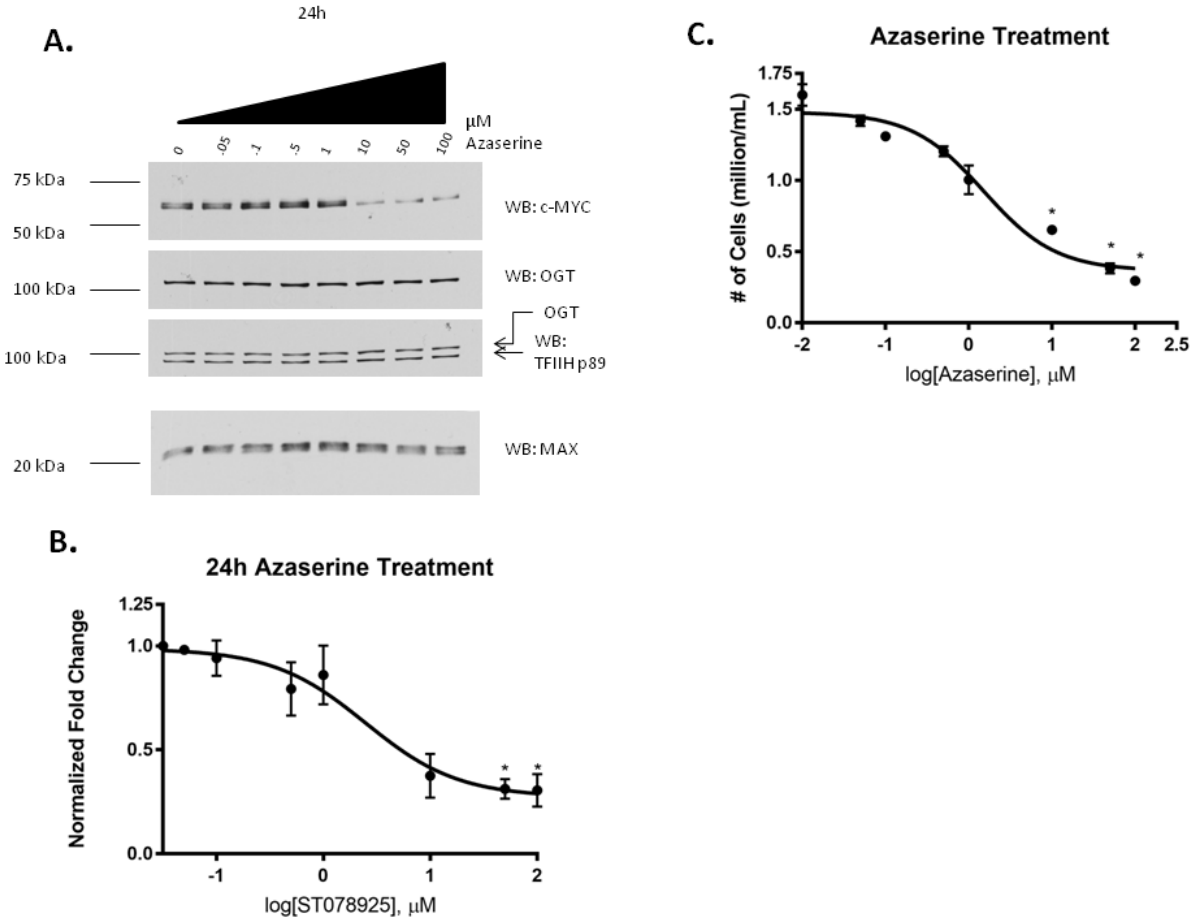

Figure 7. Azaserine reduces c-MYC protein in a dose dependent manner. (A) A representative western blot of K562 nuclear extract, from 3 independent experiments. K562 cells grown in DMEM medium are treated with a concentration gradient of Azaserine. (B) Densitometric quantification analysis of 3 independent western blot experiments. c-MYC protein levels decrease as the concentration of Azaserine increases; statistical analysis was performed by one-way ANOVA (Mean, and SEM, *p<0.05, n=3). (C) K562 cell growth is also reduced in a dose dependent manner upon Azaserine treatment at 48h; statistical analysis was performed by one-way ANOVA (Mean, and SEM, *p<0.05, n=3).

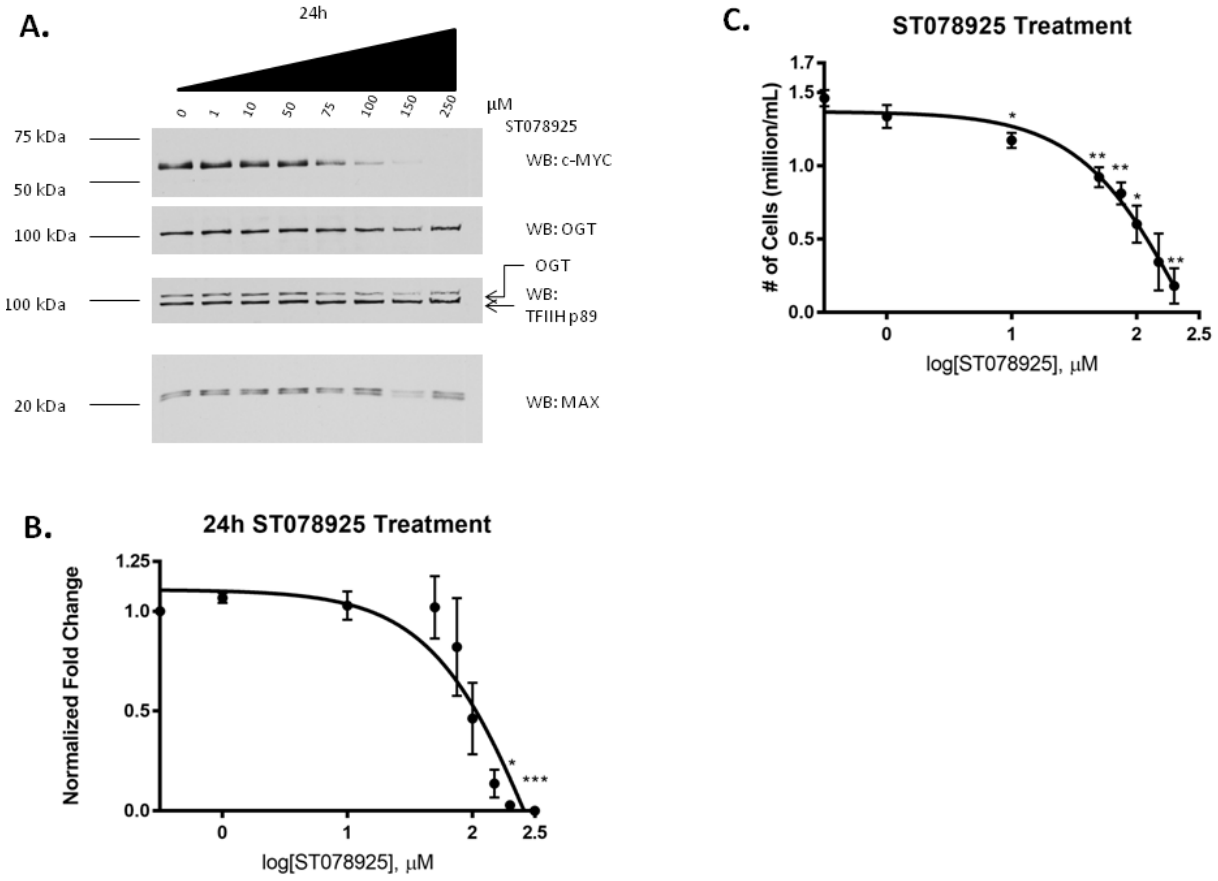

Figure 8. The specific inhibitor of OGT ST078925 reduces c-MYC protein in a dose dependent manner in K562 cells. (A) A representative western blot of K562 nuclear extract, from 3 independent experiments. K562 cells grown in DMEM medium are treated with a concentration gradient of ST078925. (B) Densitometric quantification analysis of 3 independent Western blot experiments; statistical analysis was performed by one-way ANOVA (Mean, and SEM, *p<0.05, ***p<0.001). c-MYC protein levels decrease as the concentration of ST078925 increases. (C) K562 cell growth is also reduced in a dose dependent manner upon ST078925 treatment at 48h; statistical analysis was performed by one-way ANOVA (Mean, and SEM, *p<0.05, **p<0.01, n=3).

Table 2. Table summarizing the calculated concentration of drug required to reduce the amount of c-MYC protein by 50% (IC_{50}) in K562 cells at 24h, +/- SEM, n=3.

Treatment	IC_{50} [ ] c-MYC protein
2-Deoxyglucose	1392mg/L +/-677
Azaserine	7.1µM +/-3.8
ST078925	93.2µM +/-22.9

Table 3. Table summarizing the calculated concentration of drug required to inhibit the growth of K562 normal cells by 50% relative to the vehicle treated condition (IG_{50}) at 48h of treatment. Error is +/- SEM, n=3.

Treatment	IG_{50} [ ] K562 cell growth
2-Deoxyglucose (mg/L)	1614 +/-647
Azaserine (µM)	3.3 +/- 1.3
ST078925 (µM)	92 +/- 15

These results demonstrate that the amounts of 2-deoxyglucose, Azaserine and ST078925 required to reduce 50% of c-MYC protein (IC_{50}) in K562 were 12392mg/L, 7.1µM, and 93.2 µM respectively. In addition the amounts of 2-deoxyglucose, Azaserine and ST078925 required to reduce 50% of K562 cell growth (IG_{50}), were 1614 mg/L, 3.3 µM, and 92 µM respectively.

Combining OGT inhibitors with imatinib

In order to examine the benefit of combining OGT inhibitors with imatinib on cell growth, K562 cells were either treated with an increasing concentration of imatinib alone, or in the presence of a fixed concentration of OGT inhibitor (2-DG, Azaserine, ST078925) (Fig. 9). In all cases the combination of treatments, an OGT inhibitor with Imatinib, yielded a greater reduction of cell growth at 48h.

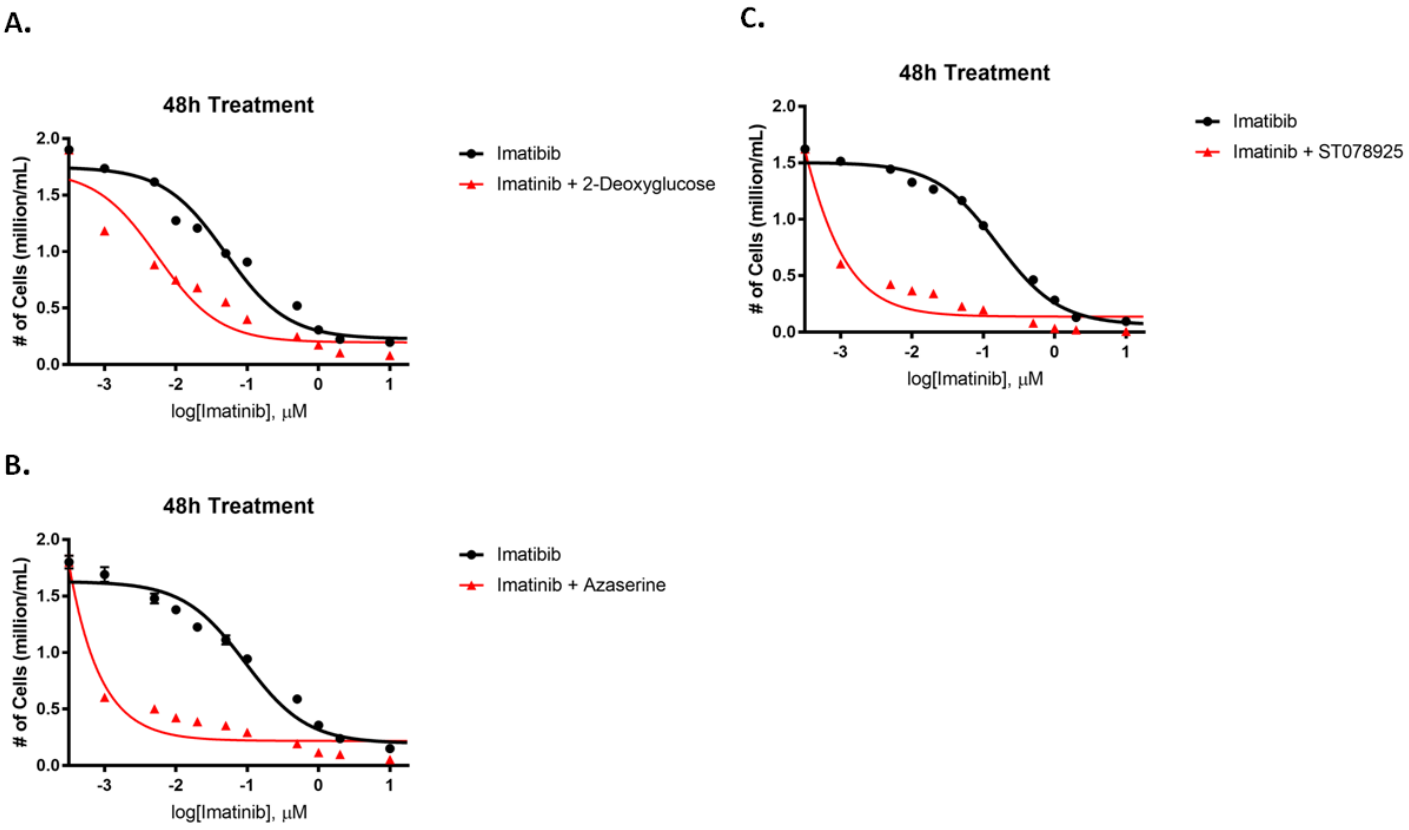

Figure 9. Cell growth curves of K562 cells treated with imatinib alone or in combination with OGT inhibitors. K562 cells were grown in DMEM and treated with either an increasing concentration of imatinib mesylate, or a combination of a fixed concentration of OGT inhibitor with an increasing concentration of imatinib Mesylate. (A) 48h cell growth curve of K562 cells treated with imatinib mesylate alone, or with 998mg/L 2-Deoxyglucose. At 48h a lower dosage of imatinib mesylate is required to achieve a reduction of cell growth if it is combined with 2-Deoxyglucose. (B) 48h cell growth curve of K562 cells treated with imatinib mesylate alone, or

with 13µM Azaserine. At 48h a lower dosage of imatinib mesylate is required to achieve a reduction of cell growth if it is combined with Azaserine. (C) 48h cell growth curve of K562 cells treated with imatinib mesylate alone, or with 125µM ST078925. At 48h a lower dosage of imatinib mesylate is required to achieve a reduction of cell growth if it is combined with Azaserine. The mean is plotted +/- SEM, n=3.

K562 Imatinib resistant cells are sensitive to OGT inhibition

Next we wanted to determine whether an imatinib resistant K562 cell line was sensitive to OGT inhibition. We used an imatinib resistant cell line grown in DMEM with 1µM imatinib mesylate. Treating the imatinib resistant K562 cells with an increasing concentration of 2-DG resulted in a dose dependent decrease of c-MYC protein (Fig. 10A). Imatinib resistant K562 cell growth also decreased in a dose dependent manner at 48h (Fig. 10B). In addition, treating the resistant K562 cells with increasing concentration of Azaserine resulted in a dose dependent decrease of c-MYC protein (Fig. 11A). Imatinib resistant K562 cell growth also decreased in a dose dependent manner at 48h (Fig. 11B). Finally, treating imatinib resistant K562 cells with increasing concentration of ST07895 resulted in a dose dependent decrease of c-MYC protein (Fig. 12A). Imatinib resistant K562 cell growth also decreased in a dose dependent manner at 48h (Fig. 12B). Together these results demonstrate that imatinib resistant cells can be treated with OGT inhibitor to inhibit their growth.

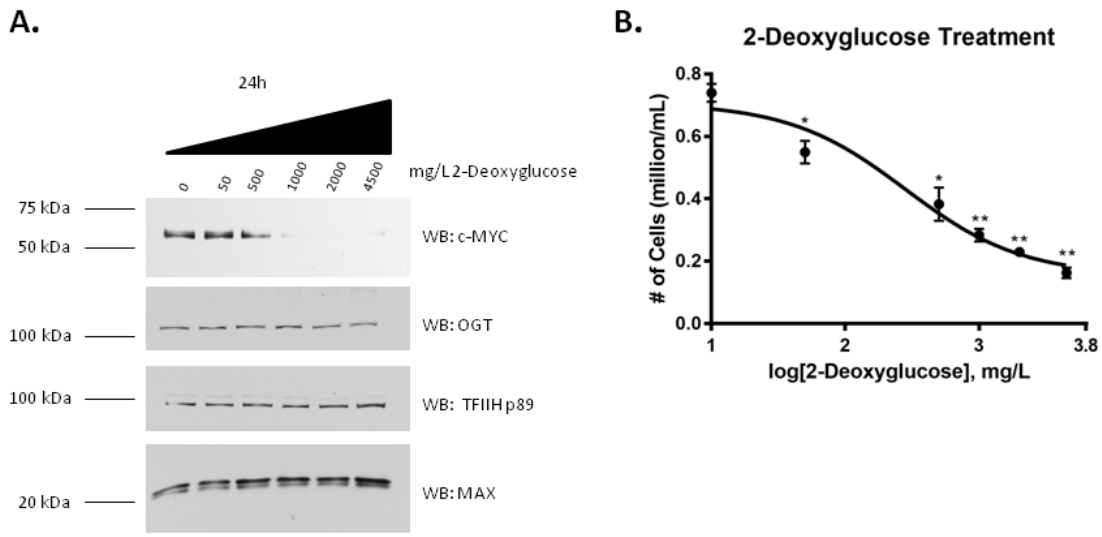

Figure 10. (A) 2-Deoxyglucose (2-DG) reduces c-MYC protein in a dose dependent manner, in imatinib resistant K562 cells. K562 Imatinib resistant cells grown in DMEM medium with 1µM imatinib mesylate are treated with a concentration gradient of 2-DG. 2-DG cannot be metabolized to produce UDP-GlcNAc, the substrate of OGT. The western blot is representative of 3 independent experiments. (B) K562 cell growth is also reduced in a dose dependent manner upon 2-DG treatment both; statistical analysis was performed by one-way ANOVA (Mean, +/- SEM, n=3 *p<0.05, **p<0.01).

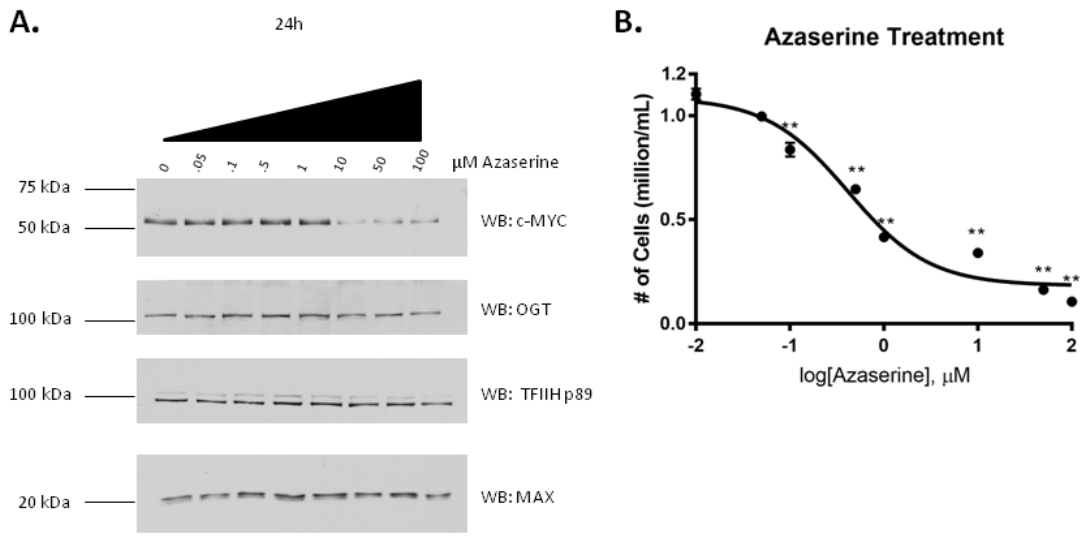

Figure 11. (A) Azaserine reduces c-MYC protein in a dose dependent manner in imatinib mesylate resistant K562 cells. The western blot is representative of 3 independent experiments. K562 imatinib resistant cells grown in DMEM medium with 1µM imatinib mesylate are treated with a concentration gradient of Azaserine. (B) K562 cell growth is also reduced in a dose dependent manner upon Azaserine treatment; statistical analysis was performed by one-way ANOVA (Mean, +/- SEM, n=3, **p<0.01).

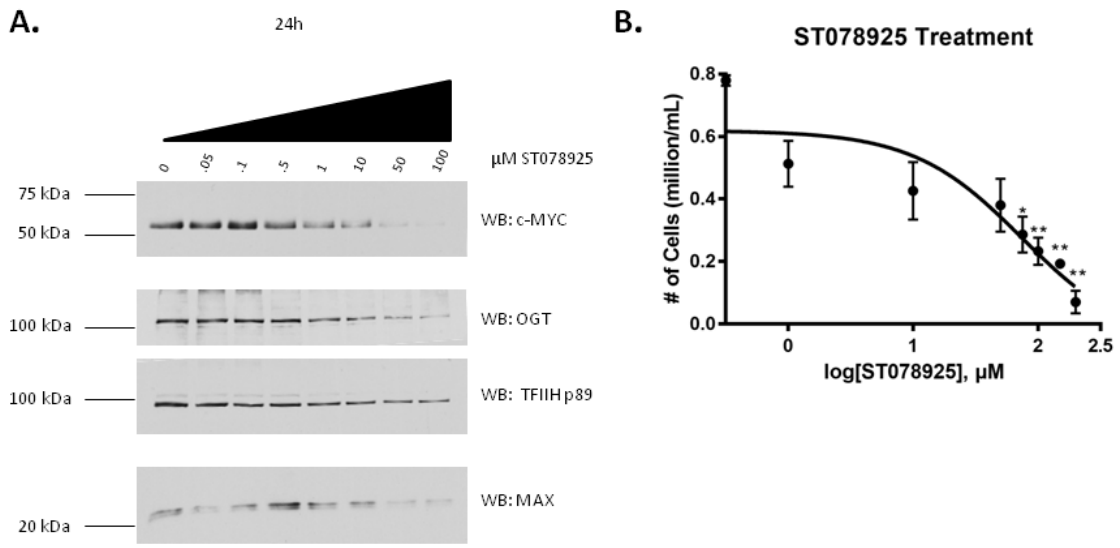

Figure 12. (A) ST078925 reduces c-MYC protein in a dose dependent manner in imatinib mesylate resistant K562 cells. Western blot is representative of 3 independent experiments. K562 imatinib resistant cells grown in DMEM medium with 1µM imatinib mesylate are treated with a concentration gradient of ST078925. (B) K562 cell growth is also reduced in a dose dependent manner upon ST078925 treatment; statistical analysis was performed by one-way ANOVA (Mean, +/- SEM, n=3, **p<0.01).

Inhibitors of OGT reduce c-MYC protein half-life in K562

The next step was to attempt to understand the mechanism of how OGT inhibitors affect c-MYC protein in K562. Firstly, we examined the stability of c-MYC protein over a period of 3 hours after treatment. We treated K562 for 30 minutes with the previously determined c-MYC 24h IC50 concentration of OGT inhibitors (2-DG, Azaserine, ST078925), or 30 minutes glucose starvation (Fig. 13). c-MYC protein was stable for a minimum of 1 hour for all of the treatments. Based on these findings, a treatment time of 30 min was chosen to determine the effect of different treatments on the half-life of c-MYC. Briefly, K562 was treated for 30 minutes with the 24h c-MYC IC50 concentration of 2-DG, Azaserine, ST078925 (previously determined by 2 independent experiments) or K562 was starved of glucose for 30 minutes. Then, either

100µg/mL of cycloheximide alone, or in combination with 10µM MG132 was added. Cycloheximide inhibits protein synthesis while MG132 inhibits protein degradation (Han & Park, 2010; Schneider-Poetsch et al., 2010). MG132 should rescue loss of protein after cycloheximide treatment if the protein is being degraded through the proteasomal degradation pathway (Anwar, Norris, & Fujita, 2011). Whole cell lysates were prepared over a period of 150 minutes and subjected to western blotting. The blots were then used to calculate the half-life of c-MYC (Fig. 14-18). The calculated half-lives are summarized in (Table 4). The treatment of K562 with OGT inhibitors or glucose starvation reduced the half life of c-MYC. This demonstrated that OGT inhibition reduced c-MYC protein stability.

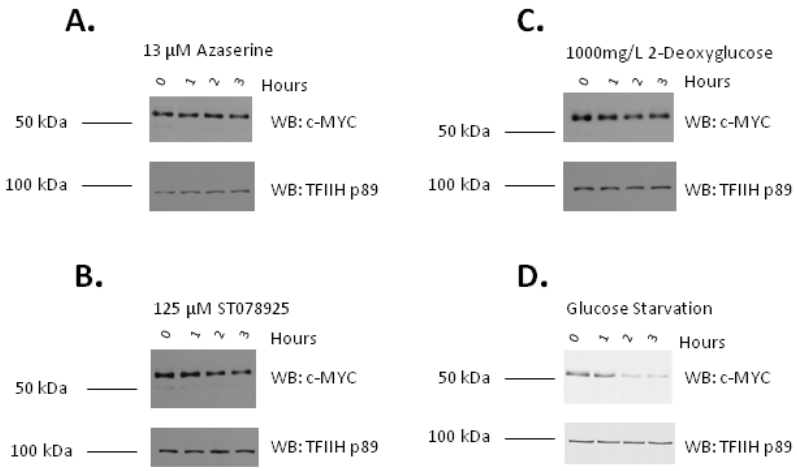

Figure 13. Short term c-MYC protein stability following drug treatment. (A,B,C) K562 cells grown in DMEM were treated with the indicated concentration of OGT inhibitors, and nuclear extracts were prepared at 0, 1, 2, and 3 hours after treatment. c-MYC protein levels appear to be stable up to at least 1 hour. (D) K562 cells were transferred from DMEM to minimum essential medium without glucose and nuclear extracts were prepared at 0, 1, 2, and 3 hours after treatment. C-MYC protein level is stable up to 1 hour.

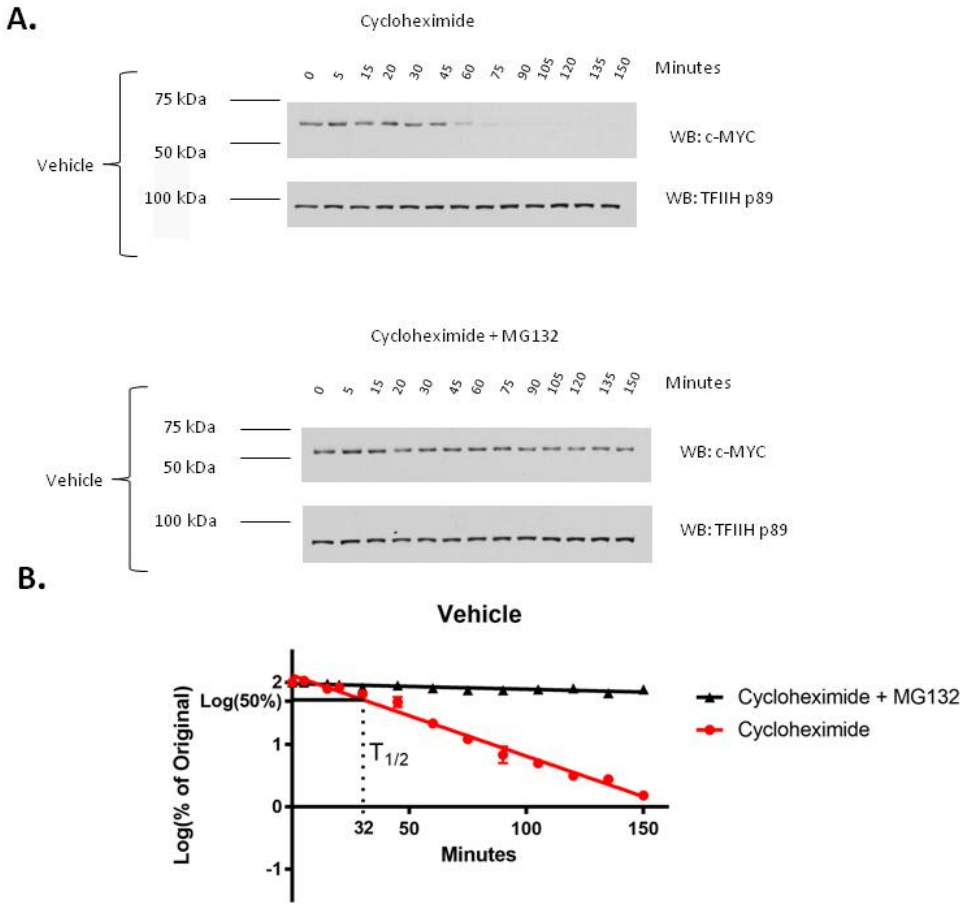

Figure 14. c-MYC half-life in vehicle treated K562 cells. (A) Western blot of K562 whole cell lysate. K562 cells grown in DMEM were resuspended in fresh DMEM media for 30 minutes. Then either 100µg/mL of cycloheximide alone, or in combination with 10µM MG132 was added. Whole cells lysates were prepared up to 150 minutes after treatment. c-MYC protein levels are diminished over time in the cycloheximide only treatment condition. The addition of MG132 rescues the phenotype. Western blots are representative of 3 independent experiments. (B) Densitometric quantification analysis of 3 independent western blot experiments. c-MYC was found to have a half life of 32 minutes (Mean, +/- SEM, n=3).

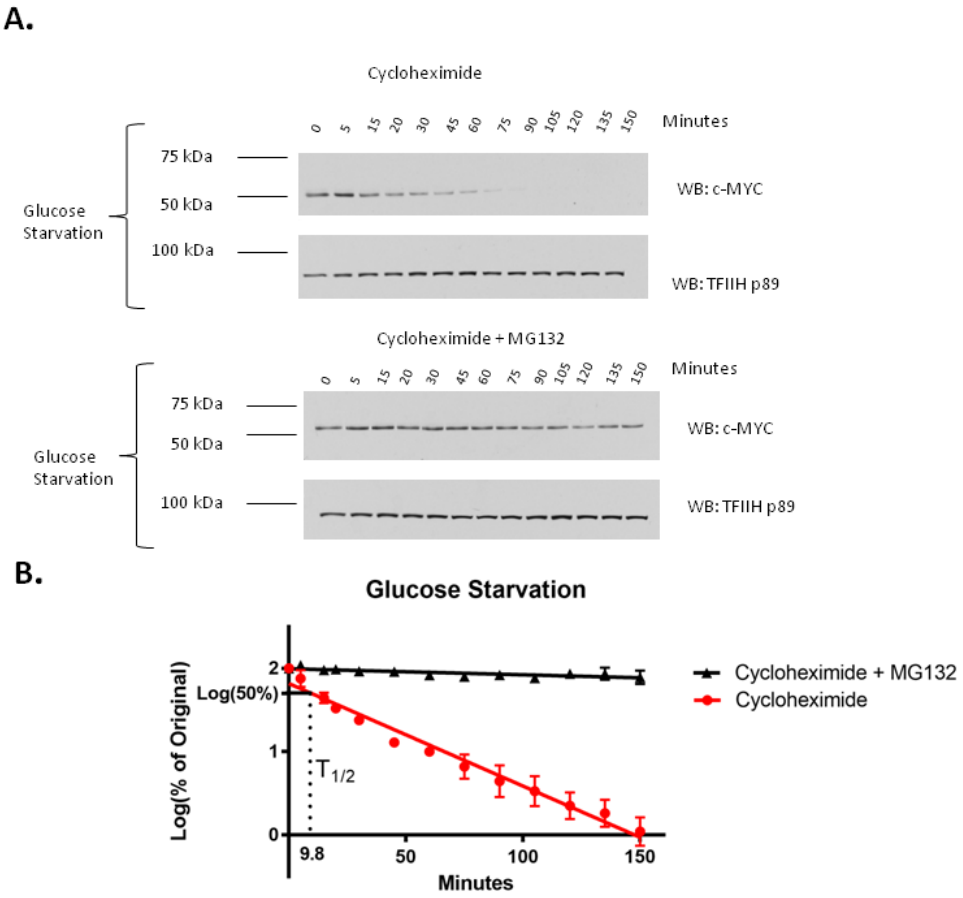

Figure 15. c-MYC half-life in glucose starved K562 cells. (A) Western blot of K562 whole cell lysate. K562 cells grown in DMEM media were resuspended in fresh glucose free media for 30 minutes. Then either 100µg/mL of cycloheximide alone, or in combination with 10µM MG132 was added. Whole cells lysates were prepared up to 150 minutes after treatment. c-MYC protein levels are diminished over time in the cycloheximide only treatment condition. The addition of MG132 rescues the phenotype. Western blots are representative of 3 independent experiments. (B) Densitometric quantification analysis of 3 independent western blot experiments. c-MYC was found to have a half life of 9.8 minutes (Mean, +/- SEM, n=3).

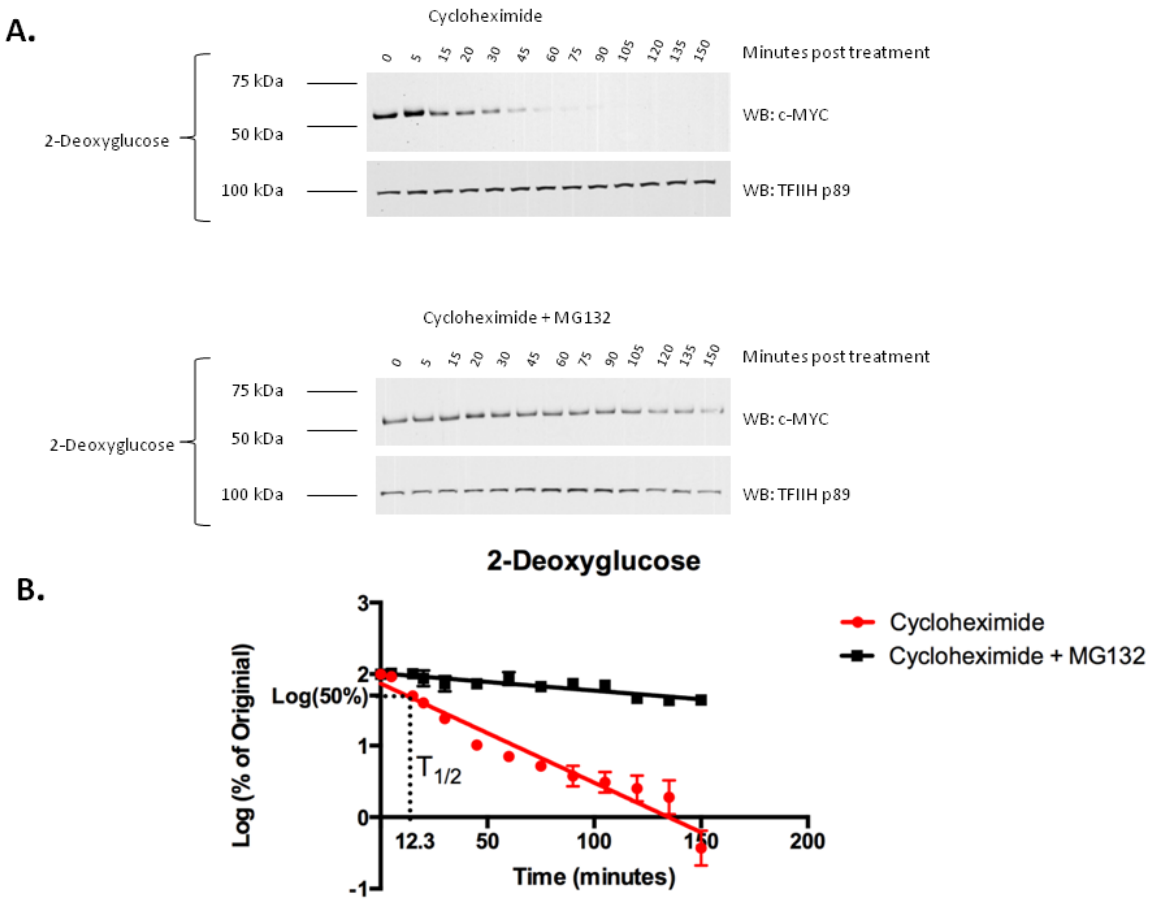

Figure 16. c-MYC half-life in 2-Deoxyglucose treated K562 cells. (A) Western blot of K562 whole cell lysate. K562 cells grown in DMEM were resuspended in fresh DMEM media with 1000mg/L of 2-Deoxyglucoxse for 30 minutes. Then either 100µg/mL of cycloheximide alone or in combination with 10µM MG132 was added. Whole cells lysates were prepared up to 150 minutes after treatment. c-MYC protein levels are diminished over time in the cycloheximide only treatment condition. The addition of MG132 rescues the phenotype. Western blots are representative of 3 independent experiments. (B) Densitometric quantification analysis of 3 independent western blot experiments. c-MYC was found to have a half life of 12.3 minutes (Mean, +/- SEM, n=3).

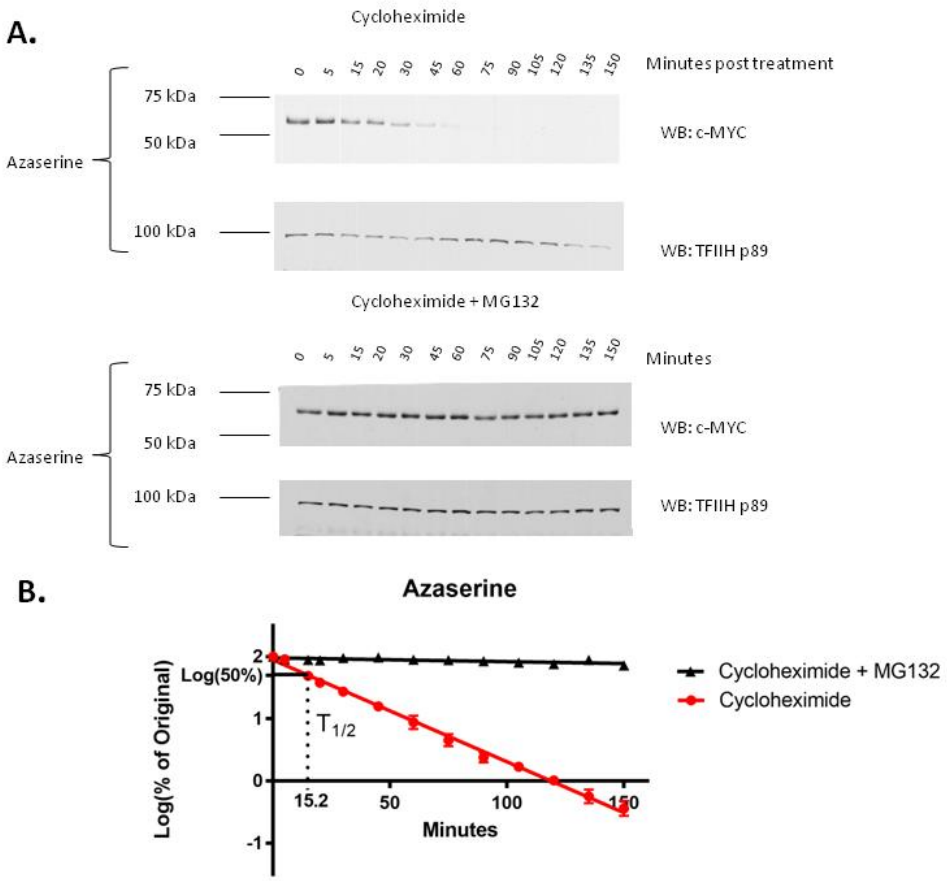

Figure 17. c-MYC half-life in Azaserine treated K562 cells (A) Western blot of K562 whole cell lysate. K562 cells grown in DMEM were resuspended in fresh DMEM media with 13µM of Azaserine for 30 minutes. Then either 100µg/mL of cycloheximide alone, or in combination with 10µM MG132 was added. Whole cells lysates were prepared up to 150 minutes after treatment. c-MYC protein levels are diminished over time in the cycloheximide only treatment condition. The addition of MG132 rescues the phenotype. Western blots are representative of 3 independent experiments. (B) Densitometric quantification analysis of 3 independent western blot experiments. c-MYC was found to have a half life of 15.2 minutes (Mean, +/- SEM, n=3).

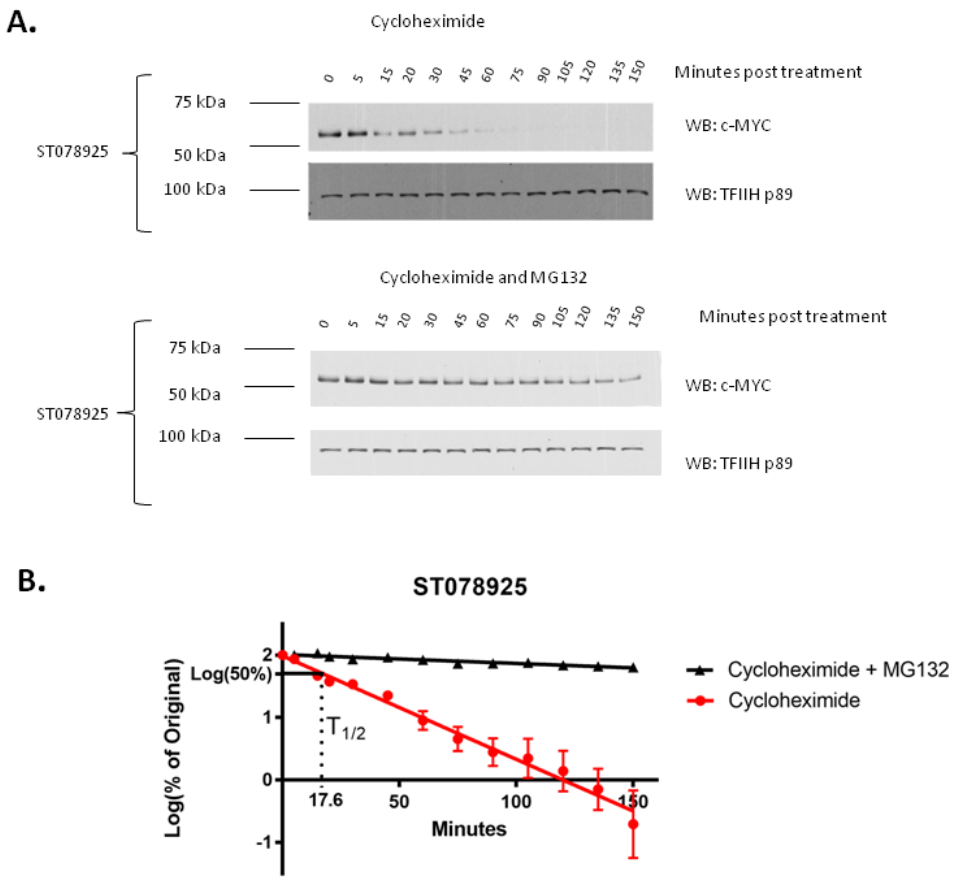

Figure 18. c-MYC half-life in ST078925 treated K562 cells. (A) Western blot of K562 whole cell lysate. K562 cells grown in DMEM were resuspended in fresh DMEM media with 125µM ST078925 for 30 minutes. Then either 100µg/mL of cycloheximide alone, or in combination with 10µM MG132 was added. Whole cells lysates were prepared up to 150 minutes after treatment. c-MYC protein levels are diminished over time in the cycloheximide only treatment condition. The addition of MG132 rescues the phenotype. Western blots are representative of 3 independent experiments. (B) Densitometric quantification analysis of 3 independent western blot experiments. c-MYC was found to have a half life of 17.6 minutes (Mean, +/- SEM, n=3).

Table 4. Table summarizing the calculated half life of c-MYC for the different experimental conditions. Glucose starvation, 2-Deoxyglucose, Azaserine, or ST078925 treatment resulted in a reduced half life of c-MYC, as expected since the treatments should interfere with c-MYC glycosylation. Mean is presented, error represents SEM, n=3.

Treatment	c-MYC Half Life (minutes)
Vehicle	32 +/-1.6
Glucose Starvation	9.8 +/-4
1000mg/L 2-Deoxyglucose	12.3 +/-2.6
13μM Azaserine	15.2 +/-0.2
125μM ST078925	17.6 +/-1.9

c-MYC and OGT colocalize on DNA

In order to examine whether or not c-MYC and OGT colocalize on specific promoter regions, we performed c-MYC and OGT chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (qPCR) in K562 (Fig. 19). A c-MYC ChIP-seq experiment has been previously performed in K562, and c-MYC enrichment is found at the promoter regions of c-MYC and OGT genes (Fig. 19A) (Raha et al., 2010). In our ChIP-qPCR experiment, both c-MYC and OGT were found to colocalize on the promoters of CCND2 and NNT, two known target genes of c-MYC (Lin et al., 2012). c-MYC and OGT were also enriched at the promoter of c-MYC and OGT genes. To test the specificity of this experiment, we performed an additional ChIP with K562 cells cultured for either 24h, or 72h in DMEM. c-MYC protein and OGT protein are downregulated at 72h compared to 24h (Fig. 20A). This made it possible to validate the specificity of the c-MYC and OGT enrichment seen by the ChIP at the target promoter regions. TBP and TFIIHp89 were used as nuclear protein loading controls. Indeed, both c-MYC and OGT were less enriched at the

target promoters in the cells cultured for 72h, compared to those cultured at 24h (Fig. 20B). This demonstrated that the ChIP is sensitive to changes in levels of c-MYC and OGT.

Finally, we wanted to examine whether c-MYC can recruit OGT to promoter regions. To test this we over-expressed FLAG-tagged c-MYC in K562, and confirmed the level of over-expression by western Blot (Fig. 21A). We then performed a c-MYC and OGT ChIP-qPCR, and found an increased enrichment of c-MYC and more importantly OGT at target gene promoter regions CCND2 and NNT, as well as the promoter regions of c-MYC and OGT genes. (Fig. 21B). This suggests that c-MYC is capable of Recruiting OGT to promoters.

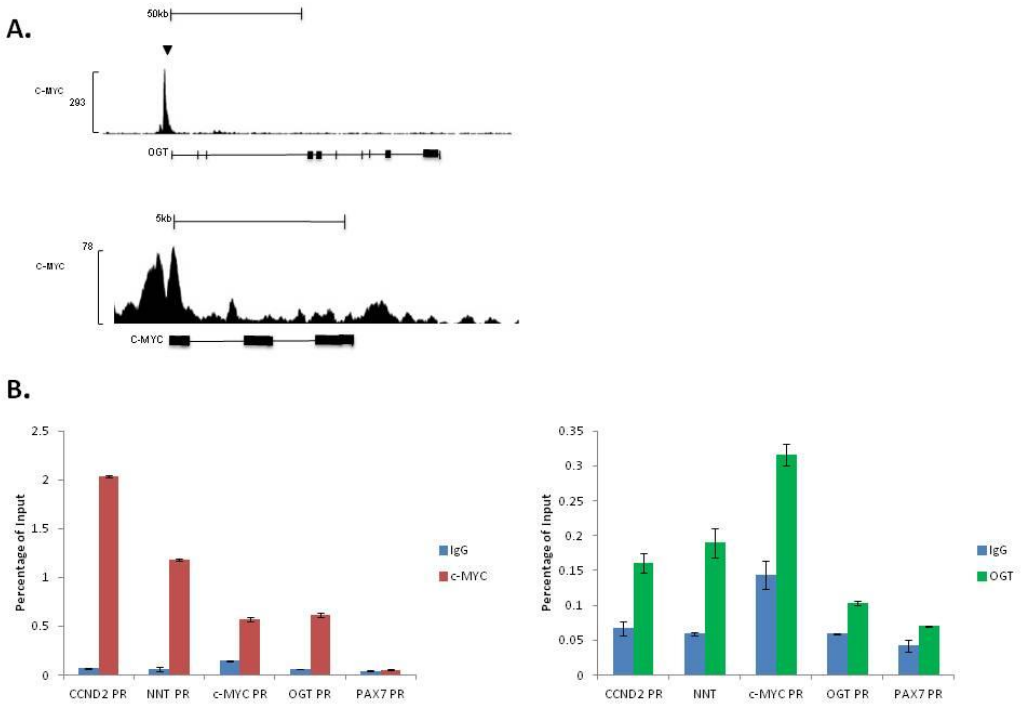

Figure 19. (A) c-MYC binding is detected at the promoters of OGT and c-MYC genes (published ChIP-seq data from (Raha et al., 2010)). (B.) c-MYC and OGT colocalize on DNA at promoters of known c-MYC target genes CCND2 , and NNT as well as the promoters of OGT and c-MYC. The PAX7 promoter region is included as a negative control. c-MYC and OGT Chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (qPCR) (ChIP-qPCR) of chromatin

from K562 cells. Chromatin was extracted from $1x10^8$ K562 cells, and 1000ug of sonicated chromatin was incubated with 5ug of the indicated antibody. Representative results are shown, error bars represent standard deviation of technical duplicates.

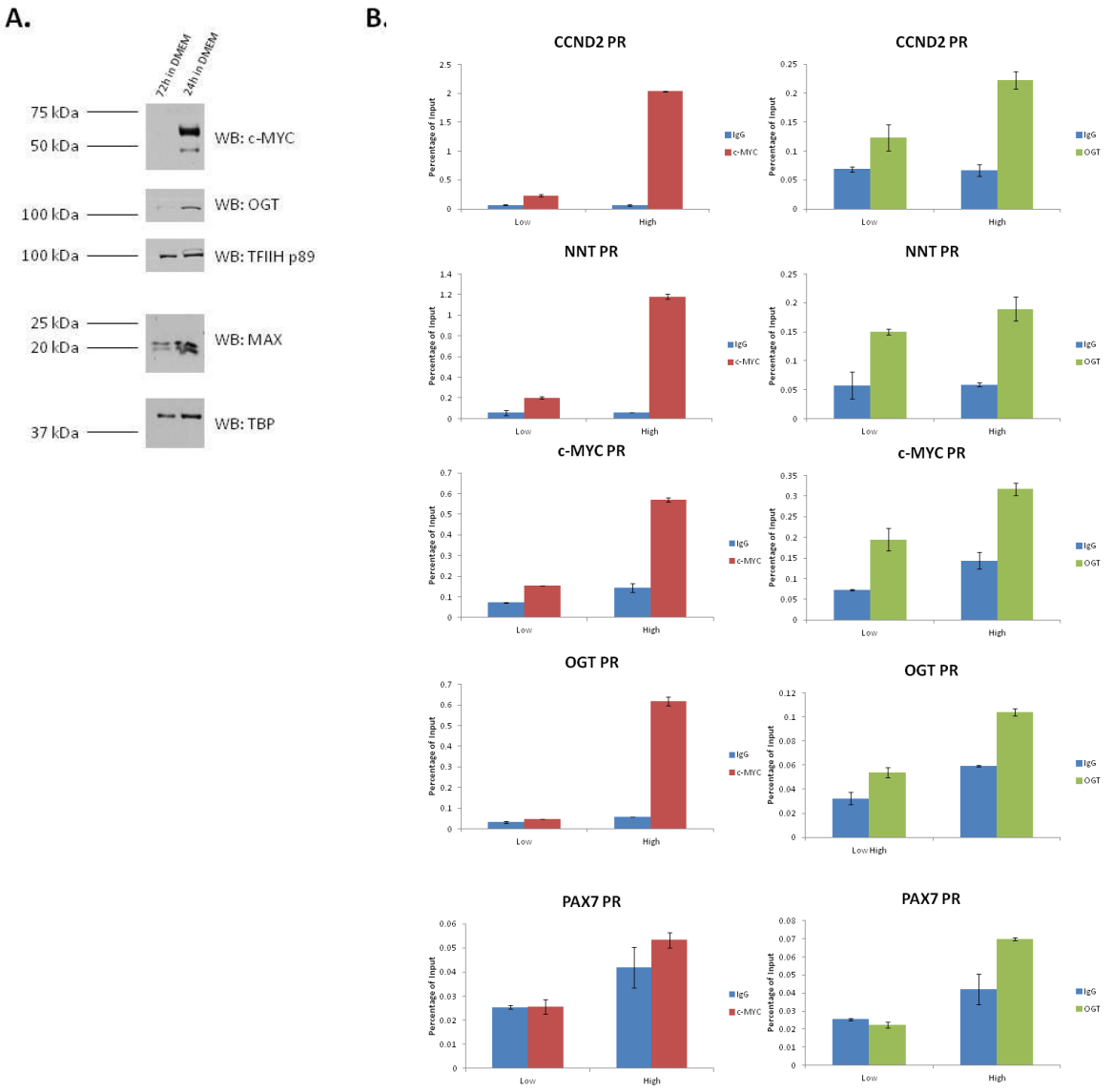

Figure 20. c-MYC ChIP is sensitive to cellular levels of c-MYC in K562 cells. (A) Western blot of nuclear extract from K562 which are either cultured for 72h in DMEM (starvation), or cultured for 24h in DMEM (no starvation). After 72h of culture, c-MYC protein level is almost abolished, and OGT protein level is severely diminished. TFIIH p89 and TBP are included as loading controls.. (B) ChIP-qPCR of starved and non-starved K562 cells. 100 million of cells from each condition were cross-linked, and used for the ChIP. c-MYC and OGT enrichment are decreased in the starvation condition at the promoters of CCND2, NNT, c-MYC and OGT. PAX7 promoter region is included as a negative control. Representative results are shown, error bars represent standard deviation of technical duplicates.

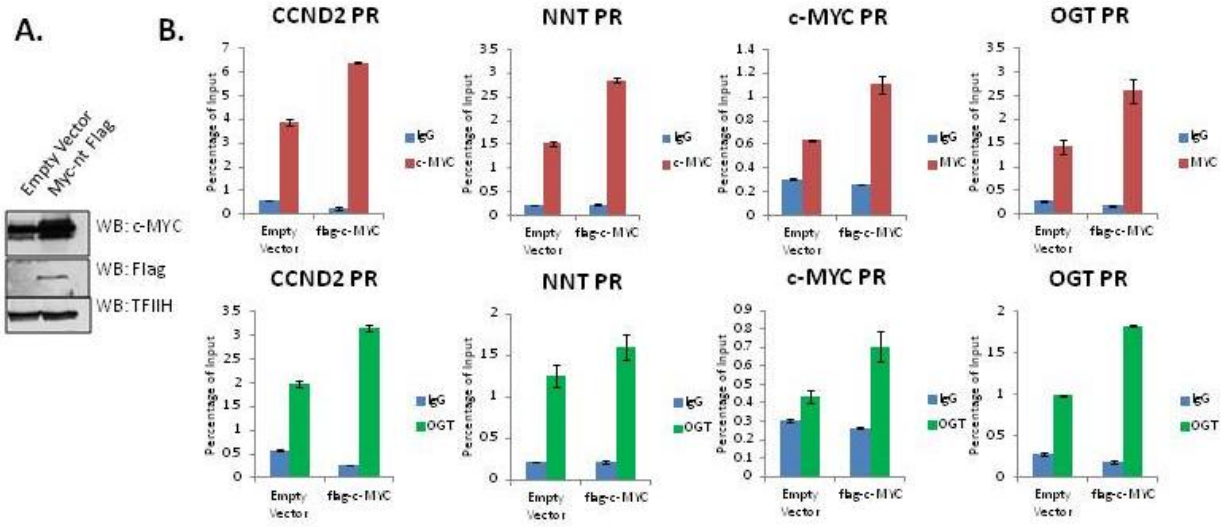

Figure 21. Increased enrichment of c-MYC and OGT when c-MYC is overexpressed in K562 cells. (A) Confirmation of FLAG-tagged-c-MYC expression in K562. (B) When c-MYC is overexpressed c-MYC and OGT enrichment is increased at c-MYC target genes. Error bars represent standard deviation of technical duplicates. c-MYC and OGT chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (qPCR) (ChIP-qPCR) of chromatin from K562 cells. Chromatin was extracted from $1x10^8$ K562 cells stably expressing flag-c-MYC, or GFP (empty vector). 1000ug of sonicated chromatin was incubated with 5ug of the indicated antibody. Red bars are for the c-MYC ChIP, and green bars are for the OGT ChIP. Representative results are shown, error bars represent standard deviation of technical duplicates.

Discussion

The oncogene c-MYC plays a role in many human cancers, including Chronic Myelogenous Leukemia (CML). Current therapies for CML which include tyrosine kinase inhibitors (TKIs) are not successful at eliminating the disease in its entirety, and relapse is often seen in patients who discontinue TKI therapy. In addition, patients can develop resistance to TKIs over time, and this is a serious problem since CML patients must remain on TKI indefinitely in order to prevent relapse (Mahon et al., 2010). Thus, finding additional therapeutic targets for CML may make it possible to prevent relapse and the development of drug resistance, if they are used in conjunction with current treatments.

c-MYC protein was shown to be aberrantly stable by an unknown mechanism in CML (Reavie et al., 2013). One possibility that we wanted to explore, was that c-MYC is stabilized post-translationally by the enzyme OGT. c-MYC can be glycosylated at Thr58, a residue which when phosphorylated by GSK-3, promotes degradation of the c-MYC protein. We thus wanted to evaluate if OGT can function to stabilize c-MYC protein in CML.

Firstly, we wanted to examine the subcellular localization of c-MYC and OGT in K562 cells. While c-MYC is a transcription factor and expected to be found within the nucleus, OGT is responsible for glycosylating both cytoplasmic and nuclear proteins, and is thus found in both cellular compartments (Smith et al., 2004; Vella et al., 2013). We were able to successfully separate nuclear, and cytoplasmic proteins from K562 cells (Fig. 2). As expected, c-MYC is localized exclusively in the nuclear fraction. In addition OGT was primarily detected in the

nucleus, while some protein was found in the cytoplasm. These results confirm that c-MYC and OGT are both found in the nucleus of K562 cells.

After confirming that c-MYC and OGT protein are found in K562 nuclear extract, we demonstrated that endogenous c-MYC and OGT can interact with each other in K562 nuclear extract. To achieve this, we immunoprecipitated (IP) c-MYC and OGT from K562 nuclear extract, and subjected the elutions to western blotting (Fig. 3). The results from these reciprocal IPs, confirm that endogenous c-MYC and OGT proteins can interact in K562 nuclear extract. c-MYC was previously shown to be glycosylated at Thr58 by sequential manual Edman degradation sequencing (Chou et al., 1995). We also wanted to test whether we could detect glycosylated c-MYC. To achieve this we performed a RL2 IP, and subjected the elutions to WB. RL2 is a pan protein O-GlcNAc antibody, and can immunoprecipitate glycosylated proteins. Following the IP, c-MYC protein could be detected in the RL2 elution. While these results strongly suggest that c-MYC is glycosylated in K562 nuclear extract, there are some limitations to the experiment. It was possible that the RL2 antibody pulled down another glycosylated protein which interacted with c-MYC. This would falsely identify that c-MYC was glycosylated. To eliminate this possibility, we attempted to perform a c-MYC IP, and detect glycosylated c-MYC using the RL2 antibody. Unfortunately, we were unsuccessful in the attempts, even after scaling up the experiment. In the future, we plan to use c-MYC IP coupled with mass spectrometry, which can be used to detect O-GlcNAcylated proteins (Chu et al., 2011)

Initially, we wanted to examine the long term stability of c-MYC protein in K562 cells. To this aim, we examined the stability of c-MYC protein levels in K562 cells over a period of 96h of

culture in normal growth media. c-MYC protein levels dropped steadily over the culture time, a result which was not expected (Fig. 4). This could be explained by the fact that as the cells grew, glucose was depleted from the media, and as a result lower levels of UDP-GlcNAc were produced, leading to reduced glycosylation and stability of c-MYC. In support of this claim, we then demonstrated that glucose levels alone affect c-MYC protein stability (Fig. 5). These results demonstrate that c-MYC protein stability depends on cell culture medium glucose concentration.

Next, we demonstrated that by either indirectly inhibiting OGT, by inhibiting enzymes at various levels of the hexosamine biosynthesis pathway (HBP), and by also directly inhibiting OGT, c-MYC protein is downregulated. In addition K562 cell growth was decreased depending on inhibitor concentration (Fig. 6-8). 2-Deoxyglucose is a non-metabolizable form of glucose that inhibits glucose-6-phosphate isomerase, and was chosen since it inhibits the HBP at an early stage (Fig. 1.1). Cells can take up 2-Deoxyglucose through glucose transporters and it accumulates in the cell, thereby reducing UDP-GlcNAc levels (Pelicano et al., 2006). Azaserine was chosen as an inhibitor as it inhibits the rate limiting enzyme of the HBP, GFAT. This greatly reduces levels of UDP-GlcNAc production (Olivier-Van Stichelen et al., 2012). Finally, ST078925 was chosen since it directly inhibits OGT through competition with its substrate UDP-GlcNAc (Capotosti et al., 2011; Gross et al., 2005). The effects of these inhibitors on cell growth, and c-MYC protein in K562 were determined. As expected, treatment with both the two indirect inhibitors of OGT and the direct inhibitor of OGT resulted in a decrease of c-MYC protein and K562 cell growth. In addition the dosage required to reduce 50% of c-MYC protein (IC_{50}) or 50% of cell growth (IG_{50}) correlated well for each drug, which suggests that the reduction of cell

growth may be mediated through the reduction of c-MYC protein levels (Tables 2 & 3). However, from these experiments it was not clear whether the indirect or direct inhibitors of OGT affected c-MYC protein stability or if c-MYC protein was down-regulated by other means.

In order to determine whether or not c-MYC protein stability was reduced post translationally, we studied the half life of endogenous c-MYC protein in K562 in vehicle treated, inhibitor treated, and glucose starvation conditions (Fig. 14-18). Cells were treated with the inhibitor of protein synthesis cycloheximide, which prevents further protein production. This allows the current protein present in the cell to degrade, and its normal half-life to be determined. The degradation can be prevented by treating the cells with MG132, which blocks ubiquitin mediated protein degradation. If MG132 can successfully rescue the effects of cycloheximide, it demonstrates that the degradation observed is mediated through the ubiquitin proteasome pathway. We showed that endogenous c-MYC protein half-life in K562 cells was around 32 minutes in the vehicle treated condition (Table 4). This was in line with a previous reporting of a c-MYC half life of 20-30 minutes depending upon cell type (Hann et al., 1985). Next, we demonstrated that c-MYC protein half-lives in K562 were decreased following treatment with 2-Deoxyglucose, Azaserine, ST078925, as well as in the case of glucose starvation. The fact that MG132 was able to rescue the decreased stability of c-MYC as a result of OGT inhibition demonstrates that the drugs work by decreasing c-MYC stability. Since OGT glycosylates c-MYC at Thr58, this suggests that the reduction of c-MYC protein observed following inhibition of OGT is a result of decreased c-MYC protein stability through OGT, perhaps through glycosylation at Thr58. In order to conclusively demonstrate that this phenomenon is mediated through OGT, mutants of c-MYC at Thr58 must be made. A Thr58 to

alanine mutant could be resistant to inhibition of OGT, as it cannot be glycosylated, and therefore should not be regulated by glycosylation. Indeed, Thr58 to alanine mutants have previously been made, and are more stable that wild-type c-MYC, which supports this claim (Funakoshi-Tago, Sumi, Kasahara, & Tago, 2013; Sears et al., 2000).

Currently, treatment of CML with imatinib presents several challenges, including resistance and side effects (Eiring et al., 2011). To combat these issues, combining imatinib with an additional treatment would potentially be beneficial in at least two ways. Firstly, it may prevent the development of resistance by lowering the chances of selecting imatinib resistant clones. Secondly, it may allow the prescription of lower doses of imatinib, which would in theory help to limit imatinib related side effects. This would be beneficial, as side effects of imatinib are cited as one of main reasons for reduced compliance to the prescription regimen. Non-compliance to the prescribed drug regimen is a serious problem as it can contribute to the development of resistance, and maintaining adequate matinib levels is essential to control the disease (Marin et al., 2010).

We demonstrated that when combined with OGT inhibitors, a lower concentration of imatinib was required in order to achieve the same decrease in cell growth achieved with imatinib treatment alone (Fig. 9). This finding suggests that OGT inhibition may be able to be combined with imatinib therapy, and allow lower dosages of imatinib to be administered to achieve the same outcome.

In order to address the concerns of overcoming imatinib resistance, we opted to use an imatinib resistant K562 cell line. We demonstrated that like regular K562 cells, the imatinib

resistant cells were sensitive to 2-Deoxyglucose, Azaserine and ST078925 treatment. Indeed, treatment with these inhibitors resulted in a down-regulation of c-MYC protein, and a reduction of cell growth in a dose dependent manner (Fig. 10-12). These findings suggest that OGT inhibition might be a suitable treatment for patients with first generation TKI (imatinib) resistance or even second generation TKI resistance.

As c-MYC is a DNA binding transcription factor, we wondered whether OGT could be detected in the same region of DNA, which would suggest that c-MYC recruits OGT to DNA. OGT has been shown to be recruited to CpG regions within gene promoters by Ten-eleven translocation protein (TET1) (Vella et al., 2013).

Therefore it is possible that c-MYC and OGT form a complex on chromatin, where OGT maintains glycosylation of c-MYC at Thr58, so that c-MYC is not targeted for degradation and remains bound to chromatin to amplify gene expression. In support of this we were able to successfully ChIP c-MYC and OGT in K562 cells (Fig. 19). The fact that OGT was able to be immunoprecipitated demonstrates that it is in close proximity to DNA. c-MYC and OGT were found in the promoter regions of known c-MYC target genes CCND2 and NNT, as well as the promoter regions of c-MYC and OGT genes (Lin et al., 2012). In addition, c-MYC ChIP-seq revealed enrichment of c-MYC at the promoter regions of OGT and c-MYC, which is also in agreement with our findings (Raha et al., 2010). In order to demonstrate the sensitivity of the ChIP, we performed both c-MYC and OGT ChIPs in conditions with high and low levels of c-MYC and OGT proteins (Fig. 20). The amounts of c-MYC and OGT were modulated by increased cell culture, as we previously discovered that c-MYC protein levels decrease over cell culture time

(Fig. 4). Interestingly OGT protein levels were also decreased in conjunction with c-MYC, through an unknown mechanism. Greater enrichments of c-MYC and OGT were detected in the condition with high c-MYC and OGT, which suggests that the ChIP experiments are sensitive to levels of c-MYC and OGT. Together, this strongly suggests that c-MYC and OGT colocalize on DNA. In the future, to confirm that c-MYC and OGT truly colocalize on DNA, a sequential immunoprecipitation should be preformed. This would conclusively demonstrate that c-MYC and OGT co-bind the same fragment of DNA. Finally, we wanted to test whether or not c-MYC could recruit OGT to chromatin. To this end, we over-expressed N-terminus FLAG-tagged c-MYC in K562 cells. We detected greater enrichment of c-MYC and OGT at target gene promoter regions (Fig. 21). The fact that there is greater enrichment of OGT when c-MYC is overexpressed suggests that c-MYC can recruit OGT to DNA. To confirm these results, one would need to knockdown c-MYC in K562, and perform a c-MYC and OGT ChIP-seq. If OGT enrichment at target genes fell in the knockdown condition, this would strongly suggest that c-MYC recruits OGT to chromatin.

Cancer cell lines derived from primary tumor cells are the most commonly used in vitro models of the disease from which they originate. While these cell lines can be useful tools for modelling diseases, they only partially recapitulate the phenotypic properties of the original tumor cells they originate from. Although many cancer cell lines maintain tumor-specific chromosomal mutations, have the same morphologies of tumour cells, and maintain expression patterns of the original cancer cells, they often have more chromosomal rearrangements, gene mutations and or gene deletions and amplifications. This is likely due to the selective pressures imposed on the original cells stemming from the in vitro culture environment which is

significantly different from the original tumor microenvironment (Ferreira, Adega, & Chaves, 2013).

In order to study the effects of OGT inhibition on CML cells, we used the CML cell line K562 as a model. K562 is the oldest established CML cell line, expresses the Philadelphia chromosome and can be forced to differentiate into precursors of granulocytic and erythroid cells from prolonged cell culture (B. B. Lozzio, Lozzio, Bamberger, & Feliu, 1981). Like primary CML cells, K562 cells are sensitive to imatinib treatment. Indeed, more recently imatinib was shown to induce apoptosis and erythroid differentiation of K562, a feature in common with primary with primary CML cells (Jacquel et al., 2007). For these reasons K562 are an acceptable model of CML in vitro.

While K562 is a suitable model, it is important to replicate the experiments we performed on K562 in primary tumor cells derived from patients with CML. This would help to circumvent some of the shortcomings of working with cell lines. It would also help to validate the findings of this study with regards to cell growth, and c-MYC protein stability following OGT inhibition.

In addition, treating both cell lines, and primary cells with drugs in tissue culture plates presents additional limitations to in vitro modelling of diseases, including CML. The cells in tissue culture are not in contact with other cell types, exposed to the niche where they are normally found, exposed to cytokines, and the drugs themselves are not subject to metabolism from the body's organs. In addition the drugs may have other cytotoxic effects that would not be detected in vitro (Weinstein, 2012). To address these limitations, it is also important to

include xenograft mouse model experiments. In such an experiment, human cells are injected into immunocompromised mice in order to generate a human tumor within the mouse's body. This is a better model of an actual tumor in that it includes effects of drug metabolism, and tumor cell microenvironment. Indeed both K562 cells and primary CML cells have been used in mouse xenograft models to assess the effects of anticancer therapeutics in vivo (Eisterer et al., 2005; Golas et al., 2003). Furthermore, using primary xenograft mouse models of CML may also allow the evaluation of therapeutics on the progression of chronic phase to blast crisis phase of CML. (Eisterer et al., 2005).

In summary, these findings support our hypothesis that c-MYC and OGT form a complex, whereby OGT stabilizes c-MYC through glycosylation, which allows for c-MYC to affect target gene expression (Fig. 22). It will be necessary in the future to perform high throughput c-MYC and OGT ChIP-seq experiments, as well as RNA-seq experiments in K562 cells, in the presence and absence of OGT inhibitors. This will allow us to establish the effects of these drugs on c-MYC and OGT genome-wide chromatin binding, and the effects on c-MYC target gene expression following drug treatment. This will provide insights into the mechanisms of inhibition of cell growth that is observed in these cells following OGT inhibitor treatment.

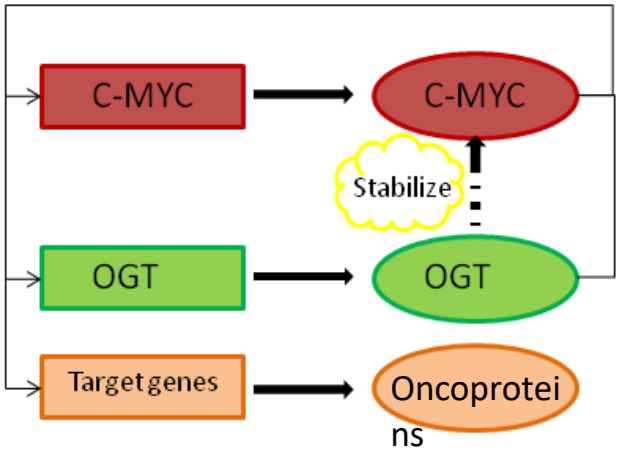

Figure 22. Model depicting c-MYC and OGT transcriptional complex. In this model c-MYC and OGT protein for a complex and regulate their own transcription as well as the transcription of target genes that will promote and maintain CML. In addition, OGT can glycosylate c-MYC thereby stabilizing it at the protein level. Therefore by inhibiting OGT it may be possible to destabilize the network and disrupt its leukemogenic program.

Conclusion

In conclusion, we have demonstrated that c-MYC protein stability in K562 cells is dependent on glucose availability and is sensitive to chemical inhibition of OGT. Treating K562 cells with either direct inhibitors of OGT, or inhibitors of the hexosamine biosynthesis pathway which supplies the substrate of OGT, led to reduced c-MYC protein levels and a concomitant reduction of K562 cell growth. We have demonstrated that the half-life of c-MYC following treatment with these inhibitors is drastically reduced. Furthermore, we have demonstrated that c-MYC and OGT colocalize at the gene promoter region of several c-MYC target genes as well as the c-MYC and OGT promoter regions. This suggests that c-MYC and OGT may function together to promote their own transcription as well as the transcription of other c-MYC target genes. Finally, we have shown that an imatinib mesylate resistant K562 cell line is sensitive to inhibition of OGT in terms of cell growth, and that c-MYC protein is also down regulated following OGT inhibition.

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