Under normoxia, 2-deoxy-D-glucose elicits cell death in select tumor types not by inhibition of glycolysis but by interfering with N-linked glycosylation

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Abstract

In tumor cells growing under hypoxia, inhibiting glycolysis with 2-deoxy-D-glucose (2-DG) leads to cell death, whereas under normoxic conditions cells similarly treated survive. Surprisingly, here we find that 2-DG is toxic in select tumor cell lines growing under normal oxygen tension. In contrast, a more potent glycolytic inhibitor, 2-fluorodeoxy-D-glucose, shows little or no toxicity in these cell types, indicating that a mechanism other than inhibition of glycolysis is responsible for their sensitivity to 2-DG under normoxia. A clue to this other mechanism comes from previous studies in which it was shown that 2-DG interferes with viral N-linked glycosylation and is reversible by exogenous addition of mannose. Similarly, we find that 2-DG interferes with N-linked glycosylation more potently in the tumor cell types that are sensitive to 2-DG under normoxia, which can be reversed by exogenous mannose. Additionally, 2-DG induces an unfolded protein response, including up-regulation of GADD153 (C/EBP-homologous protein), an unfolded protein response–specific mediator of apoptosis, more effectively in 2-DG–sensitive cells. We conclude that 2-DG seems to be toxic in select tumor cell types growing under normoxia by inhibition of N-linked glycosylation and not by glycolysis.

Because in a phase I study 2-DG is used in combination with an anticancer agent to target hypoxic cells, our results raise the possibility that in certain cases, 2-DG could be used as a single agent to selectively kill both the aerobic (via interference with glycosylation) and hypoxic (via inhibition of glycolysis) cells of a solid tumor. [Mol Cancer Ther 2007;6(11):3049–58]

Introduction

Despite angiogenesis, the metabolic demands of rapid tumor growth often outstrip the oxygen supply, which contributes to formation of hypoxic regions within most solid tumors. Consequently, the decrease in oxygen levels is shown to impede the cell cycle and thereby result in resistance to most chemotherapeutic agents and radiotherapy, which normally target rapidly proliferating cells (1, 2). However, in addition to these disadvantages for cancer treatment, hypoxia renders a tumor cell dependent on glycolysis for energy production and survival. Thus, blocking glycolysis in hypoxic tumor cells should lead to cell death. Indeed, under three different conditions of simulated hypoxia in vitro, we have shown that tumor cells can be killed by inhibitors of glycolysis (3). Moreover, inhibition of glycolysis in normally oxygenated cultured cells does not significantly affect their energy production because alternative carbon sources (i.e., amino acids and fats) can be used to drive mitochondrial oxidative phosphorylation. Therefore, glycolytic inhibitors can be used to selectively target hypoxic tumor cells without showing much toxicity to either normal or tumor cells growing aerobically (4, 5).

In fact, in vivo experiments have shown that 2-deoxy-D-glucose (2-DG; targeting slow-growing hypoxic tumor cells) increases the efficacy of the standard chemotherapeutic agents, Adriamycin and cisplatin (directed against rapidly proliferating aerobic cells), in different human tumor xenografts (6). The results of these studies as well as data from our in vitro models of hypoxia (3) have led to testing this strategy for improving chemotherapy protocols in humans in the form of a phase I clinical trial entitled ''A phase I dose escalation trial of 2-DG alone and in combination with docetaxel in subjects, with advanced solid malignancies,'' which is currently ongoing. The data from animal studies (500 mg/kg) as well as the preliminary results from the phase I clinical trial (88 mg/kg) indicate that 2-DG is well tolerated and relatively nontoxic to normally oxygenated cells and tissues (6, 7).

Although 2-DG is known to block glycolysis by competitively and allosterically inhibiting hexokinase as well as competitively inhibiting glucose phosphoisomerase (8–10), it has also been reported that in certain viruses this sugar

analogue interferes with asparagine (N)-linked glycosylation of their coat proteins (11). N-linked glycosylation is a common oligosaccharide modification of eukaryotic proteins produced by the endoplasmic reticulum. By a cotranslational process, the glycan glucose$_3$mannose$_9$ N-acetylglucosamine$_2$ (G$_3$M$_9$Gn$_2$) is transferred from a lipid-linked oligosaccharide (LLO), G$_3$M$_9$Gn$_2$-P-P-dolichol, to appropriate asparaginyl residues of nascent endoplasmic reticulum–synthesized polypeptides. Thus, perturbation of LLO synthesis results in aberrant N-linked glycosylation and interferes with glycan-dependent folding and quality control processes of the endoplasmic reticulum. In the report on 2-DG effects on viral glycoproteins, it was shown that addition of exogenous mannose could reverse the effects of 2-DG on LLO synthesis. Moreover, these investigators showed that the glucose analogue 2-fluoro-deoxy-D-glucose (2-FDG), which has been reported to be a more potent inhibitor of glycolysis than 2-DG (12), was relatively ineffective for preventing LLO synthesis, and was thereby less potent in interfering with N-linked glycosylation (13).

Preliminarily, we reported at the *AACR* annual meeting (14) that 2-DG, but not 2-FDG, was unexpectedly toxic to a select number of tumor cell lines growing under normal oxygen tension. Here, we expand those findings by investigating oxygen consumption, LLO synthesis, N-linked glycosylation, the unfolded protein response (UPR; a stress response which is a consequence of aberrant N-glycosylation) including an UPR-specific mediator of apoptosis [C/EBP-homologous protein (CHOP)/GADD153], and reversal of 2-DG effects by mannose. We conclude that in select sensitive tumor cells cultured under normoxia, 2-DG toxicity is caused by interference with N-linked glycosylation and not by inhibition of glycolysis.

Materials and methods

Cell Types

The ρ^0 cells were generated and grown as previously described (15). The SKBR3 cell line was a gift from Dr. Joseph Rosenblatt's laboratory at University of Miami (Miami, FL); tumor cell lines MDA 231 (breast), 1420 and 1469 (pancreatic), and 143B (osteosarcoma) were purchased from American Type Culture Collection (ATCC). Interestingly, the SKBR3 cell line purchased from ATCC displayed a different morphology as well as sensitivity to 2-DG than that from Dr. Rosenblatt's lab. However, this difference disappeared when the ATCC cells were passaged for 8 to 9 cycles. SKBR3 cells were grown in RPMI 1640 (Invitrogen) whereas 1420, 1469, 143B, and MDA 231 cells were grown in DMEM (Invitrogen). Medium was supplemented with 10% fetal bovine serum and glucose concentrations were kept at 2 mg/mL. Cells were grown under 5% CO$_2$ at 37°C.

Drugs and Chemicals

2-DG, oligomycin, tunicamycin, and staurosporine were purchased from Sigma-Aldrich. 2-FDG and 2-fluoro-deoxy-mannose (2-FDM) were a kind gift from Dr. Waldemar Priebe (M.D. Anderson Cancer Center, University of Texas, Houston, TX).

Oxygen Consumption

Cells were grown in 75-cm^2 flasks until they were 70% to 80% confluent, and then were trypsinized. Cells (5 × 10^6) were resuspended in 1 mL of DMEM, which did not contain glucose or fetal bovine serum. Oxygen consumption was measured with Clark electrode (Hansatech) for 10 min as previously described (16).

Models of Hypoxia

Three *in vitro* models of anaerobiosis were developed and previously described as follows: model A are tumor cells treated with 0.05 μg/mL of oligomycin (a mitochondrial inhibitor of ATP synthase); model B are ρ^0 cells that are deficient in mitochondrial DNA; and model C are cells treated in an hypoxic (0.5% O$_2$) chamber (Reming Bioinstruments; ref. 3).

Cytotoxicity Assay

Cells were incubated for 24 h at 37°C in 5% CO$_2$, at which time drug treatments began and continued for 72 h. At this time, attached cells were trypsinized and combined with their respective culture media followed by centrifugation at 400 × g for 5 min. The pellets were resuspended in 1 mL of Hanks solution and analyzed with Vi-Cell (Beckman Coulter) cell viability analyzer.

ATP Quantification Assay

ATP was measured with an ATP-lite kit (Perkin-Elmer) according to the manufacturer's directions and modified as previously described (10).

Fluorophore-Assisted Carbohydrate Electrophoresis

Cells were cultured until 90% confluent, harvested in methanol, and dried under N$_2$. LLOs were recovered in chloroform/methanol/water (10:10:3), hydrolyzed, modified with 7-amino-l,3-naphthalenedisulfonic acid (ANDS) fluorophore, and analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) as previously described (17). For *N*-glycans, the residue remaining after organic extraction was dissolved by boiling with 20 μL of 2% SDS and then diluted 10-fold with 20 mmol/L sodium phosphate buffer (pH 7.6). Ten microliters were reserved for protein assay, and the remainder was mixed gently with 20 μL of 7.5% NP40. One unit of *N*-glycanase (Calbiochem) was added, followed by incubation at 37°C for 18 h. Six hundred sixty microliters of absolute ethanol were added, and the tube was placed on ice for 15 min. After centrifugation at 10,000 × g for 15 min, the supernatant was dried under vacuum. The residue was processed for labeling with ANDS, and FACE profiling gels were prepared and run for *N*-glycans as described (17).

Western Blot Analysis

Cells were plated at 10^4/cm^2 with and without drug treatment for the indicated times. At the end of the treatment periods, cells were collected and processed as previously described (10). Gels were transferred onto nitrocellulose membranes (Amersham) and probed with anti-KDEL (Stressgen; for GRP78 and GRP94) and polyclonal anti–CHOP/GADD153 (Santa Cruz Biotechnology). After probing, membranes were processed as previously described (10).

Results

Under Normoxia, Select Tumor Cell Types Are Killed by 2-DG But Not by 2-FDG

Previously, it was reported that in cells growing anaerobically, 2-DG caused toxicity by inhibiting glycolysis, the main mechanism of ATP production under this condition (3). Surprisingly, under normal oxygen tension, we find that two tumor cell lines (SKBR3 and 1420) of different tissue origins undergo significant cell death when treated with 4 mmol/L 2-DG (Fig. 1A). In contrast, another glucose analogue, 2-fluoro-deoxy-glucose (2-FDG), previously found to be a more potent inhibitor of glycolysis than 2-DG (12), showed no toxicity in SKBR3 cells and much less toxicity than 2-DG in 1420 cells growing under normoxia (Fig. 1B). However, when oxidative phosphorylation is blocked by oligomycin, 2-FDG is more toxic than 2-DG in both cell types (Fig. 1B). Thus, our results, which show that 2-FDG causes little or no toxicity in SKBR3 and 1420 under normal oxygen tension, further suggest that the toxicity of 2-DG in these tumor lines is independent of its ability to inhibit glycolysis.

Oxygen Consumption Does Not Correlate with 2-DG Sensitivity

To directly determine whether the sensitivity to 2-DG under normoxia is due to a deficiency in mitochondrial function, oxygen consumption was measured in these select tumor cell lines using a Clark electrode. As shown in Table 1, the cell lines found to be sensitive to 2-DG under normal oxygen tension respired as well or better than the two other cell lines that were resistant to the toxic effects of this sugar analogue under these conditions. Additionally, in the two anaerobic cell models in which mitochondrial oxidative phosphorylation is dysfunctional (ρ^0 cell line and a cell line treated with 0.1 μg/mL of oligomycin), oxygen consumption was found to be markedly reduced, indicating that the respiration levels found in cells sensitive to 2-DG under normoxia correlate with functional oxidative phosphorylation. Thus, mitochondria seem be normal in tumor cells sensitive to 2-DG under normoxia, further supporting the hypothesis that inhibition of glycolysis does not seem to account for the toxic activity of 2-DG in these cells.

ATP Levels Are Decreased More by 2-FDG than by 2-DG in Sensitive Cells under Normoxia

In previous reports, it was suggested that 2-DG–induced decreases in ATP levels were the major mechanism responsible for cell death in tumor cells growing under normoxia (18). We also find that when the 2-DG–sensitive cell lines SKBR3 and 1420 are incubated with this sugar analogue, there is a significant decrease in ATP levels (Fig. 1C). However, when these cells are treated with 2-FDG, which is relatively nontoxic to these cells, ATP pools are inhibited even more than by 2-DG. Thus, reductions of ATP synthesis alone under normoxia do not seem to account for select toxicity of 2-DG.
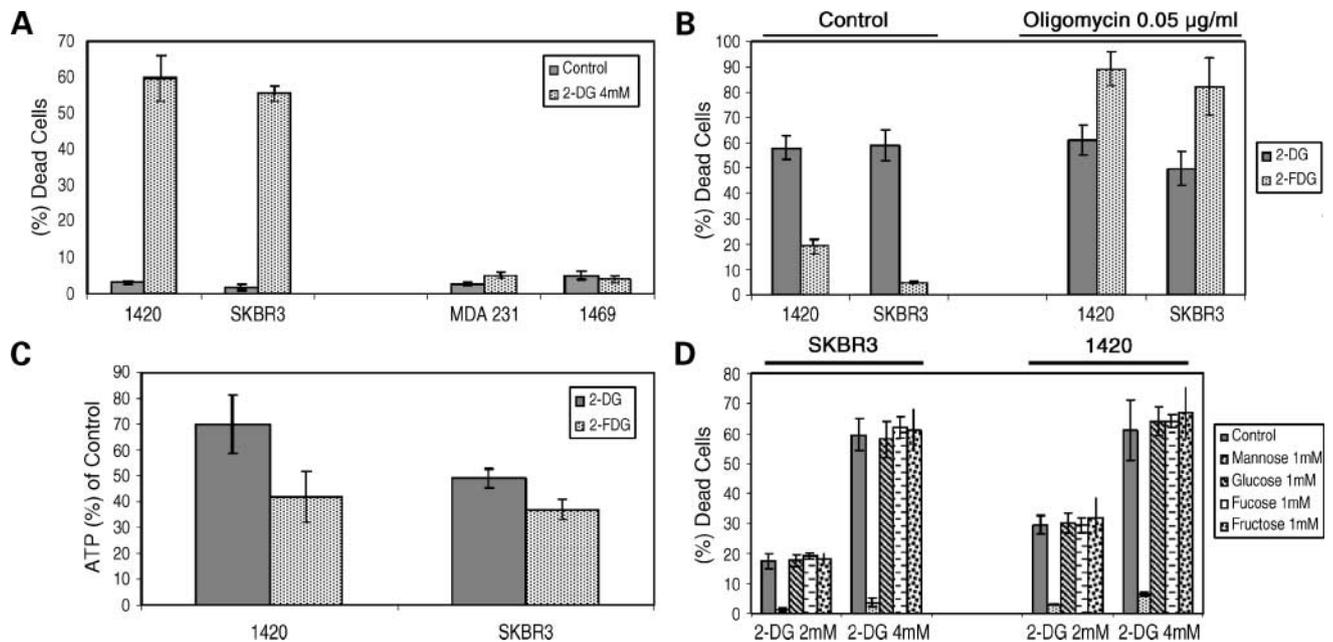
Figure 1. Effects of 2-DG and 2-FDG on ATP levels and viability in tumor cells under aerobic and anaerobic conditions: reversal by mannose. **A,** cells were treated with 4 mmol/L of 2-DG for 72 h and the percentage of dead cells was assayed by trypan bue exclusion. **B,** 2-DG – sensitive cells were treated with 4 mmol/L of 2-DG or 2-FDG under aerobic or anaerobic (oligomycin 0.05 μg/mL) conditions and the percentage of dead cells was assayed by trypan bue exclusion. **C,** in cells treated with either 4 mmol/L of 2-DG or 2-FDG under normoxic conditions, the percent reduction of ATP was calculated by dividing the amount found in treated samples by the amount in untreated controls. ATP concentrations were normalized to total protein as measured by bicinchoninic acid protein assay. To minimize complications in data interpretation arising from differential toxicity induced by 2-DG versus 2-FDG, treatment with each sugar analogue was applied for 24 h. **D,** 1420 and SKBR3 cells were treated with increasing concentrations of 2-DG in conjunction with 1 mmol/L of various sugars followed by analysis of cytotoxicity with trypan blue exclusion assays. Note that only mannose reverses 2-DG toxicity. *Columns,* average of triplicates; *bars,* SD.

Table 1. The comparison of oxygen consumption in 2-DG – sensitive versus 2-DG – resistant cell lines

Cell line	Tissue type	Average O_2 consumption (nmol/10^6 cells/min)
143B + oligomycin	Osteosarcoma with oxidative phosphorylation inhibitor	0.97 ± 0.11
ρ^0	Osteosarcoma	0.09 ± 0.004
1469	Pancreatic cancer	3.38 ± 0.32
SKBR3	Breast adenocarcinoma	2.01 ± 0.29
1420	Pancreatic adenocarcinoma	3.50 ± 0.03
MDA 231	Breast cancer	2.76 ± 0.04

Low-Dose Mannose, But Not Glucose, Reverses 2-DG Toxicity in Normoxic Cells

As mentioned above, Schwarz's group showed in the early 1970s that in viral glycoproteins, 2-DG was more potent than 2-FDG in inhibiting the assembly of LLOs, the precursors of N-linked oligosaccharides, and this inhibition could be reversed by exogenous mannose (11). Coinciding with these results, we find that the toxicity of 2-DG in cells growing under normoxia can be completely reversed by the exogenous addition of mannose, but not other sugars (i.e., glucose, fructose, and fucose), at 1 mmol/L concentrations (Fig. 1D). A trivial explanation for this result is that mannose might be interfering with 2-DG uptake. However, this possibility was ruled out in separate experiments (data not shown) in which radiolabeled 2-DG accumulation in sensitive cells did not change when exogenous mannose was added. Moreover, this interpretation is further supported by results which show that mannose does not reverse 2-DG toxicity in three distinct models of anaerobiosis (Supplementary Fig. S1).[5] Thus, it seems that mannose does not affect the ability of 2-DG to inhibit glycolysis and yet reverses the toxicity induced by this sugar analogue under normoxia. These results, together with those showing that 2-FDG shows little or no toxicity in the cell lines sensitive to 2-DG (Fig. 1B), suggest that the 2-DG–induced death in these cells is mediated by interference with N-linked glycosylation rather than by inhibition of glycolysis. It is important to note that a low dose of mannose (1 mmol/L) is sufficient to completely reverse cell death caused by 4 mmol/L 2-DG in the presence of relatively higher concentrations of glucose (12 mmol/L), whereas 1 mmol/L of additional glucose has no effect. Therefore, the competition between mannose and 2-DG seems to be the key determinant for the toxicity of this sugar analogue in cells growing under normoxia.

Schwarz et al. also showed that 2-FDM was similar to 2-DG (but somewhat less effective) in its ability to interfere with LLO synthesis. Based on this report, we investigated whether 2-FDM was toxic to cell lines that were found to be sensitive to 2-DG. As illustrated in Supplementary Fig. S2,[5] 2-FDM was similar to 2-DG (albeit less potent) in killing SKBR3 and 1420 cells. Overall, these data further suggest that interference with LLO synthesis by 2-DG and 2-FDM,

rather than inhibition of glycolysis, leads to cell death in select tumor types growing under normoxic conditions.

### 2-DG Interferes with N-Linked Glycosylation More Potently in Sensitive Cell Types

Previously, it was shown that the aberrant incorporation of 2-DG instead of mannose into LLOs was one mechanism by which 2-DG leads to disruption of LLO synthesis (11). To determine directly whether 2-DG interferes with LLO synthesis, and thereby N-linked glycosylation, the technique of FACE was used. In this procedure, which can be applied to cells grown under essentially any condition, glycans released from LLO pools by mild acid treatment, or from *N*-glycoprotein pools by enzyme treatment, are conjugated at their reducing termini with the anionic fluorophore ANDS. After electrophoresis in a mini-gel format, each ANDS-conjugated glycan can be quantitatively visualized with UV light.

Following incubation with 0.5 mmol/L 2-DG for 24 h (a lower 2-DG concentration than used in the 72 h toxicity experiments illustrated in Fig. 1), formation of mature LLO (represented by $G_3M_9Gn_2$) was significantly abrogated in the sensitive (1420 and SKBR3), but not in the resistant (1469 and MDA 231), cell lines (Fig. 2A). At a higher concentration of 2-DG (4 mmol/L), effects on LLOs were also observed in the resistant cells. When these data were quantitated, the amounts of mature LLOs ($G_3M_9Gn_2$) in both SKBR3 and 1420 cells were found to be reduced by >80% and >90% when treated with 0.5 or 4 mmol/L of 2-DG, respectively (Fig. 2C). In contrast, G3M9 was reduced in the resistant cell lines 1469 and MDA 231 by only <20% and ~80% when treated with 0.5 or 4 mmol/L of 2-DG, respectively (Fig. 2C). It should be noted that the difference of cellular material between sensitive and resistant cells due to 2-DG treatment was minimal (data not shown) and therefore could not appreciably account for lowered levels of LLO in sensitive cells. Moreover, when sensitive cells were cotreated with 0.5 mmol/L 2-DG and 1 mmol/L mannose, the synthesis of the mature LLOs was partially restored (~45% of control), which correlates with mannose reversal of 2-DG toxicity (Fig. 3A and B).

Losses of LLO by 2-DG treatment correlate with impaired N-linked glycosylation. Following both 0.5 and 4 mmol/L 2-DG treatments, less N-linked glycan was recovered from sensitive tumor cells compared with their resistant counterparts (Fig. 2B). In all cases, the impairment of N-glycosylation seemed to be less severe than the

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[5] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Figure 2. 2-DG interference with N-linked glycosylation in tumor cells growing under normoxia. A FACE technique was used to investigate the effects of 2-DG on LLO assembly (**A**) as well as on *N*-glycans (**B**). Cells were treated with either 0.5 or 4 mmol/L of 2-DG for 24 h, followed by extraction and FACE of LLOs. The standard oligosaccharides used in these studies are as follows: *G4* to *G7,* glucose oligomers; *G3M9,* mature oligosaccharide ($G_3M_9Gn_2$); *M5,* oligosaccharide intermediate (M_5Gn_2). **C,** the levels of mature LLO were quantified by measuring the density of the G3M9 bands in all cell types treated with either 0.5 or 4 mmol/L of 2-DG. The amount of LLO is shown as arbitrary units, which is calculated by the percentage of reduction in band intensity in treated as compared with control samples. *Columns,* average of two samples; *bars,* SD.
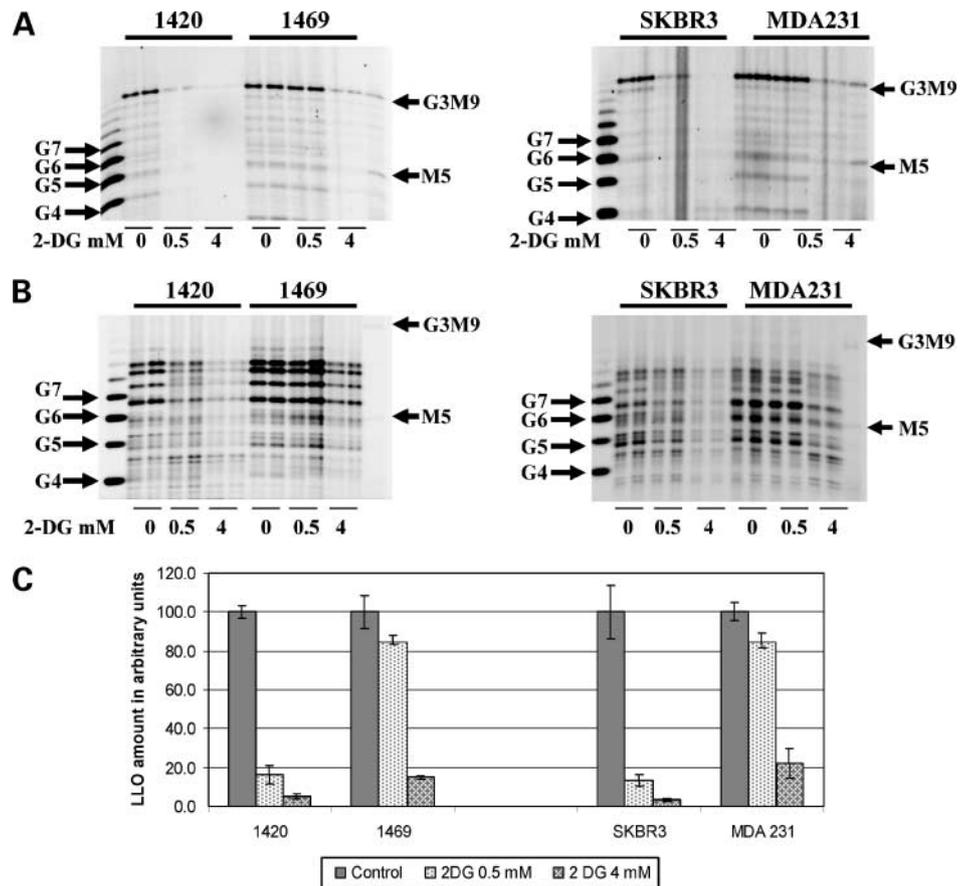
corresponding loss of LLO, an expected consequence of the high affinity of the glycan transfer system for $G_3M_9Gn_2$-P-P-dolichol. Overall, the potency of 2-DG in disrupting LLO synthesis (and thereby N-linked glycosylation), but not in inhibiting glycolysis, correlates well with its toxic activity in these four tumor cell types. This conclusion is further supported by the reversal of both 2-DG–induced LLO dysfunction and toxicity by 1 mmol/L mannose.

Sugar Analogues Induce an UPR in SKBR3 and 1420 Cells under Normoxia

Drugs that interfere with glycosylation have been shown to induce UPR, leading to increases in the protein folding capacity of endoplasmic reticulum by mechanisms including transcriptional up-regulation of chaperones such as GRP78/Bip and GRP94. As shown by Western blot, a dose of 0.5 mmol/L 2-DG, which disrupts LLO synthesis in sensitive but not resistant cells (Fig. 2A), induced a significant UPR response only in sensitive cells (Fig. 4A). When these blots were quantified, 7- to 10-fold increases in the expression of GRP78/Bip were detected in the sensitive but not in the resistant cell types (Fig. 4B). However, at higher concentrations of 2-DG (4 mmol/L), evidence of the UPR can be seen in all four cell lines (Fig. 4C and D). These data raise the possibility that cells that are resistant to 2-DG possess generally attenuated UPRs. However, this is unlikely because tunicamycin, which is known to interfere with N-linked glycosylation, induced UPR (Fig. 4C and D) and was found to be equally toxic in 2-DG–sensitive and 2-DG–resistant cell lines (data not shown).

Taken together, these results suggest that 2-DG is more potent in sensitive versus resistant cells in disrupting N-linked glycosylation, thereby leading to UPR, which correlates with the differential toxicity of 2-DG in these cell lines grown under normoxia. This conclusion was further supported by the finding that addition of 1 mmol/L of mannose, but not glucose, reverses the 2-DG up-regulation of chaperones in sensitive cells (Fig. 4C), which correlates with a similar reversal of 2-DG–induced toxicity by mannose as shown in Fig. 1D. Furthermore, when SKBR3 and 1420 cells were treated with 2-FDM under normoxia, both GRP78 and GRP94 were up-regulated, which correlates with preferential toxicity of this mannose analogue in these cells (Fig. 4C). In comparison, 2-FDG was less potent than 2-DG or 2-FDM in inducing these chaperones in SKBR3 and 1420 cells. These results correlate with previous data in viruses that 2-FDM and 2-DG, but not 2-FDG, are shown to competitively inhibit mannosyltransferase, an enzyme required for oligosaccharide assembly of viral N-linked glycoproteins (19, 20). Thus, overall these data suggest that the ability of 2-DG and 2-FDM to compete with mannose in oligosaccharide synthesis, and for 2-DG to incorporate fraudulently into oligosaccharides, is central to the toxic
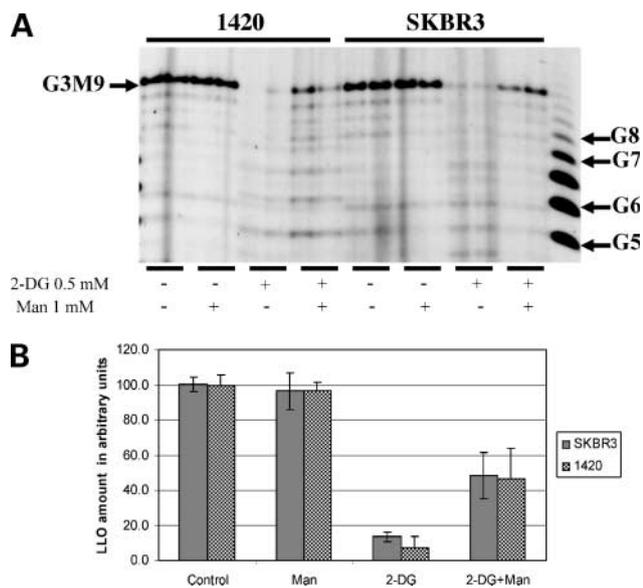
Figure 3. Reversal of the effects of 2-DG on LLO synthesis by exogenous mannose. **A,** using the FACE technique, LLO levels were investigated in two sensitive cell lines, SKBR3 and 1420, following treatment with 0.5 mmol/L 2-DG in the presence or absence of 1 mmol/L mannose for 24 h. **B,** the G3M9 band was quantified by measuring the density of the band, and the amount of LLO is shown as arbitrary units, which are calculated by percent reduction in the band intensity of treated as compared with control samples. Note that mannose is without effect on LLO synthesis in untreated control cultures. Standards are as in Fig. 2.

activity of these mannose analogues in select tumor cells growing under normoxia.

The Toxicity of 2-DG in SKBR3 and 1420 Cells Correlates with Up-regulation of CHOP/GADD153, an UPR-Specific Apoptotic Pathway

How might the 2-DG–induced dysfunction in LLO and *N*-glycan synthesis lead to cell death? It has been reported that when cells cannot overcome endoplasmic reticulum stress, an UPR-specific apoptotic pathway is induced via up-regulation of the CHOP/GADD153 gene (21, 22). In this regard, we find that 2-DG and tunicamycin up-regulate CHOP/GADD153 expression, indicating that this UPR-specific apoptotic pathway is activated by these agents (Fig. 5A). Furthermore, at this time point, the percentage of cells that are dually stained by Annexin V and propidium iodide (indicating cell death due to apoptosis) is significantly increased by 2-DG and tunicamycin (Fig. 5B). In this figure, a negative control is included, which illustrates that staurosporine, a cytotoxic agent, which neither interferes with N-linked glycosylation nor induces UPR, is found not to activate CHOP/GADD153 but kills these same cells via apoptosis. Moreover, 2-FDG yields little or no increased expression of CHOP/GADD153 in these cells (Fig. 5A). The slight activation of this UPR-specific apoptotic factor by 2-FDG in 1420 cells correlates with the small amount of apoptosis it induces in these cells (Fig. 5B) as well as UPR (Fig. 4C). Overall, our data with CHOP/GADD153 suggest that 2-DG–induced

cell death under normoxia is mediated via an UPR-specific pathway of apoptosis.

Discussion

In addition to generating ATP, glycolysis provides an important source of vital structural components required for growth and replication such as DNA and RNA sugars, as well as certain amino acids. As such, it is not surprising that when this pathway is blocked by 2-DG or 2-FDG, growth inhibition will occur even in cells respiring under normal oxygen tension. If, however, cells carry a defect in their respiratory or mitochondrial ability to produce ATP, glycolytic inhibitors then become toxic in these cell types (3, 10, 23, 24) even under normoxic conditions. To investigate whether the cell types we found to be sensitive to 2-DG under normoxia had a defect in respiration, we assayed their oxygen consumption and found them to exhibit a pattern of respiration that was similar to that of cells resistant to 2-DG (Table 1). Moreover, because 2-FDG has been shown to be a more potent inhibitor of glycolysis than 2-DG (12), cells with a mitochondrial defect would be expected to suffer greater toxicity with the former analogue. The finding that 2-FDG showed little or no toxicity in these cell types growing under normoxia (Fig. 1B) further supported our hypothesis that a mechanism other than blockage of glycolysis was responsible for 2-DG–induced toxicity.

A clue to this other mechanism came from data in which 2-DG, 2-FDG, and 2-FDM were investigated for their relative abilities to interfere with LLO synthesis and, ultimately, N-linked glycosylation of viral glycoproteins (13). The conclusion from those studies was that 2-DG and 2-FDM, because of their similarity in structure to mannose, could compete with the incorporation of this natural sugar into N-linked oligosaccaharide precursors during the process of viral glycoprotein synthesis, whereas 2-FDG could not (1, 13, 19, 20, 25, 26).

Additionally, in these studies 2-FDM was shown to be a less potent inhibitor of glycosylation than 2-DG because the latter analogue both competes with mannose and incorporates fraudulently into LLOs whereas the former competes but does not incorporate. Furthermore, 2-FDG was found to be the least effective in interfering with oligosaccharide assembly when compared with 2-DG and 2-FDM because this sugar analogue cannot compete with GDP-mannose for mannosyltransferases or incorporate into oligosaccharides. Overall, their results showed that the potency of these analogues to inhibit viral glycoprotein synthesis was in the order of 2-DG > 2-FDM > 2-FDG (11), which is similar to what we find here for the toxicity of these analogues in SKBR3 and 1420 cells growing under normoxia.

Another key finding by Schwarz's group was that the inhibitory effects of these analogues on viral glycoprotein synthesis could be reversed by exogenous addition of low-dose mannose (8). Similarly, we found that 1 mmol/L mannose reverses 2-DG and 2-FDM toxicity in both cell lines, suggesting that these mannose analogues kill select tumor cells by interfering with N-linked glycosylation.

Although mannose is a core sugar in N-linked glycosylated proteins, it can also participate in the glycolytic pathway because it can be converted to fructose-6-phosphate by phosphomannoisomerase. Thus, mannose may be reversing the toxicity of 2-DG in SKBR3 and 1420 cells by circumventing the glycolytic step, which 2-DG might purportedly inhibit (Fig. 6). However, this possibility seems to be unlikely because 1 mmol/L of mannose did not reverse cell death induced by 2-DG in all three ''hypoxic'' models, whereas it did reverse much of the inhibitory effect of 2-DG on LLO synthesis in the sensitive cells (Supplemental Fig. S1).[5] Overall, the reversal of 2-DG and 2-FDM toxicity by mannose in cells sensitive to these sugar analogues under normoxia, but not under hypoxia, further supports the hypothesis that under normoxia, mannose analogues are toxic to select tumor cells by interfering with glycosylation and not as a result of inhibition of glycolysis.

The direct effects of 2-DG on LLO synthesis and N-glycosylation in the 2-DG–sensitive and 2-DG–resistant cell pairs were investigated by the FACE technique (17), which detects changes in oligosaccharide composition. At a nontoxic dose of 2-DG (0.5 mmol/L), mature LLO ($G_3M_9Gn_2$-P-P-dolichol) formation was found to be reduced by 80% in sensitive cells but only by 20% in resistant cells, and this effect could be partially reversed by 1 mmol/L mannose (Figs. 2 and 3). However, at a toxic dose of 2-DG for sensitive cells (4 mmol/L) LLO synthesis was reduced by >95%, whereas at the same dose in resistant cells (which is

nontoxic) oligosaccharides were decreased by 80%. It should be noted that at either 0.5 mmol/L in sensitive cells or 4 mmol/L 2-DG in resistant cells, there is no toxicity although appreciable loss of N-glycosylation takes place. Thus, our data indicate that >80% reduction of LLO synthesis is required for 2-DG–mediated cell death under normoxia.

In general, reductions in LLO synthesis by 2-DG treatment were found to correlate with decreases in total N-glycan pools. Because there are numerous cellular glycoproteins with different turnover rates, it is not practical to choose a single N-glycan for quantification of the effectiveness of 2-DG on N-linked glycosylation. Nevertheless, it is clear that N-glycan pools were reduced more by 2-DG in sensitive versus resistant cells, which is in good correlation with the effects of 2-DG on LLOs.

Inhibition of N-linked glycosylation leads to disruption in the folding of glycoproteins, which are then either retained in the endoplasmic reticulum and/or degraded (27, 28). Endoplasmic reticulum accumulation of unfolded proteins results in distention of this organelle as well as perturbed protein translation. In such an event, cells initiate a signaling cascade, known as the UPR, to reestablish homeostasis in the endoplasmic reticulum. This results in a number of metabolic and molecular alterations including up-regulation of sugar transporters, increases in phospholipid synthesis, amino acid transport, and increased expression of GRP78/BiP and another molecular chaperone, GRP94 (29–32). Our findings that the expression of
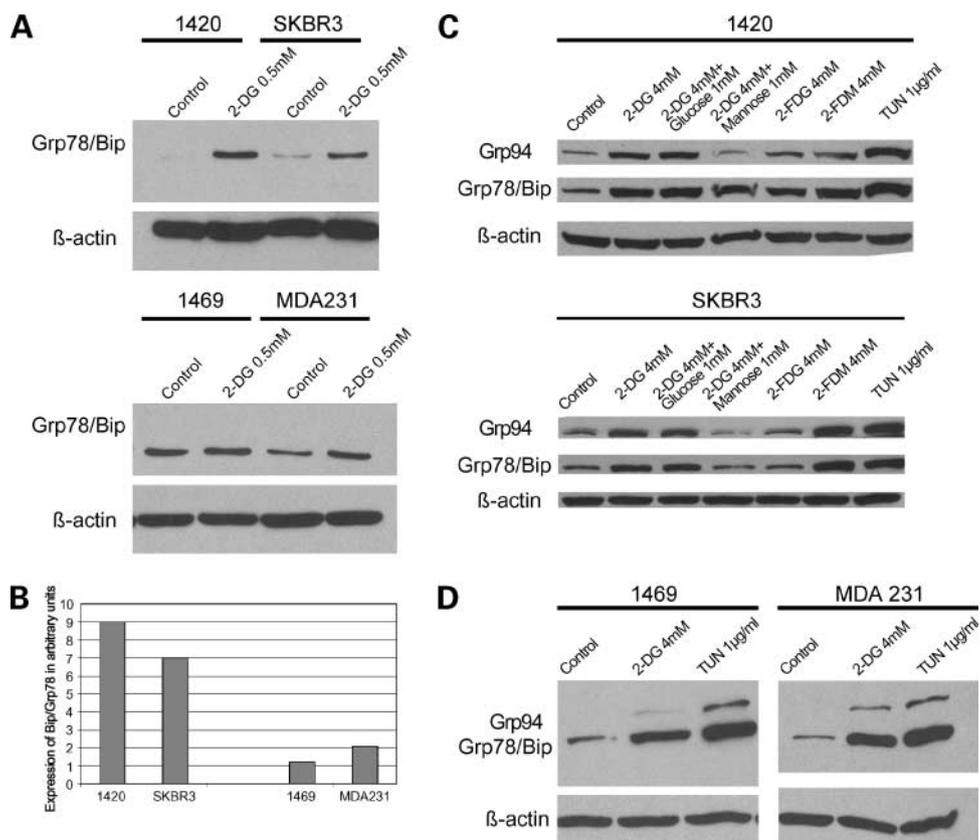
Figure 4. UPR induction by 2-DG in sensitive and resistant cells. **A,** GRP78/Bip expression was assayed by Western blot in cells treated with 0.5 mmol/L 2-DG for 24 h. Note the marked increase in GRP78/Bip expression in 1420 and SKBR3 but not in 1469 and MDA 231 cells. **B,** using a gel reader, the amount of GRP78/Bip was quantified. Induction of GRP78/Bip was calculated by dividing its amount in 2-DG–treated samples by the amount in untreated control samples. Each amount was normalized to the expression of β-actin. Western blot analysis of GRP78/Bip and GRP94 in SKBR3 and 1420 cells (**C**) or 1469 and MDA 231 cells (**D**) treated with the indicated sugar analogues or tunicamycin (*TUN*) for 24 h. β-Actin was assayed to compare the loading of samples.

GRP78 and GRP94 are up-regulated by 2-DG and 2-FDM in SKBR3 and 1420 cells under normoxia, which can be reversed by mannose, are highly consistent with the FACE data showing that these sugar analogues interfere with N-linked glycosylation. Furthermore, 2-FDG is not as effective as 2-DG and 2-FDM in inducing an UPR response in both 1420 and SKBR3 cells, coinciding with the order of our cytotoxicity results, which similarly show that 2-DG > 2-FDM > 2-FDG in killing these cells under normoxia.

The findings that tunicamycin does not select between 2-DG–sensitive and 2-DG–resistant cells indicates that differences in the UPR pathway between these cells are unlikely responsible for their differential response to 2-DG. Thus, the data in which we show that 2-DG at low dose (0.5 mmol/L) induces UPR in sensitive but not resistant cells suggests that this sugar analogue, in contrast to tunicamycin, has differential effects on glycosylation in these cell types, which accounts for its selective toxicity. At higher concentrations (4 mmol/L 2-DG), UPR seems to be equally induced in both 2-DG–sensitive and 2-DG–resistant cells. Although at this dose of 2-DG LLO synthesis is reduced more in sensitive than in resistant cells, it remains unclear why UPR seems to be the same in both cell types.

UPR is thought to be much like p53 in that DNA damage signals cell cycle arrest, activation of DNA repair enzymes, and, depending on the outcome of these processes, apoptosis. Thus, if the UPR fails to establish homeostasis within the endoplasmic reticulum, endoplasmic reticulum stress–specific apoptotic pathways are activated (33). Among the mediators of UPR-induced apoptotic pathways are caspase-4, caspase-12, and CHOP/GADD153. However, it has been shown that activation of CHOP/GADD153 is a better indicator of the endoplasmic reticulum–induced mammalian apoptotic pathway (21, 22) than activation of the others. Thus, that the expression of CHOP/GADD153, as shown in Fig. 5, is found to correlate with 2-DG– and 2-FDM–induced toxicity in SKBR3 and 1420 cells growing under normoxia, but not with that of staurosporine (a non–UPR-inducing toxic agent), further supports our hypothesis that these sugar analogues are toxic via interference with glycosylation leading to endoplasmic reticulum stress.

One possibility to explain differential 2-DG toxicity is the accumulation of higher intracellular concentrations of 2-DG metabolites in sensitive as opposed to resistant tumor cells. Using radioactive 2-DG, we indeed found that after 10-min incubation, sensitive cells were found to accumulate significantly more 2-DG than resistant cells, and this difference was even greater at 20 min (Supplementary Fig. S3).[5] Several mechanisms could account for increased accumulation of 2-DG in sensitive cells, which include higher expression of glucose transporters (34, 35), enhanced hexokinase activity (36, 37), and decreased glucose phosphatase activity (38). These possibilities are currently being investigated in our lab.

In conclusion, 2-DG and 2-FDM seem to be killing certain tumor types via interference with glycosylation leading to endoplasmic reticulum stress and endoplasmic reticulum–specific apoptotic cell death. The finding that 2-FDG does not kill these cells further supports the hypothesis that 2-DG and 2-FDM toxicity is via this pathway and not as a
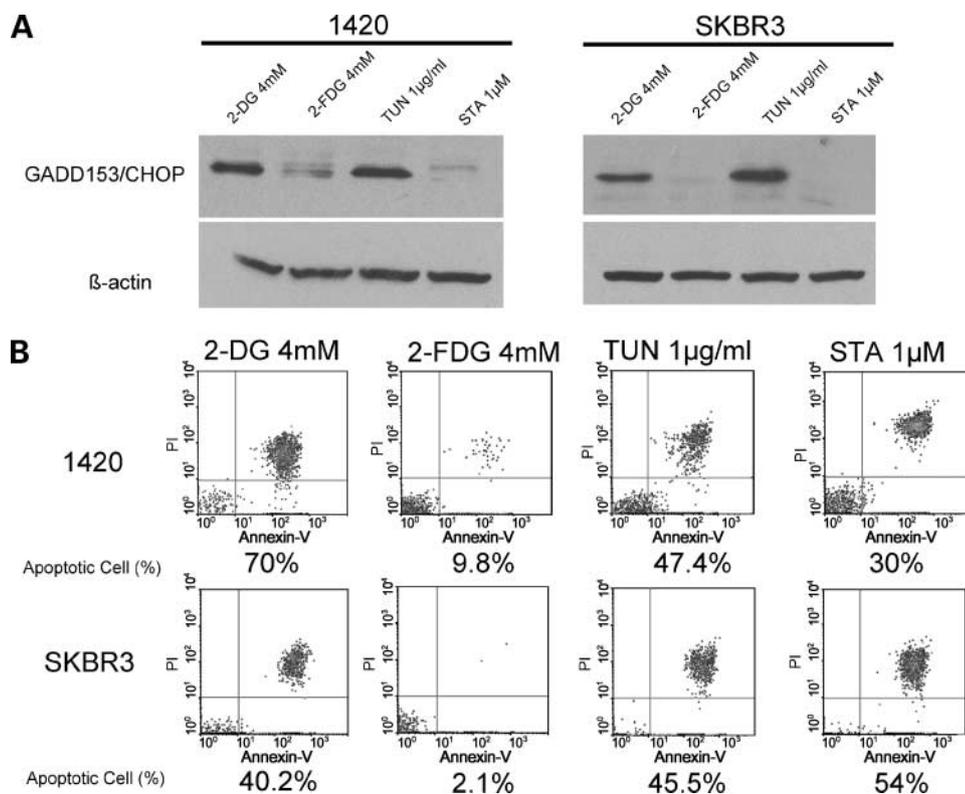
Figure 5. Assay of UPR-specific apoptotic factor CHOP/GADD153 and apoptosis in 2-DG–sensitive cells. **A,** CHOP/GADD153 expression was assayed by Western blot after 48 h of treatment with 4 mmol/L of either 2-DG or 2-FDG or 1 μg/mL of tunicamycin. Due to high toxicity, treatment with 1 μmol/L staurosporine (*STA*) was restricted to 12 h. **B,** parallel experiments were done to quantify the apoptotic cell percentage by Annexin V/propidium iodide staining and analysis via flow cytometer. The quadrants that divide cell populations into four groups (control, early apoptotic, late apoptotic, and necrotic) were set by treating these same cells with a known inducer of apoptosis, staurosporine, or an inducer of necrosis, 70% ethanol.

GLYCOLYSIS   **N-LINKED GLYCOSYLATION**
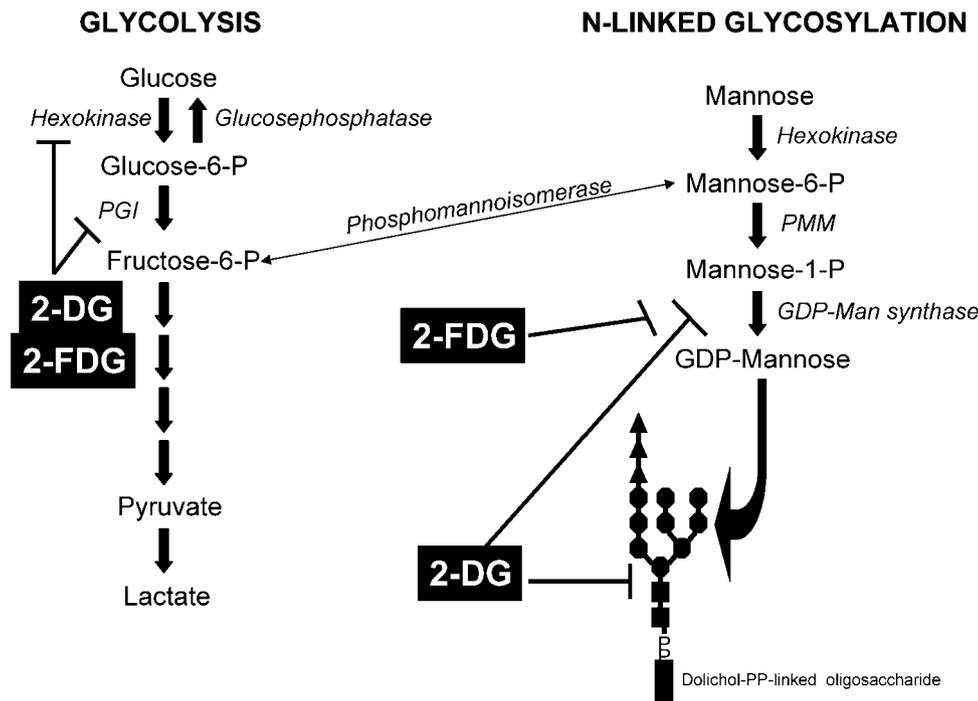
Figure 6. Overall scheme of the effects of 2-DG and 2-FDG on glycolysis and N-linked glycosylation. 2-FDG inhibits glycolysis better than does 2-DG. In contrast, 2-DG is more potent than 2-FDG in interfering with N-linked glycosylation. Due to the resemblance of 2-DG with mannose, it not only competes with mannose metabolism in this pathway but also incorporates fraudulently into dolichol-pyrophosphate (lipid) – linked oligosaccharides, which are the precursors for N-linked glycosylation. Although 2-FDG has been shown to decrease mannose metabolites, the position of the fluorine group in 2-FDG restricts it from resembling mannose and, therefore, does not have a direct inhibitory effect on mannose incorporation into LLO as does 2-DG. The greater activity of 2-DG versus 2-FDG on N-linked glycosylation correlates with its toxic activity in selected tumor types growing under normal oxygen tension, which can be reversed by addition of exogenous mannose (▲, glucose; ●, mannose; and ■, N-acetyl-glucosamine).

result of inhibition of glycolysis and ATP depletion. Currently, in phase I clinical trials, 2-DG is being used in combination with standard chemotherapeutic agents based on the concept that the latter kills the rapidly growing normoxic segments of the tumor whereas 2-DG targets the slow-growing hypoxic tumor cells. However, the findings presented in this article raise the possibility that in select solid tumors 2-DG can be used as a single agent to kill both normoxic (by interfering with glycosylation) and hypoxic (by blocking glycolysis) cell populations (Fig. 6).

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