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Development of GLUT4-selective antagonists for multiple myeloma therapy

Changyong Wei¹, Richa Bajpai¹, Horrick Sharma², Monique Heitmeier³, Atul D. Jain², Shannon M. Matulis¹, Ajay K. Nooka¹, Rama K. Mishra², Paul W. Hruz³, Gary E. Schiltz^{2,4,5}, and Mala Shanmugam¹

¹Department of Hematology and Medical Oncology, School of Medicine, Emory University, Atlanta, GA 30322

²Center for Molecular Innovation and Drug Discovery, Northwestern University, Evanston, IL 60208

³Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110

⁴Department of Pharmacology, Northwestern University, Chicago, IL 60611

⁵Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

Abstract

Cancer cells consume more glucose to fuel metabolic programs fundamental to sustaining their survival, growth and proliferation. Among the fourteen SLC2A family members, GLUTs 1 and 4 are high-affinity glucose transporters. GLUT4 (SLC2A4) is highly expressed in muscle and adipose tissue. Basally retained within the cell, GLUT4 traffics to the plasma membrane (PM) in response to insulin and exercise-stimulation. The plasma cell malignancy multiple myeloma (MM) exhibits increased constitutive expression of GLUT4 on the PM, co-opting use of GLUT4 for survival and proliferation. GLUT4 inhibition by knockdown or treatment with the FDA-approved HIV protease inhibitor ritonavir leads to cytostatic and/or cytotoxic and chemosensitizing effects in tumor cells both *in vitro* and *in vivo*. We recently reported our generation of GLUT4 homology models and virtual high-throughput screening (vHTS) to identify multiple series of novel GLUT4 antagonists. In this report, we describe our initial hit-to-lead optimization to synthesize new analogs with improved potency and selectivity for GLUT4, and the biological characterization of

Address correspondence to: Gary Schiltz, PhD., Center for Molecular Innovation and Drug Discovery, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA. (Tel): 847-467-2287, gary-schiltz@northwestern.edu or Mala Shanmugam, Ph.D., Winship Cancer Institute, School of Medicine, Emory University, 1365C Clifton Rd. NE, Suite C4002, Atlanta, GA 30322, USA. (Tel): 404-727-3005 (Fax): 404-778-4755, mala.shan@emory.edu.

ASSOCIATED CONTENT

SMILES table (CSV format) of all final compounds tested in the cell proliferation assay and their proliferation data. ¹H- and ¹³C-NMR spectra of compound **3**, **6**, and **20**. HRMS-ESI spectrum of compound **20**.

Conflict of interest

The authors declare no competing financial interest.

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these compounds in a variety of assays. We show that our lead compound (compound 20) decreases glucose uptake and cell proliferation as well as inhibits the expression of pro-survival MCL-1 in MM similar to the effect observed via knockdown of GLUT4 expression. Compound 20 is also effective at chemosensitizing multiple myeloma cell lines and patient samples to venetoclax, dexamethasone and melphalan. In sum, we report development of selective GLUT4 inhibitors lacking inhibitory activity against GLUT1 and GLUT8. We show that selective pharmacological inhibition of GLUT4 is feasible and this may represent a novel strategy for the treatment and chemosensitization of multiple myeloma to standard therapeutics.

Graphical abstract

Keywords

GLUT4; Glucose; Multiple Myeloma

1. INTRODUCTION

Tumor cells, including those of the largely fatal plasma cell malignancy multiple myeloma (MM), exhibit elevated glucose uptake [1–3], a cancer hallmark that forms the basis for clinical monitoring of myeloma using positron emission tomography (FDG-PET) [3–6]. Although the fundamental reliance of tumor cells on increased glucose catabolism for survival, proliferation and chemoresistance is well established, therapeutic targeting of this key metabolic phenotype in a tumor-specific manner has not yet been achieved [7–8]. In addition, despite the broad relevance of elevated glucose uptake in cancer cells, there is limited understanding of the transporters responsible for enabling glucose uptake in distinct tumor contexts. Previously, we determined that constitutive plasma membrane localization of the glucose transporter isoform 4 (GLUT4), plays a critical role in sustaining glucose metabolism and the proliferation and survival of myeloma cells [9–10].

The insulin-responsive facilitative glucose transporter GLUT4 exhibits limited tissue specific distribution and is most abundantly expressed in muscle and adipose tissue [11–12]. GLUT4 is normally retained within the cell, trafficking to the plasma membrane (PM) upon insulin, exercise or specific stimulation that engage the PI3K/AKT [13] or AMPK [14] pathways to recruit GLUT4 to the plasma membrane [15]. In contrast, the more ubiquitously expressed isoform GLUT1 is constitutively localized to the PM facilitating basal glucose transport. GLUT1 activity is critical for the normal function of several cell types including erythrocytes, neurons, and endothelial cells thereby accounting for transport of glucose across the blood-brain barrier [16–17]. We reported that MM cells surprisingly exhibit increased constitutive expression of GLUT4 on the PM, co-opting use of this transporter (among the 14 GLUTs) and not GLUT1 for survival and proliferation [9–10, 18]. In addition, it was also shown that GLUT4 inhibition abrogates cell proliferation and

chemoresistance *in vitro* in MM, chronic lymphocytic leukemia (CLL), solid tumor lines and *in vivo* in a xenograft model of MM [9–10, 18–19]. Roles for GLUT4 have also been suggested in human gastrointestinal tumors that exhibit enhanced PM localization of GLUT4 and weak expression of GLUT1 [20] and in breast cancers [21]. In sum, these observations suggest GLUT4 serves a unique role in both solid and liquid cancers.

The effects of GLUT4 knockdown were recapitulated by treatment with the HIV protease inhibitor ritonavir, a known GLUT4 antagonist (Figure 1) [22]. The affinity of ritonavir for GLUT4, however, is in the low micromolar range [23]. Furthermore, ritonavir also exhibits inhibitory activity against GLUT1 [23]. Efforts to develop HIV protease inhibitors devoid of GLUT4 affinity have demonstrated that the modified tripeptide oxybenzylcarbonyl-His-Phe-Phe-O-ethyl ester (zHFFe, K_i 26 μ M) mimics the core structure of ritonavir and is sufficient to selectively inhibit GLUT4 over GLUT1 [24]. The HIV protease inhibitor indinavir also has significant inhibitory activity towards GLUT4 [23, 25]. Others have developed glucose transporter inhibitors effective against a range of GLUT isoforms, including GLUT1 [26]. However, most of these compounds have had relatively modest potency at inhibiting GLUT4 and importantly have lacked selectivity for this isoform.

To generate more potent, non-competitive, reversible, and isoform-selective GLUT4 inhibitors, we previously generated an *in silico* homology model for GLUT4 and screened a library of eighteen million compounds [27]. Despite 68% homology between GLUT1 and GLUT4, a virtual screen identified two novel compounds, compound **3** and compound **17**, (and related analogues **26** and **39** were also identified) that target GLUT4 selectively [27]. Importantly, modeling suggests that these inhibitors interact with critical residues of GLUT4 (Asn176 and Ile42) known to confer selectivity of HIV protease inhibitors for inhibiting GLUT4 over GLUT1 [28]. These promising results suggest it may be possible to selectively inhibit GLUT4, and thereby produce agents that specifically target cancer cells that rely on glucose transport via GLUT4.

Despite the introduction of new therapeutics, MM remains incurable in a majority of patients due to the development of resistance linked to the inability to induce apoptosis [29–32]. Targeting GLUT4 in MM leads to apoptosis in MM cells associated with suppression of the resistance promoting BCL-2 family member MCL-1 [9, 33]. MM cells resistant to the cytotoxic effects of GLUT4 inhibition were found to induce chemosensitizing alterations in BCL-2 proteins, supporting the use of GLUT4 inhibitors as both therapeutic agents and chemosensitizers. The development of potent GLUT4 inhibitors will allow us to further elucidate glucose sustained metabolic and signaling sequelae that sustain survival in MM. These observations form the basis for the rationale that optimized GLUT4 inhibitors will offer unique tools and drug discovery leads to study and target glucose metabolism sustained by GLUT4 both *in vitro* and *in vivo*. Here, we report our initial medicinal chemistry efforts at developing our lead series of GLUT4 antagonists into more potent and selective inhibitors.

2. RESULTS

2.1 Antagonist Synthesis

In an earlier screen for specific GLUT4 inhibitors, compound **26** was identified which had an IC₅₀ value for inhibition of glucose transport through GLUT4 at 1.7 μ M and >100 μ M for GLUT1 [27]. However, compound **26** also inhibits glucose transport through GLUT8 at an IC₅₀ of 6 μ M [27]. Therefore, we wanted to develop more specific GLUT4 inhibitors with lower affinity for GLUT1 and GLUT8. Based on the structure of this hit series and molecular modeling, we determined that the tertiary amide and the pyridine ring formed important interactions with Asn176 in GLUT4. Since these appeared to form a critical hydrogen-bond anchor with the protein, the initial plan was to leave these groups intact as other modifications were explored to improve cellular potency and selectivity. The initial goal was therefore to explore variations primarily at the R₁ site on the aryl amide and the effect of different R₂ ethers. This would allow us to modify the distal ends of the molecule while keeping the central tertiary amide and pyridylmethyl groups intact to simplify SAR analysis. Furthermore, molecular modeling showed that the substituent coming from the ether was positioned near Phe307, Leu185, and Ile42, which suggested that modifications at this site would provide a way in which to improve potency and/or selectivity.

To enable a rapid and modular approach to new analogs, an efficient 3-step synthesis was developed as shown in Scheme 1. Synthesis began with reductive amination of 4-(aminomethyl) pyridine (**1**) with 3-hydroxybenzaldehyde (**2**) to provide secondary amine **3** in 68% yield. The reaction was performed in methanol with the addition of Ti(O-*i*-Pr)₄, which was found to increase the yield of the reaction. Amine **3** was coupled with the appropriate R₁ benzoic acid using standard amide coupling conditions, EDC and HOBt in DMF. Finally, ether side chains were installed by treating compound **4** with the appropriate alkyl halide and K₂CO₃ in acetone at 56 °C. Yields of the final Williamson ether reaction were generally low to modest.

2.2 Structural analogues of compound **26** decrease proliferation of MM cells

We previously identified two novel compounds that demonstrated selectivity for GLUT4 over GLUT1 [27]. A set of their close structural analogues and compound **26** [27] was selected as a lead series based on its low IC₅₀, selectivity for inhibition of glucose transport through GLUT4, and inhibition of the KMS11-GFP cell line proliferation in contrast to the GLUT1 expressing isogenic line (further described below) [27]. In order to further develop compounds with increased potency and selectivity for targeting GLUT4, compound **26** [27] was modified to obtain a set of close structural analogues. The impact of the new compounds was assessed in a high-throughput proliferation-based assay using an isogenic MM (KMS11) cell line pair overexpressing GLUT1 or GFP (as a control) (model of screen is depicted in Fig. 2A). GLUT4 expression was previously determined to be maintained equally in both GFP and GLUT1 cells and the introduction of GLUT1 was found to increase proliferation and glucose uptake [34]. We hypothesized that a GLUT4 targeting compound would be more effective in impacting growth of KMS11-GFP cells as opposed to KMS11-GLUT1 (as glucose transport inhibition would be compensated by glucose entry via GLUT1). Therefore, a compound with GLUT1 inhibitory activity would be more effective

in KMS11-GLUT1 cells and compounds not selective for GLUT1 or GLUT4 would likely impact viability of both cell types equally. It was found that a number of variations were tolerated which gave similar or better potency and/or selectivity for GLUT4 over compound **26**. The analogues synthesized and their IC₅₀ values for inhibition of proliferation of the isogenic lines are presented in Table 1. Several compounds (**8**, **9**, **17**, and **20**) that were found to elicit a greater impact on proliferation in KMS11-GFP cells compared with KMS11-GLUT1 cells were selected and their effect on cell viability evaluated by AnnexinV/DAPI staining and flow cytometric analysis. Compound **20** was selected for further evaluation as it had a greater impact on the proliferation of KMS11-GFP cells (IC₅₀=13.41 μ M) in contrast to the KMS11-GLUT1 cells (IC₅₀=21.83 μ M) in addition to eliciting greater cell death in KMS11 cells in contrast to the other compounds (supplementary Figure 1).

2.3 Compound 20 inhibits glucose uptake and GLUT4-driven proliferation in MM

We next evaluated the impact of compound **20** on glucose uptake in control and GLUT4 suppressed MM cells. GLUT4-specific shRNA efficiently reduced GLUT4 levels in JJN3 cells (Fig. 3A). Compound **20** effectively reduced [3H]2-deoxy-D-glucose (2-DOG, a glucose analog that is transported but not metabolized) uptake in JJN3 cells to a level comparable to that in JJN3 cells exhibiting GLUT4 suppression (Fig. 3B). The impact of compound **20** on the proliferation of cells exhibiting suppression of GLUT4 expression was also evaluated. The hypothesis was that GLUT4-suppressed cells would not be further growth inhibited with a GLUT4 inhibitor due to removal of the cognate target (as depicted in Fig. 2B). Indeed, GLUT4 knockdown led to suppression of proliferation as anticipated, with no further reduction in viable cell counts upon treatment with compound **20** (Fig. 3C), underscoring the specificity of compound **20** for GLUT4.

2.4 Compound 20 is a selective inhibitor of GLUT4-mediated glucose transport

Compound **20** was further screened for GLUT4 selectivity by evaluating inhibition of glucose transport in HEK293 cells exogenously over-expressing human GLUTs- 1,-2, -3, -4, or -8 that also stably express GLUT1 shRNA (except the GLUT1 overexpressing cell line) to knock down endogenous GLUT1 [35]. Preincubation of cells with a range of inhibitor, followed by a 6 minute uptake of 2-DOG, indicates that compound **20** is selective for GLUT4 over GLUTs 1, 2, 3 and 8 (Fig. 4). A summary of the IC₅₀ for inhibition of glucose transport generated with our initial vHTS hits [27] and newly developed analogues is presented in Table 2. Structures of the compounds in Table 2 are shown in our previous work [27] or in Table 1.

2.5 Compound 20 selectively binds to GLUT4 vs GLUT1

To confirm the ability of compound **20** to bind selectively to GLUT4, a previously established competitive binding assay that allows targeting of the glucose binding site of GLUTs from the cytoplasmic side was utilized [28]. Specifically, the biotinylated membrane impermeant bis-mannose photolabel ATB-BMPA was used to label low-density microsomes (LDM) prepared from Myc-tagged GLUT1- or GLUT4- overexpressing HEK293 cells in the presence or absence of inhibitor. The level of GLUT4 expression in myeloma cells is below the sensitivity of this assay, thus precluding direct assessment of drug binding in these cells.

Compound **20** reduced ATB-BMPA binding to GLUT4 to a much greater extent than to GLUT1 (~64% vs. ~35%) and with greater efficacy compared to compound **26** (Fig. 5). Interestingly, 2-DOG uptake and ATB-BMPA binding results were nearly identical in terms of GLUT1 and GLUT4 inhibition by these compounds, which strongly supports their isoform selectivity and inhibitory activity for GLUT4 over GLUT1 (Fig. 5 and [27]).

2.6 GLUT4 inhibition chemosensitizes MM to venetoclax and standard MM therapeutic agents melphalan and dexamethasone

Knockdown of GLUT4 and glucose deprivation leads to variable suppression of the pro-survival anti-apoptotic protein MCL-1 [9]. To test whether compound **20** could similarly suppress MCL-1 expression, JJN3 and L363 cells were treated with compound **20** and evaluated for MCL-1 expression. MCL-1 was significantly reduced in compound **20** treated L363 cells in contrast to the JJN3 cells (Fig. 6A), similar to that detected upon GLUT4 KD or glucose deprivation of these cell lines [9]. Cells resistant to glucose deprivation-induced cell death increase binding of pro-apoptotic BIM to BCL-2, facilitating sensitization to the BH3 mimetic venetoclax (ABT-199) [33]. Treatment of glucose-deprived resistant cells with ABT-199 releases the additional BIM bound to BCL-2, inducing apoptosis [33]. Treatment with compound **20** similarly increased sensitivity to ABT-199, as demonstrated in the L363 and JJN3 cell lines (Fig. 6B and C). The ability of compound **20** to sensitize MM patient samples to ABT-199 was also assessed. CD38⁺/CD45⁻ gated myeloma cells from myeloma patient bone marrow aspirates were evaluated for viability after 48 hrs. of treatment. 4 of 7 relapse/refractory patient samples co-treated with ABT-199 and compound **20** exhibited greater apoptosis than either drug alone (Fig. 6D). The ability of compound **20** to sensitize MM cells and induce cell death to the commonly used steroid dexamethasone (Dex) was also tested. Compound **20** treated MM.1S cells were exposed to a dose range of Dex (0.00195 μ M to 1 μ M) for 72hr (Fig. 6E). The MM.1S cell line, which is relatively insensitive to Dex, exhibited significant sensitization and cell death to a dose range of Dex upon co-treatment with Compound **20**, underscoring the significant chemosensitizing effects of GLUT4 inhibition. MM patient samples were also sensitized to the combination of 0.5 μ M dexamethasone and 10 μ M compound **20** (Fig. 6F). To rule out the cytotoxic effect of co-treatment of compound **20** with either ABT-199 or dexamethasone on other non-myeloma cell populations, we analyzed cell viability of CD38⁻/CD45⁻ and CD38⁻/CD45⁺ cells within the MM patient sample bone marrow aspirate (Fig. 6G). Compound **20** alone or in combination with either ABT-199 or Dex did not impact the viability of other normal non-myeloma cell populations with a representative analysis included in Fig. 6G. Lastly, compound **20** was also found to sensitize MM.1S cells to a dose range of the alkylating agent melphalan (Mel) that was assessed in a 72 hr treatment period (Fig. 6H).

2.7 Molecular modeling of the lead compound

To rationalize the observed SAR and work towards developing a model which would describe the binding of our lead series to GLUT4, compound **20** was docked into our homology model of GLUT4 (Fig. 7). Modeling shows a hydrogen bond between Asn176 and **20**, and the pyridine ring of **20** may also be forming a pi-pi interaction with Phe38. Furthermore, the fluorophenyl group of **20** fits within a hydrophobic pocket formed by

Phe307, Ile42, and Leu185. The SAR we observed is consistent with our compounds binding in this manner, which show that R1 groups containing polar functionality are less well tolerated (compounds 4, 9, 12). In addition, incorporating a single methylene spacer between the amide carbonyl and the R2 aromatic group produced compound **20**, which is more potent than our original hit. This activity is rationalized by the R2 aromatic group interacting with Trp404 more favorably because of the additional methylene spacer. In order to compute the binding energy of compound **20** and GLUT4 and compare with other antagonists such as indinavir and ritonavir, we computed the binding energy using the QSite module implemented in Schrödinger suite (Schrödinger, LLC, New York, NY). QSite is a mixed mode Quantum Mechanics/Molecular Mechanics (QM/MM) protocol. The QM region and MM regions were first defined by docking indinavir and ritonavir using the same docking protocols that we used to dock **20**. It was observed that Asn176 shows a potential H-bond with both indinavir and ritonavir. Furthermore, the side chains of Phe307, Phe38, and Ile42 form hydrophobic interactions with the docked poses of indinavir and ritonavir. Based on the docked poses, we defined the QM region to possess the entire residue of Asn176 and the side chains of Phe38, Phe307, and Ile42 along with the free ligand. The appropriate hydrogen caps were then assigned to the atoms of the domains separating the QM and MM regions. The QM region was subjected to density-function theory (DFT) calculations with a hybrid functional of B3LYP implemented in Schrödinger Jaguar module [36]. The rest of the GLUT4 structure was treated with molecular mechanics. The binding energies of indinavir, ritonavir, and compound **20** were found to be -17.5 kcal/mol, -13.8 kcal/mol, and -15.1 kcal/mol, respectively.

3. DISCUSSION

Glucose uptake in tumor cells is a key rate-limiting step in glucose metabolism [37]. GLUT1 is elevated in a number of cancers; however, being widely expressed and a major glucose transporter in neurons, erythrocytes, endothelial cells and consequently the blood brain barrier [16–17], it undermines the utility of targeting GLUT1 for cancer therapy. GLUT4 is expressed in muscle (skeletal and heart) and adipose tissue and plays a central role in whole body glucose homeostasis by facilitating insulin- and exercise-stimulated glucose transport [11–12]. GLUT4 is basally retained within the cell with less than 1% localized to the plasma membrane of skeletal myocytes, adipocytes and cardiac myocytes [38–39]. GLUT4 facilitates glucose transport into the cell only when it is translocated from intracellular compartments to the plasma membrane [40]. In fact, it is the inability to recruit sufficient GLUT4 to the plasma membrane in muscle and fat cells that accounts for hyperglycemia in people with diabetes mellitus [41–42].

The complex trafficking of GLUT4 to the plasma membrane is facilitated by the PI3K/AKT [13] and AMPK pathways [14]. It is therefore plausible that tumor cells dependent on sustained PI3K/AKT or AMPK activation could exhibit increased levels of constitutive GLUT4 expression on the plasma membrane to support elevated glucose uptake even in the absence of insulin stimulation. Our previous studies indeed demonstrated the presence of elevated GLUT4 in the plasma membrane of multiple myeloma cells and responsiveness of a number of solid and liquid tumor cell lines to GLUT4 inhibition [9–10, 18]. Results reported here demonstrate that targeting GLUT4 in addition to being a cytostatic and/or cytotoxic

can be used to sensitize cancer cells to existing chemotherapy (dexamethasone and melphalan) and BH3 mimetics, affording potential strategies for chemosensitization and synthetic lethality, respectively.

The normal physiologic role of GLUT4 in whole-body glucose homeostasis and consequences of genetic and pharmacologic ablation is well-established both in rodent models and humans [43–46]. Since GLUT4 is retained within the cell during unstimulated basal conditions, basal glucose levels are unchanged in whole body and muscle-specific GLUT4-null mice [43]. Whole body and muscle-specific GLUT4 null mice exhibit a reduction in fat accumulation, hyperinsulinemia and increased gluconeogenesis in the liver with time, associated with prolonged hyperglycemia and hyperinsulinemia [43]. Significant growth retardation and reduced survival is detected after six months in a whole body GLUT4-null mouse model [43]. The normal adult heart is primarily dependent on fatty acid oxidation with basal glucose metabolism supported by GLUT1-mediated glucose transport [47–48]. GLUT4 supports increased dependency of the heart on glucose metabolism during insulin stimulation [39, 49–50], ischemia [49–51], hypoxia [52], and high-frequency contraction [53] particularly in specific pathological states such as congestive heart failure and hypertrophy associated with hypertension. GLUT4 ablation in whole body or heart leads to modest cardiac hypertrophy and interstitial fibrosis associated with an increase in GLUT1 expression that is intermediate to that detected in exercise stimulated hypertrophy and pathological hypertrophy in rodents [43–46]. Most importantly, cardiac hypertrophy is detected only when GLUT4 levels are profoundly reduced i.e. greater than 95% [46]. The development of hypertrophy is attributed to prolonged hyperinsulinemia and reduction in fatty-acid oxidation in the heart [43]. However, these hypertrophic hearts exhibit normal contractile function and maintain normal blood pressure [44–46]. In contrast to the effects of chronic ablation of GLUT4 activity, extensive clinical experience with HIV protease inhibitor therapy in humans provides solid support for the clinical tolerability of short-term pharmacologic blockade. Specifically, indinavir and ritonavir, non-competitive reversible inhibitors of GLUT4 [25, 54], have been administered for over a decade in HIV patients as part of combined anti-retroviral therapies. Treatment of HIV infected patients with ritonavir or indinavir acutely promotes insulin resistance and impairs glucose clearance. These effects are fully reversible after short-term exposure [22, 55–57]. With chronic treatment, dyslipidemia mediated in part by increased fatty acid synthesis in the liver and increased lipolysis in adipose tissue attributed to GLUT4 inhibition can be observed [58–60]. While these effects are also reversible, they are indicative of the consequences of *chronic* GLUT4 inhibition. Although protease inhibitor therapy is also associated with increased long-term cardiovascular risk in HIV infected patients [61–62], it is important to note that HIV infection in itself is frequently associated with cardiovascular complications. In sum, acute or short-term administration of a GLUT4-selective inhibitor should not impact basal glucose metabolism in the normal heart, muscle and fat, or exhibit alterations in glucose and lipid metabolism leading to dyslipidemia and diabetes which are detected with long term suppression GLUT4 function. The acute risks may be tolerable in relation to the toxicity of current chemotherapy, particularly in humans with normal cardiac function. Monitoring of heart function and blood glucose levels would be warranted, as with any therapy, with

caution in administering a GLUT4 inhibitor to patients with pre-existing cardiovascular complications.

Chemoresistance in MM, leukemia, and solid tumors is largely due to tumor cell evasion of apoptosis that in turn is primarily due to the inability to release sufficient pro-apoptotic BCL-2 proteins above a threshold level required to elicit apoptosis [32]. Since BCL-2 family members are central to the development of chemotherapeutic resistance, our ability to selectively perturb glucose metabolism in cancer cells provides a method to not only suppress resistance-promoting MCL-1 expression; but also elicit synthetic lethality to BH3 mimetics like ABT-199. ABT-199 is highly effective as a single agent in CLL (which is BCL-2 dependent) [63], however only effective in a small subset of MM [64].

Dexamethasone has been used in chemotherapy in combination with various drugs, however it has several off-targets effects and the ability to utilize lower doses of dexamethasone or melphalan in combination with a GLUT4 inhibitor can be highly beneficial in MM therapy. The well-characterized effects of the FDA-approved drugs ritonavir and indinavir on GLUT4 [25, 54] are proof of principle that patients can tolerate drugs targeting GLUT4 that may cause impaired glucose uptake and hyperglycemia in chronic, high dose regimens. However, administration of these compounds in conjunction with chemotherapy cycles may greatly potentiate anti-tumor efficacy in cancers like myeloma while significantly reducing off-target effects due to shorter durations of administration. These studies thus support the use of GLUT4 inhibitors as both therapeutic agents and chemosensitizers. Optimal exploitation of this approach will be aided by the development of compounds with even greater affinity and selectivity for GLUT4.

In our previous report [27], we built a homology model of GLUT4 and used this structure to carry out an *in silico* screen to identify several novel GLUT4 antagonists. Here, our efforts at hit-to-lead chemistry to develop our initial hits into GLUT4 antagonists with improved biological properties are described. The chemistry plan was to initially explore the requirements of the ether substituent and aryl benzamide and a straightforward 3-step route that allowed functionalization of the ether position at the last step was developed. Using this approach, diverse substituted aromatic and non-aromatic groups were selected that possessed varying hydrogen-bond acceptor capacity and electron donating/withdrawing ability. This route will allow the continued exploration of SAR for this series to continue improving their biological properties. Several analogs of the aryl amide were also prepared. Interestingly, it was found that inclusion of a methylene spacer in the amide portion (e.g. compound **20**) produced increased selectivity in our phenotypic screening assay. While the methylene spacer of compound **20** improved selectivity, it caused a slight decrease in potency of glucose uptake inhibition (Table 2) relative to compound **26**. Based on the modeling (Figure 7), addition of the spacer may reduce the strength of the interaction between the aromatic group and Trp404, resulting in diminished potency. This compound was then used in other assays to show the effects of GLUT4 inhibition on a variety of MM-related properties. Calculation of the binding energy of compound **20** with known GLUT4 antagonists indinavir and ritonavir showed that compound **20** was intermediate between these two known antagonists. This result will be exploited in future work to prepare

additional analogs with varied linkers between the R₂ group and the central amide-containing core.

4. CONCLUSION

We have carried out initial hit-to-lead medicinal chemistry on our novel series of GLUT4 antagonists. These analogs explored several areas of the scaffold and initial structure-activity relationships were identified. Our lead compound possesses excellent selectivity for GLUT4 over other GLUT isoforms and moderate potency. Biological evaluation demonstrates that these compounds possess clear binding to GLUT4 to impact validated functional end points. Importantly, lead compound **20** was used to show that antagonism of GLUT4 in MM produces the anticipated inhibition of glucose-sustained MCL-1 expression in addition to exhibiting chemosensitizing effects to dexamethasone, melphalan and ABT-199.

5. EXPERIMENTAL SECTION

5.1 Chemistry

5.1.1 General Chemistry Experimental—All chemical reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. Anhydrous solvents were purchased from Sigma-Aldrich, and dried over 3 Å molecular sieves when necessary. DCM and THF were purified by passage through a bed of activated alumina. Normal phase flash column chromatography was performed using Biotage KP-Sil 50µm silica gel columns and ACS grade solvents on a Biotage Isolera flash purification system. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60 F₂₅₄ plates and visualized by UV light. Proton (¹H), and carbon (¹³C) NMR spectra were recorded on a 500MHz Bruker Avance III with direct cryoprobe spectrometer. Chemical shifts were reported in ppm (δ) and were referenced using residual non-deuterated solvent as an internal standard. The chemical shifts for ¹H-NMR and ¹³C-NMR are reported to the second decimal place. Proton coupling constants are expressed in hertz (Hz). The following abbreviations were used to denote spin multiplicity for proton NMR: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad singlet, dd = doublet of doublets, dt = doublet of triplets, quin = quintet, tt = triplet of triplets. In some cases, overlapping signals occurred in the ¹³C-NMR spectra. Low resolution liquid chromatography/mass spectrometry (LCMS) was performed on a Waters Acquity-H UPLC/MS system with a 2.1 mm × 50 mm, 1.7 µm, reversed phase BEH C18 column and LCMS grade solvents. A gradient elution from 95% water + 0.1% formic acid/5% acetonitrile + 0.1% formic acid to 95% acetonitrile + 0.1% formic acid/5% water + 0.1% formic acid over 2 min plus a further minute continuing this mixture at a flow rate of 0.85 mL/min was used as the eluent. Total ion current traces were obtained for electrospray positive and negative ionization (ESI+/ESI-). High-resolution mass spectra were obtained using an Agilent 6210 LC-TOF spectrometer in the positive ion mode using electrospray ionization with an Agilent G1312A HPLC pump and an Agilent G1367B autoinjector at the Integrated Molecular Structure Education and Research Center (IMSERC), Northwestern University.

5.1.2 Preparation of intermediates and final compounds

3-(((pyridin-4-ylmethyl)amino)methyl)phenol (3): To a solution of pyridin-4-ylmethanamine (2.49 mL, 24.57 mmol) and 3-hydroxybenzaldehyde (3.0 g, 24.57 mmol) in MeOH (60 mL) was added tetraisopropoxytitanium (9.36 mL, 31.9 mmol). The reaction was stirred for overnight (16 h) and then cooled to -78°C . Sodium borohydride (0.929 g, 24.57 mmol) was then added and reaction mixture stirred for another 1 h. The reaction was then quenched with water and extracted with ethyl acetate. The organic layer was evaporated and dried in vacuum. The reaction mixture was purified by flash chromatography (0–20% MeOH/DCM) to give the title compound (4.45 g, 85%). Rf in 10% MeOH/DCM = 0.5; MS (ESI): mass calcd. for $\text{C}_{13}\text{H}_{14}\text{N}_2\text{O}$, 214.11; m/z found, 215.18 $[\text{M}+\text{H}]^{+}$; ^1H NMR (500 MHz, CDCl_3) δ ppm 3.80 (s, 2 H), 3.87 (s, 2 H), 6.79 – 6.82 (m, 1 H), 6.86 (d, $J=6.71$ Hz, 1 H), 6.92 – 6.96 (m, 1 H), 7.23 (t, $J=7.78$ Hz, 1 H), 7.35 – 7.38 (m, 2 H), 8.56 – 8.59 (m, 2 H).

N-(3-hydroxybenzyl)-4-methoxy-N-(pyridin-4-ylmethyl)benzamide (4): To a mixture of 4-methoxybenzoic acid (1.015 g, 6.67 mmol), HOBT (1.022 g, 6.67 mmol), EDC (1.279 g, 6.67 mmol), and 3-(((pyridin-4-ylmethyl)amino)methyl)phenol (1.3 g, 6.07 mmol) in DMF (25 mL) was added Et_3N (0.863 mL, 6.19 mmol). The reaction was stirred overnight (18 h). After the reaction, water was added and reaction was extracted with ethyl acetate. The organic layer was evaporated and dried in vacuum. The crude was purified using flash chromatography (0–20% MeOH/DCM) to give the title compound (0.9 g, 43%). Rf in 5% MeOH/DCM = 0.45; MS (ESI): mass calcd. for $\text{C}_{21}\text{H}_{20}\text{N}_2\text{O}_3$, 348.15; m/z found, 349.41 $[\text{M}+\text{H}]^{+}$; ^1H NMR (500 MHz, CDCl_3) δ 3.82 (s, 3 H), 4.37 – 4.78 (m, 4 H), 6.59 – 6.78 (m, 2 H), 6.80 – 6.92 (m, 3 H), 7.12 – 7.27 (m, 3 H), 7.35 – 7.57 (m, 2 H), 8.59 (d, $J=4.27$ Hz, 2 H).

4-chloro-N-(3-hydroxybenzyl)-N-(pyridin-4-ylmethyl)benzamide (5): 3-(((pyridin-4-ylmethyl)amino)methyl)phenol (150 mg, 0.700 mmol) was dissolved in THF (2.0 mL). Then Et_3N (0.081 mL, 0.583 mmol) was added and reaction stirred for 5 min. 4-chlorobenzoyl chloride (0.075 mL, 0.583 mmol) was then added and reaction stirred overnight (12 h). After the reaction, water was added and the reaction mixture was extracted with ethyl acetate. The organic layer was evaporated and dried in vacuo. The crude was purified using flash chromatography (0–20% MeOH/DCM) to give the title compound (74 mg, 36%). MS (ESI): mass calcd. for $\text{C}_{20}\text{H}_{17}\text{ClN}_2\text{O}_2$, 352.10; m/z found, 353.36 $[\text{M}+\text{H}]^{+}$; ^1H NMR (500 MHz, CDCl_3) δ 4.32 – 4.49 (m, 2 H), 4.61 – 4.75 (m, 2 H), 6.59 – 6.80 (m, 2 H), 6.83 (d, $J=9.16$ Hz, 1 H), 7.11 – 7.27 (m, 3 H), 7.33 – 7.49 (m, 4 H), 8.59 (br. s., 2 H).

N-(3-hydroxybenzyl)-2-(4-methoxyphenyl)-N-(pyridin-4-ylmethyl)acetamide (6): To a mixture of 2-(4-methoxyphenyl)acetic acid (128 mg, 0.770 mmol), HOBT (118 mg, 0.77 mmol), EDC (148 mg, 0.77 mmol), and 3-(((pyridin-4-ylmethyl)amino)methyl)phenol (150 mg, 0.70 mmol) in DMF (2 mL) was added Et_3N (0.117 mL, 0.84 mmol). The reaction was stirred overnight (18 h). Afterwards, water was added and the reaction was extracted with ethyl acetate. The organic layer obtained was evaporated and dried in vacuum. The crude residue was used as is in the next step.

A sample was purified by preparative HPLC to allow ^1H -NMR characterization. The sample appeared as a 2:1 mixture of rotamers. ^1H NMR (500 MHz, CDCl_3) δ ppm 3.69 (s, 1 H), 3.75 (s, 1.5 H), 3.77 (s, 2 H), 3.79 (s, 3 H), 4.41 (s, 2 H), 4.44 (s, 1 H), 4.54 (s, 1 H), 4.57 (s, 2 H), 6.58 – 6.62 (m, 2 H), 6.65 (d, $J=7.63$ Hz, 0.5 H), 6.72 (s, 0.5 H), 6.77 (dd, $J=8.24, 1.83$ Hz, 0.5 H), 6.81 (d, $J=8.85$ Hz, 2 H), 6.84 – 6.88 (m, 2 H), 7.00 (d, $J=5.80$ Hz, 1H), 7.08 (d, $J=5.80$ Hz, 2 H), 7.11 – 7.15 (m, 1.5 H), 7.16 – 7.22 (m, 2 H), 8.46 – 8.49 (m, 2 H), 8.54 (d, $J=6.10$ Hz, 1 H).

Representative procedure for the synthesis of final compounds: Acetone (0.5 mL) was added to phenol (**4-6**) (50 mg, 0.144 mmol). Then K_2CO_3 (39.7 mg, 0.287 mmol, 2eq.) and bromide (0.144 mmol, 1eq.) were added and the reaction mixture was stirred at 56°C overnight. Thereafter, the reaction mixture was filtered, centrifuged, and the supernatant was evaporated to dryness. The crude was purified using prep TLC or Biotage flash column chromatography to afford the desired final compound.

4-methoxy-N-(3-(4-methylphenethoxy)benzyl)-N-(pyridin-4-ylmethyl)benzamide (7): Yield = 32%. ^1H NMR (500 MHz, CD_3OD) δ 2.29 (s, 3 H), 2.98 (t, $J=6.87$ Hz, 2 H), 3.31 (dt, $J=3.28, 1.56$ Hz, 2 H), 3.79 (s, 3 H) 4.10 (m, 2 H), 4.51 - 4.75 (m, 4 H), 6.58 – 6.67 (m, 1 H), 6.70 – 6.78 (m, 1 H), 6.82 (d, $J=6.71$ Hz, 1 H), 6.95 (d, $J=7.93$ Hz, 2 H), 7.07 – 7.11 (m, 2 H), 7.14 – 7.17 (m, 2 H), 7.22 (t, $J=7.93$ Hz, 1 H), 7.26 – 7.34 (m, 2 H), 7.46 (d, $J=7.93$ Hz, 2 H), 8.45 (d, $J=6.10$ Hz, 2 H); ^{13}C NMR (126 MHz, CDCl_3) δ 21.03, 35.30, 55.33, 68.86, 113.63, 113.88, 113.94, 127.42, 128.86, 129.19, 129.95, 134.92, 136.09, 137.86, 146.21, 150.14, 159.36, 161.00, 172.25. HRMS (ESI): m/z calculated for $\text{C}_{30}\text{H}_{30}\text{N}_2\text{O}_3 + \text{H}^+$ [$\text{M} + \text{H}^+$]: 467.2329. Found: 467.2337.

4-methoxy-N-(pyridin-4-ylmethyl)-N-(3-(3-(trifluoromethyl)phenethoxy)benzyl)benzamide (8): Yield = 12%. ^1H NMR (500 MHz, CD_3OD) δ ppm 3.17 (t, $J=6.41$ Hz, 2 H), 3.83 (s, 3 H), 4.21 (br. s., 2 H), 4.57 – 4.78 (m, 4 H), 6.62 – 6.92 (m, 3 H), 6.94 – 7.04 (m, 2 H), 7.21 – 7.56 (m, 8 H), 7.60 (d, $J=7.32$ Hz, 1 H), 7.65 (s, 1 H), 8.51 (br. s., 1 H); ^{13}C NMR (126 MHz, CD_3OD) δ 29.27, 34.94, 54.46, 67.89, 113.58, 113.65, 122.80, 122.83, 125.38, 125.42, 125.45, 128.76, 130.11, 130.37, 132.53, 140.06, 159.23, 161.38, 173.40. HRMS (ESI): m/z calculated for $\text{C}_{30}\text{H}_{27}\text{F}_3\text{N}_2\text{O}_3 + \text{H}^+$ [$\text{M} + \text{H}^+$]: 521.2047. Found: 521.2053.

4-methoxy-N-(pyridin-4-ylmethyl)-N-(3-((tetrahydro-2H-pyran-4-yl)methoxy)benzyl)benzamide (9): Yield = 52%. ^1H NMR (500 MHz, CD_3OD) δ ppm 1.45 (qd, $J=12.51, 4.58$ Hz, 2 H), 1.76 (dd, $J=12.97, 1.68$ Hz, 2 H), 1.99 – 2.12 (m, 1 H), 3.46 (td, $J=11.90, 1.83$ Hz, 2 H), 3.72 – 3.87 (m, 5 H), 3.99 (dd, $J=10.83, 3.51$ Hz, 2 H), 4.52 – 4.82 (m, 4 H), 6.64 – 6.83 (m, 1 H), 6.86 (dd, $J=8.09, 1.37$ Hz, 2 H), 6.99 (d, $J=7.02$ Hz, 2 H), 7.27 (t, $J=7.93$ Hz, 2 H), 7.35 (br. s., 1 H), 7.42 – 7.60 (m, 2 H), 8.51 (br. s., 2 H); ^{13}C NMR (126 MHz, CDCl_3) δ 29.75, 35.15, 55.37, 67.62, 72.57, 113.61, 113.91, 127.42, 128.82, 130.01, 137.86, 146.21, 150.16, 159.58, 161.05, 172.29. HRMS (ESI): m/z calculated for $\text{C}_{27}\text{H}_{30}\text{N}_2\text{O}_4 + \text{H}^+$ [$\text{M} + \text{H}^+$]: 447.2278. Found: 447.2286.

4-methoxy-N-(3-phenethoxybenzyl)-N-(pyridin-4-ylmethyl)benzamide (10): Yield = 35%. ^1H NMR (500 MHz, CD_3OD) δ ppm 3.06 (t, $J=6.87$ Hz, 2 H), 3.82 (s, 3 H), 4.16 (br. s., 2 H), 4.51 – 4.80 (m, 4 H), 6.65 – 6.80 (m, 1 H), 6.86 (d, $J=7.02$ Hz, 1 H), 6.98 (d, $J=7.63$

Hz, 2 H), 7.21 – 7.38 (m, 8 H), 7.40 – 7.58 (m, 3 H), 8.49 (br. s., 2 H); ^{13}C NMR (126 MHz, CD_3OD) δ 35.30, 54.48, 68.49, 113.66, 126.04, 127.09, 128.05, 128.48, 128.65, 129.97, 138.40, 148.81, 159.38, 161.38, 173.38. HRMS (ESI): m/z calculated for $\text{C}_{29}\text{H}_{28}\text{N}_2\text{O}_3 + \text{H}^+$ $[\text{M} + \text{H}^+]$: 453.2173. Found: 453.2179.

tert-butyl 4-((3-((4-methoxy-N-(pyridin-4-ylmethyl)benzamido)methyl)phenoxy)methyl)piperidine-1-carboxylate (11): Yield = 24%. ^1H NMR (500 MHz, CD_3OD) δ ppm 1.22 – 1.28 (m, 1H), 1.48 (s, 9 H), 1.79 – 1.81 (m, 2H), 1.93 – 1.95 (m, 1H), 2.77 (bs, 2H), 3.78 – 3.80 (m, 5H), 4.10 – 4.12 (m, 2H), 4.55 – 4.81 (m, 4 H), 6.65 – 6.80 (m, 1H), 6.86 (d, $J=8.24$ Hz, 2 H) 6.98 (d, $J=7.02$ Hz, 2 H) 7.22 – 7.38 (m, 2 H), 7.31 (bs, 1H), 7.42 – 7.62 (m, 2 H), 8.50 (br. s., 2 H); ^{13}C NMR (126 MHz, CD_3OD) δ 28.86, 30.07, 37.55, 40.10, 56.04, 67.83, 73.48, 81.12, 115.06, 115.23, 127.72, 128.64, 128.76, 128.97, 129.43, 130.01, 130.27, 131.19, 150.43, 156.68, 161.14, 162.96, 174.96. HRMS (ESI): m/z calculated for $\text{C}_{32}\text{H}_{39}\text{N}_3\text{O}_5 + \text{H}^+$ $[\text{M} + \text{H}^+]$: 546.2962. Found: 546.2967.

N-(3-(cyclohexylmethoxy)benzyl)-4-methoxy-N-(pyridin-4-ylmethyl)benzamide (12): Yield = 25%. ^1H NMR (500 MHz, CD_3OD) δ ppm 1.10 (qd, $J=12.16$, 2.90 Hz, 2 H), 1.20 – 1.40 (m, 3 H), 1.69 – 1.83 (m, 4 H), 1.88 (d, $J=13.12$ Hz, 2 H), 3.71 (s, 2 H), 3.81 (s, 3 H), 4.56 – 4.79 (m, 4 H), 6.64 – 6.79 (m, 1 H), 6.85 (d, $J=7.02$ Hz, 2 H), 7.00 (d, $J=6.41$ Hz, 2 H), 7.26 (t, $J=7.93$ Hz, 2 H), 7.29 – 7.62 (m, 4 H), 8.52 (br. s., 2 H); ^{13}C NMR (126 MHz, CD_3OD) δ 27.12, 27.79, 31.09, 39.24, 56.04, 74.65, 115.10, 115.23, 128.67, 130.06, 131.15, 139.27, 150.42, 161.36, 162.95, 174.96. HRMS (ESI): m/z calculated for $\text{C}_{28}\text{H}_{32}\text{N}_2\text{O}_3 + \text{H}^+$ $[\text{M} + \text{H}^+]$: 445.2486. Found: 445.2494.

N-(3-(4-bromophenoxy)benzyl)-4-methoxy-N-(pyridin-4-ylmethyl)benzamide (13): Yield = 18%. ^1H NMR (500 MHz, CD_3OD) δ ppm 3.04 (t, $J=6.56$ Hz, 2 H), 3.83 (s, 3 H), 4.16 (br. s., 2 H), 4.53 – 4.78 (m, 4 H), 6.59 – 6.72 (m, 1 H), 6.74 – 6.81 (m, 1 H), 6.85 – 6.86 (m, 2H), 6.93 – 7.05 (m, 2 H), 7.24 – 7.27 (m, 4 H), 7.31 – 7.39 (bs, 1H), 7.46 (d, $J=8.55$ Hz, 2H), 7.49 – 7.55 (m, 2 H) 8.51 (br. s., 1 H); ^{13}C NMR (126 MHz, CDCl_3) δ 35.17, 55.37, 68.30, 113.65, 113.91, 120.43, 127.41, 128.81, 129.01, 130.00, 130.76, 131.57, 131.79, 137.23, 137.97, 146.18, 150.19, 159.24, 161.05, 172.29. HRMS (ESI): m/z calculated for $\text{C}_{29}\text{H}_{27}\text{BrN}_2\text{O}_3 + \text{H}^+$ $[\text{M} + \text{H}^+]$: 531.1278. Found: 531.1283.

4-methoxy-N-(3-((4-nitrobenzyl)oxy)benzyl)-N-(pyridin-4-ylmethyl)benzamide (14): Yield = 35%. ^1H NMR (500 MHz, CD_3OD) δ ppm 3.82 (s, 3 H) 4.52 – 4.76 (m, 4 H), 5.23 (br. s., 2 H), 6.73 – 6.89 (m, 2 H), 6.95 – 6.98 (m, 3 H), 7.30 (t, $J=7.93$ Hz, 2 H), 7.32 – 7.49 (m, 3 H), 7.68 (d, $J=8.85$ Hz, 2 H), 8.22 – 8.29 (m, 2 H), 8.46 – 8.52 (m, 2 H); ^{13}C NMR (126 MHz, CD_3OD) δ 54.47, 68.23, 113.62, 114.23, 119.74, 122.84, 123.28, 126.99, 127.53, 128.42, 129.82, 137.99, 144.99, 147.49, 148.91, 158.76, 161.39, 173.33. HRMS (ESI): m/z calculated for $\text{C}_{28}\text{H}_{25}\text{N}_3\text{O}_5 + \text{H}^+$ $[\text{M} + \text{H}^+]$: 484.1867. Found: 484.1877.

4-methoxy-N-(pyridin-4-ylmethyl)-N-(3-((4-(trifluoromethyl)benzyl)oxy)benzyl)benzamide (15): Yield = 47%. ^1H NMR (500 MHz, CD_3OD) δ ppm 3.81 (s, 3 H), 4.56 – 4.74 (m, 4 H), 5.17 (br. s., 2 H), 6.80 (br. s., 2 H), 6.96 (d, $J=7.93$ Hz, 3H), 7.18 – 7.34 (m, 3 H), 7.45 (br. s., 2 H), 7.60 – 7.65 (m, 2 H), 7.66 – 7.72 (m, 2 H), 8.49 (br. s., 2 H); ^{13}C NMR (126 MHz,

CD₃OD) δ 54.46, 68.55, 113.65, 114.17, 123.18, 125.04, 125.33, 127.02, 127.28, 128.37, 129.38, 129.64, 129.77, 137.94, 141.90, 147.66, 148.88, 158.91, 161.38, 173.37. HRMS (ESI): m/z calculated for C₂₉H₂₅F₃N₂O₃ + H⁺ [M + H⁺]: 507.1890. Found: 507.1898.

***N*-(3-((4-fluorobenzyl)oxy)benzyl)-4-methoxy-*N*-(pyridin-4-ylmethyl)benzamide (16):**

Yield = 34%. ¹H NMR (500 MHz, CD₃OD) δ ppm 3.82 (s, 3 H), 4.51 – 4.77 (m, 4 H), 4.88 (s, 2 H), 6.67 – 6.88 (m, 2 H), 6.92 – 7.00 (m, 3 H), 7.08 – 7.14 (m, 2 H), 7.27 (t, J =8.09 Hz, 2 H), 7.33 (d, J =5.19 Hz, 1 H), 7.38 – 7.52 (m, 4 H), 8.49 (d, J =6.10 Hz, 2 H); ¹³C NMR (126 MHz, CD₃OD) δ 56.03, 70.35, 115.20, 115.73, 116.32, 116.50, 120.99, 124.48, 128.60, 129.95, 130.68, 130.75, 131.25, 134.81, 139.41, 149.25, 150.45, 174.92. HRMS (ESI): m/z calculated for C₂₈H₂₅FN₂O₃ + H⁺ [M + H⁺]: 457.1922. Found: 457.1927.

***4*-methoxy-*N*-(3-((3-methoxybenzyl)oxy)benzyl)-*N*-(pyridin-4-ylmethyl)benzamide (17):**

Yield = 27%. ¹H NMR (500 MHz, CD₃OD) δ ppm 3.79 (s, 3 H), 3.82 (s, 3 H), 4.52 – 4.72 (m, 4 H), 5.07 (s, 2 H), 6.76 (br. s., 1 H), 6.86 – 6.89 (m, 1 H), 6.92 – 7.01 (m, 6 H), 7.23 – 7.33 (m, 4 H), 7.36 – 7.50 (m, 2 H), 8.48 (br. s., 2 H); ¹³C NMR (126 MHz, CD₃OD) δ 113.98, 114.42, 114.88, 115.30, 115.95, 120.62, 124.42, 128.61, 129.95, 130.35, 130.82, 131.23, 139.06, 140.41, 149.16, 150.44, 160.70, 161.54, 162.90, 174.91. HRMS (ESI): m/z calculated for C₂₉H₂₈N₂O₄ + H⁺ [M + H⁺]: 469.2122. Found: 469.2128.

***N*-(3-(2-(4-chlorophenyl)-2-oxoethoxy)benzyl)-4-methoxy-*N*-(pyridin-4-ylmethyl)benzamide (18):**

Yield = 47%. ¹H NMR (500 MHz, CD₃OD) δ ppm 3.81 (s, 3 H), 4.51 – 4.76 (m, 4 H), 5.42 (br. s., 2 H), 6.66 – 6.88 (m, 2 H), 6.88 – 7.02 (m, 3 H), 7.19 – 7.49 (m, 4 H), 7.40 – 7.53 (m, 1 H), 7.54 – 7.60 (m, 2 H), 8.00 – 8.06 (m, 2 H), 8.47 (br. s., 2 H); ¹³C NMR (126 MHz, CDCl₃) δ 31.99, 55.32, 70.67, 126.69, 127.26, 128.53, 128.72, 129.23, 129.56, 132.67, 133.50, 140.51, 141.88, 146.11, 146.13, 150.12, 158.35, 161.00, 161.03, 172.25, 193.33. HRMS (ESI): m/z calculated for C₂₉H₂₅ClN₂O₄ + H⁺ [M + H⁺]: 501.1576. Found: 501.1586.

***4*-chloro-*N*-(3-(4-fluorophenethoxy)benzyl)-*N*-(pyridin-4-ylmethyl)benzamide (19):**

Yield = 39%. ¹H NMR (500 MHz, CD₃OD) δ ppm 3.04 (t, J =6.56 Hz, 2 H), 4.13 (br. s., 2 H), 4.50 (br. s., 2 H), 4.71 (br. s., 2H), 6.60 – 6.72 (m, 1H), 6.81 – 6.85 (m, 2H), 7.00 (t, J =8.70 Hz, 2 H), 7.18 – 7.32 (m, 5H), 7.41 – 7.49 (m, 4H), 8.46 (br. s., 2 H); ¹³C NMR (126 MHz, CDCl₃) δ 35.97, 69.94, 114.76, 115.23, 116.05, 116.22, 120.63, 124.53, 130.20, 131.32, 131.84, 131.90, 135.50, 135.97, 135.99, 137.43, 150.40, 160.91, 164.18, 173.78. HRMS (ESI): m/z calculated for C₂₈H₂₄ClFN₂O₂ + H⁺ [M + H⁺]: 475.1583. Found: 475.1586.

***N*-(3-(4-fluorophenethoxy)benzyl)-2-(4-methoxyphenyl)-*N*-(pyridin-4-ylmethyl)acetamide (20):**

Yield = 40%. ¹H NMR (500 MHz, CD₃OD) δ ppm 3.02 (t, J =6.71 Hz, 2 H), 3.72 – 3.84 (m, 5 H), 4.03 (t, J =6.71 Hz, 1 H), 4.07 (t, J =6.56 Hz, 1 H), 4.59 (s, 2 H), 4.64 (d, J =2.75 Hz, 2 H), 6.49 (s, 1 H), 6.68 – 6.74 (m, 1 H), 6.82 (d, J =8.54 Hz, 2 H), 6.88 (d, J =8.54 Hz, 1 H), 6.99 – 7.06 (m, 2 H), 7.10 – 7.22 (m, 5 H), 7.30 (ddd, J =8.54, 5.49, 2.75 Hz, 2 H), 8.42 (dd, J =17.70, 5.19 Hz, 2 H); ¹³C NMR (126 MHz, CDCl₃) δ 34.94, 34.97, 40.03, 40.22, 47.70, 48.60, 49.36, 50.94, 53.44, 55.31, 68.52, 68.64, 112.73, 113.72, 114.02, 114.29, 115.18, 115.23, 115.35, 115.40, 118.87, 120.71, 121.30, 122.78, 126.40, 126.58, 129.70, 129.80, 130.16, 130.40, 130.47, 133.82, 133.84, 133.96, 137.55, 138.34, 145.87,

146.34, 150.02, 150.36, 158.71, 159.14, 159.46, 160.75, 162.70, 171.92, 172.21. HRMS (ESI): m/z calculated for $C_{30}H_{29}FN_2O_3 + H^+$ [$M + H^+$]: 485.2235. Found: 485.2242.

***N*-(3-(2-(3-bromophenyl)-2-oxoethoxy)benzyl)-4-methoxy-*N*-(pyridin-4-ylmethyl)benzamide (21):** Yield = 36%. 1H NMR (500 MHz, CD_3OD) δ ppm 3.81 – 3.86 (m, 3 H), 4.48 – 4.81 (m, 5 H), 5.45 (br. s., 1 H), 6.71 – 6.89 (m, 2 H), 6.90 – 7.03 (m, 3 H), 7.25 – 7.35 (m, 3 H), 7.41 – 7.55 (m, 3 H), 7.84 – 7.88 (m, 1 H), 8.03 – 8.08 (m, 1 H), 8.20 (t, $J=1.68$ Hz, 1 H), 8.49 (br. s., 2 H); HRMS (ESI): m/z calculated for $C_{29}H_{25}BrN_2O_4 + H^+$ [$M + H^+$]: 545.1070. Found: 545.1072.

***4*-methoxy-*N*-(3-((3-nitrobenzyl)oxy)benzyl)-*N*-(pyridin-4-ylmethyl)benzamide (22):** Yield = 39%. 1H NMR (500 MHz, $CDCl_3$) δ ppm 3.84 (s, 3 H), 4.48 – 4.78 (m, 4 H), 5.17 (s, 2 H), 6.81 – 6.97 (m, 5 H), 7.10 – 7.25 (m, 2 H), 7.32 (t, $J=7.93$ Hz, 1 H), 7.48 (br. s., 2 H), 7.61 (t, $J=7.93$ Hz, 1 H), 7.79 (dd, $J=7.63, 0.61$ Hz, 1 H), 8.22 (dd, $J=8.09, 1.37$ Hz, 1 H), 8.34 (s, 1 H), 8.61 (d, $J=4.88$ Hz, 2 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 55.33, 68.61, 113.89, 122.11, 122.98, 127.27, 128.76, 129.62, 130.14, 133.08, 138.28, 138.91, 146.12, 148.45, 150.16, 158.65, 161.05, 172.27. HRMS (ESI): m/z calculated for $C_{28}H_{25}N_3O_5 + H^+$ [$M + H^+$]: 484.1867. Found: 484.1875.

5.2 Biological Evaluation

5.2.1 Cell Culture—JJN3, L363 and KMS11 cell lines were graciously provide by Dr. M. Kuehl (NCI, National Institutes of Health). MM.1S was obtained from Dr. S. Rosen (City of Hope, CA). KMS11-GFP- and -GLUT1-expressing cells were generated previously in our lab [27]. All cells were cultured in complete RPMI 1640 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100mg/ml streptomycin, 0.5 μ g/ml plasmocin (InvivoGen, San Diego) and maintained in a 37°C incubator with 5% CO_2 . Generation of HEK293 cells overexpressing human GLUT1 (GLUT1), human GLUT2 (GLUT2), human GLUT3 (GLUT3), human GLUT4 (GLUT4) or human GLUT8 (GLUT8) has been previously described.[35] In all HEK293 cell lines except for HEK293 overexpressing GLUT1 cells, endogenous GLUT1 was knocked down using cognate shRNA as previously described [35].

5.2.2 Drugs—Chemotherapeutic drugs were purchased from the indicated sources: Venetoclax: Biovision Inc. (Catalogue #2253-1), dexamethasone: Sigma (Catalogue #D4902) and Melphalan: Sigma (Catalogue #M2011).

5.2.3 Cell death, viability and proliferation assays— IC_{50} studies were performed using the CellTiter-Glo assay (Promega, Madison, WI). 20,000 cells in 100 μ l complete RPMI-1640 medium were plated per well in 96-well plates, with a concentration range of individual compounds. After 72 h incubation, 100 μ l Glo reagent was added to each well and luminescence measured using a Biotek Synergy 4 multimode plate reader. Cell counts were also assessed by trypan blue staining and an automatic cell counter (Biorad). For cell viability assays 0.125×10^6 cells/mL were treated with the indicated concentration of drug, washed with 1x PBS and evaluated for viability by AnnexinV/DAPI staining and flow cytometry. Data analysis was performed with the FCS express version 3 (De Novo software, Los Angeles).

5.2.4 RNA extraction and real-time PCR—RNA extraction was performed using the GeneJET RNA purification kit (Thermo Scientific). cDNA was synthesized with RevertAid First Strand cDNA synthesis kit (Thermo Scientific). Primer-probe sets to GLUT4 and GAPDH were purchased from Applied Biosystems. Real-time PCR was performed using the Applied Biosystems 7500 Real-Time PCR System.

5.2.5 Myeloma Patient Sample Processing—Primary myeloma cells were isolated from bone marrow aspirates or peripheral blood samples from consenting myeloma patients as previously described.[33] Cells subjected to various treatments were stained with anti-CD38 phycoerythrin and anti-CD45-allophycocyanin-Cy7 (BD Biosciences) to identify MM cells. All samples were collected following an Emory University Institutional Review Board-approved protocol.

5.2.6 Lentiviral production and myeloma cell transduction—Highly concentrated lentiviral vectors were generated as previously described [9]. Lentivirus was concentrated by precipitation using PEG 6000. The GLUT4 pLKO.1 shRNA (TRCN0000043632) construct was purchased from Sigma. For transduction, JJN3 cells in serum-free medium containing 2.5 µg/ml polybrene and 50µl concentrated virus were centrifuged at 1250 g for 90 min at room temperature with additional details as previously reported [9].

5.2.7 2-Deoxyglucose Uptake Measurements—Tissue culture plates were pretreated with 25 µg/ml polyethyleneimine (Fluka, catalogue number P3143) in 150 mM NaCl for 20min to let cells adhere. JJN3 cells were subsequently plated and grown to 40–60% confluency in complete RPMI-1640 medium. HEK293 GLUT overexpressing cells were plated at 400,000 cells/ml overnight. Cells were then washed with glucose-free HEPES buffer twice and starved for 30 min. The uptake of 2-[³H] deoxy-D-glucose (2-DOG) (50 µM) was measured in glucose-free HEPES buffer for 4–6 min at 37 °C as described previously [65]. Compound **20** (20 µM) was added 5–6 min prior to the addition of glucose depending on the specific assay and indicated in the figure legend. For the HEK293 GLUT overexpressing cells, non-specific uptake was measured in non-transfected HEK293 cells containing the shRNA GLUT1 knockdown and was subtracted from the experimental values. Data are plotted as percent uptake relative to unexposed HEK293 cultures. Data were normalized to fit by nonlinear regression analysis using GraphPad Prism software.

5.2.8 Isolation and photolabeling of Low Density Microsomes—LDMs were isolated from Myc-tagged GLUT1 or GLUT4 HEK293 cells as previously described [66]. Inhibitors were added to LDMs (200– 400 g) for 10 min at room temperature. Samples were then incubated for 10 min at room temperature with biotinylated ATB-BMPA (50 M final concentration) ATB-BMPA (2.5 M final concentration) and then placed on ice prior to UV irradiation. Reactions were transferred to a 24-well low protein retention culture dish (Costar, Corning, NY) and then irradiated at room temperature 5 cm from a Green Spot UV lamp for 1 min (30 s of light followed by 30s of darkness followed by 30 s of light).

5.2.9 Immunoblotting—Whole cell lysate preparation cells were incubated with RIPA lysis buffer (supplemented with phosphatase and protease inhibitors and 1% PMSF) for 15min on ice and then centrifuged at max speed 15000rpm for 20min. Supernatant is

collected and evaluated for protein expression as indicated. Antibodies to: MCL-1 (sc-819) was purchased from Santa Cruz Biotechnology and Actin from Cell Signaling Technology (CST3700s).

5.2.10 Statistical analyses—The IC₅₀ value for compounds was determined using GraphPad Prim software. Results are expressed as mean ± S.E. of at least three independent determinations, unless otherwise noted. *p*-values were determined by an unpaired student *t*-test using GraphPad Prim software (San Diego) with *p* values <0.05 considered statistically significant. Bonferroni post-tests were performed to compare replicate means where each column in a row was compared to all other columns. *p* values are indicated as follows: *p*<0.05 as *, *p*<0.01 as **, *p*<0.001 as ***.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MM	multiple myeloma
TM	transmembrane
LDM	low density microsome
EC	extracellular
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
GLUT	glucose transporter
2-DOG	2-[³ H]deoxyglucose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt

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Highlights

- A series of novel GLUT4 antagonists were designed and synthesized
- Lead compounds have high selectivity for GLUT4 versus other GLUT isoforms
- Compound **20** inhibits GLUT4-mediated glucose uptake and MM cell proliferation
- Treatment of patient samples with compound **20** causes sensitization to MM drugs

Figure 1.
Known GLUT4 antagonists

Figure 2. Screen for identifying GLUT4-selective inhibitors

(A) KMS11 isogenic myeloma cell lines expressing GFP or GLUT1 and (B) JJN3 cells expressing non-targeting pLKO.1 or GLUT4 directed shRNA are treated with test compounds for 72 hours following which viability is assessed using the Cell Titer Glo assay.

Figure 3. Compound 20 inhibits glucose uptake in JJN3 and reduces cell proliferation by targeting GLUT4

(A) JJN3 cells were transduced with non-targeting shRNA (C shRNA) or GLUT4-directed shRNA. Efficiency of knockdown was determined by qRT-PCR analysis of GLUT4 normalized to GAPDH expression. (B) Evaluation of 2-[3H]deoxyglucose (2-DOG) uptake: C shRNA and GLUT4 shRNA expressing cells from part (A) were pre-treated with Compound **20** (20 μM) for 6 min followed by measurement of 2-DOG uptake for 6 min at 37 °C; (C) Cells from part (A) were evaluated for viability/proliferation after 72 hours of Compound **20** (15 μM) treatment using trypan blue and an automated cell counter. Data, normalized to controls, represent the mean ± S.E. of three independent measurements. * indicates p value <0.05. *** indicates p value <0.001. *ns* means not statistically significant.

Figure 4. Compound 20 exhibits selectivity for inhibition of 2-deoxyglucose (2-DOG) transport through GLUT4

Following a 5-minute exposure to Compound **20**, HEK293 cells over-expressing individual human GLUTs were assayed for 2-DOG uptake for 6 minutes at 37 °C. Non-specific uptake was measured in non-transfected HEK293 cells containing the shRNA GLUT1 knockdown and was subtracted from the experimental values. Data are plotted as percent uptake relative to unexposed HEK293 cultures. Data represent the mean $\pm$ S.E. of three independent measurements and were normalized to fit by nonlinear regression analysis using GraphPad Prism software.

Figure 5. Compound 20 selectively binds to GLUT4 vs. GLUT1

DMSO (vehicle), compound **26** (20 μ M) and compound **20** (20 μ M) were added to 200 μ g LDM prepared from Myc-tagged GLUT1- or GLUT4- overexpressing HEK293 cells for 10 min at room temperature. Samples were then incubated with biotinylated ATB-BMPA (50 μ M final concentration) followed by UV irradiation. Biotinylated proteins, isolated from detergent solubilized LDM using a high-capacity streptavidin agarose resin, were analyzed by immunoblot analysis using GLUT4 or GLUT1 antibodies. GLUT proteins were quantified using an Odyssey Infrared Imaging System. Data, normalized to vehicle controls, represent the mean $\pm$ S.E. of three independent measurements. * indicates p value <0.05 , *** indicates p value <0.001 , and *ns* means not statistically significant. Compound **26** denoted with an asterisk, a previously reported GLUT4 antagonists (Mishra et al., J. Biol. Chem., 2015) was used as a positive control.

Figure 6. Compound 20 decreases MCL-1 expression and sensitizes MM cell lines and patient samples to Venetoclax (ABT-199), dexamethasone (Dex) and melphalan (Mel)

(A) JJN3 and L363 cells treated with/without compound **20** (15 μ M) for 18 hours were evaluated for expression of MCL-1 and actin (as a loading control) by immunoblot analysis of cellular lysates. One of two representative experiments is shown. (B) L363 and (C) JJN3 treated with 10 μ M compound **20** with/without indicated concentrations (0.025 μ M – 3 μ M) of ABT-199 for 72 hours were evaluated for cell death; (D) MM patient sample bone marrow aspirate buffy coat cells were similarly treated with 10 μ M compound **20** with or without 0.5 μ M ABT-199 for 48 hours. CD38-phycoerythrin and CD45-allophycocyanin-Cy7 positive myeloma cells were evaluated for cell death; (E) MM.1S cells were treated with indicated concentrations of Dex (0.00195 μ M – 1 μ M) and/or 10 μ M compound **20** for 72 followed by assessment of cell death; (F) MM patient samples treated with 10 μ M compound **20** and 0.5 μ M Dex for 48hr and evaluation of cell death; (G) Effect of compound **20** administered either alone or in combination with ABT-199 or Dex on normal cellular populations within MM patient bone marrow aspirate buffy coat cells used in (F). Data from patient #6 is representative of all patient samples evaluated; (H) MM.1S cells treated with

10 μ M compound **20** or the indicated concentrations (0.01 μ M – 4 μ M) of melphalan (Mel) or their combination for 72 hours were evaluated for cell death. Cell death was evaluated by AnnexinV/DAPI flow cytometric staining. Data in (B), (C), (E) and (H) are mean $\pm$ SEM (n=3). ** indicates *p* value <0.01, *** indicates *p* value <0.001, and *ns* means not statistically significant.

Figure 7. Docked pose of compound 20 in GLUT4

The tertiary amide and pyridine nitrogen form hydrogen bonds to Asn176. Significant hydrophobic interactions take place between the chlorophenyl group and Phe307, and the methoxyphenyl group and Trp404.

Scheme 1.

Synthesis of tertiary amide analogs of previously reported compound 26^a

^aReagents and conditions: (a) $\text{Ti}(\text{O-}i\text{-Pr})_4$, MeOH, RT, then NaBH_4 , -78°C to RT, 1h; (b) HOBt, EDCI, TEA, $R_1\text{CO}_2\text{H}$, DMF, RT, 18h; (c) K_2CO_3 , acetone, $R_2\text{-Br}$, 56°C , 18h.

Table 1
Hit-to-lead SAR: evaluation of IC₅₀ for inhibition of proliferation

Isogenic KMS11-GFP/GLUT1 overexpressing myeloma cell lines were treated with a dose range of the indicated test compound for 72 hours followed by evaluation of viable cell number by Cell Titer Glo assay. Data represent the mean $\pm$ standard error (S.E.) of three independent measurements.

Compound	R1	R2	KMS11-GFP IC ₅₀ (μM) $\pm$ S.E.	KMS11-GLUT1 IC ₅₀ (μM) $\pm$ S.E.
7			9.1 $\pm$ 0.52	12.7 $\pm$ 0.17
8			8.9 $\pm$ 0.07	15.1 $\pm$ 0.09
9			17.7 $\pm$ 0.12	25.8 $\pm$ 1.11

Compound	R1	R2	KMS11-GFP IC ₅₀ (μM) ± S.E.	KMS11-GLUT1 IC ₅₀ (μM) ± S.E.
10			16.9 ± 0.12	21.0 ± 0.51
11			5.1 ± 0.06	4.9 ± 0.06
12			19.7 ± 0.09	16.0 ± 0.17

Compound	R1	R2	KMS11-GFP IC ₅₀ (μM) ± S.E.	KMS11-GLUT1 IC ₅₀ (μM) ± S.E.
13			8.4 ± 0.13	6.5 ± 0.17
14			15.4 ± 0.45	11.7 ± 0.46
15			14.3 ± 0.17	16.8 ± 0.09

Compound	R1	R2	KMS11-GFP IC ₅₀ (μM) ± S.E.	KMS11-GLUT1 IC ₅₀ (μM) ± S.E.
16			22.7 ± 2.16	28.6 ± 0.26
17			16.9 ± 0.81	28.5 ± 0.14
18			18.5 ± 0.16	16.9 ± 1.49
19			13.1 ± 0.12	15.6 ± 0.23

Compound	R ₁	R ₂	KMS11-GFP IC ₅₀ (μM) ± S.E.	KMS11-GLUT1 IC ₅₀ (μM) ± S.E.
20			13.4 ± 0.92	21.8 ± 0.99
21			18.7 ± 1.33	21.4 ± 1.98
22			19.6 ± 0.13	20.6 ± 0.48

Table 2
Compound 20 inhibits glucose uptake by selectively targeting GLUT4

Glucose transport in HEK293 cells over-expressing GLUT1 or over-expressing GLUT4 or GLUT8 in cells lacking GLUT1 were used to evaluate inhibition of glucose transport. Compounds denoted with an asterisk were previously reported GLUT4 antagonists (Mishra et al., J. Biol. Chem., 2015) used as positive controls to assess our observed improvement in selectivity. Cells were pre-treated with compound for 5 minutes followed by evaluation of 2-DOG uptake for 6 min at 37 °C. Non-specific uptake was measured in non-transfected HEK293 cells containing the shRNA GLUT1 knockdown and was subtracted from the experimental values. Data are plotted as percent uptake relative to unexposed HEK293 cultures. IC₅₀ was determined by a non-linear regression analysis using GraphPad Prism 5 software. Data represent the mean of three independent measurements.

Compound	IC ₅₀ (inhibition of 2-DOG transport in HEK293 cells over-expressing GLUT isoforms) ± S.E.		
	GLUT1	GLUT4	GLUT8
3*	>100 μM	20 ± 0.16 μM	>100 μM
17*	>100 μM	11 ± 0.16 μM	>100 μM
26*	>100 μM	1.7 ± 0.12 μM	6 ± 0.26 μM
39*	30 ± 0.5 μM	30 ± 0.51 μM	>100 μM
20	>100 μM	18.2 ± 0.03 μM	>100 μM