HIF-1α Modulates Energy Metabolism in Cancer Cells by Inducing Over-Expression of Specific Glycolytic Isoforms

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Abstract: To develop new and more efficient anti-cancer strategies it will be important to characterize the products of transcription factor activity essential for tumorigenesis. One such factor is hypoxia-inducible factor-1α (HIF-1α), a transcription factor induced by low oxygen conditions and found in high levels in malignant solid tumors, but not in normal tissues or slow-growing tumors. In fast-growing tumors, HIF-1α is involved in the activation of numerous cellular processes including resistance against apoptosis, over-expression of drug efflux membrane pumps, vascular remodeling and angiogenesis as well as metastasis. In cancer cells, HIF-1α induces over-expression and increased activity of several glycolytic protein isoforms that differ from those found in non-malignant cells, including transporters (GLUT1, GLUT3) and enzymes (HKI, HKII, PFK-L, ALD-A, ALD-C, PGK1, ENO-α, PYK-M2, LDH-A, PFKFB-3). The enhanced tumor glycolytic flux triggered by HIF-1α also involves changes in the kinetic patterns of expressed isoforms of key glycolytic enzymes. The HIF-1α induced isoforms provide cancer cells with reduced sensitivity to physiological inhibitors, lower affinity for products and higher catalytic capacity ($Vmax_f$) in forward reactions because of marked over-expression compared to those isoforms expressed in normal tissues. Some of the HIF1α-induced glycolytic isoforms also participate in survival pathways, including transcriptional activation of H2B histone (by LDH-A), inhibition of apoptosis (by HKII) and promotion of cell migration (by ENO-α). HIF-1α action may also modulate mitochondrial function and oxygen consumption by inactivating the pyruvate dehydrogenase complex in some tumor types, or by modulating cytochrome *c* oxidase subunit 4 expression to increase oxidative phosphorylation in other cancer cell lines. In this review, the roles of HIF-1α and HIF1α-induced glycolytic enzymes are examined and it is concluded that targeting the HIF1α-induced glucose transporter and hexokinase, important to glycolytic flux control, might provide better therapeutic targets for inhibiting tumor growth and progression than targeting HIF1α itself.

Key Words: Glucose transporters, hexokinases, HIF-1α, glycolysis, mitochondria, glycolytic inhibitors, mitochondrial inhibitors.

INTRODUCTION: HIF-1

The development of hypoxic regions in solid tumors is a recurrent feature which is linked to the processes of malignant transformation, metastasis and resistance to chemo-, immuno- and radio-therapy [1, 2]. The hypoxia-inducible factor (HIF) is a key transcriptional regulator that plays a role in these processes by modulating expression of proteins involved in angiogenesis, erythropoiesis, cellular proliferation and survival, vascular remodeling, vasomotor control, and catecholamine, iron and energy metabolic pathways, thereby allowing tissues to adjust to low oxygen concentrations [3].

Although several isoforms of HIF exist including HIF-1, -2 and -3 (see below for more detail), the focus of this review will be on HIF-1 because its functions are the most well defined in relation to modifying glycolysis. The molecular biological mechanisms whereby HIF-1 acts as a transcriptional activation factor regulating gene expression have been extensively studied, since the seminal publication of Semenza *et al*., [4], and reviewed [5,6]. Thus, the present review focuses on those proteins that play key roles in controlling the changes in glycolytic flux inside cancer cells and whose isoforms are modulated by HIF-1 and hypoxia.

HIF-1 is a heterodimer that binds to promoter regions containing the DNA sequence 5′-RCGTG-3′(R= A or G) [3, 4, 7], called hypoxic responsive elements (HRE) (Fig. **1**). This transcriptional factor is comprised of two subunits, HIF-1α and HIF-1β, which both contain one beta Helix-Loop-Helix (bHLH) and two (PER-ARNT (arylhydrocarbon receptor nuclear translocator)-SIM) PAS domains in their N-terminal segments (Fig. **1**). The bHLH domain regulates DNA binding; the PAS domains regulate HIF (α + β) subunit heterodimerization and are likely to participate in the target gene selection (Fig. **1**) [7]. As HIF-1β is constitutively expressed, the activity of HIF-1 is regulated by varying the levels of HIF1α expression. Under normoxia (21 % O_2 in air ≈ 250 μM O_2 dissolved in water at sea level and 37ºC), HIF-1α content is negligible, given its half-life of 5 min, whereas treatment with desferrioxamine, an iron chelator

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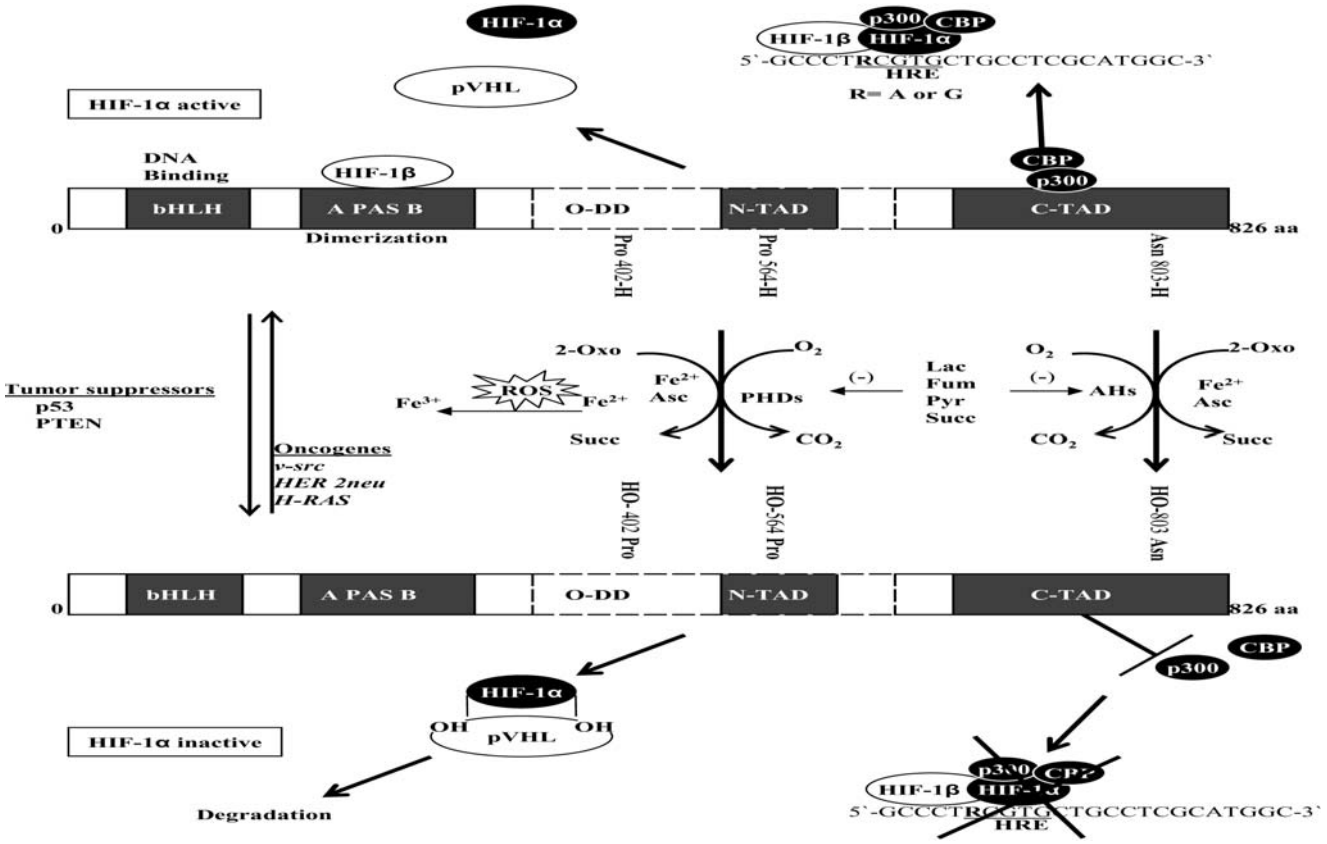


Fig. (1). Regulation of HIF-1α stability and activity.

Under normoxia, prolyl hydroxylase (PHD) hydroxylates proline (Pro) residues (402 and 564) of HIF-1α in a region called the oxygen-dependent degradation (O-DD) domain, which facilitates its interaction with the von Hippel-Lindau protein (pVHL) and hence with an ubiquitin-protein ligase complex that marks HIF-1α for destruction by the proteasome. Asparaginyl-aspartyl hydroxylases (AHs) by hydroxylating an Asn residue (803) in the carboxy-terminal transcriptional activation domain (C-TAD) of HIF-1α, inhibits the binding of cofactors, such as p300 and CBP that are required for the transcription of target genes. HIF-1α is a heterodimer that binds to hypoxic responsive elements (HRE) contained in the promoter region of the glycolytic genes. Abbreviations: 2-oxo, 2-oxoglutarate; Succ, succinate; N-TAD, amine-terminal transcriptional activation domain; Lac, lactate; Fum, fumarate; Pyr, pyruvate; Asc, ascorbate, (-), inhibition.

that mimics hypoxic conditions, the half-life becomes increased to 30 min [8].

In particular, enhanced HIF-1 expression has been detected in the majority of brain, pancreas, mammary gland, colon, ovary, lung and prostate primary tumors, and in their metastasis, but not in the majority of benign tumors or normal tissues [9-15]. Higher expression of HIF-1α correlates with poor survival in breast, head and neck, esophagus, stomach and lung cancers, although for cervical cancer this association is not so clear [15]. Both the biological complexity of the HIF system and methodological difficulties such as the criteria used to identify HIF positive cells, immunohistochemical protocols and source of tumor tissue for its experimental evaluation most probably account for any conflicting data [15].

Regulatory Mechanisms Controlling HIF-1 Activity

The von Hippel-Lindau protein (pVHL), a component of the ubiquitin ligase E3 complex, regulates HIF-1α degradation (Fig. **1**). For pVHL-HIF interaction, HIF-1α must first be hydroxylated at prolines 402 and 564 in the oxygen-dependent degradation (O-DD) domain by prolyl-4-hydroxylases (PHDs). HIF-1α transcriptional activity can also be directly inhibited by Asn 803 hydroxylation catalyzed by asparaginyl-aspartyl hydroxylases (AHs; also known as factors inhibiting HIF-1, FIHs). Hydroxylation prevents recruitment of p300/CBP co-activators that would otherwise combine together with HIF-1α, forming the active transcriptional complex [7] that binds target genes (Fig. **1**).

PHD and AH enzymatic activities both require Fe^{2+}, 2-oxoglutarate, ascorbate and oxygen (Fig. **1**). Hence, one way to reduce HIF hydroxylation would be by decreasing the oxygen level below to that required for PHDs. In this manner, it was proposed that these enzymes sense intracellular oxygen levels [16, 17] and that under hypoxic conditions, PHDs and AHs are inactivated with the result that HIF-1α becomes stabilized and activated [16].

The role of PHDs as an intracellular oxygen sensing system remains uncertain because the hydroxylase *Km* values for O_2 are reported to be much higher (> 90 μM) [18, 19] than the actual O_2 concentration that exists in the cytosol (12.5-25 μM) and in the capillaries and arterioles (20-50

μM) [17, 20-22]. Consequently, HIF-1α should not be inactivated by hydroxylation at an [O_2] of 10 μM. Under such conditions, hydroxylase activity, with a *Km* value of 100 μM should only be 9% of maximal velocity (*Vm*), which is most likely not sufficient to inactivate HIF-1α. In this regard, HIF-1α stabilization has been reported to occur in intact cells at [O_2] below 50 μM [17].

Several possibilities have been suggested to explain the apparent discrepancy between the high *Km* values of PHDs determined for O_2 and the fact that HIF-1α hydroxylation and associated degradation occurs under normoxia. For instance, kinetic studies have not taken into account the contribution of the length of the peptide substrates used in assaying PHD activity nor the role that HIF-1α substrate binding plays in facilitating oxygen binding, which may lower the actual *Km* (O_2) to more physiologically relevant values (reviewed in [23]). In addition, the expression levels of PHD2 and PHD3 are themselves increased under hypoxic conditions by HIF-1α [24, 25]. Therefore, increased PHD expression would be expected to also help reduce HIF-1α levels. In this regard, PHD2 was shown to be the most prominently expressed isoform in a large range of cancer cell lines with potent activity towards HIF-1α [24].

Mitochondrial Involvement in the Regulation of HIF-1α

An additional mechanism explaining how HIF-1α levels are increased during hypoxia is that the decrease in [O_2] causes an increase in the generation of radical oxygen species (ROS) in mitochondria by respiratory complexes I and III [26, 27]. The increased ROS induces oxidation of Fe^{2+} to Fe^{3+}, which would function to diminish the hydroxylase activity of PHD and AH (Fig. **1**). In agreement with this hypothesis, HIF-1α is not stabilized in anti-oxidant treated cells (hepatoma, smooth muscle, cardiomyocytes, gastric epithelium, renal tubule epithelium, macrophages) under hypoxia. In contrast, where ROS production is low, such as in cells lacking (a) mitochondrial DNA (rho zero, ρ^o cells), (b) cytochrome *c*, or (c) complex III Rieske iron-sulfur protein, and (d) in cells treated with stigmatellin, an inhibitor of complex III [28], HIF-1α hydroxylation proceeds efficiently under hypoxia [22, 29, 30].

Regarding the role of the respiratory chain, it has been proposed that the reduction in [O_2] during hypoxia leads to a decrease in cytochrome c oxidase (COX; complex IV) activity [31], resulting in the accumulation and overloading of the reduced intermediates, ubiquinol and semiquinone, particularly the latter, which then promote superoxide generation (see Fig. **3** for chemical structures). Specific inhibition of the respiratory complexes, by either cyanide (COX), antimycin (complex III; cytochrome b-c_1 complex), thenoyltrifluoroacetone (TTFA) or α-tocopheryl succinate (complex II; succinate dehydrogenase) or rotenone (complex I) (Fig. **3**), can also promote the generation of ROS under normoxia, because these respiratory inhibitors affect the electron transport by respiratory chain complexes to induce increased levels of semiquinone (and other free radical molecules) [32-34]. In contrast, blocking entry of energy substrates to inhibit the respiratory chain at the electron entrance level, by using malonate to inhibit complex II, phenylsuccinate or n-butylmalonate to block transport of succinate and other dicarboxylate Krebs cycle intermediates, or α-cyano-hydroxycinnamates to prevent pyruvate uptake into mitochondria (Figs. **2** and **3**), is not expected to induce generation of ROS, as these inhibitors do not directly modify the respiratory chain at the level of electron flow.

Changes in the Krebs cycle are also likely to contribute to the regulation of HIF-1α activity. The enzyme2 of the 2-oxoglutarate dehydrogenase complex (2-OGDH) can be targeted for ubiquitination-dependent degradation by Siah2, the RING finger ubiquitin-protein isopeptide ligase [35]. As Siah2 is induced by hypoxia, disruption of mitochondrial metabolism by affecting 2-OGDH would lead to loss of mitochondrial stability and cell death.

How is HIF-1α Maintained Stable and Active in Cancer Cells?

It is thought that HIF-1α in tumor cells is stabilized due to the hypoxic environment developed in certain regions, particularly in solid tumors 1 mm diameter or larger [36, 37]. Although tumors may have an active angiogenesis, unorganized, thin and fragile new vessels are formed that affect the normal dynamics of the blood flux. Consequently, some tumor sections will become excluded, leading to hypoxic regions [15, 38]. HIF-1α stabilization is also promoted by activation of certain oncogenes such as *v-src*, HER 2^{neu} and H-RAS, or by inactivation of some tumor suppressors such as p53 and PTEN [3, 6]. However, the molecular mechanisms operating in these processes have not been elucidated. A high incidence of pVHL mutations is associated with kidney and central nervous system tumors. These pVHL mutations modify or delete either the α-domain in the C-terminal region which binds to elongin-C in the proteasome, or the β-domain that interacts with the HIF-1α O-DD domain and is required for nuclear/cytosolic trafficking, preventing HIF-1α degradation (Fig. **1**) [3, 39].

Under normoxia, HIF-1α can be stabilized by the high lactate and pyruvate levels generated by active tumor glycolysis. It has been shown that these monocarboxylates, and oxaloacetate, inhibit PHD activity by competing with 2-oxoglutarate for binding [29, 40]. Similarly, mutations or down-regulation of succinate dehydrogenase (SDH) and fumarate hydratase (FH) induce a state of pseudo-hypoxia that makes cancer cells behave as if they were hypoxic, which leads to HIF-1α stabilization and enhancement [41-43]. These mutations inhibit SDH and FH activities, leading to succinate and fumarate accumulation, without associated ROS production, and to product-inhibition of hydroxylases [41-43] (Fig. **1**). Moreover, SDH and FH mutations, or their down-regulation, are associated with development of phaeochromocytomas, paragangliomas, leiomyomas, leiomyosarcomas, renal cell, gastric and colon carcinomas, and papillary thyroid cancer [22, 29, 41-43].

Additional HIF Isoforms

Three isoforms of HIF-α have been described (HIF-1α, HIF-2α/EPAS1 and HIF-3α/IPAS) and three HIF-1β isoforms (HIF-1ß/ARNT1, HIF-2ß/ARNT2, and HIF-3ß/ARNT3), although their exact relationships in forming heterodimers are not known. HIF-1α and HIF-2α/EPAS1 share similar structure, hypoxic stabilization and exclusive dimeri-

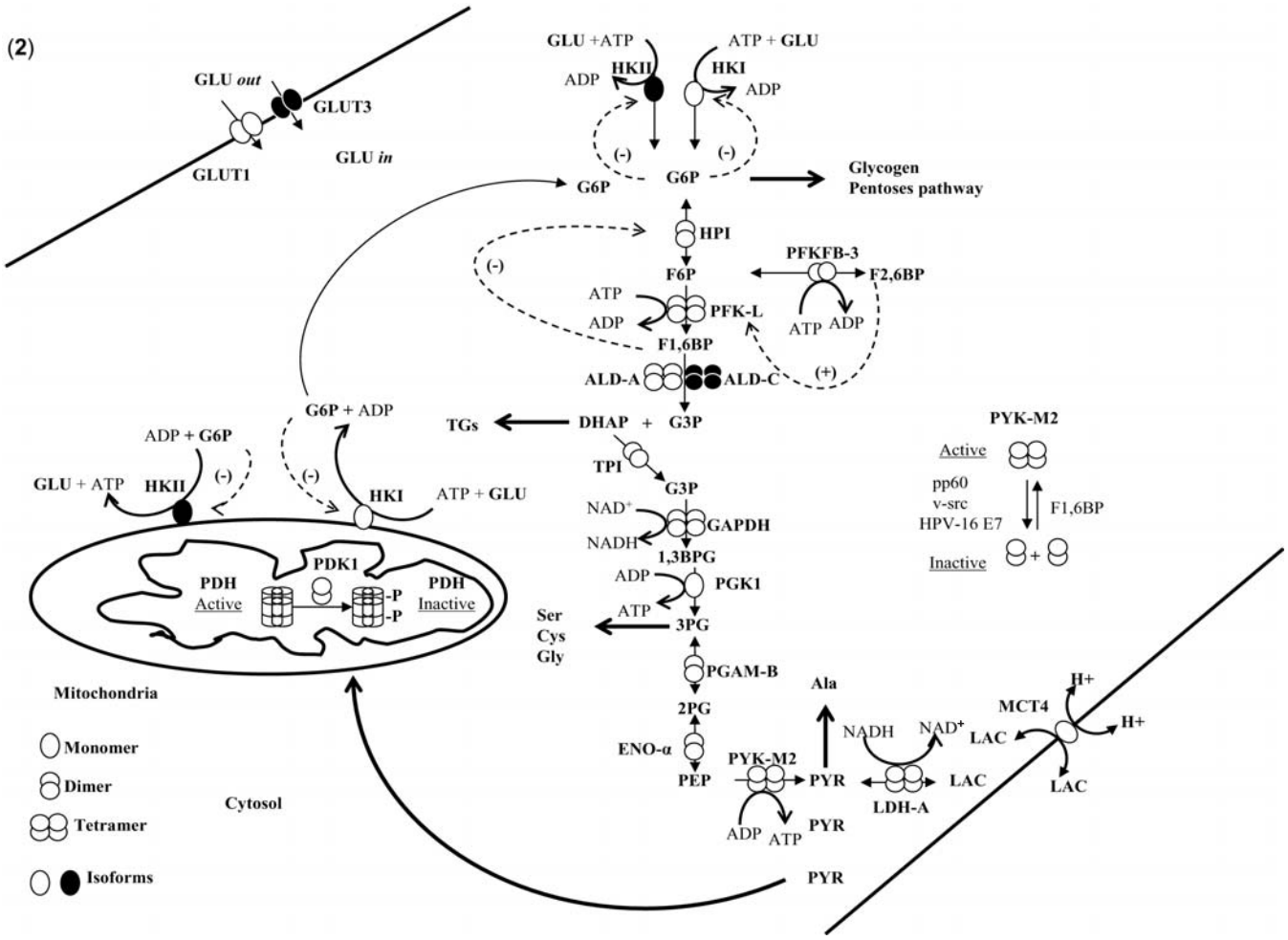


Fig. (2). Glycolytic isoforms upregulated by HIF-1 in cancer cells.

GLUT, glucose transporter; HK, hexokinase; HPI, hexosephosphate isomerase; PFK1, phosphofructokinase type 1; ALD, aldolase; PFKFB3, phosphofructokinase type II; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; GLU, glucose ; G6P, glucose 6-phosphate, F6P, fructose 6-phosphate; F2,6BP, fructose-2,6-bisphosphate; F1,6BP, fructose 1,6 bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; 1,3BPG, 1,3 bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate; TGs, triacylglycerides; Ser, serine; Cys, cysteine; Gly, glycine; Ala, alanine; (+) activation; (-) inhibition.

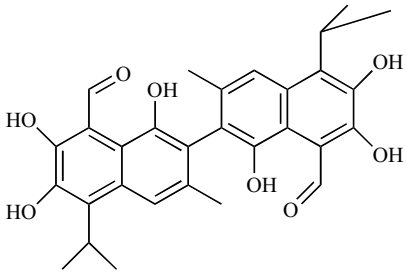
zation with HIF-1β [44]. HIF-1β may also dimerize with aryl hydrocarbon receptors, allowing cross-talk with xenobiotic metabolism. However, complexes containing HIF-2α activate a distinct subset of genes, compared to HIF-1α, that are not involved in regulating glycolytic genes [45]. HIF-2α tissue expression occurs in a limited number of non-parenchymal cells (in kidney, pancreas and brain) and parenchymal cells (in liver, intestine and heart) [45]. However, HIF-2α is also involved in tumor progression and increased expression has been observed in diverse solid tumors, including bladder, brain, breast, colon, ovary, prostate and renal carcinomas [13, 45].

The role of HIF-3α is not clear. Three splice variants can be produced from the HIF-3α gene. Isoform-2, also called IPAS (inhibitory PAS domain, a natural HIF-1α antagonist), lacks an Asn-containing transactivation domain (C-TAD), such that it acts in a dominant negative manner forming transcriptionally inactive hetero-dimers with HIF-1β, thereby preventing HIF-1α dimerizing with HIF-1β [44]. In the corneal epithelium, where the IPAS concentration is high, corneal neo-vascularization is inhibited [46]. On the other hand, HIF-1β is constitutively expressed under normoxic conditions and is slightly increased by the same effectors that upregulate HIF-1α expression (hypoxia, EGF, $CoCl_2$) [47].

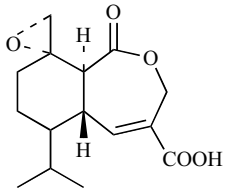
GLYCOLYSIS AND HIF-MEDIATED REGULATION

Most cancer cells show enhanced glycolytic capacity compared to their tissues of origin [48, 49]. This occurs because many of the glycolytic enzymes can be expressed as several different isoforms (Table **1**) and the isoforms expressed in cancer cells are different. This process is regulated by HIF-1α which acts as a transcriptional factor for most of the glycolytic enzymes and transporters (Figs. **1** and **2**)

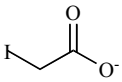
GLYCOLYTIC INHIBITORS



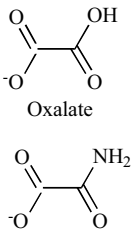
Gossypol



Koningic acid

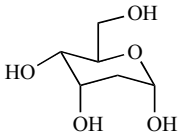


Iodoacetate

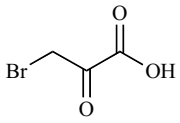


Oxalate

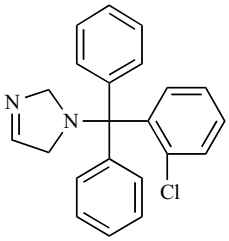
Oxamate



2-deoxyglucose

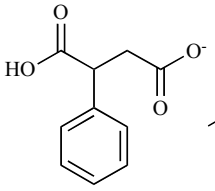


3-bromopyruvate

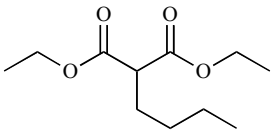


Clotrimazole

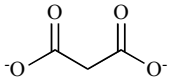
MITOCHONDRIAL INHIBITORS



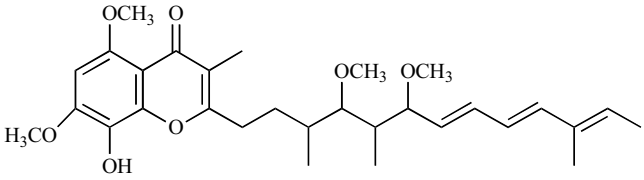
Phenylsuccinate



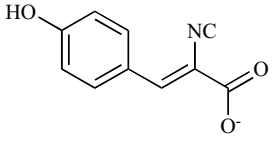
n-buthylmalonate



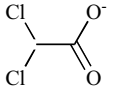
Malonate



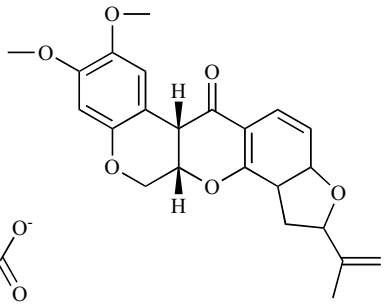
Stigmatellin



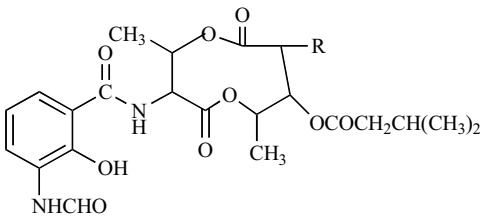
a-cyano-4-hydroxycinnamate



Dicloroacetate

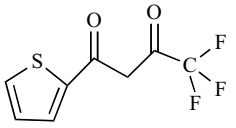


Rotenone

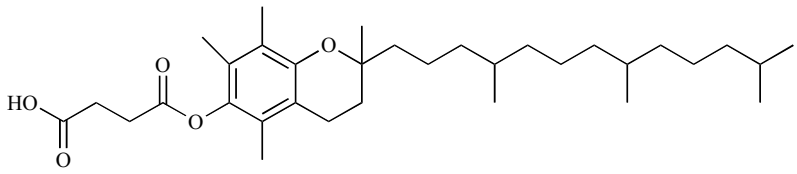


R= Hexyl: Antimycin A_1

R= Butyl: Antimycin A_3



Thenoyltrifluoroacetone



α-tocopheryl succinate

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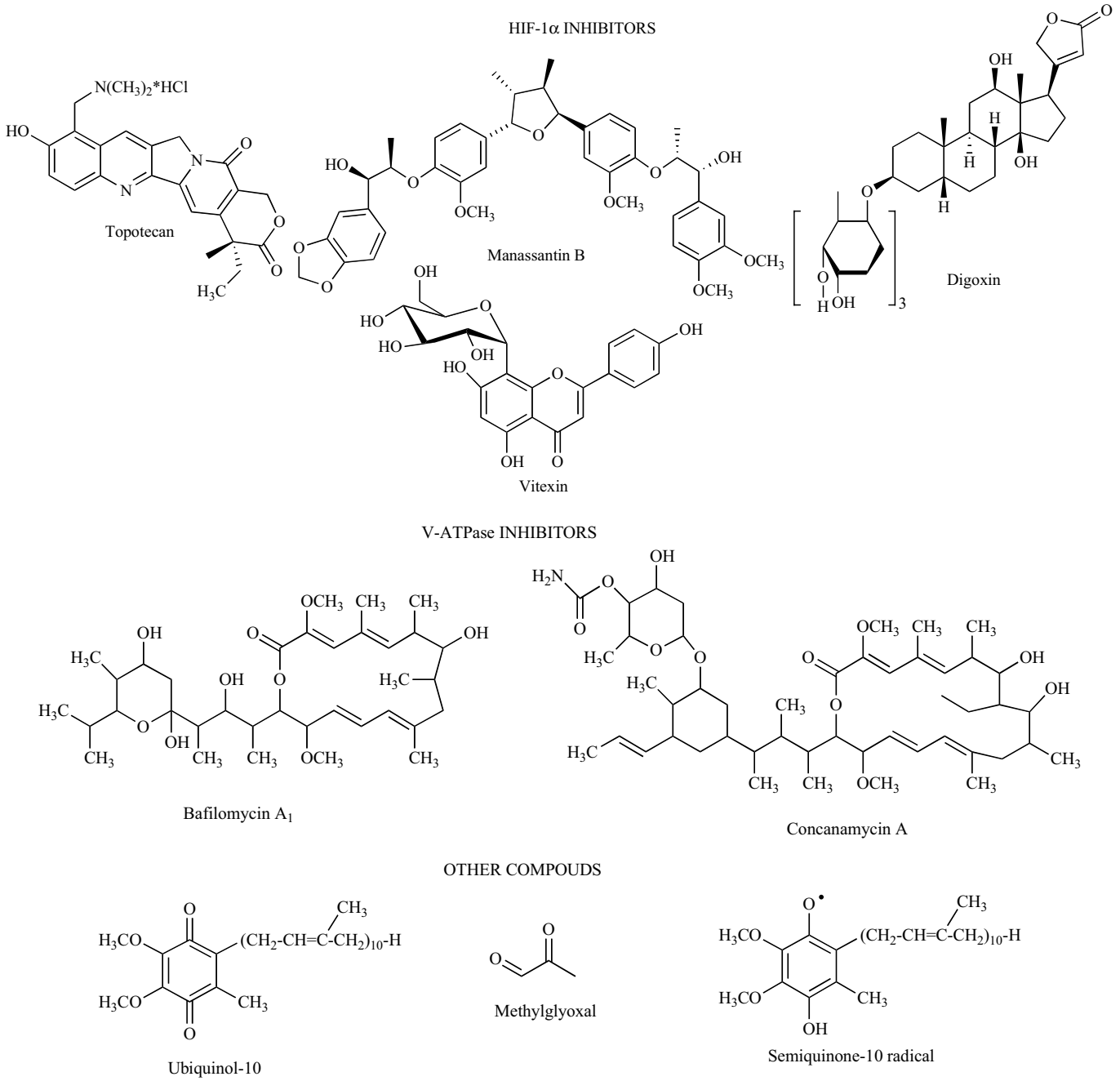

Fig. (3). Chemical structures of some anticancer drugs that block energy metabolism.

[4, 44, 50-54]. Interestingly, HIF-1α activation only increases the transcription of one particular isoform for each of the HIF-1α regulated glycolytic proteins. The following section discusses the specific HIF-1α mediated regulation of glycolysis and why some of the glycolytic isoforms may prove to be suitable drug targets for cancer therapy.

Glucose Transporters (GLUTs)

The glucose transporter family consists of three different classes. Class 1 contains four members, GLUT1-GLUT4 (Table **1**) whose preferential substrate is glucose. Class 2 and 3 transporters are selective for other carbohydrates [55]. GLUT1 and GLUT3 expression is up-regulated by HIF-1α (Fig. **2**). GLUT1 is expressed in all tissue types, whereas GLUT3 is preferentially expressed in the brain. Apparently, GLUT1 can form dimers and tetramers [56]. It has been argued that the HIF-1α-mediated GLUT1 and GLUT3 overexpression in cancer cells is related to their high glucose affinity (low *Km*) [57]. However, it is somewhat surprising that the kinetic parameters of glucose transporters have been determined only for glucose analogues such as 2-deoxyglucose(2-DOG) or 3-*O*-methyl glucose, but not for glucose itself; hence, substantial differences in the kinetic parameters have been reported: GLUT1, *Km* = 6.9-50 mM, *Vm* = 6.5-

Table 1. Isoforms of Glycolytic Proteins

Transporter or Enzyme	Genes	Isoforms	Oligomeric State	Anticancer Drugs
GLUT	4	GLUT1, GLUT2, GLUT3, GLUT4	M?, D, T	
HK	4	HKI, HKII, HKIII, HKIV	M, T	3-BrPyr, clotrimazole
HPI	1	No isoforms	D	2-DOG
PFK-1	3	PFK-L, PFK-M, PFK-P	T	clotrimazole
ALD	3	ALD-A, ALD-B, ALD-C	T	clotrimazole
TPI	1	No isoforms	D	
GAPDH	2	GAPD1, GAPD2	T	Arsenite,Goss, IAA, 3-BrPyr, Koningic acid
PGK	2	PGK1, PGK2	M	3-BrPyr
PGAM	2	PGAM-A, PGAM-B	D	Oxamate, Oxalate
ENO	3	ENO-α, ENO-β, ENO-γ	D	
PYK	2	PYK-R, PYK-L, PYK-M1, PYK-M2	T	Oxamate, Oxalate
LDH	3	LDH-A, LDH-B, LDH-C	T	Goss, Oxamate, Oxalate
PFK-2	4	PFKFB1, PFKFB2, PFKFB3, PFKFB4	D	
MCT	4	MCT1, MCT2, MCT3, MCT4	M?	

M, monomer; D, dimer; T, tetramer. IAA, iodoacetate; 2-DOG, 2-deoxyglucose; 3-BrPyr, 3-bromopyruvate; Goss, gossypol. Data taken from [55, 56, 63, 65, 73, 77, 78, 81, 100, 107, 109, 111, 116, 121, 124, 128, 130, 132].

700 pmol/min/oocyte; GLUT2, *Km* = 17-42 mM, *Vm* = 3.1-900 pmol/min/oocyte; GLUT3, *Km* = 1.8-10 mM, *Vm* = 2.2-850 pmol/min/oocyte; GLUT4, *Km* = 4.6-100 mM, *Vm* = 150 pmol/min/oocyte) [58-60]. Based on the kinetic parameters determined for 2-DOG, and the assumption that the *Km* values for 2-DOG are close to those for glucose, it can be concluded that GLUT3 is the transporter with the highest affinity and catalytic efficiency (*Vm*/*Km*; GLUT3>GLUT1> GLUT2>GLUT4), while GLUT2 over-expression would be physiologically irrelevant at normal blood glucose levels of around 5 mM.

GLUT1 is the transporter most widely over-expressed in cancer cells (Table **2**), particularly in highly proliferative and malignant tumors [55, 61]. GLUT3 is also over-expressed in lung, colon, ovary, larynx and mammary gland tumors (Table **2**); high levels of GLUT1 or GLUT3 have been used as indicators of bad prognosis [55]. Interestingly, GLUT1 and GLUT3 are one of the main controlling steps of glycolysis in some fast-growth tumor cells [62; Rodríguez-Enríquez S., Marín-Hernández A, Gallardo-Pérez J.C., Moreno-Sánchez R., unpublished data], and hence it provides a suitable therapeutic target for glycolytic and hypoxic tumors. However, inhibitors of GLUT that specifically target cancer cells have not yet been developed.

Hexokinase (HK)

Monomeric HK has four isoforms (Table **1**) with molecular masses of 100 KDa for HKI, HKII and HKIII or 50 KDa for HKIV, or glucokinase (GK). Their *Km* values for glucose range from 0.003 to 8 mM in the order of relative affinity (1/*Km*): HKIII>HKI>HKII>HKIV. The activity of isoforms I-III is strongly inhibited by the product, G6P, whereas GK is fully insensitive to this metabolite [63]. HKI and HKII genes are HIF-1α targets (Fig. **2**) [44]. HKII over-expression occurs in the majority of tumors, although in brain, testis, and head and neck tumors HKI is preferentially over-expressed (Table **2**) [64] and may form tetramers [65]. These two isoforms can bind to the external mitochondrial membrane by means of a 15 hydrophobic amino acid segment, MIASHLLAYFFTELN, in the amino-terminal region [66]. In some tumor cells, the mitochondria-bound HK accounts for 50-70% of total cellular HK [62]. However, in the majority of kinetic studies in cancer cells, the analysis of HK activity has been derived from the free or cytosolic isoform, while the contribution of membrane-bound HK has often not been evaluated, thereby underestimating the total HK activity.

Apparently, HK preferentially interacts with the membrane permeability transition (MPT) pore through the voltage-dependent anion channel (VDAC), which leads to the blocking of cytochrome c release induced by the pro-apoptotic proteins Bax and Bid and protection of cancer cells from apoptosis [66, 67]. In turn, inactivation of cyclophilin D, a matrix component of the MPT pore, induces the release of HKII from mitochondria and enhances Bax-mediated apoptosis in cancer cells [68].

Mitochondrial HKI and HKII have preferential access to ATP produced by oxidative phosphorylation because of their

Table 2. Isoforms of Glucose Transporters and Glycolytic Enzymes Expressed in Human Tumors

Isoforms	Types of tumor																									
	Liver	Pancreas	Mg	Esophagus	Brain	Kidney	Lung	Skin	Colon	Endo.	Ovarian	Cervix	Larynx	Testis	H/N	LN	Prostate	Stomach	Úterus	NS	Placenta	Eye	RL	Cartilage	BM	Thyroid
GLUT1	X	X	X	X	X	X	X	X	X	X	X	X														
GLUT3			X				X		X		X		X													
HKI					X									X	X											
HKII	X	X	X	X	X	X	X	X	X	X	X	X														
HPI	X	X			X	X	X	X			X			X	X	X	X	X	X	X						
PFK-L					X	X	X	X								X	X				X	X				
ALD-A	X	X	X			X	X	X			X			X	X		X	X	X	X	X	X				
TPI	X	X	X		X	X	X	X			X			X		X	X	X	X			X				
GAPDH	X	X	X		X	X	X	X	X		X	X		X	X	X	X	X	X	X		X	X	X		
PGK1	X	X	X		X				X					X		X	X	X	X	X	X	X			X	
PGAM-B	X		X				X		X																	
ENO-α	X	X			X	X	X	X	X		X	X		X			X	X	X	X		X				
PYK-M2	X	X	X		X	X	X	X	X		X			X	X	X	X	X	X	X	X	X	X	X		
LDH A	X		X			X		X						X	X	X	X	X	X		X	X	X			
PFKFBP3			X						X		X						X									X
MTC4	there are not reports																									

Data taken from [55, 61, 113, 114, 165, 166]. Mg, Mammary gland; Endo, endometrium; H/N, head and neck; LN, lymphatic nodules; NS, nervous system; RL, reticular lymphoma ; BM, bone marrow.

proximal location to mitochondria (Fig. **2**) [64] and, as a result, are reportedly less sensitive to inhibition by G6P [69]. However, results from our laboratory have revealed strong G6P inhibition of both mitochondrial and cytosolic HK [62] when enzyme activity was assayed under near-physiological conditions (37°C, pH 7 and concentrations of glucose and G6P ≥ 1 mM). The G6P concentration has been reported at 0.6-5 mM in tumors [62] and the inhibition constant (*Ki*) or IC_{50} values for HK vary between 20 and 210 μM [63, 70]. Consequently, the HK activity would be predicted to be strongly G6P-inhibited under such conditions (Fig. **2**). Furthermore, G6P (1 mM) induces the release of mitochondrially bound HK in both malignant and non-malignant cells [71, 72]. Hence, HK would be predominantly free in the cytosol in cancer cells with high [G6P] such as AS-30D hepatocarcinoma (G6P ≥ 5 mM), whereas in tumors with low G6P such as HeLa cells (G6P=0.6 mM), HK may be predominantly bound to mitochondrial external membrane.

It should also be pointed out that, in some studies, the relative levels of HKI and HKII activity in cytosolic fractions have very likely been under-estimated, because the ATP concentration used (3-5 mM) was not saturating given the *Km* values of 0.4-1 mM. In order to correctly estimate HK activity (*Vmax*), at least 10 times the *Km* value (≥ 10 mM ATP) should have been used for these kinetic assays. This provides an additional uncertainty in interpreting the data from the studies of others when determining the overall ratio of HK and relative contributions from cytosolic *versus* mitochondrial activity.

HKI and HKII binding to mitochondria inhibit apoptosis and ensure that mitochondrial ATP is preferentially used for hexose phosphorylation, thereby contributing to the survival advantage of tumor cells. This regulatory mechanism of tumor HK supports an essential role for the enzyme in the control of the glycolytic flux [62]. Moreover, HKII overexpression promotes enhanced glycolytic flux because HKII, together with GLUT, exerts the main control on the glycolytic rate in tumor cells [62]. Therefore, mitochondrial HKI and II make attractive targets for therapeutic intervention to suppress tumor growth.

Apparent specific inhibition of HK by 3-bromopyruvate (Table **1**; Fig. **3**) has been reported [73]. However, the cytotoxic activity against cancer cells was of low potency (IC_{50}~

50 μM) [74, 75] and other glycolytic (150 μM induces 70% inhibition of GAPDH and PGK) (Table **1**) and mitochondrial (PDH, SDH, glutamate dehydrogenase, pyruvate transporter) proteins [76, 77], as well as the mitochondrial proton leak, are also sensitive to similar low concentrations of this compound [77]. Clotrimazole (Fig. **3**) induced HK detachment from mitochondria in B16 melanoma cells, but also detached PFK-1 and ALD from the cytoskeleton in mouse LL/2 Lewis lung cancer cells, leading to diminished G6P, F1,6BP, and ATP levels, and glycolytic flux [78]. Clotrimazole reduces cellular proliferation and viability of human CT-26 colon, Lewis lung and breast MCF-7 carcinomas (IC_{50}= 50- 80 μM) [78, 79]; and the size and development of intracranial gliomas (C6 and 9L), prolonging survival in rodents [80]. Clearly, although more specific HK inhibitors are required, some of the already known HK inhibitors might assist treatment by sensitizing cancer cells to other anti-cancer drugs.

Hexosephosphate Isomerase (HPI)

HPI is a homodimer with 63 KDa subunits and with no isoforms (Table **1**) [81]. HPI is over-expressed under hypoxia through a mechanism not directly dependent on HIF-1α [54]; HIF-1 specific consensus sequence for binding in the HPI gene has not been described (Fig. **2**). Moreover, the enzyme does not seem to exert significant flux control over glycolysis [62], and hence HPI over-expression in cancer cells (Table **2**) may be related to its other less well-known functions. In addition to participating in glycolysis, HPI also promotes cell migration, proliferation and metastasis [82]. In tumor cells, HPI is expelled to the extracellular space where acts as a cytokine (*i.e.*, autocrine motility factor, AMF) thus altering several functions including tumor progression and metastasis.

HPI is inhibited by erythrose 4-phosphate (ERI4P) and F1,6 BP (*Ki* values of 0.7 and 170 μM, respectively) [83; Marín-Hernández A, Moreno-Sánchez R, Saavedra E, manuscript in preparation], which may exist at relatively high concentrations inside tumor cells (16 μM and 10-25 mM, respectively) [62, 84]. Hence, HPI modulation by ERI4P and F1,6BP can be proposed as one mechanism for limiting excessive flux through the glycolytic pathway, regulating the supply of G6P for the pentose phosphate and glycogen synthesis pathways.

2-DOG is a glucose analog recognized by glucose transporters, phosphorylated by HK and dehydrogenated by glucose 6-phosphate dehydrogenase (G6PDH). However 2-DOG (as 2-DOG6P after HK phosphorylation) is not isomerized by HPI, which it inhibits, thereby diminishing glycolytic flux (Table **1**). In addition, 2-DOG effectiveness is drastically reduced in the presence of glucose, due to the competition for GLUT, HK and G6PDH. The 2-DOG primary effect is on HPI because HPI inhibition by 2-DOG6P (*Ki*=5 mM) [85] induces, in turn, the accumulation of G6P, which is a more potent HK inhibitor (*Ki*=20-200 μM [63, 70]) than 2-DOG (*Ki*=0.4 mM) and 2DOG6P (*Ki*=4.3 mM) [86]. In consequence, glycolytic flux in the presence of 2-DOG diminishes because HK is inhibited by G6P and HPI is inhibited by 2-DOG6P.

Interestingly, 2-DOG is more toxic for osteosarcoma ρ^o cells than for the parental osteosarcoma cells (IC_{50} values of 32-100 μM and 0.6-6 mM, respectively), presumably because the ρ^o cells are only dependent on glycolysis for ATP generation [87]. 2-DOG (500 mg/kg weight) does not exhibit anticancer activity in osteosarcoma nude mouse xenografts and non-small cell lung cancer [88], which may be because they rely more on OxPhos for their energy supply. However, 2-DOG significantly enhances the anticancer activity of etoposide, camptothecin, and Hoechst-33342 in a range of other cancers, including cerebral glioma BMG-1, squamous carcinomas 4451 and 4197, and malignant glioma U-87 cells [89]. It is possible that cells treated with the other agents become heavily reliant on glycolysis and therefore become more sensitive to 2-DOG. Combining 2-DOG with adriamycin, paclitaxel or etoposide diminishes the size and proliferation of human osteosarcoma, xeno-transplanted MV522 lung carcinoma and Ehrlich hepatoma-bearing mice in comparison with tumors treated with 2-DOG or anticancer drugs, separately [88-90]. This increased sensitivity towards anticancer drugs induced by 2-DOG is attributed to the high glycolysis-dependence of the tumor for ATP supply and may result from increased demands for ATP made by the cell damaging agents.

2-DOG also affects protein glycosylation, induces accumulation of mis-folded proteins in the endoplasmic reticulum, leads to a decrease in the amount of HK associated with mitochondria and induces the expression of P-glycoprotein [91, 92]. Therefore, the drug is not a specific glycolysis inhibitor and its anticancer activity may as a result be limited.

Phosphofructokinase Type 1 (PFK-1)

PFK-1 is a homo- or hetero-tetramer of 380 KDa, with three isoforms (Table **1**). The main isoforms expressed in liver and platelets are PFK-L and PFK-P (or C), respectively, whereas skeletal muscle only has PFK-M. A mixture of the three isoforms is found in all other tissues [81], but HIF-1α only increases expression of the PFK-L isoform [4, 44].

PFK-M shows the highest affinity for F6P ($K_{0.5}$=0.6-2 mM) and it is the least sensitive to ATP inhibition. PFK-L is the least sensitive isoform to inhibition by the Krebs cycle intermediate, citrate (IC_{50}=0.18 mM). PFK-P has the lowest affinity for F6P ($K_{0.5}$=1.4-4 mM) and is more sensitive to citrate inhibition (IC_{50}=0.08 mM) [93]. Therefore, to increase flux through this enzyme (and hence increase glycolysis and ATP synthesis) tumors preferentially over-express M and L, over the P isoform, exploiting their reduced sensitivity to feed-back inhibition by ATP and citrate. A lower pH also inhibits PFK-1 activity, decreasing both the affinity for F6P and *Vm* [Moreno-Sánchez R, Marín-Hernández A, Encalada R, Saavedra E, unpublished data]. Due to their higher glycolytic flux resulting in lactic acid production, cancer cells have a more acidic cytosol and extracellular pH [94], which would decrease PFK-1 activity. Hence, it is not surprising that tumors express greater levels of PFK-1 induced by HIF-1α to compensate for the lower pH.

The role for activators such as F2,6BP and AMP, which would also be expected to promote an increased flux *via* the L and M isoforms, is currently unclear because unfortunately, detailed kinetic studies on PFK-1 are scarce. The systematic kinetic analysis of AS-30D and HeLa PFK-1 are

currently under investigation in our laboratory [Moreno-Sánchez R, Marín-Hernández A, Encalada R, Saavedra E, unpublished data]. The results have shown that the *Ki* values for ATP and citrate are 1.7 and 4-17 mM, whereas the *Ka* values for F2,6BP and AMP are 0.1-33 μM and 0.4-3 mM, respectively, in a K^+-based medium; K^+ is also a PFK-1 activator with a *Ka* of 11.5-13.5 mM. Usually, these affinity constants have been determined in reaction medium with no K^+.

The physiological concentrations of ATP (1.4-9.2 mM), AMP (0.15-3.3 mM), citrate (0.4-1.7) and F2,6BP (5-50 μM) present in tumor cells [62, 95-97] would indicate that ATP inhibition is more likely to be relevant than citrate inhibition, and that F2,6BP would prevail over AMP activation. Furthermore, it has been established that PFK-1 activation by F2,6BP overcomes ATP and citrate inhibition [62]. Hence, the high F2,6BP levels present in cancer cells [96, 97] does not support a major role for activated PFK in the flux-control of glycolysis [62]. On the other hand, PFK-1 in tumors with low expression or low F2,6BP content might still exert significant flux control of glycolysis [Marín-Hernández A, Moreno-Sánchez R, Saavedra E, manuscript in preparation].

Aldolase (ALD)

ALD is a homo-tetramer of 40 KDa subunits, with three isoforms (Table **1**). ALD-A, B, and C predominate in skeletal muscle, liver and brain, respectively. Combinations of the three isoforms are found throughout all tissues [81].

ALD-A and C are more efficient (10-20 times) than B in the forward (glycolytic) reaction [98]. ALD-B shows higher affinity for G3P and DHAP, which facilitates the reverse reaction. Thus, ALD-A and C are preferentially localized in tissues with high glycolysis such as skeletal muscle, erythrocytes and brain, whereas ALD-B is in gluconeogenic tissues such as liver and kidney. Therefore, HIF-1α up-regulates the expression of ALD-A and C (Fig. **2**) [4, 44] with ALD-A predominantly expressed in tumors (Table **2**) [61].

Triosephosphate Isomerase (TPI)

TPI is a homodimeric enzyme of 27 KDa subunits without isoforms (Table **1**), although post-translational regulation has been described [81]. TPI gene is a HIF-1α target [51] (Fig. **2**). Enzymatic activity of TPI is one of the highest found in nature and in tumors its activity is 6-61 U/mg protein, whereas other glycolytic enzymes have much lower activities in the 0.003-0.8 U/mg protein range [62]. Therefore, TPI does not exert flux control of glycolysis [62], and its elevated content in cancer most likely have a still unknown function.

TPI deficiency in patients induces an increase in DHAP concentration. The DHAP accumulation favors its non-enzymatic decomposition to methylglyoxal (Fig. **3**), which is a highly reactive aldehyde that modifies proteins and DNA. Interestingly, some studies have suggested that methylglyoxal has anticancer properties. In particular, it inhibits glycolysis and mitochondrial respiration in human leukaemia cells, but not in normal cells [99]. Therefore, the physiological meaning of an increased expression of TPI in tumor cells, induced by HIF-1α, may be related to avoiding the accumulation of DHAP, and the generation of methylglyoxal.

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

GAPDH is a homo-tetramer of 37 KDa subunits [100] with no isoforms, except for a second gene (*gapd2*) exclusively expressed in spermatozoa [101]; post-translational regulation has also been described. Its *Km* values for G3P and NAD^+ are 240 and 80 μM, respectively [Marín-Hernández A, Moreno-Sánchez R, Saavedra E, manuscript in preparation]. HIF-1α up-regulates its expression. In addition to glycolysis and gluconeogeneis, GAPDH participates in transcriptional regulation as a nuclear tRNA export protein, and in replication and repair of DNA (acting as uracyl-DNA glycosylase, by removing uracyl residues). GAPDH can also mediate endocytosis by its interaction with tubulin and it can be required for programmed neuronal cell death [82, 100]. However, the role of its nuclear translocation in cancer development and growth has not yet been established.

GAPDH is a house-keeping protein that is used in numerous studies as a cytosolic marker or control for protein loading in SDS-PAGE. However, this protein is over-expressed to variable degrees in cancer cells, and its subcellular localization varies with the cellular growth state. In quiescent cells, GAPDH is localized in the cytosol but, in proliferating cells, GAPDH is detected also in the nucleus [100]. Therefore, it is not ideal to use the GAPDH protein or mRNA as a loading control for western or northern blotting studies or as a cytosolic marker in studies with tumor cells.

Gossypol, a polyphenolic aldehyde derived from cotton seeds (Table **1**; Fig. **3**), is an inhibitor of GAPDH, but it also inhibits other NAD^+-dependent enzymes such as LDH (Table **1**) and some mitochondrial dehydrogenases (*e.g*., isocitrate dehydrogenase). Gossypol can also affect a number of cellular functions associated with cellular proliferation, including ion transport, membrane properties, glycolysis, respiration, glucose uptake, and calcium homeostasis by inhibiting calcineurin [102]. At 1-9 μM, gossypol induces growth inhibition of several human cancer cell lines (breast, cervix, melanoma, ovary, and colon) [102, 103]. Structural data and molecular modeling studies have shown the direct interaction of gossypol with Bcl-2 and Bcl-XL and support its ability to inhibit the pro-survival activity of these proteins in cancer cells, promoting apoptosis [104]. At low doses (30 mg/kg), the drug reduced tumor size by 65%, and mortality was reduced to 8% in nude mice with the human SW-13 adrenocortical carcinoma [105]. In a phase I clinical trial, gossypol decreased glial and adrenal tumor size by 10-50% [106].

Koningic acid (heptelidic acid, avocettin) is a sesquiterpene antibiotic (Fig. 3) that inhibits GAPDH activity by reacting with the essential thiol group (cysteine 149). The type of inhibition is competitive against G3P (*Ki*=1.1 μM) [107]. At 1 mg/Kg, it suppresses tumor growth of Erlich ascites *in vivo*, whereas at 36 μM (10 μg/mL), it inhibits the growth of several cancer cell lines with high glycolysis but not the growth of normal cells lines with low glycolysis [108]. However, koningic acid also shows acute toxicity in mice (at 31.5 mg/Kg or about 600 μg *per* mouse) and deleterious effects on normal cells with high glycolysis (erythrocytes) [108].

For other GAPDH inhibitors, such as arsenite (AsO_2^{1-}) and iodoacetate (Table **1**; Fig. **3**), information about their

effects on tumor cells are scarce. Arsenate ($H_2AsO_4^{1-} \leftrightarrow HAsO_4^{2-}$ at neutral pH) can also block glycolytic flux because GAPDH may use it, instead of phosphate, to form 1-arseno-3-phosphoglycerate, which is spontaneously and rapidly hydrolyzed in water back to G3P with no associated synthesis of ATP (Table **1**) [for further details see 109].

Phosphoglycerate Kinase (PGK)

PGK is a monomer of 48 KDa with two isoforms (Table **1**). PGK1 is expressed in all somatic and cancer cells (Table **2**), whereas PGK2 only appears in spermatozoids [81]. HIF-1α up-regulates expression of PGK1 (Fig. **2**) [4, 44]. As PGK does not have significant flux control of glycolysis, its over-expression in tumor cells may have other functions. Tumor cells secrete PGK, and extracellular disulfide bond reduction of plasminogen by PGK acting as a disulfide reductase leads to production of angiostatin by promoting autoproteolytic cleavage of plasminogen [110]. Angiostatin inhibits the plasma membrane FoF_1-ATPase, normally present in mitochondria but found expressed in cancer cells. As a result cytosolic acidification occurs and both angiogenesis and metastasis are inhibited.

Phosphoglycerate Mutase (PGAM)

PGAM is a dimeric enzyme consisting of A or B isoforms (AA, AB and BB) (Table **1**) each requiring 2,3-bisphosphoglycerate (2,3BPG) as a cofactor [111]. PGAM-B (homodimer of B subunits) shows a higher affinity for 3PG and 2,3BPG (*Km*= 0.5 mM and 25 μM) than PGAM-A (*Km*=0.8 mM and 60 μM, respectively) whereas the affinity for 2PG is similar in both isoforms (*Km*=0.28 mM) [112]. PGAM-B over-expression has been reported in liver, lung, colon, and mammary gland tumors (Table **1**) [113, 114]; in addition, HIF-1α regulation of PGAM-B expression has been indicated [50], suggesting that PGAM-B may play an important role in malignancy. Furthermore, it has been reported that increased expression of the two PGAM isoforms favors the proliferation and immortalization of fibroblasts, whereas decreased expression induces premature senescence [115]. This observation suggests that PGAM promotes the immortalization of cancer cells rather than affecting increased glycolysis, as this enzyme is not a flux-controlling step [62]. Hence, PGAM is not highly relevant to the theme of this review.

Enolase (ENO)

ENO is a dimeric enzyme formed from three different subunits of 82-100 KDa (Table **1**). The main isoforms are αα, αβ, ββ, αγ and γγ [116]. ENO-α (homodimer of α subunits) is distributed in most tissues whereas ENO-β and ENO-γ are expressed preferentially in skeletal muscle and brain, respectively [116]. The three isoforms show similar affinity for 2PG (*Km*= 30 μM) [117]. ENO has an essential requirement for divalent metal ions in the following order of potency: $Mg^{2+} > Zn^{2+} > Mn^{2+} > Fe^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+} > Sm^{3+} > Tb^{3+}$ [116].

HIF-1α up-regulates ENO-α expression [4, 44] to significant levels in several tumor types (Table **1**). ENO-α acts as a receptor for plasminogen which favors tumor growth and metastasis [116]. Considered together with the role of PGK discussed above as a facilitator of plasminogen activation by autoproteolysis to plasmin, which is involved in catalyzing the degradation of fibrin aggregates, the evidence suggests that the combination of these two glycolytic enzymes is likely to facilitate cancer metastasis. Again, however, these enzymes are not highly relevant to the purpose of this review.

Pyruvate Kinase (PYK)

PYK is a homo-tetramer with four isoforms (Table **1**). PYK-L is localized mainly in liver and kidney (gluconeogenic tissues) and PYK-R is expressed in erythrocytes. These two isoforms are encoded by the same gene (which has 12 exons), but are expressed from alternative promoters such that specific promoters for L and R isoforms are in exon1 and 2, respectively. The two other isoforms are PYK-M1, localized in brain, heart and skeletal muscle, and PYK-M2, which is expressed in embryonic and stem cells, leukocytes, platelets, and cancer cells (Fig. **2**). The M1 and M2 isoforms are also encoded by the same gene through alternative splicing [81].

PYK-M1 is the only isoform with no cooperative kinetics in relation to its substrate, PEP, but has the highest affinity (*Km* = 0.08 mM) whereas PYK-R exhibits the lowest affinity (*Km* = 1.4 mM) [118]. The three other isoforms (R, L and M2) that exhibit cooperative kinetics are also potently activated by F1,6BP (*Ka*= 0.06-0.4 μM), an upstream glycolytic intermediary that establishes a feed-forward regulatory mechanism. ATP strongly inhibits the activity of the L and R isoforms (*Ki*= 0.1 and 0.04 mM, respectively) but only mildly inhibits M1 and M2 activity (*Ki*= 3 and 2.5 mM, respectively). Phosphorylation of the L and R isoforms by protein kinases fully abolishes activity, whereas the M1 and M2 isoforms are not susceptible to phosphorylation and hence are not directly regulated by the action of hormone binding [118]. The kinetic properties of the M2 isoform suggests that it is highly active in tumor cells at physiological concentrations of PEP (0.1-0.3 mM), F1,6BP (0.6-25 mM) and ATP (1.4- 9.2 mM) [64, 96] and, therefore, this is not a controlling step of glycolysis [62].

HIF-1α only up-regulates PYK-M2 expression, which is the main isoform found in tumors (Table **2**). As this isoform is relatively insensitive to ATP inhibition and it is not regulated by phosphorylation, it seems clear that its over-expression is favored in tumors to attain an enhanced glycolytic flux. Furthermore, PYK-M2 undergoes a dimer (inactive)- tetramer (active) transition (Fig. **2**) which is modulated by F1,6BP and the oncoproteins pp60 -v-src and HPV-16 E7 [119]. PYK-M2 binds to tyrosine-phosphory-lated peptides which are induced in growth factor-stimulated cells [120]. The interaction of these peptides and oncoproteins with PYK-M2 induces the release of the allosteric activator F1,6BP, promoting PYK-M2 dimerization and inactivation [119, 120] (Fig. **2**); the less active dimer seems to predominate in cancer cells [119].

It has been proposed that the increase in the inactive enzyme lead to accumulation of upstream glycolytic intermediaries which are in turn channeled to synthesis of ribose and nucleic acids, proteins and lipids essential for cellular prolif-

eration [119, 120]. However, no experimentation has been performed to support this conclusion. Kinetic analysis of PYK, carried out in different rodent and human cancer cell lines, reports higher activity than in their normal counterparts, which suggests that PYK in cancer cells is not inactive and it is not in a dimeric form. Furthermore, glycolytic flux is unaffected in cancer cells by PYK inhibition because this enzyme does not exert significant flux-control [62] due to a marked over-expression, null ATP inhibition and potent F1,6BP activation; in other words, the PYK-M2 tetramer might be less abundant than the dimer, but it would still exceed the pathway demands.

Lactate Dehydrogenase (LDH)

LDH is a homo- or hetero-tetrameric enzyme of 33.5 KDa subunits with two main isoforms. LDH-A (also LDH-5 or LDH-M) is abundant in skeletal muscle, LDH-B in heart, and five other subunit combinations have been found in other tissues (Table **1**): LDH1(B_4), LDH-2 (B_3A), LDH-3 (B_2A_2), LDH-4 (BA_3) and LDH-5 (A_4). LDH-C4 is expressed exclusively in the testis and spermatozoids [121].

In glycolytic tissues such as liver and skeletal muscle, LDH-4 and 5 (isoforms with high subunit A content) are predominant. In contrast, in tissues that consume lactate (heart, kidney, erythrocytes), LDH-1 and 2 (isoforms with high subunit B content) predominate, because of their higher lactate affinity (*Km* = 4 mM) compared to the LDH-4 and 5 isoforms (*Km* = 7 mM) [122]. Not surprisingly, HIF-1α upregulates LDH-A (LDH-5) expression [4, 44] (Fig. **2**) favoring an enhanced glycolytic flux. A high LDH-A level correlates with aggressive forms of several different tumor types [123].

In addition, LDH-A over-expression stimulates other non-glycolytic functions. GAPDH and LDH-A bind to single-stranded DNA. NADH addition diminishes the formation of GAPDH- or LDH-DNA complexes indicating that the $NADH/NAD^+$ ratio may regulate DNA binding of these glycolytic enzymes [82]. GAPDH and LDH-A constitute the transcription factor complex OCA-S, which increases histone transcription (H2Bgene) to maintain the replication process and function of eukaryotic chromosomes [82].

Oxamate and oxalate (Table **1**; Fig. **3**) are classical LDH inhibitors. Oxamate is a competitive inhibitor of LDH that inhibits glycolysis (albeit at very high concentrations with an IC_{80}= 80 mM) [124], but which is much more potent as an inhibitor of tumor cell growth (IC_{50}=10-47 μM) [87]. In monolayer leukemia cultures, tumor micro-spheroids, and *in vivo* tumor models (mouse melanoma), oxalate and oxamate induce apoptosis and cellular death at sub-millimolar doses [125, 126]. Unfortunately, in these studies, the inhibitory effect on LDH and glycolysis was not determined. Oxamate and oxalate are not very specific for LDH, as they also affect other glycolytic (PGAM, PYK), and non-glycolytic enzymes including transaminases, PDH, pyruvate carboxylase and the mitochondrial pyruvate transporter [127, 128]. This makes it difficult to define the importance of LDH inhibition in the control of the glycolytic flux in cancer cells.

However, LDH-A knock down in breast cancer cells increases mitochondrial respiration and decreases mitochondrial membrane potential, compromising the ability of these tumor cells to proliferate under hypoxia [129]. The tumorigenicity of the LDH-A-deficient cells was severely diminished, and this phenotype was reversed by complementation with the human ortholog LDH-A protein. These results demonstrated that LDH-A plays a role in tumor maintenance [129], although it remains to be determined whether similar knock-down of other glycolytic steps also induces the same described phenotype.

Phosphofructokinase Type 2 (PFK-2)

PFK-2 is a bi-functional homodimeric enzyme with 52-58 KDa subunits. The enzyme, through its kinase and phosphatase activities regulates the concentration of F2,6BP, the most potent activator of PFK-1. Therefore, the PFK-2 kinase/phosphatase activity ratio determines the actual F2,6BP cellular level and the degree of PFK-1 activation. The PFK-2 activities are oppositely regulated by PEP, α-glycero-phosphate and citrate, and by protein kinase C phosphorylation, all of which inhibit the kinase activity and stimulate the phosphatase activity [130].

There are four genes (*pfkfb-1, 2, 3, and 4*) in the rat and human genomes that encode four different PFK-2 isoforms (liver, heart, placenta, and testis, respectively). It seems that HIF-1α regulates the expression of the four genes, although the specific consensus sequence for HIF-1α binding has only been described for *pfkfb-3* [53], consistent with the over-expression of the placenta-type PFK-2 in a great variety of tumors (Table **1**). A high content of the placenta-type PFK-2 promotes an increased level of F2,6BP, because the phosphatase activity of this isoform is very low (0.2 mU/mg recombinant protein), but its kinase activity is relatively high (140 mU/mg recombinant protein). The placenta-type PFK-2 kinase/phosphatase activity ratio is therefore about 710, which is the highest compared to the other isoforms (0.4-4.1). Moreover, the placenta-type PFK-2 kinase activity cannot be inhibited by phosphorylation because it lacks the required Ser residue [130].

The high F2,6BP level in cancer cells [96, 97], brought about by over-expression of the placenta–type PFK-2 overcomes ATP and citrate inhibition and induces full activation of PFK-1 [62] which favors an increased glycolytic flux.

Monocarboxylate Transporter (MCT) and Plasma Membrane H^+-ATPase

Enhanced glycolysis elevates levels of lactate and H^+, which must be actively expelled from cancer cells to keep the cytosolic pH and osmotic balance under control [131]. The MCT family consists of 9-14 members from which MCT1-MCT4 catalyze the reversible co-transport of lactate, pyruvate or ketone bodies and H^+ (Table **1**; Fig. **2**). Lactate extrusion is favored by an acidic cytosolic pH, or an alkaline extracellular pH [131, 132].

MCT1 is in all types of tissues, whereas MCT2 is mainly expressed in the liver, stomach, skin, kidney and brain, MCT3 is exclusive to the retina and MCT4 is abundantly expressed in tissues with high glycolysis such as skeletal muscle, leukocytes, testis, lung, placenta and heart. The affinity for lactate and pyruvate (*Km*= 0.7-28 mM; and 0.1-150

mM, respectively) differs among the four isoforms with MCT4 showing the lower affinity [131, 132]. MCT4 is the predominant isoform expressed in some breast cancer cell lines [133] but in oxidative tumor cells such as CaCo-2, WiDr, FaDu, SiHa and PC-3 cells and primary human cancers it is MCT1 [134, 135]. HIF-1α only up-regulates MCT4 expression [52], although inhibition of MCT1 decreases tumor growth and renders it sensitive to irradiation [135], suggesting that both isoform transporters play a relevant role in the operation of glycolysis in tumors.

A second system used to regulate the cytosolic pH is the plasma membrane V-type H^+-ATPase [132, 136]. This enzyme is over-expressed in tumors and is involved in the tumor interstitium acidification from pH 6.8 to pH 6.5 [137], which in turn promote metastasis [138]. Thus, inhibition of the V-type ATPase and cytosolic acidification can induce cell death and could constitute a promising and novel therapeutic approach. For instance, inhibiting V-ATPase with macrolide antibiotics, bafilomycins or concanamycins (Fig. **3**) [139], or down-regulating its expression [140], induce cancer cell death. Blocking other cytosolic pH regulators such as the Na^+/H^+ antiporter, the MCT family described above, or the Na^+-dependent Cl^-/HCO_3^- exchanger might also be suitable anti-cancer targets and some specific inhibitors have been found [132].

HIF-1α AND REGULATION OF MITOCHONDRIAL ENZYMES, CYTOCHROME C OXIDASE AND PYRUVATE DEHYDROGENASE KINASE

This section discusses the two mitochondrial enzymes, cytochrome c oxidase (COX; complex IV) and PDK, known to be regulated by HIF-1α. COX is the respiratory complex that consumes O_2 and is inhibited by cyanide (Fig. **3**), H_2S, CO, CO_2 or NO. COX is a dimer in which each monomer comprises 13 subunits. Subunits 1-3 are encoded by the mitochondrial DNA, are highly conserved, and constitute the catalytic core. Subunit 4 participates in the initial steps of COX assembly and binds ATP, which induces COX inhibition. HIF-1α regulates COX subunit 4-1 expression, causing an isoform switch from the usual subunit 4-2 to 4-1. The net effect is an increase in COX activity, but only a slight increase in O_2 consumption and ATP levels [141, 142], in agreement with the negligible role of COX in the control of the respiratory flux and oxidative phosphorylation rates [143].

The pyruvate dehydrogenase complex (PDH) is inhibited by phosphorylation in a reaction catalyzed by PDH kinase (PDK). Four PDK isoforms have been identified in mammalian cells (PDK1-4). These enzymes are dimers with subunits of 46 KDa [144]. PDK1 is expressed almost exclusively in heart. PDK2 is found in heart, skeletal muscle, placenta, lung, brain, kidney, pancreas and liver. Heart and skeletal muscle also express PDK3 and PDK4 [145, 146].

PDKs phosphorylate three serine residues (site 1, Ser-264; site 2, Ser-271; site 3, Ser-203) of the PDH-E1 α subunit. PDK1 can phosphorylate all three sites whereas the other isoforms only phosphorylate sites 1 and 2 [147, 148]. HIF-1α up-regulates PDK1 and has been proposed to play a major role in the inactivation of the PDH enzyme complex, thereby decreasing pyruvate oxidation through the Krebs cycle, oxygen consumption, and mitochondrial function in cancer cells [149, 150]. However, this proposal [5, 149-152] assumes that pyruvate is the main oxidizable substrate in hypoxic cancer cells and that PDH is the rate-limiting step of Krebs cycle. Moreover, complete PDH phosphorylation and inhibition has not been demonstrated. Neither has a PDK induced significant diminution in the rate of mitochondrial respiration or OxPhos nor an associated enhancement in glycolysis been shown to occur. Dichloroacetate (DCA; Fig. **3**) has been used as an inhibitor of PDK as a means to highlight the importance of PDK in tumor cell metabolism [152]. In this study, DCA was shown to induce cancer cell death by increasing ROS production and apoptosis. However, DCA probably also affects other cellular functions and hence, the role of PDK is less than certain based on these results. Furthermore, tumor mitochondria are able to oxidize several alternative energy substrates, such as glutamine, glutamate, fatty acids and ketone bodies [49], at high rates and independently of PDH complex activity.

CAN SMALL DRUG INHIBITORS OF HIF-1α ACTIVATION BE DESIGNED AND DEVELOPED AS NOVEL CANCER THERAPIES?

Given the obvious importance of HIF-1α activity to the enhanced proliferation, promotion and survival of cancer cells, it follows that inhibitors of HIF-1α would likely be important cancer therapies (reviewed in [153]). Unfortunately, many of the substances found to inhibit HIF-1α have proven too cytotoxic to be useful as drug candidates. A considerable effort has been made to identify therapeutically useful HIF-1α small drug inhibitors, many of which are natural products or synthetic compounds based on natural products. Among the most recent interesting developments are the manassantins [154] such as manassantin B (Fig. **3**), a complex dineolignan extracted from *Saururus chinensis* and *cernuus*, herbs used in Chinese and Korean folk medicine. It inhibits both the growth of hypoxic cancer cells and HIF-1α activation with nM IC_{50} values. Unlike other compounds that attack hypoxic cancer cells manassantin B has very low toxicity, and as such, is a lead compound in the development of new non-toxic anti-cancer therapeutic agents. The second interesting development is the discovery that cardiac glycosides, such as digoxin (Fig. **3**), are potent inhibitors of HIF-1α synthesis (in the submicromolar range) [155]. At low concentrations, these drugs have also been shown to inhibit tumor growth *in vivo*. These results suggest that previously difficult to treat hypoxic tumors with high HIF-1α activity may now be targeted.

CONCLUSIONS

HIF-1α is a major transcription factor regulating the genes encoding glycolytic enzymes and transporters. Its activity is mainly targeted to those glycolytic isoforms that increase pathway flux but also on other functions such as regulation of gene transcription, DNA repair, cellular migration, invasion and metastasis, and inhibition of apoptosis to favor tumor development and growth. For these reasons, HIF-1α is a logical therapeutic target for the treatment of cancer [156].

HIF-1α inhibition by either RNA interference or by the drugs vitexin or topotecan (Fig. **3**) induces a reduction in tumor growth and metastasis [157-159]. Hypoxia depresses proliferation of tumor from HIF-1α positive embryonic stem cells but, in marked contrast, it does not affect proliferation of HIF-1α-deficient (HIF-1α$^{-/-}$) tumors from embryonic stem cells [160]. In contrast, HIF-1α$^{-/-}$ astrocytes can generate tumors in the vascular-rich brain parenchyma but not in the poorly vascularized subcutaneous environment [161]. Thus, HIF-1α may have different roles in tumor growth and development. HIF-1α also participates in the developing heart and vascular system, in the working skeletal muscle, in the adaptation of ischemic cardiovascular disease, in the female reproductive tract and in osteoblast development in addition to being one of the key transcriptional factors for embryonic development and maintenance of the immune system [3, 162, 163]. Therefore, given the wide-ranging activities and potential for HIF-1α targeted drug induced toxicity, it will be essential that the multiple functions of this transcription factor should be fully elucidated before embarking on clinical trials targeting HIF-1α for the treatment of cancer. General inhibition of HIF-1α activity certainly promotes pronounced side effects [10].

The complete characterization of the HIF-1α regulated mitochondrial proteins and their functions should first be undertaken to better understand why certain isoforms are preferentially synthesized in cancers and to facilitate the identification of the best therapeutic targets.

From the perspective of flux control analysis, it appears that GLUT and HK, but not PFK-1 and PYK, provide the best targets for therapeutic intervention at the level of energy metabolism in hypoxic and glycolytic tumors. It follows that specific, potent and cell permeable inhibitors of these two controlling steps of glycolysis may prove to be preferred targets rather than HIF-1α. For specificity, it is also desirable that putative drugs should only interact with the tumor proteins and not with the non-tumor proteins. It may also be possible to exploit the more acidic extracellular pH in tumors because some compounds such as α-tocopheryl-succinate become more potent anticancer drugs at lower pH than at neutral pH [164]. For potency, preferred compounds will be those with low nanomolar range *Ki* values and drug design should consider that the compound has to penetrate into the cancer cells, for which a hydrophobic chemical segment may prove beneficial.

ACKNOWLEDGEMENTS

The present work was partially supported by CONACyT-México grant No. 80534. The authors wish to thank Prof. P.K. Ralph for his stimulating and helpful observations. AMH was the recipient of CONACyT-México fellowship 159991.

ABBREVIATIONS

HIF = Hypoxia-inducible factor

HRE = Hypoxic responsive elements

pVHL = Von Hippel-Lindau protein

PHDs = Prolyl-4-hydroxylases

AHs = Asparaginyl-aspartyl hydroxylases

ROS = Radical oxygen species

O-DD = Oxygen-dependent degradation

C-TAD = Asparagine-containing transactivation domain

TTFA = Thenoyltrifluoroacetone

SDH = Succinate dehydrogenase

FH = Fumarate hydratase

MPT = Membrane permeability transition

VDAC = Voltage-dependent anion channel

OxPhos = Oxidative phosphorylation

GLUT = Glucose transporter

HK = Hexokinase

HPI = Hexosephosphate isomerase

PFK-1 = Phosphofructokinase type 1

PFK-2 = Phosphofructokinase type 2

ALD = Aldolase

TPI = Triosephosphate isomerase

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

PGK = Phosphoglycerate kinase

PGAM = Phosphoglycerate mutase

ENO = Enolase

PYK = Pyruvate kinase

LDH = Lactate dehydrogenase

MCT = Monocarboxylate transporter

PDH = Pyruvate dehydrogenase complex

PDK = Pyruvate dehydrogenase kinase

G6PDH = Glucose 6-phosphate dehydrogenase

G6P = Glucose 6-phosphate

F6P = Fructose 6-phosphate

F2,6BP = Fructose 2, 6 bisphosphate

F1,6BP = Fructose 1,6 bisphosphate

DHAP = Dihydroxyacetone phosphate

G3P = Glyceraldehyde-3-phosphate

1,3BPG = 1,3 bisphosphoglycerate

2,3BPG = 2,3-bisphosphoglycerate

3PG = 3-phosphoglycerate

2PG = 2-phosphoglycerate

PEP = Phosphoenolpyruvate

PYR = Pyruvate

LAC = Lactate

ERI4P = Erythrose 4-phosphate

2-DOG = 2-deoxyglucose

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Received: 16 December, 2008 **Revised: 21 April, 2009** **Accepted: 23 April, 2009**