REVIEW

Glycolysis inhibition for anticancer treatment

H Pelicano[1], DS Martin[2,*], R-H Xu[3] and P Huang[1]

[1]The University of Texas MD Anderson Cancer Center, Houston, TX, USA; [2]Memorial Sloan-Kettering Cancer Center, New York, NY, USA and [3]Department of Medical Oncology, Sun Yat-sen University Cancer Center, Guangzhou, China

Most cancer cells exhibit increased glycolysis and use this metabolic pathway for generation of ATP as a main source of their energy supply. This phenomenon is known as the Warburg effect and is considered as one of the most fundamental metabolic alterations during malignant transformation. In recent years, there are significant progresses in our understanding of the underlying mechanisms and the potential therapeutic implications. Biochemical and molecular studies suggest several possible mechanisms by which this metabolic alteration may evolve during cancer development. These mechanisms include mitochondrial defects and malfunction, adaptation to hypoxic tumor microenvironment, oncogenic signaling, and abnormal expression of metabolic enzymes. Importantly, the increased dependence of cancer cells on glycolytic pathway for ATP generation provides a biochemical basis for the design of therapeutic strategies to preferentially kill cancer cells by pharmacological inhibition of glycolysis. Several small molecules have emerged that exhibit promising anticancer activity _in vitro_ and _in vivo_, as single agent or in combination with other therapeutic modalities. The glycolytic inhibitors are particularly effective against cancer cells with mitochondrial defects or under hypoxic conditions, which are frequently associated with cellular resistance to conventional anticancer drugs and radiation therapy. Because increased aerobic glycolysis is commonly seen in a wide spectrum of human cancers and hypoxia is present in most tumor microenvironment, development of novel glycolytic inhibitors as a new class of anticancer agents is likely to have broad therapeutic applications.
Oncogene (2006) **25**, 4633–4646. doi:10.1038/sj.onc.1209597

Keywords: glycolysis; Warburg effect; glycolytic inhibitor; ATP; mitochondria

Introduction

Therapeutic selectivity, or preferential killing of cancer cells without significant toxicity to normal cells, is one of the most important considerations in cancer chemotherapy. Understanding the biological differences between normal and cancer cells is essential for the design and development of anticancer drugs with selective anticancer activity. In the recent years, tremendous progress has been made in our understanding of the molecular mechanisms of cancer, in identification of specific genes and signaling pathways involved in carcinogenesis and cancer progression, and in developing chemical compounds or specific antibodies that specifically target the oncogenic molecules. Such target-specific agents have major advantages over the traditional chemotherapeutic compounds in that the targeting agents specifically interact with the key molecular players in cancer cells and have low toxicity to the normal cells. New agents with a high degree of target specificity and clinical therapeutic activity, exemplified by Gleevec (imatinib), Iressa (gefitinib), herceptin (trastuzumab), and rituximab, represent an exciting direction for cancer drug development. However, the mechanisms underlying cancer development and the disease progression are extremely complex, and it is now recognized that in many types of cancers there are multiple genetic and epigenetic alterations. Even within a specific cancer type, the malignant cell populations are heterogeneous and contain diverse genetic changes, which further alter over time because of genetic instability as the disease progresses. As such, it would be difficult to specifically kill these cancer cells by targeting a single gene. Proper combination of multiple target-specific agents may be required to effectively eliminate the entire cancer cell population. An alternative strategy to achieve both therapeutic selectivity and efficiency is to take advantage of the fundamental difference between cancer cells and normal cells in their biochemical metabolism. One of the most prominent metabolic alterations in cancer cells is the increase in aerobic glycolysis and the dependency on glycolytic pathway for ATP generation, known as the Warburg effect (Warburg _et al._, 1924; Warburg, 1930, 1956). As this metabolic alteration is frequently seen in cancer cells of various tissue origins, targeting the glycolytic pathway may preferentially kill the malignant cells and likely have broad therapeutic implications. This review article will summarize several important aspects of the glycolytic pathway in cancer, compounds that inhibit glycolysis and other relevant metabolic processes, and their potential applications in cancer treatment.
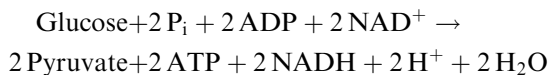
Correspondence: Dr P Huang, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA.
E-mail: phuang@mdanderson.org
*Deceased.

The glycolytic pathway

Glycolysis is a series of metabolic processes by which one molecule of glucose is catabolized to two molecules of pyruvate with a net gain of two ATP. The following equation shows the overall glycolytic reaction:

$$\text{Glucose} + 2\,P_i + 2\,\text{ADP} + 2\,\text{NAD}^+ \rightarrow$$
$$2\,\text{Pyruvate} + 2\,\text{ATP} + 2\,\text{NADH} + 2\,H^+ + 2\,H_2O$$

The glycolytic pathway is also known as the Embden–Meyerhof pathway, which has two phases, the priming phase and the energy-yielding phase. As illustrated in Figure 1, the priming phase uses two molecules of ATP to convert glucose to fructose-1,6-bisphosphate through sequential reactions catalysed by hexokinase, phosphoglucose isomerase, and phosphofructokinase. In the
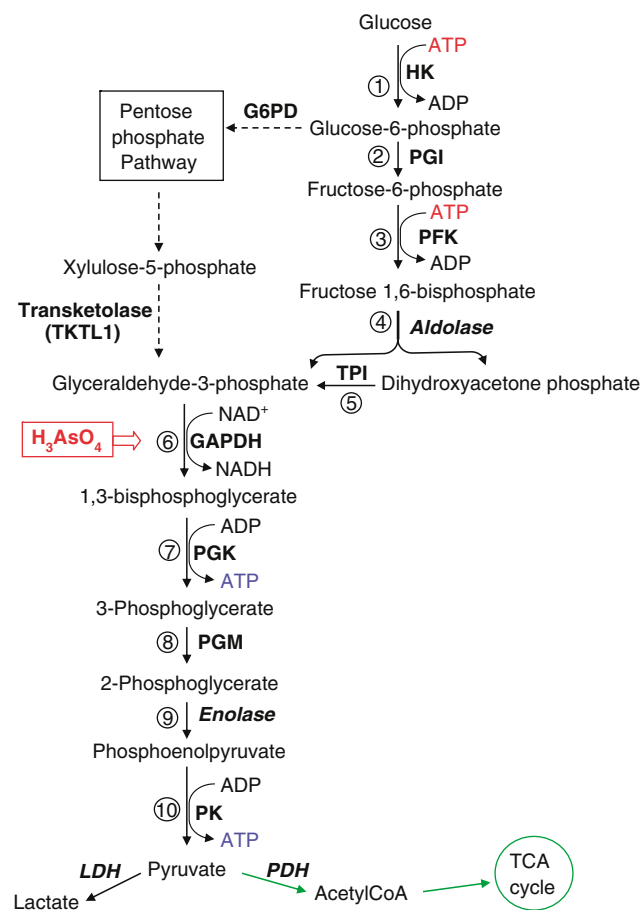
second phase, fructose-1,6-bisphosphate is further converted stepwise into pyruvate with the production of four molecules of ATP and two molecules of NADH. During this process, two ADP and two NAD^+ are consumed. In the absence of oxygen, NAD^+ is regenerated from NADH by reduction of pyruvate to lactic acid catalysed by lactate dehydrogenase (LDH). Under aerobic conditions, pyruvate can be further oxidized to CO_2 and H_2O in the mitochondria through the tricarboxylic acid (TCA) cycle and the respiratory chain, yielding large amount of ATP.

As illustrated in Figure 1, each reaction in the glycolytic pathway is catalysed by a specific enzyme or enzyme complex. In addition to their well-characterized enzymatic activities, recent studies suggest that some of the glycolytic enzymes are multi-functional proteins. For instance, hexokinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and enolase have been implicated to play a role in transcriptional regulation (Niederacher and Entian, 1991; Herrero et al., 1995; Feo et al., 2000; Rodriguez et al., 2001; Zheng et al., 2003). Hexokinase and GAPDH may regulate apoptosis (Ishitani and Chuang, 1996; Shashidharan et al., 1999; Tajima et al., 1999; Dastoor and Dreyer, 2001; Gottlob et al., 2001; Pastorino et al., 2002; Rathmell et al., 2003; Majewski et al., 2004), and glucose-6-phosphate isomerase can affect cell motility (Liotta et al., 1986; Nabi et al., 1990; Watanabe et al., 1996; Niinaka et al., 1998; Sun et al., 1999). Furthermore, although glycolysis is the classical metabolic pathway that generates pyruvate, other metabolic reactions such as conversion of xylulose-5-phosphate to glyceraldehyde-3-phosphate through the pentose phosphate pathway by transketolase also produce the metabolic intermediate that channel to the second phase of the glycolytic pathway (Figure 1, dashed arrows), yielding pyruvate and ATP. Interestingly, overexpression of the transketolase-like enzyme 1 (TKTL1) in cancer cells has recently been reported (Coy et al., 2005). The authors suggest that since transketolase regulate glucose metabolic flow into the pentose phosphate pathway, overexpression of TKTL1 may cause an increase in pentose phosphate pathway activity, leading to increased generation of glyceraldehyde-3-phosphate, which in turn is used in the energy-yielding phase of the glycolytic pathway (Coy et al., 2005).

Figure 1 Glycolytic pathway and its metabolic interconnection with the pentose phosphate pathway. The solid arrows indicate glycolytic reactions, whereas the dashed arrows show the pentose phosphate pathway. The green arrows indicate further metabolism of pyruvate downstream of glycolysis. Pentavalent arsenic compound (H_3AsO_4) abolishes ATP generation by causing arsenolysis in the glyceraldehyde-3-phosphate dehydrogenase reaction. HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; PDH: pyruvate dehydrogenase; LDH: lactate dehydrogenase.

Hexokinase

The ATP-dependent phosphorylation of glucose to form glucose-6-phosphate (G-6-P) is the first and rate-limiting reaction in glycolysis, and is catalysed by tissue-specific isoenzymes known as hexokinases. This phosphorylation converts the nonionic glucose to an anion (G-6P) that is trapped in the cells. Glucose-6-phosphate serves as the starting point for the sugar to enter the glycolic pathway or the pentose phosphate pathway (Figure 1), or for glycogen synthesis. Four mammalian isozymes of hexokinase (Types I–IV) have been identified, with the Type IV isozyme often referred to as glucokinase and found in hepatocytes. Glucokinase has a higher K_m for

glucose than other isozymes. The regulation of hexokinase and glucokinase activities is also different. Hexokinases I, II, and III are allosterically inhibited by product accumulation (G-6-P), whereas glucokinases are not. These enzyme properties favor glucose storage in the liver during times of glucose excess and peripheral glucose utilization. Hexokinases are 100 kDa molecules thought to have evolved by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase. Thus, these isozymes display internal sequence repetition, and the N- and C-terminal halves have extensive sequence similarity (Bork et al., 1993; Wilson, 1995; Cárdenas et al, 1998). Several studies demonstrate that hexokinase, particularly the Type II isoform (HK II), plays a critical role in initiating and maintaining the high glucose catabolic rates of rapidly growing tumors. Most immortalized and malignant cells display increased expression of HK II, which might contribute to elevated glycolysis (Bustamante and Pedersen, 1977; Arora et al., 1990; Rempel et al., 1996). At the genetic level, certain tumor cells exhibit increased gene copy number of Type II hexokinase. At the transcriptional level, the gene promoter shows a wide promiscuity toward multiple signals activated by glucose, insulin, hypoxic conditions, and phorbol esters, all of which enhance the rate of transcription (Mathupala et al., 1997b; Pirinen et al., 2004). It has also been suggested that the tumor suppressor p53 may be involved in regulating hexokinase gene transcription (Mathupala et al., 1997a).

At the protein level, hexokinases are either free in the cytosol or bound to the mitochondrial outer membrane (Wilson, 2003). The mitochondria-bond hexokinase seems to have the advantage of using ATP produced by oxidative phosphorylation as the substrate to phosphorylate glucose (Golshani-Hebroni and Bessman, 1997; Pastorino and Hoek, 2003). It was estimated that approximately 70% of cellular hexokinase is associated with mitochondria under basal metabolic conditions (Lynch et al, 1991). These findings suggest that oxidative phosphorylation may be efficiently coupled to the glycolytic pathway via the mitochondrial-bound hexokinase. Furthermore, hexokinase binds to the outer mitochondrial membrane at sites where the voltage-dependent anion channel (VDAC) is located (Wilson, 2003). This places the complex in close association with the adenine nucleotide translocator (ANT), which spans the inner mitochondrial membrane and facilitates the exchange of cytoplasmic ADP for mitochondrial ATP. Since recent studies suggest that VDAC/ANT may play an important role in regulating mitochondrial permeability transition and release of apoptotic factors such as cytochrome c, it is suspected that hexokinase may also participate in the apoptotic pathway. For instance, mitochondrial hexokinase activity seems to be required for the growth factor-induced cell survival, and Akt (protein kinase B) signaling appears to promote the association of hexokinase with VDAC on the mitochondrial membrane and enhance the mitochondrial hexokinase activity, leading to inhibition of apoptosis (Gottlob et al., 2001; Bryson et al., 2002). The molecular mechanism underlying the antiapoptotic effect of hexokinase is still unclear. Akt-mediated activation of mitochondrial hexokinase seems to inhibit cytochrome c release and apoptosis by antagonizing the proapoptotic function of tBid, which is an activator of apoptotic molecules Bax and Bak (Pastorino et al., 2002; Rathmell et al., 2003; Majewski et al., 2004). Analysis of mitochondrial protein complexes has revealed that glucokinase, a type IV hexokinase expressed predominantly in liver, is present in a mitochondrial complex that contains another VDAC-interacting protein BAD (Danial et al., 2003). BAD is a pro-apoptotic Bcl-2 family member that induces apoptosis by inhibiting the anti-apoptotic molecule Bcl-X_L. Transgenic mice expressing a non-phosphorylated BAD display aberrantly reduced mitochondrial glucokinase activity and reduced glucose tolerance, a condition found in diabetes (Danial et al., 2003). Thus, it appears that hexokinase and its association with mitochondrial protein complex may play important roles in the essential homeostatic processes such as glucose metabolism and apoptosis. Inhibition of this enzyme is likely to have profound effects on cellular energy metabolism and survival. Thus, hexokinase is an attractive target for anticancer agents.

Glucose-6-phosphate isomerase

The interconversion of G-6-P and fructose-6-phosphate is catalysed by phosphoglucose isomerase (PGI), which plays an important role in both the glycolytic and gluconeogenesis pathways (Harrison, 1974). Interestingly, recent studies have revealed that GPI can also function as an autocrine motility factor (AMF), which is secreted from the tumor cells to promote cell motility and proliferation (Niinaka et al., 1998; Sun et al, 1999). Autocrine motility factor and its receptor AMFR (gp78) were originally identified in melanoma and oncogene-transfected metastatic NIH3T3 cells, and the AMF/AMFR interaction seems to stimulate tumor cell migration *in vitro* and enhance metastasis and angiogenesis *in vivo* (Liotta et al, 1986; Nabi et al., 1990; Watanabe et al., 1996; Funasaka et al., 2001, 2002). Autocrine motility factor receptor is overexpressed in various metastatic tumors and is correlated with a poor prognosis (Hirono et al., 1996). The presence of GPI in serum and urine is associated with cancer progression and indicates poor prognosis (Baumann and Brand, 1988; Baumann et al., 1990; Filella et al., 1991). The expression of PGI is stimulated by hypoxia (Yoon et al., 2001; Niizeki et al., 2002). Thus, in addition to its well-defined enzymatic activity in the glycolytic pathway, GPI also functions as a cytokine extracellularly and is associated with aggressive malignant behaviors.

Phosphofructokinase

This enzyme catalyses the rate-limiting phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, using ATP as the energy source. Phosphofructokinase is allosterically regulated by 2,3-diphosphoglycerate (DPG) (Layzer et al, 1969). Three forms of phosphofructokinase, M (muscle), L (liver), and P (platelet),

have been identified in humans (Vora, 1983). The involvement of phosphofructokinase in cancer is unclear.

Aldolase

Aldolase catalyses the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Aldolase is a tetramer of identical subunits of 40 kDa each. Three distinct isoenzymes (A–C) have been identified. Interestingly, this enzyme becomes elevated in the serum of patients with certain malignant tumors (Taguchi and Takagi, 2001). Proteome analysis indicates that this enzyme is overexpressed in human lung squamous carcinoma (Li et al., 2006).

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase is well known as a classical glycolytic enzyme encoded by a 'housekeeping gene' which is constitutively expressed in most cells. This enzyme catalyses an essential redox reaction in the glycolytic pathway: conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate coupled with the reduction of NAD^+ to NADH. Glyceraldehyde-3-phosphate dehydrogenase is unique among the glycolytic enzymes because of its ability to bind NAD^+ or NADH, and also to DNA and RNA (Perucho et al., 1980; Grosse et al., 1986; Nagy et al., 2000). This unique property enables this protein to affect multiple cellular processes including endocytosis, membrane fusion, vesicular secretory, nuclear tRNA transport, and DNA replication and repair (Sirover, 2005). Nuclear GAPDH forms an Oct-1 transcriptional coactivator complex, OCA-S, which is an S-phase-dependent transactivator of the gene encoding histone H2B (Zheng et al., 2003). The DNA-binding property of GAPDH is enhanced when the $NADH/NAD^+$ ratio is low, leading to enhanced OCA-S activity and H2B expression. Interesting, GAPDH is implicated in apoptosis when it is translocated into the nucleus (Chuang et al., 2005), although the molecular mechanism responsible for its nuclear translocation and its role in cancer remain to be defined.

Phosphoglycerate kinase

This enzyme catalyses the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate coupled with the generation of ATP from ADP. Two isozymes of PGK have been identified. Phosphoglycerate kinase (PGK)-1 is ubiquitously expressed in somatic cells, whereas PGK-2 seems to express only in spermatozoa (VandeBerg, 1985). Phosphoglycerate kinase consists of two domains that are connected by a conserved hinge with the ADP/ATP-binding site located in the C-terminal domain and the phosphoglycerate-binding site in the N-terminal domain. A conformational rearrangement involving bending of the hinge occurs upon binding of both substrates, bringing them in position for phosphate transfer.

Phosphoglycerate mutase

The glycolytic enzyme phosphoglycerate mutase catalyses the interconversion of glycerate-3-phosphate and glycerate-2-phosphate. Phosphoglycerate mutase requires D-glycerate-2,3-diphosphate for activation by donating one of its phosphoryl groups to form a covalently linked phosphoryl enzyme. A recent study using proteome analysis showed that this enzyme seems to be differentially overexpressed in human lung squamous carcinoma (Li et al., 2006).

Enolase

Enolase catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate. The enzyme is highly conserved, and tissue-specific isoforms are found with minor kinetic differences (Marangos et al., 1978). Three major isoforms of enolase have been identified in mammals. The α-isoform is expressed in fetal cells and other adult cell types, the β-isoform is expressed in striated muscle, and the γ-isoform is neuron-specific. The expression of enolase is regulated both developmentally and tissue specifically, but the enzyme kinetic properties of all isoenzymes are similar. A recent proteome analysis showed that α-enolase is overexpressed in human lung squamous carcinoma (Li et al., 2006).

Pyruvate kinase

Pyruvate kinase (PK) catalyses the irreversible phosphoryl group transfer from phosphoenolpyruvate to ADP, yielding pyruvate and ATP. Pyruvate is an essential metabolic intermediate that channels into several metabolic pathways. Pyruvate kinase is a tetramer that is allosterically activated by phosphoenolpyruvate and negatively regulated by ATP. Interestingly, tumor cells in particular express the PK isoenzyme type M2 (M2-PK), which seem to regulate the proportions of glucose carbons for synthetic processes or for glycolytic energy production (Mazurek et al., 2005). The dimeric form of M2-PK is present predominantly in tumor cells (known as tumor M2-PK), and such dimerization appears to be caused by direct interaction of M2-PK with certain oncoproteins. This regulatory mechanism is thought to allow tumor cells to survive in environments with varying oxygen and nutrients (Mazurek et al., 2005).

Lactate dehydrogenase

Lactate dehydrogenase is a tetramer of A and B subunits, encoded by two separate genes. This enzyme catalyses the conversion of pyruvate to lactate coupled with an oxidation of NADH to NAD^+, which is essential for the glycolytic pathway. Interestingly, LDH-A gene is controlled by hypoxia inducible factor (HIF)-1α, whereas LDH-B gene is not regulated by low oxygen. In addition to its essential role in glucose metabolism, LDH-A isoform has been identified as a single-stranded-DNA-binding protein (Cattaneo et al., 1985; Grosse et al., 1986). Lactate dehydrogenase-5 was also identified as a DNA-helix-destabilizing protein and

speculated to be involved in transcription (Williams et al., 1985). The binding of LDH-A to single-stranded DNA is inhibited by NADH, which induces conformational change and modulates the DNA-binding activity of LDH (Cattaneo et al., 1985; Williams et al., 1985). More recent biochemical studies suggest that LDH-A and LDH-B are components of a cell-cycle-dependent transcriptional coactivator (Zheng et al, 2003). These unexpected observations indicate that a fraction of LDH might participate in DNA replication and RNA transcription.

Increase of aerobic glycolysis in cancer

The phenomenon of aerobic glycolysis increase in cancer cells was first described by Otto Warburg (1930) over 70 years ago. He showed that compared to normal cells, malignant cells exhibit significantly elevated glycolytic activity even in the presence of sufficient oxygen, and considered this phenomenon as the most fundamental metabolic alteration in malignant transformation, or 'the origin of cancer cells' (Warburg, 1956). Although the cause–effect relationship between the increase in aerobic glycolysis and the development of cancer is controversial (Zu and Guppy, 2004), increased glycolysis has been consistently observed in many cancer cells of various tissue origins (for a review, see Semenza et al., 2001), suggesting that this metabolic alteration is common in cancer. Indeed, the positron emission tomography (PET) widely used in clinical diagnosis of cancer is based on the fact that cancer cells are highly glycolytic and actively uptake glucose. The Warburg effect can be viewed as a prominent biochemical symptom of cancer cells that reflects a fundamental change in their energy metabolic activity. Several mechanisms have been suggested to affect energy metabolism and thus contribute to the Warburg effect. These mechanisms include (1) mitochondrial defects, (2) adaptation to hypoxic environment in cancer tissues, (3) oncogenic signals, and (4) abnormal expression of certain metabolic enzymes.

Table 1 provides a summary and explanations of these possible mechanisms.

Mitochondrial respiration injury
Mitochondrial respiration injury has long been suspected to be a factor responsible for increased glycolysis in cancer cells (Warburg, 1956), although the underlying molecular mechanisms remain unclear. Subsequent studies revealed that mitochondrial DNA (mtDNA) has high rates of mutations in cancer cells (for a review, see Carew and Huang, 2002; Singh, 2004; Taylor and Turnbull, 2005). For instance, frequent mtDNA mutations have been observed in prostate cancer (Chen and Kadlubar, 2004), breast cancer (Zhu et al., 2005), gastric cancer (Zhao et al., 2005), and leukemia (Carew et al., 2003). Several factors seem to contribute to the high mutation rates in mtDNA. These factors include the close physical location of mtDNA to the ROS generation sites in the mitochondria, lack of histone protection, and weak DNA repair capacity in the mitochondria. Because the mitochondria genome encodes 13 important protein components of the respiratory chain, mutations in mtDNA are likely to affect its encoded proteins and compromise the function of the respiratory chain. Since most of the mtDNA is structural gene sequence without introns, the possibility is high that a mutation in mtDNA would cause malfunction of the respiratory chain. Because oxidative phosphorylation in the mitochondria and glycolysis in the cytosol are two major metabolic pathways, by which ATP may be generated from glucose, malfunction of the mitochondrial respiratory chain would force the cells to use glycolytic pathway to generate ATP. Because the production of ATP is much more efficient through oxidative phosphorylation (36 ATP per glucose) than by glycolysis (two ATP per glucose), a small loss of respiratory function would require a substantial increase of glycolytic activity to maintain the energy balance.

Hypoxia
Hypoxia is a strong modulator of energy metabolism. Without mtDNA mutations, a functional defect in

Table 1 Potential mechanisms leading to increase in glycolysis in cancer cells

Mechanism	Explanation/example	Reference
1. Mitochondrial defects	mtDNA mutations lead to malfunction in respiration and oxidative phosphorylation	Carew and Huang (2002), Singh (2004), Taylor and Turnbull (2005)
2. Hypoxia	Adaptation to respiratory suppression owing to lack of oxygen in microenvironment	Gatenby and Gillies (2004), Brahimi-Horn and Pouyssegur (2005)
3. Oncogenic signals	Ras, Src Akt, Bcr-abl	Flier et al. (1987), Ramanathan et al. (2005) Elstrom et al. (2004), Gottlob et al. (2001) Boren et al. (2001), Serkova and Boros (2005)
4. Altered metabolic enzymes	Hexokinase II TKTL1 overexpression FH and SDH mutation	Bustamante and Pedersen (1977), Rempel et al. (1996) Coy et al. (2005) Astuti et al. (2001), Neumann et al. (2004), Pawlu et al. (2005), Selak et al. (2005)

MtDNA, mitochondrial DNA; TKTL1, transketolase-like enzyme 1; FH, fumarate hydratase; SDH, succinate dehydrogenase.

mitochondrial respiration may also force the cancer cells to use glycolytic pathway for ATP when oxygen is limited. This most likely occurs when cancer cells are in a hypoxic tissue environment. It is well documented that hypoxia is frequently present in human malignancies, especially in solid tumor tissues when the tumor mass reaches certain size and oxygen penetration becomes limited. Under such conditions, oxidative phosphorylation may not proceed normally because of insufficient oxygen, even if the mitochondria in cancer cells do not have structural defects. Increased glycolysis will result in elevated production of lactate, which leads to acidification of tumor tissue and provides a microenvironment that promotes and selects cells with malignant behaviors. Thus, increase in glycolysis may be viewed as cellular adaptation to hypoxia (Gatenby and Gillies, 2004). The cellular response to hypoxia is controlled in part by HIF-1, which activates the expression of target genes involved in angiogenesis, glucose uptake, glycolysis, growth factor signaling, apoptosis, invasion, and metastasis (Brahimi-Horn and Pouyssegur, 2005). Importantly, hypoxia has been associated with drug resistance and reduced sensitivity to radiation therapy, due in part to upregulation of HIF-1 and activation of survival molecules such as Akt and nuclear factor-κB. Therapeutic resistance associated with hypoxia is a significant problem in clinical treatment of cancer, and inhibition of glycolysis may provide a novel approach to overcoming such resistance. In fact, recent studies showed that under hypoxic conditions, cells exhibited increased sensitivity to glycolytic inhibitors 2-deoxyglucose (2-DG), oxamate, or 3-bromopyruvate (3-BrPA) (Liu et al., 2002; Maher et al., 2004; Xu et al, 2005b).

Studies using gene transfection approaches have revealed an intriguing possible mechanism by which malignant transformation by *oncogenic signals* may regulate energy metabolic pathways and renders the cancer cells highly glycolytic and become addictive to glycolysis for ATP production. Early studies in rodent cells showed that transfection with *ras* or *src* oncogenes led to a marked increase in the glucose uptake, accompanied by an increase in the expression of glucose transporter at both the mRNA and protein levels (Flier et al., 1987). In embryotic cells, H-ras was shown to stimulate glycolysis and inhibits oxygen consumption (Biaglow et al., 1997). The important role of *Ras* in promoting glycolysis was recently demonstrated in a study, in which transformation of cells by hTERT, SV-40T/t, and H-ras caused an increase in glycolysis dependency, and as the cells progressed toward a more tumorigenic state, they became more sensitive to the glycolytic inhibitor 2-DG (Ramanathan et al., 2005). Interestingly, inhibition of H-ras by *trans*-farnesylthiosalicylic acid resulted in inhibition of glycolysis and cell death in human glioblastoma U87 cells, with concomitant decrease in HIF-1α and glycolytic enzymes (Blum et al., 2005).

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which promotes malignant transformation (Karnauskas et al., 2003), has also been shown to enhance aerobic glycolysis and render cells dependent on glycolysis for survival (Elstrom et al., 2004). Several studies demonstrated that the signaling through the insulin receptor activates PI3K and Akt and result in stimulation of glucose uptake and glycolysis (Ruderman et al., 1990; Burgering and Coffer, 1995). After diffusion into cells through facilitative transport, which can be activated by Akt (Kohn et al, 1996; Rathmell et al., 2003), glucose is converted to G-6-P by hexokinase, preventing diffusion out of the cells through the bidirectional transporters. Thus, the activity of hexokinase play a key role in regulating the glucose uptake, and the activated forms of Akt have been shown to stimulate hexokinase activity (Gottlob et al., 2001; Rathmell et al., 2003). Interestingly, Akt has also been shown to phosphorylate and activate phosphofructokinase and release the inhibition of phosphofructokinase by ATP (Van Schaftingen and Hers, 1986; Deprez et al., 1997).

Another oncogene Bcr-Abl has also been implicated to play a role in glycolysis, and inhibition of Bcr-Abl by Gleevec seems to reverse the Warburg effect by switching glucose metabolism from glycolysis to mitochondrial oxidative phosphorylation (Gottschalk et al., 2004). Stable isotope-based dynamic metabolic profiling studies suggest that in myeloid cells isolated from patients, non-oxidative ribose synthesis from glucose and decreased mitochondrial glucose oxidation appear to be a metabolic signature of drug resistance and disease progression (Serkova and Boros, 2005). Together, these observations suggest that oncogenic signals may play important roles in regulation of energy metabolism, and contribute to the Warburg effect.

Alterations of enzyme expression in cancer cells have also been postulated to cause metabolic changes leading to the Warburg effect. Increase of hexokinase II expression in cancer and its possible role in promoting glycolysis are described above. Notably, it was recently observed that TKTL1, a transketolase-like enzyme, is highly expressed in a variety of human cancer tissues (Coy et al., 2005). The enzyme TKTL1 exhibits ketolase enzyme activity capable of cleaving xylulose-5-phosphate (5-carbon sugar) to glyceraldehyde-3-phosphate (3-carbon), which can then be channeled to the energy-yielding phase of the glycolytic pathway to generate ATP and lactate. The authors suggest that since transketolase regulate the glucose metabolic flow into the pentose phosphate pathway, high expression of TKTL1 would lead to an increased activity of this pathway to produce pentose-5-phosphates and NADPH needed for tumor growth, and to generate lactate through the metabolic intermediate glyceraldehyde-3-phosphate. This may provide a biochemical explanation for the Warburg effect (Coy et al., 2005). Interestingly, inhibition of transketolase by oxythiamine seems to have anticancer activity (Rais et al., 1999), suggesting an important role of the pentose pathway in cancer.

Two enzymes of the TCA cycle, fumarate hydratase (FH) and succinate dehydrogenase (SDH), play a vital role in ATP production in the mitochondria. Germline mutations in FH and SDH are associated with certain hereditary tumors such as leiomyomatosis, renal cell

carcinoma, pheochromocytoma, and paraganglioma (Astuti et al., 2001; Pollard et al., 2003; Neumann et al., 2004; Bayley et al., 2005). The exact underlying mechanisms are still poorly understood. Several mechanisms, including pseudo-hypoxia, mitochondrial dysfunction and impaired apoptosis, oxidative stress, and anabolic drive have been postulated to be involved in this predisposition to neoplasia through TCA cycle defects (Pollard et al., 2003). A recent study showed that inhibition of SDH causes an accumulation of succinate, which suppresses HIF-1α prolyl hydroxylases in the cytosol, leading to stabilization and activation of the oncogenic molecule HIF-1α (Selak et al., 2005). As SDH is an important enzyme involved in energy metabolism through the TCA cycle and mitochondrial complex II electron transport, mutations in SDH or loss of this enzyme activity would compromise the mitochondrial energy metabolism, leading to accumulation of succinate and abnormal activation of HIF-1α. Thus, SDH appears to provide a mechanistic link between mitochondrial dysfunction and oncogenic events associated with elevated HIF-1α.

In addition, reactive oxygen species (ROS) is known to damage the mitochondrial metabolic enzymes such as aconitase and α-ketoglutarate dehydrogenase, leading to a suppression of the TCA cycle (Tretter and Adam-Vizi, 2005). Thus, it is possible that the increased ROS generation in cancer cells associated with their intrinsic oxidative stress may lead to suppression of the ROS-sensitive enzymes involved in TCA cycle, forcing the cells to increase glycolysis to maintain ATP supply. Oxidative stress seems to be another biochemical characteristic of cancer cells attributed to multiple mechanisms including mitochondrial respiratory malfunction and oncogenic stress (see review, Pelicano et al., 2004).

Inhibition of glycolysis for anticancer treatment

Although the biochemical and molecular mechanisms leading to increased aerobic glycolysis in cancer cells are rather complex and can be attributed to multiple factors such as mitochondrial dysfunction, hypoxia, and oncogenic signals, the metabolic consequences seem similar: the malignant cells become additive to glycolysis and dependent on this pathway to generate ATP. Because ATP generation via glycolysis is far less efficient (two ATP per glucose) than through oxidative phosphorylation (36 ATP per glucose), cancer cells consume far more glucose than normal cells to maintain sufficient ATP supply for their active metabolism and proliferation. As such, maintaining a high level of glycolytic activity is essential for cancer cells to survive and growth. This metabolic feature has led to the hypothesis that inhibition of glycolysis may severely abolish ATP generation in cancer cells and thus may preferentially kill the malignant cells (Munoz-Pinedo et al., 2003; Izyumov et al., 2004; Xu et al., 2005b). As illustrated in Figure 2, under physiological conditions, normal cells
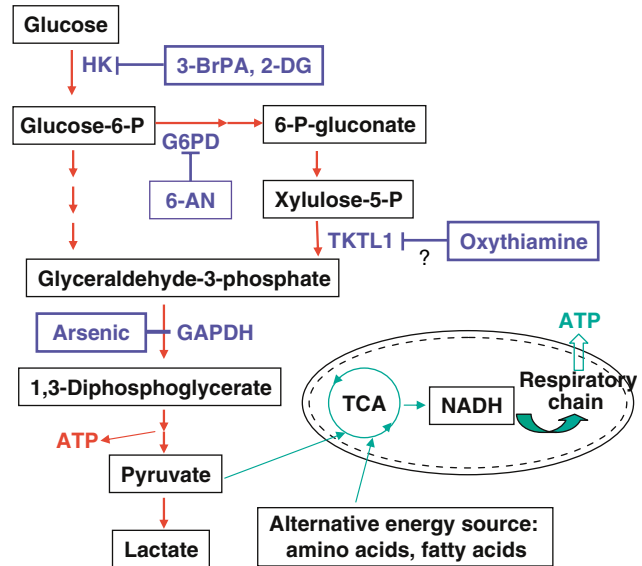

Figure 2 Metabolic basis for targeting the glycolytic pathway as an anticancer strategy. Cancer cells are more depend on glycolysis coupled with the pentose phosphate pathway (indicated by the red arrows) for ATP generation, whereas normal cells with competent mitochondrial function may use various metabolic intermediates as energy sources to effectively generate ATP through the mitochondrial oxidative phosphorylation (indicated by green arrows). Inhibition of glycolysis is expected to have a severe impact on ATP generation and preferentially affect the cancer cells. The potential target enzymes and respective inhibitors are indicated in blue. HK, hexokinase; 3-BrPA, 3-bromopyruvate; 2-DG, 2-deoxyglucose; G6PG, glucose-6-phosphate dehydrogenase; 6-AN: 6-aminonicotinamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TKTL1, transketolase-like enzyme 1.

with intact mitochondrial function can effectively use glucose and other metabolic intermediates to generate ATP through the TCA cycle and oxidative phosphorylation in the mitochondria (green arrows). However, the ability of cancer cells to use the mitochondrial respiratory machinery to generate ATP is compromised for the reason described above. This forces the cancer cells to increase their glycolytic activity to maintain sufficient ATP generation. It is postulated that such a metabolic adaptation eventually renders cancer cells highly addictive to and dependent on the glycolytic pathway (red arrows), and become vulnerable to glycolytic inhibition (Gatenby and Gillies, 2004; Xu et al., 2005b). When glycolysis is inhibited, the intact mitochondria in normal cells enable them to use alternative energy sources such as fatty acids and amino acids to produce metabolic intermediates channeled to the TCA cycle for ATP production through respiration. As such, cells with normal mitochondria are expected to be less sensitive to agents that inhibit glycolysis.

Recent studies have provided supporting evidence that inhibition of glycolysis may exert preferential effect on cells with compromised mitochondrial function due either to genetic defects or a lack of oxygen. For instance, inhibition of hexokinase by 3-BrPA causes a depletion of ATP in cancer cells, and this effect is

especially severe in cells with mitochondrial DNA deletion and respiration defects, leading to massive cell death (Ko *et al.*, 2001, 2004; Xu *et al.*, 2005b). Interestingly, inhibition of hexokinase also leads to a rapid dephosphorylation of Bcl-2-associated death promoter homolog (BAD), a molecule known to be importantly involved in both glycolysis and apoptosis (Danial *et al.*, 2003). Dephosphorylation of BAD at Ser-112 is associated with re-localization of BAX to mitochondria, cytochrome *c* release, and apoptosis (Xu *et al.*, 2005b). The same study also showed that inhibition of glycolysis effectively kills colon cancer cells (HCT116) and lymphoma cells (Raji) in hypoxic environment, in which cells exhibit high glycolytic activity and a decreased sensitivity to other anticancer agents including taxol, doxorubicine, arsenic trioxide, vincristine, and ara-C. Another glycolytic inhibitor 2-DG also exhibits preferential killing of cancer cells with mitochondrial defects or under hypoxia (Liu *et al*, 2001, 2002). Both 3-BrPA and 2-DG also show effective anticancer activity in animal tumor models (Geschwind *et al.*, 2002; Ko *et al.*, 2004; Maschek *et al*, 2004), suggesting that inhibition of glycolysis is a promising therapeutic strategy and may have broad clinical implications.

In addition, the transketolase-like enzyme TKTL1 has been shown to be overexpressed in cancer cells and may increase the activity of the pentose phosphate pathway, leading to generation of glyceraldehyde-3-phosphate and subsequent production of ATP and lactate (Coy *et al.*, 2005). Thus, inhibition of the transketolase enzyme activity may provide another mechanism to preferentially impact the energy metabolism in cancer cells. The anticancer activity of oxythiamine (an inhibitor of transketolase) observed in animal model provides supporting evidence (Rais *et al.*, 1999). If the increased glucose flow into the pentose phosphate pathway is a significant mechanism contributing to the Warburg effect, inhibitors of this pathway may be useful as potential anticancer agents.

The observations that cancer cells exhibit increased glycolysis and are more dependent on this pathway for ATP generation have led to the evaluation of glycolytic inhibitors as potential anticancer agents. Table 2 lists several compounds that inhibit glycolytic pathway or suppress the pentose phosphate pathway. Their mechanisms of action and therapeutic potential are discussed below.

2-Deoxyglucose

This compound is a glucose analog and has long been known to act as a competitive inhibitor of glucose metabolism (Brown, 1962). Upon transport into the cells, 2-DG is phosphorylated by hexokinase to 2-DG-P. However, unlike G-6-P, 2-DG-P cannot be further metabolized by phosphohexose isomerase, which converts G-6-P to fructose-6-phosphate (Weindruch *et al.*, 2001). 2-Deoxyglucose-P is trapped and accumulated in the cells, leading to inhibition of glycolysis mainly at the step of phosphorylation of glucose by hexokinase. Inhibition of this rate-limiting step by 2-DG causes a depletion of cellular ATP, leading to blockage of cell cycle progression and cell death *in vitro* (Maher *et al.*, 2004). However, the effectiveness of 2-DG is significantly affected by the presence of its natural counterpart glucose and seems to only partially reduce the availability of glucose for glycolysis. 2-Deoxyglucose also affects protein glycosylation, causes aberrant GlcNAcylation of proteins, and induces accumulation of misfolded proteins in the endoplasmic reticulum (ER), leading to ER stress response (Little *et al.*, 1994; Kang and Hwang, 2005). Interestingly, incubation of cells with 2-DG leads to a decrease in the amount of hexokinase associated with mitochondria, suggesting that this compound may also affect the mitochondrial glucose metabolism (Lynch *et al.*, 1991). *In vitro* studies show that 2-DG exhibits cytotoxic effect in cancer cells, especially those with mitochondrial respiratory defects or cells in hypoxic environment (Liu *et al.*, 2001, 2002; Maher *et al*, 2004). *In vivo*, 2-DG significantly enhances the anticancer activity of adriamycin and paclitaxel in mice bearing human osteosarcoma or non-small-cell lung cancer xenografts (Maschek *et al*, 2004). However, the same study showed that administration of 2-DG alone did not exhibit significant anticancer activity *in vivo*. A recent study showed that 2-DG induces the expression of P-glycoprotein encoded by the MDR1 gene, raising a possibility that this might help cancer cells to develop chemoresistance (Ledoux *et al.*, 2003). A clinical trial suggests that 2-DG at the doses up to 250 mg/kg appears safe for use in combination with

Table 2 Glycolytic inhibitors and compounds that modulate glycolytic metabolism

Compound status	Mechanisms of action	Drug development
2-Deoxyglucose	Inhibits phosphorylation of glucose by hexokinase	Clinical trials (I/II)
Lonidamine	Inhibits glycolysis and mitochondrial respiration Inhibits HK; disassociating HK from mitochondria	Clinical trials (II/III)
3-Bromopyruvate	Inhibits HK; acts as an alkylating agent	Pre-clinical
Imatinib	Inhibit Bcr-Abl tyrosine kinase; causes a decrease in HK and G6PD activity	Approved for clinical use
Oxythiamine	Suppresses PPP by inhibiting transketolase; inhibits pyruvate dehydrogenase	Pre-clinical

Abbreviations: HK, hexokinase; G6PG, glucose-6-phosphate dehydrogenase; PPP, pentose phosphate pathway.

radiation therapy in patients with glioblastoma multiforme (Singh et al., 2005).

Lonidamine

This compound is a derivative of indazole-3-carboxylic acid, and has been known for a long time to inhibit aerobic glycolysis in cancer cells (Floridi et al., 1981). In cell culture, lonidamine decreases oxygen consumption in both normal and neoplastic cells. Interestingly, it seems to enhance aerobic glycolysis in normal cells, but suppresses glycolysis in cancer cells, likely through inhibition of the mitochondrially bound hexokinase (Floridi et al., 1981). Importantly, in vivo administration of lonidamine to a patient with B-cell chronic leukemia resulted in a decrease of lactate production comparable to that observed in vitro (Natali et al., 1984). Subsequent studies in Ehrlich ascites tumor cells showed that lonidamine inhibits both respiration and glycolysis in a dose-dependent manner leading to a decrease in cellular ATP (Floridi et al., 1998). The same study also showed that this compound causes an increase in the intracellular content of doxorubicin in both doxorubicin-resistant and sensitive cells owing to reduced ATP availability. In human breast cancer MCF-7 cells, lonidamine enhances the cytotoxicity of several alkylating agents, including cisplatin, 4-hydroperoxycyclophosphamide, melphalan, and BCNU (Rosbe et al., 1989). The proven ability of lonidamine to inhibit energy metabolism in cancer cells and to enhance the activity of other anticancer agents has led to clinical trials (phase II–III) of this compound in combination with other anticancer agents for the treatment of breast cancer, glioblastoma multiforme, ovarian cancer, and lung cancer (De Lena et al., 2001; Di Cosimo et al., 2003; Oudard et al., 2003; Papaldo et al., 2003). Lonidamine (also known as TH-070) is also currently in phase II/III clinical trials for treatment of benign prostatic hyperplasia (BPH), given by oral drug administration.

3-Bromopyruvate

This compound is an inhibitor of hexokinase and has been shown to abolish ATP production and cause severe depletion of cellular ATP (Ko et al., 2001; Geschwind et al., 2004; Xu et al, 2005b). Like 2-DG, 3-BrPA also exhibits potent cytotoxic activity against cancer cells with mitochondrial respiratory defects and cells in hypoxic environment (Xu et al., 2005b). Associated with ATP depletion, 3-BrPA causes a rapid dephosphorylation of BAD at Ser_{112}, re-localization of BAX to mitochondria, release of cytochrome c, leading to massive cell death. Interestingly, depletion of ATP by 3-BrPA also effectively induces apoptosis in multi-drug-resistant cells, suggesting that deprivation of cellular energy supply may be a novel way to overcome multidrug resistance (Xu et al., 2005b). 3-Bromopyruvate is effective at the concentration of $100 \mu M$, which is more potent than 2-DG (effective in mM range). Combination of 3-BrPA with mTOR inhibitor seems to have synergistic effects on leukemia and lymphoma cells (Xu et al., 2005a). It should be noted that 3-BrPA is an alkylating agent, which may also interact with other molecules in the cells. Thus, its cytotoxic activity may not be exclusively attributed to inhibition of hexokinase. Animal studies showed that 3-BrPA has significant in vivo therapeutic activity against liver cancer when the compound was given by local infusion, and seems to inhibit metastasis when given intravenously (Geschwind et al., 2002; Ko et al., 2004). The significant anticancer activity of 3-BrPA warrants further evaluation for potential use in cancer treatment.

Imatinib (Gleevec)

This compound is a tyrosine kinase inhibitor designed to specifically target BCR-ABL, which is responsible for the development of chronic myeloid leukemia (CML). The Bcr-Abl oncogene is a fusion DNA sequence created by chromosome translocation and codes for a constitutively active tyrosine kinase fusion protein. The BCR-ABL-positive cells express the high-affinity glucose transporter (GLUT-1) and exhibit increased glucose uptake. Imatinib treatment decreased the activity of both hexokinase and glucose-6-phosphate dehydrogenase (G6PD) in leukemia cells, leading to suppression of aerobic glycolysis (Boren et al., 2001; Gottschalk et al., 2004; Serkova and Boros, 2005). A decrease in G6PD activity would lead to lower glucose flow into the pentose phosphate pathway, and thus deprives transformed cells of metabolic intermediates for ATP generation and substrates for macromolecule synthesis. Although imatinib is an antileukemia drug, its ability to suppress aerobic glycolysis may make it useful for the treatment of certain solid tumors.

Oxythiamine

This compound is a thiamine antagonist and inhibits transketolase and pyruvate dehydrogenase, which require thiamine pyrophosphate (TPP) as a cofactor for their enzyme activity. Early studies suggest that oxythiamine is phosphorylated to yield diphosphate ester which then acts as a strong competitive inhibitor ($K_i = 0.07 \mu M$) against the normal cofactor TPP ($K_m = 0.11 \mu M$) when highly purified pyruvate dehydrogenase was used (Strumilo et al., 1984). As transketolase is a crucial enzyme of the pentose phosphate pathway, inhibition of this enzyme would cause a suppression of the pentose phosphate pathway and thus deprives cells of the metabolic intermediate (glyceraldehyde-3-phosphate) for ATP generation and of the substrates (NADPH, ribose-phosphate) for macromolecule synthesis. This metabolic inhibition seems to be responsible, at least in part, for the significant anticancer activity observed in vitro and in vivo (Rais et al., 1999; Comin-Anduix et al., 2001). Because one of the transketolase isozyme TKTL1 has recently been found to be highly expressed in cancer cells and is considered as an important factor contributing to the Warburg effect (Coy et al., 2005), it would be of interest to explore the possibility to inhibit this enzyme as a potential anticancer strategy.

6-aminonicotinamide

The pentose phosphate pathway can also be inhibited by 6-aminonicotinamide. This compound is believed to inhibit glucose-6-phosphate dehydrogenase (G6PD), which catalyses the conversion of G-6-P 6-phosphoglu-conolactone, the first step of the pentose phosphate pathway. 6-aminonicotinamide (6-AN) has been widely used as a chemical tool in various experimental systems to study the biological consequences of inhibiting pentose phosphate pathway. Because of the essential roles of this pathway in generating reducing power (NADPH) and important metabolic intermediates (pentose-5-phosphate) for synthesis of macromolecules, it is not surprising that 6-AN exhibits anticancer activity *in vitro*, causes oxidative stress, and sensitizes cells to anticancer agents and radiation (Budihardjo et al., 1998; Varshney et al, 2003, 2005). However, 6-AN also causes neurotoxicity and other toxic side effects (Kim and Wenger, 1973; Bolin and Carlton, 1996; Penkowa et al., 2004).

Several other compounds are potentially useful to modulate glucose metabolism. *Genistein* is a natural compound found in soybean, and has been shown to decreases glucose uptake and glucose carbon incorporation into nucleic acid ribose in pancreatic adenocarcinoma cells (Boros et al., 2001). It also has inhibitory effect on tyrosine kinase and protein kinase (El-Zarruk and van den Berg, 1999; Waltron and Rozengurt, 2000), causes cell cycle arrest, and suppresses angiogenesis (Lian et al., 1998; Zhou et al., 1999). Genistein seems potentially useful as a chemosensitization and radio-sensitization agent (Garg et al., 2005). *5-Thioglucose* (5-TG) is an analog of glucose and has an inhibitory effect on glucose uptake and hexokinase. Inhibition of glycolysis by 5-TG occurs rapidly, and is competitive with respect to glucose. *Mannoheptulose* is another non-metabolizable glucose analog with anticancer effect. This compound was shown to inhibit glucokinase, decrease glucose uptake, and suppress tumor cell growth (Board et al., 1995; Xu et al., 1995). *α-Chlorohydrin* inhibits GAPDH, causing an increase in the cellular fructose-1,6-bisphosphate and triosephosphates, and a depletion of ATP. This compound has been shown to have antifertility effect due to its ability to affect energy metabolism in sperm (Jelks and Miller, 2001), although recent evidence suggest that sperm can remain motile with normal ATP concentrations despite inhibition of GAPDH by this compound (Ford, 2006). *Ornidazole* also inhibit GAPDH and triosephosphate isomerase. The conversion of ornidazole to 3-chlorolactate in rats suggests an action similar to that of α-chlorohydrin (Jones and Cooper, 1997). *Oxalate* was considered as an *in vitro* inhibitor of lactate dehydrogenase, monophosphoglycerate mutase, and pyruvate kinase, but studies in red cells suggest that the site of oxalate action is at the reaction catalysed by pyruvate kinase, and the apparent inhibition of the glyceraldehyde phosphate dehydrogenase step is due to an increase in the NADH/NAD ratio (Beutler et al., 1997). The pentavalent *arsenic compounds* can abolish ATP generation by causing arsenolysis during the GAPDH-catalysing reaction in the glycolytic

pathway (Figure 1, step 6), preventing the generation of 1,3-bisphosphoglycerate, although the GAPDH activity is not directly inhibited. *Glufosfamide* is a conjugate of D-glucose with the active metabolite of isophosphoramide mustard. This novel compound utilizes the elevated glucose uptake of tumor cells expressing the SAAT1 glucose transporter for entering the cells (Veyhl et al., 1998). Glufosfamide does not require metabolic activation in the liver and the active moiety is released upon entry into tumour cells. This compound has been tested for its therapeutic activity against head and neck cancer, pancreatic adenocarcinoma, and non-small-cell lung cancer (Briasoulis et al., 2003; Dollner et al., 2004; Giaccone et al., 2004). Glufosfamide represent a novel oxazaphosphorine analog that uses the glucose transporter system for cellular entry to damage nuclear DNA (Seker et al., 2000).

Combination of glycolytic inhibition and other anticancer agents

Although cancer cells exhibit increased glycolysis and depend more on this pathway for ATP generation, inhibition of glycolysis alone may not be sufficient to effectively kill the malignant cells. It has been suggested that ATP depletion should reach certain thresholds in order to trigger cell death by apoptosis or necrosis processes, with a depletion of 25–70% ATP leading to apoptosis, and an over 85% ATP depletion causing necrosis (Lieberthal et al., 1998). Since all cancer cells contain mitochondria, some degree of ATP generation through oxidative phosphorylation is still possible when glycolysis is inhibited. This may compromise the efficiency of glycolytic inhibitors to deplete cellular ATP. One way to achieve a high level of ATP depletion and improve therapeutic activity is to combine multiple ATP-depleting agents with different mechanisms of action (Martin et al., 2001). Indeed, early studies showed that the combination of N-(phosphonacetyl)-L-aspartate (PALA), 6-methylmercaptopurine riboside (MMPR), and 6-aminonicotinamide (6-AN) is an effective ATP-depleting regimen that increases the anticancer activity of radiation, adriamycin, or taxol (Koutcher et al., 1993; Martin et al., 1994, 1996). Combination of glycolytic inhibitor 2-deoxyglucose with adriamycin or paclitaxel also resulted in a significant increase of *in vivo* therapeutic activity in animal tumor models bearing osteosarcoma or non-small-cell lung cancer xenografts (Maschek et al., 2004).

Interestingly, a recent study showed that cells using aerobic glycolysis to support their bioenergetics undergo rapid ATP depletion and necrotic cell death in response to activation of poly(ADP-ribose) polymerase (PARP) when treated with DNA-alkylating agents (Zong et al., 2004). Activation of PARP by DNA-damaging agents leads to a rapid consumption of NAD^+, which is a co-factor necessary for one of the glycolytic reactions (reaction # 6, Figure 1). Thus, any DNA damage that activates PARP would indirectly inhibit glycolysis

through depletion of NAD$^+$. Furthermore, since repair of DNA require ATP as the energy source, inhibition of ATP generation by suppression of glycolytic pathway would severely compromise cellular ability to repair DNA damage. Thus, combination of glycolytic inhibitors and DNA-damaging agents seems to be an attractive therapeutic strategy to effectively kill cancer cells.

Combination of inhibitors of glycolytic pathway and pentose phosphate pathway has also been tested for their ability to cause radiosensitization (Varshney et al., 2005). Combination of 2-DG and 6-AN (an inhibitor of G6PD that catalyses the rate-limiting step of the pentose pathway) caused a profound decrease in the cellular glutathione content and enhanced radiation damage, leading to mitotic and apoptotic cell death (Varshney et al., 2005). Based on earlier studies showing that 2-DG could enhance the efficacy of radiotherapy in experimental models, Singh et al. (2005) conducted a clinical trial to examine the tolerance and safety of escalating 2-DG dose in combination with radiotherapy in glioblastoma multiforme patients. Their study showed that administration of 2-DG at the doses up to 250 mg/kg in combination with fractions of radiation (5 Gy/fraction/week) is safe and could be tolerated in glioblastoma patients without acute toxicity and late radiation damage to the brain. The authors suggest that further clinical studies to evaluate the efficacy of this combined treatment are warranted.

Summary and future perspectives

Cancer cell commonly exhibit increased aerobic glycolysis. This biological adaptation to metabolic changes owing to mitochondrial dysfunction, hypoxia, and oncogenic signals renders the malignant cells addictive to glycolysis and dependent on this pathway for ATP generation. These alterations in energy metabolism and the associated increased expression of glycolytic enzymes and other pro-survival molecules provide a survival advantage for the cancer cells. Furthermore, the acidic tumor microenvironment associated with accumulation of lactate owing to increased glycolysis provides a tissue environment for selection of cancer cells with high survival capacity and malignant behaviors. These biological alterations present a major challenge in cancer treatment, as exemplified by the facts that cancer cells in hypoxic environment become resistance to chemotherapeutic agents and radiation therapy. However, the increased dependency of cancer cells on glycolysis for energy generation also provides a biochemical basis to preferentially kill the malignant cells by inhibition of glycolysis. Recent studies have provided compelling evidence showing that cancer cells with mitochondrial defects or under hypoxia are highly sensitive to glycolysis inhibition. Several glycolytic inhibitors have been shown to have promising anticancer activity in vitro and in vivo, and some of them have entered clinical trials.

It should be recognized, however, that there are potential concerns and challenges in using glycolytic inhibitors for cancer treatment. It is known that certain normal tissues including brain, retinae, and testis also use glucose as the main energy source. Inhibition of glycolysis may be potentially toxic to these tissues. It is unclear whether these normal tissues can effectively use alternative energy sources (fatty acids, amino acids, etc.) to generate sufficient ATP through mitochondrial metabolism to support their cellular function when the glycolytic pathway is inhibited during therapy. A recent clinical trial suggested that the use of 2-DG at the doses up to 250 mg/kg is safe (Singh et al., 2005). One way to minimize the potential neurotoxicity is to develop glycolytic inhibitors that do not cross the blood–brain barrier. Another potential problem is that the glycolytic inhibitors currently available are not very potent, and high concentrations are required. 3-Bromopyruvate is not stable in solution. Thus, development of new generations of glycolytic inhibitors with high potency, stability, and good safety profiles represent an important research area in this field. It is also important to evaluate the combination effects of glycolytic inhibitors and other therapeutic modalities such as chemotherapeutic agents and radiation, and develop optimal combination regimens for effective treatment of cancer.

References

Arora KK, Fanciulli M, Pedersen PL. (1990). J Biol Chem **265**: 6481–6488.

Astuti D, Douglas F, Lennard TW, Aligianis IA, Woodward ER, Evans DG et al. (2001). Lancet **357**: 1181–1182.

Baumann M, Brand K. (1988). Cancer Res **48**: 7018–7021.

Baumann M, Kappl A, Lang T, Brand K, Siegfried W, Paterok E. (1990). Cancer Investig **8**: 351–356.

Bayley JP, Devilee P, Taschner PE. (2005). BMC Med Genet **6**: 39–44.

Beutler E, West C, Britton HA, Harris J, Forman L. (1997). Blood Cells Mol Dis **23**: 402–409.

Biaglow JE, Cerniglia G, Tuttle S, Bakanauskas V, Stevens C, McKenna G. (1997). Biochem Biophys Res Commun **235**: 739–742.

Blum R, Jacob-Hirsch J, Amariglio N, Rechavi G, Kloog Y. (2005). Cancer Res **65**: 999–1006.

Board M, Colquhoun A, Newsholme E, High A. (1995). Cancer Res **55**: 3278–3285.

Bolin DC, Carlton WW. (1996). Vet Hum Toxicol **38**: 85–88.

Boren J, Cascante M, Marin S, Comín-Anduix B, Centelles JJ, Lim S et al. (2001). J Biol Chem **276**: 37747–37753.

Bork P, Sander C, Valencia A. (1993). Prot Sci **2**: 31–40.

Boros LG, Bassilian S, Lim S, Lee WN. (2001). *Pancreas* **22**: 1–7.

Brahimi-Horn MC, Pouyssegur J. (2005). *Int Rev Cytol* **242**: 157–213.

Briasoulis E, Pavlidis N, Terret C, Bauer J, Fiedler W, Schoffski P et al. (2003). *Eur J Cancer* **39**: 2334–2340.

Brown J. (1962). *Metabolism* **11**: 1098–1112.

Bryson JM, Coy PE, Gottlob K, Hay N, Robey RB. (2002). *J Biol Chem* **277**: 11392–11400.

Budihardjo II, Walker DL, Svingen PA, Buckwalter CA, Desnoyers S, Eckdahl S et al. (1998). *Clin Cancer Res* **4**: 117–130.

Burgering BM, Coffer PJ. (1995). *Nature* **376**: 599–602.

Bustamante E, Pedersen PL. (1977). *Proc Natl Acad Sci USA* **74**: 3735–3739.

Cárdenas ML, Cornish-Bowden A, Ureta T. (1998). *Biochim Biophys Acta* **1401**: 242–264.

Carew JS, Huang P. (2002). *Mol Cancer* **1**: 9–20.

Carew JS, Zhou Y, Albitar M, Carew JD, Keating MJ, Huang P. (2003). *Leukemia* **17**: 1437–1447.

Cattaneo A, Biocca S, Corvaja N, Calissano P. (1985). *Exp Cell Res* **161**: 130–140.

Chen JZ, Kadlubar FF. (2004). *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **22**: 1–12.

Chuang DM, Hough C, Senatorov VV. (2005). *Annu Rev Pharmacol Toxicol* **45**: 269–290.

Comin-Anduix B, Boren J, Martinez S, Moro C, Centelles JJ, Trebukhina R et al. (2001). *Eur J Biochem* **268**: 4177–4182.

Coy JF, Dressler D, Wilde J, Schubert P. (2005). *Clin Lab* **51**: 257–273.

Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM et al. (2003). *Nature* **424**: 952–956.

Dastoor Z, Dreyer JL. (2001). *J Cell Sci* **114**: 1643–1653.

De Lena M, Lorusso V, Latorre A, Fanizza G, Gargano G, Caporusso L et al. (2001). *Eur J Cancer* **37**: 364–368.

Deprez J, Vertommen D, Alessi DR, Hue L, Rider MH. (1997). *J Biol Chem* **272**: 17269–17275.

Di Cosimo S, Ferretti G, Papaldo P, Carlini P, Fabi A, Cognetti F. (2003). *Drugs Today (Barc)* **39**: 157–174.

Dollner R, Dietz A, Kopun M, Helbig M, Wallner F, Granzow C. (2004). *Anticancer Res* **24**: 2947–2951.

Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, et al. (2004). *Cancer Res* **64**: 3892–3899.

El-Zarruk AA, van den Berg HW. (1999). *Cancer Lett* **142**: 185–193.

Feo S, Arcuri D, Piddini E, Passantino R, Giallongo A. (2000). *FEBS Lett* **473**: 47–52.

Filella X, Molina R, Jo J, Mas E, Ballesta AM. (1991). *Tumour Biol* **12**: 360–367.

Flier JS, Mueckler MM, Usher P, Lodish HF. (1987). *Science* **235**: 1492–1495.

Floridi A, Bruno T, Miccadei S, Fanciulli M, Federico A, Paggi MG. (1998). *Biochem Pharmacol* **56**: 841–849.

Floridi A, Paggi MG, Marcante ML, Silvestrini B, Caputo A, De Martino C. (1981). *J Natl Cancer Inst* **66**: 497–499.

Ford WC. (2006). *Hum Reprod Update* **12**: 269–274.

Funasaka T, Haga A, Raz A, Nagase H. (2001). *Biochem Biophys Res Commun* **285**: 118–128.

Funasaka T, Haga A, Raz A, Nagase H. (2002). *Int J Cancer* **101**: 217–223.

Garg AK, Buchholz TA, Aggarwal BB. (2005). *Antioxid Redox Signal* **7**: 1630–1647.

Gatenby RA, Gillies RJ. (2004). *Nat Rev Cancer* **4**: 891–899.

Geschwind JF, Georgiades CS, Ko YH, Pedersen PL. (2004). *Expert Rev Anticancer Ther* **4**: 449–457.

Geschwind JF, Ko YH, Torbenson MS, Magee C, Pedersen PL. (2002). *Cancer Res* **62**: 3909–3913.

Giaccone G, Smit EF, de Jonge M, Dansin E, Briasoulis E, Ardizzoni A et al. (2004). *Eur J Cancer* **40**: 667–672.

Golshani-Hebroni SG, Bessman SP. (1997). *J Bioenerg Biomembr* **29**: 331–338.

Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. (2001). *Genes Dev* **15**: 1406–1418.

Gottschalk S, Anderson N, Hainz C, Eckhardt SG, Serkova NJ. (2004). *Clin Cancer Res* **10**: 6661–6668.

Grosse F, Nasheuer HP, Scholtissek S, Schomburg U. (1986). *Eur J Biochem* **160**: 459–467.

Harrison RA. (1974). *Anal Biochem* **61**: 500–507.

Herrero P, Galindez J, Ruiz N, Martinez-Campa C, Moreno F. (1995). *Yeast* **11**: 137–144.

Hirono Y, Fushida S, Yonemura Y, Yamamoto H, Watanabe H, Raz A. (1996). *Br J Cancer* **74**: 2003–2007.

Ishitani R, Chuang DM. (1996). *Proc Natl Acad Sci USA* **93**: 9937–9941.

Izyumov DS, Avetisyan AV, Pletjushkina OY, Sakharov DV, Wirtz KW, Chernyak BV et al. (2004). *Biochim Biophys Acta* **1658**: 141–147.

Jelks KB, Miller MG. (2001). *Toxicol Sci* **62**: 115–123.

Jones AR, Cooper TG. (1997). *Xenobiotica* **27**: 711–721.

Kang HT, Hwang ES. (2005). *Life Sci* **78**: 1392–1399.

Karnauskas R, Niu Q, Talapatra S, Plas DR, Greene ME, Crispino JD et al. (2003). *Oncogene* **22**: 688–698.

Kim SU, Wenger BS. (1973). *Acta Neuropathol (Berl)* **26**: 259–264.

Ko YH, Pedersen PL, Geschwind JF. (2001). *Cancer Lett* **173**: 83–91.

Ko YH, Smith BL, Wang Y, Pomper MG, Rini DA, Torbenson MS et al. (2004). *Biochem Biophys Res Commun* **324**: 269–275.

Kohn AD, Summers SA, Birnbaum MJ, Roth RA. (1996). *J Biol Chem* **271**: 31372–31378.

Koutcher JA, Alfieri AA, Stolfi RL, Devitt ML, Colofiore JR, Nord LD et al. (1993). *Cancer Res* **53**: 3518–3523.

Layzer RB, Rowland LP, Bank WJ. (1969). *J Biol Chem* **244**: 3823–3831.

Ledoux S, Yang R, Friedlander G, Laouari D. (2003). *Cancer Res* **63**: 7284–7290.

Li C, Xiao Z, Chen Z, Zhang X, Li J, Wu X et al. (2006). *Proteomics* **6**: 547–558.

Lian F, Bhuiyan M, Li YW, Wall N, Kraut M, Sarkar FH. (1998). *Nutr Cancer* **31**: 184–191.

Lieberthal W, Menza SA, Levine JS. (1998). *Am J Physiol Renal Physiol* **274**: F315–F327.

Liotta LA, Mandler R, Murano G, Katz DA, Gordon RK, Chiang PK et al. (1986). *Proc Natl Acad Sci USA* **83**: 3302–3306.

Little E, Ramakrishnan M, Roy B, Gazit G, Lee AS. (1994). *Crit Rev Eukaryotic Gene Expression* **4**: 1–18.

Liu H, Hu YP, Savaraj N, Priebe W, Lampidis TJ. (2001). *Biochemistry* **40**: 5542–5547.

Liu H, Savaraj N, Priebe W, Lampidis TJ. (2002). *Biochem Pharmacol* **64**: 1745–1751.

Lynch RM, Fogarty KE, Fay FS. (1991). *J Cell Biol* **112**: 385–395.

Maher JC, Krishan A, Lampidis TJ. (2004). *Cancer Chemother Pharmacol* **53**: 116–122.

Majewski N, Nogueira V, Robey RB, Hay N. (2004). *Mol Cell Biol* **24**: 730–740.

Marangos PJ, Parma AM, Goodwin FK. (1978). *J Neurochem* **31**: 727–732.

Martin DS, Spriggs D, Koutcher JA. (2001). *Apoptosis* **6**: 125–131.

Martin DS, Stolfi RL, Colofiore JR, Nord LD. (1996). *Anticancer Drugs* **7**: 655–659.

Martin DS, Stolfi RL, Colofiore JR, Nord LD, Sternberg S. (1994). *Cancer Invest* **12**: 296–307.

Maschek G, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF et al. (2004). *Cancer Res* **64**: 31–34.

Mathupala SP, Heese C, Pedersen PL. (1997a). *J Biol Chem* **272**: 22776–22780.

Mathupala SP, Rempel A, Pedersen PL. (1997b). *J Bioenerg Biomembr* **29**: 339–343.

Mazurek S, Boschek CB, Hugo F, Eigenbrodt E. (2005). *Semin Cancer Biol* **15**: 300–308.

Munoz-Pinedo C, Ruiz-Ruiz C, Ruiz de Almodovar C, Palacios C, Lopez-Rivas A. (2003). *J Biol Chem* **278**: 12759–12768.

Nabi IR, Watanabe H, Raz A. (1990). *Cancer Res* **50**: 409–414.

Nagy E, Henics T, Eckert M, Miseta A, Lightowlers RN, Kellermayer M. (2000). *Biochem Biophys Res Commun* **275**: 253–260.

Natali PG, Salsano F, Viora M, Nista A, Malorni W, Marolla A et al. (1984). *Oncology* **41**: 7–14.

Neumann HP, Pawlu C, Peczkowska M, Bausch B, McWhinney SR, Muresan M, et al., European-American Paraganglioma Study Group. (2004). *J Am Med Assoc* **292**: 943–951.

Niederacher D, Entian KD. (1991). *Eur J Biochem* **200**: 311–319.

Niinaka Y, Paku S, Haga A, Watanabe H, Raz A. (1998). *Cancer Res* **58**: 2667–2674.

Niizeki H, Kobayashi M, Horiuchi I, Akakura N, Chen J, Wang J et al. (2002). *Br J Cancer* **86**: 1914–1919.

Oudard S, Carpentier A, Banu E, Fauchon F, Celerier D, Poupon MF et al. (2003). *J Neurooncol* **63**: 81–86.

Papaldo P, Lopez M, Cortesi E, Cammilluzzi E, Antimi M, Terzoli E et al. (2003). *J Clin Oncol* **21**: 3462–3468.

Pastorino JG, Hoek JB. (2003). *Curr Med Chem* **10**: 1535–1551.

Pastorino JG, Shulga N, Hoek JB. (2002). *J Biol Chem* **277**: 7610–7618.

Pawlu C, Bausch B, Hartmut PH. (2005). *Familial Cancer* **4**: 49–54.

Pelicano H, Carney D, Huang P. (2004). *Drug Resist Update* **7**: 97–110.

Penkowa M, Quintana A, Carrasco J, Giralt M, Molinero A, Hidalgo J. (2004). *J Neurosci Res* **77**: 35–53.

Perucho M, Salas J, Salas ML. (1980). *Biochim Biophys Acta* **606**: 181–195.

Pirinen E, Heikkinen S, Malkki M, Deeb SS, Janne J, Laakso M. (2004). *Biochim Biophys Acta* **1676**: 149–154.

Pollard PJ, Wortham NC, Tomlinson IP. (2003). *Ann Med* **35**: 632–639.

Rais B, Comin B, Puigjaner J, Brandes JL, Creppy E, Saboureau D et al. (1999). *FEBS Lett* **456**: 113–118.

Ramanathan A, Wang C, Schreiber SL. (2005). *Proc Natl Acad Sci USA* **102**: 5992–5997.

Rathmell JC, Fox CJ, Plas DR, Hammerman PS, Cinalli RM, Thompson CB. (2003). *Mol Cell Biol* **23**: 7315–7328.

Rempel A, Mathupala SP, Griffin CA, Hawkins AL, Pedersen PL. (1996). *Cancer Res* **56**: 2468–2471.

Rodriguez A, De La Cera T, Herrero P, Moreno F. (2001). *Biochem J* **355**: 625–631.

Rosbe KW, Brann TW, Holden SA, Teicher BA, Frei III E. (1989). *Cancer Chemother Pharmacol* **25**: 32–36.

Ruderman NB, Kapeller R, White MF, Cantley LC. (1990). *Proc Natl Acad Sci USA* **87**: 1411–1415.

Seker H, Bertram B, Burkle A, Kaina B, Pohl J, Koepsell H et al. (2000). *Br J Cancer* **82**: 629–634.

Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD et al. (2005). *Cancer Cell* **7**: 77–85.

Semenza GL, Artemov D, Bedi A, Bhujwalla Z, Chiles K, Feldser D et al. (2001). *Novartis Found Symp* **240**: 251260.

Serkova N, Boros LG. (2005). *Am J Pharmacogenomics* **5**: 293–302.

Serkova N, Boros LG. (2005). *Am J Pharmacogenomics* **5**: 293–302.

Shashidharan P, Chalmers-Redman RM, Carlile GW, Rodic V, Gurvich N, Yuen T et al. (1999). *NeuroReport* **10**: 1149–1153.

Singh D, Banerji AK, Dwarakanath BS, Tripathi RP, Gupta JP, Mathew TL et al. (2005). *Strahlenther Onkol* **181**: 507–514.

Singh KK. (2004). *Ann NY Acad Sci* **1019**: 260–264.

Sirover MA. (2005). *J Cell Biochem* **95**: 45–52.

Strumilo SA, Senkevich SB, Vinogradov VV. (1984). *Biomed Biochim Acta* **43**: 159–163.

Sun YJ, Chou CC, Chen WS, Wu RT, Meng M, Hsiao CD. (1999). *Proc Natl Acad Sci USA* **96**: 5412–5417.

Taguchi K, Takagi Y. (2001). *Rinsh Byori* **116**: 117–124.

Tajima H, Tsuchiya K, Yamada M, Kondo K, Katsube N, Ishitani R. (1999). *Neuro Report* **10**: 2029–2033.

Taylor RW, Turnbull DM. (2005). *Nat Rev Genet* **6**: 389–402.

Tretter L, Adam-Vizi V. (2005). *Philos Trans R Soc Lond B Biol Sci* **360**: 2335–2345.

VandeBerg JL. (1985). *Isozymes Curr Top Biol Med Res* **12**: 133–187.

Van Schaftingen E, Hers HG. (1986). *Eur J Biochem* **159**: 359–365.

Varshney R, Adhikari JS, Dwarakanath BS. (2003). *Indian J Exp Biol* **41**: 1384–1391.

Varshney R, Dwarakanath B, Jain V. (2005). *Int J Radiat Biol* **81**: 397–408.

Veyhl M, Wagner K, Volk C, Gorboulev V, Baumgarten K, Weber WM et al. (1998). *Proc Natl Acad Sci USA* **95**: 2914–2919.

Vora S. (1983). *Isozymes Curr Top Biol Med Res* **11**: 3–23.

Waltron RT, Rozengurt E. (2000). *J Biol Chem* **275**: 17114–17121.

Warburg O. (1930). *The Metabolism of Tumors*. Costable: London.

Warburg O. (1956). *Science* **123**: 309–314.

Warburg O, Posener K, Negelein E. (1924). *Biochem Z* **152**: 309–344.

Watanabe H, Takehana K, Date M, Shinozaki T, Raz A. (1996). *Cancer Res* **56**: 2960–2963.

Weindruch R, Keenan KP, Carney JM, Fernandes G, Feuers RJ, Floyd RA et al. (2001). *J Gerontol A Biol Sci Med Sci* **56**: 20–33.

Williams KR, Reddigari S, Patel GL. (1985). *Proc Natl Acad Sci USA* **82**: 5260–5264.

Wilson JE. (1995). *Rev Physiol Biochem Pharmacol* **126**: 65–198.

Wilson JE. (2003). *J Exp Biol* **206**: 2049–2057.

Xu LZ, Weber IT, Harrison RW, Gidh-Jain M, Pilkis SJ. (1995). *Biochemistry* **34**: 6083–6092.

Xu RH, Pelicano H, Zhang H, Giles FJ, Keating MJ, Huang P. (2005a). *Leukemia* **19**: 2153–2158.

Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN
  et al. (2005b). *Cancer Res* **65**: 613–621.
Yoon DY, Buchler P, Saarikoski ST, Hines OJ, Reber HA,
  Hankinson O. (2001). *Biochem Biophys Res Commun* **288**:
  882–886.
Zhao YB, Yang HY, Zhang XW, Chen GY. (2005). *World J
  Gastroenterol* **11**: 3304–3306.
Zheng L, Roeder RG, Luo Y. (2003). *Cell* **114**: 255–266.

Zhou JR, Gugger ET, Tanaka T, Guo Y, Blackburn GL,
  Clinton SK. (1999). *J Nutr* **129**: 1628–1635.
Zhu W, Qin W, Bradley P, Wessel A, Puckett CL, Sauter ER.
  (2005). *Carcinogenesis* **26**: 145–152.
Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson
  CB. (2004). *Genes Dev* **18**: 1272–1282.
Zu XL, Guppy M. (2004). *Biochem Biophys Res Commun* **313**:
  459–465.