Regulation of Death Induction and Chemosensitizing Action of 3-Bromopyruvate in Myeloid Leukemia Cells: Energy Depletion, Oxidative Stress, and Protein Kinase Activity Modulation

Eva Calviño, María Cristina Estañ, Carlos Sánchez-Martín, Rocío Brea, Elena de Blas, María del Carmen Boyano-Adánez, Eduardo Rial, and Patricio Aller

Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain (E.C., M.C.E., C.S.-M., R.B., E.B., E.R., P.A.); and Departamento de Biología de Sistemas, Unidad de Bioquímica y Biología Molecular, Facultad de Medicina y Ciencias de la Salud, Universidad de Alcalá, Alcalá de Henares, Spain (M.C.-B.-A.)

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ABSTRACT

3-Bromopyruvate (3-BrP) is an alkyllating, energy-depleting drug that is of interest in antitumor therapies, although the mechanisms underlying its cytotoxicity are ill-defined. We show here that 3-BrP causes concentration-dependent cell death of HL60 and other human myeloid leukemia cells, inducing both apoptosis and necrosis at 20–30 μM and a pure necrotic response at 60 μM. Low concentrations of 3-BrP (10–20 μM) brought about a rapid inhibition of glycolysis, which at higher concentrations was followed by the inhibition of mitochondrial respiration. The combination of these effects causes concentration-dependent ATP depletion, although this cannot explain the lethality at intermediate 3-BrP concentrations (20–30 μM). The oxidative stress caused by exposure to 3-BrP was evident as a moderate overproduction of reactive oxygen species and a concentration-dependent depletion of glutathione, which was an important determinant of 3-BrP toxicity. In addition, 3-BrP caused glutathione-dependent stimulation of p38 mitogen-activated protein kinase (MAPK), mitogen-induced extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK), and protein kinase B (Akt)/mammalian target of rapamycin/p70S6K phosphorylation or activation, as well as rapid LKB-1/AMP (AMPK) activation, which was later followed by Akt-mediated inactivation. Experiments with pharmacological inhibitors revealed that p38 MAPK activation enhances 3-BrP toxicity, which is conversely restrained by ERK and Akt activity. Finally, 3-BrP was seen to cooperate with antitumor agents like arsenic trioxide and curcumin in causing cell death, a response apparently mediated by both the generation of oxidative stress induced by 3-BrP and the attenuation of Akt and ERK activation by curcumin. In summary, 3-BrP cytotoxicity is the result of several combined regulatory mechanisms that might represent important targets to improve therapeutic efficacy.

Introduction

The assumption that many cancers rely on glycolysis as a prominent source of energy, even under high oxygen tension conditions (“aerobic glycolysis” or the “Warburg effect”), has drawn attention to this process as an important target for the development of novel antitumor therapies (Cardaci et al., 2012a). For example, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid (lonidamine) and the glucose analog 2-deoxy-D-glucose (2-DG) have selective effects on tumors in culture and in animal models, also producing promising results in...
clinical trials. Nevertheless, these agents are not normally very effective when used in monotherapy; thus, their clinical use may be restricted to combined therapies as radiosensitizing and chemosensitizing drugs (Di Cosimo et al., 2003; Dwarkanath and Jain, 2009). In recent years, much attention has been focused on the action of 3-bromopyruvate (3-BrP), which appears to be a powerful energy-depleting agent and a potentially efficacious anticancer drug (for a brief historical review, see Ko et al., 2012). 3-BrP is a small alkylating agent that reacts with cysteine residues in proteins (Oronsky et al., 2012), and it has an intense cytoreductive effect on tumor-derived cell lines, causing apoptosis or necrosis, and eradicates tumors in animal models (reviewed by Ganapathy-Kanniappan et al., 2010, 2013).

Glyceraldehyde-3-phosphate-dehydrogenase has been identified as the main target of 3-BrP, provoking the inhibition of glycolysis and ATP depletion (Ganapathy-Kanniappan et al., 2010, 2013). However, with its alkylating capacity, 3-BrP may also react with many other molecules, influencing other cellular processes that are important for cell viability (Shoshan, 2012). For instance, 3-BrP inhibits the glycolytic enzymes 3-phosphoglycerate kinase and type II hexokinase, albeit with lower efficacy, as well as succinic dehydrogenase, thereby affecting mitochondrial respiration (Pereira da Silva et al., 2009). In addition, 3-BrP was reported to cause oxidative stress by stimulating the production of intracellular reactive oxygen species (ROS) (Kim et al., 2008) and by reducing intracellular glutathione (GSH) levels (Qin et al., 2010). It may also induce endoplasmic reticulum stress (Ganapathy-Kanniappan et al., 2010) and autophagy (Davidescu et al., 2012). Furthermore, 3-BrP can reverse multidrug resistance by inhibiting ATP-binding cassette transporter activity (Nakano et al., 2012). However, the capacity of 3-BrP to modulate protein kinase-related signaling pathways that are known to regulate cell death has been poorly studied, and the data available are somewhat conflicting (e.g., Bhardwaj et al., 2010; Lee et al., 2011).

The response of tumor cells to anticancer drugs, including energy-depleting agents, depends greatly on the environmental conditions and on the intrinsic metabolic characteristics of the cell model used. In this regard, the action of 3-BrP has been studied mainly in cell models derived from solid tumors, and little information is available from leukemia cell models, which often have a poor glycolytic phenotype (Xu et al., 2005; Berridge et al., 2010). 3-BrP was found to provoke dose-dependent apoptosis or necrosis in human acute myeloid leukemia (AML) cells, and the severity of cell death correlated closely with the intensity of ATP depletion (Xu et al., 2005). However, we recently found that moderate ATP depletion neither caused a loss of cell viability, nor could it explain the proapoptotic (chemosensitizing) capacity of the glycolytic inhibitor 2-DG in leukemia cells (Estar et al., 2012). With these precedents in mind, we set out to investigate how the induction of cell death is regulated by 3-BrP in HL60 cells and in other cell models of human AML. We conclude that moderate (20–60 μM) concentrations of 3-BrP effectively induce dose-dependent apoptosis and necrotic cell death that cannot be unequivocally explained by a single mechanism. Instead, this cell death appears to be the result of a combination of influences on glycolysis- and mitochondrial respiration–dependent ATP depletion, oxidative stress, and the modulation of proapoptotic and defensive protein kinase signaling pathways.
Flow Cytometry. The analysis of samples was carried out on an EPICS XL flow cytometer (Coulter, Hialeah, FL) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to fluorescein isothiocyanate (FITC), H$_2$DCFDA, calcein-AM, and Rh123 were collected with a 525-nm band-pass filter and the signals corresponding to DHE and PI with a 620-nm band pass filter. A total of 10$^4$ cells were scored in cell cycle and annexin V-FITC/PI assays, and 5 $\times$ 10$^4$ cells in the other assays.

Cell Viability, Cell Cycle, Apoptosis, and Necrosis. Cell viability was determined by the MTT colorimetric assay. Cell-cycle phase distribution was routinely determined by cell permeabilization followed by PI staining and flow cytometry analysis. This technique also provided an estimation of the frequency of apoptotic cells, characterized by low (sub-G$_1$) DNA content. In addition, apoptosis was evaluated by chromatin condensation or fragmentation, as determined by cell permeabilization, followed by DAPI staining and microscopy examination. The criterion used for necrosis (either genuine, “primary” necrosis or apoptosis-derived, “secondary” necrosis) was the loss of plasma membrane integrity, as determined by free PI uptake into nonpermeabilized cells and flow cytometry analysis. A detailed description of all of these techniques can be found in earlier works (Sánchez et al., 2010; Estañ et al., 2012, and references therein), and hence is omitted here. In addition, apoptosis and necrosis were determined simultaneously by double labeling with annexin V-FITC and PI and flow cytometry measurement using an AnoAlert annexin V-FITC kit (Clontech, Mountain View, CA) following the procedure indicated by the manufacturer. In this case, annexin V-positive/PI-negative cells were considered early apoptotic cells, annexin V-negative/PI-positive cells as genuine necrotic cells, and annexin-V negative/PI-positive cells as genuine necrotic cells.

Mitochondrial Membrane Permeabilization and Dissipation of the Membrane Potential. Inner mitochondrial membrane permeabilization (mIMP) was assessed by the calcein-AM/CoCl$_2$ method and flow cytometry, and the dissipation of mitochondrial membrane potential (ΔΨm) was measured using Rh123 and flow cytometry, as described elsewhere (Calviño et al., 2011). Control assays proving the adequacy of the techniques used were presented in the same article.

Oxygen Consumption and the Rate of Extracellular Acidification. The oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR, a proxy for lactate production) were determined using an XF24 instrument (Seahorse Bioscience, North Billerica, MA) (Wu et al., 2007a). HL60 cells were plated in 24-well Seahorse culture plates coated with 3.5 μg/cm$^2$ Cell-Tak (BD Biosciences, Bedford, MA), according to the manufacturer’s protocol. For the XF24 assay, RPMI 1640 growth medium was replaced by unbuffered RPMI 1640 medium (Sigma-Aldrich) containing 11.1 mM glucose and 2.05 mM L-glutamine supplemented with 1% fetal bovine serum. Subsequently, 10$^5$ cells per well were seeded in 100 μl of assay medium, and they were sedimented by low-speed centrifugation. The cells were allowed to attach for 30 minutes at 37°C in a CO$_2$-free incubator, and thereafter, the wells were supplemented with another 400 μl of warm RPMI 1640 medium and left for another 20 minutes at 37°C before placing the culture plate in the instrument. Basal oxygen consumption was recorded over 24 minutes, and subsequently, 1 μM oligomycin, 600 nM FCCP, and 400 nM FCCP were added. At the end of the run, 1 μM rotenone plus 1 μM antimycin A were added to determine the mitochondria-independent oxygen consumption. Four basal rates and three response rates (after the addition of each compound) were measured, averaged, and the averaged OCR in the presence of rotenone and antimycin A was subtracted. The basal rate of glycolysis was estimated from the ECAR determinations under the same conditions (Wu et al., 2007a).

ATP Measurements. Intracellular ATP content was measured using the ATP Bioluminescence Assay Kit ASII (Roche, Mannheim, Germany). At the end of the treatments, samples of approximately 10$^6$ cells were washed once with PBS and then processed according to the manufacturer’s protocol. The ATP-derived fluorescent signal was measured on a Varioskan Flash microplate reader (Thermo Fisher Scientific Inc., Waltham, MA). Cell samples were collected in parallel to take into account possible variations in protein content, and the ATP values were normalized accordingly.

ROS and GSH levels. The rate of ROS formation was estimated in a Varioskan Flash microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using the fluorescent probe H$_2$DCFDA (10 μM), a nonspecific ROS indicator (Eruslanov and Kusmartsev, 2010). Cells (3 $\times$ 10$^4$ per well) were seeded in 96-well plates in RPMI 1640 medium without serum and phenol red. The cells were pretreated for 1 hour at 37°C with the different drug concentrations; at the end of the incubation, the fluorescent probe was added automatically to each well. The fluorescence intensity was recorded for 50 minutes, taking measurements every 2 minutes, and the maximum rate of the increase in fluorescence was calculated after subtracting the fluorescence obtained in the absence of cells.

The intracellular accumulation of ROS was measured in an EPICS XL flow cytometer (Coulter, Hialeah, FL) using H$_2$DCFDA and the anion superoxide-specific probe DHE (Wardman, 2007). At the end of the drug treatments, the cells were labeled (30 minutes at 37°C) with the corresponding probe (5 μM), washed extensively with ice-cold PBS, resuspended in ice-cold RPMI 1640 medium, and analyzed by flow cytometry. Internal controls using unlabeled cells indicated that there was no Br-BrP or ATO autofluorescence in any of the conditions assessed.

The intracellular GSH content was determined in a Varioskan Flash microplate reader at excitation wavelength of 390 nm and emission wavelength of 520 nm, using the fluorescent probe monochlorobimane (Fernández-Checka and Kaplowitz, 1990). At the end of the treatments, samples of approximately 5 $\times$ 10$^4$ cells were washed and resuspended in 400 μl of PBS containing 2 mM monochlorobimane. After a 30-minute incubation at 37°C in the dark, the cells were centrifuged and resuspended in 400 μl of PBS, and 100-μl aliquots were taken to estimate the fluorescence. Cell samples were collected in parallel to take into account possible variations in protein content, and the GSH values were normalized accordingly.

Immunoblotting. Cells were collected by centrifugation and washed with PBS, and total protein extracts were obtained by lysing them for 20 minutes at 4°C in a buffer consisting of 20 mM Tris-HCl (pH 7.5) containing 137 mM NaCl, 10% (v/v) glycerol, and 1% Nonidet P-40 and supplemented with a protease inhibitor cocktail, 1 mM sodium orthovanadate, and 10 mM NaF. After centrifugation for 15 minutes at 10,000 g, the supernatants were collected, and samples containing equal amounts of proteins were resolved by SDS-PAGE. The proteins were then transferred to membranes and immunodetected, as previously described (Galán et al., 2000).

Data Presentation. Except when indicated, all experiments were repeated at least three times, and as a rule, the results are expressed as the mean value ± S.D. The significance of the differences between the experimental conditions was calculated using the Student’s t test, and positive differences are indicated by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

Cell Growth and Cell Death. We first assessed the capacity of 3-BrP to decrease the viability of HL60 AML cells and to provoke apoptotic and/or necrotic death when administered for 24 hours at concentrations ranging from 10 to 60 μM. At concentrations above 10 μM, 3-BrP clearly affected cell growth, as evident in the MTT assay (Fig. 1A). Flow cytometry indicated that intermediate drug concentrations (20–30 μM) provoked apoptosis in a fraction of cells, as witnessed by the appearance of cells with a sub-G$_1$ DNA

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content (Fig. 1, B and C). At these concentrations 3-BrP also disrupted the plasma membrane in another fraction of the cells, a characteristic of necrosis, as indicated by the uptake of free PI (Fig. 1, D and E). By contrast, the highest concentration of 3-BrP (60 μM) produced an almost exclusively necrotic response (Fig. 1, D and E) with negligible apoptosis (Fig. 1, B and C). Likewise, dose-dependent apoptotic and necrotic responses were also obtained with human U939 and NB4 cells with similar efficacy as in HL60 cells (data not shown). It was noteworthy that exposure to the pan-caspase inhibitor z-VAD-fmk greatly reduced the number of apoptotic (sub-G1 DNA) cells (Fig. 1, B and C), but it had little effect on the frequency of PI-permeable cells (Fig. 1, D and E), indicating that this represents a genuine necrotic response rather than apoptosis-derived “secondary” necrosis (or late apoptosis). By contrast, treatment with the glycolytic inhibitor 2-DG (20–40 mM), used for comparison, also caused dose-dependent apoptosis and free PI uptake, but in this case, PI-permeability was almost totally suppressed by z-VAD-fmk, suggesting that this represented late apoptosis instead of genuine necrosis (see Supplemental Fig. 1). The apoptosis provoked in the presence of 30 μM 3-BrP was corroborated by measuring chromatin condensation or fragmentation (data not shown), phosphatidylserine translocation (measured as annexin V staining, Fig. 1F), and caspase-3 cleavage or activation (Fig. 1G), with negligible annexin V and caspase cleavage after exposure to 60 μM 3-BrP. Time-course assays showed that apoptosis was first evident after approximately 8 hours in the presence of 30 μM 3-BrP and that it augmented thereafter. By contrast, necrosis was detected even earlier in cells maintained in the presence of 60 μM 3-BrP (Supplemental Fig. 2A). Finally, 3-BrP rapidly caused dose-dependent permeabilization of the mIMP, as revealed by the decrease in calcein retention (Supplemental Fig. 3A), followed by the dissipation of the mitochondrial membrane potential (ΔΨm) witnessed by Rh123 staining (Supplemental Fig. 3B). Similar mitochondrial dysfunction was also observed with other glycolytic inhibitors (Calviño et al., 2011; Estañ et al., 2012) and could represent a determinant for the induction of cell death.

**Energy Metabolism.** Since 3-BrP may affect the activities of glycolytic enzymes and those involved in mitochondrial metabolism, it was important to examine the impact of 3-BrP on cellular energy levels. The use of [3-3H]glucose in the presence or absence of 3-BrP demonstrated that 3-BrP inhibited glucose utilization and glycolysis, as evidenced by the decrease in [3-3H]glucose incorporation into 2-DG (Supplemental Fig. 2B), consistent with the findings of previous studies on the effects of 3-BrP on glycolysis and the induction of cell death. These findings suggest that 3-BrP may act as a potential therapeutic agent for the treatment of leukemia, particularly in the context of targeting glycolytic pathways and mitochondrial dysfunction.
respiration (Pereira da Silva et al., 2009), we investigated the relative capacity of 3-BrP to affect these processes in HL60 cells. Accordingly, we simultaneously measured the OCR and the ECAR (a proxy for lactate production) of these cells as an estimate of the rates of mitochondrial respiration and glycolysis, respectively. In HL60 cells, lactate production was rapidly abolished in the presence of 3-BrP, and although there was a 75% inhibition of the lactate produced by 20 μM 3-BrP (Fig. 2A), mitochondrial respiration was less affected, and only approximately 35% inhibition was evident at the same concentration (Fig. 2B). This difference was even greater when only the oligomycin-sensitive component was considered (Fig. 2D), suggesting that oxidative phosphorylation probably relied on nonglycolytic substrates like glutamine and fatty acids. The different sensitivity of these two processes, lactate formation and respiration, is reflected as a progressive increase in the OCR/ECAR ratio (Fig. 2C). Finally, 60 μM 3-BrP caused complete inhibition of both lactate production and respiration (Fig. 2, A and B).

There is evidence that ATP depletion may compromise cell viability, leading to either apoptosis or necrosis in function of the extent of depletion (Lieberthal et al., 1998; Lemasters et al., 2002). Since the effects of 3-BrP on glycolysis and respiration ought to be reflected in the generation of ATP, we examined the changes in total intracellular ATP content caused by exposure to 20–60 μM 3-BrP. A concentration-dependent decrease in ATP levels was evident after exposure to 3-BrP (Fig. 2E), which roughly correlated with the cell death observed (see Fig. 1). At the highest 3-BrP concentration (60 μM), ATP fell below the limits considered as minimal to permit activation of the apoptotic machinery (20%–25% of the control levels: Leist et al., 1997; Lieberthal et al., 1998), which is consistent with the total absence of apoptosis and the generation of a pure necrotic response. By contrast, the functional relevance of the partial decrease in ATP (approximately 50%) provoked in the presence of 20–30 μM 3-BrP is unclear. In fact, treatment with 10 mM 2-DG (included for comparison) caused a similar depletion of ATP (Fig. 2E and Supplemental Fig. 4A), but the lethality was negligible (see Supplemental Fig. 1).

Oxidative Stress. To analyze the capacity of 3-BrP to produce oxidative stress, we measured ROS generation and GSH depletion and the potential relevance of these effects as determinants of apoptosis or necrosis in HL60 cells. Exposure for 50 minutes to 30 μM 3-BrP stimulated the rate of ROS generation approximately 3-fold, as evident by fluorimetry using the nonspecific fluorescent probe H_2DCFDA, whereas treatment with a nontoxic concentration of 3-BrP (10 μM) had no effect (Fig. 3A). Other respiratory inhibitors also increased the rate of ROS generation to some extent, namely, rotenone (complex I) and antimycin A (complex III). In addition, exposure for 3 and 6 hours to 30 μM 3-BrP resulted in a mild yet significant increase in intracellular ROS accumulation, as evident in flow cytometry assays using H_2DCFDA, as well as with the anion superoxide indicator DHE (Fig. 3, B and C). Exposure for 3 hours to 20–60 μM 3-BrP caused a dose-dependent depletion of GSH, which almost totally disappeared at 60 μM. This effect persisted for at least 24 hours at the intermediate (30 μM) concentration (Fig. 4A). To further examine the importance of GSH depletion as a regulator of 3-BrP-provoked toxicity, experiments were performed with BSO, an inhibitor of the rate-limiting enzyme for GSH biosynthesis, γ-glutamyl cysteine synthetase (Griffith and Meister, 1979), and with the permeable GSH derivative GSH-OEt. Treatment for 24 hours with 1 mM BSO alone progressively decreased the intracellular GSH content to levels similar to those detected in the presence of 30 μM 3-BrP (Fig. 4B). Notably, this partial GSH depletion was not toxic per se, because BSO did not cause apoptosis or necrosis, nor did it affect the cell-cycle distribution even after a 48-hour exposure (Fig. 4, C and D, and data not shown). However, pre-exposure

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**Fig. 2. Effect of 3-BrP on the energy metabolism of HL60 cells. (A–D)** Analysis of lactate formation and mitochondrial respiration rates after a 60-minute exposure to 3-BrP. The extracellular acidification rate (ECAR, a proxy for the rate of lactate formation) and the rate of oxygen consumption (OCR) were determined on a Seahorse XF24 metabolic flux analyzer. (A and B) Basal ECAR and OCR, respectively. (C) Ratio between the basal OCR and ECAR. (D) Mitochondrial ATP turnover determined from the oligomycin-sensitive component of the basal rate of respiration. Data points represent the mean ± S.D. of at least three independent experiments performed in sextuplicate. (E) Intracellular ATP content, measured in a luminometric assay of HL60 cells, exposed to the indicated concentrations of 3-BrP for the times shown. Treatment of 3 hours with 10 mM 2-DG was included for comparison, and treatment of 3 hours with 10 μM oligomycin in glucose-free medium (Glu-7/Oligo) was used as a control. The results (the mean ± S.D. of at least four independent determinations) are expressed in relation to untreated (Cont) cells (approximate ATP content 20 nmol/10⁶ cells). Asterisks indicate significant differences in relation to the controls: *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 3. ROS generation and accumulation in HL60 cells. (A) The rate of ROS formation was estimated from the increase in fluorescence of the cell-permeant redox-sensitive fluorescent indicator H_{2}DCFDA measured with a microplate reader. Fluorescence was monitored for 50 minutes after 1-hour exposure to either 10 or 30 μM 3-BrP. The results represent the mean ± S.E.M. of six independent measurements. (B) Relative intracellular accumulation of ROS after 3 and 6 hours in the presence of 30 μM 3-BrP, measured by flow cytometry using the H_{2}DCFDA (nonspecific) and DHE (anion superoxide-specific) probes. The results represent the mean ± S.D. of at least four independent determinations. All results are expressed in relation to untreated cells (Cont, arbitrary value of 1.0). Some flow cytometry histograms at 3 hours are represented as examples in (C). Asterisks indicate the significant differences relative to the controls: *P < 0.05; **P < 0.01.

for 24 hours to BSO greatly potentiated 3-BrP toxicity, as witnessed by the increase in the frequency of apoptotic and/or necrotic cells in the presence of 10 and 20 μM 3-BrP and by the partial decrease in apoptosis with a concomitantly higher incidence of necrosis following exposure to 30 μM 3-BrP (Fig. 4, C and D). In other experiments, cells were preloaded for 4 hours with 5 mM GSH-OEt, which had to be eliminated before treatment with 3-BrP to avoid any possible direct interactions between 3-BrP and GSH in the culture medium. Despite this technical limitation, GSH-OEt to some extent attenuated GSH depletion (Fig. 4E); also, it reduced the apoptosis and necrosis caused by 30 μM 3-BrP (Fig. 4, F and G). Taken together, these results indicate that although moderate GSH depletion is not toxic per se, it is a determinant of 3-BrP toxicity in the leukemia cell model. Significantly, comparative experiments showed that 10–30 mM 2-DG also caused a slight, dose-dependent decrease in GSH (Supplemental Fig. 4B) and that preincubation with 1 mM BSO increased the frequency of apoptotic and PI-permeable cells in 2-DG-treated cultures, but this effect was much weaker than in the case of 3-BrP (Supplemental Fig. 4, C and D).

Protein Kinase Modulation. p38 MAPK has been characterized as a protein kinase responsive to moderate oxidative stress (Kurata et al., 2000), whereas AMPK is activated by oxidative stress and by agents causing ATP depletion (Hardie, 2007). Moreover, recent studies showed that 3-BrP may affect ERK and Akt activation, although this phenomenon appeared to be dependent on the cell model studied (Bhardwaj et al., 2010; Davidescu et al., 2012). For these reasons, we analyzed the effect of 3-BrP on the phosphorylation-activation of these kinases and their possible regulatory role in the induction of apoptosis or necrosis in leukemic cells. The results are represented in Fig. 5A. Exposing HL60 cells to 20–60 μM 3-BrP for 4 and 8 hours caused a dose-dependent phosphorylation of p38 MAPK. Similarly, 3-BrP caused a dose-dependent phosphorylation of Akt and, except at a concentration of 60 μM, of the downstream mTOR and p70S6K kinases. 3-BrP also stimulated ERK phosphorylation, although as a function of the time of treatment. Conversely, 3-BrP caused a dose-dependent dephosphorylation-inactivation of AMPK, as well as that of the upstream LKB-1 and downstream ACC kinases. Of note, after 8 hours in the presence of 3-BrP (30 and 60 μM), some AMPK degradation was evident (see double bands in the blot), an effect that was also observed on long-term treatments with other glycolytic inhibitors (Estaèn et al., 2012). With minor quantitative differences, similar modifications of protein kinases were obtained using NB4 instead of HL60 cells (data not shown).

Because AMPK inactivation by a metabolic inhibitor may be contemplated as an unusual response, and also because Akt and AMPK are thought to be antagonistic kinases (Jin et al., 2007; Lee and Park, 2010; Kuznetsov et al., 2011), we investigated the possible Akt-AMPK interaction in our model system. Hence, HL60 cells were subjected to short treatments (from 15 minutes to 2 hours) with 30 μM 3-BrP, or they were treated for 4 hours with 3-BrP in combination with the PI3K/Akt inhibitor LY294002. After 15 minutes in the presence of 30 μM 3-BrP, AMPK was mildly hyperphosphorylated, a response that was reversed after a 30-minute exposure and coinciding with the initiation of Akt phosphorylation (Fig. 5B). Moreover, cotreatment with LY294002 not only suppressed Akt phosphorylation, but it also prevented the decrease in AMPK phosphorylation, which increased to levels even higher than those in untreated cells (Fig. 5C). Together, these results indicate that Akt activation regulates AMPK inactivation in 3-BrP-treated cells.

The possible influence of 3-BrP-provoked GSH depletion on protein kinase activation was analyzed in cells preloaded with GSH-OEt prior to exposure to 30 μM 3-BrP. GSH-OEt prevented or attenuated the increase in p38 MAPK, Akt, and ERK phosphorylation (Fig. 5D), further evidence that GSH depletion is an important early regulatory event for 3-BrP toxicity.

Akt and ERKs are normally considered defensive, anti-apoptotic kinases, whereas p38 MAPK may exert proapoptotic or antiapoptotic functions (Wada and Penninger, 2004; Steelman et al., 2008). The regulatory role of these kinases on 3-BrP toxicity was investigated in HL60 cells using specific pharmacological inhibitors, namely, the p38 MAPK inhibitors BIRB 796 (0.1 μM) and SB203580 (10 μM), the Akt inhibitor triciribine (AktiV, 10 μM), the MEK/ERK inhibitor U0126 (5 μM), and the AMPK inhibitor compound C (CC, 5 μM). The optimal concentrations of these inhibitors (effective kinase inhibition and low toxicity) were selected from our previous studies or in preliminary assays (data not shown). Cotreatment with BIRB 796 and (with lower efficacy) SB203580 reduced both the apoptosis and necrosis induced by 3-BrP, although this response was enhanced in the presence of AktiV.
and U0126 (Fig. 6). These results indicate that p38 MAPK activation facilitates 3-BrP toxicity, and Akt and ERK activation probably fulfills a defensive role by reducing excess drug toxicity. Moreover, although 3-BrP per se was sufficient to downregulate AMPK, cotreatment with the AMPK inhibitor compound C (5 μM) further enhanced the apoptosis and necrosis induced by 3-BrP (Fig. 6). This result indirectly suggests that AMPK plays a defensive role and, hence, that its inactivation by 3-BrP facilitates drug toxicity.

**Cooperation of 3-BrP with Other Antitumor Drugs.**
It was earlier reported that cotreatment with 3-BrP increased the toxicity of some conventional antitumor agents (Ihrlund et al., 2008) or decreased the cell’s resistance to them (Xu et al., 2005; Hulleman et al., 2009). We hypothesized that ROS overproduction and, especially, GSH depletion by 3-BrP, could serve to increase the toxicity of oxidant-sensitive antitumor drugs (i.e., drugs that cause greater lethality under environmental oxidizing conditions) and, conversely, that the potential inhibition of defensive kinases by some antitumor drugs might enhance 3-BrP toxicity. For this reason, and based on our preceding studies, we studied the effect of 3-BrP in combination with the antileukemic agent ATO, which is highly sensitive to oxidative stress, as well as with the phenolic agent curcumin that inhibits Akt activation in myeloid cells (Sánchez et al., 2010 and references therein). Treatment of 24 hours with 2 μM ATO or 7 μM curcumin alone caused negligible cell death (apoptosis or necrosis). However, ATO and curcumin cooperated with 3-BrP to cause apoptosis or necrosis, with higher efficacy (at 20 μM 3-BrP) in the case of ATO (Fig. 7, A and B). These results correlated with the increase in apoptosis generated by ATO and curcumin after preincubation with BSO, which, as indicated, decreased GSH levels without causing cell toxicity per se, again with a higher efficacy (at 20 μM 3-BrP) in the case of ATO (Fig. 7, C and D). It is noteworthy that we did not detect cooperation in the induction of apoptosis or necrosis between 3-BrP and the DNA-damaging agent cisplatin (4 μM), which correlated with the inability of BSO to potentiate cisplatin lethality (Fig. 7, A–D). Finally, exposure to curcumin for 4 hours strongly attenuated the Akt and ERK phosphorylation provoked by 3-BrP, whereas ATO only slightly attenuated Akt
phosphorylation. Conversely, curcumin and ATO caused little or no effect on the increase in p38 MAPK phosphorylation provoked by 3-BrP, respectively (Fig. 7E). Hence, it would appear that the pro-oxidant action of 3-BrP may serve to potentiate curcumin and particularly ATO toxicity and that the inactivation of the defensive kinases by curcumin may serve to potentiate 3-BrP toxicity.

**Discussion**

The results presented here indicate that 3-BrP causes apoptotic and/or necrotic cell death in HL60 and other (U937, NB4) human myeloid leukemia cell lines. Cell death occurred within a narrow range of drug concentrations, since no lethality was observed at 10 μM, reaching approximately 50% death cells at 30–60 μM, namely, a mixture of apoptotic and necrotic cells at 30 μM and only necrosis at 60 μM. To adequately interpret these results, it must be considered that the efficacy of antitumor drugs is strongly dependent on the environmental conditions and metabolic characteristics of the cell model analyzed. In particular, it has been reported that the moderate lethality of 3-BrP on HL60 and other cancer cells grown under standard (normoxic) culture conditions was accentuated when the cells were switched to a more glycolytic dependence, as in mutants defective in mitochondrial respiration or by culturing under hypoxic conditions (Xu et al., 2005). This is a relevant observation, since blood flow limitation and subsequent hypoxia affect not only solid tumors but also leukemic cells at the bone marrow (Jensen et al., 2000). Therefore, the underlying study should be interpreted as an attempt to obtain new insight into the mode of action of 3-BrP in the particular setting of respiratory-sufficient AML cell lines.

Elucidating the mechanisms responsible for 3-BrP toxicity is difficult, given that as an alkylating drug it may affect multiple molecular targets that are important for cell proliferation and viability. It is generally assumed that ATP depletion is the main determinant of 3-BrP toxicity and 3-BrP was also reported to inhibit glyceraldehyde-3-phosphate-dehydrogenase as a primary target (Ganapathy-Kanniappan et al., 2010, 2013). Indeed, glycolysis appears to be affected by the lowest concentration of 3-BrP assayed here (10 μM), although with no effect on viability. At double this concentration (20 μM), the preservation of oxidative phosphorylation when lactate formation is almost completely depressed can be interpreted as the result of the inhibition of the glycolytic flow and the switch to mitochondrial oxidation of alternative substrates like glutamine or fatty acids (Samudio et al., 2010). The inhibition of mitochondrial respiration at the maximal concentration (60 μM) is likely to reflect the effect of 3-BrP on the respiratory chain (Pereira da Silva et al., 2009). This latter response is consistent with the almost total collapse of ATP levels and the execution of a pure necrotic response, since apoptosis is an energy-dependent process requiring ATP (Leist et al., 1997). By contrast, the partial ATP depletion at lower 3-BrP concentrations (20–30 μM) may not adequately explain cell death, since similar ATP depletion was caused by 10 mM 2-DG without provoking significant apoptosis or necrosis. Indeed, we previously reported that the moderate depletion of ATP caused by glucose starvation reduced the rate of cell proliferation without causing cell death in HL60 cells (Estañ et al., 2012). Notably, 3-BrP does not induce
apoptosis or necrosis of glioblastoma cells, but rather it activates autophagy in association with ATP depletion and a decrease in the Δψm (Macchioni et al., 2011). In our experiments, 20–30 μM 3-BrP decreased ATP and induced mIMP, and preliminary observations also point to a decrease in p62/SQSTM1 levels (data not shown) that is a characteristic of autophagy. Because autophagy is considered a prosurvival response opposing apoptosis and necrosis (Wu et al., 2009; Han et al., 2011), an attractive possibility is that the intensity of apoptosis or necrosis in 3-BrP-treated AML cells is restrained by a concomitant autophagic response, a hypothesis that requires further study.

The generation of oxidative stress was postulated as another possible determinant of 3-BrP lethality (Kim et al., 2008; Qin et al., 2010; Cardaci et al., 2012b). In our cells, 3-BrP (30 μM) increased the rate of ROS generation, an effect mimicked by rotenone or antimycin A, and hence probably due to inhibition of the mitochondrial respiratory chain, leading to mild intracellular ROS accumulation. Nonetheless, the functional relevance of ROS accumulation is unclear. The ROS scavenging agent N-acetyl-L-cysteine can attenuate 3-BrP toxicity (Ihrlund et al., 2008; Kim et al., 2008; Qin et al., 2010; and our unpublished observations). However, protection by N-acetyl-cysteine, a thiolic agent, might largely be a consequence of 3-BrP titration as opposed to true antioxidant protection. Moreover, agents such as genistein (50 μM: Sánchez et al., 2008), curcumin (7.5 μM: Sánchez et al., 2010), and BSO (results not shown) also cause moderate ROS over-accumulation in AML cells, and yet their individual lethality is negligible.

3-BrP also caused a dose-dependent GSH depletion in HL60 cells, with almost total abrogation at 60 μM. GSH depletion could be the consequence of carrier-mediated extrusion, which was characterized as an early event in apoptosis (Ghibelli et al., 1998). Alternatively, 3-BrP may affect the pentose phosphate pathway (Filomeni et al., 2011; Cardaci et al., 2012a), reducing NADPH formation and limiting GSH regeneration. Whatever the case, attenuation of the decrease in GSH by preincubation with GSH-OEt diminished 3-BrP lethality; conversely, lethality was potentiated dramatically by preincubation with BSO, indicating that GSH depletion is an important regulatory event in this pathway. Significantly, partial GSH depletion (as obtained with 30 μM 3-BrP) is not toxic per se, since BSO alone also caused a decrease in GSH but not cell death, and preincubation with BSO had a minimal effect on 2-DG lethality. Thus, the dependence of 3-BrP toxicity on GSH could be explained by the decrease in the intracellular GSH pool rendering thiol residues in targeted proteins unprotected, thereby making them more prone to attack by 3-BrP. Alternatively, in the event of a direct 3-BrP-GSH interaction (as yet not demonstrated), a decrease in the intracellular GSH pool could increase the amount of free active intracellular 3-BrP and, hence, toxicity.

The capacity of 3-BrP to affect protein kinase pathways has been little studied, and the data available to date are inconsistent. Indeed, 3-BrP was reported to mildly stimulate Akt activation in hippocampal neurons (Lee et al., 2011), to mildly repress Akt/mTOR without significantly affecting ERK in Panc-1 pancreatic cancer cells (Bhardwaj et al., 2010), and to repress Akt but activate ERK in GL15 glioblastoma cells (Davidescu et al., 2012). Our data indicate that 3-BrP provokes a rapid, dose-dependent stimulation of both ERK and Akt/mTOR phosphorylation-activation. Akt and ERK activation likely plays a defensive role by restricting excessive toxicity, since cotreatment with pharmacological inhibitors of these kinases augments apoptosis or necrosis provoked by 3-BrP. Interestingly, the potentiation of apoptosis/necrosis by the MEK/ERK inhibitor might support the hypothesis of a role for defensive autophagy, since autophagy is normally positively regulated by MEK/ERK (Han et al., 2011; Davidescu et al., 2012). However, this hypothesis could not explain the potentiation of apoptosis or necrosis by the Akt inhibitor since the Akt/mTOR pathway commonly exerts negative regulation on the autophagic response (Wu et al., 2009; Davidescu et al., 2012). In addition, 3-BrP stimulated p38 MAPK phosphorylation which, because of its property as an oxidative stress-inducible kinase (Kurata, 2000; Sánchez et al., 2008), is consistent with the capacity of 3-BrP to cause ROS overproduction or the depletion of GSH. The activation of this kinase positively regulates drug toxicity as judged by the attenuation of apoptosis and necrosis by p38 MAPK inhibitors. The behavior of AMPK was more complex since...
this kinase suffered a slight, transient activation before decreasing below control levels. The initial AMPK activation is consistent with the drop in ATP levels, whereas its subsequent downregulation and of the upstream LKB-1 and downstream ACC kinases) could be mediated by antagonistic Akt activation (Jin et al., 2007; Lee and Park, 2010; Kuznetsov et al., 2011). Indeed, our experiments demonstrated opposing behavior of Akt and AMPK and also indicated that AMPK was not inhibited but that it was overactivated when 3-BrP was combined with a PI3K/Akt kinase inhibitor. Nonetheless, additional mechanisms, such as potential inhibition of protein phosphatase 2A by 3-BrP (which tightly controls AMPK phosphorylation: Wu et al., 2007b, and our unpublished observations) cannot be ruled out.

In addition to its evident toxicity as a single agent, 3-BrP was earlier characterized as an effective chemosensitizing drug. This effect was considered to be a consequence of energy depletion, which could affect ATP-dependent processes such as DNA repair on treatment with DNA-damaging agents (Ihrlund et al., 2008) or pump-mediated drug export in multidrug-resistant (MDR) cells (Xu et al., 2005; Nakano et al., 2012). We combined 3-BrP with the antileukemic agent ATO and the phenolic agent curcumin for the following reasons: 3-BrP causes oxidative stress, and its toxicity is increased on Akt and ERK inhibition (our present results); ATO toxicity is augmented in pro-oxidant environments, either constitutive or experimentally induced ROS elevation (Yi et al., 2002; Díaz et al., 2005) or GSH decrease (Dai et al., 1999; Yang et al., 1999); and phenolic agents such as curcumin frequently downregulate Akt (Lin, 2007; Sánchez et al., 2010).

We found that 3-BrP cooperates with ATO and curcumin to cause apoptosis and necrosis in HL60 cells, which corresponds with the capacity of the GSH depleting-agent BSO to increase the lethality of ATO (and, to a lesser extent, of curcumin), as well as with the capacity of curcumin to prevent 3-BrP-provoked Akt or ERK activation. The potential interest of these combinatory treatments warrants consideration, since ATO is a clinically relevant antileukemic drug (Wang and Chen, 2008) and curcumin is a potential anticancer agent (Anand et al., 2008), although their clinical efficacy as individual agents is frequently poor.
cannot be unequivocally explained by a single factor; rather, it appears to be the result of a combination of mechanisms that include glycolysis- and mitochondrial respiration–dependent ATP depletion, oxidative stress (especially GSH depletion), and alterations to proapoptotic and defensive protein kinase signaling pathways. GSH depletion and defensive protein kinase modulation may also explain the increased 3-BrP toxicity in combination with other anticancer drugs. Although this is an in vitro preclinical research study, a better understanding of these mechanisms might ultimately help to improve the use of 3-BrP as a therapeutic agent.

Authorship Contributions
Participated in research design: Rial, Aller.
Conducted experiments: Cañiño, Estaña, Sánchez-Martín, Brea, de Blas, Boyano-Adánez, Rial, Aller.
Performed data analysis: Rial, Aller.
Wrote or contributed to the writing of the manuscript: Boyano-Adánez, Rial, Aller.

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Address correspondence to: Dr. Patricio Aller, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040-Madrid, Spain. E-mail: aller@cib.csic.es.