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Targeting glycogen metabolism in bladder cancer

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Abstract

Metabolism has been a heavily investigated topic in cancer research for the past decade. Although the role of aerobic glycolysis (the Warburg effect) in cancer has been extensively studied, abnormalities in other metabolic pathways are only just being understood in cancer. One such pathway is glycogen metabolism; its involvement in cancer development, particularly in urothelial malignancies, and possible ways of exploiting aberrations in this process for treatment are currently being studied. New research shows that the glycogen debranching enzyme amylo-α-1,6-glucosidase, 4-α-glucanotransferase (AGL) is a novel tumour suppressor in bladder cancer. Loss of AGL leads to rapid proliferation of bladder cancer cells. Another enzyme involved in glycogen debranching, glycogen phosphorylase, has been shown to be a tumour promoter in cancer, including in prostate cancer. Studies demonstrate that bladder cancer cells in which AGL expression is lost are more metabolically active than cells with intact AGL expression, and these cells are more sensitive to inhibition of both glycolysis and glycine synthesis—two targetable pathways. As a tumour promoter and enzyme, glycogen phosphorylase can be directly targeted, and preclinical inhibitor studies are promising. However, few of these glycogen phosphorylase inhibitors have been tested for cancer treatment in the clinical setting. Several possible limitations to the targeting of AGL and glycogen phosphorylase might also exist.

Introduction

In the past 10–20 years, much attention in cancer research has been given to the differences in metabolism between cancer and normal cells to discover whether these differences

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promote tumour progression and whether altered metabolism can be exploited for treatment.[1–3] A considerable amount of this research has focused on the Warburg effect. This phenomenon encompasses a drastic increase in glucose uptake and glucose metabolism in cancer cells via glycolysis to lactate, instead of through oxidative phosphorylation, even in the presence of plentiful oxygen in the cell (termed aerobic glycolysis).[4] This metabolic switch is accomplished by upregulation of the activity and expression of glucose transporters on the cell membrane and the enzymes involved in glycolysis.[5–8] In cancer, the primary role of glycolysis is not to increase energy production, but to increase the supply of precursor molecules into other metabolic pathways, thus providing the necessary cellular building blocks for rapidly proliferating cancer cells.[2] Many glycolytic enzymes are important in cancer progression and survival,[9–12] and some, such as lactate dehydrogenase A[13] and the phospho-fructokinase 2 isoform expressed from *PFKFB3*,[14] are viable targets for therapy.

Although they have received less attention than glycolysis, other metabolic pathways, such as the tricarboxylic acid cycle and fatty acid metabolism, are also altered in cancer, with increased flux through these pathways providing energy or cellular metabolites necessary for cell proliferation.[5,15–17] In addition (or as an alternative) to glucose, cancer cells can increase glutamine import.[16] Once intracellular, glutamine is converted to glutamate and enters the tricarboxylic acid cycle to provide an alternate carbon source for the cells in the production of ATP, amino acids, and nucleic acids. Mutations in tricarboxylic acid cycle enzymes also cause a shift in metabolism from the tricarboxylic acid cycle to glycolysis.[18–20] Mutations in fumarate hydratase and succinate dehydrogenase lead to reduced degradation of the hypoxia-inducible factor 1 (HIF-1), the transcription factor that induces the expression of many glycolytic enzymes, increasing glycolytic flux and the Warburg phenotype.[21,22] Abnormal lipid metabolism has also been observed in tumours,[15,17,23] with increased expression of enzymes involved in fatty acid metabolism, such as ATP-citrate lyase,[24] fatty acid synthase (FAS),[25–27] and mono-acylglycerol lipase.[28] Many cancer cells produce fatty acids *de novo* instead of primarily using exogenously imported fatty acids, as is the case in normal tissue.[15,23] Some cancers, such as prostate cancer,[26] glioblastoma,[23] and ovarian cancer,[29] are dependent on fatty acid metabolism to maintain growth. Enzymes involved in these pathways might provide novel therapeutic targets for these malignancies.

Regardless of mechanism, changes in energy metabolism are observed across all cancer types, and this ‘reprogramming’ has been defined as a hallmark of cancer.[30] Hence, understanding cancer metabolism might provide us with new avenues of disease treatment, which might even be tailored to individual patients, depending on which changes in metabolism are observed.

Key points

- Aerobic glycolysis (the Warburg effect) has been extensively studied in urological and other cancer models, but changes in other metabolic pathways also warrant investigation

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- Glycogen metabolism is one metabolic pathway that is altered in cancer and is just beginning to be understood
- Loss of the glycogen debranching enzyme (AGL) leads to increased proliferation of bladder cancer cells *in vitro* and *in vivo*, and has prognostic value in patients with bladder cancer
- Glycogen phosphorylase, involved in glycogen breakdown, is important for cancer cell survival in some tissues
- Glycogen phosphorylase and metabolic alterations resulting from reduced AGL expression might represent new targets in the treatment of urological cancers

In this Review, we discuss the role of glycogen metabolism in cancer development and growth with a focus specifically on urothelial bladder cancer, using our studies on the role of the glycogen debranching enzyme amylo-α-1,6-glucosidase, 4-α-glucanotransferase (AGL) in bladder cancer growth and disease progression.[31] We describe the genes, enzymes and mechanisms involved in this metabolic pathway and how alterations of enzymatic function influence cancer viability. In addition, we will consider possible future strategies for targeting the glycogen metabolism and relevant safety aspects.

Targeting bladder cancer metabolism

In 2015, an estimated 74,690 individuals will be diagnosed with bladder cancer and 15,580 will die from the disease in the USA alone.[32] Many genetic and epigenetic alterations are implicated in bladder cancer tumorigenesis, progression, and metastasis, yet few functional drivers of these processes have been defined. However, such functional delineation is critical for providing novel strategies of therapeutic intervention. The metabolism of bladder cancer cells is one such avenue that can be exploited.

Glucose metabolism in bladder cancer

Glucose uptake is increased in bladder cancer via over-expression of glucose transporter type 1 (GLUT-1).[33] GLUT-1 has a high affinity for glucose, and is the glucose transporter that is most often upregulated across all cancer types.[34] In bladder cancer, high expression of GLUT-1 correlates with disease progression,[33,35] and is also associated with poor survival: the 5-year survival of patients with high GLUT-1 expression is 32% compared with 72% for patients with lower than median GLUT-1 expression.[36] In addition, GLUT-1 is not expressed in normal urothelium or benign bladder papillomas,[33,35] in contrast to non-muscle-invasive urothelial carcinoma and muscle-invasive disease.[33] Similarly, in renal cell carcinoma (RCC), GLUT-1 is expressed in tumours, but not in normal tissue.[37,38]

GLUT-1 activity is dependent on its location in the cell; membrane localization is critical for glucose import.[39,40] Although changes in localization have been noted in some cancers, such as ovarian cancer[41] and lymphoma,[42] no information regarding such changes is available for urologic cancers. Although glucose metabolism in cancer, including bladder cancer, has been extensively studied, the role of glycogen metabolism in cancer is not well understood.

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Glycogen metabolism in bladder cancer

Overview of glycogen metabolism

Glycogen, a large, branched polymer of glucose monomers, is primarily found in the liver, heart and muscle, and it is responsible for glucose storage within the cell (Figure 1). Glucose is converted to glucose 1-phosphate (G1P) through glycolysis and added to a growing glycogen chain by *GYS1*-expressed glycogen synthase (GYS1).[43]

When exogenous glucose is limited, glycogen is metabolized (Figure 2). Most glucose molecules in the polymer are linked by α-1,4-glycosidic bonds, whereas the branch points of the glycogen molecule are linked by α-1,6-glycosidic bonds.[43] Glycogen phosphorylase is responsible for the cleavage of the α-1,4-bonds and releases G1P monomers that can enter the glycolytic pathway or pentose phosphate pathway, or are further metabolized to free glucose to enter the bloodstream for export to other tissues. However, glycogen phosphorylase cannot break the α-1,6-glycosidic bonds at glycogen branch points. When four glucose monomers remain before the branch point, AGL (using its transferase activity) moves three of the glucose monomers to another glycogen chain via an α-1,4 linkage and cleaves the α-1,6-glycosidic bond at the branch point via its glucosidase activity, releasing one free glucose molecule and eliminating the branch point.[44] Glycogen phosphorylase resumes releasing G1P at the end of the newly elongated glycogen branch.[44]

Glycogen breakdown via glycogen phosphorylase (as well as glycogen synthesis through GYS1) is a highly regulated and complicated process. Glycogen phosphorylase and GYS1 are allosterically regulated via concentrations of AMP, ATP, glucose 6-phosphate (G6P) and glucose, and are enzymatically regulated via phosphatidylinositol-4,5-bisphosphate 3-kinase, glycogen synthase kinase-3 β, protein kinase A and protein phosphatase 1.[45] Less is known about the regulation of AGL, although AGL is known to interact with 5' AMP-activated protein kinase.[46] However, this interaction does not affect AGL enzymatic activity, but rather affects 5' AMP-activated protein kinase activity.[46] The exact function of this interaction in cells is unknown.

Alterations in cancers

Observational studies in the 1980s showed that several cancer cell models, including bladder, breast, colon, glioblastoma, kidney, melanoma, ovarian and uterine, contain high levels of glycogen.[47,48] In 2012, glycogen synthesis genes were shown to be induced in a HIF-1-dependent manner in colon carcinoma, RCC, ovarian carcinoma, and cervical cancer cell lines,[49,50] resulting in a repletion of glycogen stores, which cancer cells can use to provide glucose for anaerobic glycolysis when they encounter transient energy starvation conditions.[49,51] In the same year, exposure of U87 glioblastoma, MCF-7 breast, and HCT116 colon cancer cells to hypoxia induced expression of two glycogen metabolism proteins, GYS1 and the liver isoform of glycogen phosphorylase.[52] Glycogen phosphorylase isoforms (liver form [PYGL], brain form [PYGB] and muscle form [PYGM]), along with AGL, are responsible for glycogenolysis (Figure 1). Follow-up experiments revealed that loss of PYGL in these three tumour cell lines resulted in increased reactive oxygen species, leading to senescence and cell death (Box 1, Figure 3).[52] PYGB is also important for cancer

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cell survival.[53,54] In glucose-starvation-resistant pancreatic cancer cells, PYGB is necessary for the resistance; inhibition or loss of PYGB results in cell death upon glucose starvation, indicating that glycogen breakdown is important to the survival process.[53] Similarly, in gastric cancer cell lines in serum starvation conditions, PYGB phosphorylation and activity, as well as glycogen breakdown, are increased, with concurrent increased cell survival and decreased apoptosis.[54] These studies suggest that glycogen phosphorylase isoforms, and glycogen metabolism in general, might provide novel therapeutic targets in cancer.

Box 1

Two glycogenolysis enzymes with opposite effects in cancer

Glycogen phosphorylase supports the growth of cancer cells, while AGL inhibits cancer cell growth. These two enzymes have opposite effects on cancer, despite being involved in the same process (glycogenolysis) owing to the nonenzymatic ('moonlighting') function of AGL. Glycogen phosphorylase supports cancer growth and proliferation by breaking down glycogen into glucose for cancer cells to use in other metabolic pathways. When glycogen phosphorylase is inhibited, glycogenolysis and, in turn, catabolic pathways, are blocked, resulting in cell damage and death.[52,80,92] Glycogen phosphorylase is the rate limiting enzyme of glycogenolysis and is responsible for the breakdown of the majority of the glycogen in the cell; hence, a loss of the enzyme has dramatic effects on glycogenolysis and energy status of the cell (Figure 2).[104] In the absence of AGL, cells can break down a large amount of glycogen through glycogen phosphorylase alone, thus, reducing the impact of AGL loss on cellular glucose metabolism. We have clearly shown that AGL enzymatic activity is not required for its role in cancer, as enzymatically dead AGL mutants retain the tumour-suppressive function of wild-type AGL in bladder cancer models.[31] Thus, speculating that some currently undefined secondary function of AGL is responsible for the inhibition of cancer growth is exciting. When AGL is lost, this inhibition is relieved, allowing cancer cells to grow faster. These moonlighting functions are not uncommon for enzymes in cancer. For example, aldolase A,[12] pyruvate kinase PKM isoform M2,[105] and glucose-6-phosphate isomerase (also called autocrine motility factor)[106] all have roles in cancer in addition to their enzymatic functions. Studies are currently underway in our laboratory to determine the underlying mechanism of this moonlighting function of AGL and how it relates to bladder cancer.

Specific changes in bladder cancer

Results of a study from our group suggest that genes involved in glycogen metabolism also have an important role in bladder cancer.[31] In these experiments, an *in vivo* short-hairpin RNA (shRNA) functional screen was used to find novel tumour suppressors of bladder cancer.[31] Mice were inoculated with human bladder cancer cells that had been transduced with a whole-genome, pooled shRNA library at cell concentrations that do not normally produce tumours. In the mice that developed tumours, next-generation sequencing identified shRNA constructs present in the tumours, uncovering AGL as a novel tumour suppressor. This result was validated in multiple bladder cancer cell lines.[31] As expected, in cells with

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AGL loss, we observed a decrease in normal cellular glycogen with a concurrent increase in abnormal glycogen structures (such as limit dextrin), an increase in glucose uptake, and a corresponding increase in lactate production. AGL depletion led to increased proliferation and anchorage-independent growth *in vitro*, as well as xenograft tumour growth *in vivo*.[31]

Autosomal recessive inheritance of two mutated copies of the *AGL* gene results in glycogen storage disease type III (GSDIII), one of 14 genetic glycogen storage diseases that result in severe metabolic disorders.[55] Symptoms of GSDIII include hepatomegaly, cardiomyopathy, exercise intolerance, and progressive muscle weakness caused by the inability to metabolize glycogen. Biochemically, lack of AGL leads to accumulation of limit dextrin in the affected tissues.

In contrast to glycogen phosphorylase, which seems to be necessary for proliferation in cancer cell lines,[52] it is loss of AGL that promotes bladder cancer growth. To further investigate this phenomenon, we utilized enzymatically inactive AGL mutants with point mutations in the glucosidase or transferase domains.[56] Interestingly, in bladder cancer cells transduced with shRNA against AGL, which resulted in decreased AGL expression and increased cell proliferation, transfection with the enzymatically inactive AGL mutants reversed the increased proliferation, and cell growth levels were similar to cells transfected with wild-type AGL, showing that the role of AGL in tumour growth is independent of its enzymatic function.[31] Furthermore, loss of glycogen phosphorylase did not affect bladder cancer models: depletion of neither of the two isoforms that are expressed in the bladder cancer cell lines tested (PYGL and PYGB —PYGM is not expressed in these cells) changed the growth of these cells.[31] These experiments strongly suggest that AGL does not influence bladder tumour growth through its enzymatic function, but rather that the AGL protein has other unknown nonenzymatic (‘moonlighting’) functions, possibly as a scaffolding protein that drives tumour growth. Perhaps this phenomenon explains why one enzyme involved in glycogenolysis seems to aid in cancer cell proliferation (glycogen phosphorylase), while the other (AGL) seems to inhibit proliferation (Box 1).

Further investigation into the role of AGL in bladder tumour growth revealed that metabolic reprogramming occurs in cells with AGL loss.[31] Initial experiments showed that cells with AGL loss have increased glucose uptake and lactate production,[31] as well as increased GLUT-1 translocation to the membrane and reduced oxygen consumption (D. Theodorescu, unpublished work), pointing to decreased oxidative phosphorylation. To find specific metabolic pathways that are affected by AGL loss, we used ^{13}C-labelled glucose to trace and analyse which metabolites accumulate in cells with reduced AGL.[31] Although total glucose uptake was increased in these cells, glucose-to-lactate conversion was decreased, suggesting that more glucose is being shunted to pathways resulting in macromolecules required for proliferation.

We also observed an increase in glycine levels in cells with reduced AGL expression, which was particularly interesting, as the growth media contained sufficient glycine. At the same time, serine, the precursor of glycine, was unchanged compared with control cells, strongly suggesting that the serine-to-glycine conversion pathway is reprogrammed with AGL loss. The metabolism of serine to glycine is catalyzed by two serine hydroxymethyltransferase

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enzymes, the *SHMT1*-expressed cytosolic form (SHMT1) and the *SHMT2*-expressed mitochondrial form (SHMT2). A study published in 2012 highlighted the importance of SHMT2 specifically in rapidly proliferating cancer cells.[57] Similar to these results, we observed an increase in SHMT2 levels in bladder cancer cells with AGL loss.[31] Therefore, we depleted SHMT2 in bladder cancer cells in the presence and absence of AGL expression and found that loss of SHMT2 abrogates the growth advantage of bladder cancer cells without AGL, suggesting that these cells are dependent on glycine synthesis.[31] Consistent with increased glycine synthesis, purine ring synthesis of nucleotides (for which glycine is a precursor) increased, suggesting that a glycine-driven increase in nucleotide synthesis contributes to enhanced cell proliferation in cancers with low AGL expression.[31]

AGL and clinical outcomes

In addition to its role as a suppressor of tumour growth in experimental models, measurement of AGL expression has prognostic value in bladder cancer. Analysis of both mRNA and protein expression data from patient sample datasets showed that low AGL expression was associated with clinically aggressive bladder cancer.[31] *AGL* mRNA expression was significantly reduced in tumours compared with normal bladder urothelium in three independent datasets (P <0.001, P <0.001 and P = 0.04, respectively), and low *AGL* mRNA expression was associated with high stage and high grade in three of three datasets and with poor patient survival in two of three datasets. Consistent with mRNA data, analysis of AGL protein expression in patient tumour samples and normal urothelium showed that AGL protein expression was also lower in bladder tumours compared with normal urothelium, and patients with low AGL protein expression had reduced recurrence-free survival.[31] In addition, a further decrease in AGL protein expression was observed in metastases compared with primary tumours.[31] Taken together, these data suggest that reduced AGL expression in tumours is associated with more aggressive disease in comparison to tumours with normal AGL expression.

We also investigated the relationship between *AGL* and *SHMT2* mRNA expression and found that expression of these two mRNAs was significantly inversely correlated in two of three datasets studied (P = 0.02 and P <0.001) and approached significance in the third (P = 0.08).[31] *SHMT2* mRNA expression was also increased in malignant but not normal urothelial tissue in two of three datasets, and high *SHMT2* mRNA levels correlated with poor patient survival.

These studies of clinical patient samples, along with our data in animal models, indicate that glycine synthesis is clinically relevant in patients with low AGL expression, and might provide a new therapeutic target in this subgroup of patients with bladder cancer.

Relationship between GSDIII and cancer

Although it seems clear that loss of AGL is a driver of bladder cancer growth and that low AGL expression is a predictor of poor outcome in patients with bladder cancer, the role of AGL in carcinogenesis is not known. Patients with GSDIII[58] could provide us with some insight into the role of AGL in bladder cancer or other cancers. A higher incidence of bladder cancer in patients with GSDIII might suggest that AGL loss is involved in

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transformation of normal bladder epithelia. Hence, we investigated whether a link between GSDIII and bladder cancer exists.[31]

Bladder cancer typically develops in older individuals (median age at diagnosis 72 years),[59] with an increased risk through smoking and exposure to industrial chemicals.[60] Two databases of patients with GSDIII exist: an international cohort consisting of around 175 patients,[61] and one cohort from the Faroe Islands, Denmark,[62] where 1 in 22 individuals is a carrier of an *AGL* gene founder mutation related to GSDIII and 1 in 800 have the disease (D. Weinstein, personal communication). The data from the Faroe Islands population is also included in the international cohort. To date, most individuals in these cohorts are not old enough to have developed bladder cancer, and information about their smoking status is also not gathered. In fact, no case of bladder cancer has been reported in either database (T. Derks and D. Weinstein, personal communications). Thus, given the small number of individuals included in these cohorts, no conclusion can be drawn regarding the risk of bladder cancer development in patients with GSDIII. However, our study[31] suggests the need for increased vigilance in older patients with GSDIII who develop symptoms suggestive but not specific of bladder cancer, such as microscopic haematuria.

In contrast to bladder cancer, hepatocellular carcinoma has been observed in patients with GSDs, including GSDIII;[63–68] however, whether these patients have a higher risk for HCC in comparison with the nonaffected population is unclear. The current working hypothesis is that hepatocellular carcinoma results from chronic hepatic inflammation and/or cirrhosis.[69,70] Although cirrhosis is less common in patients with GSDIII than in patients with other GSDs,[65] pre-existing cirrhosis is present in all cases of hepatocellular carcinoma reported in patients with GSDIII.[64] Nevertheless, a biochemical mechanism has been proposed for the induction of liver cancer in patients with GSDIII: owing to the absence of AGL, patients with GSDIII cannot produce sufficient G6P from glycogenolysis and, instead, gluconeogenesis is required to support the G6P requirement for various metabolic processes.[71] However, β-oxidation of fatty acids would need to increase to provide energy and substrates for this increase in the gluconeogenic pathway with loss of AGL,[71] which could lead to an increase in reactive oxygen species, resulting in DNA damage, inflammation, or other contributors to carcinogenesis. However, to our knowledge, the existence of such a pathway or its effect on carcinogenesis has not been demonstrated in any animal models of cancer and, thus, remains speculative.

Therapeutic and targeting options

As for all cancers, personalized medicine is being optimized for patients with urological cancers depending on their tumour expression profiles.[72–76] In patients with viscerally metastatic bladder cancer, the current standard of treatment is platinum-based chemotherapy, with a median survival after treatment of approximately 1 year.[77] Although our studies have demonstrated that AGL is a prognostic marker in bladder cancer,[31] whether AGL levels can predict responses to various treatments has not yet been tested. We hypothesize that AGL expression could serve as an important bio-marker in selecting patients with bladder cancer for personalized treatment. Specifically, patients with low AGL expression in either non-muscle-invasive or muscle-invasive tumours might benefit from

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stage-appropriate neoadjuvant or adjuvant therapies.[78,79] In addition, other approaches, such as targeting metabolic vulnerabilities specific to AGL loss, could also be envisioned. Furthermore, glycogen phosphorylase, the other enzyme involved in glycogen breakdown and shown to be important for growth in many *in vitro* tumour models,[52–54] including prostate,[80] might also be a viable therapeutic target in other urological cancers.

Options for tumours with low AGL expression

Several innovative therapeutic opportunities exist for patients with urological cancers and low AGL expression and various drugs and compounds are being studied (Figure 1).

Glucose transporter and glycolytic inhibitors—Entrance of glucose into the cell via glucose transporters is the rate limiting step in glucose metabolism.[34] Increased glucose transporter translocation to the plasma membrane is also a major factor driving the aggressive tumorigenic phenotype of cancer cells.[34,81] In bladder cancer cells with reduced AGL expression, we observed an increase in glucose uptake and lactate production, indicating an increase in aerobic glycolysis.[31] Indeed, in cells with low AGL, GLUT-1 translocation to the membrane was increased (D. Theodorescu, unpublished work) and growth was inhibited in response to glucose deprivation and glycolysis inhibition by 2-deoxy-D-glucose.[31] Based on these data, we speculate that AGL-depleted bladder cancer cells are specifically vulnerable to inhibition of glucose uptake and glycolysis.

Phloretin, a natural phenol derivative that blocks glucose uptake through GLUT-1 and GLUT-2,[82] can sensitize cancer cells to chemotherapy.[83] The small molecule WZB117 specifically inhibits glucose transport through GLUT-1 and inhibits growth *in vitro* and *in vivo* in non-small-cell lung cancer and breast cancer models.[84] Cisplatin, a drug commonly used to treat bladder cancer, showed a synergistic anticancer effect in combination with GLUT-1 inhibitor WZB117 in lung and breast tumour models.[84,85] Cisplatin resistance in cancer cells has also been attributed to increase in aerobic glycolysis.[86] Furthermore, downstream glycolytic inhibitors, such as lonidamine, 3-bromopyruvate and 2-deoxy-D-glucose, negatively regulated tumour growth by preventing the activity of the glycolytic enzyme hexokinase.[87,88] Combination of 2-deoxy-D-glucose with anticancer drugs adriamycin, paclitaxel or daunorubicin (three chemotherapeutics already used to treat urological cancers, however, with variable success)[89] resulted in increased therapeutic sensitivity in osteosarcoma, non-small-cell lung cancer, and leukaemia cell lines through an increase in cell death *in vitro* and a decrease in tumour growth in xenografts.[83,88,90] These combination therapies have not been tested in either urological cancers or specifically in cancers with abnormal glycogen metabolism, which is where we propose they would be most effective. Importantly, such combinations might be options for patients who cannot tolerate platinum-based agents.

SHMT2—In our study,[31] depletion of SHMT2 expression using RNA interference reduced the increased growth exhibited by AGL-depleted cells. Whether this association between AGL and SHMT2 is direct or a consequence of increased growth and metabolic rate in general has not yet been established. Nevertheless, SHMT2 is upregulated in many cancer cell types that proliferate rapidly,[57] and, being an enzyme, represents a targetable protein.

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Inhibitors of SHMT2, such as aminomethylphosphonate, are currently under investigation for their effect on thymidylate synthesis in cells,[91] but the research into inhibition of SHMT2 is new and no animal or clinical trials exist. On the basis of our data,[31] cells and/or tumours with low AGL should be selected for such treatments.

Targeting glycogen phosphorylase

In addition to cancer, deregulation of glycogen phosphorylase has been observed in type 2 diabetes and other metabolic disorders.[45] Accordingly, many pharmaceutical companies are interested in developing glycogen phosphorylase inhibitors, and a number of studies on these inhibitors have been recently published and inhibitors have been patented.[45] Although many of these compounds show efficacy in rat models of type 2 diabetes,[45] few have been tested in patients with type 2 diabetes, and even fewer have been tested in *in vitro* or xenograft cancer models.

One encouraging preclinical study in human pancreatic cancer cells showed that inhibition of glycogen phosphorylase with the compound CP-320626 led to the blockade of glycogen metabolism and, in turn, a reduced ability of the cells to recycle glucose into the necessary pentose phosphate pathways and *de novo* fatty acid synthesis pathways, which are required to support rapidly proliferating cancers.[92] The inhibition resulted in a corresponding reduction of proliferation and increase in apoptosis in the pancreatic cancer cells, with no effect on normal and slowly proliferating human fibroblasts.[92] Subsequent studies showed that this drug also has effects on the mitogen-activated protein kinase and NF-κB pathways, suggesting pervasive effects of these drugs in cells, either through or independent of glycogen phosphorylase inhibition.[93]

Other inhibitors of glycogen phosphorylase, CP-91149 and flavopiridol,[94–96] inhibit glycogen phosphorylase by binding at the subunit interface, stabilizing the inactive form of the enzyme, or at the nucleotide binding site, which allosterically inhibits the enzyme, respectively. Only one glycogen phosphorylase inhibitor, flavopiridol, has been tested in clinical trials for the treatment of malignancies, including prostate cancer, renal cell carcinoma and colorectal cancer.[97–99] However, as flavopiridol is also a cyclin-dependent kinase inhibitor,[100] whether its antitumorigenic activity is due to inhibition of cyclin-dependent kinase or glycogen phosphorylase is unclear. Nevertheless, in clinical trials, treatment with flavopiridol in combination with other drugs was safe and showed some efficacy, including in urological cancers.[97–99] Glycogen phosphorylase might therefore be a viable therapeutic option in urological cancers with overactive glycogen and glucose metabolism.

Another aspect to consider when targeting enzymes involved in glycogenolysis is any possible adverse effect on the liver, where glycogen is primarily stored and metabolized. We do not anticipate that inhibition of these enzymes will be fatal, owing to the existence of people living with GSDs. However, patients with GSD certainly have symptoms that affect their liver, so effects on the liver must be closely monitored if these drugs proceed to clinical trials.

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Conclusions

Although glycolysis in cancer, including urological cancers, has been extensively studied, alternative metabolic pathways have not been fully investigated. Here, we propose the idea that glycogen metabolism enzymes have important roles in urological cancers, and that targeting these enzymes and/or downstream molecules that are affected by changes in glycogen metabolism might provide new and interesting therapeutic targets.

In particular, we have discussed our findings on AGL in bladder cancer, the effect of AGL loss on metabolism, and possible therapeutic targets that might become viable for bladder cancer treatment. Of course, more work is needed to determine how loss of AGL expression exerts its effects in bladder cancer, as this mechanism is not yet defined. Genome-wide expression and proteomic studies are ongoing to gain further insight into how AGL loss drives bladder cancer and, particularly, which other metabolic pathways might be perturbed upon AGL loss. The relationship of AGL with expression of SHMT2 also requires further investigation.

Many inhibitors of glucose uptake and glycolysis are available, but very few have been tested in urological cancers. We have shown that bladder cancer cells with absent AGL expression might actually be more dependent on glucose uptake and glycine synthesis than cells with normal AGL levels,[31] and might therefore be more sensitive to these inhibitors. Testing these inhibitors in bladder cancer cells with low AGL expression levels will be important. Furthermore, knockout mouse models of AGL now exist,[101,102] providing a new tool for examining the effect of decreased AGL expression on bladder carcinogenesis and possibly treatments for patients with bladder cancer and AGL loss.

Glycogen phosphorylase could provide a more direct target for treatment of cancer than AGL; however, whether glycogen phosphorylase activity and expression have a major role in urological cancers remains to be seen. Although drugs targeting glycogen phosphorylase exist, many have not been tested in the treatment of cancer, and the issues of specificity and accompanying toxicity will require investigation. The three isoforms of glycogen phosphorylase (muscle, brain and liver) have around 80% sequence identity.[103] Thus, isoform specificity of any inhibitors of glycogen phosphorylases is important. The different isoforms of glycogen phosphorylase are upregulated in different types of cancers, but the enzymes are required for glycogen metabolism in all cells in the body, hence, inhibition of all glycogen phosphorylase isoforms could result in overwhelming toxicity.

Although hurdles still exist, the investigation of glycogen phosphorylase, AGL, and other glycogen metabolism enzymes has resulted in the discovery of tentative biomarkers of tumour progression and novel therapeutic targets. Now, sufficient data exists to warrant further investigation into the role of glycogen metabolism in cancer to determine whether it will provide a major therapeutic advance in bladder and other urological cancers.

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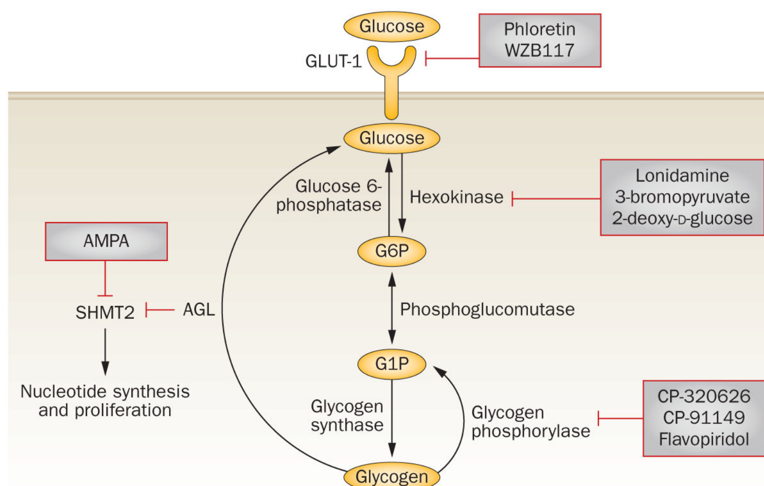

Figure 1.

Glycogen metabolism pathways and drug targets. Glucose enters the cell primarily through GLUT-1. Intracellular glucose is converted to G6P by hexokinase and isomerized to G1P by phosphoglucomutase. G1P is then added to an existing glycogen molecule through a series of steps ending with glycogen synthase. When the cell requires glucose, glycogen phosphorylase can convert glycogen back to G1P, which can then be converted back to glucose through phosphoglucomutase and glucose 6-phosphatase. AGL can produce more free glucose via its glucosidase function. In addition, AGL (via an unknown function) inhibits the enzyme SHMT2 that is important for proliferation in cancer cells. Several compounds that target various steps in glycogen metabolism are currently under investigation. Abbreviations: AGL, glycogen debranching enzyme; AMPA, amino methyl phosphonate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GLUT-1, glucose transporter type 1; SHMT2, mitochondrial serine hydroxymethyltransferase.

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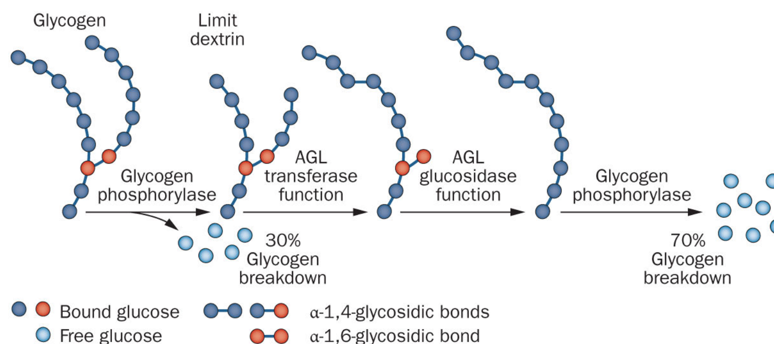

Figure 2.

Glycogenolysis. As glycogen is degraded, glycogen phosphorylase breaks α-1,4-glycosidic bonds until there are four glucose units remaining before a branch point. When AGL is missing from the cell, this process happens at all branch points, producing limit dextrin. Up to 30% of the glucose in a glycogen molecule can be released without AGL. When AGL is present, AGL continues glycogenolysis via its two enzymatic functions: the AGL transferase activity transfers a trisaccharide unit from one branch to another, exposing the α-1,6-glycosidic branch point. AGL glucosidase activity hydrolyses the 1–6 linkages, releasing the remaining glucose monomer and eliminating the branch point. Glycogen phosphorylase then resumes the breakdown of the newly elongated glycogen branch. Abbreviation: AGL, glycogen debranching enzyme.

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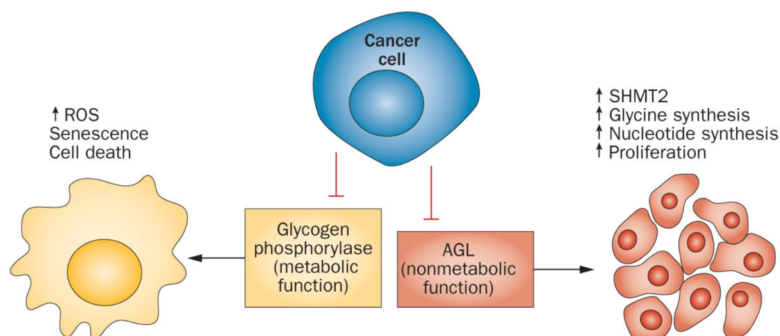

Figure 3.

Differences between glycogen phosphorylase and AGL loss in cancer. When glycogen phosphorylase is lost in cancer cells, intracellular ROS levels increase, leading to senescence and cell death.[52] This effect is due to the metabolic function of glycogen phosphorylase, as glycogen degradation via glycogen phosphorylase is important for optimal function of the pentose phosphate pathway.[52] By contrast, when AGL is lost, SHMT2 (an enzyme responsible for glycine synthesis) is upregulated, leading to increased nucleotide synthesis and increased proliferation. This effect is due to a currently undiscovered 'moonlighting' function of AGL.[31] Abbreviations: ↑, increase or upregulation; AGL, glycogen debranching enzyme; ROS, reactive oxygen species; SHMT2, mitochondrial serine hydroxymethyltransferase.