6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol, a specific glutathione *S*-transferase inhibitor, overcomes the multidrug resistance (MDR)-associated protein 1–mediated MDR in small cell lung cancer

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Abstract

In the present work, we have investigated the antitumor activity of 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) on aggressive small cell lung cancer. NBDHEX not only is cytotoxic toward the parental small cell lung cancer H69 cell line (LC_{50} of 2.3 ± 0.6 μmol/L) but also overcomes the multidrug resistance of its variant, H69AR, which overexpresses the ATP-binding cassette transporter multidrug resistance – associated protein 1 (MRP1; LC_{50} of 4.5 ± 0.9 μmol/L). Drug efflux experiments, done in the presence of a specific inhibitor of MRP1, confirmed that NBDHEX is not a substrate for this export pump. Interestingly, NBDHEX triggers two different types of cell death: a caspase-dependent apoptosis in the H69AR cells and a necrotic phenotype in the parental H69 cells. The apoptotic pathway triggered by NBDHEX in H69AR cells is associated with c-Jun NH_2-terminal kinase and c-Jun activation, whereas glutathione oxidation and activation of $p38^{MAPK}$ is observed in the NBDHEX-treated H69 cells. In contrast to the parental cells, the higher propensity to die through apoptosis of the H69AR cell line may be related to the lower expression of the antiapoptotic protein Bcl-2. Therefore, down-regulation of a factor crucial for cell survival makes H69AR cells more sensitive to the cytotoxic action of NBDHEX, which is not a MRP1 substrate. We have previously shown that NBDHEX is cytotoxic toward P-glycoprotein – overexpressing tumor cell lines. Therefore, NBDHEX seems a very promising compound in the search for new molecules able to overcome the ATP-binding cassette family of proteins, one of the major mechanisms of multidrug resistance in cancer cells. [Mol Cancer Ther 2008;7(2):371 – 9]

Introduction

Lung cancer is the leading cause of cancer-related deaths and is classified clinically into two major histologic types: small cell lung cancer (SCLC), which accounts for ∼25% of cases, and non-SCLC, which accounts for the remaining 75%. SCLC is a particularly aggressive form of lung cancer and has an extremely poor prognosis (1, 2). Current information suggests that, in most cells, defects in apoptosis signaling are the most critical determinants for tumor development and progression (3, 4). The antiapoptotic protein Bcl-2 is a critical survival factor for SCLC cells, and it has been shown that down-regulation of Bcl-2 induces apoptosis and enhances chemosensitivity in these tumor cells (5, 6). Moreover, after an initial response to chemotherapy, there is a relapse in most patients with SCLC, and their tumors become largely refractory to further treatments. P-glycoprotein (P-gp) and the multidrug resistance (MDR)-associated protein 1 (MRP1) are the most extensively studied ATP-binding cassette transporters and are often regarded as the prototypes for understanding the cell-based mechanisms of MDR (7–9). The clinical importance of P-gp and MRP1 stems from the fact that, in parallel with their overexpression, the tumor cells simultaneously exhibit acquired cross-resistance to diverse chemotherapeutic agents, resulting in the failure of chemotherapy for many cancers. To study the MDR phenotype of SCLC, Mirsk et al. (10) have developed a drug-resistant SCLC cell line by selection with Adriamycin. This cell line, designated H69AR, does not overexpress P-gp. Thus, it provides a model system for investigating MRP1-mediated MDR. We have recently reported that 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) is not a substrate of the P-gp export pump and both P-gp–overexpressing tumor cells and their parental cell lines are efficiently committed to death by this molecule (11). NBDHEX is a nonpolar compound that behaves as a suicide inhibitor for glutathione *S*-transferases (GST). In fact, it is conjugated with reduced glutathione (GSH), so

leading to a stable σ complex in the GST active site (12). The MRP1 export pump is able to transport hydrophobic anions, including drugs conjugated with GSH (GS-X pump), glucuronide, and sulfate. In addition, the MRP1 pump removes unmodified anticancer agents cotransported with GSH (13, 14). Therefore, the anionic adduct between NBDHEX and GSH could represent a substrate for the MRP1 transporter and it may be extruded from the cell. In the present work, we have analyzed the activity of NBDHEX against both H69 tumor cells and their Adriamycin-resistant counterpart H69AR, which overexpresses MRP1.

Materials and Methods

Cell Lines and MDR Typing with Monoclonal Antibodies

Human SCLC H69 and its Adriamycin-selected, multidrug-resistant H69AR cell variant were kindly provided by Dr. Susan P. Cole (Department of Pathology, Queen's University, Kingston, Ontario, Canada). Cells were grown in RPMI 1640 supplemented with 10% FCS and antibiotics. H69AR cells were maintained by alternate feedings with drug-free medium or medium containing 1.7 μmol/L Adriamycin. The stability of the MDR phenotype was periodically assessed by testing the MRP1 expression with the MRP1-specific monoclonal antibody MRPm6 (Vinci-Biochem; ref. 15). MDR cells were also typed for possible P-gp expression with the monoclonal antibody MM4.17, which recognizes an extracellular P-gp epitope (16).

Cell Viability Test

Cell viability testing was done using the sulforhodamine B assay (17). In brief, H69 and H69AR cells were placed in 96-well microtiter plates at a density of 0.7×10^6/mL and then incubated 48 h with NBDHEX at the desired final concentration (from 0.05 to 20 μmol/L). A positive control was also done by treating both H69 and H69AR cells with 10 μmol/L puromycin, a dose that causes 100% cell death after 48 h (data not shown). After 48 h of incubation, cell growth was evaluated by an *in situ* cell fixation procedure followed by sulforhodamine B staining of proteins. In order not to exceed the absorbance linearity range, a suboptimal wavelength (490 nm) was used to read the samples (17). The absorbance value of the positive control was subtracted from that of both NBDHEX-treated and untreated control samples. The dose-response profile obtained fulfills the LC_{50} for NBDHEX (the concentration used to obtain 50% cellular mortality).

Cell Treatments

Treatments were done on cells cultured at 0.8×10^6/mL. NBDHEX was synthesized as reported by Ricci et al. (12). Stock solutions of this compound were prepared in DMSO and diluted to the appropriate concentration in RPMI 1640 cell medium just before use.

The caspase-dependent nature of the NBDHEX-induced apoptosis was confirmed by measuring caspase activity and apoptosis in cells treated with the general caspase inhibitor Z-VAD-*fmk* at a concentration of 40 μmol/L. The inhibitor was added to the cell culture 1 h before the addition of 3 μmol/L NBDHEX and maintained throughout the experimental time.

The protective effect of GSH on the NBDHEX-induced necrosis (H69) and apoptosis (H69AR) was determined after the addition to the cells of 1 mmol/L glutathione ethylester 1 h before the addition of 5 μmol/L NBDHEX.

Flow Cytometric Detection of Functional Drug Efflux

Cells were incubated for 1 h in the presence of 1.7 μmol/L Adriamycin or 10 μmol/L NBDHEX in the presence or absence of 50 μmol/L MK571 (VinciBiochem), a specific inhibitor of MRP1 (18). Then, cells were incubated for 1 h in drug-free medium with or without MK571 to give the opportunity for efflux or efflux blocking to occur. Efflux was stopped by pelleting the cells and adding ice-cold medium. Fluorescence of Adriamycin or NBDHEX was analyzed by a FACSCalibur flow cytometer (BD Biosciences) equipped with an argon laser source.

Determination of Apoptosis and Necrosis

Percentages of apoptosis and necrosis were determined on cells treated for 24 h with different concentrations of NBDHEX (from 0 to 5 μmol/L).

For apoptosis determination, aliquots of cells were stained for 10 min with Hoechst 33342 and examined by fluorescence microscopy. For necrosis determination, aliquots of cells were stained with trypan blue and examined by microscopy using a Neubauer hemacytometer. Alternatively, cells were stained with 50 μg/mL propidium iodide, permeabilized with NP40 (0.1%, w/v), and analyzed by a FACSCalibur instrument.

Glutathione Determination

The intracellular levels of both reduced (GSH) and oxidized form (GSSG) of glutathione were determined at different times from the treatment with 3 μmol/L NBDHEX, as previously reported (19). Data are expressed as nmol of GSH or GSSG equivalents/mg of protein.

Enzyme Activity

GST and caspase-3 activities were measured as described previously (20). Protein concentration was determined by the bicinchoninic acid protein assay reagent (Pierce).

Western Blotting Analysis

Immunoblotting analysis was done on H69 and H69AR cell lines. At each time point analyzed, pellets of untreated control cells and cells treated with 3 μmol/L NBDHEX were lysed for Western blot analyses as described previously (20). Twenty micrograms of proteins were loaded on 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). Monoclonal anti–phosphorylated c-Jun NH$_2$-terminal kinase (JNK) and anti–phosphorylated c-Jun (1:500; Santa Cruz Biotechnology); polyclonal anti-JNK and anti–c-Jun (1:1,000; Upstate Biotechnology), anti-p38^{MAPK} (1:1,000; Santa Cruz Biotechnology), anti–phosphorylated isoform of p38^{MAPK} (1:1,000; Cell Signaling Technology and New England Biolabs), anti–β-actin (1:5,000; Sigma), and anti–α-tubulin; and monoclonal anti–Bcl-2 (1:500; Dako) and anti–GSTP1-1 (1:2,000; Calbiochem) were used as primary antibodies. The protein complex formed on incubation with specific
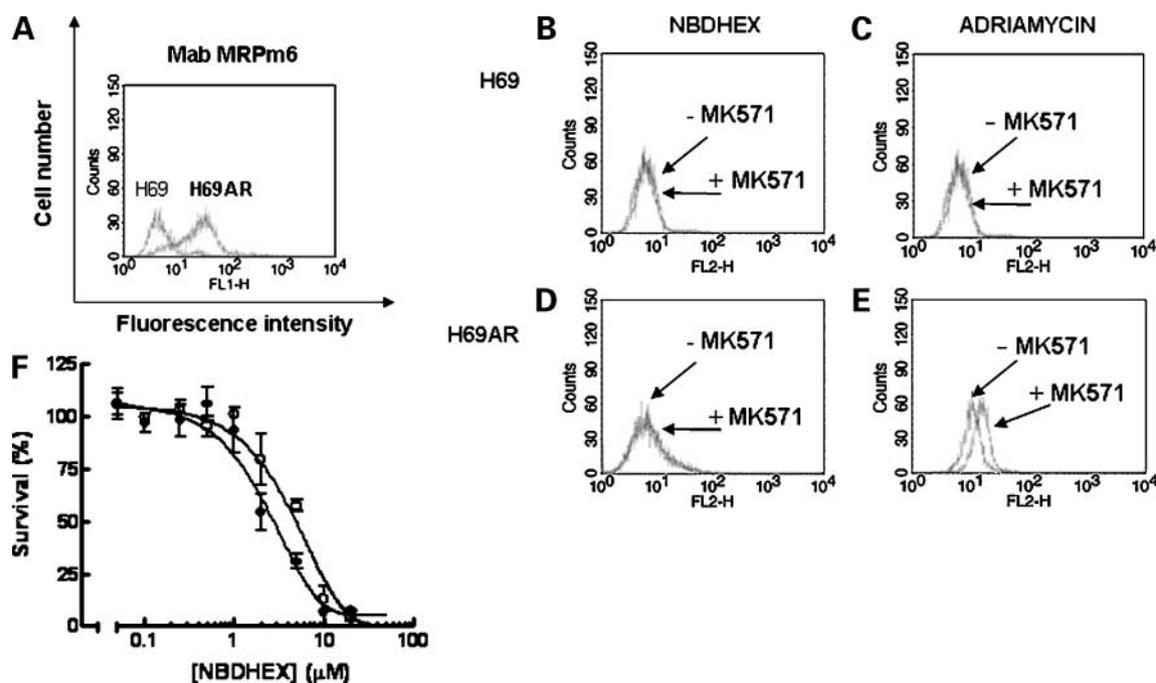
Figure 1. NBDHEX is not a substrate for MRP1 export pump and shows cytotoxic activity toward both H69 and H69AR cell lines. **A,** MRP1 expression was evaluated by flow cytometric analyses of H69 and H69AR cells incubated with monoclonal antibody (*Mab*) MRPm6. The effect of 50 μmol/L MRP1 inhibitor MK571 was determined on both H69 and H69AR cell lines. Cells were treated with either 10 μmol/L NBDHEX or 1.7 μmol/L Adriamycin alone or in combination with MK571. **B,** H69 with NBDHEX and with NBDHEX and MK571. **C,** H69 with Adriamycin and with Adriamycin and MK571. **D,** H69AR with NBDHEX and with NBDHEX and MK571. **E,** H69AR with Adriamycin and with Adriamycin and MK571. **F,** cell survival at different NBDHEX concentrations (from 0.05 to 20 μmol/L) was determined by sulforhodamine B assay after 48 h of treatment. The dose-response profile for NBDHEX yields LC$_{50}$s of 2.3 ± 0.6 μmol/L with H69 cells (●) and 4.5 ± 0.9 μmol/L with H69AR cell line (○; $P < 0.05$). *Points,* mean of three independent experiments, each done in quadruplicate; *bars,* SD.

secondary antibodies (1:10,000; Bio-Rad) was identified using FluorChem Imaging System (Alpha Innotech) after incubation with ChemiGlow chemiluminescence substrate (Alpha Innotech).

Densitometric analyses of proteins were done using the software Quantity One (Bio-Rad), and protein levels were normalized to the density of related β-actin bands.

Data Presentation

All of the experiments were repeated at least thrice. Results are presented as mean ± SD. Statistical evaluation was done, where appropriate, using the Student's *t* test or ANOVA followed by correction with Bonferroni's *t* test. The criterion for statistical significance used was $P < 0.05$.

Results

NBDHEX Is Not a Substrate of MRP1

Flow cytometry studies, conducted by specific antibodies, show that MRP1 is not present in the drug-sensitive H69 parental cell line. On the other hand, this export pump is highly expressed in the MDR cell variant H69AR, as assessed by the intracellular detection of the MRPm6 epitope by its cognate monoclonal antibody (Fig. 1A). In contrast, P-gp was not detected in both cell lines by the anti–P-gp monoclonal antibody MM4.17 (data not shown). Thus, the H69 and H69AR drug-sensitive/resistant cell pair provides a model system for investigating MRP1-mediated MDR. To clarify if NBDHEX efflux could be mediated by MRP1, we did experiments in the presence of MK571. This molecule is known to specifically inhibit the MRP1-mediated transport, so inducing intracellular accumulation of MRP1 substrates such as Adriamycin. As expected, MK571 inhibited Adriamycin efflux in H69AR cells (Fig. 1E), although it did not affect the intracellular concentration of Adriamycin in the sensitive variant (Fig. 1C). Conversely, MK571 did not affect the intracellular concentration of NBDHEX in both sensitive and resistant cell lines (Fig. 1B and D, respectively).

Moreover, a recently isolated NBDHEX-resistant cell variant of the human T-lymphoblastic leukemia CCRF-CEM does not show detectable MRP1 or P-gp multidrug transporters (data not shown).[7] These findings confirm the fact that NBDHEX is not a substrate for these export pumps.

Cytotoxic Effect of NBDHEX on SCLC Cell Lines

The ability of NBDHEX to cause cell death in H69 and H69AR cells is affected by an increase of cell density. Thus, taking advantage of the wide range of linearity of the sulforhodamine B assay, the NBDHEX cytotoxic activity

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[7] M.L. Dupuis, in preparation.

has been determined by using a cell density comparable with that used in the apoptosis/necrosis experiments. Under these experimental conditions, the dose-response profiles revealed a good cytotoxic activity both in sensitive H69 cell line (LC_{50} of 2.3 ± 0.6 μmol/L) and in its Adriamycin-resistant counterpart H69AR (LC_{50} of 4.5 ± 0.9 μmol/L; Fig. 1F).

NBDHEX Induces Necrosis in H69 Cells and a Dose-Dependent Apoptosis in the H69AR Cell Line

To characterize the type of cell death induced by NBDHEX, we evaluated the amount of apoptotic/necrotic cells in both H69AR and H69 cell lines after incubation with the drug. Figure 2 reports the percentages of cells undergoing necrosis (Fig. 2A) or apoptosis (Fig. 2B and C) after incubation with NBDHEX. The occurrence of apoptosis in H69AR was revealed by the presence of cells with fragmented chromatin (data not shown). Time course analysis of cell death showed that apoptosis occurred within 3 h and continued to increase throughout the incubation period. Moreover, a clear dose-dependent effect was observed after 24 h of incubation with different amounts of NBDHEX. By contrast, the H69 cell line seemed quite insensitive to the treatment up to ~1 μmol/L NBDHEX, whereas a further increase of drug concentration caused cell death by necrosis as determined by trypan blue staining. The necrotic H69 cells exhibited cytoplasmic swelling and diffuse nonfragmented
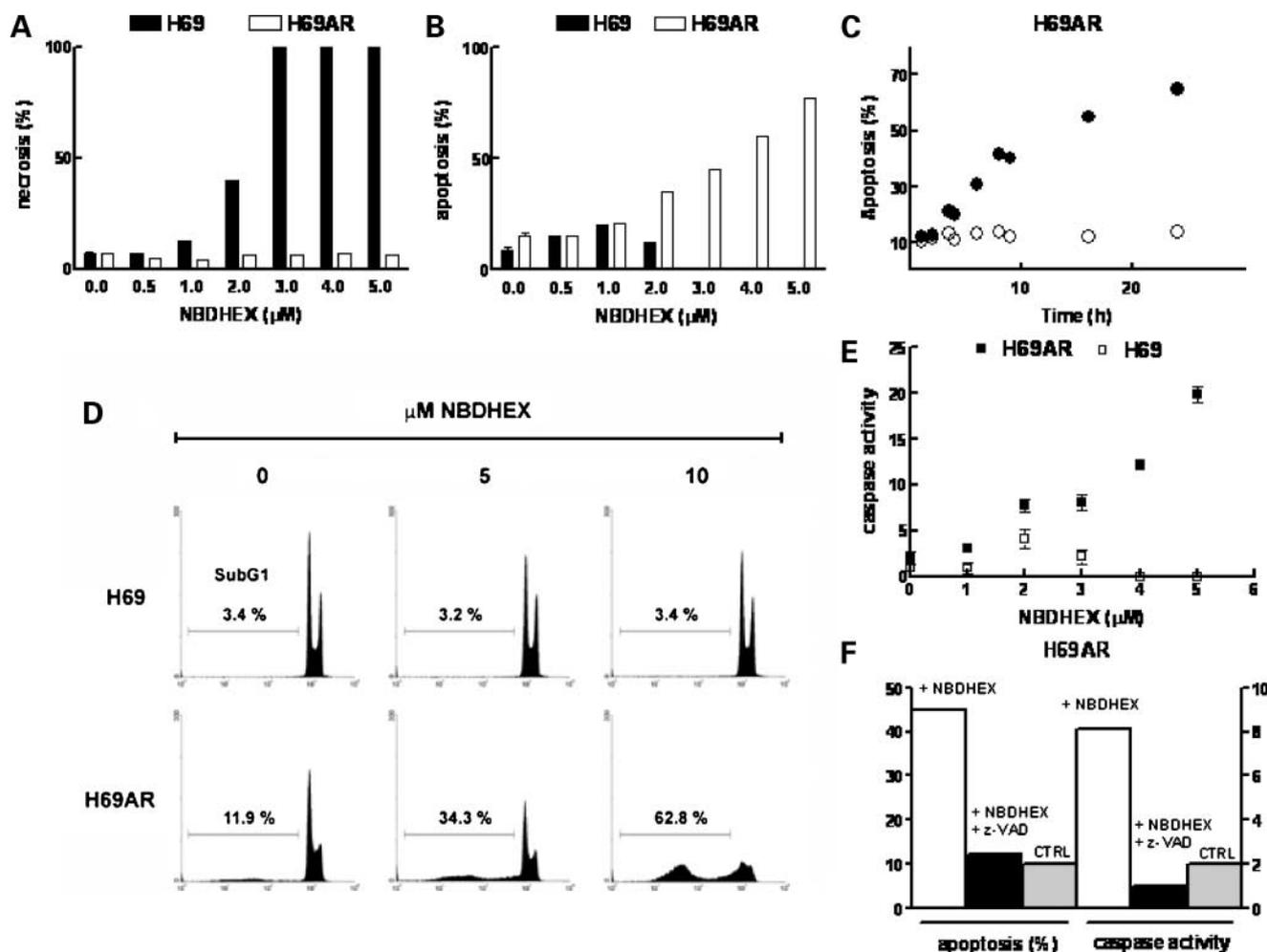

Figure 2. Cell death induction by NBDHEX. Cell death was determined in both H69 and H69AR cells after 24 h of incubation with several amounts of NBDHEX (from 0 to 5 μmol/L). The histograms represent the frequency of necrotic (**A**) and apoptotic (**B**) SCLC cells as determined by trypan blue permeability and nuclear staining with Hoechst 33342, respectively. Results are expressed as a percentage of total cells and are representative of three independent experiments. **C,** time course of apoptosis triggered by 3 μmol/L NBDHEX in H69AR cells. ●, NBDHEX-treated cells; ○, untreated control cells. **D,** flow cytometric analysis done on both H69 and H69AR cells incubated with different amounts of NBDHEX (from 0 to 10 μmol/L). After 6 h of treatment, cell distribution, according to their DNA content, was measured after cell permeabilization and propidium iodide staining. The fraction of cells with sub-G_1 DNA content (apoptotic cells) was calculated using WinMDI version 2.8 software and is indicated in each profile. **E,** increase of caspase-3 activity in H69 and H69AR cell lines after 24 h of treatment with several amounts of NBDHEX (from 0 to 5 μmol/L). Activity is expressed as fluorescence change measured within 1 min from the substrate addition, normalized per number of cells. *Points,* mean of triplicate independent determinations; *bars,* SD. **F,** caspase-3 activity and apoptosis were also determined, after 24 h of NBDHEX treatment, on H69AR cells preincubated with 40 μmol/L of caspase inhibitor Z-VAD-*fmk* before the addition of 3 μmol/L NBDHEX and maintained throughout the experiment. Results are representative of three independent experiments that gave similar results.

Figure 3. NBDHEX-induced apoptosis in H69AR cells is associated with the activation of JNK/c-Jun pathway. H69-sensitive and H69AR cells were treated with 3 μmol/L NBDHEX. The levels of JNK, c-Jun, and their phosphorylated isoforms were detected at indicated time points on total protein extracts (20 μg) by Western blot analysis. α-Tubulin was used as loading control. The immunoblot shown is from one experiment representative of three that gave similar results.
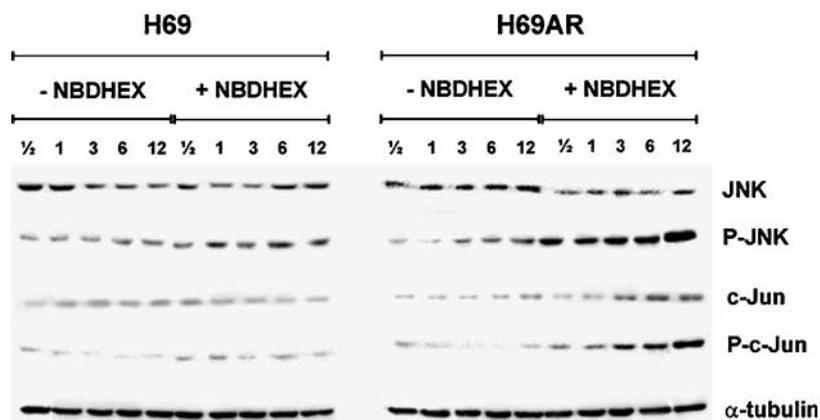
chromatin, suggesting that the phenomenon observed is primary necrosis instead of apoptosis-derived secondary necrosis (data not shown). To corroborate this hypothesis, we did cytofluorimetric analysis of both H69 and H69AR cells on staining with propidium iodide. H69AR treated with different amounts of NBDHEX showed a dose-dependent increase of cell population with hypodiploid (sub-G_1) nuclei, in which DNA cleavage occurred as the ultimate step of apoptosis. Conversely, this trend was absent in the parental H69 cells, which showed no increase in sub-G_1 peak when treated with the same amounts of NBDHEX (Fig. 2D). Taken together, these results indicate that the observed necrosis in H69 cell line is a bona fide apoptosis-independent, primary necrosis.

Activation of the caspase family proteases is also a central biochemical event occurring only in apoptotic cells. Therefore, the specific proteolytic activity of caspase-3 was assayed at intervals after drug treatment in both H69 and H69AR cell lines. Figure 2E shows caspase-3 activity after 24 h of incubation with different amounts of NBDHEX. H69 cells showed only a slight increase in caspase-3 activity after 2 μmol/L NBDHEX treatment, which quickly dropped to background levels by increasing drug concentration. In contrast, caspase-3 activity was already observed in H69AR cells treated with 1 μmol/L NBDHEX and increased progressively with the increase of NBDHEX concentration. Caspase-3 inactivation by the general caspase inhibitor Z-VAD-*fmk* fully prevented apoptosis in H69AR cells (Fig. 2F), so confirming the caspase involvement in the apoptotic response of cells exposed to NBDHEX.

NBDHEX-Induced Apoptosis Is Associated with the JNK/c-Jun Activation

Mitogen-activated protein kinases (MAPK) are important components of signaling pathways, which transduce extracellular stimuli into intracellular responses. It has been reported that NBDHEX is able to induce the activation of JNK pathway in a variety of cancer cells (11, 20). Therefore, the effect of NBDHEX on the phosphorylation/activation of this MAPK was examined both in H69 cell line and in its Adriamycin-resistant counterpart. As shown in Fig. 3, treatment with NBDHEX increased the phosphory-

lation of JNK in H69AR cells in a time-dependent fashion; phosphorylated JNK activation occurred as early as 30 min and remained at high levels for at least 12 h. c-Jun, a direct downstream transcription factor activated by JNK, was also increased in the H69AR cells after 3 h of NBDHEX treatment, suggesting an involvement of the JNK/c-Jun pathway in the NBDHEX-induced apoptosis.

In contrast, neither change in the intensity of the immunoreactive band of phosphorylated JNK nor c-Jun activation was detected in the NBDHEX-treated H69 cells. It is well known that the GST isoenzyme GSTP1-1 inhibits JNK activity by forming a GSTP1-1-JNK heterocomplex (21). Therefore, to rule out the possibility that a higher GSTP1-1 expression in H69 cells could be responsible for the lack of JNK activation (11), the amount of this enzyme was determined both in the H69 cell line and in its MDR counterpart. No differences in the GSTP1-1 levels were observed between these cell lines (Fig. 4A). Neither were any significant changes in the GST activity observed between NBDHEX-treated and untreated control cells up to 6 h of incubation (Fig. 4B). These findings indicate that NBDHEX triggers a different cell death mechanism in H69 cells.

Intracellular Redox Status and p38^{MAPK} Activation

To evaluate the effects induced by NBDHEX on pro-apoptotic members of MAPK family, we next analyzed the change in the phosphorylation/activation of p38^{MAPK}. Immunoblot analysis of H69 cell line showed a marked increase in phosphorylation of p38^{MAPK}, which occurred within 30 min of drug treatment, whereas NBDHEX induced only a negligible increase of p38^{MAPK} phosphorylation in the H69AR cell line (Fig. 5A). It has been suggested that p38^{MAPK} senses very low changes of the intracellular redox state (22). Thus, to show a possible change of this redox system after NBDHEX treatment, we measured the intracellular content of both reduced and disulfide form of glutathione, the most abundant low molecular weight antioxidant within the cell. Parental H69 cell line showed an average intracellular concentration of GSH of ~60 nmol/mg protein, a value 3-fold higher than that of the Adriamycin-resistant counterpart (~20 nmol/mg protein; Fig. 5B). Moreover, a time-dependent increase of GSSG content was detected in the NBDHEX-treated H69

cell line but not in the H69AR cells (Fig. 5C). This increase was not due to a reduced activity of the NADPH-dependent glutathione disulfide reductase that continuously reduces GSSG back into GSH. In fact, H69 cells show a GSH reductase activity of 0.090 unit/mg (µmol/min/mg a pH 7.6), which is comparable with the value found in the resistant H69AR cell line (0.080 unit/mg). It is known that the intracellular redox status depends on the relative amounts of the GSH and GSSG (22) and that the oxidation of a limited amount of GSH into GSSG can drastically change this ratio, so leading to activation of $p38^{MAPK}$ and subsequent apoptosis. This was already reported for the K562 cell line treated with NBDHEX (20). However, the imbalance of the intracellular redox state and the activation of $p38^{MAPK}$ found in H69 cells were not followed by the apoptotic cascade, and in this case, cells died by necrosis.
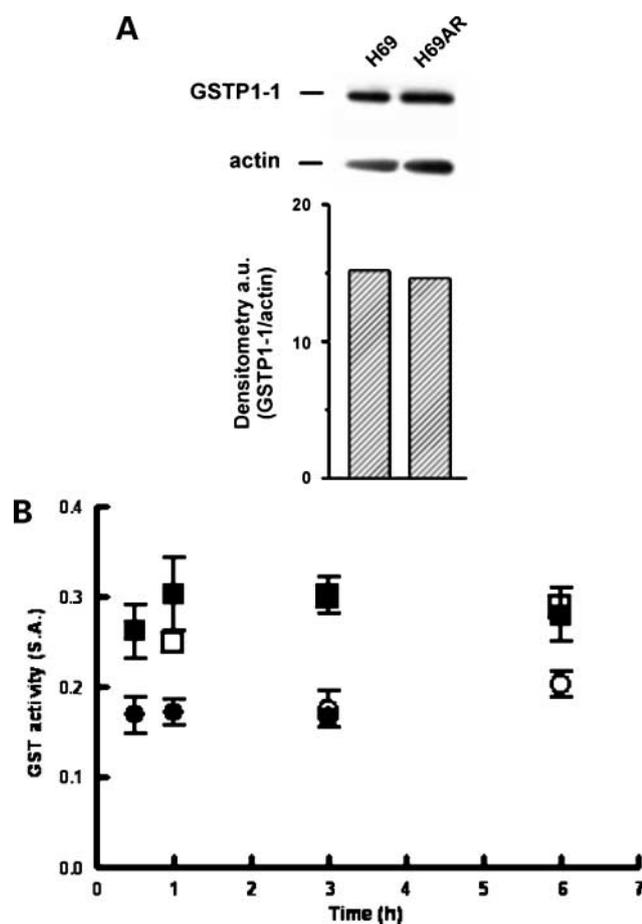

Figure 4. GST content in SCLC cell lines. **A,** 20 µg of total protein extracts from H69 and H69AR cells were used for detection of GSTP1-1 protein content by Western blot. β-Actin was used as loading control. Immunoblot is from one experiment representative of three that gave similar results. Density of immunoreactive bands (reported below the immunoblot) was calculated using the software Quantity One, normalized for β-actin and reported as arbitrary units (*a.u.*). **B,** GST specific activity (S.A. = µmol·min^{-1}·mg^{-1}) determined at indicated time points in H69 (●) and H69AR (■) cells after 3 µmol/L NBDHEX treatment; untreated control cells are also reported (○, H69; □, H69AR). *Points,* mean of triplicate independent determinations; *bars,* SD.

Effect of GSH Increase on Necrosis and Apoptosis Induced by NBDHEX

An increase in intracellular oxidation may determine the selection between apoptosis and necrosis. In particular, suppression of apoptosis and the induction of necrosis is often associated with the intracellular GSH content (23, 24). Therefore, we tested the possibility of switching the NBDHEX-induced necrosis in apoptosis by increasing the intracellular level of GSH. We incubated H69 cells with glutathione ethylester, a permeant compound that directly increases the intracellular levels of GSH (25). Figure 5E shows that treatment with glutathione ethylester significantly prevented NBDHEX-mediated necrosis. In fact, the percentage of necrotic cells, after 24 h of treatment with 5 µmol/L NBDHEX, decreased from 100% to 36% in the presence of 1 mmol/L glutathione ethylester, although no switch from necrosis to apoptosis was observed. On the other hand, an increase of the intracellular levels of GSH partially improved (~25%) the survival of H69AR cells after 24 h of treatment with 5 µmol/L NBDHEX (Fig. 5D).

Bcl-2 Content

SCLCs, and particularly H69 cells, are known to express high levels of the antiapoptotic protein Bcl-2 (26). This was confirmed by Western blot analysis, which conversely showed a very low amount of Bcl-2 protein in the MPR1-overexpressing H69AR cell line (Fig. 6). It can be hypothesized that the strong down-regulation of this antiapoptotic protein in H69AR cells may favor the occurrence of apoptosis following NBDHEX treatment.

Discussion

SCLC is characterized by a highly aggressive clinical course and a strong tendency to develop distant metastases. This makes chemotherapy more effective than surgical or radiation therapy. Consequently, we have investigated the efficacy of the nitrobenzoxadiazole-derivative NBDHEX on the human SCLC H69 cell line and on its MDR variant H69AR. Surprisingly, we found that NBDHEX was cytotoxic in both cell lines with LC_{50}s in the micromolar range. Interestingly, this drug triggers two different types of cell death: a caspase-dependent apoptotic pathway in the Adriamycin-resistant H69AR and a necrotic phenotype in the parental H69 cell line. To explain this result, some of the internal cell factors that counteract apoptosis in lung cancer have been analyzed. We found higher GSH levels and Bcl-2 overexpression in H69 cells compared with levels found in the Adriamycin-resistant H69AR cells characterized by an almost undetectable Bcl-2 content. Such evidence matches several studies showing a positive correlation between elevated Bcl-2 and GSH levels and suppression of apoptosis (27, 28). The important role that these antiapoptotic factors play in lung cancer progression is confirmed by the action mechanism of Adriamycin, which triggers apoptosis in H69 cells by down-regulation of Bcl-2 expression and depletion of GSH (29). However, under our experimental conditions, neither GSH nor Bcl-2 content decreased in H69 cells after treatment
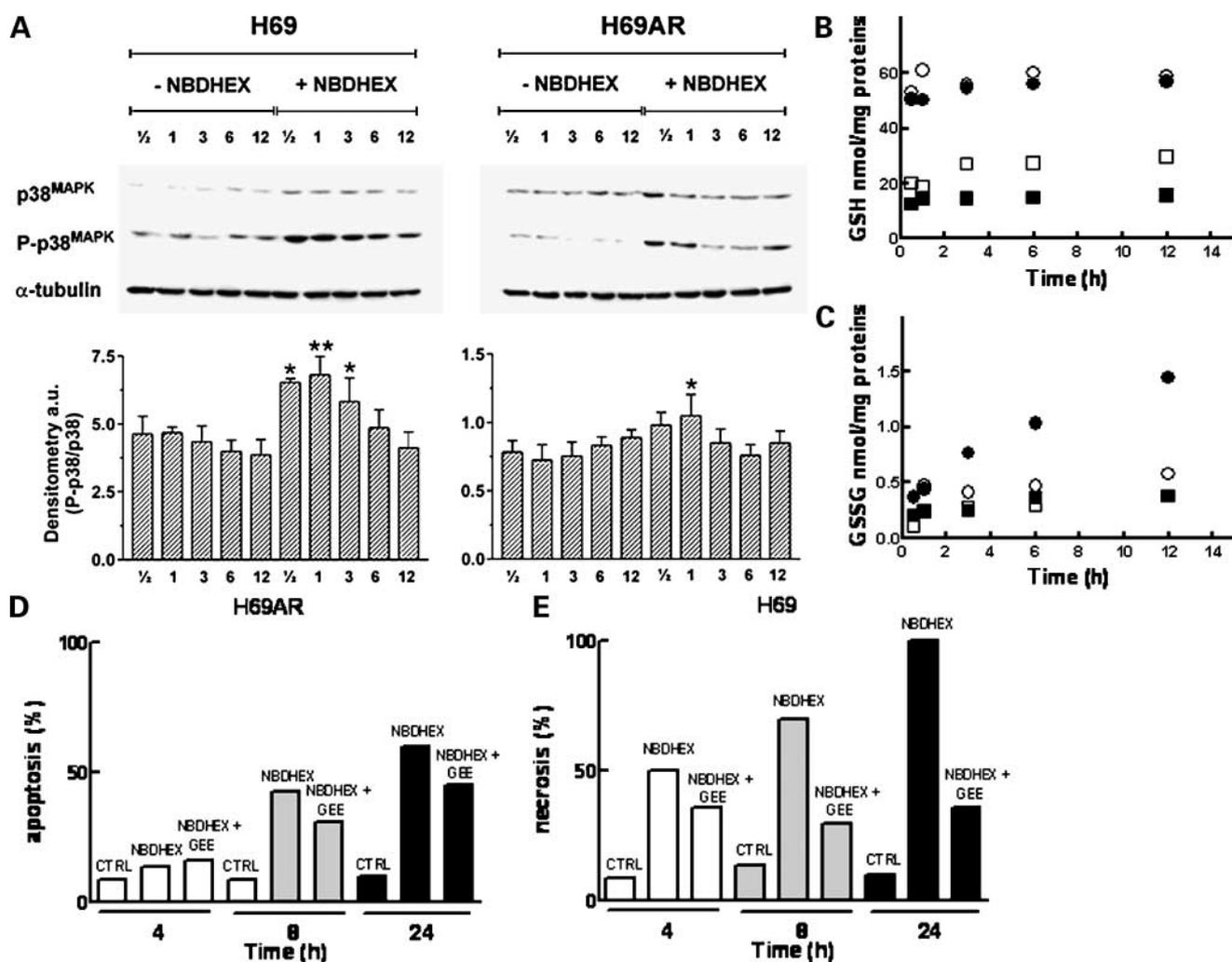
Figure 5. Redox status and p38[MAPK] activation in SCLC cells. **A,** levels of p38[MAPK] and its phosphorylated isoform were detected at indicated time points in both H69 and H69AR cells treated with 3 μmol/L NBDHEX. α-Tubulin was used as loading control. The immunoblot shown is from one experiment representative of three that gave similar results. The density of immunoreactive bands (reported below the immunoblot) was calculated using the software Quantity One, normalized for α-tubulin and reported as arbitrary units of the ratio between the phosphorylated and unphosphorylated form of p38 (*P-p38/p38*). *Starred columns,* significant increase relative to the controls (*, $P < 0.05$; **, $P < 0.01$). Intracellular GSH (**B**) and GSSG (**C**) levels were analyzed in H69 (●) and H69AR (■) cells at the indicated times after 3 μmol/L NBDHEX treatment. Untreated control cells were also analyzed (○, H69; □, H69AR). Data are expressed as nmol of GSH or GSSG equivalents/mg total protein and are representative of three independent experiments that gave similar results. H69AR (**D**) and H69 (**E**) cell lines were preincubated with 1 mmol/L glutathione ethylester (*GEE*) for 1 h before the addition of 5 μmol/L NBDHEX. At the indicated times, the percentage of apoptosis in H69AR cells was determined by flow cytometric analysis after cell permeabilization and propidium iodide staining. The percentage of necrosis in H69 cells was determined after staining with trypan blue. Results are representative of three independent experiments that gave similar results.

with NBDHEX (Figs. 5 and 6, respectively). Moreover, an increase of intracellular GSH concentration did not switch necrosis to apoptosis, suggesting that the amount of intracellular GSH does not control by itself the mode of cell death in H69 cell line. It has also been reported that Bcl-2 influences intracellular signaling cascades, thereby preventing apoptosis. In particular, Bcl-2 blocks the activation of the JNK signaling pathway by various apoptotic stresses (i.e., staurosporine UV light and anisomycin) by suppressing the stimulation of the upstream protein kinase MAPK kinase kinase (30, 31). These findings may explain the lack of JNK/c-Jun activation and the absence of apoptosis in H69

cells despite their being provided with an effective apoptotic machinery. The resulting necrosis observed after NBDHEX treatment may constitute a default cell death pathway induced when the activation of specific signal transduction cascades leading to cell death is inhibited (32). In spite of the adverse effects of necrosis, it could be therapeutically advantageous to trigger cell death by necrosis in lung cancer cells selected to resist apoptosis and characterized by extremely poor prognosis.

As far as MDR H69AR cells are concerned, we have found that the MRP1 transporter overexpressed in this cell line does not counteract the cytotoxicity of NBDHEX,
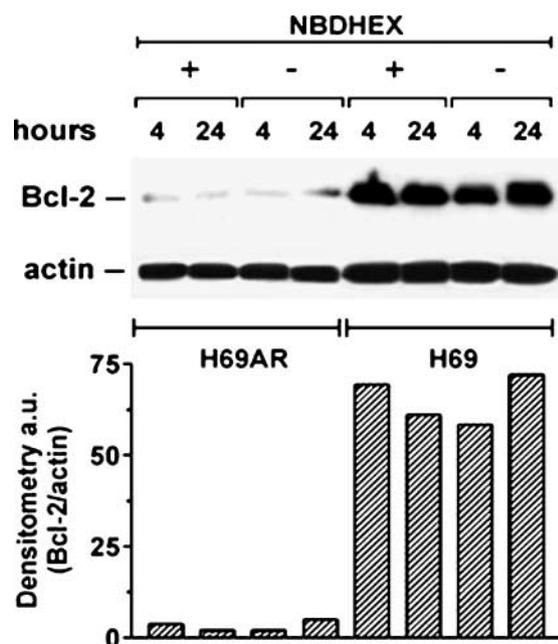
Figure 6. Bcl-2 expression in SCLC cells. The expression of Bcl-2 was examined in both H69 and H69AR cells by immunoblot analysis. At the indicated times, total protein extracts (20 μg) from untreated and 3 μmol/L NBDHEX – treated cells were subjected to Western blot analysis. β-Actin was used as loading control. The density of immunoreactive bands (reported below the immunoblot) was calculated using the software Quantity One, normalized for β-actin and reported as arbitrary units.

which is not a substrate for this pump. NBDHEX induces a caspase-dependent apoptosis and this process may be promoted by the very low amount of Bcl-2 found in H69AR cells. The decrease of this antiapoptotic protein seems linked to the MDR phenotype. In fact, previous data show the concomitant down-regulation of *Bcl-2* gene in response to a significant overexpression of the *MRP1* gene in H69 cells (33, 34). Moreover, it has been shown that the amount of intracellular GSH levels is a critical factor for MRP1-expressing cells. In particular, MRP1 expression causes a reduction in cellular GSH levels and this makes H69AR cells hypersensitive to drugs, such as verapamil and flavonoids, which increase the MRP1-mediated GSH transport. Accordingly, addition of GSH to the medium efficiently prevents apoptosis induced by these drugs in MRP1-expressing cells (35, 36). Under our experimental conditions, we observed that an increase in intracellular GSH levels partially improved the survival of H69AR cells treated with NBDHEX. Moreover, NBDHEX does not seem to affect the correct redox equilibrium of this cell line. In fact, no decrease in GSH levels and no accumulation of oxidized glutathione are observed during NBDHEX treatment (Fig. 5). We cannot rule out the hypothesis that GSH could be extruded from the cells under our experimental conditions; however, this probably occurs in a long time frame so as to allow the cell to induce GSH neosynthesis. Therefore, the role played by GSH in the apoptosis of NBDHEX-treated H69AR cells is not completely under-

stood at this time and will be the goal of future investigations. Whatever the case, a down-regulation of factors crucial for cell survival makes H69AR cells more sensitive to the cytotoxic action of drugs that are not MRP1 substrates. Actually, MRP1 transports not only a wide variety of neutral hydrophobic compounds but also numerous glutathione, glucuronate, and sulfate conjugates (13), and only a few anticancer drugs are poor substrates (37) or not substrates of MRP1 at all (38). Thus, many attempts have been made to identify agents able to reverse the MDR mediated by MRP1 (39). However, many of these molecules in clinical trials revealed intrinsic toxicity at doses effective in reversing MDR (40). Finally, to enhance the efficacy of chemotherapy in MDR lung cancer, complex strategies based on the simultaneous administration of an anticancer drug and of antisense oligonucleotides targeting antiapoptotic defense have been proposed (41). Taken together, all this evidence emphasizes the potential anticancer role of NBDHEX, a compound that is effective against drug-resistant tumors overexpressing both P-gp (11) and MRP1 and that also shows low toxicity *in vivo* (20).

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