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

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ABBREVIATIONS: ACC, phospho-acetyl-CoA carboxylase; Akt, protein kinase B; Akt\textsuperscript{V}, Akt inhibitor \textsuperscript{V}, triciribine, 5-Dihydro-5-methyl-1-\textbeta-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphtylen-3-amine hydrate; AMPK, AMP-activated kinase; AML, acute myeloid leukemia; ATO, arsenic trioxide; BIRB 796, 1-5-\texttextsuperscript{t}er-t-Butyl-2-p-tolyl-2H-pyrazol-3-yl-3-[4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl] urea; 3-BrP, 3-bromopyruvate; BSO, DL-buthionine-S,R-sulfoximine; calcein-AM, calcein O,O'-diacetate tetrakis(acetoxymethyl) ester; CC, Compound C, 6-[4(2-Piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine; CDDP, cisplatin, cisplatinum(II)-diammine dichloride; DAPI, 4,6-diamino-2-phenylindole; 2-DG, 2-deoxy-D-glucose; DHE, dihydroethidium; ECAR, extracellular acidification rate; ERK, extracellular signal-regulated kinase; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; FITC, fluorescein isothiocyanate; GSH, reduced glutathione; GSH-Oet, GSH-ethyl ester; H\textsubscript{2}DCFDA,
dichlorodihydrofluorescein diacetate; LKB-1, liver kinase B1; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; MEK, mitogen-induced extracellular kinase/extracellular signal-regulated kinase; MTT, 3(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; OCR, oxygen consumption rate; PBS, phosphate buffered saline; PI3K, phosphatidylinositol 3-kinase; PI, propidium iodide; Rh123, rhodamine 123; ROS, reactive oxygen species; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; U0126, 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; z-VAD-fmk, Z-Val-Ala-Asp(OMe)-CH₂F.

**Recommended Section:** Cellular and Molecular.
ABSTRACT

3-Bromopyruvate (3-BrP) is an alkylating, energy-depleting drug that is of interest in anti-tumor 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 over-production 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-MAPK, MEK/ERK and Akt/mTOR/p70S6K phosphorylation/activation, as well as rapid LKB-1/AMPK activation that 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 anti-tumor 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 a 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 anti-tumor therapies (Cardaci et al., 2012a). For example, 1-(2,4-Dichlorobenzyl)-1H-indazole-3-carboxylic acid (lonidamine) and the glucose analogue 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 and thus, their clinical use may be restricted to combined therapies as radio- and chemo-sensitizing drugs (Di Cosimo et al., 2003; Dwarakanath 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 anti-tumor 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 cyto-reductive effect on tumor-derived cell lines, causing apoptosis and/or necrosis, and eradicating tumors in animal models (reviewed by Ganapthy-Kaniappan et al., 2010, 2013, among others).

Glyceraldehyde-3-phosphate-dehydrogenase has been identified as the main target of 3-BrP, provoking the inhibition of glycolysis and ATP depletion (Ganapthy-Kaniappan et al., 2010, 2013). However, due to 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 succinate 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 (Ganapthy-Kaniappan et al., 2010) and autophagy (Davidescu et al., 2012). Furthermore, 3-BrP can reverse multidrug resistance by inhibiting ABC 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 anti-cancer drugs, including energy-depleting agents,
depends very much on the environmental conditions and on the intrinsic metabolic
characteristics of the cell model used. In this regard, the action of 3-BrP has mainly been
studied in cell models derived from solid tumors, while there is little information from leukemia
cell models that 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 caused
neither a loss of cell viability, nor could it explain the pro-apoptotic (chemo-sensitizing) capacity
of the glycolytic inhibitor 2-DG in leukemia cells (Estañ 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 pro-apoptotic and defensive
protein kinase signaling pathways.
Materials and Methods

Reagents and Antibodies. All components for cell culture were obtained from Invitrogen, Inc. (Carlsbad, CA). 4,6-diamino-2-phenylindole (DAPI) was obtained from Serva (Heidelberg, Germany), dichlorodihydrofluorescein diacetate (H$_2$DCFDA) and monochlorobimane from Molecular Probes, Inc. (Eugene, OR), and dihydroethidium (DHE, supplied as a 5 mM solution in dimethyl sulfoxide) from Invitrogen, Inc. The kinase inhibitors 6-[4(2-Piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine (Compound C, CC), 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), 2-(4-Morpholiny1)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 5-Dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphtylen-3-amine hydrate (triciribine hydrate, Akt inhibitor V, Akti,V), 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), and the caspase inhibitor Z-Val-Ala-Asp(OMe)-CH$_2$F (z-VAD-fmk), were obtained from Calbiochem (Darmstad, Germany), and the kinase inhibitor 1-5-tert-Butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl] urea (Doramapimod, BIRB 796) from Shelleck (Houston, TX). Rabbit anti-human AMPKα, phospho-Acetyl-CoA Carboxylase (Ser79), p38 MAP kinase, phospho-p38 MAP kinase (Thr180/Tyr182), p44/42 MAP kinase, phospho-p44/p42 MAPK (Thr202/Tyr204), Akt, phospho-Akt (Ser473) (D9E) XP™, phospho-mTOR (Ser2448), and caspase-3 polyclonal antibodies (pAbs); rabbit anti-human phospho-AMPKα (Thr172) (40H9), and phospho-LKB1 (Ser428) (C6743), monoclonal antibodies; and mouse anti-human phospho-p70 S6 kinase (Thr389) (1A5) (p70S6K) monoclonal antibody, were obtained from Cell Signaling Technology Inc (Danvers, MA); and peroxidase-conjugated immunoglobulin G antibodies from DAKO Diagnostics, S.A. (Barcelona, Spain). All other non-mentioned reagents and antibodies were from Sigma (Madrid, Spain).

Cells and Treatments. The human cell lines HL60 and U937 (acute myeloid leukemia, AML) and NB4 (acute promyelocytic leukemia) were grown in standard RPMI 1640 medium (containing 2.05 mM L-glutamine and 11.11 mM D-glucose) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics in a humidified 5% CO$_2$ atmosphere at 37°C. Cells were routinely maintained under logarithmic growth by passing them every 2-3 days. Under these conditions, the approximate doubling times were 18 h for HL60 and U937, and 24 h for
NB4. Except when necessary, to avoid manipulations which could affect basal protein kinase activation, 24 h before treatments the cell density was adjusted to $10^5$ (for 16-24 h treatments) or to $2 \times 10^5$ (for 0.5-8 h treatments) cells/ml using a mixture of conditioned and fresh medium, and the cells remained undisturbed until the time of drug administration.

Calcein O,O'-diacetate tetrakis(acetoxymethyl) ester (calcein-AM) was commercially obtained as a 4 mM solution in dimethyl sulfoxide. Stock solutions of curcumin (20 mM) H$_2$DCFDA (5 mM), CC, U0126, LY294002 and Akt$m$ (20 mM each), BIRB 796 (0.1 mM), z-VAD-fmk (25 mM), monochlorobimane (200 mM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 mM), rotenone (1 mM), and antimycin A (1 mM) were prepared in dimethyl sulfoxide. Rhodamine 123 (Rh123) was dissolved in ethanol at 1 mg/ml. 3(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2$H$-tetrazolium bromide (MTT) was dissolved at 5 mg/ml in PBS. Oligomycin (31.6 mM) was prepared in RPMI 1640. A stock solution of cisplatinum(II)-diammine dichloride (cisplatin, CDDP, 3.3 mM) was prepared in distilled water. All these solutions were stored at –20°C. Stock solutions of DAPI (10 g/ml) and propidium iodide (PI, 1 mg/ml) were prepared in PBS. Arsenic trioxide (ATO) was initially dissolved in a small amount of 1 N NaOH, and then diluted with PBS to give a final concentration of 10 mM. These solutions were stored at 4°C. 3-Bromopyruvate was freshly prepared at 30 mM in PBS, and the pH of the solution was adjusted at 7.2 with NaOH. Reduced glutathione ethyl ester (GSH-OEt, 100 mM) and DL-buthionine-$S,R$-sulfoximine (BSO, 50 mM) were freshly prepared in distilled water.

**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 $5 \times 10^3$ 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-G₁) DNA content. In addition, apoptosis was evaluated by chromatin condensation/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 non-permeabilized cells and flow cytometry analysis. A detailed description of all 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 was determined simultaneously by double labeling with annexin V-FITC and PI and flow cytometry measurement using an ApoAlert 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 as early apoptotic cells, annexin V-positive/PI-positive cells as late apoptotic and/or 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/CoCl₂ 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 Seahorse Bioscience instrument (North Billerica, MA) (Wu et al., 2007). HL60 cells were plated in 24-well Seahorse culture plates coated with 3.5 μg/cm² Cell-Tak™ (BD Biosciences, Bedford, MA), according to the manufacturer’s protocol. For the XF24 assay, RPMI growth media was replaced by un-buffered RPMI (Sigma) containing 11.1 mM glucose and 2.05 mM L-glutamine supplemented with 1% fetal bovine serum. Subsequently, 10⁵ 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 CO₂-free incubator and thereafter the wells were supplemented with another 400 μl of warm RPMI medium and left for another 20 min 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 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., 2007).

**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 10⁶ 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 H₂DCFDA (10 μM), a non-specific ROS indicator (Eruslanov and Kusmartsev, 2010). Cells (3x10⁴ per well) were seeded in 96-well plates in RPMI without serum and phenol red. The cells were pre-treated for 1 h at 37°C with the different drug concentrations and, at the end of the incubation, the fluorescent probe was added automatically to each well. The fluorescence intensity was recorded for 50 min, taking measurements every 2 min, 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 H₂DCFDA and the anion superoxide-specific probe DHE.
At the end of the drug treatments the cells were labeled (30 min at 37°C) with the corresponding probe (5 µM), they were washed extensively with cold PBS, re-suspended in cold RPMI medium, and analyzed by flow cytometry. Internal controls using unlabelled cells indicated that there was no 3-BrP and ATO auto-fluorescence in any of the conditions assayed.

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-Checa and Kaplowitz, 1990). At the end of the treatments, samples of approximately 5x10⁶ cells were washed and re-suspended in 400 µl of PBS containing 2 mM monochlorobimane. After a 30 min incubation at 37°C in the dark, the cells were centrifuged and re-suspended in 400 µl 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, washed with PBS and total protein extracts were obtained by lysing them for 20 min 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 min at 10,000g and 4°C, the supernatants were collected, and samples containing equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. 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 ± SD. 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 or necrotic death when administered for 24 h 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-G₁ DNA content (Fig. 1B,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. 1D,E). By contrast, the highest concentration of 3-BrP (60 μM) produced an almost exclusively necrotic response (Fig. 1D,E) with negligible apoptosis (Fig. 1B,C). Likewise, dose-dependent apoptotic and necrotic responses were also obtained with human U939 and NB4 cells with a 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. 1B,C), but it had little effect on the frequency of PI-permeable cells (Fig. 1D,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-fm, suggesting that this represented late apoptosis instead of genuine necrosis (see supplementary Fig. 1). The apoptosis provoked in the presence of 30 μM 3-BrP was corroborated by measuring chromatin condensation/fragmentation (data not shown), phosphatidylserine translocation in the plasma membrane (measured as annexin V staining, Fig. 1F) and caspase-3 cleavage/activation (Fig. 1G), with negligible annexin V staining and caspase cleavage following exposure to 60 μM 3-BrP. Time-course assays showed that apoptosis was first evident after approximately 8 h 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 (supplementary Fig. 2A). Finally, 3-BrP rapidly caused dose-dependent permeabilization of the inner mitochondrial membrane (mIMP), as revealed by the decrease in calcein retention (supplementary Fig. 3A),
followed by the dissipation of the mitochondrial membrane potential ($\Delta \Psi_m$) witnessed by Rh123 staining (supplementary 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 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 while there was a 75% inhibition of the lactate produced by 20 $\mu$M 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 non-glycolytic 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 $\mu$M 3-BrP caused complete inhibition of both lactate production and respiration (Fig. 2A,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 $\mu$M 3-BrP. A concentration-dependent decrease in ATP levels was evident following exposure to 3-BrP (Fig. 2E), which roughly correlated with the cell death observed (see Fig. 1). At the highest 3-BrP concentration (60 $\mu$M), 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 $\mu$M 3-BrP is unclear.
In fact, treatment with 10 mM 2-DG (included for comparison) caused a similar depletion of ATP (Fig. 2E and supplementary Fig. 4A), but the lethality was negligible (see supplementary 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/necrosis in HL60 cells. Exposure for 50 min to 30 μM 3-BrP stimulated the rate of ROS generation approximately 3-fold, as evident by fluorimetry using the non-specific fluorescent probe H$_2$DCFDA, while treatment with a non-toxic 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 h to 30 μM 3-BrP resulted in a mild yet significant increase in intracellular ROS accumulation, as evident in flow cytometry assays using H$_2$DCFDA, as well as with the anion superoxide indicator DHE (Fig. 3 B,C).

Exposure for 3 h 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 h 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 h with 1 mM BSO alone progressively decreased the intracellular GSH content to similar levels as those detected in the presence of 30 μM 3-BrP (Fig. 4B). Notably, this partial GSH depletion was not toxic per se, since BSO did not cause apoptosis or necrosis, nor did it affect the cell cycle distribution even after a 48 h exposure (Fig. 4C,D, and data not shown). However, pre-exposure for 24 h 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. 4C,D). In other experiments, cells were pre-loaded for 4 h 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) and also, it reduced the apoptosis
and necrosis caused by 30 μM 3-BrP (Fig. 4F,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 (supplementary Fig. 4B), and that pre-incubation 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 (supplementary Fig. 4C,D).

**Protein Kinase Modulation.** MAPK p38 has been characterized as a protein kinase responsive to moderate oxidative stress (Kurata, 2000), while 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 on the induction of apoptosis/necrosis in leukemic cells. The results are represented in Fig. 5A. Exposing HL60 cells to 20-60 μM 3-BrP for 4 and 8 h caused a dose-dependent phosphorylation/activation of p38-MAPK. Similarly, 3-BrP caused a dose-dependent activation of Akt and, except at a concentration of 60 μM, of the downstream mTOR and p70S6K kinases. 3-BrP also stimulated ERK phosphorylation, although in function of the time of treatment. Conversely, 3-BrP caused a dose-dependent de-phosphorylation/inactivation of AMPK, as well as that of the upstream LKB-1 and downstream ACC kinases. Of note, after 8 h 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 upon long-term treatments with other glycolytic inhibitors (Estañ et al., 2012). With minor quantitative differences, similar modifications of protein kinases were obtained using NB4 instead of HL60 cells (data not shown).

Since 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 min to 2 h) with 30 μM 3-BrP, or they were treated for 4 h with 3-BrP in combination with the
PI3K/Akt inhibitor LY294002. After 15 min in the presence of 30 μM 3-BrP, AMPK was mildly hyper-phosphorylated, a response that was reversed after a 30 min exposure and coinciding with the initiation of Akt phosphorylation (Fig. 5B). Moreover, co-treatment with LY294002 not only suppressed Akt phosphorylation but also, it prevented the decrease in AMPK phosphorylation, which rose 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 pre-loaded 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 as defensive, anti-apoptotic kinases, while p38-MAPK may exert pro- or anti-apoptotic 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). Co-treatment with BIRB 796 and (with lower efficacy) SB203580 reduced both the apoptosis and necrosis induced by 3-BrP, while this response was enhanced in the presence of AktiV and U0126 (Fig. 6). These results indicate that p38-MAPK activation facilitates 3-BrP toxicity, while Akt and ERK activation probably fulfills a defensive role by reducing excess drug toxicity. Moreover, although 3-BrP per se was sufficient to down-regulate AMPK, co-treatment 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 Anti-tumor Drugs.** It was earlier reported that co-treatment with 3-BrP increased the toxicity of some conventional anti-tumor 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 over-production and especially, GSH depletion by 3-BrP, could serve to increase the toxicity of oxidant-sensitive anti-tumor drugs (i.e.: drugs that cause greater lethality under environmental oxidizing conditions), and conversely, that the potential inhibition of defensive kinases by some anti-tumor 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 anti-leukemic 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 for 24 h 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 and/or necrosis, with higher efficacy (at 20 μM 3-BrP) in the case of ATO (Fig. 7A,B). These results correlated with the increase in apoptosis generated by ATO and curcumin following pre-incubation with BSO, which as indicated above decreased GSH levels without causing cell toxicity per se, again with a higher efficacy in the case of ATO (Fig. 7C,D). It is noteworthy that we did not detect co-operation 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. 7A-D). Finally, exposure to curcumin for 4 h strongly attenuated the Akt and ERK phosphorylation provoked by 3-BrP, while 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 anti-tumor 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 alkylation 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-Kanniapan 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. Since autophagy is considered as a pro-survival response opposing apoptosis and necrosis (Wu et al., 2009; Han et al., 2011), an attractive possibility is that the intensity of apoptosis/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-L-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, 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 pre-incubation with GSH-OEt diminished 3-BrP lethality, while conversely, lethality was potentiated dramatically by pre-incubation 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 pre-incubation 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 indicates 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 co-treatment with pharmacological inhibitors of these kinases augments apoptosis and/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/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, due to its property as an oxidative stress-inducible kinase (Kurata et al., 2000; Sánchez et al., 2008), is consistent with the capacity of 3-BrP to cause ROS over-production and/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, while its subsequent down-regulation (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 over-activated 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., 2007, 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 chemo-sensitizing drug. This effect was considered to be a consequence of energy depletion, which could affect ATP-dependent processes such as DNA repair upon 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 anti-leukemic agent ATO and the phenolic agent curcumin for the following reasons: 3-BrP causes oxidative stress, and its toxicity is increased upon 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 like 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 and/or ERK activation. The potential interest of these combinatory treatments warrants consideration, since ATO is a clinically relevant anti-leukemic drug (Wang and Chen, 2008) and curcumin is a potential anti-cancer agent (Anand et al., 2008), although their clinical efficacy as individual agents is frequently poor.
A diagram summarizing the effects of 3-BrP in respiratory-sufficient human AML cells is presented in Fig.8. 3-BrP appears as a powerful cytoreductive drug, causing concentration-dependent apoptosis and/or necrosis. The cytotoxic action of 3-BrP cannot be unequivocally explained by a single factor but 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 pro-apoptotic 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 anti-tumor drugs. While this is an in vitro pre-clinical 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, and Aller

Conducted experiments: Calviño, Estañ, Sánchez-Martín, Brea, de Blas, Boyano-Adánez, Rial, and Aller.

Performed data analysis: Rial and Aller.

Wrote or contributed to the writing of the manuscript: Boyano-Adánez, Rial, and Aller
References


Footnotes

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FIGURE LEGENDS

**Fig. 1.** Effect of 3-BrP on HL60 cell growth, death (apoptosis and necrosis) and cell cycle progression. Cells were incubated for 24 h with 3-BrP at a concentration of 10, 20, 30 or 60 μM (which for convenience are indicated as sub-headings). Where indicated, the cells were co-incubated with the pan-caspase inhibitor z-VAD-fmk (50 μM). (A) Changes in cell growth, as indicated by the MTT assay. Absorption values are expressed in relation to untreated cultures. (B) Frequency of apoptosis, as determined by sub-G₁ DNA content. (C) Examples of flow cytometry histograms showing the distribution of cells in different phases of the cell cycle and the fraction of apoptotic cells (Ap). (D) Frequency of cells permeable to propidium iodide (PI⁺), indicating the plasma membrane disruption that is characteristic of necrosis. (E) Examples of flow cytometry histograms showing the fraction of PI-permeable cells. (F) Frequency of cells exhibiting phosphatidylserine translocation, as measured by cell surface annexin V binding, and free PI uptake: cells positive for annexin V and negative for PI are considered as early apoptotic; those negative for annexin V and positive for PI as genuine necrotic; and the population positive for both annexin V and PI probably represents a mixture of late apoptotic and necrotic cells. (G) Caspase-3 cleavage/activation, determined in immunoblots after 16 h of treatment. Actin is included as a loading control. The bar charts in (A, B and D) indicate the mean ± SD of at least three independent determinations. The results in (C) and (E) are representative of one of three, and those in (F) and (G) of one of two determinations, with essentially similar results. Asterisks indicate statistically significant (*** p<0.001) differences between the indicated pair of values (n.s., non-significant).

**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 min 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 ± SD 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 for 3 h with 10 mM 2-DG was included for comparison, and treatment for 3 h with 10 μM oligomycin in glucose-free medium (Glu/Oligo) was used as a control. The results (the mean ± SD 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 increase in fluorescence of the cell-permeant redox-sensitive fluorescent indicator H₂DCFDA measured with a microplate reader. Fluorescence was monitored for 50 min after 1 h exposure to either 10 or 30 μM 3-BrP or 5 min after the addition of either 2 μM rotenone (Rot) or 2 μM antimycin A (AntA). The results represent the mean ± SEM of six independent measurements. (B) Relative intracellular accumulation of ROS after 3 and 6 h in the presence of 30 μM 3-BrP, measured by flow cytometry using the H₂DCFDA (non-specific) and DHE (anion superoxide-specific) probes. The results represent the mean ± SD 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 h are represented as examples in (C). Asterisks indicate the significant differences relative to the controls: * p<0.05; ** p<0.01.

Fig. 4. Alterations in GSH content, and the effect of GSH modulators. (A,B) Changes in intracellular GSH content in HL60 cells, as determined by monochlorobimane derivatization. Cells were exposed to the indicated concentrations of 3-BrP (A) or to 1 mM BSO (B) for the times indicated. The results are shown relative to the untreated (Cont) cells (approximate GSH content, 10 nmol/10⁶ cells). (C,D) Frequency of apoptotic (C) and PI-permeable (D) cells in cultures treated for 48 h with 1 mM BSO alone, or in cultures pre-incubated in the absence (-) or
the presence (+) of BSO for 24 h, and then exposed to different concentrations of 3-BrP for another 24 h. (E-G) Effects of GSH pre-loading. Cells were pre-loaded (+GSH) or not (-) with 5 mM GSH-OEt for 4 h, washed extensively with PBS and finally exposed to 30 μM 3-BrP for the desired times. (E) Intracellular GSH levels in untreated cells (Cont), at the end GSH-OEt loading (GSH), or after a 3 h exposure to 3-BrP. (F,G) Frequency of apoptotic and PI-permeable cells after exposure to 3-BrP for 24 h. All the results are the mean ± SD of at least three independent determinations. Asterisks indicate significant differences between the indicated pair of values: * p<0.05; ** p<0.01; *** p<0.001 (n.s., non-significant). For other conditions see legend to Fig. 1.

Fig. 5. Protein kinase activation. (A) Relative levels of phosphorylated (P) and total (T) p38-MAPK, Akt, mTOR, p70S6K, ERK, LKB-1, AMPK and ACC, in untreated HL60 cells (Cont) and in cells treated for 4 and 8 h with the indicated concentrations of 3-BrP, as determined in immunoblots. The levels of β-actin were assessed as control of loading. (B) Relative levels of phosphorylated and total Akt and AMPK after brief exposure 3-BrP, demonstrating the opposite behavior of these kinases. (C) Relative levels of phosphorylated Akt, and phosphorylated and total AMPK following a 4 h exposure to 3-BrP, alone or in conjunction with the PI3K/Akt inhibitor LY294002 (LY, 30 μM). The kinase inhibitor was applied 1 h before 3-BrP. (D) Relative levels of phosphorylated p38-MAPK, Akt and ERK upon treatment for 4 h with 3-BrP, either with or without GSH-OEt pre-loading (following the same experimental design as in Fig. 4E-G).

Fig. 6. Effect of protein kinase inhibitors. (A,B) Frequency of apoptotic (A) and PI-permeable (B) HL60 cells following a 16 h exposure to the p38-MAPK inhibitors SB203580 (SB, 10 μM) and BIRB-796 (BIRB, 0.1 μM), the Akt inhibitor triciribine (AktiV, 10 μM), the MEK/ERK inhibitor U0126 (U, 5 μM), the AMPK inhibitor compound C (CC, 5 μM), and 20 or 30 μM 3-BrP, either alone (-) or in combination (+) with the kinase inhibitors. In the combined treatments, the inhibitors were applied 1 h before exposing the cells to 3-BrP. The results represent the mean ± SD of at least four independent determinations. In the case of p38-MAPK inhibitors, the
asterisks indicate significant differences between 3-BrP alone and 3-BrP+SB or 3-BrP+BIRB: * p<0.05; ** p<0.01 (n.s., non-significant). In the case of Akt, MEK/ERK and AMPK inhibitors, symbols indicate that values in the combined treatment (3-BrP+inhibitor) are equivalent to (#) or significantly higher (* p<0.05) than the sum of the values in the corresponding individual treatments (e.g., 3-BrP_20/Akt\_V in relation to the sum of 3-BrP_20 alone plus Akt\_V alone, etc.). For other conditions, see legend to Fig. 1.

**Fig. 7.** Effects of 3-BrP in combination with other anti-tumor drugs. (A-D) Frequency of apoptotic (A,C) and PI-permeable (B,D) HL60 cells following exposure for 24 h to arsenic trioxide (ATO, 2 μM), curcumin (Cur, 7 μM), and cisplatin (CDDP, 4 μM), either alone or in combination with 20 or 30 μM 3-BrP, or with 1 μM BSO. In (A,B), 3-BrP was added simultaneously to ATO, Cur or CDDP. In (C,D), the cells were pre-incubated for 24 h with BSO, and then exposed to ATO, Cur or CDDP for another 24 h in the presence of BSO. The symbol (-) indicates incubation for 24 h with 3-BrP alone or for 48 h with BSO alone. The results represent the mean ± SD of at least three independent determinations. Symbols indicate that values in the combined treatment are not higher (#) or are significantly higher than the sum of values in the corresponding individual treatments (e.g., ATO/3-BrP_20 in relation to the sum of ATO alone plus 3-BrP_20 alone, etc.): * p<0.05; ** p<0.01; *** p<0.001. (E) Levels of phosphorylated and total p38-MAPK, Akt and ERK after a 4 h treatment with 3-BrP, ATO and curcumin, alone or in combination. For other conditions see legends to Figs. 1 and 4.

**Fig. 8.** Effects of 3-BrP in AML cells. The diagram summarizes the mechanisms which regulate death induction by 3-BrP, alone and in combination with other anti-tumor drugs. 3-BrP causes: (i) dose-dependent inhibition of glycolysis and mitochondria respiration, leading to ATP
depletion; (ii) dose-dependent GSH depletion; and (iii) p38-MAPK, MEK/ERK and Akt/mTOR activation, and Akt-dependent LKB-1/AMPK inhibition, which either facilitate (+) or decrease (-) drug lethality. These alterations cause cell death, either apoptotic/necrotic or purely necrotic, depending on the severity of effects (e.g., intensity of ATP and GSH depletion). (iv) GSH depletion mediates or potentiates (+) other effects of 3-BrP (e.g., protein kinase activity alterations and possibly (?) glycolysis/respiration inhibition). Moreover, GSH depletion exacerbates the toxicity of oxidative stress-sensitive drugs such as arsenic trioxide (ATO) and curcumin (Curc). This effect, together with the curcumin-provoked attenuation of ERK and Akt defensive kinase activities, may explain the increased lethality of the combined treatments. Thick upward and downward arrows indicate increase or decrease, respectively, of either expression levels or activities.
Fig. 2
Fig. 3
Fig. 5
Fig. 7
Regulation of death induction and chemosensitizing action of 3-bromopyruvate in myeloid leukemia cells. Energy depletion, oxidative stress and protein kinase activation.

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Supplementary Fig. 1. Cell death induction by 2-deoxy-D-glucose (2-DG) in HL60 cells. (A) Frequency of apoptosis, measured by sub-G₁ DNA content, and (B) plasma membrane disruption, as indicated by free PI uptake, following a 24 h exposure to 2-DG at concentrations ranging from 10 to 40 mM (indicated as sub-headings), with or without the caspase inhibitor z-VAD-fmk. Asterisks indicate significant differences between each pair of values: ** p<0.01; *** p<0.001. Observe that, by contrast to the case of 3-BrP (Fig. 1D,E), the caspase inhibitor almost totally suppressed PI-permeability, indicating that it likely represents late apoptosis instead of genuine necrotic response.
Supplementary. Fig. 2. (A) Frequency apoptosis, measured by sub-G₁ DNA content, and (B) plasma membrane disruption, as indicated by free PI uptake, at different times of treatment with the indicated concentrations of 3-BrP in HL60 cells. For other details, see legend to Fig. 1.
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Supplementary Fig. 3. Inner mitochondria membrane permeabilization (mIMP) and mitochondrial membrane potential ($\Delta \Psi_m$) dissipation, in HL60 cells exposed to the indicated concentrations of 3-BrP. Panel (A) shows mIMP at 3 h exposure, indicated by the decrease in calcein retention capacity, using labeling with calcein-AM/CoCl$_2$ and flow cytometry. Panel (B) shows $\Delta \Psi_m$ dissipation at 16 h exposure, indicated by the decrease in Rh123-derived fluorescence, measured by flow cytometry. For further details, see Materials and Methods. The figure provides a representative example of one of three determinations, with qualitatively similar results.
Supplementary Fig. 4. Effects of 2-deoxy-D-glucose (2-DG) on intracellular ATP and GSH levels in HL60 cells. (A) Intracellular ATP content after a 3 h exposure to the indicated concentrations of 2-DG. Treatment with 10 μM oligomycin in glucose-free medium (Glu−/Oligo) was used as a control. The results are expressed in relation to untreated (Cont) cells. Observe that 10 mM 2-DG causes considerable ATP depletion, but negligible cell death (see Sup. Fig. 1). (B) Changes in intracellular GSH content after a 3 h exposure to the indicated concentrations of 2-DG. The results are represented in relation to untreated (Cont) cells. (C,D) Frequency of apoptotic (C) and PI-permeable cells (D) upon treatment for 48 h with 1 mM BSO alone, or for 24 h with 30 mM 2-DG, either alone (-) or with 1 mM BSO. In this case BSO was applied 24 h before 3-BrP, and maintained during the 2-DG treatment. Observe that the potentiation by BSO of 2-DG lethality is much lower than in the case of 3-BrP (Fig. 4C). Asterisks indicate significant differences in relation to the controls (A,B) or between the indicated pair of values (C,D): * p<0.05; ** p<0.01; *** p<0.001 (n.s., non-significant).