Targeting GLUT1 in acute myeloid leukemia to overcome cytarabine resistance

A key alteration in cancer metabolism is an increase in glucose uptake mediated by the glucose transporter (GLUT). Otto Warburg observed already in the 1950s that glycolysis was increased in many tumors, and this is now called the Warburg effect. Interestingly, a distinct glucose metabolic signature was recently described for acute myeloid leukemia (AML), showing that enhanced glycolysis correlates with decreased sensitivity for chemotherapy (cytarabine, Ara-C) and poor prognosis. AML is the most common acute leukemia in adults and is associated with poor survival, especially in patients >60 years, an age group in which only 5-15% are cured. Moreover, older patients who cannot tolerate intensive chemotherapy have a median overall survival of only 5-10 months. Thus, novel therapeutic approaches are needed to improve the cure rates of AML. Interestingly, defective GLUT1-mediated glucose uptake was shown to impair AML cell proliferation, and transplantation of GLUT1-deleted murine AML cells attenuated AML development in mice, suggesting that GLUT1 plays an important role in AML. Thus, targeting GLUT1 may represent a novel therapeutic vulnerability in AML by overcoming Ara-C resistance. However, there are still no clinically available drugs targeting GLUT, which may partly be due to the lack of suitable in vitro drug-screening systems. Here we present a detailed structural and functional analysis of compounds that inhibit glucose transporters and sensitize AML cells for chemotherapy.

GLUT1 is an integral membrane protein consisting of 12 transmembrane helices and an intracellular domain, which transports glucose depending on the concentration gradient (Figure 1A). Measuring the activity of membrane proteins such as GLUT1, which transport uncharged substrates, is challenging due to the lack of an easily accessible readout. However, we have developed a system by which purified glucose transporters are reconstituted in vitro into giant vesicles reporting their transport activity using fluorescence microscopy. This allows glucose uptake to be measured without any interference from other proteins by having the purified transporters imbedded in a lipid-bilayer mimicking the size and curvature of mammalian cells. Applying this method, putative GLUT1 inhibitors PGL-13, PGL-14 and PGL-27 (Figure 1B), were validated and benchmarked against the well-known GLUT1 inhibitors WZB-117 and cytochalasin B (CB). A clear decrease in glucose uptake was detected for PGL-13 and PGL-14, but not for PGL-27 (Figure 1C).

To rationalize these results, molecular modeling studies including docking, molecular dynamics (MD) simulations and ligand-protein binding energy evaluations were carried out. The structure of GLUT1 has previously been determined in complex with CB and phenylalanine (Figure 1D). The docking solutions could be clustered into three binding modes, and for each cluster the docking solution with the best estimated binding energy was selected as a representative potential binding mode. To assess the reliability of the predicted binding modes, the three ligand-protein complexes (complex 1-3, Online Supplementary Figure S1A-C) were subjected to MD simulations. In parallel, the same MD protocol was applied to the GLUT1-PGL-14 complex predicted from our previous docking studies based on the GLUT1 homology model in a partially occluded inward-facing conformation, where the ligand is bound to the intracellular domain of GLUT1 (complex 4, Online Supplementary Figure S1D). In 3 out of 4 GLUT1-PGL-14 complexes studied (complex 2-4), the ligand maintained its binding mode predicted by docking showing an average root-mean square deviation (RMSD) of its disposition during the MD simulations below 1.5 Å (Figure 1D). In particular, the binding disposition of compound PGL-14 in the intracellular site (complex 4) was remarkably stable, with an average RMSD of about 0.7 Å. Combined, these analyses suggest that PGL-14 likely interacts with GLUT1 in a partially occluded, inward-facing conformation at the intracellular domain. This is an interesting feature that distinguishes the PGL from competitive inhibitors of GLUT1, for instance CB that is known to bind to the transmembrane area. However, we cannot exclude the possibility of PGL compounds having two potential GLUT1 binding sites: the transmembranes and the intracellular binding site (Figure 1E). A refined binding mode was generated for PGL-14, revealing that the interactions are essentially as previously described, with a salicylketoxime ring sandwiched between the side chains of E146 and R212, forming a π-π stacking interaction with the latter residue, as well as H-bond interactions by the functional groups of the ligand to the backbone of the protein (Figure 1F). As PGL-27 did not have inhibitory effects (Figure 1C), the presence of a meta-methyl group in the terminal phenolic ring of PGL-27 (Figure 1B) could result in less favorable binding affinity as it requires displacement of the water molecule that is forming a water-bridged interaction between the ligand and the protein (Figure 1F), and might additionally result in steric clashes.

To experimentally investigate the modeled binding sites, we monitored intrinsic fluorescence quenching for the two binding-site tryptophans upon PGL binding. Measurements showed a decrease in fluorescence intensity, suggesting binding to the transmembrane domain (Figure 1G and H). However, at higher concentrations, a red shift in the emission spectra and an increase in fluorescence intensity was observed (Figure 1G and H). Based on the MD simulations, such behavior could imply PGL binding to two sites (Figure 1D and E) with different affinities. The decrease in fluorescence shows binding in the transmembrane part, while the red shift could result from a secondary conformational event in the intracellular domain, as structural changes accompanying PGL binding could have an overall effect. Indeed, a recent study demonstrated that the intracellular domain of GLUT1 is highly mobile and that its conformational flexibility is strongly coupled to other parts of the protein.

To evaluate whether the PGL compounds are specific for GLUT1, and if targeting GLUT1 can sensitize AML cells for chemotherapy, myeloid leukemia-derived cell lines were screened for GLUT1 expression (Online Supplementary Figure S2). THP-1 cells expressed significant amounts of GLUT1 in contrast to KG-1 cells (Figure 2A and B). Thus, comparison between THP-1 and KG-1 cells is an applicable model system to validate the specificity of PGL towards GLUT1 and their sensitization effects for Ara-C treatment. First, the effect on cell viability by Ara-C, PGL-13 and PGL-14 was assessed at increasing concentrations in THP-1 and KG-1 cells (by ATP-assay) and IC25 values were determined (Online Supplementary Figure S3A-C). Subsequently, co-treatments with Ara-C and PGLs at IC25 were evaluated for potential synergistic or additive effects. The combinatorial
Figure 1. Inhibition of glucose uptake via GLUT1 by PGL compounds through interference with the intracellular domain. (A) Cartoon representation of GLUT1 in complex with cytochalasin B (CB) (PDB ID 5EQI). N- and C-terminal domains are colored in wheat and green, respectively, and the intracellular domain is shown in magenta. The bound CB is shown in stick representation in yellow. (B) Structures of PGL-13, PGL-14 and PGL-27. (C) Inhibition of glucose uptake by selected compounds measured in giant vesicles. Results demonstrate mean normalized to the DMSO control (CTRL), n=5. (D) Molecular dynamics (MD) simulation of representative binding modes of PGL-14 at GLUT1 (complex 1-3) and at homology model of GLUT1 (complex 4). (E) Two PGL binding sites, transmembrane and intracellular, predicted by docking of PGL-14. Complex 2 with predicted inhibitor binding site at GLUT1 inward open conformation overlapping with a glucose/CB binding site (PDB ID 5EQI). Complex 4 with a predicted intracellular binding site at homology model of GLUT1 in a partially occluded inward-facing conformation. (F) Minimized average structure of PGL-14 within the intracellular binding site of GLUT1, derived from the last 15 ns of MD simulation. Hydrogen bonds are represented as dashed lines. (G and H) Intrinsic fluorescence spectra for purified GLUT1 at different concentrations of the inhibitors (G) PGL-13 and (H) PGL-14, with excitation wavelength at 295 nm.
Figure 2. Sensitization to cytarabine (Ara-C) by inhibition of glucose uptake. (A) Immunofluorescence of GLUT1 expression (magenta) in THP-1 cells and KG-1 cells. Nucleus stained with DAPI. 60x magnification, scale bars=30 μm. (B) Western blot of THP-1 and KG-1 whole lysates; GAPDH as the loading control. (C and D) Relative inhibition of cell viability in THP-1 (magenta) and KG-1 (blue) cells using Ara-C and (C) PGL-13 or (D) PGL-14, alone or in combination at IC25. Co-treatment effects were designated synergistic or additive/no effect as determined by the Bliss independence model through calculation of the combination index (CI). SSynergy (CI<1) and is based on CI-values (C) 0.64 and (D) 0.78. (E and F) Relative inhibition of cell viability in THP-1 cells using Ara-C, Brequinar (BQR) and (E) PGL-13 or (F) PGL-14, alone or in combination. Combination of PGL with Ara-C gave values (E) CI=0.76 and (F) CI=0.90. All co-treatment values show mean±standard deviation normalized to a DMSO control, n=3-5. Dotted lines show theoretical IC25 values.
effect was considered synergistic when the combination index (CI) had a value < 1. An inhibitory synergistic effect was detected for PGL-13 (CI=0.64) and PGL-14 (CI=0.78) in combination with Ara-C for THP-1, but not for KG-1 (CI=1.24 and CI=1.10 for PGL-13 and PGL-14, respectively) (Figure 2C and D and Online Supplementary Figure S4A and B). A similar inhibitory effect for the combination of doxorubicin with PGL-13 (CI=0.53) or PGL-14 (CI=0.76) was detected in THP-1 cells (Online Supplementary Figures S5D and S4C and D). To confirm the specificity of the PGL, THP-1 cells were also co-treated with maltose (a disaccharide known to bind to GLUT1 but which is non-transportable) and Ara-C, showing a clear inhibitory synergistic effect (CI=0.82) (Online Supplementary Figures S3E and S4E). This suggests a sensitization effect by both PGL compounds in myeloid leukemia cells with high GLUT1 expression. However, also Mono-Mac-6 (MM6) cells with a clear but lower GLUT1 expression level compared to THP-1 (Online Supplementary Figure S2A and B) displayed synergistic inhibitory effects for the PGL with Ara-C (CI=0.62 and CI=0.53, for PGL-13 and PGL-14, respectively), suggesting that the presence of GLUT1, rather than the amounts, is contributing to sensitization effects (Online Supplementary Figures S3A-C and S4F and G).

A mechanistic explanation for the observed synergistic effects could be that Ara-C inhibits the Akt-pathway resulting in increased dependence on aerobic glycolysis that in turn is inhibited by PGL. This is supported by the observations that PGL-13 can restore repression of increased glycolysis caused by Akt inhibitors,1 and by the suggestions that Ara-C can inhibit phosphorylation of Akt and the downstream mTOR pathway in AML.12 However, Ara-C resistant AML cells have been shown to rather be dependent on oxidative phosphorylation (OXPHOS) than glycolysis.13 Thus, to validate the hypothesis, we also targeted dihydroorotate dehydrogenase (DHODH) a known putative metabolic target for Brequinar, a well-studied DHODH inhibitor, provides electrons for OXPHOS. A distinct inhibition of proliferation in THP-1 cells in combination with Ara-C (Figure 2E and F, and Online Supplementary Figures S3F and S5A-D). In addition, when combining PGL, Brequinar and Ara-C the outcome was similar as to when only PGL and Ara-C were combined (Figure 2E and F, and Online Supplementary Figures S3F and S5A-D). Taken together, this suggests that AML cells with high levels of glucose transporters are particularly vulnerable towards blocking glycolysis in combination with chemotherapy, suggesting that the PGL class of compounds should be further evaluated for AML therapy.

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