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SHORT COMMUNICATION

The glutathione *S*-transferase inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol overcomes the MDR1-P-glycoprotein and MRP1-mediated multidrug resistance in acute myeloid leukemia cells

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Abstract
Purpose There has been an ever growing interest in the search for new anti-tumor compounds that do not interact with MDR1-Pgp and MRP1 drug transporters and so circumvent the effect of these proteins conferring multidrug resistance (MDR) and poor prognosis in AML patients. We have investigated the cytotoxic activity of the strong glutathione *S*-transferase (GST) inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) on AML (HL60) cell lines.
Methods Functional drug efflux studies and cell proliferation assays were performed on both sensitive and MDR AML (HL60) cells after incubation with NBDHEX. Moreover, the mode of cell death (apoptosis vs. necrosis) as well as the correlation between NBDHEX susceptibility and GST activity or Bcl-2 expression was investigated.
Results NBDHEX is not a substrate of either MDR1-Pgp or MRP1 efflux pumps; in fact, it is not only cytotoxic toward the parental HL60 cell line, but also overcomes the MDR phenotype of its HL60/DNR and HL60/ADR variants.
Conclusions The data herein reported show that NBDHEX mediates efficient killing of both MDR1-Pgp and MRP1 over-expressing AML cells. Therefore, this drug can potentially be used as an effective agent for treating MDR in AML patients.

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Introduction

The major problem in the treatment of patients with AML is the occurrence of resistance to structurally and functionally unrelated chemotherapeutic agents, called multidrug resistance (MDR) [1]. One of the known MDR mechanisms is the overexpression of the ATP-binding cassette (ABC) efflux pumps [2]. MDR1-P-glycoprotein (MDR1-Pgp), the best characterized human drug efflux pump, has been associated with poor treatment outcome in AML patients [3]. Besides MDR1-Pgp, the multidrug resistance protein 1 (MRP1) also contributes to the observed resistance in AML [3]. Alternative transporter proteins, which include MRP1 homologues and the breast cancer resistance protein (BCRP), have been shown to be expressed at variable levels in AML patient cells, although their relative roles in conferring the MDR phenotype in AML remain undefined [4, 5].

As most anticancer agents (anthracyclines and epipodophyllotoxins) subject to MDR efflux are currently irreplaceable in AML chemotherapy regimens, an attractive solution for improving response to therapy can be the development of new classes of anti-AML agents that do not interact with the multidrug ABC transporters. We have previously shown the antitumoral properties of the nitrobenzofurazane derivative 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), which is a strong inhibitor of the glutathione *S*-transferase (GST) family [6]. GST catalyze the conjugation with GSH of many anticancer drugs that can be efficiently removed from the cell by specific export pumps [7]. Moreover, GST (in particular the isoenzyme GSTP1-1)

forms a complex with JNK in non-stressed cells, so inhibiting apoptosis mediated by this MAPK. We have reported that NBDHEX promotes the dissociation of GSTP1-1 from the GSTP1-1–JNK complex resulting in cell death by apoptosis [8]. Recently, we have also shown that NBDHEX overcomes the MDR phenotype, due to its effectiveness against different drug resistant tumor cell lines [9–11]. Therefore, in this study, we investigated the possible occurrence of a NBDHEX-mediated cytotoxicity in the AML HL-60 cell line and in its MDR variants overexpressing either the MDR1-Pgp or the MRP1 ABC drug-efflux system. Our data show that NBDHEX is not a substrate of these export pumps. In this way, the parental drug-sensitive HL-60 cell line as well as its resistant variants is efficiently committed to death by this molecule. These findings indicate that NBDHEX may be a novel compound highly effective at killing AML cells even in the presence of ABC transporters conferring the MDR phenotype.

Materials and methods

Cells and MDR phenotype

Human HL-60 cell line and its MDR variants were kindly provided by Dr. Tang [12]. HL-60 cells were selected for either MDR1-Pgp (HL60/DNR) or MRP1 (HL60/ADR)-mediated drug resistance by a progressive adaptation to increasing concentrations of adriamycin or daunorubicin. Cells were grown in basic medium (BM) consisting of RPMI 1640 medium (Hyclone, Logan, Utah) supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 U/mL). HL60 MDR cells were maintained by alternate feedings with drug-free medium or medium containing drugs as previously reported [12]. The stability of the MDR phenotype was periodically assessed by evaluating MDR1-Pgp and MRP1 expression using specific mAbs MM4.17 [13] and MRPm6 (VinciBiochem, Florence, Italy) according to previously described procedures [13, 14]. The level of mAbs binding to cells was determined by flow-cytometry analysis (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

Flow cytometric detection of functional drug efflux

The cells (1×10^6) were incubated for 1 h at 37°C in the presence of 8.5 μM doxorubicin (Ebewe, Rome, Italy) or 10 μM NBDHEX (synthesized as previously reported [6]) in the absence or presence of MDR inhibitors: 5 μM Verapamil (Abbott-Knoll Pharmaceuticals, Latina, Italy) was used to inhibit MDR1-Pgp drug efflux, while 50 μM MK571 (VinciBiochem) was used as specific inhibitor of MRP1 function. Cells were then incubated for 1 h in a drug-free medium with or without MDR inhibitors to enable efflux or efflux-blocking to occur. Efflux was stopped by pelleting the cells and adding ice-cold medium. Taking advantage from the intrinsic fluorescence of doxorubicin and NBDHEX, the cellular drug content was determined by a FACSCalibur (Becton Dickinson) equipped with an argon laser source.

Cell proliferation assay

An evaluation of cell viability at different drug concentrations was determined by PreMix WST-1 kit (Vinci Biochem) according to the manufacturer's instructions. The cells were placed in 96-well microtiter plates (200 μL/well) at a density of 4×10^5 cells/mL in BM containing scalar concentrations of NBDHEX (from 0 to 50 μM) for 24 h at 37°C and 5% CO_2. After the incubation period, 20 μL/well of PreMix WST-1 reagent was added and the cells incubated for a further 1–4 h in a humidified atmosphere (37°C, 5% CO_2). Finally the absorbance of the samples against a background control as blank was measured using a microtiter plate ELISA reader (Model 680, Biorad, Hercules, CA) (450 nm).The dose-response profile obtained fulfills the LC_{50} value (the drug concentration used to obtain 50% cellular mortality) for each cell line assayed.

Determination of apoptosis

Percentages of apoptosis and necrosis were obtained by Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (MBL international, Woburn, MA) according to the manufacturer's instructions. Briefly, the cells were seeded in 24 well plates (4×10^5 cells/mL) in BM with different concentrations of NBDHEX (from 0 and 10 μM) for different times. Afterward, the cells were washed in PBS and resuspended in binding buffer containing 0.5 μg/mL of Annexin V-FITC and 2 μg/mL propidium iodide (PI). The simultaneous application of PI as a DNA stain is required to discriminate necrotic from apoptotic cells. The samples were incubated at room temperature (RT) for 15 min in the dark before analysis of the stained cells by FACSCalibur (Becton Dickinson).

GST activity and Bcl2-expression

Glutathione *S*-transferase activity was measured at 25°C as described previously [8]. The standard assay mixture contained 1 mM GSH, 1 mM CDNB and 0.1 mM EDTA in 1 mL (final volume) of 0.1 M potassium phosphate buffer, pH 6.5. The activity was determined spectrophotometrically at 340 nm (where the product of reaction absorbs $\varepsilon = 9{,}600\ M^{-1}\ cm^{-1}$) within 10 s from addition of the substrates. Protein concentration was determined by the

bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). For Bcl-2 expression at each time point analyzed, lysates of untreated control cells and cells treated with 5 μM NBDHEX were runned for Western blot analyses as described previously [10]. Thirty micrograms of proteins were loaded on 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). Anti-Bcl-2 mAb (R&D System, Minneapolis, MN) was used at 0.1 μg/mL (4°C, overnight) and anti-β-actin (Sigma-Aldrich, St. Louis, MO) was used at 0.2 μg/mL (RT, 1 h). Specific binding was detected by incubation with an horseradish peroxidase-conjugated anti-mouse Ig (Dako, Glostrup, Denmark, 0.2 μg/mL at RT 1 h). Membranes were visualized by enhanced chemiluminescence kit (Pierce).

Data presentation

All the experiments were repeated at least thrice. The significance was assessed by Student's *t* test. The criterion for statistical significance used was $P < 0.05$.

Results and discussion

HL60 cell lines characterization

The parental HL60 cell line and its MDR variants were tested for ABC transporter expression using mAbs MM4.17 and MRP1m6 specifically recognizing external and cytoplasmic domains of MDR1-Pgp and MRP1 (Fig. 1a). We found that the parental drug-sensitive HL60 cells are completely negative for MDR1-Pgp, while a small fraction of cells (from 10 to 15%) proved to be MRP1 positive. In addition, the MDR cell variant named HL60/ADR showed a non-homogenous over-expression of MRP1, suggesting a differential induction of MRP1 gene by drug selection procedure of the parental HL60 cells. Finally, we found that the HL60/DNR cells, obtained using a highly stringent drug selection procedure, are characterized by a very high level of MDR1-Pgp and the absence of MRP1 expression.

MDR1-Pgp and MRP1 did not affect the NBDHEX efflux

To clarify if NBDHEX efflux could be mediated by MDR1-Pgp or MRP1, we performed efflux experiments in the presence of Verapamil or MK-571. These drugs are known to inhibit the MDR1-Pgp (Verapamil) and MRP1 (MK-571) export pumps so inducing intracellular accumulation of MDR substrates such as doxorubicin. As expected, Verapamil and MK-571 inhibited doxorubicin efflux in HL60/DNR and HL-60/ADR, respectively, although they did not affect the intracellular concentration of doxorubicin in the sensitive variant HL60 cells (Fig. 1b). Conversely, Verapamil and MK-571 did not affect the intracellular concentration of NBDHEX in parental HL60 cell line as well as in its MDR variants HL60/DNR and HL60/ADR. These findings are consistent with the fact that NBDHEX is not a substrate of the MDR1-Pgp or MRP1 export pumps.

NBDHEX induces a dose- dependent apoptosis and necrosis in AML HL60 cell lines

The NBDHEX cytotoxic activity has been determined by seeding 4×10^5 cells/mL in the presence of drug concentration outlined in Fig. 1c. Under these experimental conditions, the dose-response profiles, obtained after 24 h, revealed a good cytotoxic activity in the parental HL60 cells with an LC_{50} value of 2.9 ± 0.3 μM. Interestingly, a similar result was obtained with the multidrug resistant variants; in fact, LC_{50} values of 2.6 ± 0.2 μM and 3.4 ± 0.6 μM were calculated for HL60/DNR and HL60/ADR, respectively. The statistical analysis indicates that there are no significant differences between the MDR HL60 variants and the parental HL60 cell line in regard to their NBDHEX susceptibility. It has been shown that NBDHEX triggers a caspase-dependent apoptosis in several tumor cell lines [8–11], while death by necrosis was observed in one cell line (H69) overexpressing Bcl-2 [10]. Therefore, Annexin V-FITC and PI staining was used to characterize the type of cell death occurring in both sensitive and MDR HL60 cell lines cultured in the presence of different concentrations of the drug. Figure 2a reports the percentage of cells undergoing apoptosis or necrosis after 24 h incubation with different concentration of NBDHEX. A clear dose-dependent effect was observed in both the HL60 cells and the MDR variants (HL60/DNR and HL60/ADR). However, the parental cell line and its MDR variant over-expressing MDR1-Pgp (HL60/DNR) seem to have an higher propensity to die through an apoptotic pathway, in contrast to the HL60/ADR cells that mainly showed a necrotic phenotype. A drug concentration higher than 5 μM caused an increase of cell death by apoptosis also in the HL60/ADR cells; however, the percent of apoptosis was negligible compared to that observed in HL60 and HL60/DNR cell lines. The distribution of Annexin V +/PI+ or Annexin V−/PI+ necrotic cells sub-fractions suggested that the necrosis recorded for HL60 and HL60/DNR is essentially a secondary necrosis following death by apoptosis (data not shown). Time course analysis of cell death corroborates these observations. In fact, apoptosis induced by 5 μM NBDHEX occurred within 4 h and continued to increase throughout the incubation period reaching a peak at 8 h in both HL60 and HL60/DNR cells. By contrast, in the MRP1 expressing HL60/ADR cells, apoptosis became clearly visible after 24 h of treatment (Fig. 2b).

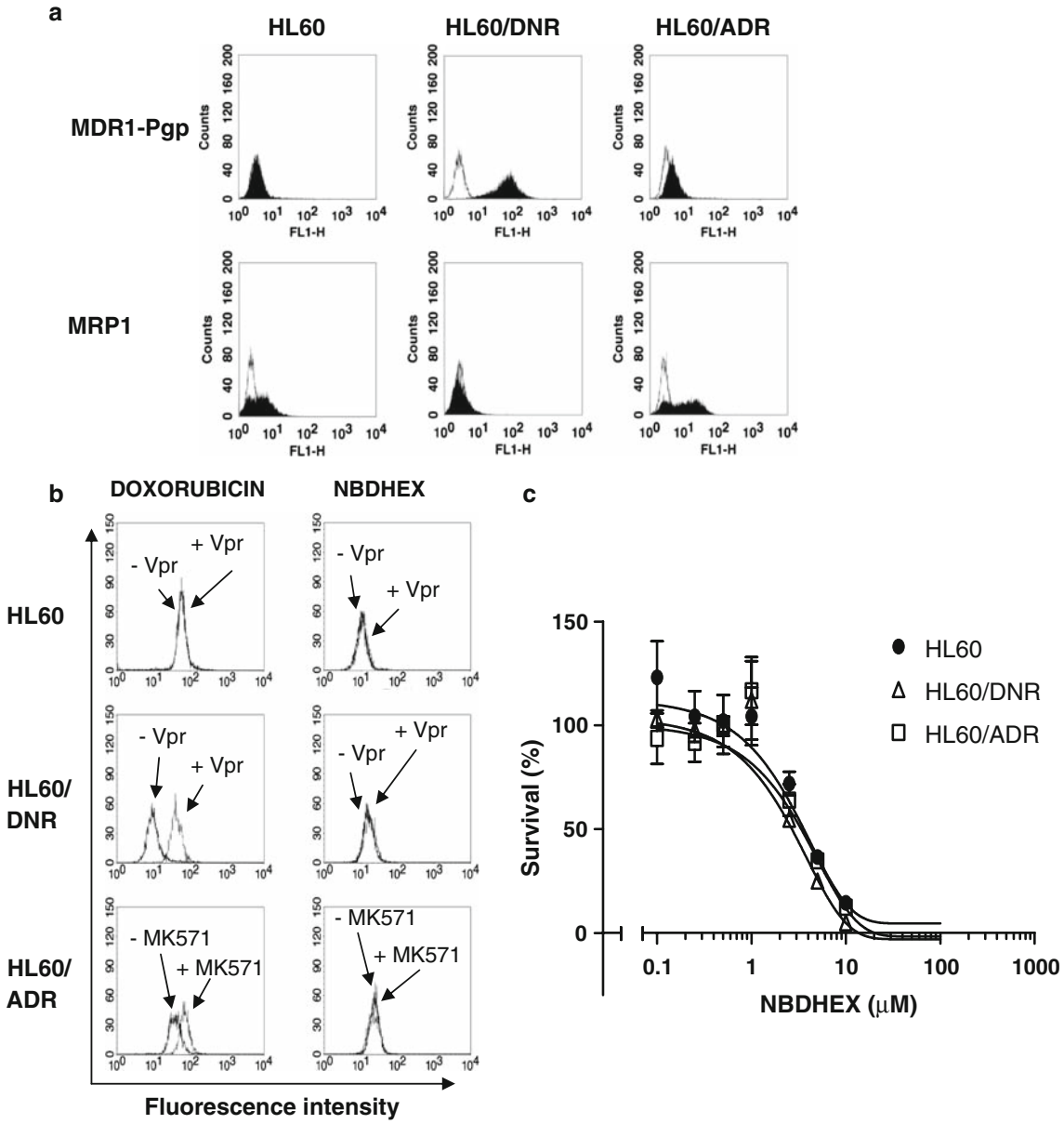

Fig. 1 NBDHEX is not a substrate for MDR1-Pgp and MRP1 export pumps and show cytotoxic activity toward both parental HL60 and its MDR variants HL60/ADR and HL60/DNR cell lines. **a** MDR1-Pgp and MRP1 expression were evaluated by flow cytometric analyses of HL60 cells incubated with either MM4.17 or MRPm6 mAbs specific for MDR1-Pgp and MRP1, respectively. **b** The efflux of NBDHEX and doxorubicin were determined in the presence of the MDR1-Pgp and MRP1 inhibitors, Verapamil (Vpr) and MK-571. **c** Cell survival was determined by WST-1 assay after 24 h treatment with NBDHEX at the indicated concentrations. The dose-response profiles gave similar values for HL60/DNR ($LC_{50} = 2.6 \pm 0.2$ μM) and HL60/ADR (LC_{50} 3.4 ± 0.6 μM) cell lines which are also comparable to that found in the parental HL60 ($LC_{50} = 2.9 \pm 0.3$ μM) cell line. Points, mean of three independent experiments, each done in triplicate; *bars*, SD

GST activity and Bcl-2 content in AML

In the attempt to explain the different cell death induced by NBDHEX in HL60/ADR cells, we determined the amount of two proteins that are frequently over-expressed in drug resistant tumors and are known to antagonize apoptosis, i.e. GST and Bcl-2. In HL60/ADR, the GST activity was significantly higher than that found in both the parental HL60 and the MDR1-Pgp overexpressing resistant variant, suggesting a possible link between the GST level and the degree of necrosis found in HL60 cell lines (Fig. 2d). However, a very impressive difference was found in the concentration of the antiapoptotic protein Bcl-2. In fact, Western blot analysis (Fig. 2c) showed an elevated expression of Bcl-2 in the HL60/ADR cells, while Bcl-2 was almost undetectable in the parental drug sensitive HL60 cells and in its MDR1-Pgp overexpressing variant. In addition, under our experimental conditions, the expression of Bcl-2

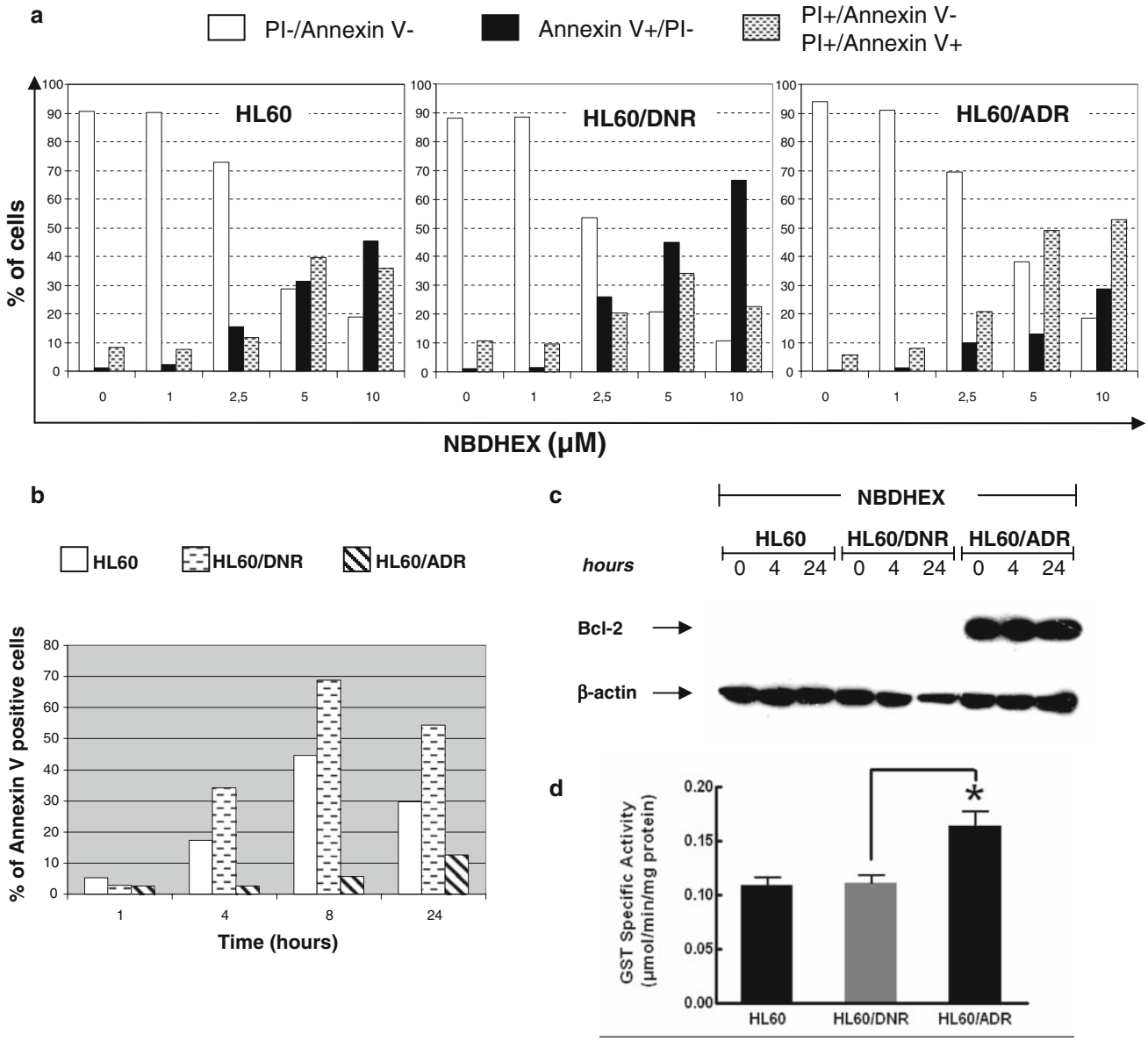

Fig. 2 Cell death characterization and anti-apoptotic proteins determination. **a** Cell death was determined in HL-60 cells after 24 h of incubation with several amounts of NBDHEX (from 0 to 10 μM). The histograms represent the frequency of living (PI−/Annexin V−), apoptotic (Annexin V+/PI−) and necrotic (PI+/Annexin **V**− and PI+/Annexin V+) HL60 cells. Results are expressed as a percentage of total cells and are representative of three independent experiments that gave similar results. **b** Time course of apoptosis (determined by Annexin V-FITC staining) triggered by 5 μM NBDHEX in HL-60 cells. Results are representative of three independent experiments that gave similar results. **c** The expression of Bcl-2 before and after treatment with NBDHEX (5 μM) was examined in HL60 cells by immunoblot analysis. At the indicated times, total protein extracts (30 μg) from untreated and NBDHEX-treated cells were subjected to Western blot analysis. *β*-actin was used as loading control. **d** The specific activity of GST was determined in the cell extracts as reported in "Material and methods" $^{*}P < 0.05$

remained constant in HL60/ADR cells up to 24 h of treatment with NBDHEX (Fig. 2c). Then, it can be hypothesized that the high down regulation of this anti-apoptotic protein in HL60 and HL60/DNR cells may strongly contribute to their major propensity to die by apoptosis. These results match with previously published data showing a predominant necrotic phenotype, after NBDHEX treatment, in cells with high levels of Bcl-2 [10].

Concluding remarks

Our findings confirm and extend the NBDHEX potential in the treatment of tumors and particularly of AML, which are often characterized by high levels of MDR1-Pgp or MRP1 in elderly or relapsed patients. The mechanism of cell death triggered by NBDHEX has been previously described [9, 10]. This compound is an efficient inhibitor of GST [6], a

family of enzymes also involved in the cancer drug resistance phenomenon [15, 16]. Until now, traditional GST inhibitors have been mainly used to prevent the multidrug resistance phenomenon in patients undergoing treatment with other chemotherapeutic agents. NBDHEX, besides its possible utilization in association with other chemotherapeutic agents, is itself toxic for AML cells. The resulting necrosis observed in HL60/ADR cells after NBDHEX treatment, in spite of its adverse effects, could be balanced by the therapeutic benefits represented by the death of cells overexpressing cellular factors, such as Bcl-2 and MRP1, that counteract chemotherapy and cause an extremely poor prognosis.

All these observations together with the low toxicity of NBDHEX previously described in in vivo studies [8] indicate that this drug may be a novel and highly effective compound in selectively killing AML cells and their MDR variants.

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