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# Forum Original Research Communication

# Glucose 6-Phosphate Dehydrogenase Overexpression Models Glucose Deprivation and Sensitizes Lymphoma Cells to Apoptosis

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## ABSTRACT

**Glucocorticoids are one component of combined treatment regimens for many types of lymphoma due to their ability to induce apoptosis in lymphoid cells. In WEHI7.2 murine thymic lymphoma cells, altering catalase and glutathione peroxidase activity by transfection or the use of chemical agents modulates the ability of glucocorticoids to induce apoptosis. This suggests that the oxidative stress response is important in determining the glucocorticoid sensitivity of the cells. For glutathione peroxidase and catalase to detoxify reactive oxygen species (ROS), reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) are ultimately required. The major source of NADPH in the cell is glucose 6-phosphate dehydrogenase (G6PDH). Therefore, we created G6PDH-overexpressing WEHI7.2 variants to test whether G6PDH activity is a key determinant of glucocorticoid sensitivity in WEHI7.2 cells. G6PDH-overexpressing WEHI7.2 cells were more sensitive to oxidative stress and glucocorticoids. The G6PDH-overexpressing WEHI7.2 variants appeared similar to cells undergoing glucose deprivation with decreased adenosine triphosphate (ATP) synthesis by the mitochondria and increased basal levels of ROS. Overexpression of G6PDH also sensitized the cells to other standard lymphoma chemotherapeutics including cyclophosphamide, doxorubicin, and vincristine. The decreased ATP and elevated ROS due to G6PDH overexpression may be key factors in increasing the sensitivity of the WEHI7.2 cells to lymphoma chemotherapeutics.** *Antioxid. Redox Signal.* 8, 1315–1327.

## INTRODUCTION

Leukemias and lymphomas show a heterogeneous response to standard therapies. The biggest challenges for effective treatment are to understand why therapy fails for some patients and to design new treatment strategies to improve prognosis in these patients. A more thorough understanding of the mechanism of drug action and the cellular determinants of chemoresistance is critical to increasing drug efficacy.

Glucocorticoids are one component of standard therapy for many leukemias and lymphomas because of their ability to induce apoptosis in lymphoid cells (44, 48). Glucocorticoid-induced apoptosis depends on the binding of the steroid to a cytosolic receptor and translocation of the steroid–receptor complex to the nucleus, where it exerts its effect on transcription (26). An unknown series of events then transpires (the signaling phase), resulting in the release of cytochrome *c* from the mitochondria (the committed step), formation of the apoptosome, and activation of caspases (the execution phase) (22). Lack of functional glucocorticoid receptors is one source of resistance both in the clinic and in cell culture (12, 19, 49, 29). Increases in the antiapoptotic Bcl-2 family members can also cause resistance in cell culture (13, 39) and cor-

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relate with poor survival in some lymphomas (18). However, resistance can be found in the absence of receptor alterations (28, 69) or Bcl-2 increases (2, 58). Therefore, additional factors must contribute to steroid resistance in lymphoid cells.

Several types of evidence suggest that reactive oxygen species (ROS) play a critical role in the signaling phase of steroid-induced apoptosis. Results from previous studies have shown that: (a) treatment of thymocytes with chemical antioxidants or antioxidant defense enzymes protects against glucocorticoid-induced apoptosis (5, 6, 36, 38, 51, 60, 61, 67); (b) treatment with metal chelators inhibits this type of cell death (45, 67); and (c) culture of thymocytes under hypoxic conditions is generally protective (5, 38, 53). By using WEHI7.2 murine thymic lymphoma tissue-culture cells treated with dexamethasone, a synthetic glucocorticoid, we have shown that cells with an increased antioxidant defense are resistant to glucocorticoids. Treatment of the cells with selenium to increase glutathione peroxidase, selection of a $H_2O_2$-resistant cell population, or overexpression of catalase increased both the $H_2O_2$-removal capability of the cells and steroid resistance (5, 57, 58). Overexpression of thioredoxin also is protective (4). Thioredoxin is a small protein that transfers reducing equivalents to maintain protein sulfhydrals in the reduced form (50). One of the functions of thioredoxin is to regenerate the reducing capacity of the peroxiredoxins after they have detoxified $H_2O_2$ (8). In all of these cases, we have traced the resistance to the signaling phase of apoptosis, before cytochrome *c* release. These data suggest that the redox environment of the cell and the ability to metabolize $H_2O_2$ influences the ability of glucocorticoids to signal for apoptosis in lymphoid cells.

The ability of each of the antioxidant defenses mentioned earlier to operate in the WEHI7.2 cells depends on a supply of reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). Glutathione peroxidase uses reducing equivalents from glutathione to metabolize $H_2O_2$, resulting in glutathione disulfide. Glutathione is regenerated via glutathione reductase by using reducing equivalents from NADPH (11). Regeneration of the reduced form of thioredoxin also depends on NADPH (8). Catalase does not use NADPH directly to detoxify $H_2O_2$; however, NADPH binds to and stabilizes the catalase molecule (27).

The major supply of NADPH in the cell is from glucose 6-phosphate dehydrogenase (G6PDH), the first and rate-limiting step in the pentose phosphate pathway (PPP) (41). G6PDH activity increases in response to oxidative stress (55, 62). Increasing G6PDH activity by overexpression protects numerous cell types against oxidants (15, 16, 31, 46, 56). Overexpression of this enzyme in NIH3T3 cells is also oncogenic in nude mice (30). Because the effectiveness of each of the changes in the antioxidant defense described in the WEHI7.2 cells could depend ultimately on the NADPH production in the cell, this suggests the possibility that the oxidative stress resistance and thus glucocorticoid resistance could depend on G6PDH activity. Therefore, we designed the current study to determine whether increased G6PDH could protect against glucocorticoid-induced apoptosis and oxidants in WEHI7.2 cells. From a drug-design standpoint, this could be important because it would suggest a novel single target to inactivate the multiple overlapping and semiredundant $H_2O_2$-removal mechanisms associated with drug resistance in the WEHI7.2 cells.

## MATERIALS AND METHODS

### *Cell culture and transfections*

The mouse thymic lymphoma–derived WEHI7.2 parental cell line is a subclone of the WEHI7 cell line (10), which was established from thymic lymphomas induced by repeated irradiation in BALB/c mice (21). Cells were maintained, in suspension, in Dulbecco's Modified Eagle Medium–low glucose (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT) at 37°C in a 5% $CO_2$ humidified environment. Cells were maintained in exponential growth at a density between 0.01 and $2 \times 10^6$ cells/ml.

G6PDH-overexpressing cells were constructed by stably transfecting WEHI7.2 cells with a vector containing human G6PDH (31); the cDNA was excised from the original vector and inserted into the *EcoR*1 sites in the pcDNA3 vector (Invitrogen). Cells were transfected by electroporation, as described (57), and a clone pool was selected by growth in 800 μg/ml G418 (Life Technologies Inc., Grand Island, NY). Individual clones were selected from this pool by using limiting dilution. Twenty-five individual clones were screened for increased G6PDH expression by reverse transcription–polymerase chain reaction (RT-PCR). Three of these clones were selected for further study. Vector-only overexpressing clones were selected by limiting dilution in the presence of drug. All transfectants were maintained in 800 μg/ml G418 (Life Technologies, Inc., Grand Island, NY). Before each experiment, cells were cultured in medium in the absence of G418 for 1 week.

### *Chemicals and drugs*

All chemicals and drugs were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise specified.

### *RT-PCR*

Total cytoplasmic mRNA was extracted by using the Qiagen RNeasy Protect Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's specifications. RT-PCR primers and probes for G6PDH were designed based on the human G6PDH cDNA sequence (NM_000402) by using the assays by design service (Applied Biosystems Inc., Foster City, CA). The sequences of the oligonucleotides were as follows: forward primer, 5′-GGCAAGGCCCTGAACGA-3′; reverse primer, 5′-CGGCCACATCATGGAACTG-3′; probe, 5′-CCTCACCTCGGCCTTG-3′. The PCR product from this reaction is 54 base pairs. The 18S ribosomal subunit probe/primer set was obtained from Applied Biosystems, Inc., and used as the normalization and loading control. RT-PCR was carried out by using the TaqMan one-step reagents and protocol (Applied Biosystems, Inc.) with the appropriate probe/primer set by using the GeneAmp 5700 sequence-detection system and accompanying software (Applied

Biosystems, Inc.). The PCR products were visualized by separation with a pre-cast 12% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) with ethidium bromide staining.

### *Enzyme activities*

G6PDH activity was measured by using a modified version of the tetrazolium salt method described by Winzer (66). In brief, an aliquot of exponentially growing cells was incubated in the assay medium (66) with a final concentration of 6 m*M* glucose-6-phosphate for 30 min at room temperature. The assay medium was removed, and cells were lysed with 200 μl DMSO. Absorbance was read at 600 nm by using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) and activity calculated by using a molar extinction coefficient of 38,500 *M*/cm. Catalase activity was measured as previously described (57). For glutathione reductase activity, cell lysates were prepared as for catalase activity measurements, and glutathione reductase activity was measured by monitoring the disappearance of NADPH in the presence of glutathione disulfide (7). Activity was calculated by using an extinction coefficient of 6.3 m*M*/cm. All enzyme activities were normalized to cellular protein as measured by the BCA Protein assay kit (Pierce, Rockford, IL).

### *Measurement of glutathione, glutathione disulfide and NADP(H)*

Glutathione and glutathione disulfide were measured as dansyl derivatives by using high-performance liquid chromatography (HPLC) and fluorometric detection as described by Jones *et al*. (25). NADP(H) was extracted under alkaline conditions and measured by using the enzymatic cycling method of Jacobson *et al*. (24), which depends on the oxidation of thiazolyl blue. All measurements were normalized to cellular protein measured as described earlier.

### *Apoptosis measurements*

Sensitivity to dexamethasone was determined by incubating cells in a final concentration of 1 μ*M* dexamethasone in an ethanol vehicle (final concentration of ethanol, 0.01%) or an equivalent amount of vehicle alone. Viable cell number was determined by dye exclusion, as described previously (57).

The fraction of apoptotic cells after dexamethasone treatment was determined by using the apoptosis detection kit (R & D Systems, Inc., Minneapolis, MN) according to the manufacturer's specifications. Cellular fluorescence was measured and analyzed by using a FACscan flow cytometer with CELLQuest software (Becton Dickinson, San Jose, CA). Cells that were positive for annexin V staining and negative for propidium iodide staining were considered apoptotic. The percentage of apoptotic cells in the presence of dexamethasone was corrected for that in the vehicle-treated cells for each cell variant. Spontaneous apoptosis was measured by removing dead cells from the culture with Ficoll-Plaque Plus (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer's instructions. Cells were maintained in normal growth medium for 1 week, and the percentage of apoptosis was determined in untreated cells by using the Apoptosis Detection Kit (R & D Systems, Inc.). Ten thousand cells were analyzed per sample.

Caspase-3 activity was measured by using a caspase-3 colorimetric assay kit (R & D Systems Inc.) according to the manufacturer's specifications. Absorbance was measured by using the Microplate EL-311 plate reader with Kineticalc v2.03 software (Bio Tek Instruments Inc. Winooski, VT). Caspase-3 activity was normalized for cellular protein, measured as described earlier. Caspase-3 activity in the presence of dexamethasone was corrected for that in vehicle-treated cells for each cell variant.

Increased frequency of cells with apoptotic morphology due to dexamethasone treatment was verified by microscopic examination of dexamethasone- and vehicle-treated cells. Cells were fixed onto slides by using the Shandon Cytoblock Kit with the Cytospin 2 centrifuge (Shandon Inc., Pittsburgh, PA) and then stained by using the Diff-Quik staining kit (Dade Behring Inc., Newark, DE). Shrunken cells with nuclear condensation and apoptotic bodies were considered apoptotic (57).

### *ROS measurements*

Cells were washed with DMEM containing 0.5% calf serum (Hyclone Laboratories) and then incubated for 2 h at 37°C in a 5% $CO_2$ humidified environment in 0.5% serum DMEM containing 5 μ*M* 5-(and-6)-carboxy-2′7′-dichlorofluorescein diacetate (cDCFH) (C-369) or 20 μ*M* 2′7′-dichlorodihydrofluorescein diacetate (DCFH) (D-399) (Molecular Probes, Inc., Eugene, OR). Both dyes are taken into the cell and the acetate groups removed. cDCFH will fluoresce once the acetate groups are removed, but DCFH fluoresces only in the presence of ROS. Thirty minutes before analysis, 5 μg/ml propidium iodide was added to the medium. Cells were analyzed by using a FACScan flow cytometer with Cell Quest software (Becton Dickinson). Ten thousand cells were analyzed per sample. Cells that were propidium iodide positive were excluded from analysis. Fluorescence due to DCFH was corrected for the relative cDCFH fluorescence to account for differences in dye uptake between the variants and the WEHI7.2 cells.

### *ATP measurements*

ATP was measured by using the Bioluminescent Somatic Cell Assay Kit (Sigma-Aldrich Chemical Co.) according to the manufacturer's instructions, as previously described (59). To measure ATP without mitochondrial ATP production, cells were treated with 10 μ*M* carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), an uncoupler of mitochondrial respiration, for 2 h before cell harvest (59). ATP measurements were normalized to cell number.

### *$EC_{50}$ measurements*

Cells were grown in a range of drug or oxidant concentrations for 48 h. The relative cell number was then measured by using the Non-radioactive Cell Proliferation Assay (Promega Corp., Madison, WI), and the fraction control absorbance calculated as previously described (14). The $EC_{50}$ was calculated as the concentration at which the absorbance was 50% that of the control. For each cell variant at least three independent plates were assayed.

### *Statistics*

Sample means were compared by *t* tests assuming unequal variances by using the algorithm in Excel (Microsoft Corp., Redmond, WA) or the formula from Moore and McCabe (40). Significance was set at $p \leq 0.05$.

## RESULTS

### *Overexpression of G6PDH increases enzyme activity and NADP(H)*

We established WEHI7.2 cells that stably overexpressed G6PDH to test whether G6PDH activity is a key determinant of glucocorticoid sensitivity in these cells. Based on the screening of 25 drug-resistant clones by RT-PCR, we selected three clones and a clone pool that represented different levels of overexpression to test our hypothesis. The clone pool contains an unknown number of drug-resistant clones and was used as a control for clonal variation.

Overexpression of the G6PDH transgene resulted in significantly increased G6PDH activity (Fig. 1A). The G6PDH clone pool showed a 1.8-fold increase in activity compared with the parental WEHI7.2 cells. Clones G6PDH2 and G6PDH15 had slightly higher activities than the pool, a 1.9- and 2.3-fold increase, respectively.

G6PDH is the rate-limiting step in the formation of NADPH via the PPP (41). Therefore, increased G6PDH is expected to increase intracellular NADP(H). Overexpression of G6PDH significantly increased the intracellular NADP(H) (Fig. 1B). The G6PDH clone pool, G6PDH2, and G6PDH15 transfectants showed 1.2-, 1.3-, and 1.5-fold increased NADP(H), respectively, compared with the WEHI7.2 cells. The vector only–transfected clone, Neo2, did not show an increase in either G6PDH activity or intracellular NADP(H).

### *Elevated G6PDH activity increases sensitivity to dexamethasone-induced apoptosis*

To compare the glucocorticoid sensitivity of the parental and G6PDH-overexpressing cells, we treated the cells with dexamethasone, a synthetic glucocorticoid. The G6PDH-overexpressing cells showed an accelerated loss of viable cells from the cell culture compared with the WEHI7.2 and vector-only (Neo2) transfectants (Fig. 2A). No increase in cell death due to vehicle (EtOH) alone was seen (data not shown).

The data in Fig. 2A indicate that dexamethasone is causing increased cell death in the G6PDH-overexpressing cells; however, they do not address the mechanism of cell death. To determine whether the cells were undergoing increased apoptosis in these cultures, we used three independent assessments of apoptosis. First, we measured phosphatidylserine exposure by relative annexin binding; this event occurs concomitant with cytochrome *c* release in WEHI7.2 cells (57, 59). After 18 h in dexamethasone, greater phosphatidylserine exposure was found in the G6PDH-overexpressing cells than in the WEHI7.2 cells (Fig. 2B). Second, we measured caspase-3 activity as an indication of the activation of an apoptosis-specific enzyme (22). We also found significantly increased caspase-3 activity in the G6PDH-overexpressing cells compared with the WEHI7.2 and Neo2 cells after a 12-h dexamethasone treatment (Fig. 2C). The highest caspase-3 activity in the G6PDH pool cell culture is consistent with the most rapid loss of viable cells in that culture after dexamethasone treatment. Finally, we saw an increase in cells displaying apoptotic morphology in the G6PDH-overexpressing cell cultures after a 24-h treatment with dexamethasone compared with the WEHI7.2 and Neo2 cell cultures (data not shown). These data suggest that overexpression of G6PDH sensitizes WEHI7.2 cells to dexamethasone-induced apoptosis.

### *Loss of G6PDH transgene expression correlates with loss of dexamethasone sensitivity*

During the initial screening of the selection drug-resistant clones after the G6PDH transfection, G6PDH clone 1 (G6PDH1) showed the greatest transgene expression by RT-PCR. G6PDH1 was more sensitive to dexamethasone-induced apoptosis than the WEHI7.2 cells when the first study was completed (week 1; Fig. 3A). Over time in continuous culture, the sensitivity to dexamethasone decreased. By week 5, the sensitivity of G6PDH1 was similar to that seen in the WEHI7.2 cells (Fig. 3A). In response to dexamethasone, caspase-3 activity, which was initially higher than WEHI7.2 values at week 1, was similar to that in WEHI7.2 cells by week 5 (data not shown). A comparison of the RT-PCR product by using RNA isolated before week 1 and after week 5 showed that we could no longer detect the presence of the transgene (Fig. 3B). This lack of amplification was similar to that seen in the WEHI7.2 cell RNA. G6PDH activity in the G6PDH1 cell sample collected after week 5 did not significantly differ from WEHI7.2 values (data not shown). These data corroborate the finding that the increased steroid sensitivity is due to the increased G6PDH expression.

### *Increased G6PDH does not increase glutathione in WEHI7.2 cells*

Studies in other cell types suggest that increased G6PDH activity and NADPH production generally protect cells by increasing the glutathione content (31, 46); therefore we compared the glutathione in the parental and G6PDH-overexpressing cells. As shown in Table 1, the glutathione content was not increased in the G6PDH-overexpressing cells. All the G6PDH-overexpressing cells had lower glutathione than the WEHI7.2 cells, although the G6PDH clone pool and G6PDH15 appeared to be within the normal glutathione range because the values in these cells were similar to those in the Neo2 clone. The glutathione in the G6PDH2 cells was significantly lower than in any of the other cell variants. No differences were found in glutathione disulfide or glutathione reductase activity. Glutathione peroxidase activity was undetectable because of a lack of sufficient selenium in the medium (data not shown).

NADPH also functions to stabilize catalase (27), so we tested whether G6PDH overexpression altered catalase activity. Unexpectedly, as shown in the last column of Table 1, catalase activity was significantly decreased in the G6PDH-overexpressing clones. The G6PDH clone pool, G6PDH2,

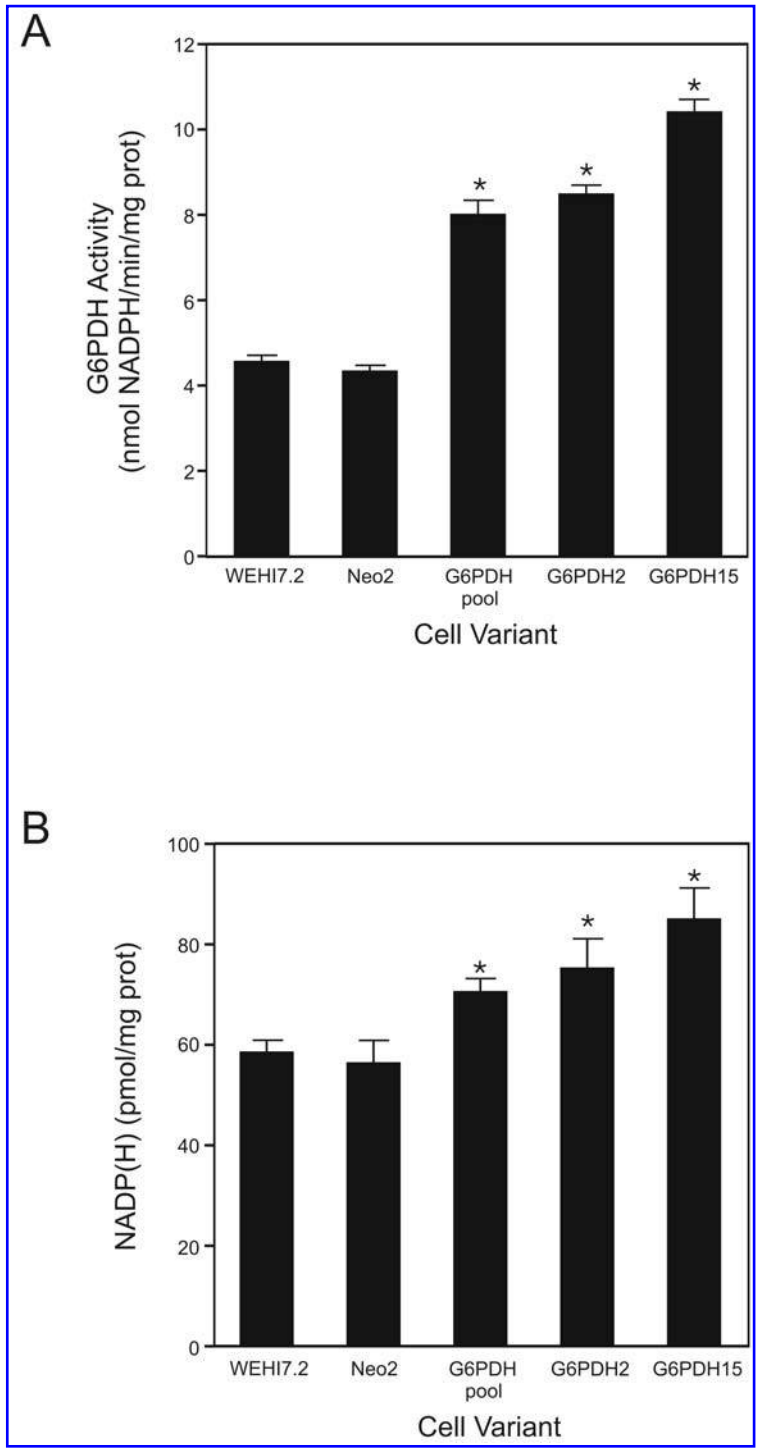


**FIG. 1. Overexpression of G6PDH increases activity and intracellular NADP(H). A:** Comparison of the specific activity of G6PDH in the parental, WEHI7.2, cells to that in the vector-only transfectants (Neo2), the G6PDH clone pool (G6PDH pool) and two G6PDH-overexpressing clones (G6PDH2 and G6PDH15). **B:** Comparison of intracellular NADP(H) in the parental, vector only-, and G6PDH-overexpressing transfectants. The bars represent the mean of six to 10 independent measurements + SEM. *Significantly different from WEHI7.2 values ($p \leq 0.05$).

and G6PDH15 had 1.54-, 2.27-, and 1.35-fold less catalase than the WEHI7.2 cells, respectively. Catalase activity in the Neo2 clone was similar to that in the WEHI7.2 cells.

### *G6PDH transfectants show signs of increased oxidative stress*

The profile of the G6PDH-overexpressing cells shown in Table 2 is not the expected profile of cells with an increased capacity to detoxify ROS. In our previous studies, we found that a change as small as a 1.4-fold increase in catalase significantly increased the resistance to oxidants (57, 58). This suggests that the decrease in the antioxidant defense in the G6PDH-overexpressing clones might be indicative of oxidative stress. To determine whether the G6PDH-overexpressing cells were oxidatively stressed, we compared the ROS production by using the ROS-sensitive dye, DCFH. As shown in Fig. 4, the ROS in the Neo2 cells was similar to that in the

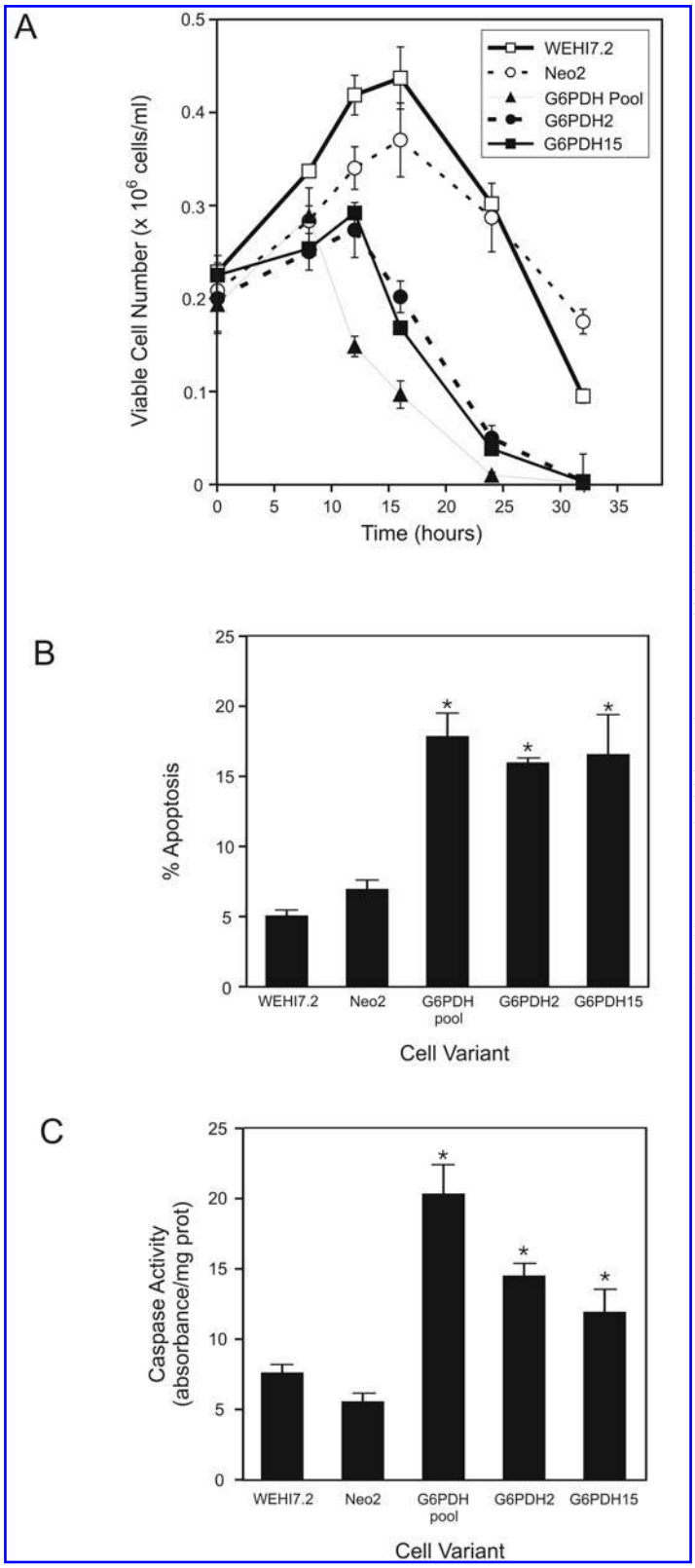


**FIG. 2. Overexpression of G6PDH increases sensitivity to glucocorticoid-induced apoptosis.** **A:** Comparison of viable cell number over time after the addition of 1 μ*M* dexamethasone at time 0. Viable cells were defined as those that excluded dye. Each point represents the mean ± SEM ($n$ = 3). This is a representative experiment that has been replicated. **B:** Comparison of the percentage of apoptotic cells in the cell culture 18 h after the addition of dexamethasone. Annexin-positive, propidium iodide–negative cells were considered apoptotic. Each bar represents the mean percentage of apoptosis in the presence of dexamethasone corrected for the apoptosis in the vehicle (EtOH)-treated cultures + SEM ($n$ = 9). **C:** Comparison of the relative caspase-3 activity after a 12-h treatment with dexamethasone. Each bar represents the mean caspase activity in the presence of dexamethasone corrected for the caspase activity in EtOH-treated cells + SEM ($n$ = 3). *Significantly different from WEHI7.2 values ($p \leq 0.05$).

WEHI7.2 cells; however, the ROS production was increased in the G6PDH-overexpressing cells. The increase in DCF fluorescence is not due to differential dye uptake because all the values were corrected for the fluorescence of 5-(and-6)-carboxy-2′7′dichlorofluorescein diacetate (C-369; Molecular Probes), a dye that fluoresces in the cell independent of ROS production. These data suggest that G6PDH overexpression causes oxidative stress in the WEHI7.2 cells.

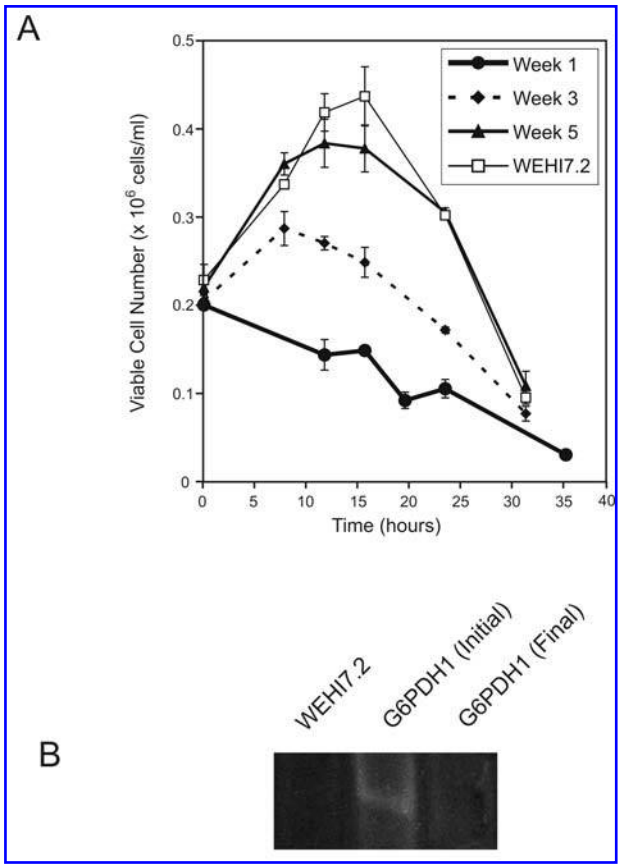


**FIG. 3. Loss of G6DPH overexpression correlates with loss of glucocorticoid sensitivity.** **A:** G6PDH clone 1 (G6PDH1) was maintained in continuous culture and tested for sensitivity to dexamethasone at the indicated weeks. The plots indicate the viable cell number over time after the addition of 1 μ*M* dexamethasone at time 0. Viable cells were defined as those that excluded dye. Each point represents the mean ± SEM ($n$ = 3). WEHI7.2 cells are included as a control. **B:** RT-PCR analysis of G6PDH expression in WEHI7.2 cells and in G6PDH1 cells before week 1 (initial) and after week 5 (final).

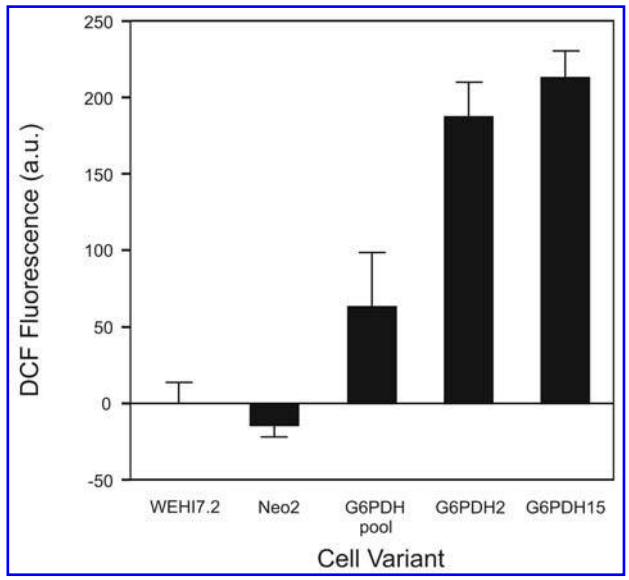


**FIG. 4. Overexpression of G6PDH increases oxidative stress.** Comparison of the mean ROS production (DCF fluorescence) in WEHI7.2, vector-only (Neo2), and G6PDH-overexpressing cells. Values are corrected for dye uptake by using an ROS-independent fluorescent dye as described in Methods. Values are normalized to WEHI7.2 cell ROS production set at 0. Error bars represent SEM after correction and normalization ($n$ = 9). The error bars for the WEHI7.2 cells represent the SEM of the DCF fluorescence without correction or normalization.

### *ATP production is altered in the G6PDH transfectants*

One possible explanation for the elevated ROS is that increasing the G6PDH expression increases the competition for glucose entering the cell. Once glucose enters the cell, it is phosphorylated and then goes through glycolysis or through the PPP (41). Increased movement of glucose through the PPP could result in decreased glucose available for ATP production, similar to the effect of glucose deprivation. Glucose deprivation results in increased ROS production in many cell types (1, 3, 35, 52). As shown in Fig. 5A, cellular ATP content was decreased slightly in the G6PDH clone pool and to a greater extent in the G6PDH2 and G6PDH15 cells. Cells in culture produce ATP via aerobic glycolysis, with lactate as the end product, or via mitochondrial respiration (37). By treating the cells with CCCP, an uncoupler of mitochondrial respiration, we were able to determine the relative contribution to the ATP pool from aero-

TABLE 1. COMPARISON OF GLUTATHIONE SYSTEM AND PEROXIDE REMOVAL CAPABILITIES IN THE G6PDH-OVEREXPRESSING CELLS, VECTOR ONLY-TRANSFECTANTS, AND THE PARENTAL WEHI7.2 CELLS

| *Cell variant* | *Glutathione (nmol/mg prot)* | *Glutathione disulfide (nmol/mg prot)* | *Glutathione reductase (μmol NADPH/min/mg prot)* | *Catalase (μmol $H_2O_2$/min/mg prot)* |
|---|---|---|---|---|
| WEHI7.2 | 96.45 ± 2.72 | 1.14 ± 0.038 | 43.76 ± 1.37 | 21.67 ± 1.13 |
| Neo2 | 81.32 ± 1.85* | 1.36 ± 0.082* | 48.78 ± 0.90* | 20.28 ± 1.45 |
| G6PDH pool | 84.09 ± 2.90* | 1.24 ± 0.114 | 47.83 ± 1.25 | 14.08 ± 0.73* |
| G6PDH2 | 64.34 ± 2.19* | 1.10 ± 0.078 | 41.45 ± 1.68 | 9.56 ± 0.99* |
| G6PDH15 | 85.21 ± 2.68* | 1.14 ± 0.098 | 45.00 ± 2.87 | 16.07 ± 0.72* |

Values represent mean ± S.E.M. (n = 6).
*Denotes significantly different from WEHI7.2 values ($p \leq 0.05$).

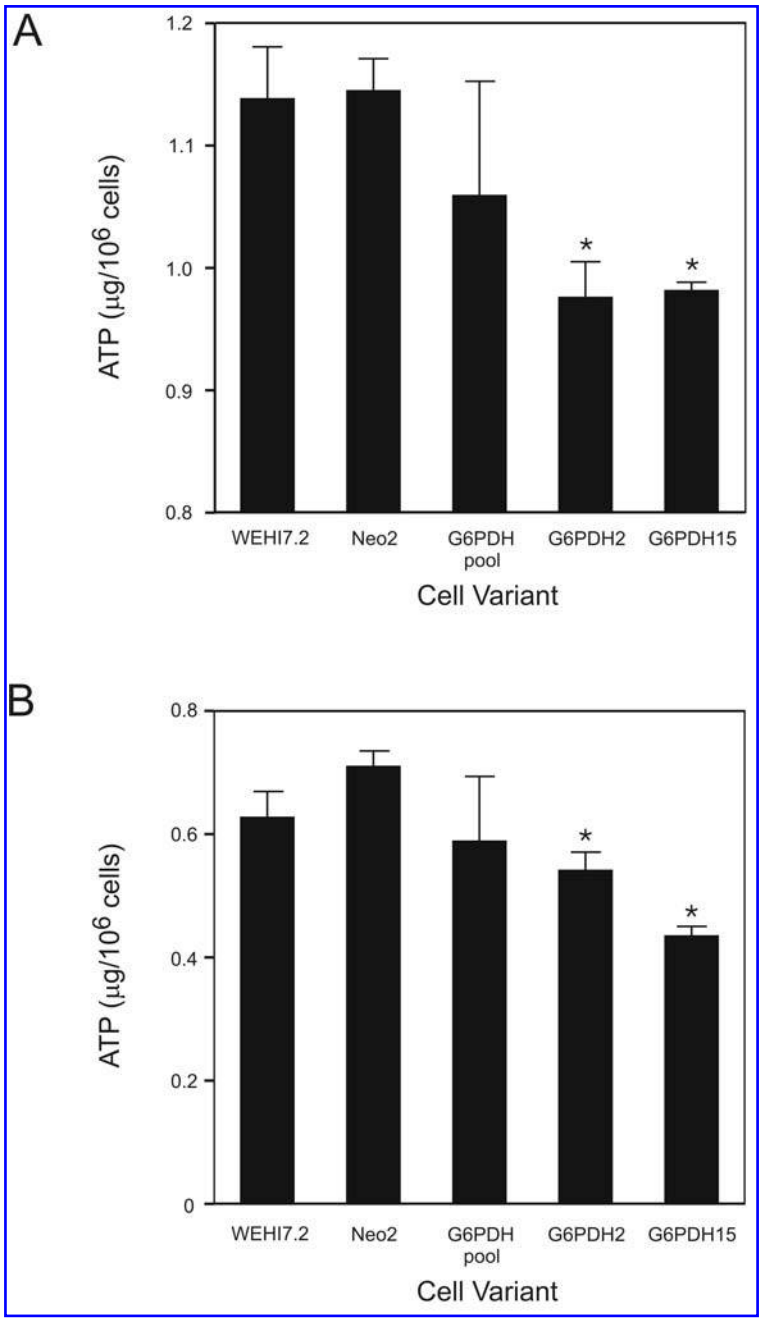


**FIG. 5. Overexpression of G6PDH decreases mitochondrial ATP production.** **A:** Comparison of the ATP pool in the WEHI7.2, vector-only (Neo2), and G6PDH overexpressing cells. **B:** Comparison of the contribution to the ATP pool by mitochondrial respiration measured by calculating the difference in the ATP in the cells in the presence and absence of CCCP, an uncoupler of mitochondrial respiration. Values represent the mean + SEM ($n$ = 9). *Significantly lower than WEHI7.2 values ($p \leq 0.05$).

TABLE 2. COMPARISON OF THE SPONTANEOUS APOPTOSIS AND THE $EC_{50}$ FOR HYDROGEN PEROXIDE AND LYMPHOMA CHEMOTHERAPEUTICS IN THE G6PDH-OVEREXPRESSING CELLS, VECTOR ONLY-TRANSFECTANTS, AND THE WEHI7.2 PARENTAL CELLS

| *Cell variant* | *Apoptosis (%)* | *$H_2O_2$ (μM)* | *Cyclophosphamide (mM)* | *Doxorubicin (nM)* | *Vincristine (nM)* |
|---|---|---|---|---|---|
| WEHI7.2 | 10.15 ± 0.89 | 121.06 ± 7.76 | 2.14 ± 0.31 | 8.12 ± 0.19 | 1.49 ± 0.12 |
| Neo2 | 9.12 ± 0.66 | 128.62 ± 8.31 | 2.45 ± 0.43 | 7.16 ± 0.27* | 1.14 ± 0.13 |
| G6PDH pool | 13.67 ± 1.34* | 83.89 ± 9.03* | 1.18 ± 0.07 | 4.07 ± 0.29* | 0.90 ± 0.08* |
| G6PDH2 | 18.44 ± 3.75* | 80.80 ± 4.54* | 1.28 ± 0.09 | 6.69 ± 0.38* | 1.30 ± 0.05 |
| G6PDH15 | 20.12 ± 1.13* | 74.25 ± 3.18* | 1.21 ± 0.12* | 4.46 ± 0.54* | 0.88 ± 0.03* |

Values represent mean ± S.E.M.: (n = 6) (apoptosis) n = 3 or 4 ($EC_{50}$ measurements).
*Denotes significantly different from WEHI7.2 values ($p \leq 0.05$).

bic glycolysis and mitochondrial respiration. The contribution to the ATP pool from aerobic glycolysis was similar in all the cell variants (data not shown). However, the contribution to the ATP pool from mitochondrial respiration was decreased slightly in the G6PDH clone pool and significantly in G6PDH2 and G6PDH15 compared with the WEHI7.2 cells (Fig. 5B). The relative production of ATP from the mitochondria was inversely proportional to the production of ROS and the G6PDH overexpression.

### *Overexpression of G6PDH sensitizes cells to hydrogen peroxide and chemotherapeutic drugs*

The G6PDH-overexpressing cells showed significantly increased spontaneous apoptosis in culture (Table 2), so our next step was to test whether they were more sensitive to oxidants and lymphoma chemotherapeutic drugs. All three transfectants were more sensitive to $H_2O_2$ than the parental or vector-only transfected cells (Table 2). The transfectants were also more sensitive to doxorubicin and cyclophosphamide than the parental WEHI7.2 cells. G6PDH15 was significantly more sensitive to cyclophosphamide, whereas the cyclophosphamide sensitivity of the G6PDH clone pool and G6PDH2 approached significance, $p = 0.056$ for each. Finally, the G6PDH clone pool and G6PDH15 were significantly more sensitive than the WEHI7.2 cells to vincristine. These data indicate the G6PDH transfectants are generally more sensitive to oxidants and multiple other agents.

## DISCUSSION

These data suggest that overexpression of G6PDH in WEHI7.2 cells models glucose deprivation. In the G6PDH-overexpressing cells, we think that the elevated G6PDH increases the proportion of glucose shunted through the PPP at the expense of glucose used for the production of ATP. This results in decreased ATP synthesis by the mitochondria and an increase in ROS in the G6PDH transfectants. The increased ROS and/or the decreased ATP may sensitize the cells to glucocorticoid-induced apoptosis as well as hydrogen peroxide and other lymphoma chemotherapeutics. Both increased ROS and decreased ATP synthesis occur in other cell types as a result of glucose deprivation (1, 17, 35, 52, 63).

The G6PDH-overexpressing cells may be particularly sensitized to glucocorticoids because in lymphoid cells, including WEHI7.2 cells, glucocorticoids shut off glucose uptake (9, 20, 41, 59). Once glucose import is shut down in the G6PDH-overexpressing cells, the glucose-deprivation effect would be amplified because even less glucose would be available in those cells to synthesize ATP. The resultant loss in ATP and increased ROS would occur more quickly in the G6PDH-overexpressing cells. This could explain the accelerated loss of these cells from the culture in the presence of dexamethasone.

The suggestion that overexpression of G6PDH increases ROS by modeling glucose deprivation is one potential explanation of the observations in the current study; however, other possibilities exist. Some enzymes that produce ROS consume NADPH. For example, NADPH is the source of electrons for the reaction catalyzed by NADPH oxidases, which function to produce ROS (41). NADPH is also involved in the reaction mechanism of the cytochrome P-450 enzymes, which function primarily to metabolize xenobiotics. Cytochrome P-450 enzymes are considered significant sources of ROS both in the presence and absence of substrates because of the uncoupling that occurs between NADPH consumption and substrate metabolism (68). An increase in NADPH could potentially increase ROS production by these enzymes, particularly if NADPH were previously available in limited quantities. Although the source of the increased ROS in the G6PDH-overexpressing cells remains to be determined, the increased baseline ROS may amplify the glucocorticoid-generated proapoptotic signals.

In other cell types, the mechanism by which G6PDH protects against oxidants is through increased glutathione and presumably the glutathione peroxidase system (31, 46). In the G6PDH-overexpressing WEHI7.2 cells, the antioxidant defense system is compromised; glutathione is not elevated, and catalase is decreased. This is reflected in the increased sensitivity to hydrogen peroxide seen in these cells. The compromised antioxidant defense system is expected to lower the threshold of oxidant exposure required to activate redox-sensitive signaling pathways that respond to oxidative stress and lead to oxidant-induced cell death.

In the G6PDH-overexpressing cells, glutathione may be used by multiple enzymes to mitigate the consequences of the higher observed basal ROS production (e.g., glutathione S-transferases conjugating glutathione to ROS-damage products) (54). No increase in GSSG was seen in the G6PDH-overexpressing cells, suggesting that if oxidation of glutathione is occurring, either the glutathione reductase and NADPH are sufficient to reduce GSSG to glutathione, or the GSSG is exported (47). The lower glutathione in the G6PDH transfectants may contribute to the glucocorticoid sensitivity because it has been shown that decreased GSH sensitizes thymocytes to glucocorticoid-induced apoptosis (65).

Lower catalase activity levels may also be a key to the increased sensitivity of the G6PDH-overexpressing cells to dexamethasone. A 1.4-fold increase in catalase is enough to cause significant steroid resistance in WEHI7.2 cells (57, 58). The decrease in catalase seen in the G6PDH cells ranged from 1.35- to 2.27-fold less than that in the parental cells. Based on our previous results, a change of this magnitude is within a range to cause a phenotypic effect. The higher basal ROS levels could contribute to the decreased catalase because the catalase protein is less stable in the presence of excess $H_2O_2$ (27). Catalase is also strongly regulated at the transcriptional level by the redox-sensitive transcription factor, NF-Y (43). NF-Y must be in a reduced form to bind and activate transcription (42); therefore excess ROS may inhibit catalase transcription.

We do not have an explanation for the G6PDH pool being more sensitive to glucocorticoids than G6PDH2 or G6PDH15, although the G6PDH expression is lower in the pool than in the two clones; however, several possibilities exist. The clone pool contains an unknown number of clones, some of which may be more sensitive to glucocorticoids because of additional factors besides G6PDH overexpression.

Glucocorticoid-induced apoptosis depends on the activation/repression of a gene network; thus multiple components contribute to the sensitivity to the steroid treatment (13). Alternatively, G6PDH2 and G6PDH15 are chronically exposed to higher oxidative stress than that in the clone pool. Partial adaptation to oxidative stress may have occurred to increase slightly the steroid resistance in G6PDH2 and G6PDH15 compared with the G6PDH clone pool. However, the partial adaptation still does not compensate for the effect of the G6PDH overexpression (*i.e.*, G6PDH2 and G6PDH15 are still more sensitive to steroids than the WEHI7.2 cells). Another possibility is that the amount the ROS increases with dexamethasone treatment is key in determining the speed with which apoptosis is initiated. In thymocytes, treatment with dexamethasone causes an increase in ROS from complex III in the mitochondria (60). In G6PDH2 and G6PDH15, the basal ROS production is already elevated. The most likely source of the ROS is increased leak from the mitochondria, as seen in glucose deprivation because of the lack of normal electron flow through the electron-transport chain (1). If basal ROS from the mitochondria is already elevated, as in the G6PDH2 and G6PDH15 clones, it may not be possible for it to increase as much or as quickly in these clones compared with the clone pool. Additional explanations are certainly possible, and the exact mechanism remains to be determined.

G6PDH-overexpression sensitizes the WEHI7.2 cells to multiple lymphoma chemotherapeutic drugs, which act via different mechanisms. Because G6PDH overexpression in the WEHI7.2 cells models glucose deprivation, this suggests that agents that cause glucose deprivation could sensitize lymphoma cells to other drugs. Glucocorticoid treatment of lymphoid cells decreases glucose uptake (9, 20, 41, 59). This suggests that use of glucocorticoids in a combined chemotherapy can both (a) induce apoptosis outright; and (b) act as a sensitizer for other chemotherapeutics by shutting down glucose uptake.

## PERSPECTIVES

We predict, based on the current study, that the redox changes caused by glucose deprivation alter steps in the signaling phase of apoptosis to increase apoptosis sensitivity in response to treatment with several lymphoma chemotherapeutic drugs. If this is true, agents that cause glucose deprivation could increase lymphoma treatment efficacy if they are used in a combined chemotherapy regimen. Glucose analogues such as 2-deoxy-D-glucose (2DG) are good candidates for this purpose because they are effective at inhibiting glucose metabolism both in cell culture and *in vivo* and are preferentially taken up by the tumor cells (23, 32, 34). Upon uptake, 2DG is phosphorylated by hexokinase, effectively trapping it in the cell where it cannot be further metabolized to synthesize ATP and acts as a competitive inhibitor of glycolysis (64). In lymphoma, the preferential uptake and trapping of 2DG-$PO_4$ has been exploited for diagnostics. Fluoride-labeled 2DG is currently used in positron emission tomography (PET) scans to image lymphoma and to assess chemotherapeutic response (23). Use of 2DG for treatment purposes has also been evaluated in a limited way in leukemia patients. A bolus treatment of 2DG was effective in decreasing glucose metabolism in leukemia cells isolated from patients and well tolerated, but as a single agent did not alter the course of the disease (32, 33). These data, combined with the data from the current study, suggest that use of 2DG or similar agents in combination therapy might enhance treatment efficacy in leukemia and lymphoma.

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## ABBREVIATIONS

CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; cDCFH, 5-(and -6)-carboxy-2′7′-dichlorofluorescein; DCF(H), 2′7′-dichlorodihydrofluorescein; 2DG, 2-deoxy-D-glucose; DMEM, Dulbecco's Modified Eagle Medium; G6PDH, glucose 6-phosphate dehydrogenase; G6PDH pool, pool of WEHI7.2 clones overexpressing G6PDH; G6PDH1, G6PDH2, G6PDH15, WEHI7.2 clones overexpressing G6PDH; Neo2, vector only-overexpressing WEHI7.2 cells; PET, positron emission tomography; PPP, pentose phosphate pathway; ROS, reactive oxygen species.

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