Inhibition of Glycolysis in Cancer Cells: A Novel Strategy to Overcome Drug Resistance Associated with Mitochondrial Respiratory Defect and Hypoxia

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Abstract

Cancer cells generally exhibit increased glycolysis for ATP generation (the Warburg effect) due in part to mitochondrial respiration injury and hypoxia, which are frequently associated with resistance to therapeutic agents. Here, we report that inhibition of glycolysis severely depletes ATP in cancer cells, especially in clones of cancer cells with mitochondrial respiration defects, and leads to rapid dephosphorylation of the glycolysis-apoptosis integrating molecule BAD at Ser^{112}, relocalization of BAX to mitochondria, and massive cell death. Importantly, inhibition of glycolysis effectively kills colon cancer cells and lymphoma cells in a hypoxic environment in which the cancer cells exhibit high glycolytic activity and decreased sensitivity to common anticancer agents. Depletion of ATP by glycolytic inhibition also potently induced apoptosis in multidrug-resistant cells, suggesting that deprivation of cellular energy supply may be an effective way to overcome multidrug resistance. Our study shows a promising therapeutic strategy to effectively kill cancer cells and overcome drug resistance. Because the Warburg effect and hypoxia are frequently seen in human cancers, these findings may have broad clinical implications. (Cancer Res 2005; 65(2): 613-21)

Introduction

Over 70 years ago, Warburg (1) observed that cancer cells frequently exhibit increased glycolysis and depend largely on this metabolic pathway for generation of ATP to meet their energy needs. He attributed this metabolic alteration to mitochondrial "respiration injury" and considered this as the most fundamental metabolic alteration in malignant transformation or "the origin of cancer cells" (2). During the past several decades, the Warburg effect has been consistently observed in a wide spectrum of human cancers, although the underlying biochemical and molecular mechanisms are extremely complex and remain to be defined. Among the possible mechanisms, mitochondrial malfunction and hypoxia in the tumor microenvironment are considered two major factors contributing to the Warburg effect. However, whether the increase of glycolytic activity in cancer cells is mainly due to inherent metabolic alterations or due to anaerobic environment in the tumor tissues remains controversial (3).

Under physiologic conditions, generation of ATP through oxidative phosphorylation in the mitochondria is an efficient and preferred metabolic process, which produces far more ATP molecules from a given amount of glucose compared with glycolysis. However, when the ability of cells to generate ATP through mitochondrial oxidative phosphorylation is compromised, cells are able to adapt alternative metabolic pathways, such as increasing glycolytic activity, to maintain their energy supply. Mitochondrial respiratory function can be negatively affected by multiple factors, including mutations in mitochondrial DNA (mtDNA), malfunction of the electron transport chain, aberrant expression of enzymes involved in energy metabolism, and insufficient oxygen available in the cellular microenvironment. It is known that mtDNA contains a displacement loop, and the coding gene sequence for 13 important protein components of the mitochondrial respiratory complexes without introns. Mutations in mtDNA are likely to cause alterations of the encoded protein and compromise the respiratory chain function. Thus, the frequent mtDNA mutations observed in a variety of human cancers are thought to contribute to respiratory malfunction in cancer cells (4–6). The constant generation of reactive oxygen species within the mitochondria and the increased free radical stress in cancer cells may cause further damage to both mtDNA and the electron transport chain, thus amplifying respiratory malfunctions and dependency on glycolysis (7).

Hypoxia is another important factor that contributes to the Warburg effect. The fast growth of cancer cells and rapid expansion of the tumor mass usually outpace new vascular generation, resulting in an insufficient blood supply to certain area of the tumor tissues. Such a hypoxic environment within the tumor mass limits the availability of oxygen for use in mitochondrial respiration and synthesis of ATP and forces the cancer cells to up-regulate the glycolytic pathway as the main route of energy production (8). The ability of oxygen to regulate glucose metabolism is know as the Pasteur effect and is mediated through several pathways involving various kinases (9, 10). In this case, the increased glycolytic activity in cancer cells is not necessarily due to intrinsic mitochondrial defects but is induced by the tumor microenvironment through a series of metabolic adaptation processes, including preferentially increased expression of enzymes required for glycolysis (11).

Although the underlying mechanisms responsible for the Warburg effect are rather complex and can be attributed to a variety of factors, such as mitochondrial defects and hypoxia, the metabolic consequences seem similar. The compromised ability of cancer cells to generate ATP through oxidative phosphorylation

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forces the cells to increase glycolysis to maintain their energy supply and thus renders cancer cells highly dependent on this metabolic pathway for survival. As such, it is conceivable that the metabolic alterations in malignant cells may be exploited to serve as a biochemical basis to develop therapeutic strategies to target this metabolic abnormality. One possibility is to inhibit glycolysis and preferentially kill the cancer cells that are dependent on glycolytic pathway for ATP generation.

Several agents, including 2-deoxyglucose and arsenate compounds, have long been known to abolish ATP generation through the glycolytic pathway. For instance, 2-deoxyglucose is an analogue of glucose and is able to bind and suppress hexokinase II, an enzyme that catalyzes the initial metabolic step in the conversion of glucose to glucose-6-phosphate during glycolysis (12). Inhibition of this rate-limiting step by 2-deoxyglucose causes a depletion of cellular ATP, leading to blockage of cell cycle progression and cell death *in vitro*, and exhibits antitumor activity *in vivo* (13, 14). However, the effectiveness of 2-deoxyglucose is significantly affected by the presence of its natural counterpart glucose and seems to only partially reduce the availability of glucose for glycolysis (12). In contrast, the pentavalent arsenic compounds do not directly inhibit glycolysis but abolish net ATP generation by causing arsenolysis in the glyceraldehyde-3-phosphate dehydrogenase reaction, preventing the generation of 1,3-bisphosphoglycerate. However, the pentavalent arsenic compounds seem to have limited specificity due to other toxic effects. More recent studies showed that 3-bromopyruvate (3-BrPA) is a potent inhibitor of hexokinase II and effectively inhibits glycolysis (12, 15). This compound is effective in killing liver cancer cells in the rabbit VX2 tumor implantation animal tumor model when given by local infusion (16).

In the present study, we used defined experimental systems to examine the potential therapeutic implications of the Warburg effect by testing the sensitivity of cancer cells to glycolytic inhibition under hypoxic and normoxic conditions and of cancer cells with genetic mitochondrial defects. We showed that cancer cells with such respiratory abnormalities were less sensitive to chemotherapeutic agents commonly used in the clinical treatment of cancer but could be effectively killed by inhibition of glycolysis using 3-BrPA. We also observed that inhibition of glycolysis caused a rapid dephosphorylation of BAD protein at Ser^{112}, leading to BAX localization to mitochondria and massive cell death. Importantly, cells that express a multidrug-resistant (MDR) phenotype still remain sensitive to inhibition of glycolysis. Because alterations in energy metabolism and increased dependency on glycolysis are commonly seen in cancer cells, our findings suggest potential broad therapeutic implications of using glycolytic inhibitors for cancer treatment.

Materials and Methods

Chemicals and Reagents. 3-BrPA was purchased from Sigma (St. Louis, MO). The compound was dissolved into water and neutralized with NaOH immediately before use in cell culture. [^{3}H]2-deoxyglucose and [^{3}H]2-leucine were purchased from Amersham (Piscataway, NJ). Mouse monoclonal anti-pBAD(Ser^{112}), rabbit anti-caspase-3, rabbit polyclonal anti-pAkt(Ser^{473}), and rabbit polyclonal anti-BAD antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal anti–cytochrome *c* was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-BAX was purchased from BD Biosciences (San Diego, CA).

Cells and Cell Culture. Human leukemia cell line HL-60 and human lymphoma cell line Raji were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. The mitochondrial defective clones (ρ^- cells) HL-60/C6F and Raji/C8 were derived as described previously (17, 18) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 50 mmol/L uridine, and additional 2.7% glucose. Human colon cancer HCT116 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. A hypoxic culture condition was created by incubating cells in a sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with 5% CO_2 and 95% N_2. Because the culture flasks contained ambient oxygen at the beginning of the experiments, the final oxygen content in the hypoxia chamber was ~ 0.5% to 1.0% after achieving air equilibrium.

Cytotoxicity Assays. Cell growth inhibition was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay using MTT reagent in 96-well plates. After 72 hours of drug incubation, 50 μL MTT reagent was added to each well and incubated for an additional 4 hours. The plates were then centrifuged (1,500 × *g*, 5 minutes) and the supernatant was removed. The cell pellets were dissolved in 200 μL DMSO. Absorbance was determined using a MultiSkan plate reader (LabSystems, Helsinki, Finland) at a wavelength of 570 nm. Apoptotic or necrotic cell death was determined by flow cytometric analysis of cells double stained with Annexin V-FITC and propidium iodide (PI) using an assay kit from BD PharMingen (San Diego, CA). Briefly, after drug incubation, cells were collected, washed with cold PBS, and suspended in Annexin V binding buffer. The cells were stained with Annexin V-FITC for 15 minutes at room temperature in the dark, washed, and stained with PI. The samples were analyzed with a FACSCalibur flow cytometer with CellQuest Pro software.

Measurement of Cellular ATP Pool. Cells were incubated with various concentrations of 3-BrPA for indicated time intervals. Intracellular nucleotides were extracted from the cells with 0.4 N perchloric acid, neutralized with concentrated KOH, and quantitated as described previously (19). Briefly, nucleotides in the cell extracts were analyzed by HPLC using an anion exchange Partisil-10 SAX column with an elution flow rate of 1.5 mL/min over a 50-minute buffer gradient [curve 5, buffer A: 0.005 mol/L $NH_4H_2PO_4$ (pH 2.8); buffer B: 0.5 mol/L $NH_4H_2PO_4$ + 0.25 mol/L KCl (pH 2.8)]. The eluting flow was continuously monitored by UV absorption at 262 nm, and the peaks of nucleoside triphosphates were quantitated by electronic integration with reference to external standards. The intracellular ATP contents were calculated and normalized by equal cell number and expressed as percentage of the control cells.

Determination of Glycolytic Activity. The cellular glycolytic activity under various experimental conditions was evaluated by measuring the glycolytic index, which was calculated by the formula: glycolytic index = $(L \times G) / O$, where L is the cellular lactate generation rate, G is glucose uptake rate, and O is the oxygen consumption rate. To determine the cellular lactate production, cells in exponential growth phase were washed and incubated with fresh medium for the indicated times. Aliquots of the culture medium were removed for the analysis of lactate content using an Accutrend lactate analyzer with a linear range of standard lactate concentrations according to the procedures recommended by the manufacturer (Roche, Mannheim, Germany). Cellular glucose uptake was measured by incubating cells in glucose-free RPMI 1640 with 0.2 Ci/mL [^{3}H]2-deoxyglucose (specific activity, 40 Ci/mmol) for 60 minutes. After the cells were washed with ice-cold PBS, the radioactivity in the cell pellets was quantified by liquid scintillation counting. To determine cellular oxygen consumption, cells were resuspended in 1 mL fresh warm medium pre-equilibrated with 21% oxygen and placed in the sealed respiration chamber equipped with a thermostat control, a microstirring device, and a Clark-type oxygen electrode disc (Oxytherm, Hansatech Instrument, Cambridge, United Kingdom). The oxygen content in the cell suspension medium was constantly monitored and oxygen consumption rate was determined as described previously (17).

Western Blot Analysis. Proteins in whole cell lysates, mitochondrial fractions, or cytosolic fractions were resolved by electrophoresis on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were

blotted for molecules of interest with anti-BAD (1:1,000), anti-pBAD(Ser^{112}) (1:1,000), anti-pAkt/Ser^{473} (1:1,000), anti-BAX (1:1,000), anti–cytochrome *c* (1:1,000), or anti-caspase-3 (1:1,000) overnight. The bound primary antibodies were detected using appropriate horseradish peroxidase–conjugated secondary antibodies followed by detection with a SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL). For sequential blotting, the membranes were stripped with a stripping buffer (Pierce) followed by reblotting with proper antibodies. To prepare mitochondrial and cytosolic fractions, cells were harvested, washed once with ice-cold PBS, and resuspended in 3 volumes of isolation buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L DTT, 250 mmol/L sucrose, and a cocktail of protease inhibitors]. After keeping in an ice bath for 10 minutes, the cell suspension was homogenized with 15 strokes in a 2 mL glass homogenizer. The samples were centrifuged twice at 1,500 $\times$ *g* at 4°C for 5 minutes to remove nuclei, unbroken cells, and cell debris. The supernatants were centrifuged again at 15,000 $\times$ *g* for 15 minutes to separate the mitochondrial fraction (pellets) and the cytosolic fraction (supernatants). The mitochondria pellet was washed once with the isolation buffer before use.

Results

Alterations of Drug Sensitivity in Cancer Cells with Respiratory Deficiency or under Hypoxic Conditions. We first established *in vitro* experimental systems to examine the effects of mitochondrial defects and hypoxia, two major underlying factors of the Warburg effect, on cellular sensitivity to various anticancer agents. Clones of cells with mitochondrial respiratory defects were derived from human leukemia cells (HL-60) and human lymphoma cells (Raji) by preferentially damaging the mtDNA. The procedures to derive respiration-defect clones (ρ^- cells) and their biochemical and molecular characterization were described previously (17, 18). One such ρ^- clone, C6F derived from HL-60 cells, contained multiple mutations in their mtDNA and were completely defective in mitochondrial respiration (17). Transmission electron microscopy analysis revealed that mitochondria in C6F cells are abnormally swollen, with pale matrix and disorganized cristae (Fig. 1*A*). Biochemical analysis showed that C6F cells consumed little oxygen (Fig. 1*B*), indicating that these cells were severely deficient in mitochondrial respiration. Compared with the parental HL-60 cells, C6F cells were more active in glucose uptake ($P = 0.008$; Fig. 1*C*) and produced significantly more lactate (end product of glycolytic metabolism), which was released and accumulated in the culture medium ($P = 0.002$; Fig. 1*D*). The lack of oxygen consumption, increase in glucose uptake, and lactate accumulation in C6F cells indicate that the glycolytic pathway is highly active in these cells. Using these three major biochemical variables, we calculated that the glycolytic indexes for the HL-60 parental cells and C6F cells were

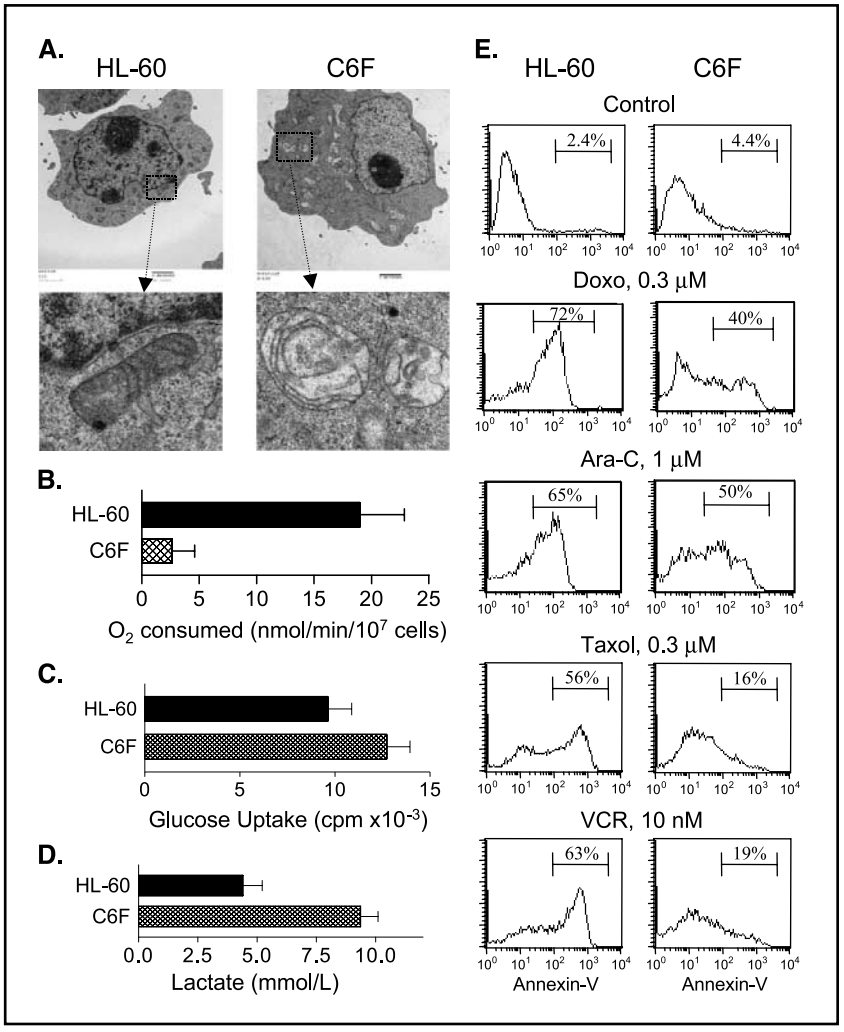

Figure 1. Characterization of cancer cells with mitochondrial defects and alterations in drug sensitivity. *A,* comparison of mitochondrial morphology in HL-60 and the respiratory-deficient C6F clone by transmission electron microscopy analysis. Note the swollen mitochondria with significant pale matrix and disorganized cristae in C6F cells. *B,* oxygen consumption in HL-60 and C6F cells. *C,* comparison of glucose uptake in HL-60 and C6F cells. Cells (~2.5 $\times$ 10^6) were incubated in 5 mL RPMI 1640 (glucose-free) with 0.2 Ci/mL [^{3}H]2-deoxyglucose for 1 hour, and the uptake of radioactivity was determined as described in MATERIALS AND METHODS. *Columns,* mean of three independent experiments; *bars,* SD. *D,* lactate production in HL-60 and C6F cells. Cells in exponential growth phase were resuspended in RPMI 1640 at the density of 5 $\times$ 10^5/mL and incubated for 24 hours, and the lactate concentration in the culture medium was measured as described in MATERIALS AND METHODS. *Columns,* mean of three independent experiments; *bars,* SD. *E,* comparison of drug sensitivity in HL-60 and C6F cells. Cells were incubated with the indicated concentrations of doxorubicin (*doxo*), ara-C, taxol, and vincristine (*VCR*) for 24 hours, and cell death was analyzed by flow cytometry for positive Annexin V staining.

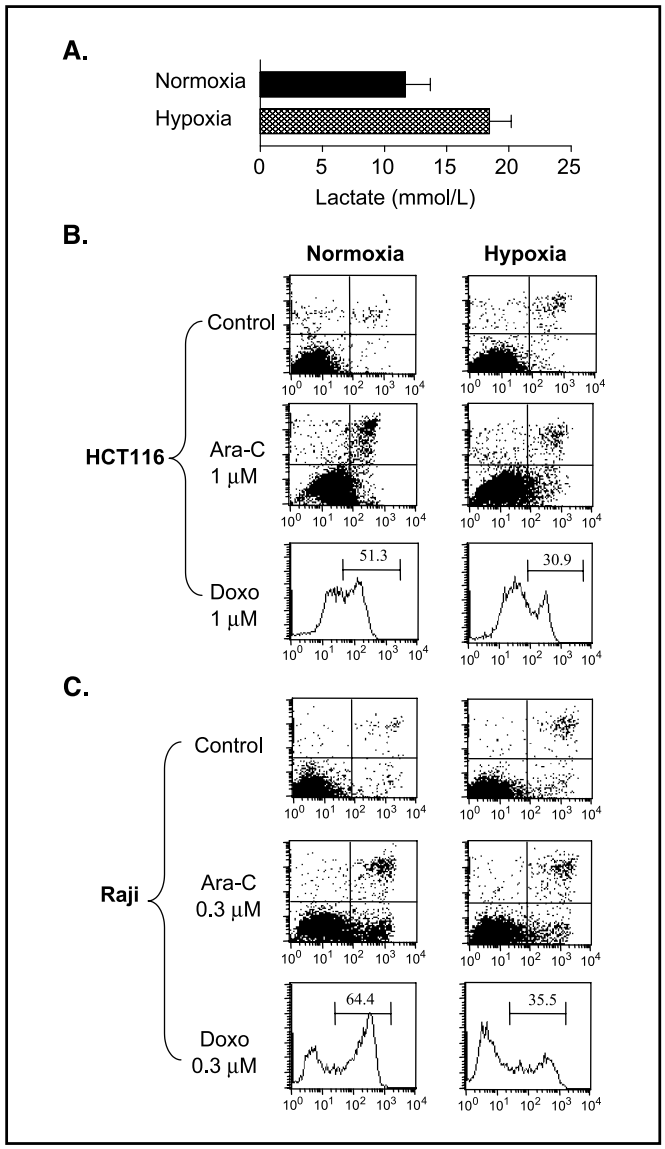

Figure 2. Hypoxia caused an increase in glycolytic activity and a decrease in drug sensitivity. *A,* lactate generation in HCT116 cells under normoxic and hypoxic conditions. HCT116 were seeded in culture flasks at a density of 1×10^6 cells per flask (T-25) and incubated for 24 hours under normoxic conditions. The culture medium was then replaced with fresh medium, and the samples were incubated for 36 hours under either normoxic or hypoxic conditions. Lactate concentration in the culture medium was measured as described in MATERIALS AND METHODS, and the rate of lactate production was calculated. *Columns,* mean of three independent experiments; *bars,* SD. *B,* comparison of drug sensitivity under normoxic and hypoxic conditions in HCT116 cells. Cells were treated with ara-C (1 μmol/L) or doxorubicin (1 μmol/L) for 36 hours under normoxic or hypoxic conditions, and cell death was measured by flow cytometric analysis as described in MATERIALS AND METHODS. *C,* comparison of drug sensitivity under normoxic and hypoxic conditions in Raji cells. Cells were treated with ara-C (0.3 μmol/L) or doxorubicin (0.3 μmol/L) for 24 hours under normoxic or hypoxic conditions. Cell death was measured by flow cytometric analysis.

2.4 and 45.6, respectively. Similar biochemical alterations indicative of highly increased glycolysis were also observed in the ρ^- cell clones derived from the Raji cells.

Because mitochondria play important roles in energy metabolism as well as in regulation of apoptosis, we then examined the effect of mitochondrial respiratory defects on the cellular response to anticancer agents by comparing the drug sensitivity of HL-60 and C6F cells. As shown in Fig. 1*E*, the respiration-deficient C6F cells were substantially less sensitive than the parental cells to several anticancer agents commonly used in the clinic. These therapeutic agents include 1-β-D-arabinofuranosylcytosine (ara-C), doxorubicin (Adriamycin), taxol, and vincristine. There was a consistent decrease of drug sensitivity in C6F cells for each agent tested, with the most reduced sensitivity observed with taxol and vincristine. Similarly, reduced drug sensitivity was also observed in the ρ^- cells derived from Raji cells (data not shown). Thus, mitochondrial respiration deficiency not only caused increased glycolysis but also blunted the cellular responses to anticancer agents.

Hypoxia is another major factor that causes cancer cells to adapt glycolysis to generate ATP. We next tested if this metabolic adaptation might, as in the cells with mitochondrial defects, also alter the cellular sensitivity to anticancer agents. The lymphoma cell line Raji and a solid tumor cell line HCT116 (human colon cancer) were used to test this possibility. As illustrated in Fig. 2, cells incubated under hypoxic conditions produced a significantly greater amount of lactate than under normoxia during the same incubation period ($P = 0.0015$; Fig. 2*A*), confirming an increase in glycolytic activity when cells were deprived of oxygen. This metabolic adaptation was accompanied by a reduced sensitivity to doxorubicin and ara-C. This decrease in drug sensitivity was consistently observed in both HCT116 cells (Fig. 2*B*) and Raji cells (Fig. 2*C*). These data, together with the observations in the ρ^- cells, suggest that cancer cells with increased glycolysis, due either to mitochondrial genetic defects or to environmental hypoxia, become less sensitive to common anticancer agents. This reduced sensitivity was not due to a general slowing down of the cell death process, because these cells exhibited a greater sensitivity to glycolytic inhibition under similar incubation conditions (see below).

Inhibition of Glycolysis Effectively Kills Cancer Cells with Respiratory Defects. The above observations prompted us to explore new strategies to effectively kill cancer cells and overcome drug resistance associated with hypoxia or mitochondrial defects. One logical approach is to inhibit glycolysis and thus block the major metabolic pathway by which the respiration-deficient cancer cells generate their ATP. 3-BrPA, a known inhibitor of hexokinase II with a potent inhibitory effect on glycolysis (12, 15, 16), was used in our study to test this possibility. As shown in Fig. 3*A* and *B*, incubation of HL-60 cells with 3-BrPA caused a concentration- and time-dependent depletion of the cellular ATP pool, confirming its ability to block energy metabolism in this cell line. 3-BrPA also caused massive cell death in a concentration- and time-dependent manner as evidenced by positive Annexin V staining and loss of membrane integrity (positive PI staining) revealed by flow cytometry analysis (Fig. 3*C* and *D*). The ability of this compound to induce cell death was correlated with its ability to deplete cellular ATP. It should be noted that a nearly complete depletion of ATP occurred 3 to 6 hours after cells were incubated with 300 μmol/L 3-BrPA (Fig. 3*B*), whereas significant cell death was not observed until 12 to 24 hours (Fig. 3*D*), indicating that ATP depletion occurred before the onset of cell death.

Because the proapoptotic Bcl-2 family member BAD has been shown to play an important role in integrating glycolysis and apoptosis (20), we tested the possibility that inhibition of glycolysis by 3-BrPA might affect the proapoptotic function of BAD. As shown in Fig. 4*A*, incubation of HL-60 cells with this compound caused a concentration- and time-dependent dephosphorylation of BAD at Ser^{112}, with a complete dephosphorylation

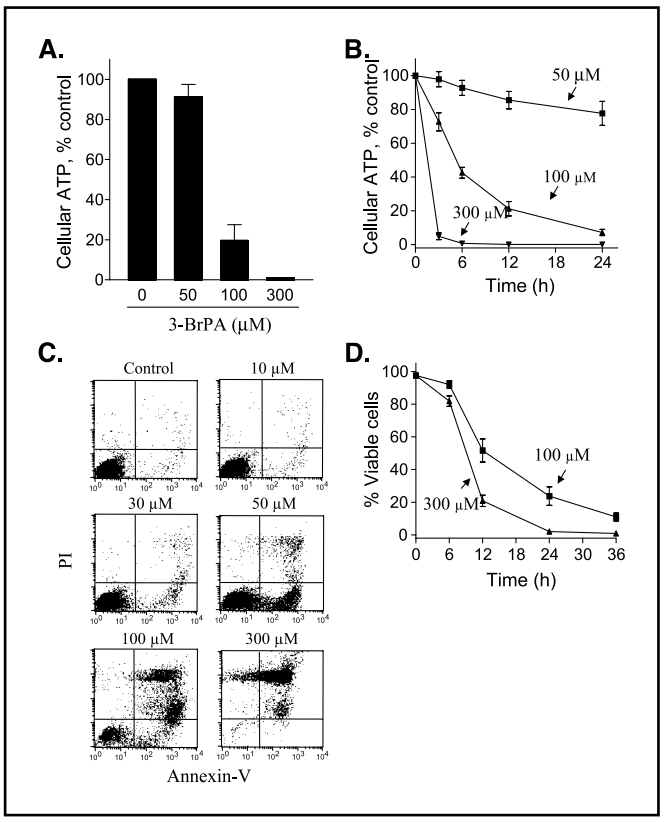

Figure 3. Induction of ATP depletion and cell death in HL-60 cells by 3-BrPA. *A,* HL-60 cells were treated with the indicated concentrations of 3-BrPA for 12 hours. Intracellular ATP was measured by HPLC analysis as described in MATERIALS AND METHODS and expressed as percentage of the control. ATP content of the control cells was 2.6 ± 0.1 nmol per 10^6 cells. *Columns,* mean of three independent experiments; *bars,* SD. *B,* time-dependent depletion of ATP in HL-60 cells treated with 3-BrPA. Cells were incubated with the indicated concentrations of 3-BrPA for various times, and cellular ATP was determined as described above. *Points,* mean of three independent experiments; *bars,* SD. *C,* concentration-dependent cell death in HL-60 cells treated with 3-BrPA. Cell death was measured by flow cytometric analysis of cells double stained with Annexin V and PI. *D,* time-dependent apoptosis in HL-60 cells treated with 3-BrPA. Cells were incubated with the indicated concentrations of 3-BrPA for various times and analyzed by flow cytometry after double staining with Annexin V and PI. *Points,* mean of three independent experiments; *bars,* SD.

8 hours after drug incubation. Interestingly, 3-BrPA did not cause a decrease in total BAD protein. Instead, there was a detectable increase of total BAD after the drug incubation (Fig. 4*A*). The inhibitory effect of 3-BrPA on BAD phosphorylation was also observed under hypoxic conditions and seemed to be specific for BAD because the phosphorylation status of another protein Akt was not decreased under identical conditions (Fig. 4*B*). It is known that the BH3 domain-only member BAD requires the multidomain proapoptotic members such as BAX to trigger the mitochondrial apoptotic pathway and that Bcl-2 and Bcl-X_L can sequester these proapoptotic factors by forming complexes to prevent apoptosis (21). We speculated that the increased amount of dephosphorylated BAD in cells treated with 3-BrPA might displace BAX from this sequestering complex, allowing BAX to localize to the mitochondria and cause a change in membrane permeability and the release of cytochrome *c*. Western blot analysis of the mitochondrial and cytosolic protein fractions showed that, when BAD was dephosphorylated, there was a concurrent increase of BAX protein in the mitochondrial fraction, accompanied by a simultaneous decrease of cytosolic BAX (Fig. 4*C*), suggesting a translocation of BAX to the mitochondria. The release of cytochrome *c* from the mitochondria to the cytosol and cleavage of caspase-3 were also detected at these time points (Fig. 3*C* and *D*). These data suggest that inhibition of glycolysis significantly affected the phosphorylation status of BAD, leading to BAX translocation to the mitochondria.

Because respiration-deficient cancer cells are highly dependent on glycolysis, we reasoned that inhibition of glycolysis by 3-BrPA might have a profound effect on cells with respiratory defects. Two clones of the ρ^- cells, C6F and C8 derived from parental HL-60 and Raji cells, respectively, were used to test this possibility. As shown

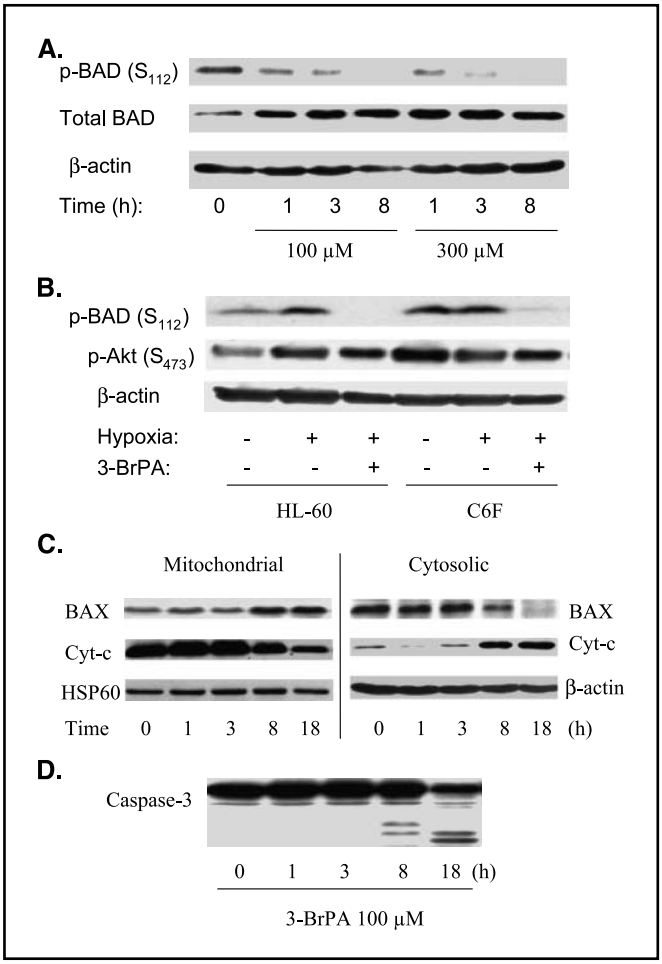

Figure 4. Dephosphorylation of BAD and alterations of mitochondrial and cytosolic BAX and cytochrome *c* in HL-60 cells treated with 3-BrPA. *A,* HL-60 cells were incubated with 100-300 μmol/L 3-BrPA for the indicated times. Equal amounts of total cellular proteins from each sample were resolved by SDS-PAGE and blotted for total BAD protein, pBAD(Ser^{112}), and β-actin. *B,* effect of 3-BrPA on the phosphorylation status of BAD and Akt. HL-60 and C6F cells were treated with 100 μmol/L 3-BrPA under hypoxic or normoxic conditions for 8 hours as indicated. Cell lysates were subjected to Western blot analysis using anti-pBAD(Ser^{112}), anti-pAkt(Ser^{473}), and anti-β-actin antibodies. *C,* translocation of BAX to mitochondria and release of cytochrome *c* to cytosol in cells treated with 3-BrPA. Mitochondrial and cytosolic fractions were isolated from HL-60 cells treated with 100 μmol/L 3-BrPA for the indicated times and blotted for BAX and cytochrome *c* (*Cyt-c*) as described in MATERIALS AND METHODS. Mitochondrial HSP60 protein and cytosolic β-actin were also blotted as protein loading controls. *D,* cytosolic fractions were blotted for caspase-3 using an antibody that detects both procaspase-3 and cleaved fragments.

in Fig. 5*A* and *B*, inhibition of glycolysis by 3-BrPA (100 μmol/L) caused a significantly greater depletion of ATP in both ρ^- clones compared with their respective parental cell lines. A higher concentration (300 μmol/L) led to complete depletion of ATP in both ρ^- cells and parental cells. The ρ^- cells were also more sensitive to 3-BrPA-mediated BAD dephosphorylation, which occurred 1 to 3 hours after drug incubation (Fig. 5*C* and *D*). Importantly, analysis of cytotoxicity revealed that both ρ^- clones were substantially more sensitive to 3-BrPA than their parental cells (Fig. 5*E* and *G*). For instance, incubation of C6F or C8 cells with 100 μmol/L 3-BrPA for 24 hours caused cell death in most all of the ρ^- cells, whereas a portion of the parental cells still remained intact after the same drug incubation (Fig. 5*E*). This is in direct contrast with the results shown in Fig. 1, in which the respiration-defect cells were less sensitive to common anticancer agents, such as doxorubicin, ara-C, and taxol.

Inhibition of Glycolysis Effectively Kills Cancer Cells under Hypoxic Conditions. The findings shown in Fig. 5 prompted us to further test the possibility to use 3-BrPA to effectively kill cancer cells under hypoxic conditions, which also rendered the cells dependent on glycolysis and less sensitive to common anticancer agents (Fig. 2). Incubation of lymphoma cells (Raji) and colon cancer cells (HCT116) with 3-BrPA under hypoxic conditions caused a significant inhibition of glycolysis as shown by the decrease of lactate production (Fig. 6*A* and *B*). Both cell lines were more sensitive to 3-BrPA under hypoxic conditions than under normoxic conditions as shown by a substantial increase in percentage of cells with Annexin V/PI–positive staining (Fig. 6*C* and *D*).

MDR Cells Remain Sensitive to Glycolytic Inhibition. Because inhibition of glycolysis by 3-BrPA causes a severe depletion of cellular ATP, we speculated that this compound might be able to effectively kill MDR cells, which use ATP-dependent molecular pumps to export various anticancer drugs out of the cells. To test this possibility, we used a previously characterized MDR cell line HL-60/AR derived from HL-60 cells (22) and examined its sensitivity to 3-BrPA in comparison with several common anticancer agents. As expected, the MDR cells HL-60/AR are highly resistant to both doxorubicin and vincristine, with the IC_{50} values ~1,000 times greater than that of the parental cells (Fig. 7*A* and *B*). The HL-60/AR cells also exhibited reduced sensitivity to ara-C (Fig. 7*C*). In contrast, these MDR cells remained sensitive to 3-BrPA, which caused similar inhibitory effect in both parental HL-60 cells and HL-60/AR cells (MTT assay; Fig. 7*D*). Using an acute apoptotic assay (Annexin V/PI staining), we further showed that 50 μmol/L 3-BrPA, which alone caused only <10% apoptotic cells in 24 hours, was able to substantially increase the cytotoxic effect of doxorubicin, vincristine, or ara-C in HL-60/AR cells (Fig. 7*E*).

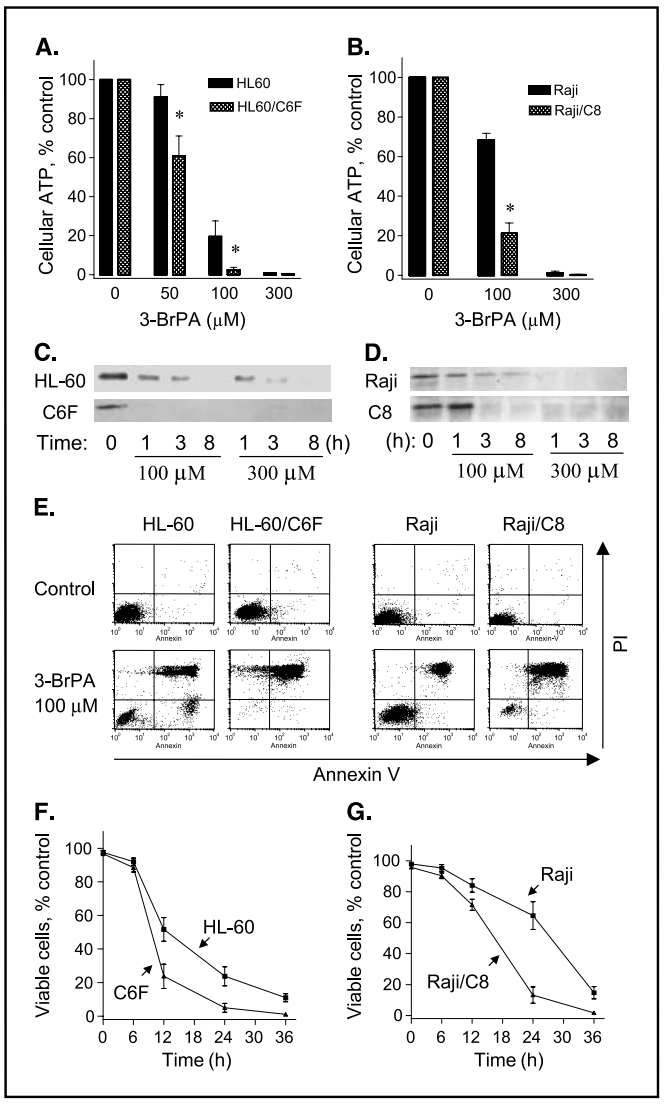

Figure 5. Induction of ATP depletion and cell death by 3-BrPA in mitochondrial-deficient cells in comparison with parental cell lines. *A,* HL-60 cells and ρ^- C6F cells were incubated with the indicated concentrations of 3-BrPA for 12 hours, and cellular ATP was determined as described in MATERIALS AND METHODS. ATP content was 2.6 ± 0.1 nmol per 10^6 cells in HL-60 control cells and 2.2 ± 0.1 nmol per 10^6 cells in C6F control cells. *B,* Raji cells and C8 (ρ^-) cells were incubated with the indicated concentrations of 3-BrPA for 12 hours. Cellular ATP was analyzed by HPLC. ATP content was 3.2 ± 0.2 nmol per 10^6 cells in Raji control cells and 2.2 ± 0.2 nmol per 10^6 cells in C8 control cells. *, $P < 0.05$, difference between ρ^- cells and parental cells. *C,* comparison of 3-BrPA-induced BAD dephosphorylation (Ser^{112}) in HL-60 and C6F cells. *D,* comparison of 3-BrPA-induced BAD dephosphorylation in Raji and C8 cells. *E,* mitochondrial-deficient cells (C6F and C8) were more sensitive to 3-BrPA than their parental cells (HL-60 and Raji, respectively) and exhibited massive cell death after the drug incubation (100 μmol/L, 24 hours). Cell death was measured by flow cytometric analysis after the cells were double stained with Annexin V and PI. *F* and *G,* comparison of time-dependent cell death in ρ^- cells with the parental cells. Viable cells were defined as Annexin V/PI double-negative cells.

Discussion

The Warburg effect, or increased dependency on glycolysis in cancer cells, has been a long-standing observation (2, 23). Despite the consistent findings of this metabolic alteration in a wide spectrum of human cancers, the therapeutic implications of the Warburg effect still remain speculative. At the biochemical level, the Warburg effect is generally attributed to "respiration injury" or malfunction of the mitochondrial oxidative phosphorylation, forcing the cells to use the glycolytic pathway to generate ATP. The major factors contributing to the Warburg effect include mitochondrial metabolic defects, due in part to mtDNA mutations and/or aberrant expression of metabolic enzymes, and hypoxic environment that limits oxygen supply to tumor mass. Unfortunately, the metabolic adaptation in response to these alterations is associated with reduced sensitivity to common anticancer agents. Hypoxia is also known to cause resistance to radiation therapy due in part to induction of hypoxia-inducible factor-1α protein accumulation (24–27). This imposes a serious challenge in clinical

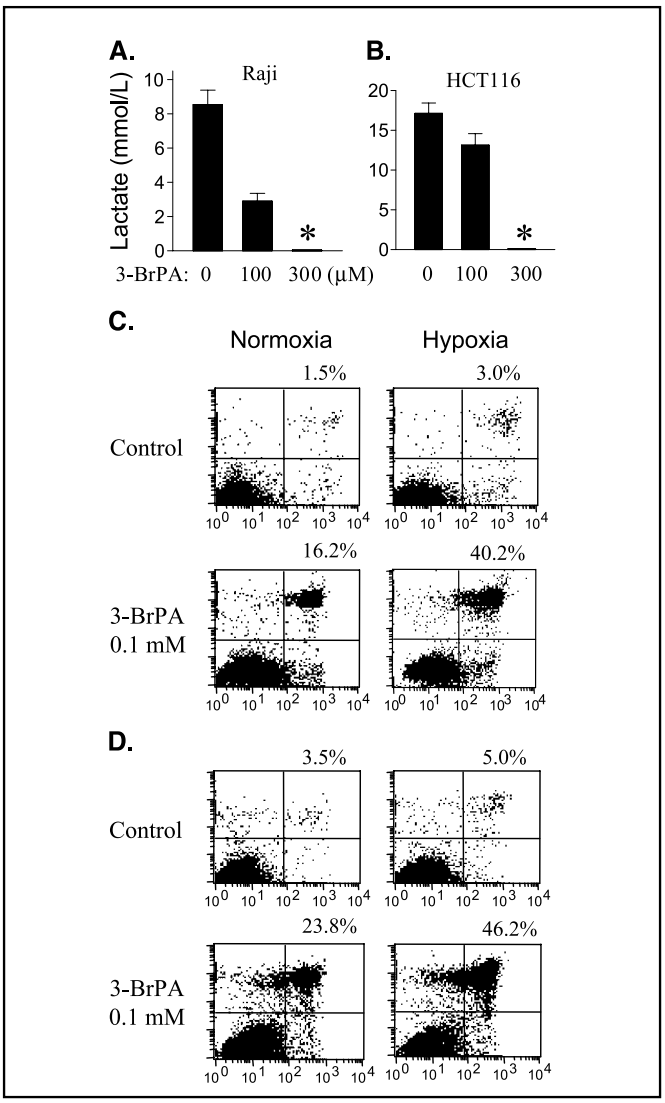

Figure 6. Cancer cells under hypoxic conditions were more sensitive to glycolytic inhibition by 3-BrPA. *A,* inhibition of lactate generation by 3-BrPA in Raji cells under hypoxic conditions. Raji cells (5×10^5) were resuspended in 1 mL fresh medium and incubated under hypoxia condition for 24 hours in the presence of the indicated concentrations of 3-BrPA. Lactate levels in the culture medium were measured as described in MATERIALS AND METHODS. *Columns,* mean of three independent experiments; *bars,* SD. **,* lactate levels below the limit of detection. *B,* HCT116 cells (1×10^6) were seeded in a culture plate and incubated for 24 hours. The culture medium was then replaced with 10 mL fresh medium, and the cells were incubated under hypoxic conditions for 36 hours in the presence of the indicated concentrations of 3-BrPA. Lactate levels in the culture medium were measured as described in MATERIALS AND METHODS. *Columns,* mean of three independent experiments; *bars,* SD. *C,* Raji cells under hypoxic conditions were more sensitive to 3-BrPA (100 μmol/L, 24 hours) than under normoxic conditions. *Number on the top right corner,* % dead cells. *D,* HCT116 cells under hypoxic conditions were more sensitive to 3-BrPA (100 μmol/L, 36 hours) than under normoxia condition. Cell death was measured by flow cytometric analysis after the cells were double stained with Annexin V and PI.

treatment of cancer. Thus, it is important to develop new agents and test novel therapeutic strategies to effectively kill cancer cells and overcome drug resistance associated with hypoxia and mitochondrial respiratory defects.

In the current study, we hypothesized that the increased dependency on glycolysis in cancer cells with respiration defects or under hypoxic conditions could be exploited for therapeutic benefits and tested this hypothesis in defined experimental systems using 3-BrPA as a pharmacologic tool to inhibit glycolysis. Mechanistically, it is logical to speculate that cells with mitochondrial defects or in a hypoxic environment are heavily dependent on glycolysis and are likely more sensitive to glycolytic inhibition. Indeed, we showed that two separate clones of respiration-deficient cells derived from human leukemia or lymphoma cells or the parental cells cultured under hypoxic conditions all exhibited reduced sensitivity to common anticancer agents, such as doxorubicin, vincristine, ara-C, and taxol (Figs. 1 and 2). In sharp contrast, these cells showed increased sensitivity to glycolytic inhibition by 3-BrPA (Figs. 5 and 6). These data provide a proof of principle that it is possible to use glycolytic inhibitors to effectively kill cancer cells and overcome drug resistance associated with the Warburg effect.

It is evident that depletion of cellular ATP is the key event

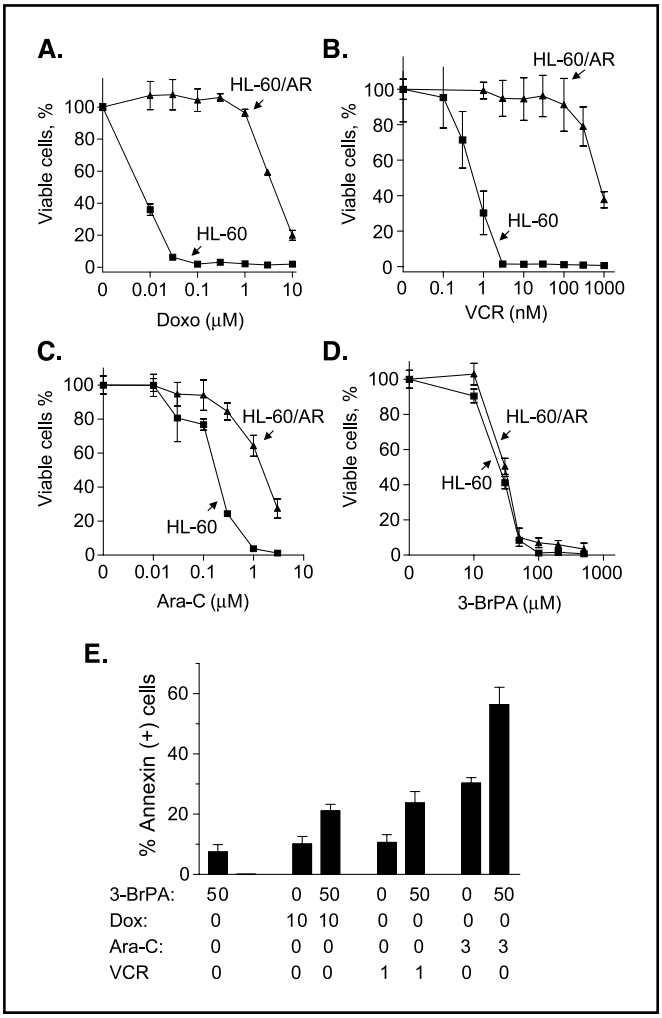

Figure 7. Comparison of HL-60 and MDR HL-60/AR cells in response to doxorubicin (*A*), vincristine (*B*), ara-C (*C*), and 3-BrPA (*D*). Cells were incubated with the indicated concentrations of each compound, and cell viability was measured by MTT assay. *E,* 3-BrPA enhanced the cytotoxic effects of doxorubicin, ara-C, and vincristine in MDR HL-60/AR cells. Cells were incubated with the indicated concentrations of each compound in the presence or absence of a moderately toxic concentration of 3-BrPA (50 μmol/L) for 24 hours. Cell death was measured by flow cytometric analysis after the cells were double stained with Annexin V and PI. *Columns,* average and range of two independent experiments.

responsible for the cytotoxic action of 3-BrPA. Data in Figs. 3 and 5 showed that the drug-induced cell death was closely associated with ATP depletion, which occurred before the appearance of cell death. An important finding of this study was that inhibition of glycolysis by 3-BrPA led to the dephosphorylation of BAD, a protein recently found to integrate glycolysis and apoptosis in the mitochondria (20). It is known that the antiapoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, are able to bind BAD and BAX and sequester them in protein complexes to prevent triggering of apoptosis (21). We now showed that inhibition of glycolysis caused dephosphorylation of BAD, leading to translocation of BAX to the mitochondria, loss of mitochondrial membrane integrity, release of cytochrome *c*, and activation of the apoptotic cascades. This is consistent with the proapoptotic role of dephosphorylated BAD and BAX (28–30).

It should be noted that previous studies have suggested that apoptosis is an ATP-dependent process and that depletion of ATP tends to cause cell death by necrosis (31–34). Interestingly, cell death induced by 3-BrPA seems to contain both apoptotic and necrotic components (double-positive in Annexin V/PI staining). When the 3-BrPA concentration was relatively low (50 μmol/L) and ATP depletion was moderate, mainly apoptosis was observed as evidenced by a large population of cells with positive Annexin V staining and negative PI staining (Fig. 3*C*). The amount of ATP that remained in the cells under these conditions seemed to be sufficient to support the execution of the apoptotic process. However, at higher drug concentrations (≥100 μmol/L), 3-BrPA caused a severe ATP depletion and massive necrosis, because the majority of the cells were positively stained with PI, an indication of loss of membrane integrity during necrosis (Figs. 3, 5, and 6).

Depletion of cellular ATP by inhibition of glycolysis seems to be effective in killing cancer cells with a MDR phenotype. It is known that cells expressing MDR proteins, such as MDR or MRP, require ATP as the energy source to pump the drug substrates out of the cells (35, 36). Thus, it is possible to overcome such drug resistance by depletion of cellular ATP, causing the pump to fail. The observations that cells with MDR phenotype still remained sensitive to 3-BrPA and that combination of 3-BrPA with doxorubicin, vincristine, or ara-C enhanced the activity of each drug against the drug-resistant cells (HL-60/AR) are consistent with this hypothesis. Thus, glycolytic inhibition may have therapeutic implications in overcoming multidrug resistance.

The mechanism by which 3-BrPA inhibits glycolysis is mediated mainly through its inhibition of hexokinase II, the key enzyme catalyzing the first step of the glycolytic pathway. It is also known that hexokinase II is highly expressed in malignant cells compared with normal cells (12, 37), probably reflecting the necessity of high glycolytic metabolism to maintain sufficient ATP supply in the tumor cells. As such, the use of glycolytic inhibitors, such as 3-BrPA, as anticancer agents might provide a preferred therapeutic selectivity. The ability of 3-BrPA to preferentially kill cancer cells with mitochondrial defects and tumor cells in a hypoxic environment provides a biochemical basis to further develop this class of compounds as novel anticancer agents with potentially promising therapeutic activity and selectivity. It should be noted, however, that 3-BrPA is relatively unstable and shows significant inhibition of glycolysis only at relatively high concentrations (100-300 μmol/L) in cell culture. This issue needs to be carefully considered and addressed in the future development of better glycolytic inhibitors for the clinical treatment of cancer. Nevertheless, our study suggests that inhibition of glycolysis is an effective strategy to kill cancer cells and overcome drug resistance associated with mitochondrial defects and hypoxic conditions. This novel approach may have broad applications in cancer treatment, considering the prevalent Warburg effect observed in a wide spectrum of human cancers.

Acknowledgments

Received 9/27/2004; revised 11/2/2004; accepted 11/11/2004.

Grant support: National Cancer Institute, NIH grants CA85563, CA100428, CA109041, and CA81534 and American Legion Auxiliary fellowship (J.S. Carew).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Bert Vogelstein for kindly providing the human colon cancer HCT116 cells and Min Du for her expert assistance in HPLC analysis of cellular ATP.

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