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Heterogeneity of glycolysis in cancers and therapeutic opportunities

Marc O. Warmoes, Jason W. Locasale *

Division of Nutritional Sciences, Cornell University, Ithaca, NY, United States

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ABSTRACT

Upregulated glycolysis, both in normoxic and hypoxic environments, is a nearly universal trait of cancer cells. The enormous difference in glucose metabolism offers a target for therapeutic intervention with a potentially low toxicity profile. The past decade has seen a steep rise in the development and clinical assessment of small molecules that target glycolysis. The enzymes in glycolysis have a highly heterogeneous nature that allows for the different bioenergetic, biosynthetic, and signaling demands needed for various tissue functions. In cancers, these properties enable them to respond to the variable requirements of cell survival, proliferation and adaptation to nutrient availability. Heterogeneity in glycolysis occurs through the expression of different isoforms, posttranslational modifications that affect the kinetic and regulatory properties of the enzyme. In this review, we will explore this vast heterogeneity of glycolysis and discuss how this information might be exploited to better target glucose metabolism and offer possibilities for biomarker development.

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Abbreviations: UDP-GlcNAc, uridine diphospho-N-acetylglucosamine; HK, hexokinase; BrPyr, 3-bromopyruvic acid; EC, endothelial cells; MCT, monocarboxylate transporter; 2DG, 2-deoxyglucose; F26P, fructose-2,6-bisphosphate; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; O-GlcNAc, O-linked β-N-acetylglucosamine; OGT, O-linked N-acetylglucosamine (GlcNAc) transferase; AMPK, AMP-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; GLUT1, glucose transporter 1; SER, serine; PPP, pentose phosphate pathway; SAICAR, succinyl-5-aminoimidazole-4-carboxamide-1-ribose-5′-phosphate; PTM, posttranslational modification; Kpg, lysine-phosphoglycerate; 13BGP, 1,3-biphosphoglycerate; MCA, metabolic control analysis.

* Corresponding author at: 108 Savage Hall, Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, United States. Tel.: +1 607 255 5114.
E-mail address: locasale@cornell.edu (J.W. Locasale).

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1. Introduction

Increased glucose metabolism is one of the distinguishing features between normal cells and highly proliferating cells like cancer, stem and immune cells [1]. Recognized by Otto Warburg over 80 years ago, one of the main features is the observation that growing cells secrete lactate while consuming large amounts of glucose (the Warburg Effect) [2]. The Warburg Effect occurs both through the activity of oncogenes and tumor suppressor genes [3] and through adaptations to the tumor microenvironment [4]. Pre-clinical and clinical research into cancer metabolism has shown that small molecules that interfere with different metabolic pathways can indeed have marked effects on tumor proliferation, either alone [5–7] or in combination with longstanding chemotherapies [8–11]. In fact, some of the oldest classes of chemotherapy are anti-metabolites that interfere with one-carbon metabolism and nucleotide synthesis such as 5-fluorouracil (5-FU) and methotrexate [12]. Even though many of these drugs target one-carbon metabolism and nucleotide synthesis, the last decade has seen additional development of agents that target glycolysis. There are several rationales for targeting glycolysis. First, high glycolytic flux leads to new biology including the selective diversion of carbon into several anabolic pathways and thus provides precursors for nucleotide, protein and fatty acids synthesis and the maintenance of signal transduction processes that occur through changing the levels of metabolites [2]. Second, the rate at which glycolysis is upregulated in cancer cells compared to normal cells creates an opportunity to selectively target tumors [13]. Last, the high flux leading to lactate secretion has also been linked to promote favorable non-cell autonomous conditions for uncontrolled proliferation such as evasion of the immune system [14] and induction of angiogenesis and metastasis [14] and these processes may be targeted by altering glycolysis. Nevertheless, there are still many unanswered questions on how to target glycolysis more effectively.

2. Heterogeneity in glycolysis

Heterogeneity in glycolysis is a typical feature of eukaryotic cells living either as interacting cell populations or as multi-cellular organisms [15]. This offers cells from various tissues or populations control to express the enzyme with the optimal kinetic and regulatory properties needed for the specific tissue or population function. Specifically, for multi-cellular organisms, this serves three main functions. Firstly, it allows for a coordinated control of glucose homeostasis within the body [16]. Second, it also allows an organisms cells to respond adequately to different forms of oxygen or glucose stress, such as hypoxia [15], ischemia [17] or changes in diet [16]. Lastly, glycolytic heterogeneity that allows for a high glycolytic rate, offers a subset of cells that need to sustain increased levels of proliferation during certain stages of physiological processes like angiogenesis [18], immune activation [19] and stem cell growth [20] (also see above). Research has shown that these isoenzymes also display a wide variety in expression according to tissue specific tumor development [21–23]. The functional basis for this tumor specific heterogeneity can to a certain degree be explained by the metabolic features of the tissue of origin [21]. There is also evidence that glycolytic isoenzymes are differentially regulated during the cell cycle [24]. Because proliferation rate and glycolysis also show a certain degree of correlation, this therefore explains another contributing factor to glycolytic heterogeneity in tumors with varying proliferation rates [21].

Many glycolytic isoenzymes are transcribed from different gene loci (see Table 1 and Fig. 1). This offers cells the first level of heterogeneity. Additional heterogeneity is introduced because a specific gene locus can also give rise to different splice-forms. Some of these splice forms have only been predicted computationally or identified from preliminary large scale RNA-sequencing and still await further experimental validation [25]. Especially, N-terminal splicing seems to be common for most glycolytic enzymes, with one of the most notable exceptions being the pyruvate kinase muscle isozyme (PKM) (see Table 1). These isoenzymes have different enzyme kinetic parameters such as Michaelis constants (see Table 1) and turnover rates. In addition many isoforms of glycolytic enzymes experience different allosteric regulation of small molecules. Furthermore, these enzymes have different regulation of their levels by promoters [26,27], non-coding RNA [28,29], expression of splice factors [30], etc. Further complexity could also be generated by the fact that glycolytic enzymes undergo a myriad of posttranslational modifications (PTMs) that are regulated by signal transduction events [31–40]. In some instances, modifications could allow for the exertion of 'moon-lighting' functionalities such as modulation of gene-expression in the nucleus [41,42] or inhibition of apoptosis [43]. According to the phosphositeplus database [44], the most prevalent modifications are serine-, threonine- and tyrosine-phosphorylation, acetylation and ubiquitination (see Table 1). Nevertheless, despite these intriguing possibilities attributed to enzyme PTMs, it is not clear whether the stoichiometry of these modifications can ever reach sufficient levels to have a substantial impact on glycolytic flux [38,45].

Together the extent of molecular diversity within glycolysis (see Fig. 1) offers the ability to rapidly tune enzyme kinetics to adapt to given environmental demands. In addition, this diversity also allows for a tremendously heterogeneous array of possibilities for flux through glycolysis and its control.

3. Therapeutic opportunities in glycolysis

Targeting glucose metabolism can occur both through altering systemic metabolism and through directly targeting enzymes in the diseased cell. For systemic metabolism examples include suppressing of hepatic gluconeogenesis by anti-diabetic agents like metformin [46–48], or the administration of the ketogenic diet [49–51] which enforces increased reliance on lipid oxidation as an energy source. Targeting glucose metabolism directly in the tumor is an alternative to this approach and aims to alter the biosynthetic, bioenergetic, and signaling processes that are differentially occurring as a result of the enhanced cell autonomous glucose metabolism [2,52]. Both approaches are complementary and are actively being explored in the clinic [49,53–55].

One approach to target glycolysis directly is to consider a single anti-glycolytic agent that targets a single enzyme with high specificity. This approach is promising in that it would offer potentially less toxicity but leads to difficulties in establishing whether there is sufficient efficacy, especially if tumors can develop resistance by expressing an alternate isoform of the targeted enzyme or establishing some other bypass mechanism. An additional approach is to consider anti-glycolytic agents that have multiple targets, both within the glycolysis pathway and in other pathways [56]. In response to this, some have argued for the development of "dirty drugs" or cocktails of specific molecules that are able to simultaneously target multiple nodes within the network and will be more effective [57,58].

Often it is difficult to classify (anti-glycolytic) agents clearly into either class because specificity of small molecules for their proposed targets is usually dose dependent [59]. Moreover, identifying off targets effects or exact mechanism of action within a cell is also impeded by technical limitations [59,60]. In the light of this uncertainty, it is interesting to describe the research into the mechanism of action of 3-bromopyruvic acid (BrPyr). Originally

Table 1
Various tissue specific, genetic, biochemical and kinetic properties of glycolytic enzymes.

Enzyme (predominant complex formation)	Isoforms with separate gene loci	Gene locus	Splice forms (N-terminal)	Km substrate (mM)	Cancer associated isoform	Organ specific expression	Posttranslational modifications			
							P	Ac	Ub	Other
HK (monomer, dimer, tetramer)	HK1	10q22	4 (3)	0.03		Most tissues	16	4	18	0
	HK2	2p13	/	0.37	×	Insulin-sensitive tissues	10	0	15	0
	HK3	5q35.2	/	0.003			12	0	2	0
	HK4 (glucokinase)	7p15.3-p15.1	3 (2)	10		Liver, pancreas	1	1	11	6
GPI (dimer)	GPI	19q13.1	2	0.4	×	Most tissues	20	7	20	1
PFK1 (homo- and hetero-tetramers)	PFKM	12q13.3	3	0.6–2 (most sensitive to F26P)	×	Muscle, heart, brain	15	4	16	0
	PFKL	21q22.3	2	0.35-0.55 (not in combination with L and M subunits)	×	Liver, kidney	9	3	5	0
	PFKP	10p15.3-p15.2	2 (1)	1.4–4 (lowest affinity for F6P)	×	Platelet	15	7	22	1
PFK2 (tetramers through dimerization of dimers)	PFKFB1	Xp11.21	/	0.015 (F6P)/0.0005 (F26P)		Liver, adipose tissue, proliferating cells	10	1	5	0
	PFKFB2	1q31	2	0.03 (F6P)/0.07 (F26P)		Heart, kidney	15	0	0	0
	PFKFB3	10p15.1	4 (2)	0.03 (F6P)/0.13 (F26P)	×××	All organs, most in muscle, most hypoxia responsive	18	0	4	0
	PFKFB4	3p22-p21		0.09 (F6P)/0.02 (F26P)	×	Testis	3	0	1	0
ALDO (homotetramer)	ALDOA	16p11.2	2 (1)	0.03	×	Embryonic, most tissues, high in muscle and erythrocytes	29	13	13	2
	ALDOB	9q21.3-q22.2	/	0.003	×	Liver, the kidney cortex	12	10	15	0
	ALDOC	17cen-q12	/	0.01	×	Brain, nervous tissue and smooth and heart muscle	14	6	13	0
TPI1 (homodimer)	TPI1	12p13	3 (2)	1.6 (DHAP)/0.51 (G3P)	×××	Most tissues	18	8	11	0
GAPDH (homotetramer)	GAPDH	12p13	2 (1)	0.19	×××	Most tissues	41	17	25	1
	GAPDHS	19q13.12	/			Testis	13	2	7	2
PGK (monomer)	PGK1	Xq13.3	/	0.079	×××	Most tissues	25	20	30	0
	PGK2	6p12.3	/			Spermatogenesis	3	6	3	0
PGAM (monomer)	PGAM1	1p31	2 (1)	0.19	×××	Most tissues	20	8	15	0
	PGAM2	4p14	/			Muscle, embryonic	8	1	4	0
ENO (homo- and heterodimers)	ENO1 (ENOα)	1p36.2	2 (1)	0.03	×××	Almost all human tissue	35	31	31	0
	ENO2 (ENOγ)	12p13	/	0.03	×	Neuron and neuroendocrine tissues	14	1	3	0
	ENO3 (ENOβ)	17p13.2	3	0.03		Muscle tissues	15	1	14	0
PK (active tetrameric form and nearly inactive dimeric)	PKM	15q22	3 (1)	0.03–0.5 (PKM2)/0.033 PKM1)	×××	All somatic tissues (The M1 isoform mostly in muscle and brain, and the M2 isoform during embryogenesis, in adipose tissue and pancreas)	42	14	29	1
	PKLR	1q21	2 (1)	0.3 (PKR)		Liver, kidney, small intestine and erythrocytes	6	0	1	0

Isoform and spliceform information was collected from Uniprot (version 144) [151], chromosome locations from the NCBI website [152] and posttranslational modification sites from the phosphositeplus database (release date May 5 2014) [44]. Kinetic information was gathered from: HK [81,153,154], GPI [155], PFK1 [133], PFK2 [156], ALDO [157,158], TPI1 [155], GAPDH [155], PGK [155], PGAM [155], ENO [159], PK [160,161]. Tissue specificity: HK [81,153,154], GPI, PFK1 [133], PFK2 [91,156,162], ALDO [158], TPI1, GAPDH [163], PGK, PGAM [164], ENO [159,165,166], PK [114].

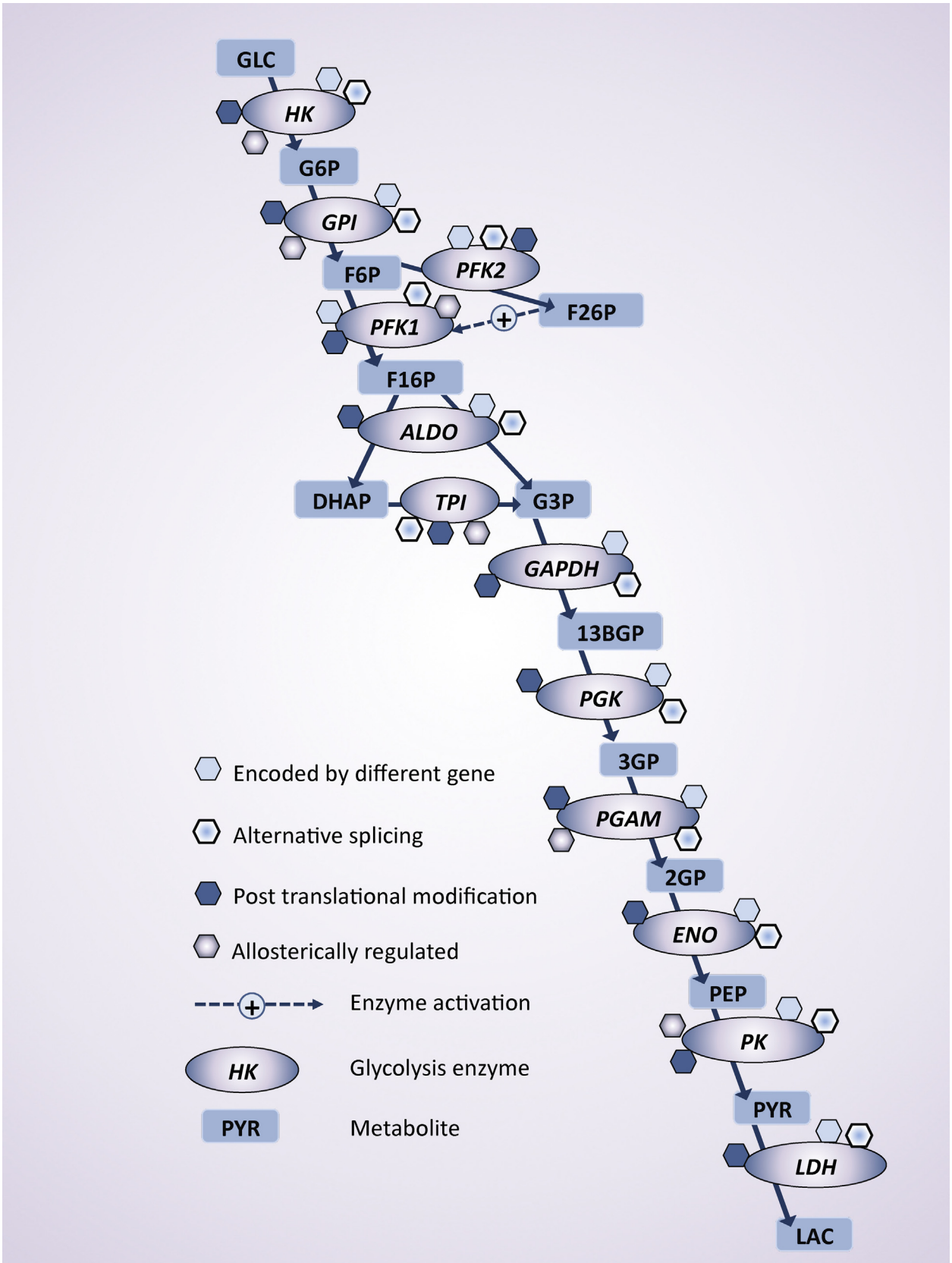

Fig. 1. Heterogeneity for glycolytic enzymes. Heterogeneity on glycolytic enzymes can be imposed by differences in gene encoding, spliceforms and posttranslational modifications. This heterogeneity can alter kinetic and functional properties of the enzymes and also modify their response to various allosteric interactions and anti-glycolytic agents. *Abbreviations*: HK, hexokinase; BrPyr, 3-bromopyruvic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G6P, glucose-6-phosphate; PFK, phosphofructokinase; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; PGAM, phosphoglycerate mutase; F16P, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate, F26P, fructose-2,6-bisphosphate; PK, pyruvate kinase; ENO, Enolase; ALDO, Aldolase; PGI, Glucose-6-phosphate isomerase; GLC, glucose; TPI, triosephosphate isomerase; PGK, phosphoglycerate kinase; LDH, lactate dehydrogenase; LAC, lactate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate.

identified as a an inhibitor of hexokinase, BrPyr is a small molecule and because of its structural homology has been described as a lactate and pyruvate homologue [61]. It is also an alkylating agent subject to nucleophilic attack and displacement of the alkyl bromide by a broad class of compounds [61]. Because of these properties BrPyr is highly reactive and therefore probably acts on multiple targets, although RNA/DNA alkylation, a common feature seen in most alkylating agent used in cancer therapy, has to our knowledge not been observed [62]. Three of the most frequently proposed targets of BrPyr include HK2 (hexokinase 2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex A (SDHA) [62]. Pre-clinical studies have shown impressive results in eradicating tumors [61,63,64] and clinical trials are ongoing. Part of this efficacy might be explained that BrPyr alkylates predominantly the thiol group of cysteine that is often part of the catalytic mechanism of many metabolic enzymes but also mediates the stability of protein bridges through the formation of cysteine bridges. This highly reactive nature suggests that once localized in cells, BrPyr may function non-selectively and independent of context. However, its transport into cells could be selectively mediated by the lactate transporter monocarboxylate transporter 1 (MCT1) as has been proposed. In this scenario, expression of MCT1 would serve as a biomarker for the predictive response of the anti-tumor effects of BrPyr [65]. Moreover, the overall low expression of MCT1 in normal cells could explain its apparent low level of toxicity in preclinical models. The generality of this model is unclear and the possibility nevertheless remains that BrPyr exerts cytotoxicity through its combinatorial targeting of defined enzymes in glycolysis and functions to selectively inhibit defined states of glucose metabolism that are observed in tumor cells. Of note, current chemotherapies used in the clinic disrupt a large number of biological processes that sustain cancer development and growth [66–71]. However, these therapies although efficacious, are also highly toxic [72–74]. Uptake of BrPyr by MCT1 could also seem in contradiction with the high glycolytic rate and lactate secretion associated with the Warburg effect, since MCT1 is a lactate importer under certain glucose/oxygen concentrations. Recent research into lactate transporters has shown that certain tumors are made up of symbiological populations where some fractions secrete lactate via the MCT4 exporter, while other fractions take up lactate as an energy source via the MCT1 importer [75,76]. This process is thought to depend on oxygen and glucose concentration gradients within the tumor whereby MCT1 positive cells can develop the ability to migrate towards areas of increased glucose concentrations [75]. These subpopulations with different levels of MCT1/4 expression can therefore have implications not only for the efficacy of BrPyr treatment, but also whether drugs that are highly selective for either MCT1 or MCT4 or a pan MCT1/4 inhibitor will show the most efficacy [14].

Several outstanding reviews have described the use of anti-glycolytic agents focusing predominantly on targeting the glycolytic enzymes themselves [6,7,77]. Here we will focus more specifically on the heterogeneity of glycolysis and how different realizations of glycolysis might have targetable liabilities. In this review, we will discuss this apparent heterogeneity for key glycolytic enzymes, its impact on enzymatic functionality and the use of some promising anti-glycolytic agents.

4. Targeting heterogeneous glycolytic enzymes

4.1. Hexokinases

For hexokinase (HK) both HK1 and HK2 isoenzymes are expressed in cancer cells, but HK2 appears more utilized in tumors [78–80]. All three HKs are sensitive to product inhibition by G6P. Interestingly, for HK2 and HK3, product inhibition is enhanced by elevated levels of inorganic phosphate (Pi), while for HK1, Pi has an antagonizing effect on product inhibition [81]. Patra et al. showed that specific depletion of the HK2 isoform in tumor has been shown to reduce tumor growth in preclinical models [79]. The authors further demonstrated that a systemic depletion of HK2 was well tolerated, suggesting that HK2 specific inhibitors could target HK2 expressing tumors with potentially limited toxicity.

A number of small molecules have been proposed as inhibitors of hexokinases. Once considered a HK inhibitor, BrPyr has been shown to have many (metabolic) targets (see above), and research into its mechanisms of action are ongoing. Another well described hexokinase inhibitor is 2-deoxyglucose (2DG). 2DG is taken up through glucose transporters where it acts as a competitive inhibitor of hexokinases and gets metabolized into 2-DG-6-phosphate (2DG-6P). This inhibition results in decreases glycolytic flux, ATP-depletion and eventually cell death [82]. 2DG-6P, a phosphorylated molecule that does not diffuse through the plasma membrane, cannot be metabolized by glucose-6-phosphate isomerase (PGI) or through the pentose phosphate pathway. The subsequent buildup of 2DG-6P, a reactive phosphate, also has toxic effects on the cell. 2DG-6P buildup continues also in part since the Ki for product inhibition of 2DG-6P for hexokinase is much larger compared to G6P [83].

Currently, to our knowledge no HK2 specific inhibitors have been developed but the differences in Km and protein sequences suggests this could be feasible. HK1 and HK2 also behave differently towards product inhibition in relation to concentrations of inorganic phosphate [79,81].

4.2. Phosphofructokinases (PFK's) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatases (PFKFB's)

Phosphofructokinase catalyzes the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F16P) and represents the second irreversible step in glycolysis. Phosphofructokinases are transcribed form three different genes and form a homo- or hetero tetrameric complexes called PFK1 [84]. Phosphofructokinases display some of the most complex regulation within glycolysis. PFK1 is activated by fructose-2,6-bisphosphate (F26P), ADP/AMP and ammonium ions [85] and inhibited by ATP and citrate. F26P is synthesized by tetrameric complexes called PFK2 of which the components are encoded by PFKFB genes [86]. Especially, the activation by F26P has caught widespread attention as inhibition of PFKFB enzymes can also down regulate glycolysis [87]. It is interesting to note here that ammonia is an allosteric activator of PFK1 [88]. Ammonia can be derived from deamination of glutamine, an amino acid that is taken up in large quantities by many tumor types and is one of the main components of cell culture media [89].

The PFK1 subunits are transcribed from three different genes called PFKM (muscle), PFKL (liver) and PFKP (platelet). While the PFKM is the only isoform to be tissue specific, the other three isoforms have been found to show distinct expression in other tissues [90]. The three isoforms share a relatively low sequence similarity between 66% and 70%. Although substrate affinities are relatively similar (see Table 1), the isoforms have marked difference in response to the allosteric activator by F26P. Changes in PFK1 isoform composition have been associated with cancer progression [22,23].

For PFKFB's, the PFKFB4 and especially the PFKFB3 isoforms seem to be the most relevant for cancer tissues [91] and are also induced by hypoxia [92]. One of the most well studied PFKFB3 inhibitors is 3-PO [93,94], which has been shown to lower F-2,6-BP and the glucose uptake in cancer cells. 3-PO was developed using computational methods in combination with a PFKFB3 enzymatic

assay and appears to be specific for PFKFB3, but the extent of this specificity warrants further investigation [91,94]. Recent research into PFKFB3 provides us with an example of how this isoenzyme is not only relevant for cancer cells but is also involved in angiogenesis along the VEGF gradient [95,96]. Quiescent endothelial cells (ECs) already have a high baseline level of glycolysis likely functioning to avoid diversion of oxygen away from tissues that require oxygenation [97]. During vessel sprouting however, activated endothelial cells upregulate glycolysis to meet the demands cell proliferation and migration. Blockade of PFKFB3 by 3-PO was also able to reduce vessel sprouting. Intriguingly, this blockade was also able to abrogate the residual hyper-branching of proliferating ECs after loss of directed growth along the VEGF gradient due to Notch and VEGFR2 inhibition [96]. Such a synergism holds promise for more effective angiogenic therapy, especially taking into account the relative low toxicity profile of 3-PO.

PFK's also undergo acetylations and phosphorylation's [44]. PFKFB3 activity can be enhanced through a Ser461 phosphorylation by activated AMPK. AMPK activation and its role in cancer has an expansive literature described in detail elsewhere [98,99]. PFKFB3 is also phosphorylated by protein kinase A (PKA) at Ser461, enhancing its kinase activity and increasing the activity of PFK1 [100]. Acetylations have been shown to destabilize or inactivate a number of glycolytic enzymes. This could explain the strong synergistic effect found between the glycolytic inhibitor 2-DG and lysine-(K)-deacetylase (KDAC) inhibitors [101]. Protein kinase C (PKC) phosphorylation of PFKFB3 at Ser461 (the same site as AMPK and PKA phosphorylation) also induces up-regulation of its kinase activity and activation of PFK1 [100]. PFK1 can also be activated through Akt kinase phosphorylation of PGK2 [91,102]. Akt also phosphorylates and activates the glycolytic enzymes HK and glucose transporter 1 (GLUT1) [103,104]. Phosphorylation of PFK1's could interfere with allosteric inhibition by lactate and has been proposed as a driver for the Warburg effect, as it abrogates one the main negative feedback loops for fermentative glycolysis [90]. Yi et al. recently described a O-linked β-N-acetylglucosamine (O-GlcNAc) modification on a serine residues (Ser529) for PFKM which is induced under hypoxia or glucose deprivation [105]. They further demonstrated that this modification is dynamically controlled by its substrate, uridine diphospho-N-acetylglucosamine (UDP-GlcNAc), a key integrator of several biosynthetic and bioenergetic pathways. This modification was shown to inhibit PFK1 activity and redirected glycolytic flux into the biosynthetic pentose phosphate pathway. This finding is especially intriguing, since PFKM (85-kDa) has been shown to be post-translationally cleaved, resulting in a constitutively active 47-kDa N-terminal fragment [106]. The cleavage of the C-terminal domain not only contains the sites for allosteric inhibition with citrate and ATP, but also the site for inhibition by O-GlcNAc modification on Ser529 [107]. PFKM also gets phosphorylated during mitosis on Ser667, which is also located on the cleaved off C-terminal, and would thus also remove any regulation imposed on PFKM during mitosis [44]. For O-GlcNAc modifications only one enzyme has been discovered so far, namely O-linked N-acetylglucosamine transferase (OGT) [108]. OGT inhibitors could therefore provide a suitable synergistic drug for anti-glycolysis treatment. Both glucose and glutamine participate in UDP-GlcNAc synthesis. How the increased uptake of both these nutrients relates to O-GlcNAc modification is currently not known, but it has been postulated that decreased activity of several glycolytic enzymes by O-GlcNAc modification diverts part of the glycolytic flux into anabolic pathways [109].

4.3. Phosphoglycerate mutases

Phosphoglycerate mutases (PGAM's) catalyze the reversible reaction of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) but has also been implicated in the conversion of phosphoenolpyruvate (PEP) to pyruvate (PYR) [110]. This transfer of PEP to a histidine residue (His11) in the catalytic site actually increases the mutase function of PGAM1. This alternative conversion is thought to occur more efficiently at higher PEP concentrations generated by the less active downstream PKM2 spliceforms the PKM enzyme, the main converter of PEP to pyruvate in cells. Such a mechanism maintains glycolytic flux, but ensures a buildup of glycolytic intermediates needed for anabolic pathways.

PGAM is acetylated and enhances it enzymatic activity [111]. Its substrate, 3-PG can inhibit the pentose phosphate pathway [112,113]. Thus increased priming by high PEP levels might ensure a more efficient catalytic turnover that balances the anabolic fluxes into the pentose phosphate pathway and serine synthesis. This regulatory behavior therefore makes PGAM1 a suitable target to inhibit cancer metabolism. A recently developed small molecule inhibitor, PGMI-004A resulted in increased 3-PG and decreased 2-PG levels in cancer cells, which resulted in significantly decreased glycolysis, pentose phosphate pathway flux and biosynthesis, as well as attenuated cell proliferation and tumor growth [113].

4.4. Pyruvate kinases

Pyruvate kinases (PK) perform the last major step in glycolysis whereby PEP is transformed into pyruvate together with the generation of one ATP molecule from ADP. PK are transcribed from two different gene locus's that are designated by the gene names PKLR and PKM. PKLR gives rise to the liver and erythrocyte specific enzymes, whereby the differential expression among the two cell types is accomplished by N-terminal splicing [114]. Whether PKLR isoforms contribute to tumor glycolysis is difficult to assess [114], but the similar Km of PKLR and PKM2 (see Table 1) suggest this could be possible. The PKM enzyme is transcribed from a single gene locus located on chromosome 15 but has two well described isoforms that are of major importance to cancer metabolic specific adaptations. The isoform occurring in most normal tissues is PKM1 and has the lowest Km for PEP. PKM1 form highly constitutively active tetramers protein complexes. PKM2 (in its dimeric form) on the other hand has a lower affinity for PEP and is almost universally expressed in cancer cell alongside PKM1. Besides cancer specific expression, PKM2 has several other properties that set it aside from PKM1 and could make it an interesting target to interfere with cancer metabolism. PKM2 has allosteric binding sites that are regulated by the glycolytic intermediate as F16BP [115]. More recently, PKM2 activation has been linked to serine biosynthesis, when it was shown that PKM2 can be activated by serine [116] and a small molecule intermediate from the purine synthesis pathway called succinyl-5-aminoimidazole-4-carboxamide-1-ribose-5′-phosphate (SAICAR) [117].

The requirements of pyruvate kinase activity and expression of any given isoform is thus a complex subject. Strikingly, tumors were shown to grow in complete absence of activity [118] and deletion of PKM2 could in some instances accelerate tumor growth [119]. In addition, activators of PKM2, like TEP-46 [120], have been shown to reduce tumor growth. Together, these findings indicate that decreased flux through the pyruvate kinase step in glycolysis could have selective advantages for tumor growth. Because PKM2 has lower catalytic activity, it is thought to build up glycolytic intermediates that can be used for anabolic pathways. Activation of PKM thus depletes these intermediates and results in reduced cell proliferation. Buildup of anabolic intermediates has recently been identified a consequence of a newly identified PTM that seems especially prevalent on glycolytic enzymes. What is especially intriguing is that this PTM is established by 1,3-biphosphoglycerate

(13BPG), the product of GAPDH [121]. Accumulation of highly reactive 1,3-BPG results in modification of lysine to lysine-phosphoglycerate (Kpg). This modification not only results in auto-modification of GAPDH itself, but also in modification of the glycolytic enzymes PGAM1, enolase 1 (ENO1) and PKM2. This modification can then lead to significant alterations in enzyme activity and excessive buildup of glycolytic intermediates. How Kpg modification alters PKM2 activity is currently not know but since exon10 is thought to be involved in the formation of dimer and tetramers, there could be an effect on enzyme kinetics.

5. Conclusions and future perspectives

The large heterogeneity in glycolytic enzymes offered by different genes, spliceforms and posttranslational modifications gives cancer cells an array of tools to react differently upon treatment with anti-glycolytic agents. This heterogeneity also makes it possible to give marked different responses to allosteric regulators that originate within glycolysis and other metabolic pathways and have consequences for drugs that target these allosteric sites. Lastly, this heterogeneity also provides cancer cells with rapid switches between the bioenergetics, signaling and biosynthetic needs. Current technical and computational advances in the assessment of glycolytic heterogeneity hold promise to better target this heterogeneity and thereby improve therapeutic outcomes.

5.1. Assessment of the heterogeneity in glycolytic enzymes and its dynamic properties

Several reports on differential enzyme expression between different tumor types have already surfaced indicating their relevance in cancer metabolism. Most of these studies have used transcription profiling [21] or protein expression assays [122,123]. Although we have some knowledge of how these isoenzymes are expressed in different normal tissues (see Table 1), our understanding of how these isoenzymes are expressed between different tumors or within a tumor is often not well defined. Also the large number of (predicted) spliceforms has seen relatively little functional characterization. With the advent of next-generation sequencing [124] and (targeted) proteomics [122,125], a better understanding of the quantitative nature of isoenzyme expression is to be expected and could serve as basis for biomarker discovery for anti-glycolytic agents. However, the relevant phenotypic consequence of the alteration in mRNA and protein expression is a change in metabolism that is characterized by changes in metabolite concentrations and fluxes [126,127]. Several in vivo imaging strategies are now commonplace in assessing changes in flux through glycolysis by using positron emission tomography with radioactive glucose (FDG-PET) [128] or by advanced technologies that exploit the properties of heavy labeled, hyperpolarized nutrients such as choline, acetate, and glutamine [129,130]. High resolution mass spectrometry offers additional possibilities for characterizing the state of glycolysis in a tumor through either profiling with metabolomics the state of a biopsy or a biofluid such as plasma or sera [131].

Better kinetic characterization of glycolytic enzymes especially when isoforms are expressed in parallel can be helpful to elucidate the dynamic behavior of glycolysis in various (tumor) cell types though mathematical modeling using techniques such as metabolic control analysis (MCA) [132,133] and can find use in identifying better drug targets [134,135].

A better understanding of the influence and functionality of isoenzyme expression can also be attained by flux studies using isotope tracers [136]. Furthermore, understanding concentration patterns of metabolites in glycolysis in the context of basal rates of glycolysis and their changes upon partial inhibition by therapeutics will serve as a helpful guideline for therapeutic efficacy [137]. In addition, excretory fluxes that may be altered as a result of manipulating fluxes in glycolysis could result in the production of metabolites that could be measured in tumors or in biofluids. The measurement of these molecules could allow for amplification of signals that are derived from the internal state of glycolysis. Such measurements may be useful for identifying biomarkers that so far have been difficult to identify. Together such studies could also determine drug efficacy [138,139], highlight rewiring in metabolic pathways in sensitive and resistant tumor cells and provide crucial metabolic biomarkers of drug efficacy [140].

We will also need to make use of cell culture and in vivo models that are more representative of the spatial and temporal differences in pH, pO_2 and nutrient and hormonal concentrations that exist in tumors and different tissues. Recent studies have shown that under these conditions tumor (subpopulations) exist with marked heterogeneity in isoenzyme expression and flux [76,141,142].

5.2. Opportunities for targeting glycolytic heterogeneity

The techniques described above to resolve the quantitative and dynamic properties of glycolytic heterogeneity holds promise to improve therapeutic intervention. By providing insight into the proliferation strategies of cells within the tumor environment opportunities exist to highlight points of liability that current anti-glycolytic agents and/or systemic strategies could exploit [143]. Currently, it is not well established what region of a solid tumor should be targeted by anti-glycolysis agents. One option is to target the well oxygenated/high glucose vascularized component of the tumor [75,144], but additional focus on targeting cells in nutrient deprived areas such as the hypoxic core of the tumor has also been considered [145]. Differences in pH and nutrient availability are essential parameters for controlling flux through glycolysis and these properties affect both the pharmacological properties of the compound [146] and the control properties of glycolysis [141]. Other variables including genetic status and availability of growth factors are also likely key mediators of the regional response to targeting glycolysis.

Additional insight will also be gained by a better understanding of glycolytic heterogeneity in tumor associated physiological process like angiogenesis [95,96] and inhibition of immune activation [147,148]. Moreover, by the use of integrative approaches that combine experimental and computational techniques, a better understanding of tumor and organism interactions is to be expected [149,150].

Currently almost every enzyme or transporter involved in glycolysis is subject of intensive research as an anti-cancer target by small molecules [77]. It is now becoming clear that the expression of different isoenzymes and regulation of their PTMs could have profound influences on the efficacy of these drugs. Only a minority of this glycolytic heterogeneity has been functionally characterized and provides opportunities for more optimal targeting of cancer metabolism and biomarker development for current therapeutic strategies.

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