Hypersensitization of Tumor Cells to Glycolytic Inhibitors†

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ABSTRACT: The slow growth of cells in the inner core of solid tumors presents a form of multidrug resistance to most of the standard chemotherapeutic agents, which target the outer more rapidly dividing cells. However, the anaerobic environment of the more centrally located tumor cells also provides an opportunity to exploit their dependence on glycolysis for therapeutic gain. We have developed two in vitro models to investigate this possibility. Model A represents osteosarcoma wild-type (wt) cells treated with agents which inhibit mitochondrial oxidative phosphorylation (Oxphos) by interacting with complexes I, III, and V of the electron transport chain in different ways, i.e., rhodamine 123 (Rho 123), rotenone, antimycin A, and oligomycin. All of these agents were found to hypersensitize wt cells to the glycolytic inhibitor 2-deoxyglucose. Cells treated with Rho 123 also become hypersensitive to oxamate, an analogue of pyruvate, which blocks the step of glycolysis that converts pyruvate to lactic acid. Model B is ρ^0 cells which have lost their mitochondrial DNA and therefore cannot undergo Oxphos. These cells are 10 and 4.9 times more sensitive to 2-deoxyglucose and oxamate, respectively, than wt cells. Lactic acid levels, which are a measure of anaerobic metabolism, were found to be >3 times higher in ρ^0 than in wt cells. Moreover, when wt cells were treated with Rho 123, lactic acid amounts increased as a function of increasing Rho 123 doses. Under similar Rho 123 treatment, ρ^0 cells did not increase their lactic acid levels. These data confirm that cell models A and B are similarly sensitive to glycolytic inhibitors due to their dependence on anaerobic metabolism. Overall, our in vitro results suggest that glycolytic inhibitors could be used to specifically target the slow-growing cells of a tumor and thereby increase the efficacy of current chemotherapeutic and irradiation protocols designed to kill rapidly dividing cells. Moreover, glycolytic inhibitors could be particularly useful in combination with anti-angiogenic agents, which, a priori, should make tumors more anaerobic.

Although Warburg (*1*) originally proposed that tumor cells depend less on mitochondrial function for ATP production and more on glycolysis than normal cells, there has been little definitive data to date to confirm this hypothesis. There are data, however, which indicate that cells, tumor or normal, when compromised aerobically switch to anaerobic metabolism, thereby increasing their uptake and utilization of glucose (*2*). We believe that it is this process that accounts for the successful use of 2-deoxyglucose (2-dg),[1] an analogue of glucose, for localizing tumors in vivo (*2*). The specific uptake of increased amounts of glucose or glucose analogues is most likely attributable to the anaerobic (hypoxic) environment of inner core tumor cells and not to their intrinsic nature or to their inherent inability to undergo normal Oxphos. Thus, since a cell that is functioning normally relies mainly on Oxphos for its supply of ATP, when this mechanism is compromised or absent, as in the case of hypoxia, glycolysis (the only other way of producing ATP) automatically increases.

The slow growth of inner core tumor cells makes them resistant to most of the currently used anticancer agents which target rapidly dividing cells. Thus, anaerobically metabolizing, slow-growing tumor cells present another form of multidrug resistance (MDR) in addition to the MDR mechanisms previously identified, i.e., the ABC cassette family of transporter proteins P-GP and MRP (*3*, *4*), topoisomerase (*5*), and inhibition of apoptosis (*6*). Anaerobiosis, however, also presents an opportunity for selectively attacking slow-growing tumor cells by exploiting their hypothetical collateral sensitivities to inhibitors of glycolysis.

To investigate this concept, we have developed two anaerobic in vitro cell model systems, designated A and B. Model A represents tumor cells treated with agents that interfere with Oxphos such as Rho 123, rotenone, antimycin A, and oligomycin. Model B is ρ^0 cells which have lost their mitochondrial DNA and therefore cannot undergo Oxphos (*7*, *8*). These anaerobic cell models also serve as in vitro tools to determine whether test drugs in intact cells utilize functional mitochondria as cytotoxic targets.

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[1] Abbreviations: Rho 123, rhodamine 123; wt, wild type; 2-dg, 2-deoxyglucose; MDR, multidrug resistance; MRP, multidrug resistance related proetin; Oxphos, oxidative phosphorylation; Dox, doxorubicin; DMEM, Dulbecco's minimal essential medium.

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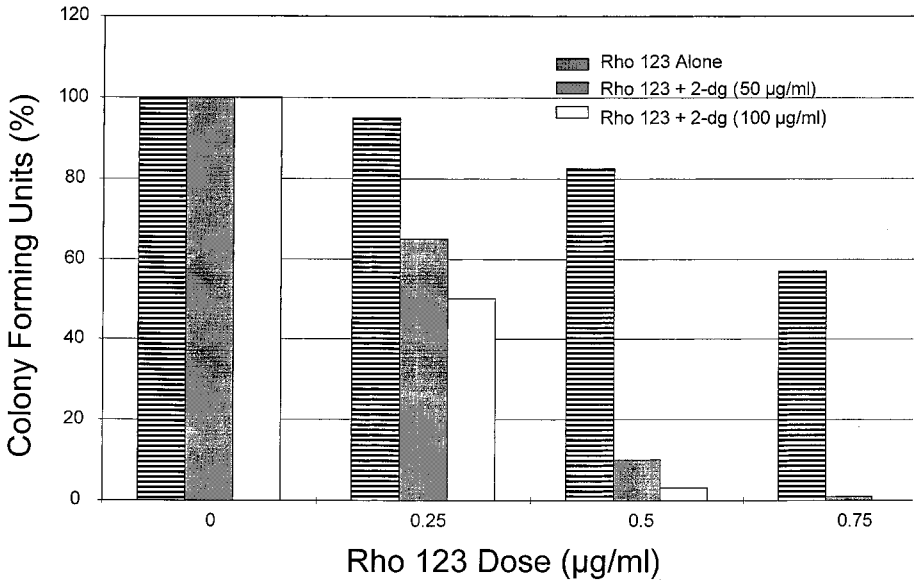

FIGURE 1: Clonogenic survival assays. Osteosarcoma cell line 143b was continuously treated for 10 days, separately (striped column) or in combination with rhodamine 123 and 2-deoxyglucose (50 μg/mL, dark column, or 100 μg/mL, white column) (model A). Note the increased hypersensitization of tumor cells to 50 and 100 μg/mL 2-deoxyglucose as a function of increasing rhodamine 123 doses. SE for all values < 15%.

MATERIALS AND METHODS

Cell Types. An osteosarcoma cell line 143b (wt) was exposed to ethidium bromide for prolonged periods, and a mutant cell line with complete loss of mtDNA (ρ^0) was selected (*7*). Since the ρ^0 cells are uridine and pyruvate auxotrophs, they were grown in DMEM supplemented with 10% fetal calf serum, 50 μg/mL uridine, 100 mM sodium pyruvate, and 10 μg/mL gentamycin. To maintain standard experimental conditions, the parental cell line (wt) was grown in the same medium.

Growth Inhibiton and Clonogenic Assays. For growth inhibition assays 1 mL of cells was seeded at 4×10^4/mL in 24-welled plates, and drugs were applied 24 h later. Drug treatments were continuous for 72 h at 37 °C and 5% CO_2, at which time trypan blue exclusion cell counts were conducted by hemocytometer. Inhibitory doses of 50% were calculated for each drug tested.

For clonogenic assays 2 mL of cells was seeded at 150/mL in 6-welled plates, and drugs were applied 24 h later. Drug treatments were continuous for 10−14 days at 37 °C and 5% CO_2, at which time separate individual colonies had grown large enough to be visible to the eye. Fixing the colonies (70% ethanol), rinsing in PBS, and staining with trypan blue (0.4%) allowed for calculation of cytotoxic drug doses.

Drugs. Rho 123, safranin O, 2-dg, oxamate, vinblastine, doxorubicin (Dox), Taxol, rotenone, antimycin A, and oligomycin were obtained from Sigma, St. Louis, MO.

Lactic Acid Assay. Lactic acid is measured by adding 0.025 mL of deproteinated medium, from treated or nontreated cultures, to a reaction mixture containing 0.1 mL of lactic dehydrogenase (1000 units/mL), 2 mL of glycine buffer (glycine, 0.6 mol/L, and hydrazine, pH 9.2), and 1.66 mg/mL NAD. Deproteinization occurs by treating 0.5 mL of medium from test cultures with 1 mL of perchloric acid at 8% w/v, vortexing for 30 s, then exposing this mixture to 4 °C for 5 min, and centrifuging at 1500*g* for 10 min. The supernate is centrifuged three times more, and 0.025 mL of a final clear supernate is used for lactic acid determinations as above. Formation of NADH is 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 are run in triplicate.

RESULTS

Anaerobic Cell Model A: (I) Rho 123 Hypersensitizes wt Cells to Glycolytic Inhibitors. Since Rho 123 has been shown to uncouple ATP synthesis from electron transport in isolated mitochondrial preparations (*9*), if wt cells are treated with a dose of Rho 123 which inhibits Oxphos, then these cells should theoretically become hypersensitive to inhibitors of glycolysis, i.e., 2-dg and oxamate.

Figure 1 illustrates that wt cells treated with as much as 100 μg/mL 2-dg are unaffected in their ability to form colonies. However, when wt cells are cotreated with Rho 123 at 0.5 and 0.25 μg/mL, they become hypersensitive to 50 and 100 μg/mL 2-dg. Similarly, Figure 2 shows that wt cells treated at 0.5 and 0.1 μg/mL Rho 123 become hypersensitive to 10 mg/mL oxamate, an analogue of pyruvate, which blocks glycolysis by competitively inhibiting lactic dehydrogenase. These results can best be explained by considering that Rho 123, at the doses used, uncouples mitochondria, and thus the cell must rely on glycolysis for ATP production. Consequently, under these conditions cells become hypersensitive to glycolytic inhibitors.

(II) Other Inhibitors of Oxphos Hypersensitize Cells to Glycolytic Inhibitors. The site I inhibitor, rotenone, the ATP synthetase blocker, oligomycin, and the complex III inhibitor, antimycin A, show similar abilities to Rho 123 for hypersensitizing cells to 2-deoxyglucose. Thus, as can be seen in Figure 3, these mitochondrial inhibitors, at low doses, hypersensitize wt cells to 2-dg in the following order of potency: oligomycin > antimycin A > rotenone. On the other hand, when wt cells are exposed to the chemotherapeutic agent, Dox, no hypersensitization to 2-dg is observed (Figure 4). Similar negative results were obtained with two

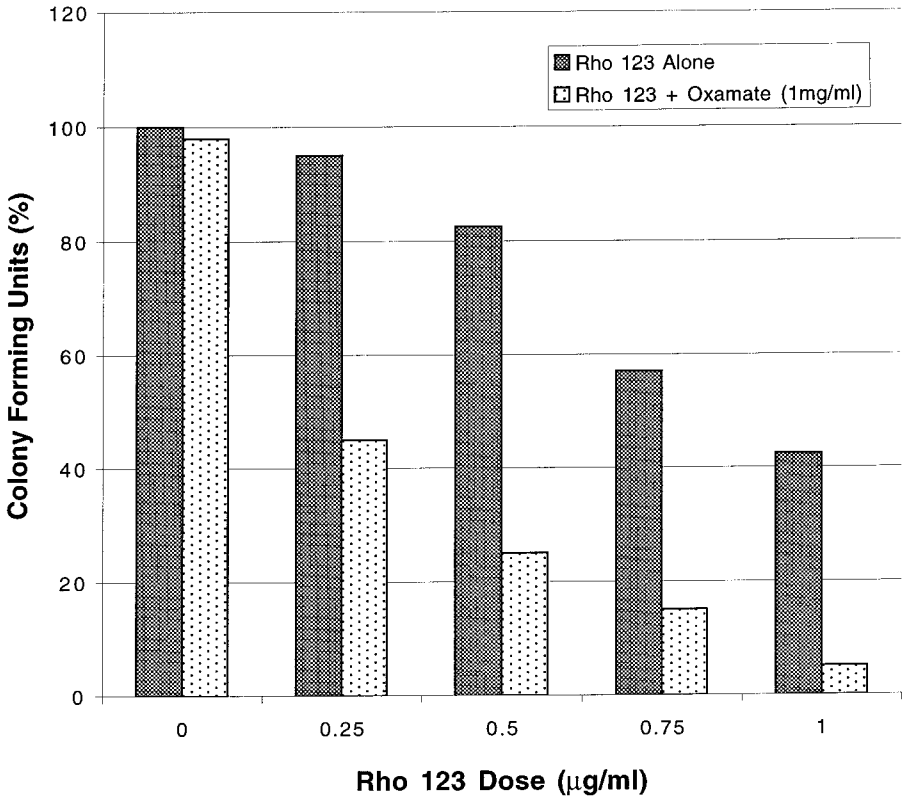

FIGURE 2: Clonogenic survival assays. Osteosarcoma cell line 143b was continuously treated for 10 days, separately (gray column) or in combination (dotted column) with rhodamine 123 and oxamate (1000 μg/mL) (model A). Note the increased hypersensitization of tumor cells to oxamate as a function of increasing rhodamine 123 doses. SE for all values < 15%.

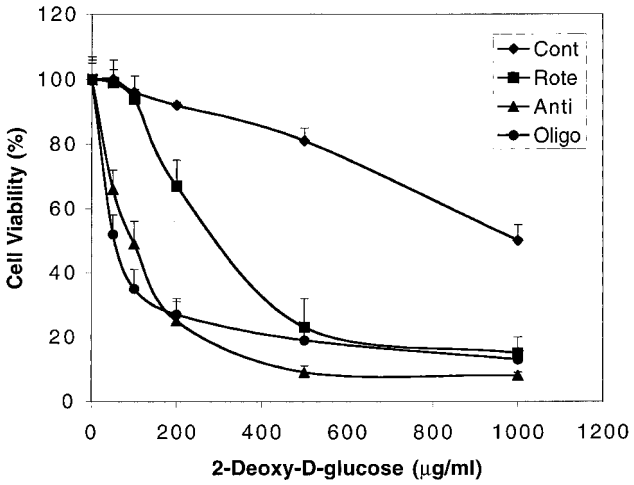

FIGURE 3: Growth inhibition assays. Osteosarcoma cell line 143b was continuously treated for 3 days, alone with increasing doses of 2-dg (diamond) or in combination with rotenone (square) at 0.05 μg/mL, antimycin A (triangle) at 1.0 μg/mL, or oligomycin (circle) at 0.05 μg/mL (model A). Note the hypersensitization of tumor cells to 2-dg for all three inhibitors of mitochondrial function.

other chemotherapeutic agents, Taxol and vinblastine (data not shown).

Anaerobic Cell Model B: ρ^0 Cells Are Hypersensitive to Glycolytic Inhibitors but Resistant to Rho 123. Since ρ^0 cells cannot undergo Oxphos, their only source of ATP is generated by metabolizing glucose via glycolysis. Thus, it appears that these cells, when compared to normally respiring cells, wt, should a priori be more sensitive to glycolytic inhibitors. Indeed, Table 1 shows in growth inhibitory assays that ρ^0 cells are 10 times more sensitive to 2-dg and 4.9 times more sensitive to oxamate than wt cells. On the other hand, since ρ^0 cells cannot undergo Oxphos, they generate a lower $\Delta\psi_{mt}$ than wt cells (*12*, *13*). Thus, as has been previously

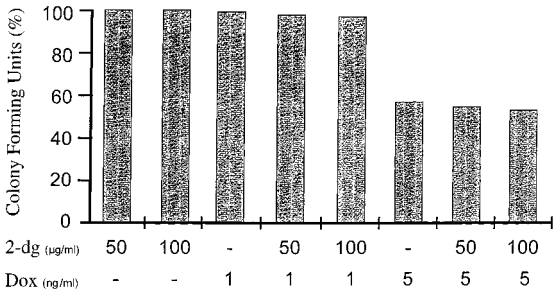

FIGURE 4: Clonogenic survival assays. Osteosarcoma cell line 143b was continuously treated for 10 days, separately or in combination with doxorubicin (Dox) and 2-dg (model A). Note the absence of hypersensitization of tumor cells to 2-dg as a function of increasing Dox doses. SE for all values < 15%.

Table 1: 50% Growth Inhibition Dose (μg/mL)[a]

drugs	cells: wild type	cells: ρ^0	ratio
rhodamine 123	1.0	50	50
saffranin O	0.35	2	6
doxorubicin	0.01	0.01	1
vinblastine	0.00075	0.00075	1
Taxol	0.05	0.05	1
2-deoxyglucose	1000	100	0.1
oxamate	5.3	1.1	0.2
antimycin	5.0	6.0	1
oligomycin	3.8	3.9	1.0
rotenone	0.07	0.08	1.1

[a] SE < 15% for all values.

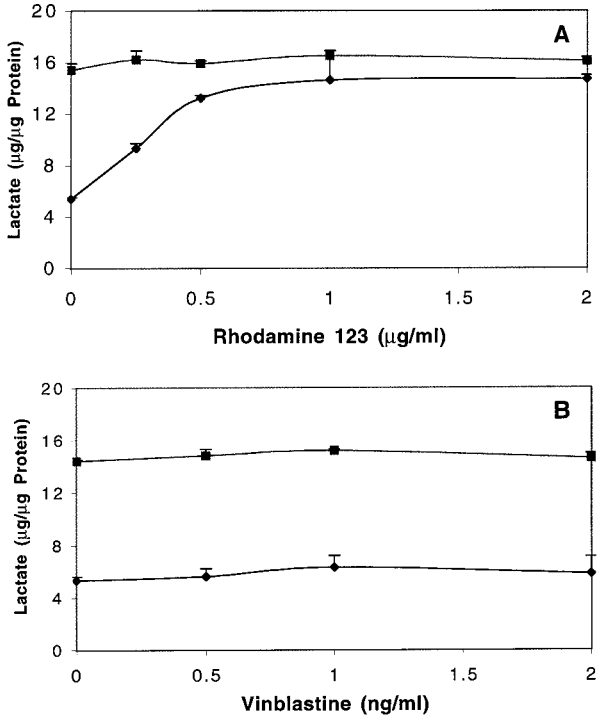

FIGURE 5: Lactic acid production in untreated and continuously treated (3 days) cells with either rhodamine 123 (A) or vinblastine (B). In panel A note that the lactic acid levels in wt cells (diamond) are significantly lower than those in ρ^0 (square) and increase as a function of increasing Rho 123 concentrations (A). Under similar Rho 123 concentrations, ρ^0 cells show no significant changes in lactic acid production (A). In panel B note that both wt (diamond) and ρ^0 (square) cells show no alterations in initial lactic acid levels when exposed to different concentrations of vinblastine.

reported, ρ^0 cells accumulate less of the positively charged dye, Rho 123, than wt (*11*), which would at least partially explain its relative resistance (50-fold) to this dye, as shown in Table 1. Similarly, ρ^0 cells are found to be resistant to safranin O, another lipophilic cationic dye, which localizes preferentially in mitochondria due to $\Delta\psi_{mt}$ (*12*) (Table 1).

Interestingly, although rotenone, antimycin A, and oligomycin are known to affect Oxphos in isolated mitochondrial preparations, and were found to sensitize wt cells to 2-dg (Figure 3), there were no significant differences detected between wt and ρ^0 cells in growth inhibitory assays with these drugs (Table 1). As expected, the nonmitochondrial localizing drugs such as Dox, vinblastine, and Taxol similarly showed no significant ID_{50} differences between ρ^0 and wt cells.

Lactic Acid Levels Correlate with Hypersensitivity to Glycolytic Inhibitors in Cell Models A and B. Since lactic acid is an end product of anaerobic metabolism, measurement of this metabolite could provide information on the mechanisms involved with inhibition of Oxphos and hypersensitization to glycolytic inhibitors. Indeed, we find that ρ^0 cells, which cannot undergo Oxphos due to their deficiency in mitochondrial DNA, and therefore metabolize glucose anaerobically, produce >3 times the amount of lactic acid than wt cells, which with fully functional mitochondria undergo aerobic metabolism (Figure 5A). Additionally, since their mitochondria are already shut off aerobically, ρ^0 cells do not respond to Rho 123, and thus their lactic acid levels remain the same regardless of the Rho 123 dose (Figure 5A).

Moreover, lactic acid levels in wt cells were found to increase as a function of increasing Rho 123 doses (model A) (Figure 5A), which further confirms our hypothesis that Rho 123 is acting in intact cells as it does in isolated mitochondria, i.e., as an uncoupler of Oxphos. Thereby, cells treated with this agent metabolize anaerobically and become hypersensitive to blockers of glycolysis.

Table 2: Effects of High and Low Glucose on Growth Inhibitory Potencies of 2-dg and Oxamate in wt and ρ^0 Cells

	ID 50S (mg/mL) in	
	wt (SD)	ρ^0 (SD)
2-dg in high glucose (4.5 mg/mL)	1.0 (0.2)	0.1 (0.2)
2-dg in low glucose (1 mg/mL)	0.325 (0.03)	0.032 (0.001)
oxamate in high glucose (4.5 mg/mL)	5.3 (0.6)	1.1 (0.3)
oxamate in low glucose (1 mg/mL)	6.0 (0.9)	1.9 (0.3)

Further clarification of our anaerobic models is provided by the results that when wt cells are treated with the chemotherapeutic agent vinblastine, which has no known direct effects on Oxphos, there is no increase in lactic acid levels (Figure 5B). Preliminarily, we have found similar negative results on lactic acid levels with Taxol and Dox, drugs which also do not select between ρ^0 and wt cells in growth inhibitory studies and do not hypersensitize wt cells to glycolytic inhibitors (data not shown).

Effects of High and Low Glucose on the Potency of 2-dg and Oxamate. Since 2-dg directly competes with glucose for glucose receptors found in the plasma membrane, and oxamate does not, we were interested in determining how glucose levels would affect the potencies of either of these compounds in inhibiting the growth of ρ^0 and wt cells. In Table 2 it is shown that cells grown in low glucose (1 mg/mL) are three times more sensitive to 2-dg than at high glucose (4.5 mg/mL) while oxamate shows less dependency on glucose levels. In fact, for oxamate both wt and ρ^0 cells seem to be a little more sensitive under high glucose growth conditions.

DISCUSSION

The development of hypoxia in solid tumors is a complex process that is thought to result from a number of different factors which include (a) long diffusion distances between tumor cells and blood vessels (*14*) and (b) transient blockage in, or collapse of, tumor blood vessels (*15*). Arterioles feeding tumor capillaries have been shown to carry 60% less oxygen than arterioles feeding normal tissues (*16*), which is believed to be due to tissue acidosis in tumors where there is high cell proliferation leading to shifts in the ability of hemoglobin to carry oxygen. In normal tissues, however, because of the abundance of arteriolar supply, this does not occur (*17*).

Clearly, in most solid tumors the demand for oxygen outstrips its supply, and hence resultant hypoxia ensues. The complex factors noted above overlap in both chronic and acute forms of hypoxia, which contribute to the overall gradient distribution of anoxia present to varying degrees within solid tumors (*17*). It is within this gradient we believe that cells, as a function of the lowered amount of oxygen available, rely more on glycolysis for production of ATP and survival. This is to be distinguished from Warburg's hypothesis that tumor cells are intrinsically more glycolytic than normal cells (*1*). Certainly, there are variations in glycolytic rate among normal as well as tumor cell types. In

fact, soon after Warburg's original report, Dickens in a series of reports showed oxidative rates in normal and tumor tissue slices to vary but to fall within the same range (*18*). In the work presented here, using two in vitro models we show that when cells are in an anaerobic environment (model A) or when they are intrinsically reliant on anaerobiosis for their survival as in the case of ρ^0 cells (model B), both are hypersensitive to glycolytic inhibitors.

Data with model A illustrates that when cells are treated with a variety of agents which interfere with the function of mitochondria and with the ultimate formation of ATP via Oxphos, i.e., Rho 123, safranin O, rotenone, antimycin A, and oligomycin, they become hypersensitive to the glycolytic inhibitors, 2-dg and oxamate (Figures 1−3). Although each of the mitochondrial agents used in these experiments has a distinct effect on mitochondrial function (as noted above in Results), they all show an ability to hypersensitize tumor cells to inhibitors of the glycolytic pathway. Similarly, they all also induce increases in lactic acid levels of tumor cells (Figure 4 for Rho 123 and data not shown for the other compounds). Thus, these results support the interpretation that when Oxphos is compromised, cells switch over to glycolysis (generating higher lactic acid levels) and thereby become more sensitive to glycolytic inhibitors, i.e., 2-dg and oxamate.

Normally, mitochondrial and nuclear DNAs code for proteins which act in concert to produce fully formed and functional mitochondria. Mitochondrial DNA, which represents less than 1% of total cellular DNA, encodes 37 genes specifying 13 polypeptides, 22 transfer RNAs, and two ribosomal RNAs (*20*). All 13 polypeptides are components of the ATP-generating Oxphos system (*19*, *20*). Hence, the ρ^0 mutant cell line, having lost the ability to produce these 13 polypeptides, cannot perform Oxphos. Thus, this cell line, designated as model B, grows anaerobically and shows similar hypersensitivity to glycolytic inhibitors (2-dg and oxamate) as model A (Table 1).

As shown previously, the ρ^0 cell which cannot undergo Oxphos and its wt parental counterpart which does, comprise a useful in vitro model for gaining information on whether a given compound uses functional mitochondria as a target for cytotoxicity (*11*). Moreover, these cell pairs were shown not to express the MDR1 gene and expressed low, similar amounts of MRP1 (*11*). Consequently, cytotoxicity or growth inhibition results with this model are not complicated by differential drug transport between ρ^0 and wt cells. Thus, Dox, vinblastine, and Taxol showed no selectivity in toxicity between wt and ρ^0 cells (Table 1), indicating that functional mitochondria were not targets for the growth inhibitory activity of these agents.

However, Rho 123 and safranin O were found to be significantly more potent in wt than in ρ^0 cells. This result can be explained by the cationic nature of these compounds, which leads to their preferential localization in mitochondria due to the high electronegative $\Delta\psi_{mt}$ of this organelle (*21*). Both of these compounds have been shown in isolated mitochondrial preparations to act as uncouplers (*9*, *10*). Thus, since ρ^0 cells intrinsically cannot undergo Oxphos and have lower $\Delta\psi_{mt}$ than wt (*12*, *13*), they accumulate less of these cations and thereby do not succumb to their uncoupling effects. It is only at higher doses where targets and cell processes other than mitochondrial Oxphos are affected that growth inhibition ensues. In contrast, oligomycin, antimycin A, and rotenone, agents that are known to inhibit distinct functions of mitochondria which involve and affect Oxphos, do not selectively distinguish between ρ^0 and wt cells in growth inhibition assays. Each of these compounds has been shown to have effects on various other cell processes, and the data presented here indicate that, at toxic doses, functions other than mitochondrial are more likely involved as drug targets. Clearly, at low doses, in wt cells (model A) (Figure 3) these agents do act on Oxphos, thereby hypersensitizing them to the glycolytic inhibitor 2-dg. Although not presented here, data with these agents indicate that each one of them, at low doses, increases lactic acid levels in wt cells. Thus, it appears that these agents, in whole cells, block Oxphos, switching the cells from aerobic to anaerobic metabolism and therby hypersensitizing them to glycolytic inhibitors.

Moreover, the increased lactic acid levels found in ρ^0 cells (Figure 5) verify their glycolytic nature, which correlates with their hypersensitivity to inhibitors of this pathway. The fact that lactic acid levels did not increase when ρ^0 cells were exposed to increasing concentrations of Rho 123 further substantiates that (1) Oxphos is not functioning in these cells and (2) the increase in lactic acid when wt cells are treated with increasing doses of Rho 123, or other mitochondrial inhibitors, is indeed due to their inhibitory effects on Oxphos and not because of some nonspecific effects. This latter point is also supported by the negative results on lactic acid levels found in wt cells when treated with the nonmitochondrial agent vinblastine (Figure 5B). Thus, overall, the lactic acid results provide further evidence that the hypersensitivity that models A and B display toward glycolytic inhibitors is due to a shift into anaerobic metabolism. These data, therefore, are key in further unifying anaerobic models A and B, even though they are distinct.

The observation that both models A and B showed hypersensitivity to inhibitors which interfered with different steps of the glycolytic pathway prompted experiments in which glucose levels were altered. It is known that 2-dg directly competes with glucose for glucose receptors found in the plasma membrane (*2*) and oxamate does not. Thus, the results in which we find that wt and ρ^0 cells grown in low glucose (1 mg/mL) are three times more sensitive to 2-dg than at high glucose (4.5 mg/mL) can be explained by the competition that is known to occur between 2-dg and glucose for glut receptors. On the other hand, the results with oxamate which showed less dependence on glucose levels, and in fact both ρ^0 and wt cells showed increased sensitivity to oxamate at high glucose, could be explained by considering that glycolysis occurs faster under high glucose levels and thus cells would be more sensitive to inhibitors that did not compete with glucose for Glut receptors such as oxamate. Overall, these data (Table 2) further support that 2-dg and oxamate are interacting at different steps of the glycolytic pathway to inhibit cell growth.

Previously, we reported that the combination of 2-dg and Rho 123 was more effective than each drug alone in killing human breast (MCF-7) carcinoma cells (*22*) in vitro. Thus, with our results here in osteosarcoma cells, it appears that the effect of manipulating Oxphos and glycolysis has general application to different tumor cell types. Moreover, we showed that this combination resulted in cures in mice implanted with ascites tumors (*23*).

In conclusion, the data presented in this paper provide an in vitro rationale for overcoming the multidrug resistance of the slower growing cells found in solid tumors, which due to their hypoxic environment metabolize anaerobically and thereby become hypersensitive to glycolytic inhibitors. We propose that the addition of such inhibitors to standard chemotherapy protocols, which are geared toward attacking the rapidly dividing aerobic metabolizing tumor cells, will increase their efficacies. This strategy can also be applied to the irradiation of tumors. Since it is known that, during irradiation, free radicals are not formed under hypoxic conditions, tumor cells in this environment are the most likely to escape damage. Thus, addition of glycolytic inhibitors to irradiation protocols should increase overall treatment efficacy by naturally targeting the surviving hypoxic population of tumor cells. Moreover, with the recent interest and success of anti-angiogenic agents in the treatment of cancer, the use of glycolytic inhibitors should also increase the efficacy of this emerging new treatment. Again, the reasoning here is that tumors will become more hypoxic if blood supplies are reduced or eliminated, and this in turn should lead to more anaerobically metabolizing tumor cells which are naturally hypersensitive to glycolytic inhibitors.

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