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A Strong Glutathione *S*-Transferase Inhibitor Overcomes the P-glycoprotein-mediated Resistance in Tumor Cells

*6-(7-NITRO-2,1,3-BENZOXADIAZOL-4-YLTHIO)HEXANOL (NBDHEX) TRIGGERS A CASPASE-DEPENDENT APOPTOSIS IN MDR1-EXPRESSING LEUKEMIA CELLS**

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The new glutathione *S*-transferase inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) is cytotoxic toward P-glycoprotein-overexpressing tumor cell lines, *i.e.* CEM-VBL10, CEM-VBL100, and U-2 OS/DX^{580}. The mechanism of cell death triggered by NBDHEX has been deeply investigated in leukemia cell lines. Kinetic data indicate a similar NBDHEX membrane permeability between multidrug resistance cells and their sensitive counterpart revealing that NBDHEX is not a substrate of the P-glycoprotein export pump. Unexpectedly, this molecule promotes a caspase-dependent apoptosis that is unusual in the P-glycoprotein-overexpressing cells. The primary event of the apoptotic pathway is the dissociation of glutathione *S*-transferase P1-1 from the complex with c-Jun N-terminal kinase. Interestingly, leukemia MDR1-expressing cells show lower LC$_{50}$ values and a higher degree of apoptosis and caspase-3 activity than their drug-sensitive counterparts. The increased susceptibility of the multidrug resistance cells toward the NBDHEX action may be related to a lower content of glutathione *S*-transferase P1-1. Given the low toxicity of NBDHEX *in vivo*, this compound may represent an attractive basis for the selective treatment of MDR1 P-glycoprotein-positive tumors.

The development of resistance to multiple chemotherapeutic drugs is a common clinical problem that limits the therapeutic potential of anticancer drugs in clinical oncology. This complex phenomenon, known as multidrug resistance (MDR),[2] is often associated with the overexpression of MDR1 P-glycoprotein (P-gp) at the plasma membrane of tumor cells (1). This 170-kDa glycoprotein utilizes the energy of ATP hydrolysis to transport a wide range of compounds and drugs across membranes out of the cytosol thereby reducing their intracellular bioavailability and hence their toxicity (2). In humans, *MDR1* gene overexpression causes cancer cells to become resistant to a variety of anticancer drugs, including vinblastine, doxorubicin, etoposide, and teniposide as well as many other cytotoxic agents (3). This low specificity represents the major problem in the development of new drugs directed against tumor cells showing an intrinsic or acquired MDR phenotype. We have recently designed and synthesized new 7-nitro-2,1,3-benzoxadiazole derivatives characterized by strong cytotoxic activity toward several cancer cell lines (LC$_{50}$ about 1 μM) and low toxicity in mice (up to 125 mg/kg) (4). Among them, NBDHEX is characterized by the presence of a thioether bridge connecting the benzoxadiazole ring with an aliphatic chain (5). This compound is an efficient inhibitor of glutathione *S*-transferases (GSTs), a family of enzymes also involved in the cancer drug resistance phenomenon (6, 7). Specifically, the isoenzyme GSTP1-1 forms a heterocomplex with c-Jun N-terminal kinase (JNK) that efficiently inhibits the stress-signaling cascade mediated by JNK (8, 9). We have recently shown in leukemia cell lines (4) that NBDHEX promotes GSTP1-1 dissociation from the GSTP1-1-JNK complex resulting in cell death by apoptosis. In this study we investigated the NBDHEX-mediated cytotoxicity in tumor cell lines overexpressing the MDR1 P-gp export pump. Our data show that NBDHEX is not a substrate of this ABC transporter, and both parental tumor cells (CCRF-CEM and U-2 OS) and their selected P-gp overexpressing variants (CEM-VBL10, CEM-VBL100, and U-2 OS/DX^{580}) are efficiently committed to death by this molecule. These findings indicate that NBDHEX may be a novel and very effective compound in selectively killing tumor cells and their chemo-resistant variants showing an intrinsic or acquired MDR phenotype.

EXPERIMENTAL PROCEDURES

Cells and Their MDR Phenotype—For this study we used the following human cell lines: human acute T-lymphoblastoid leukemia CCRF-CEM, human osteosarcoma U-2 OS and their

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[2] The abbreviations used are: MDR, multidrug resistance; GST, glutathione *S*-transferase; NBDHEX, 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol; P-gp, P-glycoprotein; VBL, vinblastine; JNK, c-Jun N-terminal kinase; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; PBS, phosphate-buffered saline.

P-gp overexpressing variants, CEM-VBL10 and CEM-VBL100, which show different relative resistance levels to vinblastine (CEM-VBL10 $<10^4$ and CEM-VBL100 $>10^6$ P-gp molecules per cell) (10, 11), and U-2 OS/DX^{580} cell lines resistant to doxorubicin (12).

All cell lines were cultured using RPMI 1640 medium enriched with 10% fetal bovine serum and antibiotics. Adherent cells (U-2 OS and U-2 OS/DX^{580}) were detached from a flask using trypsin. All components were purchased from Hyclone (Logan, UT). Periodically, MDR-expressing cells were selected by routine administration of vinblastine sulfate (VBL) (Lilly) at the final concentration of 11 nM (10 ng/ml) and 110 nM (100 ng/ml) for CEM-VBL10 and CEM-VBL100 cells, respectively, or with 1 μM doxorubicin (Sigma) for U-2 OS/DX^{580} cells. The cells were also typed (every 2–3 weeks) for P-gp expression. Briefly, living/intact CCRF-CEM and U-2 OS cell lines and their MDR variants were stained with the monoclonal antibody to MDR1 (MM4.17 directed to extracellular P-gp domain (13)) (Chemicon), and then cells were washed and labeled with fluorescein isothiocyanate-labeled secondary mouse antibody and subjected to FACSCalibur flow cytometer (BD Biosciences) analysis.

Cell Treatments—Treatments were performed on cells cultured at 1×10^6 cells/ml. NBDHEX was synthesized as reported by Ricci *et al.* (5). Stock solutions of this compound were prepared in Me_2SO and diluted to the appropriate concentration in RPMI 1640 cell medium just before use. The cytotoxic drug doxorubicin was used at 17 μM final concentration. Treatments with the cell-permeable JNK inhibitor SP600125 (Calbiochem-Novabiochem) were performed at a final concentration of 20 μM, because lower concentrations did not show significant inhibition and higher concentrations were toxic. Treatment with the pan-caspase inhibitor Z-VAD-fmk (Vinci-Biochem, Florence, Italy) was performed at a concentration of 40 μM. The P-gp inhibitor verapamil (Abbott) was used at concentration of 5 μM. All the inhibitors were added 1 h before the drug (NBDHEX or doxorubicin) treatment and maintained throughout the experiment.

Analysis of Cell Viability and Apoptosis—The NBDHEX cytotoxicity was determined in all cell lines by the sulforhodamine B assay (Sigma) (4). The NBDHEX-induced apoptosis was measured after cell staining with the DNA-specific dye Hoechst 33342 (Sigma) and analysis of nuclear fragmentation with a fluorescence microscope.

Intracellular Localization of NBDHEX—NBDHEX localization was evaluated by taking advantage of its intrinsic fluorescence (5). U-2 OS/DX^{580} cells, grown on WillCo-dishes® (WillCo Wells B.V., Amsterdam, The Netherlands) for 24 h, were incubated with 25 μM NBDHEX, the lowest concentration that allowed intracellular drug detection. At different times of incubation, cells were observed under living conditions by laser scanning confocal microscopy. Both untreated and NBDHEX treated U-2 OS/DX^{580} cells were also specifically stained for vacuoles with acridine orange (Sigma) as reported previously (14). The observations were carried out with a Leica TCS SP2 spectral confocal microscope; NBDHEX was excited with a 488 nm argon laser line, and emission lines were collected after passage through a DD 488/543 filter in a spectral window ranging from 520 to 550 nm. The acridine orange was excited at 488 nm; the emitted green light (515–565 nm) and red light (>590 nm) were simultaneously registered in sequential double fluorescence mode after passage through the DD 488/543 filter.

Inhibition of P-gp Function—CEM-VBL10, CEM-VBL100, U-2 OS/DX^{580}, and their parental cell lines were treated with either 10 μM NBDHEX or 17 μM doxorubicin for 1 h at 37 °C in the presence or absence of the P-gp inhibitor verapamil (5 μM). The cells were then washed in PBS and resuspended in drug-free medium, in the presence or in the absence of verapamil. After 1 h at 37 °C, cells were washed with ice-cold PBS and analyzed by a FACSCalibur flow cytometer (BD Biosciences) to evaluate either NBDHEX or doxorubicin intracellular retention.

Kinetics of NBDHEX Uptake and Release in Leukemia Cell Lines—CCRF-CEM, CEM-VBL10, and CEM-VBL100 cells were incubated with either 2 or 10 μM NBDHEX at 37 °C. To measure the drug uptake at different incubation times, aliquots of cells were washed in ice-cold PBS and analyzed by flow cytometry. To determine the rate of release, cells were incubated with NBDHEX for 1 h at 37 °C, washed, and resuspended in drug-free medium. At different time points aliquots of cells were washed in ice-cold PBS and analyzed for the drug content by flow cytometry. To determine the induction of apoptosis at different times of NBDHEX exposure, at the indicated times aliquots of either CEM-VBL100 or CCRF-CEM cells were washed and cultured in drug-free medium; the degree of apoptosis was determined after 9 and 24 h, respectively.

Western Blot Analyses of Leukemia Cell Lines—CCRF-CEM, CEM-VBL10, and CEM-VBL100 cells were treated with 2 μM NBDHEX. At each time point analyzed, the cell pellets were lysed for Western blot analyses as described previously (4). Proteins (20 μg) were loaded on 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). Polyclonal anti-JNK, anti-caspase-8 (1:1,000; Upstate Biotechnology, Inc., Lake Placid, NY), anti-caspase-9, anti-cleaved caspase-3 (1:1,000; Cell Signaling Technology, New England Biolabs, Beverly, MA), and monoclonal anti-phospho-JNK isoform (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin (1:5,000; Sigma) were used as primary antibodies.

Mitochondria- and cytosol-enriched fractions were obtained as described previously (15). 10 and 20 μg of proteins from mitochondria and cytosol, respectively, were loaded on 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Polyclonal anti-Bax (1:2,000; Santa Cruz Biotechnology) and monoclonal anti-cytochrome *c* (1:2,000; Pharmingen) were used as primary antibodies. Mitochondrial Hsp60, used as loading and extraction control, was recognized by a monoclonal anti-Hsp60 (1:5,000; Stressgen, Victoria, Canada). The specific protein complex formed upon appropriate secondary antibody (Bio-Rad) treatment (1:10,000) was identified using Fluorchem Imaging system (Alpha Innotech, San Leandro, CA) after incubation with ChemiGlow chemiluminescence substrate (Alpha Innotech).

Enzyme Activities in Leukemia Cell Lines—GST and caspase-3 activities were measured as described previously (4). Protein concentration was determined by the bicinchoninic acid protein assay reagent (Pierce).

TABLE 1
Cytotoxicity effect of NBDHEX

LC_{50} values were determined by SRB assay in tumor cell lines incubated with NBDHEX for 48 h. Data are calculated as the mean ± S.D. of three independent determinations. Significant differences *versus* parental cells (*, $p < 0.05$; **, $p < 0.01$) were analyzed by the Student's *t* test. LC_{50} represents the NBDHEX concentration, which inhibited growth by 50%.

Cell lines	CCRF-CEM	CEM-VBL10	CEM-VBL100	U-2 OS	U-2 OS/DX^{580}
LC_{50} (μM)	0.33 ± 0.03	0.25 ± 0.01*	0.19 ± 0.01**	1.16 ± 0.18	1.65 ± 0.23

RESULTS AND DISCUSSION

NBDHEX Induces Cell Death in P-gp Overexpressing Cells—NBDHEX, a compound that induces cell death in several nonresistant tumor cell lines (4), is also cytotoxic toward the P-gp overexpressing cells. In particular, NBDHEX shows LC_{50} values in the micromolar or even nanomolar range for U-2 OS/DX^{580}, CEM-VBL10, and CEM-VBL100 cell lines (see Table 1), independently of the acquired resistance to chemotherapeutic agents. This suggests that NBDHEX is able to circumvent the drug resistance associated with the overexpression of the P-gp export pump. Actually, NBDHEX appears to commit the MDR cell lines to death even more efficiently than the corresponding sensitive cell lines as suggested by the slight but significant increased cytotoxicity observed with the CEM-VBL10 and CEM-VBL100 cells compared with the parental cell line (Table 1).

Intracellular Localization of NBDHEX—To clarify the NBDHEX mechanism, the intracellular localization of this drug was investigated in the adherent osteosarcoma U-2 OS/DX^{580} cell line overexpressing P-gp by the laser scanning confocal microscopy technique. After 5 min of treatment with 25 μM NBDHEX the inherent fluorescence of this compound was detected almost exclusively in the cytoplasm of the cells, whereas nuclei appeared completely negative (Fig. 1, *panel A*). This picture did not change after 45, 60, or 120 min of incubation in the presence of the drug, and only a weak signal was detectable in the nuclear matrix (Fig. 1, *panel A*). At 300 min of treatment some cells showed cytoplasmic vacuoles lacking in signal (Fig. 1, *panel A arrowheads*) and have faintly fluorescent nuclei (*arrows*). The observed vacuolation of the cytoplasm could be ascribed to morphological alterations associated with the apoptotic mechanism of damage (16). In fact, the analysis of U-2 OS/DX^{580} cells treated with 25 μM NBDHEX for 300 min and stained with acridine orange demonstrated that the vacuolation was concomitant with the condensation and marginalization of the chromatin (data not shown).

P-gp Does Not Affect the NBDHEX Efflux—P-gp molecules are undetectable on the parental drug-sensitive cells, although their number progressively increases in MDR variants (11). Because the transmembrane regions of this protein are rich in highly conserved aromatic amino acids residues (17), NBDHEX could fall within the wide range of nonpolar compounds recognized as substrates by this ABC transporter. Moreover, P-gp binds its substrates via hydrogen bond formation (18, 19), and oxygen and nitrogen atoms present in the NBDHEX structure are potential hydrogen-bonding acceptors. To clarify if NBDHEX efflux could be mediated by P-gp, we performed efflux experiments in the presence of verapamil. This drug is known to inhibit the P-gp export pump inducing intracellular accumu-

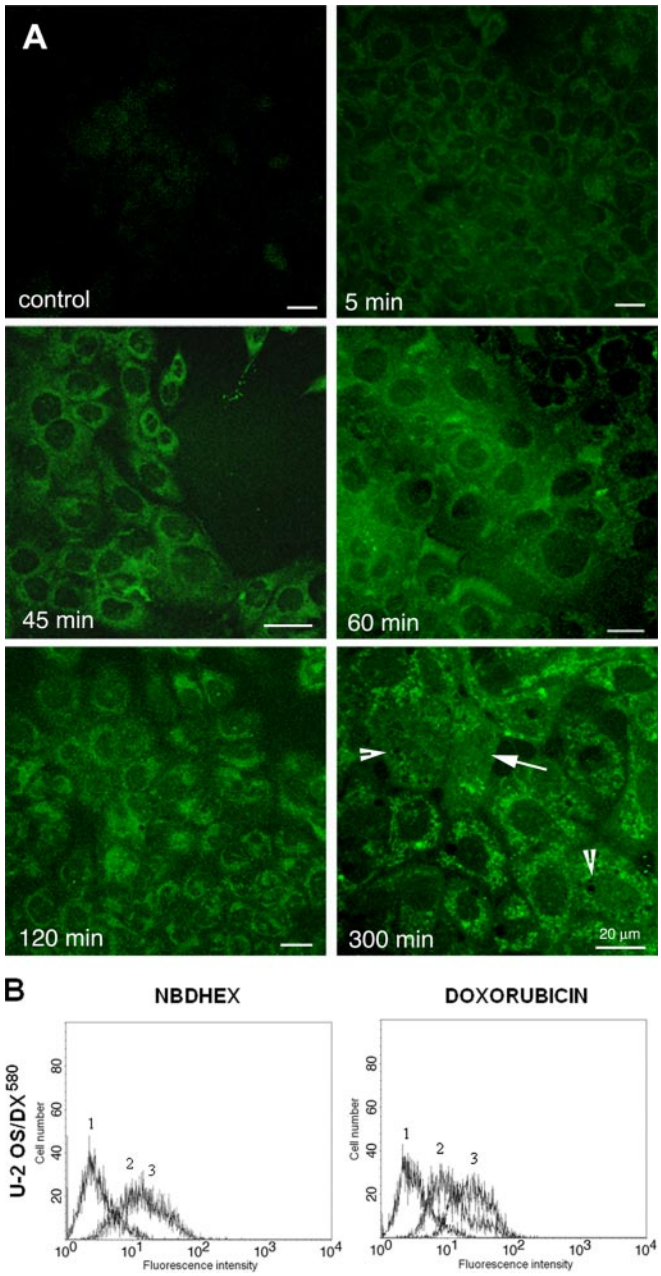

FIGURE 1. **Laser scanning confocal microscopy analysis of U-2 OS/DX^{580} cells treated with NBDHEX.** *Panel A,* U-2 OS/DX^{580} cells treated with 25 μM NBDHEX for 5, 45, 60, 120, and 300 min; untreated U-2 OS/DX^{580} control cells were also reported. At 300 min of NBDHEX treatment, some cells showed cytoplasmic vacuoli lacking in signal (*arrowheads*) and had faintly fluorescent nuclei (*arrows*). *Panel B,* cytofluorimetric analysis of the drug efflux in U-2 OS/DX^{580} cells in the absence and in the presence of the P-gp inhibitor verapamil. Cells were treated for 1 h at 37 °C with either 10 μM NBDHEX or 17 μM doxorubicin alone or in combination with 5 μM verapamil. The cells were then washed in PBS and cultured in drug-free medium for 1 more hour at 37 °C. U-2 OS/DX^{580} cells in drug-free medium (*1*), with NBDHEX (or doxorubicin) (*2*), with NBDHEX (or doxorubicin) and verapamil (*3*).

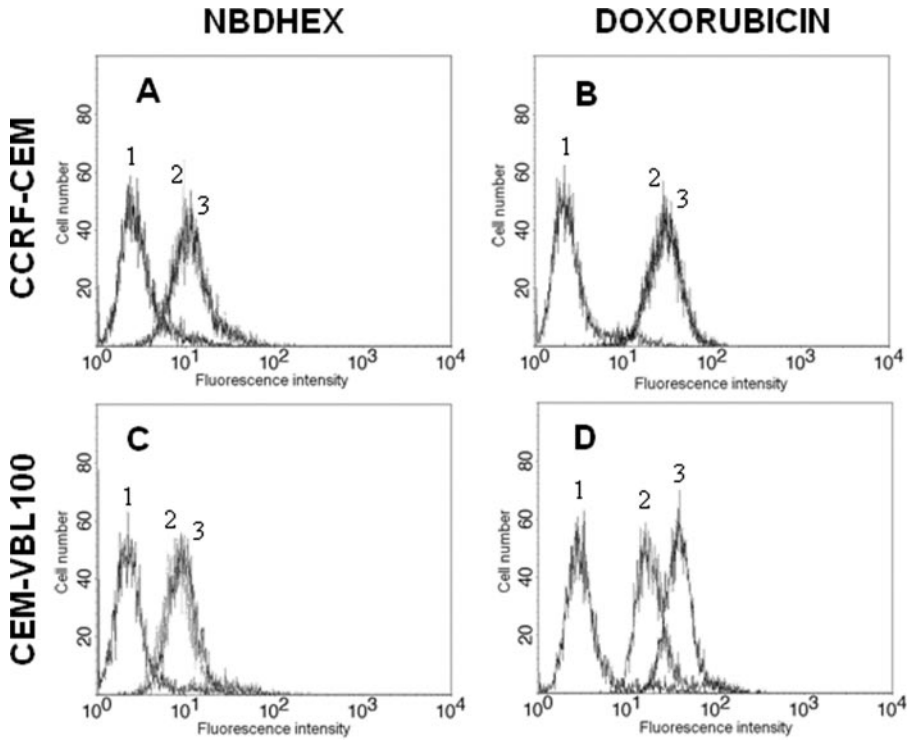

FIGURE 2. **Effect of the P-gp inhibitor verapamil on leukemia cell lines.** CCRF-CEM and CEM-VBL100 were treated with either 10 μM NBDHEX or 17 μM doxorubicin alone or in combination with 5 μM verapamil as reported above. *Panel A,* CCRF-CEM in drug-free medium (*1*), with NBDHEX (*2*), and with NBDHEX and verapamil (*3*). *Panel B,* CCRF-CEM in drug-free medium (*1*), with doxorubicin (*2*), and with doxorubicin and verapamil (*3*). *Panel C,* CEM-VBL100 in drug-free medium (*1*), with NBDHEX (*2*), and with NBDHEX and verapamil (*3*). *Panel D,* CEM-VBL100 in drug-free medium (*1*), with doxorubicin (*2*), and with doxorubicin and verapamil (*3*).

lation of P-gp substrates such as doxorubicin (20). As expected, verapamil inhibited doxorubicin efflux in both U-2 OS/DX^{580} and CEM-VBL100 cells (see Fig. 1, *panel B,* and Fig. 2, *panel D,* respectively), although it did not affect the intracellular concentration of doxorubicin in the sensitive variants (Fig. 2, *panel B*). Conversely, verapamil did not affect the intracellular concentration of NBDHEX in resistant and sensitive cell lines (see Fig. 1, *panel B,* and Fig. 2, *panels A* and *C*). These findings could be consistent with the fact that either NBDHEX is not a substrate for the P-gp export pump or a very fast inward diffusion kinetics of NBDHEX overcomes the P-gp-mediated efflux.

Kinetics of NBDHEX Uptake and Efflux in Leukemia Cell Lines—To clarify this question, we determined the drug uptake and release kinetics in both sensitive and resistant leukemia cell lines. NBDHEX uptake data fit well to a first order equation that fulfills a half-time value of about 2.9 min with CCRF-CEM cells (see Fig. 3, *panel A,* with 2 and 10 μM NBDHEX concentrations). A comparable value (about 3.5 min) was found for the MDR variant CEM-VBL100 (Fig. 3, *panel B,* with 2 and 10 μM NBDHEX concentrations). It follows that the intracellular equilibrium concentration is reached after only 15–30 min of NBDHEX treatment in both sensitive and P-gp overexpressing cell lines. Moreover, the drug efflux is slow in all tested cell lines and follows a first order kinetics, with $t_{1/2}$ values of about 50–60 min, with both 2 and 10 μM NBDHEX (Fig. 3, *panels C* and *D,* for CCRF-CEM and CEM-VBL100, respectively). Overall, these experiments show no differences in the NBDHEX drug uptake and efflux kinetics between P-gp overexpressing cell lines and their sensitive counterpart. As expected, the intracellular NBDHEX concentration measured at equilibrium is very similar in both sensitive and MDR cell lines suggesting that NBDHEX is not transported by the pump but crosses the plasma membrane by passive diffusion. An important consequence of the discrepancy between the rates of drug uptake and release is that NBDHEX accumulates inside the cells up to a concentration at least 1 order of magnitude higher than that present in the medium. In other words, when NBDHEX is present at 0.3 μM concentration in the medium (corresponding to the LC_{50} value found for this drug in leukemia cells), the intracellular concentration should be about 3 μM. In addition, the kinetics of NBDHEX uptake has been correlated to the apoptotic event by evaluating the degree of apoptosis induced by different amounts of NBDHEX diffused in the cytoplasm. It is evident from Fig. 3 (*panels A* and *B, insets*) that NBDHEX uptake kinetics parallel the degree of apoptosis showing a direct and dose-dependent cytotoxic effect of NBDHEX even at low intracellular concentrations.

NBDHEX Induces a Caspase-dependent Apoptosis—It is well known that P-gp overexpression confers resistance to a wide range of caspase-dependent apoptotic agents not only by removing drugs from the cell but also by inhibiting the activation of proteases involved in apoptotic signaling (21). Only a few drugs are reported to overcome the MDR phenotype induced by this ABC transporter, and most of them are molecules that induce cell death in a caspase-independent manner (22–25). We reported previously that NBDHEX induces a caspase-dependent apoptosis in CCRF-CEM cells (4). Therefore, it was crucial to obtain evidence of the occurrence of a caspase-dependent apoptotic pathway even in the P-gp overexpressing leukemia cells. After 3 h of NBDHEX treatment, caspase-3 activity was readily evident in the resistant cell lines, and its time course followed the increase of apoptotic cells number (Fig. 4, *panel A*). In addition, after 9 h of NBDHEX treatment, CEM-VBL10 and CEM-VBL100 cell lines showed an apoptosis extent and caspase-3 activity 2- and 5-fold higher, respectively, than the parental CCRF-CEM cell line (Fig. 4, *panel A*). The caspase-dependent nature of the NBDHEX-induced apoptosis was further confirmed by the general caspase inhibitor Z-VAD-fmk which prevents cell death in both CCRF-CEM and in the P-gp overexpressing cell lines (Fig. 4, *panel B*).

NBDHEX Induces Apoptosis via the Mitochondrial Pathway—Subsequently, we wanted to see if CCRF-CEM, and the drug-resistant variants CEM-VBL10 and CEM-VBL100, activate the receptor-mediated (extrinsic) and/or the

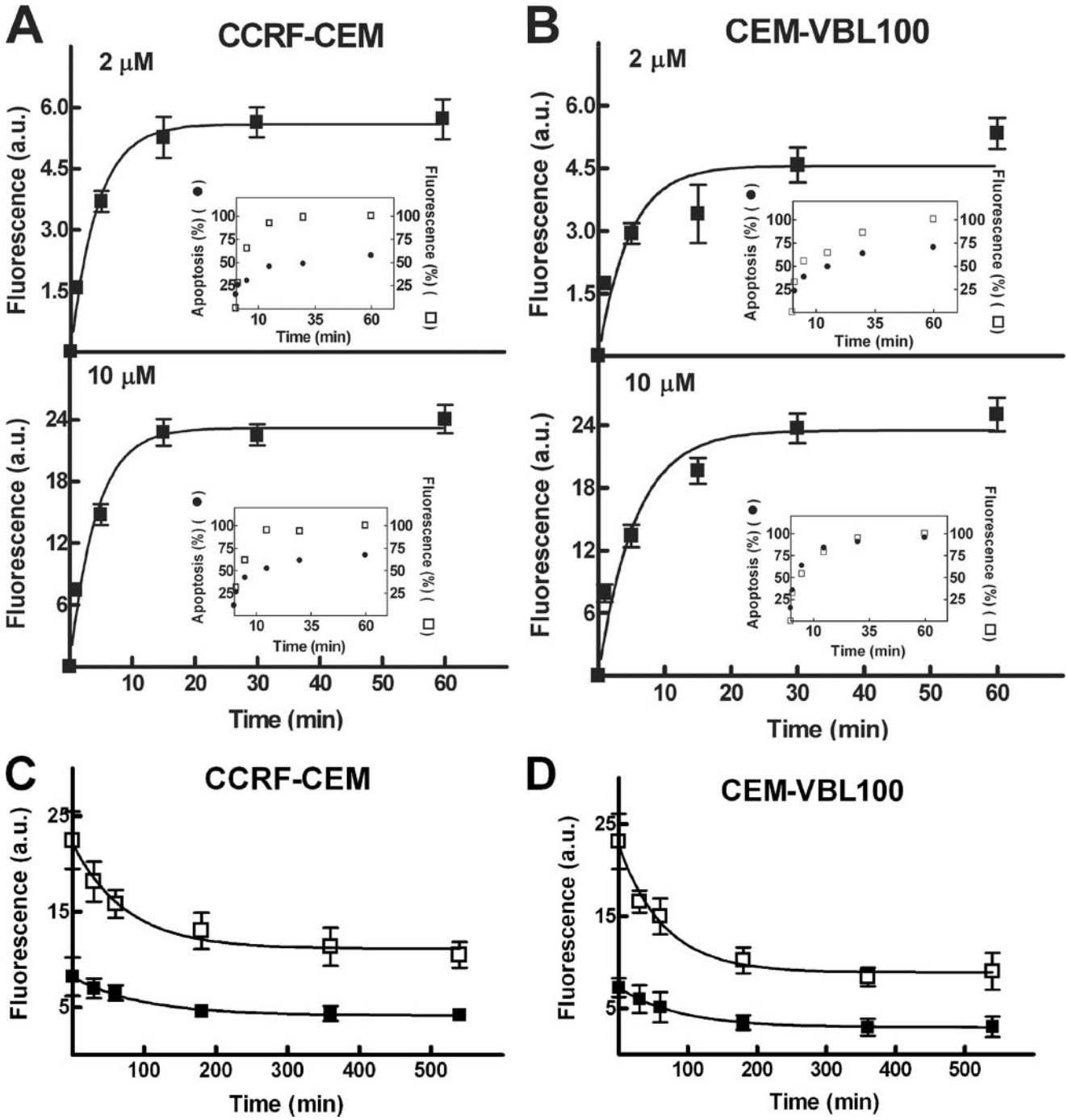

FIGURE 3. **Kinetics of NBDHEX uptake and release.** Kinetics of drug uptake was measured at 37 °C in CCRF-CEM (*panel A*) and in CEM-VBL100 cells (*panel B*) treated with either 2 or 10 μM NBDHEX. At different incubation times (1, 5, 15, 30, and 60 min), aliquots of cells were washed in ice-cold PBS and analyzed by a flow cytometer. Data are reported as mean fluorescence intensity of NBDHEX-treated cells (with control untreated cells fluorescence subtracted). Lines are the best fit of the experimental data to the following equation: $F = F_{max}(1 - \exp(-kt))$, where F is the mean fluorescence of the cells observed at a given incubation time, and F_{max} is the maximum mean fluorescence value at equilibrium. *Insets* in *panels A* and *B* show the percent of apoptosis (●) evaluated in cells incubated for 1, 5, 15, 30, and 60 min with either 2 or 10 μM NBDHEX. At the indicated time, cells were resuspended in drug-free medium, and apoptosis was determined after 9 and 24 h in CEM-VBL100 and CCRF-CEM, respectively. For comparison, the uptake kinetics, expressed as percent of fluorescence, is also reported (□). Drug efflux experiments were performed with CCRF-CEM (*panel C*) and CEM-VBL100 cells (*panel D*). Cells were incubated with either 2 μM (■) or 10 μM ((□) NBDHEX for 1 h at 37 °C. The cells were then washed and incubated in drug-free medium for 30, 60, 180, 360, and 540 min at 37 °C. At each time, cells were washed in ice-cold PBS and analyzed by flow cytometer. Data are reported as mean fluorescence intensity of NBDHEX-treated cells (with control untreated cells fluorescence subtracted). Lines are the best fit of the experimental data to the following equation: $F = ((F_{max} - F_o)\exp(-kt)) + F_o$, where F_{max} is the initial mean fluorescence of the cells, and F_o is the final mean fluorescence at equilibrium. Results are the mean (*bars* ± S.D.) of triplicate independent determinations. *a.u.*, arbitrary units.

mitochondria-dependent (intrinsic) apoptotic pathway upon NBDHEX treatment. Western blot analyses of mitochondrial and cytosolic fractions after 4 h of NBDHEX treatment show that Bax accumulated into mitochondrial extract together with the release of cytochrome *c* within the cytosol. These phenomena were associated with the complete disap-

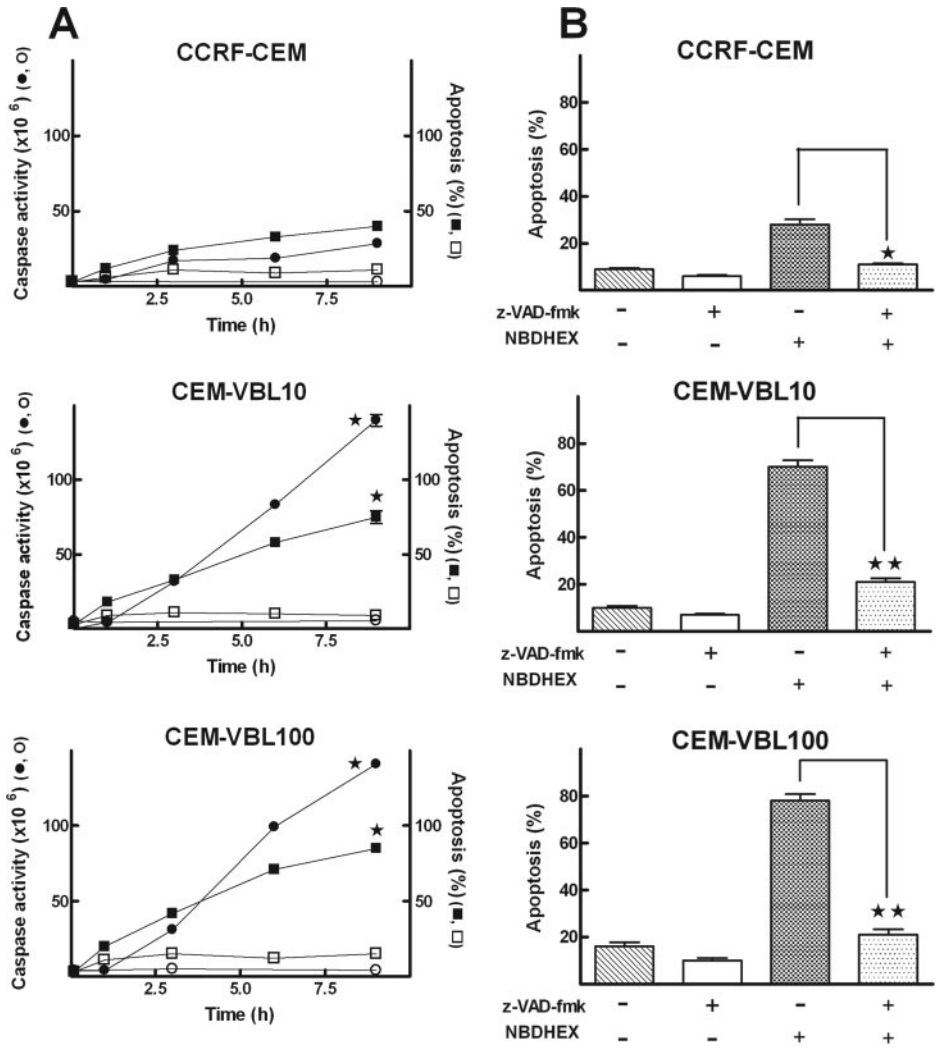

FIGURE 4. **Time course of apoptosis induced by NBDHEX.** *Panel A,* apoptosis and caspase-3 activity were determined at different incubation times with 2 μM NBDHEX in CCRF-CEM, CEM-VBL10, and CEM-VBL100 cell lines. Caspase-3 activity in NBDHEX-treated cells (●) and untreated control cells (○) is shown. The degree of apoptosis, in NBDHEX-treated cells (■) and untreated control cells (□) is shown. Caspase-3 activity is expressed as fluorescence change measured within 1 min from the substrate addition, normalized per number of cells. Data points represent the mean (*bars* ± S.D.) of quintuplicate independent determinations. Significant differences *versus* parental cells (*, $p < 0.001$) were analyzed by the *t* test. *Panel B,* apoptosis was determined in CCRF-CEM, CEM-VBL10, and CEM-VBL100 cells pretreated with 40 μM caspase inhibitor Z-VAD-fmk before the addition of 2 μM NBDHEX and maintained throughout the experiment. The percentage of apoptosis was determined after 9 h of NBDHEX treatment by fluorescence microscope analysis of nuclei stained with Hoechst. *Columns* indicate means ($n = 3$); bars ± S.D. *, $p < 0.01$; **, $p < 0.001$.

pearance of the cytosolic Bax counterpart and with a drastic decrease of mitochondrial cytochrome *c* (Fig. 5, *panel A*). Although these events occurred in all the cell lines, the kinetics of translocation in/out the mitochondria seemed to be different as follows: slow in CCRF-CEM and very rapid in CEM-VBL10 and CEM-VBL100. In fact, the mitochondrial amount of Bax almost disappeared in CEM-VBL100 after 8 h of treatment, at which time the extent of apoptotic cells was about 80–90% of the total population. The results obtained so far indicate the activation of the mitochondrial pathway as preferential route to trigger apoptosis upon NBDHEX treatment. To further confirm this issue, we determined by Western blot analyses the levels and the cleavage of both pro-caspase-8 and pro-caspase-9. Fig. 5 (*panel B*) shows that pro-caspase-8 was not affected by NBDHEX treatment because neither change in the expression level nor appearance of cleaved bands (data not shown) was observed. Concomitantly, a rapid and sustained activation of caspase-9 was observed, whereas the inactive pro-form of this enzyme completely disappeared in CEM-VBL10 and CEM-VBL100. This trend was confirmed by Western blot analyses of cleaved caspase-3 (Fig. 5, *panel B*); in fact, although present in all the cell lines examined, the intensity of the immunoreactive band of the caspase-3 active form was inversely related to vinblastine resistance supporting the evidence for high caspase activity measured in the NBDHEX-treated MDR cell lines (Fig. 4, *panel A*).

NBDHEX Triggers JNK Activation—Recently we reported that NBDHEX triggers apoptosis in CCRF-CEM cells by promoting the dissociation of GSTP1-1 from the GSTP1-1-JNK complex (4), which is located in the cytoplasm where NBDHEX was detected (see Fig. 1, *panel A*). To verify if this event occurred also in the MDR cells, we performed a comparative Western blot analysis of cells treated with 2 μM NBDHEX. Densitometric analysis shows a rapid and sustained increase of the phospho-active form of JNK up to 6 h of treatment in all cell lines tested and compared with the untreated control cells (Fig. 5, *panel C*). Actually, the JNK signal transduction pathway appears more efficiently activated in the MDR cell lines; we detected a 4-fold increase in the p-JNK levels after 3 h of incubation with NBDHEX in CCRF-CEM ($p < 0.01$; $n = 4$) and a 6–8-fold increase after 6 h of treatment in both CEM-VBL10 ($p < 0.001$; $n = 3$) and CEM-VBL100 ($p < 0.001$; $n = 3$) when compared with the untreated control cells. The crucial role played by JNK in the activation of the apoptotic signal is also confirmed by experiments carried out in the presence of the specific JNK inhibitor SP600125 (Fig. 6). SP600125 protects both MDR cell lines and CCRF-CEM cells from apoptosis, in which activation of JNK is the early event of the apoptotic pathway triggered by NBDHEX (4).

GST Activity Decreases in P-gp Overexpressing Leukemia Cells—Previous evidence shows an increase of JNK activation in response to pro-apoptotic stimuli in cells with a suppressed GSTP1-1 expression (8, 26). Therefore, we checked to see if the amount of GST in CCRF-CEM varied from that in the MDR cell lines. GST activity was 0.23 ± 0.05 units/mg in CCRF-CEM

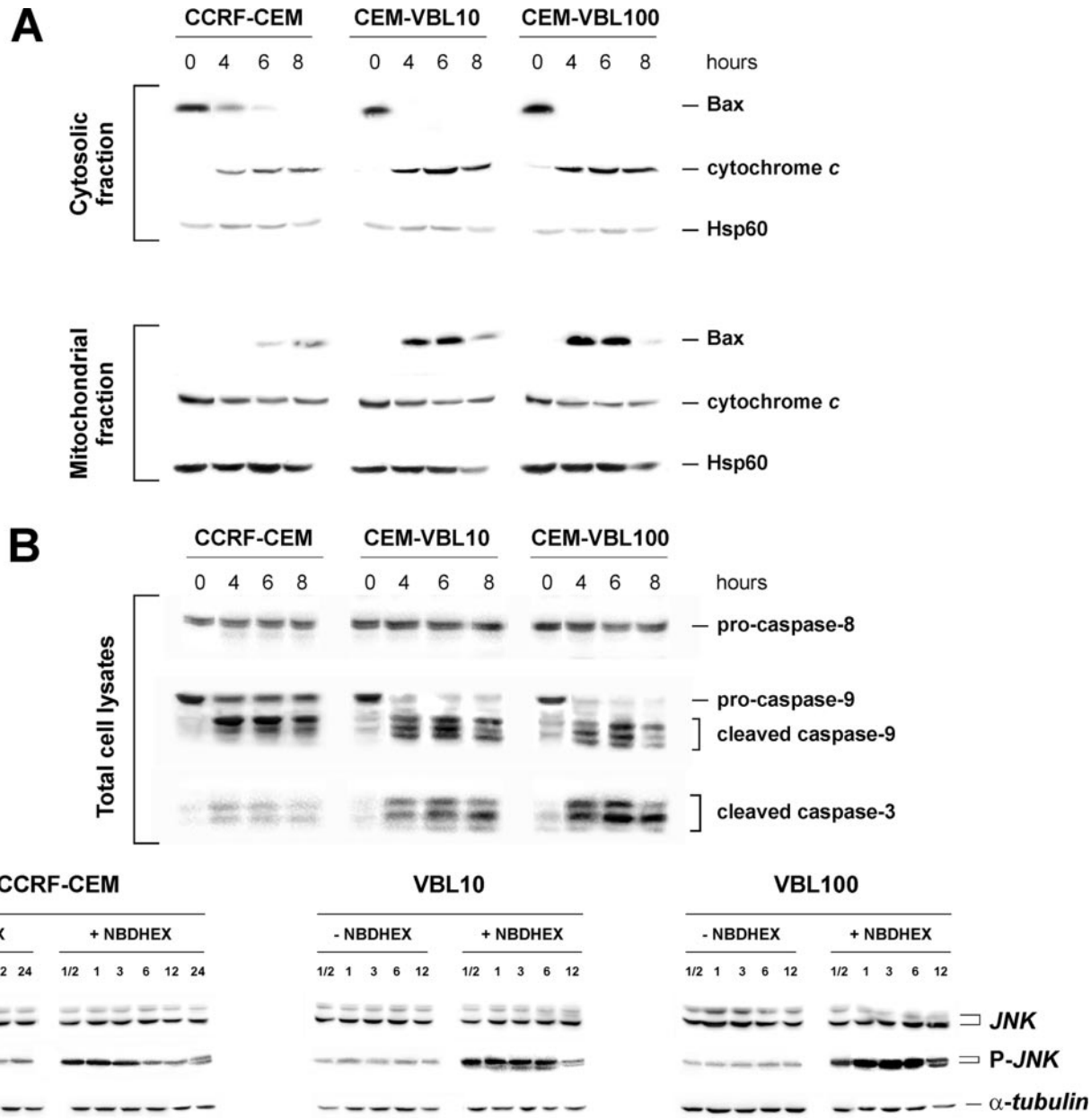

FIGURE 5. **NBDHEX induces apoptosis through mitochondrial pathway and JNK activation.** *Panel A,* Western blot analysis of Bax and cytochrome *c* was performed at the indicated times on cytosolic (20 μg of proteins) and mitochondrial (10 μg of proteins) fractions of CCRF-CEM, CEM-VBL10, and CEM-VBL100 treated with 2 μM NBDHEX. Mitochondrial HSP60 was used as loading and extraction control. *Panel B,* 20 μg of cytosolic proteins of CCRF-CEM, CEM-VBL10, and CEM-VBL100 treated with 2 μM NBDHEX were loaded onto each lane for detection of inactive (pro) and active (cleaved) forms of caspase-3, -8, and -9. *Panel C,* 20 μg of cell extracts from CCRF-CEM, CEM-VBL10, and CEM-VBL100, at different times from the addition of 2 μM NBDHEX, were subjected to immunoblotting analysis to detect JNK and its phospho-activated isoform, p-JNK. β-Actin was used as loading control. p-JNK increased up to 3 h of incubation with NBDHEX in CCRF-CEM ($p < 0.01$; $n = 4$) and up to 6 h in both CEM-VBL10 ($p < 0.001$; $n = 3$) and CEM-VBL100 ($p < 0.001$; $n = 3$) when compared with the untreated control cells.

cells, 0.12 ± 0.03 in CEM-VBL10, and 0.14 ± 0.03 units/mg in CEM-VBL100. Thus, GST activity was found 2-fold decreased in the P-gp overexpressing cell lines compared with the parental cell line ($n = 4$, $p < 0.01$ and $p < 0.05$ for CEM-VBL10 and CEM-VBL100, respectively). Overall, the present data support the idea that the decreased amount of GSTP1-1 measured in the P-gp overexpressing cells may account for the higher levels of the phospho-active form of JNK and for the greater susceptibility toward the NBDHEX action.

Concluding Remarks—In this work we investigated the interaction of NBDHEX with tumor cell lines that developed a multidrug-resistant phenotype by overexpressing P-gp. This protein, besides its drug efflux activity, is known to protect tumor cells against a range of different stimuli that function by activating the caspase pathway. Notably, NBDHEX is able to overcome these drug-resistant mechanisms inducing cell death at submicromolar concentrations. NBDHEX kills MDR tumor cells following two independent strategies. First, NBDHEX is not recognized as a substrate by the P-gp export pump, and it accumulates inside the cells. Second, NBDHEX activates in the P-gp overexpressing cells a caspase-dependent apoptotic pathway as observed in the parental cell line. These data are very

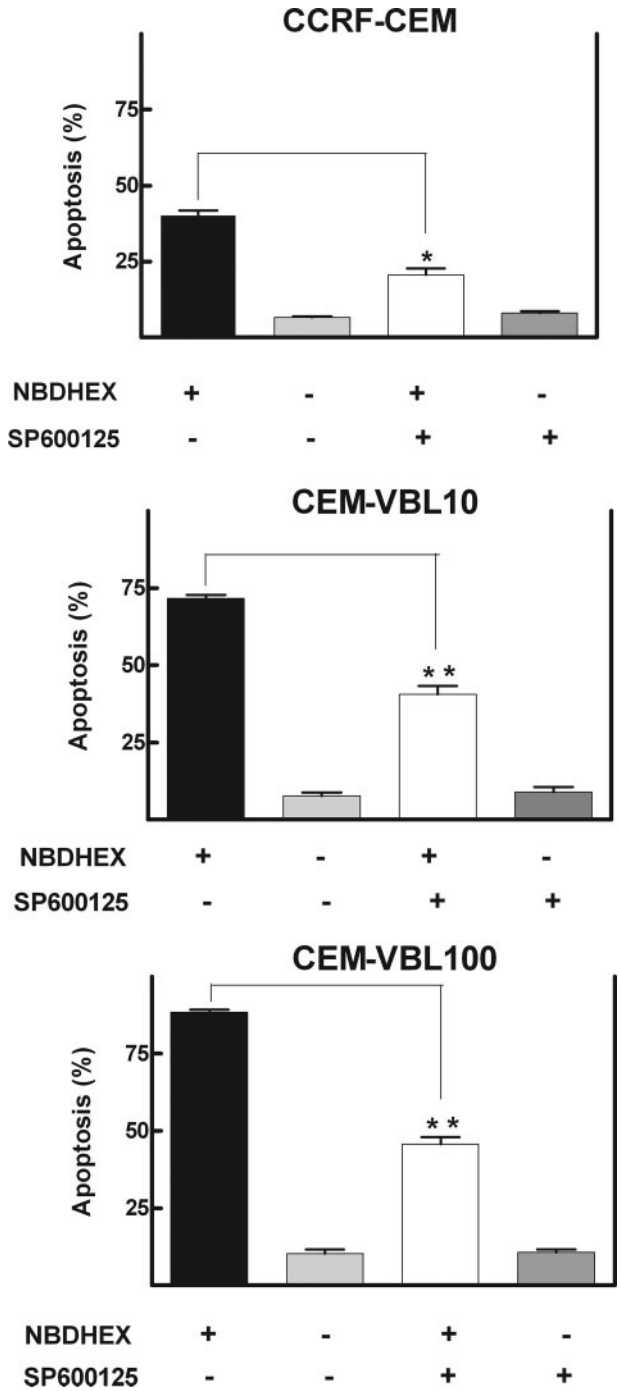

FIGURE 6. **Effect of JNK inhibitor on the NBDHEX-induced apoptosis.** CCRF-CEM, CEM-VBL10, and CEM-VBL100 cell lines were preincubated with 20 μM JNK inhibitor SP600125 before the addition of 2 μM NBDHEX and maintained throughout the experiment. The percentage of apoptosis was determined after 9 h of treatment by fluorescence microscopy after nuclear staining with Hoechst. *Columns*, means ($n = 3$); *bars* ± S.D. *, $p < 0.01$; **, $p < 0.001$.

peculiar because most of the few drugs that circumvent the P-gp system, such as suberoylanilide hydroxamic acid, staurosporine, curcumin, and hexamethylene bisacetamide, induce a caspase-independent apoptosis (22–25). Considering that the mitogen-activated protein kinases-mediated pathways are preferentially activated in transformed cells and that NBDHEX escapes recognition by the P-gp protein pump, this drug may represent an attractive basis for the selective treatment of tumors.

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