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REVIEW

Sugar-free approaches to cancer cell killing

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Tumors show an increased rate of glucose uptake and utilization. For this reason, glucose analogs are used to visualize tumors by the positron emission tomography technique, and inhibitors of glycolytic metabolism are being tested in clinical trials. Upregulation of glycolysis confers several advantages to tumor cells: it promotes tumor growth and has also been shown to interfere with cell death at multiple levels. Enforcement of glycolysis inhibits apoptosis induced by cytokine deprivation. Conversely, antiglycolytic agents enhance cell death induced by radio- and chemotherapy. Synergistic effects are likely due to regulation of the apoptotic machinery, as glucose regulates activation and levels of proapoptotic BH3-only proteins such as Bim, Bad, Puma and Noxa, as well as the antiapoptotic Bcl-2 family of proteins. Moreover, inhibition of glucose metabolism sensitizes cells to death ligands. Glucose deprivation and antiglycolytic drugs induce tumor cell death, which can proceed through necrosis or through mitochondrial or caspase-8-mediated apoptosis. We will discuss how oncogenic pathways involved in metabolic stress signaling, such as p53, AMPK (adenosine monophosphate-activated protein kinase) and Akt/mTOR (mammalian target of rapamycin), influence sensitivity to inhibition of glucose metabolism. Finally, we will analyze the rationale for the use of antiglycolytic inhibitors in the clinic, either as single agents or as a part of combination therapies.
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Metabolic transformation: cancer's friend and foe

Tumors have a special metabolism. Among the many differences with non-transformed tissues, perhaps the most relevant is that they rely on glucose as a source of energy and carbon. Tumors seek glucose so avidly that they can be visualized by the positron emission tomography technique using a glucose analog as a tracer. It has been known for decades that cancer cells show an increased uptake of glucose and utilization of glycolysis instead of respiration, even in the presence of oxygen ('the Warburg Effect'). It was thought that the glycolytic switch was caused by defects in mitochondrial respiration due to mutations in mitochondrial DNA. Currently, however, the prevailing view is that tumor cells are dependent on glucose because they use glucose to produce metabolites required to grow; in particular, nucleic acids and fatty acids (DeBerardinis *et al*., 2008; Vander Heiden *et al*., 2009). A high rate of glucose utilization is not exclusive of tumor cells, and other highly proliferative cells such as T lymphocytes also switch to aerobic glycolysis when stimulated to proliferate (Michalek and Rathmell, 2010). Alterations in metabolism allow cells to grow faster than if they used most of the glucose to produce adenosine monophosphate (ATP) through respiration.

The switch to glycolysis is promoted by signaling pathways that stimulate proliferation, which are frequently disregulated in tumors. 'Metabolic transformation' is thus linked to oncogenic transformation, as it is driven by oncogenes. A few examples are cited as follows: oncogenic Ras or activation of the PI3K/Akt pathway promotes glycolysis. Akt stimulates membrane localization of glucose transporters and enhances transcription or activity of glycolytic enzymes such as hexokinase and phosphofructokinase (Gottlob *et al*., 2001; Rathmell *et al*., 2003). K-Ras promotes transcription of several glycolytic enzymes (Chiaradonna *et al*., 2006). Conversely, tumor suppressors such as PTEN (inhibitor of the PI3K/Akt pathway) or p53 downregulate glycolysis. Activation of p53 alters the balance between glycolysis and oxidative phosphorylation through several mechanisms. p53 slows down glycolysis by downregulating the expression of glucose transporters and upregulating fructose-2,6-bisphosphatase TIGAR (TP53-induced glycolysis and apoptosis regulator), and it promotes oxidative phosphorylation by upregulating SCO2, a protein required for the function of the respiratory chain (Vousden and Ryan, 2009). Therefore, inactivation of p53 could be partially responsible for the increased aerobic glycolysis in transformed cells.

'Metabolic transformation' involves the regulation of many metabolic pathways. Indeed, glucose is not the only molecule that tumor cells require to grow. For instance, oncogenic *myc* promotes the use of the amino acid glutamine, which cells can use to produce not only proteins but also ATP and nucleic acids (DeBerardinis *et al*., 2007; Gao *et al*., 2009). Growing cells require to synthesize new lipids, nucleic acids and proteins, which

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means that inhibition of many metabolic pathways, such as fatty acid synthesis or nucleotide synthesis, could promote tumor cell death. Indeed, drugs that interfere with nucleic acid metabolism, such as methotrexate or 5-FU, have been used for decades to treat tumors. We could thus interfere with tumor metabolism at multiple levels: either depleting tumor cells of their preferred nutrients or inhibiting intracellular catabolic or biosynthetic pathways altered in tumors (Pelicano *et al*., 2006; Kroemer and Pouyssegur, 2008; Pathania *et al*., 2009; Tennant *et al*., 2010). In the present review, we will focus on glucose metabolism as a target for tumor therapy. A number of studies suggest that interference with glucose metabolism is a suitable strategy against cancer: glycolytic inhibitors such as 2-deoxyglucose (2-DG) or 3-bromopyruvate have been shown to promote tumor cell death and tumor regression in animals. Moreover, lonidamine, a glycolytic inhibitor, has been approved for use as an antitumor drug. Perhaps, however, antiglycolytic drugs may show more promising results in combination with other therapies. As we will discuss later, inhibition of glycolysis sensitizes cells to death ligands, such as TRAIL, and promotes an imbalance of Bcl-2 proteins, which sensitizes cells to chemotherapy.

Sensing glucose deprivation

Growth and survival factors promote glucose utilization. When stimulated to grow by growth factors, cells take glucose avidly and use it to produce fatty acids, nucleic acids and proteins, which are the main building blocks of the cell (DeBerardinis *et al*., 2008). Non-transformed cells are thus programmed to stop growing when growth factors are not present, and also when they lack nutrients.

At the organism level, a lack of nutrients is sensed by specific tissues, which produce hormones, provoking a feeling of hunger and prompting animals to search for food. In mammalian cells, nutrient levels are mostly sensed indirectly, by changes in the levels of metabolites from these nutrients. An important factor in sensing and reacting to lack of glucose is the adenosine monophosphate-activated protein kinase (AMPK). AMPK is activated by a decrease in the ATP/adenosine monophosphate ratio, which is a good indicator of energetic stress. This kinase phosphorylates a number of substrates that switch metabolism of the cell toward catabolic instead of biosynthetic pathways. When cells lack glucose, AMPK promotes downregulation of biosynthetic genes and utilization of stored fatty acids as a source of energy (Hardie, 2007). Moreover, AMPK activates p53 and stabilizes the cell cycle inhibitor p27 to cause cell cycle arrest (Figure 1) (Liang *et al*., 2007; Jones and Thompson, 2009).

One of the targets of AMPK is the master orchestrator of cell growth and proliferation, the mammalian target of rapamycin (mTOR) (Wang and Proud, 2009). mTOR is a kinase that is constitutively active in proliferating cells owing to its activation by the PI3-kinase/Akt pathway. When mTOR is inactive, cells stop growing, mostly because of its effect on protein

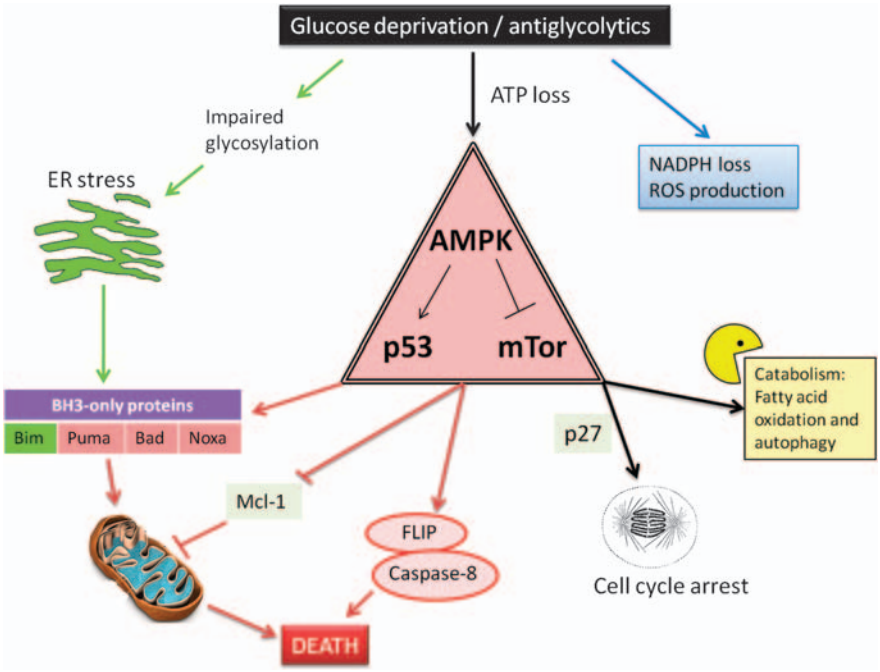

Figure 1 Inhibition of glucose metabolism promotes catabolism, cell cycle arrest or cell death. Glucose deprivation or treatment with antiglycolytics leads to loss of ATP, impaired protein glycosylation and oxidative stress. ATP depletion inactivates mTor and activates AMPK: this kinase induces cell cycle arrest through p27. AMPK also inhibits mTor and activates p53. Inhibition of mTor leads to autophagy, and AMPK and p53 promote induction of BH3-only proteins and cell death. Impaired glycosylation results in ER stress and subsequent induction of some proapoptotic BH3-only proteins. Inhibition of glucose metabolism can induce cell death through the mitochondrial pathway or through caspase-8, and it can promote downregulation of FLIP, thus sensitizing cells to death receptors.

synthesis. If cells lack glucose, the resulting energetic stress leads to mTOR inactivation and cells cease to synthesize proteins (Jones and Thompson, 2009). The mechanism that leads to mTOR inactivation on glucose withdrawal is still incompletely characterized. It was first demonstrated to be AMPK dependent, but recently it has been reported that agents that reduce ATP levels can inactivate mTOR in the absence of AMPK (Gwinn *et al*., 2008; Kalender *et al*., 2010).

Besides ATP levels, other good indicators of low glucose availability and energy stress are a decrease in the NADH/NAD^+ ratio and a general decrease in protein acetylation. NAD^+, the levels of which increase on inhibition of glycolysis, activates the sirtuin SIRT1. This protein promotes chromatin compaction and repression of gene expression. Repression of ribosomal RNA transcription by SIRT1 helps shut down ribosomal synthesis and protein translation (Murayama *et al*., 2008). On the other hand, acetylation, which is a post-transcriptional modification of many proteins, is influenced by glucose levels, as acetyl groups are derived from glucose metabolism. Histones, which regulate gene expression, and several metabolic enzymes are regulated by acetylation and are thus responsive to the presence of glucose (Wellen *et al*., 2009; Zhao *et al*., 2010).

Lack of glucose is additionally detected by the carbohydrate response element-binding protein (ChREBP), which is a transcription factor of the basic helix-loop-helix leucine zipper family (Uyeda and Repa, 2006). In the presence of glucose, ChREBP activates genes with carbohydrate-responsive elements and promotes glycolysis. Downregulation of ChREBP switches cell metabolism from aerobic glycolysis to respiration and inhibits proliferation of tumor cells (Tong *et al*., 2009).

Cells react to lack of glucose by switching to alternative energy sources

As discussed above, when subjected to glucose deprivation or energetic stress, cells cease proliferation and protein synthesis through activation of AMPK and p53, and through inactivation of mTOR. These same pathways stimulate alternative means of obtaining energy and building blocks. The aim is not only to minimize the use of resources required for proliferation but also to use energy reserves to maintain homeostatic functions. One of such energy reserves is fatty acids. In response to glucose deprivation, AMPK not only impairs fatty acid synthesis but also promotes fatty acid oxidation. Fatty acid oxidation has been shown to help glioblastoma cells survive deprivation of glucose (Buzzai *et al*., 2005).

In the presence of antiglycolytics, tumor cells can frequently use amino acids as a source of energy and carbon. The amino acid glutamine can be incorporated into the mitochondrial Krebs cycle and can be used to generate fatty acids, nucleotides and energy. When deprived of glucose, glioblastoma cells have been shown to turn to glutamine utilization, and if glutamine metabolism is impaired, cells cannot survive in the absence of glucose (Yang *et al*., 2009). Thus, when glucose is not available, cells turn to amino acids and fatty acids to try to maintain energy levels and homeostatic functions.

A conserved response to nutrient and oxygen starvation is autophagy. Autophagy is required for cells to survive nutrient deprivation (Boya *et al*., 2005). Most reports about the role of autophagy in response to nutrient deprivation have focused on cells subjected to complete starvation: depletion of amino acids, serum, glucose and vitamins. However, autophagy is thought to be activated in response to lack of glucose, and it has been shown to be induced and required for survival of prostate cells after treatment with the antiglycolytic 2-DG (DiPaola *et al*., 2008). Moreover, when apoptosis-deficient cells are subjected to glucose deprivation under hypoxia, autophagy becomes essential to maintain cell survival (Degenhardt *et al*., 2006).

Lack of glucose can also promote the unfolded protein response, which is a responsive mechanism to endoplasmic reticulum (ER) stress (Figure 1). The reason is that glucose is required for glycosylation. When proteins cannot be glycosylated, misfolded proteins accumulate, and the unfolded protein response is engaged. Indeed, ER stress, rather than energetic stress, may be the leading cause of cell death on treatment of some tumor cells with antiglycolytics such as 2-DG (Kurtoglu *et al*., 2007).

Oncogenes promote sensitivity to glucose deprivation

Proliferating cells react to glucose unavailability by shutting down biosynthetic pathways and actively promoting cell cycle arrest. However, tumor cells frequently present hyperactivation of proliferation-associated pathways and mutations in cell cycle inhibitors. This makes cells hypersensitive to glucose deprivation, as the cell is stimulated to proliferate but it lacks the building blocks needed for this purpose. For instance, hyperactivation of the oncogenic Akt/mTOR pathway promotes sensitivity to glucose withdrawal (Elstrom *et al*., 2004). Downstream of Akt, hyperactivation of mTOR, for instance, owing to the lack of tumor suppressor TSC1/2, promotes sensitivity to starvation. In this context, cells become unable to stop anabolism, and this leads to energy stress and cell death (Choo *et al*., 2010).

Survival of cells undergoing energetic stress requires AMPK to stop anabolism and promote catabolism and cell cycle arrest. Many tumor cells lack LKB1, which is the main kinase involved in activation of AMPK. As cells without LKB1 cannot respond adequately to energetic stress, they are hypersensitive to energy-depleting agents (Shaw *et al*., 2004). Downstream of AMPK, glucose deprivation promotes activation of p53 and p27. Both p53 and p27 promote cell cycle arrest and induce autophagy, which contributes to their prosurvival effects in the absence of glucose (Jones *et al*., 2005; Liang *et al*., 2007; Tasdemir *et al*., 2008). For this reason, the tumor suppressor p53, which is mutated in a variety

of tumors, has been shown to confer resistance rather than sensitivity to metabolic inhibitors. This is the reverse of that observed with most chemotherapeutic agents, which require p53 to kill cells. Thus, p53-deficient tumors are likely to be more sensitive than p53-positive tumors or non-transformed cells to antiglycolytics. However, more studies are required to clarify the role of p53 in cell death induced by glucose deprivation, as proapoptotic effects of this protein have also been reported (Zhao *et al*., 2008).

Glucose deprivation and antiglycolytics induce cell cycle arrest or cell death

The most widely used inhibitor of glycolysis is 2-deoxy-D-glucose (2-DG, DOG). This molecule is a non-metabolizable glucose analog, which is phosphorylated by hexokinase in the first step of glycolysis, but it cannot be further metabolized. Cell responses to 2-DG or to glucose deprivation are not identical (Kang and Hwang, 2006), but, in general, 2-DG produces the same effects as glucose deprivation in terms of AMPK activation, mTOR inactivation, cell cycle arrest and cell death. It has been known for decades that 2-DG, similar to glucose starvation, promotes cell cycle arrest and tumor cell death (Table 1). The effects of glucose deprivation and 2-DG are stronger under hypoxia than in normoxia, indicating that in the absence of oxygen, cells rely on anerobic glycolysis and thus become more dependent on glucose (Liu *et al*., 2002; Maher *et al*., 2004). As tumors are frequently subjected to hypoxia, drugs that kill better under hypoxia are particularly interesting for tumor treatment. Indeed, 2-DG has been tested in animal models and in clinical trials (Tables 1 and 2), in which it offered moderate success and was proven to be well tolerated and relatively non-toxic to normal tissues (Raez *et al*., 2005).

Glucose deprivation has been described to kill cells either by apoptosis or by necrosis. Apoptosis is a form of cell death orchestrated by caspases, which are proteases that degrade hundreds of substrates involved in cell homeostasis or in structural functions. When cells die by apoptosis, they are phagocytosed rapidly with no spillage of cytoplasmic content (Taylor *et al*., 2008). In contrast, necrosis occurs with rupture of the plasma membrane, which promotes inflammation. Necrosis cannot be prevented by the use of caspase inhibitors, and can only be inhibited, in certain cases, by inhibitors of RIPK1 (TP53-induced glycolysis and apoptosis regulator) or antioxidants. Although 2-DG usually kills cells by apoptosis, glucose deprivation has been shown to kill some cells by necrosis. For instance, when subjected to glucose deprivation under hypoxia, immortalized baby mouse kidney cells transfected with constitutively active Akt die by apoptosis. In contrast, their Bax-, Bak-deficient counterparts die by necrosis (Degenhardt *et al*., 2006). Bax and Bak are molecules required for one of the two main pathways of apoptosis: the intrinsic or mitochondrial pathway. These experiments suggested that cells usually die through the mitochondrial apoptotic pathway, but when this pathway is impaired, cells die by necrosis. However, glucose deprivation promotes apoptotic cell death in SV40-transformed, Bax-, Bak-deficient mouse embryonic fibroblasts. This form of cell death was dependent on caspase-8, which is a caspase involved in the non-mitochondrial, extrinsic apoptotic pathway (Caro-Maldonado *et al*., 2010). Caspase-8 was also shown to participate in the death of HeLa cells by glucose starvation. Thus, glucose deprivation can induce mitochondrial apoptosis, caspase-8-dependent apoptosis or necrosis (Table 1).

Antiapoptotic Bcl-2 proteins regulate cell death by lack of glucose

Members of the Bcl-2 family of proteins are regulators of apoptosis with a well-documented implication in cancer (Frenzel *et al*., 2009). This family is divided into three subfamilies of proteins, depending on the types and number of BH (BCL-2 homology) domains they contain. BH1-3 proteins such as Bak and Bax are the main inducers of apoptosis through the direct permeabilization of the mitochondrial membrane. The second group contains BH1-4 domains and are antiapoptotic proteins. Finally, the third subgroup of Bcl-2 proteins contains only the BH3 domain and are proapoptotic (Youle and Strasser, 2008).

A number of reports indicate that cell death induced by glucose deprivation in tumor or untransformed cells is inhibited by antiapoptotic proteins of the Bcl-2 family, indicating that glucose deprivation promotes mitochondrial apoptosis (Table 1). For instance, a study in the multidrug-resistant breast carcinoma MCF-7 line showed that, on glucose deprivation, these cells undergo apoptosis, which could be inhibited by Bcl-2 overexpression (Lee *et al*., 1997). In addition, in Ba/F3 hematopoietic cells, the stable expression of the antiapoptotic Bcl-2 homolog Bcl-xL was shown to protect cells from apoptosis caused by IL-3 starvation under low-glucose conditions (Gonin-Giraud *et al*., 2002).

Mcl-1, another member of the antiapoptotic Bcl-2 family, has also been shown to have a role in apoptosis induced by glucose deprivation, as reduction of Mcl-1 levels sensitized the acute T-cell leukemia cell line Jurkat to glucose withdrawal (Alves *et al*., 2006). Moreover, this short-lived protein was shown to be metabolically regulated. Rathmell and colleagues demonstrated that enforcement of glycolysis in hematopoietic cells leads to stabilization of Mcl-1 by inhibiting its degradation (Zhao *et al*., 2007). On the contrary, as discussed above, glucose deprivation or treatment with 2-DG results in AMPK-dependent mTor inactivation; this was shown to inhibit translation of Mcl-1 (Pradelli *et al*., 2010). Thus, Mcl-1 is controlled at translational and post-translational levels by the glycolytic metabolism. As we will discuss later, glucose restriction sensitizes cells to a number of proapoptotic stimuli, and it is possible that downregulation of Mcl-1 has a key role in this sensitization.

Table 1 Cell death induced by inhibition of glucose metabolism

Stimulus	*Response*	*Molecules implicated*	*Cell/tumor type*	*References*
Glucose deprivation	Apoptosis	Bcl-xL (protective)	IL-3 dependent FL5.12 and Ba/F3 cells	Gonin-Giraud *et al.* (2002); Vander Heiden *et al.* (2001)
		Bcl-2 (protective)	Breast carcinoma MCF-7/ADR (multidrug resistant); rat-myc-transformed fibroblasts	Lee *et al.* (1997)
		Mcl-1 (protective), Noxa	human T cells and leukemia	Alves *et al.* (2006)
		Caspase-8	MEFs Bax$^{-/-}$ Bak $^{-/-}$	Caro-Maldonado *et al.* (2010)
		Bad	Mouse liver	Danial *et al.* (2003)
		Bim, PUMA, p53 (pro-apoptotic)	IL-3 dependent FL5.12, Primary T lymphocytes	Zhao *et al.* (2008)
	Cell death	p53 (protective)	Primary mouse fibroblasts	Jones *et al.* (2005)
	Cell death with necrotic phenotype		Human fibroblasts	Yuneva *et al.* (2007)
		ARK5	Hepatoma HepG2	Suzuki *et al.* (2003)
Glucose deprivation + hypoxia	Necrosis, apoptosis	AKT, Bax, Bak	iBMK: immortalized kidney epithelial cells	Degenhardt *et al.* (2006)
2-deoxyglucose	Apoptosis		Breast cancer cell lines (MCF7), ovarian carcinoma (IGROV1, IGROV1-R10, U251), mesothelioma (MSTO-211 H), Head and neck squamous cancer (SQ2OB, SCC61)	Aft *et al.* (2002); Zhang *et al.* (2006); Kaplan *et al.* (1990)
		Beclin (protective)	Prostate cancer cells	DiPaola *et al.* (2008)
		Bcl-2 (protective)	Rat1a-Myc transformed fibroblasts, nontransformed CB33 lymphoblastoid cells, Burkitt lymphoma cell lines	Shim *et al.* (1998)
	Cell cycle blockade without apoptosis		Ovarian carcinoma (SKOV3), mesothelioma (NCI-H28), Glioblastoma (GL15), Head and neck squamous cancer (SCC12B2)	Zhang *et al.* (2006)
2-deoxyglucose	Tumor regression		Fibrosarcoma, animal model	Kern and Norton (1987)
2-deoxyglucose			Clinical trial: Prostate Cancer. Intracranial metastases	NCT00633087: 2008–2010. Phase: I/II study: Safety of 2-deoxyglucose in the treatment of advanced solid tumors and hormone refractory prostate cancer. Status: suspended NCT00247403: 2005–2009. Phase I Study: safety study of 2-DG with stereotactic Radiosurgery for treatment of intracranial metastases. Status: pharmaceutical company no longer manufactures drug.
3-BrPA	Tumor erradication		Hepatocarcinoma (animal model)	Geschwind *et al.* (2002); Ko *et al.* (2004)
Lonidamine	Cytotoxicity		Breast-carcinoma MCF7 cells	Rosbe *et al.* (1989)
Lonidamine			Clinical trials: Benign Prostatic hyperplasia	NCT00435448, NCT00237536, 2005–2009. Phase II/III studies: efficacy and safety of lonidamine for the treatment of symptomatic benign prostatic hyperplasia. Status: terminated

Abbreviation: DG, deoxyglucose.

Glucose regulates BH3-only proteins

The so-called 'BH3-only' proteins regulate apoptosis by sensing signals emanating from various cellular processes and inducing the activation of Bax/Bak, either directly or indirectly by inhibiting antiapoptotic Bcl-2 proteins. In both cases, these proteins trigger Bax and Bak to cause cytochrome *c* release and subsequent activation of the caspase cascade. BH3-only proteins have key roles in tumorigenesis and in sensitivity to chemotherapy. A few of these proteins have been shown to have a role in glucose deprivation-induced apoptosis in different models (Figure 2).

Bim has been implicated in cell death induction in various cell types and tissues in response to different stimuli, notably growth factors or cytokine withdrawal.

Table 2 Combination of inhibition of glycolysis and therapeutic agents

Anti-glycolytic stimulus	*Therapeutic stimulus*	*Cell type*	*Effect*	*Reference*
3-BrPA	Doxorubicin, vincristine, ara-C	Leukemia cells and hepatocarcinoma	Sensitization to cell death (apoptosis, necrosis)	Xu *et al*. (2005)
2-deoxyglucose	Histone deacetylase inhibitors (trichostatin A, sodium butyrate)	Brain, breast, and cervix cancer cells	Sensitization to apoptosis	Egler *et al*. (2008)
	Metformin	Prostate cancer	Sensitization to cell death *in vitro*	Ben Sahra *et al*. (2010)
	Cisplatin/carboplatin	Ovarian cancer cell lines, human epidermoid carcinoma A431, and colon carcinoma HT-29	Sensitization to cell death/ apoptosis	Yamada *et al*. (1999); Hernlund *et al*. (2009)
	Prednisolone/ dexamethasone	Leukemia cells (Jurkat, Molt4)	Sensitization to cell death	Hulleman *et al*. (2009).
2-deoxyglucose	Adriamycin, Paclitaxel	Osteosarcoma 143b, Lung cancer (MV522, NSCLC) xenograft	Inhibition of tumor growth (*in vivo*)	Maschek *et al*. (2004)
2-deoxyglucose	Docetaxel	Clinical trial (phase I): Advanced solid tumors (lung, breast, pancreatic, head and neck, gastric cancer)	Low toxicity; some anti-tumor effects.	NCT00096707: 2004–2009 Study: dose escalation trial of 2-deoxy-D-glucose alone and in combination with docetaxel in subjects with advanced solid malignancies Raez *et al*. (2007)
2-deoxyglucose, glucose deprivation	TRAIL, TNF, Fas cross-linking	MCF7, U937, Hela, SKW6.4, Jurkat	Sensitization to apoptosis	Munoz-Pinedo *et al*. (2003); Nam *et al*. (2002); Pradelli *et al*. (2010)
2-deoxyglucose	γ irradiation	Malignant glioma cells, melanoma, Hela	Sensitization to cell death	Dwarkanath *et al*. (2001); Jain *et al*. (1985)
		Murine tumor (Ehrlich ascites)	Inhibition of tumor growth (*in vivo*); enhanced animal survival	Dwarakanath *et al*. (1999); Latz *et al*. (1993)
		Glioblastoma (Phase I/II study)	Combination well tolerated; no toxicity and late radiation damage to the normal brain	Singh *et al*. (2005)
Glucose deprivation	Thymidylate synthase inhibitor 5-FUdR	Histiocytic lymphoma U937	Inhibition of apoptosis	Munoz-Pinedo *et al*. (2004).
2-deoxyglucose	Antitumor drugs (etoposide, camptothecin and mitomycin C)	Leukemia (U937)	Inhibition of apoptosis	Haga *et al*. (1998)
	Doxorubicin	Normal epithelium	Inhibition of apoptosis *in vivo*	Thakkar and Potten (1993)
Lonidamine	Cisplatin	Breast cancer *in vitro* and mammary carcinoma *in vivo*	Sensitization to cell death	Teicher *et al*. (1991)
	Epirubicin and cyclophosphamide	Breast cancer (Phase I/II)	Active and well tolerated.	Papaldo *et al*. (2003)
	Diazepam	Glioblastoma (Phase II study)	No complete or partial response. 50% stabilizations	Oudard *et al*. (2003)
	Cisplatin/carboplatin/ paclitaxel	Ovarian cancer (Phase II study)	Active and well tolerated	De Lena *et al*. (2001); Di Cosimo (2003)

Abbreviation: TNF, tumor necrosis factor.

Bim is also the main BH3-only protein associated with ER-stress-induced cell death. It was demonstrated in various cell lines that, on induction of ER stress Bim was upregulated, both transcriptionally by the transcription factor CHOP and post-translationally through its dephosphorylation by PP2a and subsequent stabilization (Puthalakath *et al*., 2007). One of the consequences of glucose deprivation is the activation of the unfolded protein response in the ER; the reason behind it is not clear yet, but it is thought that it could be caused both by the drop in ATP levels and by the impairment of protein glycosylation (reviewed in (Kaufman *et al*., 2002)). Consistent with this, Bim was shown to be induced along with Puma in hematopoietic cells exposed to glucose deprivation (Zhao *et al*., 2008). This suggests that the induction of Bim under glucose deprivation could be due to the induction of ER stress. Conversely, in hematopoietic cells, enforcement of glycolysis caused Mcl-1 upregulation and inhibition of Bim-induced apoptosis (Zhao *et al*., 2007).

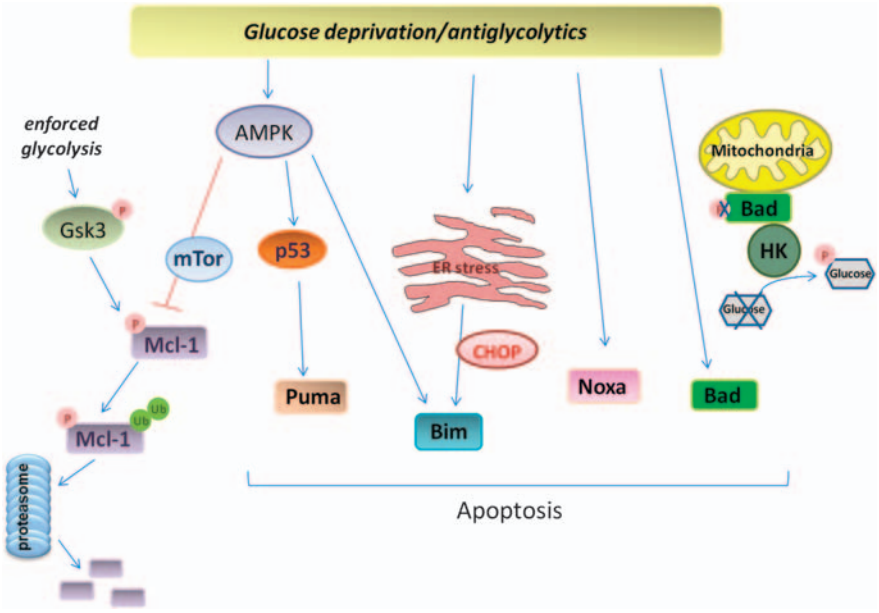

Figure 2 Regulation of the mitochondrial apoptotic pathway by inhibition of glycolysis. Glucose deprivation or treatment with 2-DG leads to AMPK-dependent mTor inactivation with the subsequent inhibition of Mcl-1 translation. Conversely, enforcement of glycolysis leads to GSK3 inactivation, and this impairs Mcl-1 degradation by the proteasome. The activation of AMPK results in p53 activation and upregulation of the BH3-only protein Puma. Bim is induced not only by AMPK activation but also by the ER stress response through the action of the transcription factor CHOP. In addition, Noxa participates in apoptosis on glucose withdrawal in activated T cells and in leukemic cell lines, possibly by inhibiting Mcl-1. Finally, Bad is regulated post-translationally on glucose deprivation through inhibition of its phosphorylation. Unphosphorylated Bad is then able to interact with Bcl-2 family members and induce apoptosis. On the contrary, Bad also interacts with a glucose hexokinase (HK), and Bad phosphorylation is crucial for the kinase activity of the hexokinase.

In addition, this BH3-only protein has been recently implicated in apoptosis induced by excitotoxic stress in primary neurons (Concannon *et al*., 2010). This stimulus promotes a drop in ATP cellular levels and subsequent activation of AMPK, which were shown to induce transcription of Bim (Concannon *et al*., 2010). In summary, Bim was shown to be induced both by ER stress and by AMPK activation, two consequences of glucose deprivation.

PUMA is upregulated in a p53-dependent manner after DNA damage and growth factor withdrawal (Nakano and Vousden, 2001). A study in murine hematopoietic cells demonstrated that PUMA was metabolically regulated and was induced on glucose deprivation through the activation of p53 (Zhao *et al*., 2008). In this model, p53 was activated, but its expression was not upregulated. Downregulation of PUMA with small interfering RNA was sufficient to reduce death induced by glucose deprivation, indicating that PUMA mediates glucose withdrawal-induced cell death. Consequently, high glucose metabolism seemed to protect from interleukin-3 deprivation by suppressing upregulation of Bim and PUMA, and, as we will discuss later, by stabilizing Mcl-1.

Noxa is another BH3-only protein under the control of p53. Noxa has been shown to participate in cell death induced by glucose deprivation, both in primary and tumor cells (Alves *et al*., 2006). In this study, Alves *et al*. looked at the response of rapidly dividing hematopoietic cells to glucose limitation. In their model, Noxa was shown to be involved in apoptosis after glucose withdrawal, as downregulation of Noxa conferred a competitive survival advantage to primary T cells, as well as to leukemic cell lines, under low glucose conditions. The authors hypothesized that the role of Noxa in this setting is related to its ability to neutralize the antiapoptotic Bcl-2 homolog, Mcl-1. Indeed, the downregulation of Mcl-1 rendered these cells more sensitive to glucose deprivation, as described earlier. Moreover, Noxa has been shown to redistribute to mitochondria on glucose deprivation. Translocation is linked to its dephosphorylation, which triggers its release from a large cytosolic complex, which also contains a glycolytic transferase (A Kelekar, personal communication). These intriguing results suggest that Noxa, similar to Bad, may have a role both in death induced by glucose deprivation and in glucose metabolism.

Bad is the BH3-only protein that has been most directly linked to glucose metabolism (Danial, 2008). Bad-deficient murine hepatocytes deprived of glucose are strikingly protected from cell death compared with their normal counterparts (Danial *et al*., 2003). These results suggest that Bad functions as a proapoptotic BH3-only protein in response to glucose deprivation. However, it is possible that Bad promotes cell death in this context indirectly, by regulating glucose metabolism. In liver and pancreatic β-cell mitochondria, Bad associates with a protein complex that contains hexokinase IV, responsible for the first step of glycolysis. Furthermore, studies on Bad−/− mice showed that Bad was necessary for

blood glucose sensing. These mice display glucose intolerance and a defect in insulin production by pancreatic islets (Danial *et al*., 2008). Intriguingly, Bad BH3 motive, which regulates cell death induced by some stimuli, was shown to be required for the activity of the hexokinase.

Bad had previously been shown to be regulated post-translationally by phosphorylation. Its phosphorylation by growth factor-activated kinases triggers its binding to 14-3-3 proteins, thus inhibiting its interaction with Bcl-2 antiapoptotic proteins and impeding its proapoptotic functions (Zha *et al*., 1996). It was shown by Danial *et al*. (2003) that phosphorylation of the BH3 domain of Bad promotes hexokinase IV activity. On the other hand, glucose deprivation promotes dephosphorylation of the BH3 motive, correlating with apoptosis induction. Furthermore, treatment of human leukemic cells with a glycolytic inhibitor (3-BrPA) led to Bad dephosphorylation and Bax oligomerization on mitochondria (Danial *et al*., 2003; Xu *et al*., 2005). Thus, the phosphorylation status of Bad seems to have opposite effects on its two main functions: phosphorylation promotes glycolysis while inhibiting apoptosis. In this context, it is unclear whether Bad participates directly in apoptosis induced by inhibition of glucose metabolism, functioning as a classical BH3-only protein. Instead, it is possible that it is the role of Bad in the maintenance of glucose metabolism that accounts for the protection of Bad-deficient hepatocytes from glucose withdrawal.

Glycolysis protects from deprivation of growth factors

Growth/survival factors promote glucose metabolism. Conversely, a high glycolytic rate has been shown to protect cells from cell death induced by survival factor withdrawal. One of the first studies that suggested the protective role of high glucose metabolism from growth factor deprivation is that by Thompson and colleagues (Vander Heiden *et al*., 2001). They demonstrated that by increasing glucose uptake through Glut1 overexpression in hematopoietic cells, they could efficiently delay apoptosis induced by interleukin-3 withdrawal. In addition, it was shown that glucose uptake and phosphorylation are required by constitutively active Akt to protect from interleukin-3 withdrawal (Rathmell *et al*., 2003). The process of deciphering the mechanism behind it has begun. Under normal glucose conditions, withdrawal of interleukin-3 leads to Mcl-1 phosphorylation by kinase GSK3, which promotes degradation of Mcl-1 by the proteasome and leads to cell death (Maurer *et al*., 2006). However, enforcement of glucose metabolism maintains GSK3α and β phosphorylated and thus inactive, and this promotes stability of Mcl-1 (Zhao *et al*., 2007; Figure 2). Moreover, although PUMA is induced by growth factor withdrawal, enforcement of glucose uptake and metabolism were shown to attenuate PUMA induction (Zhao *et al*., 2008). It was thus suggested that the high glycolytic metabolism observed in tumor cells and described as the Warburg effect was in fact a protective strategy to cope with growth factor limitation.

2-DG sensitizes to radio- and chemotherapy

Glycolytic inhibitors 2-DG, lonidamine and 3-bromopyruvate have shown encouraging results as antitumor agents in animal models. 2-DG has been studied in several clinical trials that indicate its safety in humans (Tables 1 and 2). Although some reports indicate some antitumor effects, currently, to our knowledge, there are no undergoing clinical trials using 2-DG as a single agent. However, its low toxicity, together with a good number of *in vitro* experiments, suggests that this compound may be useful in combination with chemotherapeutic drugs, as it sensitizes tumor cells to common chemotherapeutic drugs (Table 2). A number of hypotheses have been proposed to explain this effect. It is possible that glycolytic inhibitors reduce the ability of cells to repair damage caused by other drugs. Alternatively, reduction of ATP levels could impair the function of the ATP-dependent multidrug resistance-associated pumps. Moreover, as discussed earlier, inhibitors of glycolysis regulate apoptotic proteins, thus lowering the threshold for a cell to undergo apoptosis. As discussed above, glucose deprivation or treatment with 2-DG regulates levels or activity of p53, Mcl-1, PUMA, Noxa and Bad proteins, which have been associated with sensitivity to chemotherapy (Frenzel *et al*., 2009).

Several studies have shown that the glucose analog 2-DG improves responses to radiotherapy (Table 2). For example, high concentrations of 2-DG sensitize malignant glioma cells or HeLa to radiation. Interestingly, the use of 2-DG did not promote radiotoxicity of normal peripheral blood leukocytes, splenocytes or thymocytes (Kalia *et al*., 1982; Jain *et al*., 1985; Dwarkanath *et al*., 2001; Swamy *et al*., 2005). Moreover, the same results were obtained in animal experiments. In implanted murine Ehrlich ascites or sarcoma, the administration of 2-DG ($\geqslant$1 g/kg) before or immediately after irradiation increases tumor cell death and improves animal survival (Dwarakanath *et al*., 1999). However, in normal bone marrow and spleen, a similar dose of 2-DG confers radioprotection (Singh *et al*., 1990). These studies promoted the development of a clinical trial that confirmed that the combination of 2-DG with radiation was well tolerated in patients (Singh *et al*., 2005).

Chemoprotection of normal tissues by 2-DG or fasting

As seen earlier, 2-DG sensitizes to chemo- and radiotherapy. One possible negative effect of using antiglycolytic agents in combination with other therapies would be the possibility that untransformed cells would also be sensitized. As discussed above, 2-DG sensitizes tumor cells, but not non-transformed cells, to radiotherapy. Moreover, a number of studies have reported encouraging results, which indicate that 2-DG protects normal tissues from chemotherapy. In this sense, an *in vivo* study showed that the treatment with 2-DG concomitantly with doxorubicin led to inhibition of apoptosis induced by this DNA-damaging drug in intestinal cells (Thakkar and Potten, 1993). Moreover,

a differential effect of the influence of low glucose on chemotherapy resistance between normal and tumor cells has been observed (Raffaghello *et al*., 2008). Low glucose protected only primary glial cells, but not glioblastoma cancer cell lines, from cyclophosphamide.

Raffaghello *et al*. subjected mice to starvation to study its effect on chemotherapy-induced toxicity. Strikingly, 2 days of starvation protected mice from high doses of etoposide, which are usually very toxic. Treatment with etoposide after starvation promoted survival of mice injected with human neuroblastoma, suggesting that a short period of starvation would allow patients to receive higher, more effective doses of chemotherapy.

However, it should be noted that antagonistic effects of antiglycolytic agents with other drugs have also been reported in tumor cell lines. It was observed that glucose deprivation could inhibit apoptosis induced by DNA-damaging agents such as VP-16 (etoposide), adryamycin or camptothecin (Yun *et al*., 1995; Tomida *et al*., 1996). Furthermore, studies in U937 cells show that pretreatment with 2-DG also confers resistance to cell death induced by DNA-damaging agents (Haga *et al*., 1998; Munoz-Pinedo *et al*., 2004). Nevertheless, no common mechanisms of resistance have been described in these different studies. Yun and colleagues linked the resistance to a decrease in topoisomerase II cellular levels, although other studies show steady state levels of topoisomerase-DNA complexes and postulate that the inhibition occurs downstream of the DNA damage, but upstream of cytochrome *c* release from mitochondria.

It is possible that, as most of these drugs need the cells to be in a highly proliferative state in order to kill them, the cell cycle arrest caused by glucose deprivation impairs their effects. In the same line, another effect of glucose metabolism inhibitors that could explain the inhibition of death is the inhibition of *de novo* protein synthesis, which is required by most of these drugs to induce apoptosis. Nevertheless, more studies, especially *in vivo* studies, are needed in order to determine whether the targeting of glucose metabolism in combination with certain DNA-damaging drugs could cause antagonist effects.

Sensitization to death receptors

Death ligands such as Fas/CD95 ligand, TNF and TRAIL kill cells through the extrinsic pathway of apoptosis. This pathway is engaged when these proteins bind to their receptors on the surface of the cell and promote their oligomerization. Oligomerization then triggers the recruitment of several molecules to the Death Inducing Signaling Complex to which caspase-8 binds. Caspase-8 is activated through oligomerization, and can directly cleave and activate caspase-3, thus killing the cell. TRAIL is a promising death ligand that is being studied in clinical trials because of its ability to kill tumors but not normal cells (Johnstone *et al*., 2008). However, proteins of the apoptotic extrinsic pathway are very frequently upregulated or downregulated in tumors, and some tumor cells are resistant to low doses of TRAIL. *In vitro* studies have shown that glucose withdrawal or treatment with 2-DG can overcome resistance to TRAIL, TNF-α and Fas ligation in several cell lines (Nam *et al*., 2002; Munoz-Pinedo *et al*., 2003; Pradelli *et al*., 2010) (Table 2). At the molecular level, these results could be explained, at least in some cell lines, by the effects of glucose levels on the inhibitors of apoptosis FLIP and Mcl-1. These two proteins have a short half-life, and glucose deprivation promotes inhibition of their synthesis.

Synergistic effects with TRAIL suggest that glycolytic inhibitors could be combined with TRAIL agonists to improve its therapeutic effects. One important question, however, is whether inhibition of glucose metabolism sensitizes normal human cells to death receptors, which will likely cause unwanted side effects. A study suggests that this may not be the case. Schimmer and colleagues identified a compound that sensitizes tumor cells to death ligands (Wood *et al*., 2008). This compound, fasentin, inhibits glucose uptake, possibly by direct inhibition of glucose transporters. Importantly, fasentin did not sensitize normal resting or phytohemagglutinin-stimulated peripheral blood mononuclear cells to cross-linking of Fas receptors (Wood *et al*., 2008). However, to our knowledge, it has not been studied whether glycolytic inhibitors would sensitize non-transformed cells to TRAIL.

Conclusions and future perspectives

Nutrients are required for life, and nutrient availability regulates cell death. Control of cell death by metabolism is likely to occur at multiple levels, from regulation of stability and activation of BH3-only proteins to regulation of caspase activation at the Death Inducing Signaling Complex. This is probably a well-conserved phenomenon across evolution. Yuan and colleagues performed a genome-wide RNAi screen to identify genes that regulate caspase activation in Drosophila (Yi *et al*., 2007), and they found that many of the genes that regulated apoptosis were genes involved in metabolism; from carbohydrate metabolism to fatty acid synthesis.

Glucose dependency of tumor cells is exploited for diagnostic purposes. The fact that tumor cells require more glucose than their normal counterparts, and that they frequently undergo cell death when treated with antiglycolytics, has promoted clinical trial development. It should be noted, however, that antiglycolytic drugs—similar to most chemotherapeutic drugs—may have a number of undesired side effects, as there are many tissues that use glucose as fuel; among these, the brain and highly proliferating cells, such as those of the immune system (particularly activated lymphocytes), may be the most sensitive to inhibition of glycolysis. Some results discussed earlier are encouraging because they suggest that inhibition of glucose metabolism is less toxic to normal cells than to tumor cells when combined with radio- or chemotherapy. However, we should improve our understanding of how tumor metabolism

differs from metabolism of normal cells. In this sense, a matter of relevance is whether specific oncogenes confer more sensitivity than others to inhibition of specific metabolic pathways. For instance, tumors with deregulated myc expression may be less sensitive to inhibition of glycolysis than tumors with overactivation of the Akt pathway. Instead, myc-overexpressing tumors could be more susceptible to inhibition of mitochondrial/glutamine metabolism (Yuneva *et al*., 2007; Fan *et al*., 2010).

Surprisingly little is known about the apoptotic pathways involved in tumor cell death in response to starvation. Several reports described above have studied the apoptotic role of caspase-8 or specific BH3-only proteins in a few tumor cell lines or in non-transformed cells. Aside from these few reports, we do not know the specific pathways by which glucose deprivation or antiglycolytics kill tumor cells. Many tumor cells have been shown to die by necrosis when deprived of glucose. Some forms of necrosis are specifically regulated by molecules such as RIPK1; the knowledge of possible molecules involved in necrosis of tumor cells would open the possibility to control it by drugs. Moreover, it would be of interest to identify the key molecules involved in sensitization of tumor cells to radio- and chemotherapy in order to design better combinations with antiglycolytics. All these studies also open the possibility that temporary starvation will synergize with chemotherapy. It is known that caloric restriction prolongs life. However, to determine whether this would hold true for cancer patients would require many more experiments and clinical trials.

Conflict of interest

The authors declare no conflict of interest.

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