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# Hypoxia increases tumor cell sensitivity to glycolytic inhibitors: a strategy for solid tumor therapy (Model C)

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**Abstract**

Previously, we reported that two distinct *in vitro* tumor cell models of hypoxia (Models A and B) are hypersensitive to glycolytic inhibitors such as 2-deoxy-D-glucose (2-DG) and oxamate [Liu *et al*., Biochemistry 2001;40:5542–7]. Model A consists of osteosarcoma cells (143B) treated with agents that interfere with mitochondrial oxidative phosphorylation (OxPhos), and Model B represents $\rho^0$ cells, a variant derived from 143B cells, which, due to their deficiency in mitochondrial DNA, cannot perform OxPhos. Extending these studies, we report here on Model C, which is composed of 143B cells grown under various levels of external $O_2$ (0, 0.1, 0.5, 1, 5, 10, and 21%). At the lower levels of $O_2$ that we tested, 143B cells were hypersensitive to 2-DG and oxamate when compared with cells grown at a normal level of $O_2$. In contrast, 143B cells under hypoxic or aerobic conditions showed equal sensitivity to a standard chemotherapeutic agent, vinblastine. Furthermore, when treated under reduced $O_2$ amounts, $\rho^0$ cells displayed no hypersensitivity to 2-DG and, in fact, were slightly more resistant than under aerobic conditions. At 0–5% $O_2$ levels, untreated 143B cells displayed reduced growth and elevated lactic acid levels, while $\rho^0$ cell growth remained unaffected except at 0% $O_2$ where growth was inhibited by 19%. The results with Model C are in agreement with our previous data using Models A and B, and extend these studies by illustrating that within a wide range of hypoxia the growth of tumor cells is retarded and that these slow-growing cells become hypersensitized to glycolytic inhibitors. Taken together with Models A and B, the data with Model C support our hypothesis that the hypoxic environment of slow-growing cells found in the inner core of solid tumors renders them amenable to selective targeting with glycolytic inhibitors.


*Keywords:* Glycolysis; Mitochondria; Hypoxia; 2-Deoxy-D-glucose; Oxamate

## 1. Introduction

Although attempts are being made to identify gene targets that are specific to tumor cells, currently the majority of anti-cancer agents and irradiation treatments used clinically attack the most rapidly dividing cells in the body, regardless of whether they are tumor or normal cells. Thus, the acute dose-limiting toxicity of these modalities of treatment is found in normal tissues which contain the most rapidly dividing cells, i.e. bone marrow and the gastrointestinal tract. Consequently, a major obstacle in the treatment of most solid tumors is the presence of slow-growing cells.

Since slow-proliferating tumor cells are found in the inner core of solid tumors, they are compromised in their accessibility to oxygen and rely more on glycolysis for ATP synthesis and survival. This is in contrast to what Warburg [1] proposed in 1930—that all tumor cells are naturally glycolytic and differ from normal cells in their usage of the glycolytic pathway. To investigate the hypothesis that the environment of tumor cells plays a major role in their metabolism and to devise a strategy to exploit this phenomenon for therapeutic gain, we developed two *in vitro* models (A and B), which mimic the hypoxic conditions found in the inner core of most solid tumors [2,3]. Hypoxic Model A consists of osteosarcoma cells (143B) treated with agents that interfere with mitochondrial OxPhos, and Model B represents $\rho^0$ cells, which, due to their deficiency in mitochondrial DNA, cannot perform OxPhos [2,3]. Data reported with both models indicate that

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*Abbreviations:* OxPhos, oxidative phosphorylation; 2-DG, 2-deoxy-D-glucose; HIF, hypoxic inducible factor; WT, wild type.

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cells, which are compromised in their ability to undergo OxPhos, become hypersensitive to glycolytic inhibitors such as 2-DG and oxamate. That the hypersensitization is due to the cell's switching from aerobic to anaerobic metabolism is supported by the findings that Models A and B produce increased levels of lactic acid [3]. Thus, our *in vitro* models, in which mitochondria are unable to normally perform OxPhos, reflect what should happen to cells found in the hypoxic regions of solid tumors.

To test this possibility more directly, we have developed a third *in vitro* model (Model C), in which tumor cells are exposed to decreasing concentrations of oxygen. Under these conditions, cell growth, sensitivity to glycolytic inhibitors, and conversion from aerobic to anaerobic metabolism were investigated. The following is a description of these studies and a discussion of their relevance to the overall goal of developing a strategy for overcoming a formidable form of multidrug resistance in solid tumors that occurs as a consequence of slow-growing cells.

## 2. Materials and methods

### 2.1. Cell types

An osteosarcoma cell line 143B (WT) was exposed to ethidium bromide for prolonged periods, and a mutant cell line (designated 206) with complete loss of mtDNA ($\rho^0$) was selected [4]. Since the $\rho^0$ cells are uridine and pyruvate auxotrophs, they were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/mL of uridine, 100 mM sodium pyruvate, and 10 μg/mL of gentamycin. To maintain standard experimental conditions, the parental cell line (WT) was grown in the same medium.

### 2.2. Growth inhibition

For growth inhibition assays, cells (1 mL) were seeded at $4 \times 10^4$/mL in 24-well plates, and drugs were applied 24 hr later. Drug treatments were continuous for 72 hr at 37° and 5% $CO_2$ at which time trypan blue exclusion cell counts were performed using a hemocytometer. Inhibitory concentrations of 50% were calculated for each drug tested.

### 2.3. Drugs

2-DG, oxamate, and vinblastine were obtained from the Sigma Chemical Co.

### 2.4. Lactic acid assay

Lactic acid was measured by adding 0.025 mL of deproteinated medium, from treated or non-treated cultures, to a reaction mixture containing 0.1 mL of lactic dehydrogenase (1000 U/mL), 2 mL of glycine buffer (glycine, 0.6 mol/L and hydrazine, pH 9.2), and 1.66 mg/mL of NAD. Deproteinization was brought about by treating 0.5 mL of the medium from test cultures with 1 mL of perchloric acid (8%, w/v), vortexing for 30 s, then exposing this mixture to 4° for 5 min, and centrifuging at 1500 *g* for 10 min at room temperature. The supernate was centrifuged three more times, and 0.025 mL of a final clear supernate was used for lactic acid determinations, as above. Formation of NADH was measured with a Beckman DU r 520 UV/vis spectrophotometer at 340 nm, which directly corresponds to lactic acid levels as determined by a lactate standard curve. Samples were run in triplicate.

### 2.5. Hypoxia

To study sensitivity to glycolytic inhibitors and other drugs under hypoxic conditions, cells were seeded in 24-well plates and placed in a Pro-Ox *in vitro* chamber attached to a model 110 oxygen controller (Reming Bioinstruments Co.). A mixture of 95% nitrogen and 5% $CO_2$ was used to perfuse the chamber to achieve the desired oxygen levels (21 to 0.1%).

## 3. Results

### 3.1. Growth inhibition and lactic acid production under various levels of hypoxia

Results in Table 1 demonstrate that when WT cells were exposed to decreasing levels of external oxygen ($O_2$), going from normoxic (21%) to anoxic (0%) conditions, at 5% $O_2$ and lower their growth was inhibited. The degree of inhibition correlated generally with the decrease in $O_2$ so that at 0% the cells were inhibited the most. In contrast, the growth of $\rho^0$ cells that were already metabolizing anaerobically, due to their deficiency in mitochondrial DNA, was unaffected by variances in external $O_2$ except at 0% where growth was inhibited by 19%. Lactate, an indicator of anaerobic metabolism, increased in WT cells as a function of decreasing $O_2$ levels (Table 2). Thus, exposure to 10% oxygen seems to initiate cells to change their metabolism

Table 1
Effect of oxygen levels on cell growth

| % Oxygen | Growth (% of control) | |
|---|---|---|
| | 143B (WT) | 206 ($\rho^0$) |
| 21 | 100 | 100 |
| 10 | 99 ± 1[a] | 101 ± 7 |
| 5 | 85 ± 7 | 110 ± 9 |
| 1 | 73 ± 3 | 108 ± 9 |
| 0.5 | 64 ± 8 | 103 ± 0 |
| 0.1 | 58 ± 0 | 113 ± 10 |
| 0 | 48 ± 4 | 81 ± 7 |

[a] Average of two separate experiments ± range.

Table 2
Effect of oxygen levels on lactate production

| % Oxygen | Lactate (% of control) | |
|---|---|---|
| | 143B (WT) | 206 ($\rho^0$) |
| 21 | 100 | 100 |
| 10 | 139 ± 1[a] | 108 ± 5 |
| 5 | 146 ± 12 | 101 ± 12 |
| 1 | 142 ± 3 | 101 ± 4 |
| 0.5 | 179 ± 36 | 97 ± 1 |
| 0.1 | 174 ± 2 | 95 ± 4 |
| 0 | 212 ± 27 | 106 ± 5 |

[a] Average of two separate experiments ± range.

from fully aerobic to partially anaerobic. As expected, $\rho^0$ cells did not change their lactate production significantly at any of the $O_2$ levels tested (Table 2).

### 3.2. Cell sensitivity to 2-DG under various degrees of hypoxia

Since in previous studies we found that increased sensitivity to glycolytic inhibitors correlated with increased lactic acid production in Models A and B [3], here in Model C we exposed WT cells to a concentration of 2-DG that yielded 58% growth inhibition under normoxic conditions and then varied the level of $O_2$ exposure. It can be seen in Fig. 1 that at every hypoxic oxygen level beginning with 10% $O_2$, WT cells became increasingly hypersensitized to 2-DG as a function of decreasing $O_2$ concentrations, whereas in $\rho^0$ cells the same level of heightened hypersensitivity to 2-DG was maintained regardless of the level of $O_2$ (Fig. 2). Fig. 3 illustrates that when WT cells were exposed continuously for 3 days to a standard chemotherapeutic agent, vinblastine, a drug that does not inhibit glycolysis, $O_2$ levels had no effect on the sensitivity of the cells to this drug. The data points in the figures were derived from calculating the percentage of growth compared with that of untreated controls grown under each of the oxygen levels tested.

### 3.3. Cell sensitivity to oxamate during hypoxia

To determine whether by lowering oxygen WT cells could become hypersensitive to an inhibitor that affects glycolysis at a different step of the pathway than 2-DG, cells were treated with different concentrations of oxamate. Oxamate, an analog of pyruvate, binds lactic dehydrogenase, thereby inhibiting the step of the glycolytic pathway that converts pyruvate to lactate. Fig. 4 shows that, compared with cells under normoxic conditions, cells exposed to 1% $O_2$ were hypersensitized to oxamate at all concentrations used. In Fig. 5, similar results at this $O_2$ concentration were observed when 2-DG was used as the inhibitor.

## 4. Discussion

Oxygen deprivation has been shown previously to occur in both a population of tumor cells located in solid tumors whose own blood supply was defective, as well as in newly growing tumors that, due to $O_2$ diffusion problems, produce angiogenic factors [5–10]. Our present studies demonstrate that when tumor cells are exposed to environmental conditions of lower than normal oxygen concentrations they become hypersensitized to the glycolytic inhibitors 2-DG and oxamate (Figs. 1, 4, and 5). The fact that WT cells showed a basal level of lactate production under normoxic conditions that could be increased as a function of decreasing $O_2$ levels (Table 2) argues favorably

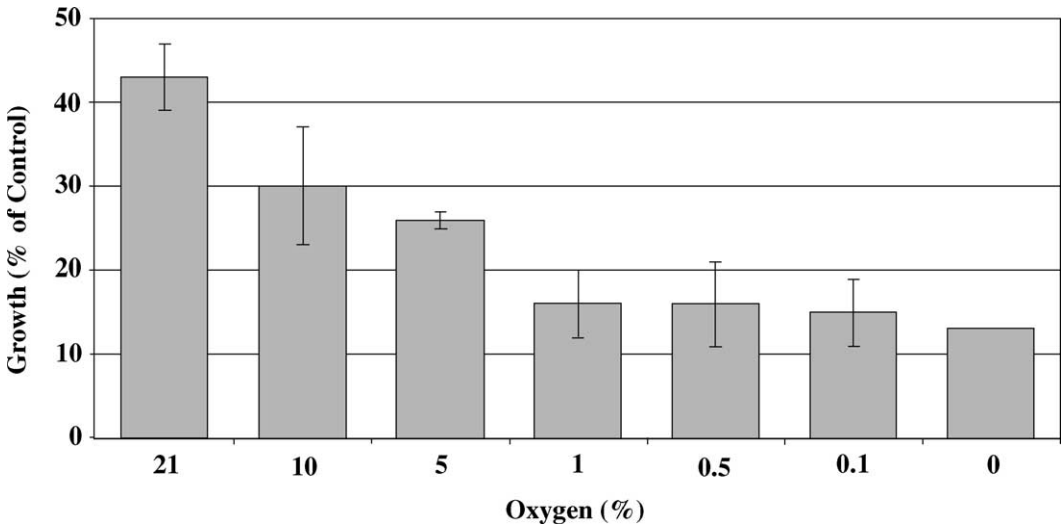


Fig. 1. Growth inhibition of WT osteosarcoma cells treated with 2 mg/mL of 2-DG under decreasing levels of oxygen. Note the hypersensitivity to 2-DG as oxygen levels decrease. Each point is calculated as the percent growth of untreated controls at each respective oxygen concentration and is the mean ± SD of triplicate samples.

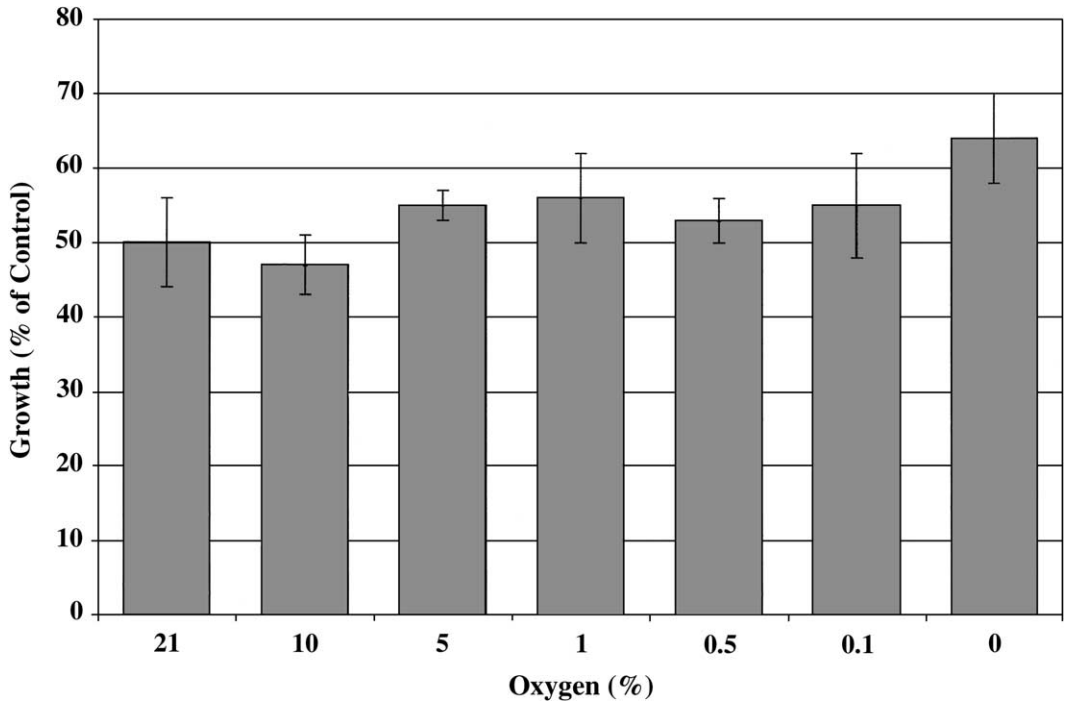


Fig. 2. Growth inhibition of $\rho^0$ cells treated with 100 μg/mL of 2-DG under decreasing levels of oxygen. Note the absence of increased sensitivity to 2-DG as oxygen levels decrease. Each point is calculated as the percent growth of untreated controls at each respective oxygen concentration and is the mean ± SD of triplicate samples.

that it is the environment to which tumor cells are exposed that changes their metabolism from aerobic to anaerobic. This point of view is in agreement with numerous *in vitro* and *in vivo* angiogenic studies which show that tumors or tumor cells deprived of $O_2$ (1% and lower) activate an hypoxic inducible factor (HIF) [11,12], which in turn activates various genes necessary for the cell to convert from aerobic to anaerobic metabolism [13–16]. Among the proteins that are up-regulated are the glut transporters [13,14] responsible for glucose uptake in most eukaryotic cells and the enzymes of the glycolytic pathway [15,16]. These reports further support the hypothesis that it is the

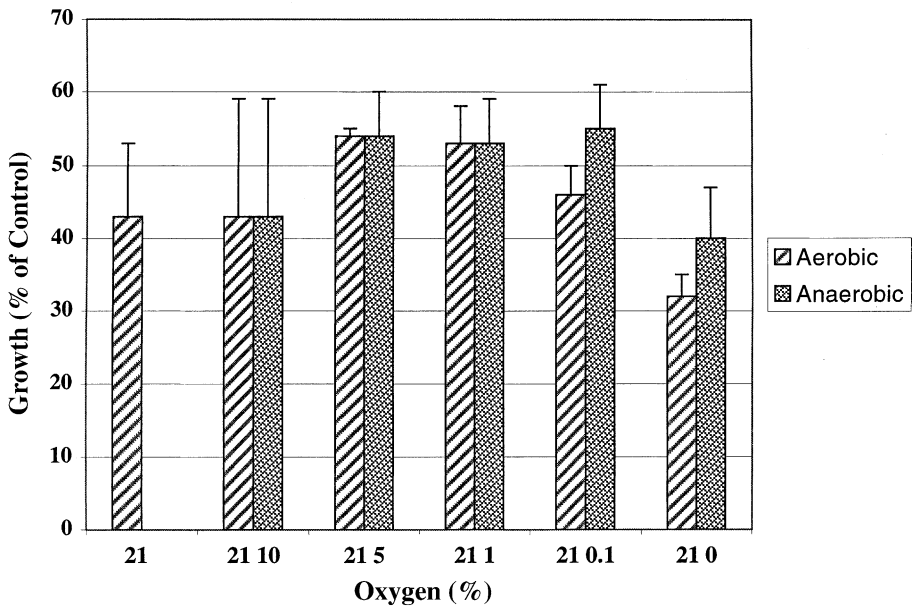


Fig. 3. Growth inhibition of WT osteosarcoma cells treated with 0.002 μg/mL of vinblastine under decreasing levels of oxygen. Note the absence of increased sensitivity to 2-DG as oxygen levels decrease. Each point is calculated as the percent growth of untreated controls at each respective oxygen concentration and is the mean ± SD of triplicate samples.

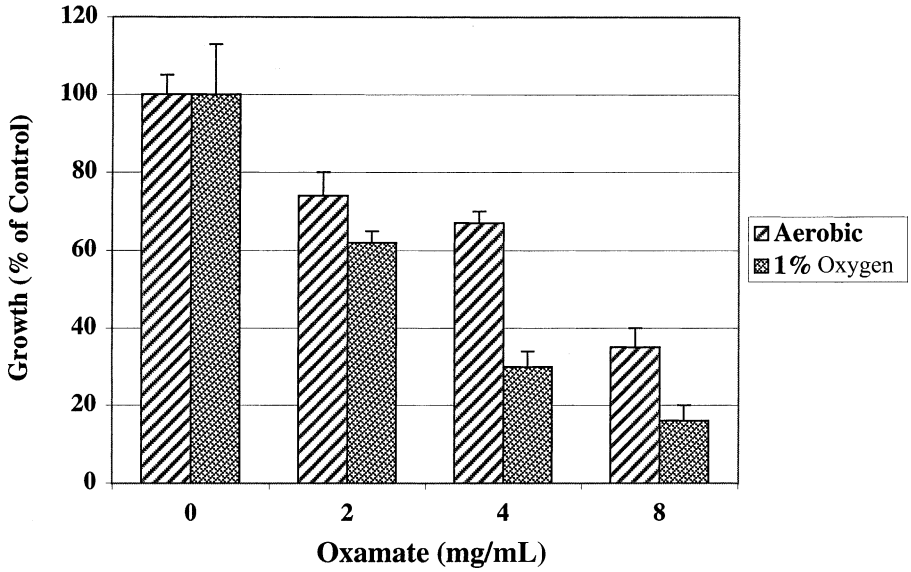


Fig. 4. Growth inhibition of WT osteosarcoma cells treated under normoxic and hypoxic (1% $O_2$) conditions with increasing amounts of oxamate. Note the increased hypersensitivity of WT cells to oxamate under hypoxic conditions. Each point is calculated as the percent growth of untreated controls at each respective oxygen concentration and is the mean ± SD of triplicate samples.

environmental conditions that a given population of tumor cells are exposed to within solid tumors which converts them from aerobic to anaerobic metabolism and not necessarily that all cells within a given tumor are glycolytic, as Warburg originally proposed [1].

On the other hand, it is reasonable to expect that certain tumors could have intrinsic properties that would influence their metabolic pathways and render them more glycolytic than others. In fact, recently, a report on pancreatic tumors showed that of 20 different pancreatic cell lines studied, 15 had HIF constitutively turned on [17]. This was in contrast to 5 tumors from other tissues, i.e. ovarian, breast, and hepatic, which did not, and required hypoxic conditions to up-regulate HIF. Although the molecular mechanisms by which the pancreatic cell lines constitutively express HIF are not explored in this report, one possibility is that mitochondrial deficiencies similar to what occurs in $\rho^0$ cells [2,4] occur in this type of tumor more frequently than in the others, accounting for this difference. Another possibility comes from the report that absence of the von Hippel–Landau tumor suppressor gene has been shown to confer a high stable level of HIFα in the presence of

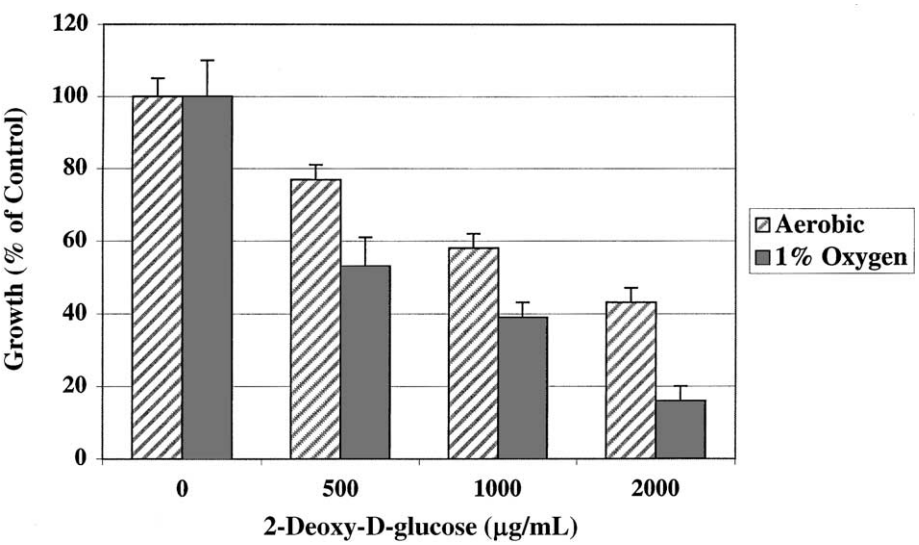


Fig. 5. Growth inhibition of WT osteosarcoma cells treated under normoxic and hypoxic (1% $O_2$) conditions with increasing amounts of 2-DG. Note the increased hypersensitivity of WT cells to 2-DG under hypoxic conditions. Each point is calculated as the percent growth of untreated controls at each respective oxygen concentration and is the mean ± SD of triplicate samples.

normal oxygen [18]. Clearly, further studies need to be performed to clarify the role that defects in mitochondrial DNA and/or suppressor genes may play on HIF activity under normoxic conditions in this tumor type.

The overall goal of our studies is to develop therapies that are involved in targeting the slow-growing cells of solid tumors. To this end, the data presented in Table 1 demonstrate that as oxygen levels were reduced, the growth of WT cells was slowed. Under these slow-growing conditions, we show that hypersensitivity to glycolytic inhibitors increased as a function of decreasing oxygen levels. Moreover, the fact that lactic acid levels increased accordingly agrees with the interpretation that these slow-growing cells have changed their metabolism from aerobic to partially anaerobic.

The decrease in growth at each lowered oxygen level when WT cells were exposed to a constant 2-DG concentration of 2 mg/mL (Fig. 1) was calculated as a percent of the growth of cells that were untreated at each respective oxygen concentration. Thus, although lower oxygen alone reduced the growth of WT cells, the percentage of growth inhibition was increased at each oxygen concentration when cells were exposed to 2-DG. This result is in contrast to what occurs with $\rho^0$ cells that are already unable to perform OxPhos and thus their growth in the presence of lowered oxygen, or sensitivity to 2-DG, was unaffected by lowered oxygen levels. It should be noted that when $\rho^0$ cells were exposed to 0% oxygen alone, cell growth was reduced by 19% (Table 1), which indicates that processes other than OxPhos, which require oxygen, such as enzymatic reactions involving oxidases, are necessary for cell growth and/or survival. These data and interpretation are supported by a recent study in which it was shown that $\rho^0$ cells consume oxygen, albeit much less than WT cells [19].

Our results with vinblastine show that although WT cells were slowed in their growth at lower oxygen levels, they were equally sensitive to this drug whether they were under normoxic or hypoxic conditions (Fig. 3). These results can be explained by considering that vinblastine was applied continuously throughout the 3-day experimental period and thus even though less cells may have gone through a replication cycle under hypoxia versus normoxia, enough drug-exposure time was given to affect similar numbers of cells under both conditions. This is in contrast to what occurs *in vivo* where a cancer chemotherapeutic agent such as vinblastine, which targets the machinery of replicating cells, is applied for a short period and thus affects only those cells that are in the $G_2$/M phase during the exposure time. Thus, a greater number of cells that are dividing rapidly will be in this phase than those that are slow- or non-dividing.

The fact that tumor cells under low oxygen become hypersensitive to both 2-DG and oxamate, inhibitors of glycolysis that interfere with different steps of the glycolytic pathway, is in agreement with our previous results in Models A and B and further support the overall concept that glycolytic inhibitors can be used to target slow-growing cells in solid tumors because they are growing under hypoxic conditions. Since anti-angiogenic drugs should *a priori* make tumors more hypoxic, the addition of glycolytic inhibitors to these protocols should enhance their efficacy. Moreover, since the rapidly growing cells of the aerobic portion of solid tumors are targeted by the chemotherapeutic agents as well as irradiation protocols currently used clinically, we believe that the addition of glycolytic inhibitors to these protocols, targeting the hypoxic portion of these tumors, containing the slow-growing cells, will increase the overall efficacy of these treatments. Finally, our *in vitro* findings demonstrating that there is a wide range of lower than normal oxygen levels (10 to 0%) that begin shifting tumor cells from aerobic to anaerobic metabolism, consequently rendering them sensitive to glycolytic inhibitors, suggest that the gradient of hypoxia that exists within solid tumors also predisposes a wide range of cells to be hypersensitive to glycolytic inhibitors. Thus, the *in vitro* data presented here provide a clear rationale for applying this strategy *in vivo* to target the slow-growing cells of solid tumors.

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