

Effect of 2-Deoxy-D-glucose on Various Malignant Cell Lines *In Vitro*

XIAO DONG ZHANG¹, EDWIGE DESLANDES¹, MARIE VILLEDIEU¹, LAURENT POULAIN¹, MARILYNE DUVAL¹, PASCAL GAUDUCHON¹, LAURENT SCHWARTZ² and PHILIPPE ICARD^{1,3}

¹Groupe Régional d'Etudes sur le Cancer (Greca),

Unité «Biologie et Thérapies Innovantes des Cancers Localement Agressifs», CLCC François Baclesse, Caen;

²Service de Radiothérapie, Hôpital de La Pitié-Salpêtrière, Paris;

³Service de Chirurgie Thoracique et Cardio-Vasculaire, CHU de Caen, France

Abstract. *Background:* 2-Deoxy-D-glucose (2-DG) is an analog of glucose that is preferentially captured by tumors and is accumulated in transformed cells, because the phosphorylated molecule (2-DG-6P) cannot be metabolized or diffused outside the cells. Targeted with a fluorine atom, 18F-DG is currently used to visualize malignant tumors (PET scan). Although cancer cells have been reported to be strongly dependent on glycolysis (Warburg effect), very few reports have studied the inhibitory effects of 2-DG on cancer. *Materials and Methods:* Our objective was to study, in a large panel of human malignant cells of various origins (ovarian, squamous, cerebral, hepatic, colonic and mesothelial), if the inhibitory activity of 2DG against tumor growth could be considered a general phenomenon and to determine its effect on the cell cycle. *Results:* Four types of response in the different cell lines were observed when cells were cultured in the presence of 2-DG (5 mM) continuous exposure: proliferation slow down; proliferation arrest without signs of apoptosis; strong cell cycle arrest accompanied by moderate apoptosis induction; massive apoptosis. *Conclusion:* 2-DG appears as an interesting new therapeutic agent against cancer *in vitro*, and should be tested in *in vivo* studies.

Although it is well known that glycolysis is accelerated in transformed cells, it is still under debate as to whether the energy production in such cells is partially or totally dependent on glycolysis.

Some authors suggested that the increase of glycolytic activity in cancer cells is due to an adaptation to the anaerobic microenvironment of some part of the tumour tissues (1, 2), whereas others considered it the consequence

of an abnormal oxidative phosphorylation (2) due to a primary metabolic or genomic disorder (1, 2). In any case, areas of hypoxic cells are often considered regions of slowly proliferating cells particularly resistant to conventional therapies. Hypoxia leads to the production of hypoxia-inducible factors (HIF) that stimulate enhanced anaerobic glycolysis (1). So, a link between hypoxia resistance to cell death and glycolysis is proposed. Since these cells mainly rely on glycolysis for survival, they could be hypersensitive to glycolytic inhibitors, as suggested by recent studies (3).

Since glycolysis is enhanced in transformed cells and provides, at least in part or in total, the energy for cell proliferation, the antiglycolytic strategy appears as a promising new anti-cancer therapy. However, few reports studied the inhibitory effects of 2-deoxy-D-glucose (2-DG) on cancer cells, either *in vitro* (3-6) or *in vivo* (7, 8).

2-DG is a glucose analog that is preferentially captured by cancer cells. Targeted with a fluorine atom, 18F-DG is currently used to visualize cancer tumors (PET scan). 2-DG blocks the first step of glycolysis. It is phosphorylated by hexokinase and this phosphorylated molecule (2-DG-6P) cannot be metabolized. It accumulates in the cells, because the reverse reaction is also blocked (4), as is hexokinase II, a key enzyme in the glycolytic pathway that is highly expressed in malignant cells compared with normal cells (9, 10). It is noteworthy that glycolysis provides several metabolic intermediates, such as the hexose monophosphates, which are precursors of the nucleic acids.

Our objectives were to determine in a large panel of human malignant cells of various origins if the inhibition activity of 2-DG against cancer cell growth could be considered a general phenomenon and to specify the nature of this effect.

Materials and Methods

Cell lines. Twelve human tumor cell lines of various origin were used for the experiments: 3 ovarian carcinoma cell lines (SKOV3, IGROV1, IGROV1-R10), 3 head and neck squamous cancer cell

Correspondence to: Xiao Dong Zhang, Greca, CLCC François Baclesse, Avenue Général Harris, 14076, Caen cedex 05, France. e-mail: x.zhang@baclesse.fr

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lines (SCC61, SQ20B, SCC12B2), 2 glioblastoma cell lines (U251, GL15), 2 mesothelioma cell lines (MSTO-211H, and NCI-H28), 1 hepatocarcinoma cell line (HepG2) and 1 colonic cell line (HT29D4).

The IGROVI and SKOV3 cell lines were established from human ovarian adenocarcinoma. IGROV1 was kindly provided by Dr. J. Bénard (IGR, Villejuif, France). SKOV3 and HepG2 was obtained from ECACC (Cerdic, Nice, France). An IGROVI subline (IGROVI-R10), presenting a 10-fold resistance to cisplatin compared to the parental cell line, was established by successive exposures to increasing doses of cisplatin, as described previously (11). HT29D4 was kindly provided by M. Marvaldi (Institut Paoletti-Calmette, Marseille, France). The mesothelioma cell lines MSTO-211H and NCI-H28, were obtained from the American Type Culture Collection (ATCC). The three tumor cell lines established from human head and neck squamous cell carcinomas were kindly provided by D. Bernard Géry (Centre François Baclesse, Caen, France): one was a radiosensitive cell line (SCC61) and two were relatively radioresistant cell lines (SQ20B and SCC12B2).

The SKOV3, IGROV1, IGROV1-R10, SCC61, SQ20B, SCC12B2, U251, MSTO-211H and NCI-H28 cell lines were maintained in RPMI 1640 medium (Gibco BRL, Lyon, France) supplemented with 20 mM Hepes, 10% heatinactivated fetal calf serum (Gibco BRL), 33 mM sodium bicarbonate (Gibco BRL) and with glutamax. Other cell lines (GL15, HepG2 and HT29D4) were grown in DMEM (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum, 4.5 mg/ml glucose, 1 mM sodium pyruvate, 33 mM sodium bicarbonate (Gibco BRL) and glutamax.

Chemicals. 2-DG was manufactured by Syntheval Chemical Laboratory (Caen, France). The 2-DG solution was prepared extemporaneously in culture media.

2-DG exposure. Cells (5×10^5 / 25 cm² flask) were continuously exposed to 5 mM of purified 2-DG. Cell viability was evaluated by the trypan blue exclusion method at various times after exposure (day 0, 2, 4, 7). All experiments were performed in duplicate.

Analysis of DNA cellular content by flow cytometry

a) Preparation of cells. After 2-DG treatment, the detached cells were collected separately. Adherent cells were then harvested by trypsin/EDTA dissociation. Adherent and detached cells were pooled and washed in PBS before being fixed in 70% ethanol and stored at -20°C until analysis. Before flow cytometry analysis, the cells were washed in PBS and incubated for 30 min at 37°C temperature in PBS in order to allow the release of low molecular weight (m.w.) DNA, characteristic of apoptotic cells, as recommended by Darzynkiewicz *et al.* (6). After centrifugation at 4000 xg for 10 min, the cell pellets were resuspended and stained with propidium iodide (PI) using the DNA Prep Coulter Reagent Kit (Beckman-Coulter) at a final density of 10⁶ cells/ml.

b) Instrument settings. The samples were analyzed using an EPICS XL flow cytometer (Beckman Coulter) equipped with an argon laser at 15 mW. PI-stained cells were analyzed using a 488 nm excitation. A 620 nm band pass filter was put on the red fluorescence of PI. Computerized gating was applied on the side and forward scatter to exclude very small debris and on pulse width and integral peak of red fluorescence to eliminate aggregates. All samples were analyzed at a flow rate lower than 100 events per second and with a sheath pressure of 30 psi.

Table I. Percentage of growth inhibition of various cell lines observed after 7 days of exposure to 2-deoxy-D-glucose (2-DG).

Origin	Name	% of growth inhibition after 2-DG exposure (7days)
Ovarian carcinoma	SKOV3	94.3
	IGROV1	94.2
	IGROV-R10	79.7
Mesothelioma	MSTO-211H	98
	NCI-H28	94
Glioblastoma	U251	95.7
	GL15	74.2
Head and neck squamous cancer	SCC12B2	95.1
	SQ20B	93.2
	SCC61	87.1
Hepatocarcinoma	HepG2	66.1
Colon cancer	HT29D4	63.7

c) Data analysis. EXPO 32 Acquisition Software (Beckman Coulter) was run for data acquisition.

Morphological characterization of apoptotic cells

Nuclear staining with diamidino-2-phenylindol (DAPI). The cells previously fixed in 70% ethanol were collected on a polylysine-coated glass slide by cytocentrifugation. The slides were incubated at room temperature in a solution of 1 µg/ml DAPI (Boehringer Mannheim, Germany) in water. After 30 min, they were extensively washed in distilled water and mounted in Mowiol (Calbiochem, Meudon, France). The slides were then observed in a Leica fluorescent microscope equipped with an ultraviolet (UV) filter.

Western immunoblotting. Cells were rinsed with ice cold PBS and lysed in 150 mM NaCl, 50 mM Tris-HCl pH 8, 1% Triton X100, 4 mM PMSF, 2 mM Aprotinin, 5 mM EDTA, 10 mM NaF, 10 mM NaPPI, 1 mM Na₃VO₄ for 30 min on ice. Lysates were clarified by centrifugation at 10,000 xg for 10 min at 4°C and protein concentrations were determined using the Bradford assay (Bio-rad, Hercules, USA). Equal amounts of total cellular protein (20 µg) were resolved in a Bis-tris-HCL buffered (pH 6,4) 4-12% polyacrylamide gel (NuPAGE® Novex® 4-12% Bis-tris gel, Invitrogen, Cergy Pontoise, France) for 35 min at 200V and electrophoretically transferred to a PVDF membrane (Millipore, Saint-Quentin-en-Yvelines, France) for 1 h and 15 min at 30V. The membrane was blocked for 1 h at room temperature in T-TBS (132 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.05% Tween 20) supplemented with 5% non-fat dry milk. The membrane was incubated for 1 h at room temperature in T-TBS-milk with the following primary antibodies: anti-PARP (1:1000, Cell-signaling Technology, Beverly, CA USA), anti-caspase-3 (1:1000, BD Biosciences Pharmingen, Le Pont de Claix, France), anti-cleaved caspase-3 (1:1000, Cell-signaling Technology, Beverly, CA, USA) and anti-α-tubulin (1:3000, Sigma, Saint Louis, MO, USA). After three washes with T-TBS, the membrane was incubated for 1 h at room temperature in T-TBS-milk with the adequate peroxidase conjugated secondary antibody (Anti-rabbit IgG, Cell-signaling Technology) and anti-mouse IgG, (Amersham, Orsay, France). After 3 washes with

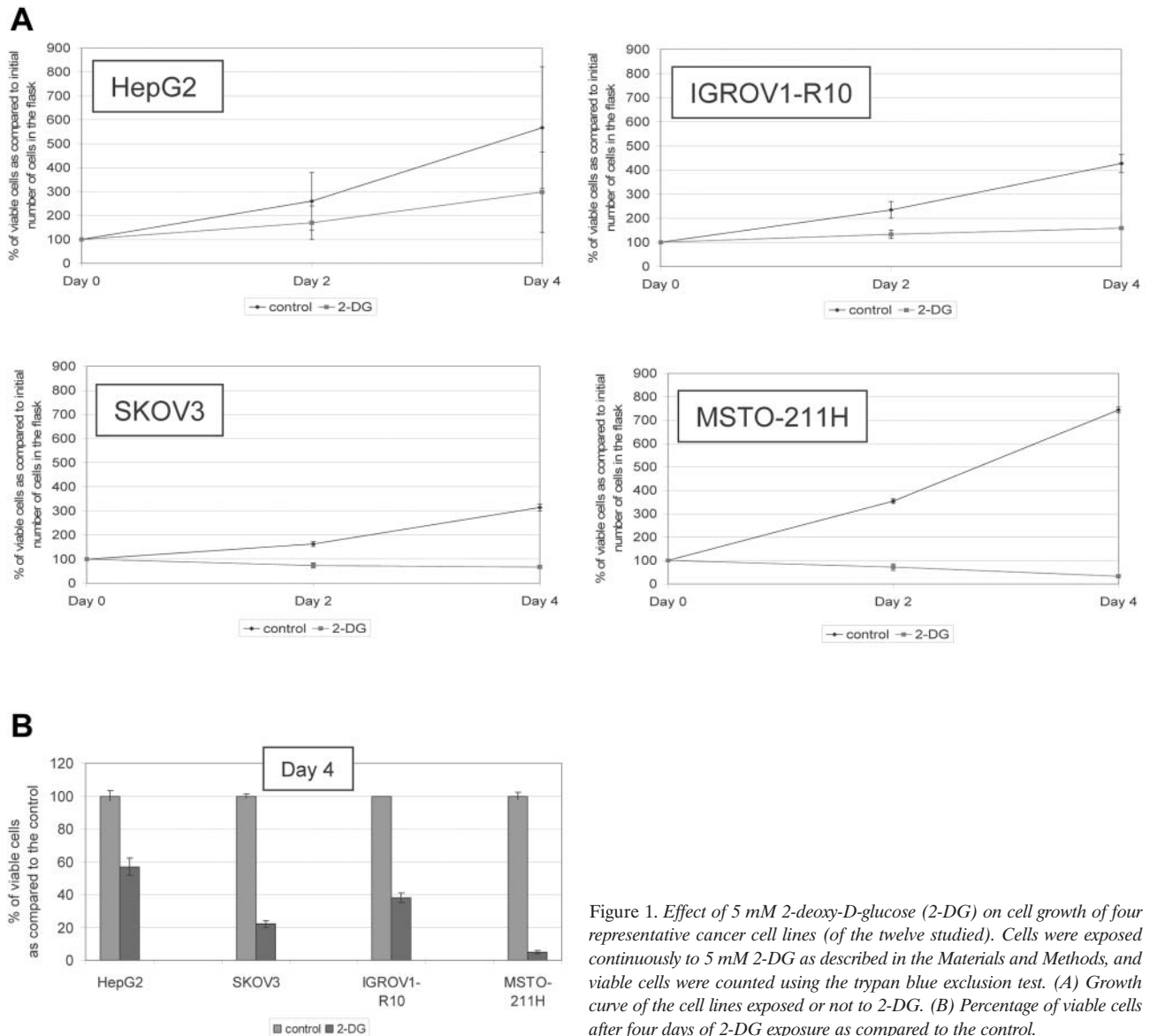


Figure 1. Effect of 5 mM 2-deoxy-D-glucose (2-DG) on cell growth of four representative cancer cell lines (of the twelve studied). Cells were exposed continuously to 5 mM 2-DG as described in the Materials and Methods, and viable cells were counted using the trypan blue exclusion test. (A) Growth curve of the cell lines exposed or not to 2-DG. (B) Percentage of viable cells after four days of 2-DG exposure as compared to the control.

T-TBS and one with TBS, the immunoreactivity was detected by enhanced chemiluminescence (ECL kit, Amersham, Orsay, France).

Results

Effect of 2-DG on cell growth and viability. The effect of 2-DG on the growth of a panel of twelve tumor cell lines of various origin was studied, as described in the Materials and Methods section. Three distinct phenomena were observed in response to 2-DG exposure (Figure 1A) in a selection of representative cell lines (hepatic HepG2, ovarian SKOV3 and IGROV1-R10 and mesothelioma MSTO-211H). In some cases, 2-DG only induced a slight slow-down in cell proliferation, whereas in other cases, it led to drastic growth

arrest, associated or not to cell death. The growth inhibition, observed in all cell lines, ranged between 40 and 95% compared to the control (Figure 1B and Table I) and was maintained in the majority of cases for at least one week. However, in two cases (HepG2 and HT29D4), this slow-down was only transient, and cells recovered a normal proliferation pattern after 4 days. In some cell lines (MSTO-211H for instance), the observed cytostatic effect became cytotoxic after two days.

Effect of 2-DG on cell cycle and apoptosis. As exemplified in Figure 2, cellular and nuclear morphologies, as well as the DNA content histogram confirmed that various responses could be observed after 2-DG exposure. In the HepG2 cells,

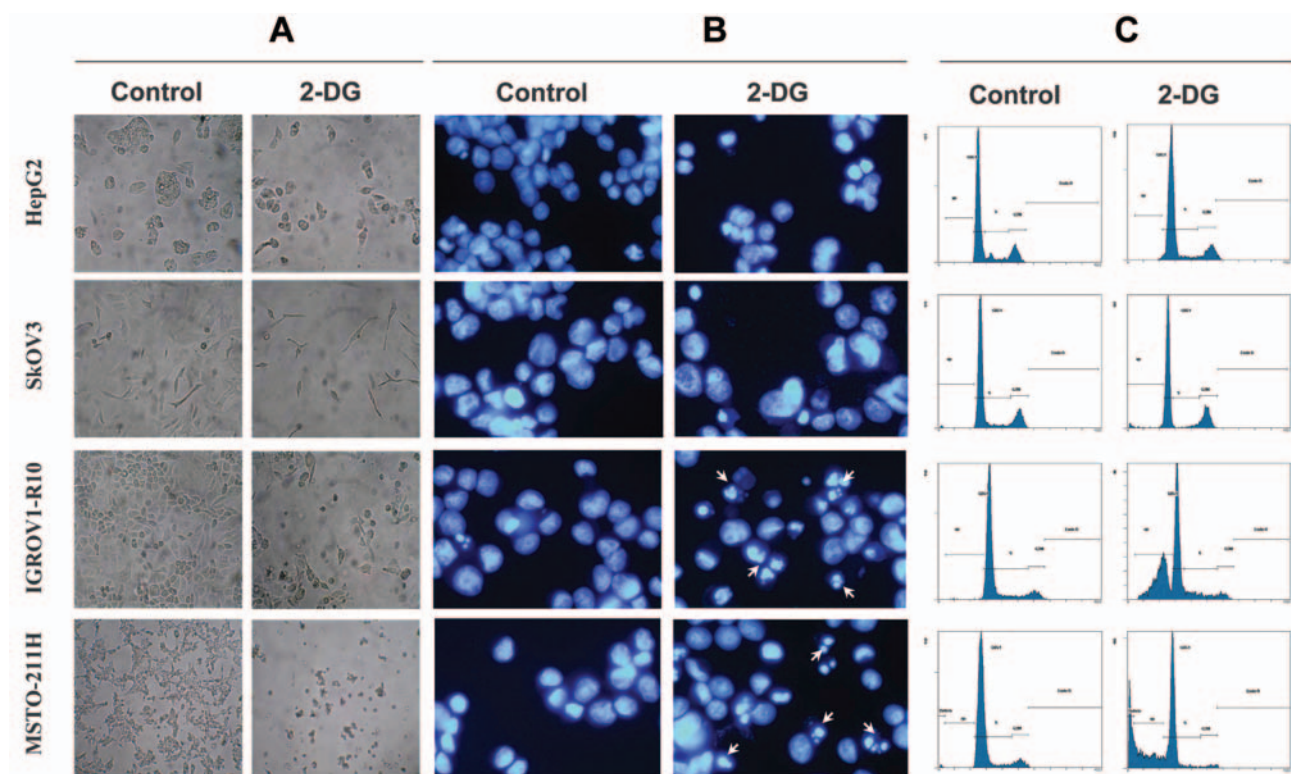


Figure 2. Effect of 2-deoxy-D-glucose (2-DG) exposure on cell morphology (A), nuclear morphology (B) and cell cycle repartition (C) in four representative cell lines, four days after the beginning of the exposure. Arrows indicate condensed and fragmented nuclei.

in which the growth slow-down was weak, the nuclear morphology appeared unaltered (Figure 2B) and the flow cytometry analysis showed that 2-DG exposure did not noticeably modify the cell cycle repartition compared to the control (Figure 2C). In the SKOV3 cells, in which 2-DG induced a strong cell growth arrest, a cell cycle blockade in G0/G1- and G2/M-phases and a complete disappearance of S-phase were observed (Figure 2C). However, no sign of apoptosis was observed in this cell line. In contrast, in the IGROV1-R10 and MSTO-211H cells, cell death features, such as cell shrinking and detachment were clearly detectable (Figure 2A and B). Moreover, DNA content histograms showed a sub-G1 peak (Figure 2C) and numerous nuclear condensations and fragmentations were observed after DAPI staining (Figure 2B). These results were collectively highly suggestive of the apoptotic nature of cell death. Moreover, in cells demonstrating a sub-G1 peak on DNA histograms after exposure to 2-DG, the apoptotic nature of cell death was confirmed by the demonstration of PARP cleavage and caspase-3 activation (Figure 3).

It should be noted that in the IGROV1-R10 cells, this apoptotic cell death probably occurred concomitantly to the proliferation of other cells, as suggested by the growth curve (since the total number of cells increased slightly after

exposure, Figure 1), by the subsistence of S- and G2/M-phases on DNA histogram (Figure 2C) and by the presence of proliferating clones in the flasks neighboring apoptotic clones (Figure 2A). On the contrary, 2-DG induced massive cell death in MSTO-211H and a drastic reduction in the number of viable cells occurred after 2-DG exposure (Figure 1A and B).

Thus, four types of response to 2-DG exposure, summarized in Table II, were observed in the different cell lines: (i) cell proliferation was not arrested, but only slowed down; (ii) cell proliferation was clearly stopped, but no sign of apoptosis was detectable; (iii) strong cell cycle arrest was accompanied by moderate apoptosis induction; (iv) cells mainly underwent apoptosis.

Discussion

Although Otto Warburg showed in the past that cancer cells, despite aerobic conditions, undergo accelerated glycolysis with the production of lactate (similar to that observed in yeasts) (2), few studies have attempted to interfere with cancer growth by inhibiting glycolysis (3-8, 11) and this anti-glycolytic strategy is not currently applied in clinical practice.

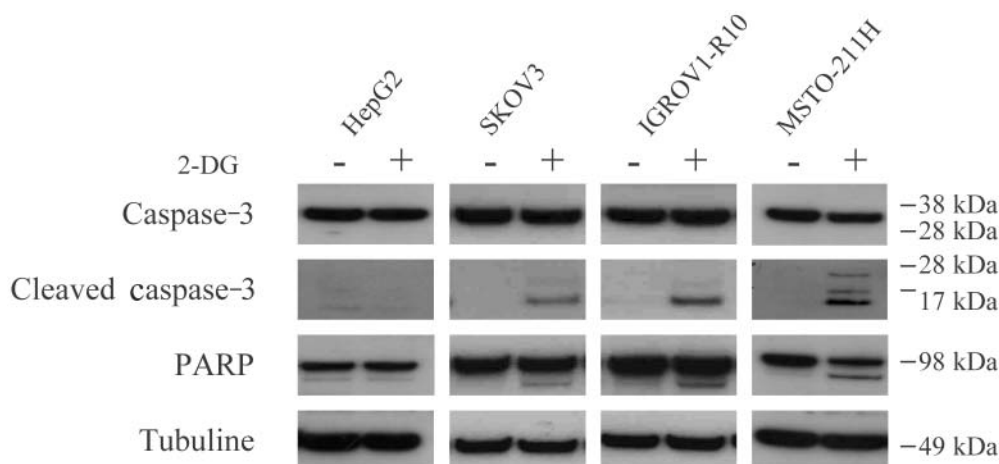


Figure 3. Effect of 2-deoxy-D-glucose (2-DG) exposure on PARP cleavage and caspase-3 activation as analyzed by Western blotting in four representative cell lines.

2-DG blocks cancer cell growth. Although previous studies have focussed on the ability of 2-DG to block cell growth, this effect was only in a few cell lines. Our study showed that the inhibition of transformed cell proliferation under 2-DG seemed to be quite universal. In our large panel of transformed cells, all cultures which received 2-DG at a concentration of 5 mM displayed slowed down or halted division. We chose the dosage of 5 mM of 2-DG, because it appeared empirically the best compromise with regard to previous reports that described an inhibition of cultures with 2-DG at doses ranging from 1 to 10 mM (4, 6).

In all our experiments, 2-DG slowed-down the cell cycle progression, demonstrating a cytostatic effect. It is noteworthy that the growth of some cell lines resistant to cisplatin (SKOV3) or to radiation therapy (SQ20B, SCC12B2) was stopped by 2-DG, sometimes with significant cytotoxicity.

Cell cycle perturbations occurred in most of the studied cell lines, affecting both G1/S and G2/M transition, occasionally leading to a "freezing" of the DNA content histogram. The observed effect appeared to be dependent on the cell line. The G0/G1 blockade observed in SKOV3 and MSTO-211H for instance, either associated with apoptotic induction or not, is in agreement with the results obtained on human histiocytic lymphoma U 937 by Halicka *et al.* (1995) (4, 6).

Cell death occurred in variable levels following 2-DG exposure to a concentration of 5 mM. Although six cell lines did not undergo apoptosis after 2-DG exposure, the six remaining cell lines presented moderate to high levels of apoptosis, as demonstrated by morphological modifications, altered nuclear morphology, sub-G1 peak occurrence, caspase-3 activation and PARP cleavage. Thus, in some cell lines, 2-DG may also exert a cytotoxic effect.

Table II. Classification of the twelve cell lines in terms of their cellular response to 2-deoxy-D-glucose (2-DG).

Response	Cell line
Proliferation slowed down	HT29D4, HepG2
Strong cell cycle blockade without apoptosis	SKOV3, SCC12B2, GL15, NCI-H28
Cell cycle arrest associated with moderate apoptosis	IGROV1-R10, U251
Strong apoptosis	IGROV1, SCC61, SQ20B, MSTO-211H

Interestingly, our results indicate that at least two cell lines (1 hepatocarcinoma cell line (HepG2) and 1 colonic cell line (HT29D4)) were "resistant" to 2-DG: after a transient cell growth arrest (2-4 days), these cells recovered a proliferation pattern and escaped the 2-DG induced initial blockage. This resistance could be due to the elimination of the drug outside of the cells as in normal cells, or to the occurrence of an alternative pathway constitutive of an adaptive metabolism. Anyway, the IC 50% should be determined for each cell line in order to assess whether the cytotoxic effect was dose-dependent.

How does 2-DG block cancer cell growth? Glycolysis inhibition may favor apoptosis. It was recently demonstrated that inhibition of glucose metabolism promoted the activation of both mitochondria-dependent and -independent pathways, both leading to apoptosis (12). This was also the case in our study, since 2-DG provoked caspase-dependent pathway death in some cultures.

Several authors suggested that the link between glucose deprivation and apoptosis might reside at the hexokinase level (13, 14), which constitutes a target of 2-DG (5). It was reported that in liver mitochondria of mice, glucokinase (hexokinase IV) resides with the pro-apoptotic protein Bad in a mitochondrial complex (with the protein phosphatase PP1), that interacts both with glycolysis and apoptosis (13). It was recently reported (14) that inhibition of hexokinase with 3-bromopyruvate caused apoptosis in two human leukaemia and lymphoma cell lines. Inhibition of hexokinase led to dephosphorylation of Bad, causing the translocation of the pro-apoptotic factor Bax to the mitochondria, thus, activating the release of cytochrome c and the caspase cascade (4, 6).

Considering these different elements, further investigations should be performed to identify the mechanisms underlying the "resistance" to 2-DG, such as the effect of increased concentrations of 2-DG, the phosphorylation status of Bad in response to 2-DG or the over-expression of anti-apoptotic proteins of the Bcl-2 family in resistant cells.

In summary, in response to 5 mM 2-DG, the growth of all the studied tumour cell lines of various origins was slowed down and half of these lines underwent apoptosis. Some of these lines were resistant to chemo and/or radiotherapy. In the other cell lines, the mechanisms responsible for the "resistance" to 2-DG should be further investigated.

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