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ABT-737 accelerates butyrate-induced death of HL-60 cells. Involvement of mitochondrial apoptosis pathway

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Abstract. The aim of presented study was to determine effect of NaBu in combination with ABT-737 on cell survival of leukemic cell line HL-60. In addition, analysis of molecular mechanism of NaBu action with a focus on mitochondrial apoptosis was performed. Both NaBu and ABT-737 are inducing death of HL-60 cells with different kinetics. ABT-737-induced cell death is fast while NaBu-induced death preceded by cell cycle arrest in G2 phase is rather slow. Cell viability was significantly decreased after 48 hours of incubation with 2 and 5 mmol/l of NaBu while it was significantly decreased after 24 hours of incubation with 1 μmol/l of ABT-737 combined with 2 and 5 mmol/l of NaBu. Incubation of HL-60 cells with NaBu was associated with increased level of pro-apoptotic protein BIM_{EL} and decreased levels of anti-apoptotic proteins of Bcl-2 family as well as GRP78 involved in ER stress signalling. It seems that ABT-737 accelerates NaBu-induced death of HL-60 cells due to mitochondrial apoptosis resulting from ABT-737-mediated inhibition of functions and NaBu-induced decrease of the levels of anti-apoptotic Bcl-2 family proteins as well as due to accelerated decrease of GRP78 observed after the treatment of cells with combination of NaBu and ABT-737. The effect of combination of both drugs on survival of HL-60 cells seems to be synergistic at high concentrations of NaBu (2 and 5 mmol/) while it is rather antagonistic at concentrations of NaBu less than 1 mmol/l. Finally, it might be assumed that NaBu is capable to induce cell death with mechanisms independent from mitochondrial apoptosis.

Key words: HL-60 — Sodium butyrate — ABT-737 — Bcl-2 family proteins — GRP78

Introduction

Butyrate that is naturally produced in the human colon by the bacterial fermentation of dietary fibres is a non-toxic short-chain fatty acid with a broad physiologic impact (Fauser et al. 2011). Besides its ability to serve as an energetic substrate for some cells, it can inhibit cell growth, by cell cycle arrest either in G1 or G2 phase, and promote differentiation in several normal and tumour cells (Pajak et al. 2007). These effects were explained by evidence that butyrate acts as an inhibitor of histone deacetylases class I and IIa (Bolden et al. 2006; Carew et al. 2008), thereby inducing histone hyperacetylation, chromatin relaxation and changes in the expression of several different regulatory proteins (Carew et al. 2008; Marks 2010). In particular, it has been documented that butyrate can induce cell-cycle arrest by increasing the expression of $p21^{WAF/CIP1}$ (Rosato et al. 2002) and $p27^{Kip1}$ (Litvak et al. 1998). In addition, butyrate-dependent induction of retinoblastoma (Rb) protein hypophosphorylation was included as p21-independent mechanism of butyrate-induced cell cycle arrest in G1 phase (Vaziri et al. 1998). Apart from the effects on the cell cycle and differentiation, butyrate can also induce cell death in many cancer cell lines (Pajak et al. 2007), including leukaemic HL-60 cells (Calabresse et al. 1993; Filippovich et al. 1994; Zimra et al. 1997; Rosato et al. 2003). It has been suggested that a cell death effect of butyrate related to the initiation of p53-independent mitochondrial apoptosis correlates with down-regulation of anti-apoptotic proteins of Bcl-2 family and up-regulation of pro-apoptotic proteins of Bcl-2 family (Bolden et al. 2006; Carew et al. 2008; Marks 2010) as well as activation of caspase 9 and caspase 3 (Shao et al. 2004; Vrba et al. 2010). In addition to mitochondrial apoptosis, butyrate can induce another types of cell death namely autophagy (Shao et al. 2004; Tang et al. 2011).

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ABT-737 is a synthetic small BH3-only mimetic molecule that has the capacity to bind to the hydrophobic clefts of Bcl-2, Bcl-X_L, and Bcl-w (Oltersdorf et al. 2005). Binding of ABT-737 to Bcl-2, Bcl-X_L, and Bcl-w disrupts their anti-apoptotic functions and is associated with death of various tumour cells whereas normal cells exhibit minimal sensitivity to ABT-737 (Chauhan et al. 2007). With respect to leukaemia, ABT-737 effectively kills leukaemic blasts, progenitor and stem cells without affecting normal hematopoietic cells (Konopleva et al. 2006). Since ABT-737 effectively binds to the Bcl-2 and Bcl-X_L but exhibits minimal affinity to Mcl-1 (Oltersdorf et al. 2005), sensitivity of tumour cells to ABT-737 is significantly determined by the relative expression levels of Bcl-2/ Bcl-X_L *versus* Mcl-1 (Konopleva et al. 2006; van Delft et al. 2006). In hand with this, down-regulation of Mcl-1 expression dramatically enhances ABT-737 cytotoxicity (Chen et al. 2007; Lin et al. 2007). On the other hand, ABT-737 displaces BIM from the BH3-binding pocket of Bcl-2 that is associated with BIM-mediated activation of Bax and consequent initiation of mitochondrial apoptosis (Del Gazo Moore et al. 2007). It seems that the extent of Bcl-2 bound to Bim, rather than total Bcl-2 expression levels, may determine cellular sensitivity to ABT-737 (Deng et al. 2007). In hand with this, ABT-737 has been shown to interact with certain anticancer agents capable of up-regulating BIM, (Kuroda et al. 2006; Zhang et al. 2008) including histone deacetylase inhibitors (HDACi) (Chen et al. 2009). However, ABT-737 could synergize with HDACi *in vitro* to kill lymphoma cells over-expressing Bcl-2 and Bcl-X_L (Whitecross et al. 2009). In addition to mitochondrial apoptosis initiation, it seems that ABT-737 may exhibit additional cellular activities like induction of cell-cycle arrest and consequent senescence (Song et al. 2011) or multiple pathways culminating in autophagy (Malik et al. 2011).

The aim of presented study was to determine effect of sodium butyrate (NaBu) in combination with ABT-737 on survival of leukaemic cell line HL-60 with a focus on kinetics of cellular action of both drugs. In addition, analysis of NaBu impact on the level of selected proteins of Bcl-2 family was performed in time- and concentration-dependent manner.

Materials and Methods

Sodium butyrate, propiodium iodide, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecylsulphate (SDS) (all from Sigma Aldrich), ABT-737 (Abbott Laboratories). Rabbit polyclonal antibodies raised against Bax (Santa Cruz Biotechnology, SC-439), Bcl-X_L (Santa Cruz Biotechnology, SC-7195), BIM (Santa Cruz Biotechnology, SC-11425), γ-H2AX (Santa Cruz Biotechnology, SC-101696) and Mcl-1 (Santa Cruz Biotechnology, SC-20679). Mouse monoclonal antibodies raised against Bcl-2 (Santa Cruz Biotechnology, SC-7382), LC3β (Santa Cruz Biotechnology, SC-271625) and β-actin (Santa Cruz Biotechnology, SC-47778). Goat polyclonal antibody raised against GRP78 (Santa Cruz Biotechnology, SC-1051). Mouse anti-goat (SC-2354), mouse anti-rabbit (SC-2357) and goat anti-mouse (SC-2005) secondary antibodies conjugated with horse radish peroxidase.

Cell culture

HL-60 cells (ATCC) were routinely maintained in IMDM medium supplemented with 20% fetal bovine serum, 1% penicillin-streptomycin (all PAA) at an optimal cell density of 0.5×10^6 cells/ml at 37°C and 5% CO_2 humidified atmosphere. The medium was changed every 3 days.

Cell viability assay

HL-60 cells were incubated 24–72 h with or without various concentrations of NaBu and/or ABT-737. Cells were seeded in 96-well plates at a concentration of 0.5×10^6 *per* ml. At the end of incubation the absorbance of formazan, which results from oxidation of added MTT by vital cells, was determined spectrophotometrically using microplate reader BioRad 2010. The relative viability of the cells was determined as ratio of optical density of formazan produced by treated cells to optical density of formazan produced by non-treated control cells and expressed as percent of control. The cell growth was determined as time course of optical density of formazan produced by particular cell population.

Cell cycle analysis

After treatment of HL-60 cells with 1, 2 and 5 mmol/l NaBu and/or 1 μmol/l ABT-737 for 24–72 h, the cells (1×10^6) were fixed in 70% ethanol for 1 hour at 4°C and washed in PBS with 2% FBS and 0.01% NaN_3. The cells were incubated with 75 μl 1 mg/ml RNase A for 30 min at 37°C, then 20 μg/ml of propidium iodide was added and incubated 30 min at RT in the dark. Cells were analyzed using MACSQuant flow cytometer (Miltenyi Biotec).

Western blotting

Isolation of proteins was performed by extraction with TriReagent (Invitrogen) according to manufacturer's instructions. Extracted proteins were separated on 12% SDS-polyacrylamide gels under reducing conditions with 30 μg of protein loaded *per* lane, transferred to nitrocellulose membrane *via* semidry transfer and probed with antibodies specific to Bcl-X_L, Bcl-2, Bax, BIM, Mcl-1, GRP78, LC3β, γ-H2AX and β-actin (Santa Cruz Biotechnology). After incubation of membranes with particular secondary antibodies, im-

munopositive bands were visualized using the chemiluminiscent substrate SuperSignal West Pico (Thermo Scientific) and Chemidoc XRS system (BioRad). Specific bands were documented by Quantity One software (BioRad).

Combined drug effect analysis

The combination effect and potential synergy were evaluated from quantitative analysis of dose-effect relationships, as described (Abrams et al. 2010) and a combination index was calculated using the CalcuSyn software (Biosoft, Cambridge, UK). This method of analysis generally defines CI values of 0.9 to 1.1 as additive, 0.3 to 0.9 as synergistic, and <0.3 as strongly synergistic, whereas values >1.1 are considered antagonistic.

Statistical analysis

All statistical analyses were done using GraphPad InStat V2.04a (GraphPad Software). The unpaired Tukey's test was used to determine differences between viability of control and treated cells. Significance level was set at $p < 0.05$.

Results

Incubation of HL-60 cells with different concentrations of NaBu for 24, 48 and 72 hours has revealed time- and concentration-dependent impact of NaBu on relative viability of HL-60 cells (Fig. 1A). NaBu in concentrations 2 and 5 mmol/l led to the significant decrease of relative viability of HL-60 cells which was dominant after 48 hours of incubation. The estimated lethal concentrations of NaBu leading effectively to decrease of HL-60 cell population to 50% of control (LC_{50}) were 1.75 ± 0.18 and 1.25 ± 0.14 mmol/l after 48 and 72 hours, respectively. On the other hand, the impact of ABT-737 on viability of HL-60 cells is faster and significant decrease of relative cell viability was already observed after 24 hours of incubation (Fig. 1B) at concentrations of ABT-737 more than 1 μmol/l. The LC_{50} values for ABT-737 were estimated to be 2.24 ± 0.08, 1.93 ± 0.04, and 1.45 ± 0.03 μmol/l after 24, 48 and 72 hours, respectively.

In order to test effect of NaBu in combination with ABT-737 two fixed concentrations of ABT-737 (0.1 and 1 μmol/l) were used whereas concentrations of NaBu were varied in complete investigated range. ABT-737 in concentration 0.1 μmol/l decreased relative viability of HL-60 cells to 88.8 ± 5.9% ($p > 0.05$), 95.8 ± 3.3 % ($p > 0.05$) and 79.3 ± 2.7% ($p < 0.05$) after 24, 48 and 72 hours, respectively. However, we did not observe significant difference in the effect of NaBu on relative viability of HL-60 cells incubated in the absence or presence of 0.1 μmol/l ABT-737 (Fig. 2A, 2B and 2C). ABT-737 in concentration 1 μmol/l decreased relative viability of HL-60 cells to 76.9 ± 7.5 % ($p < 0.05$), 72.3 ± 3.6% ($p < 0.01$) and 60.9 ± 2.2% ($p < 0.001$) after 24, 48 and 72 hours, respectively. After 24 hours of incubation, significant difference in the effect of NaBu on relative viability of HL-60 cells was observed between cells incubated with NaBu only and cells incubated with NaBu in combination with 1 μmol/l ABT-737 (Fig. 2A). At this time, the relative viability of HL-60 cells in the presence of 1 μmol/l of ABT-737 was significantly decreased at concentrations of NaBu 2 and 5 mmol/l as compared to the relative viability of HL-60 cells incubated with NaBu only (43.7 ± 6.1% *versus* 87 ± 3.9% and 36 ± 4.6% *versus* 76.7 ± 1.2%, respectively). After 48 and 72

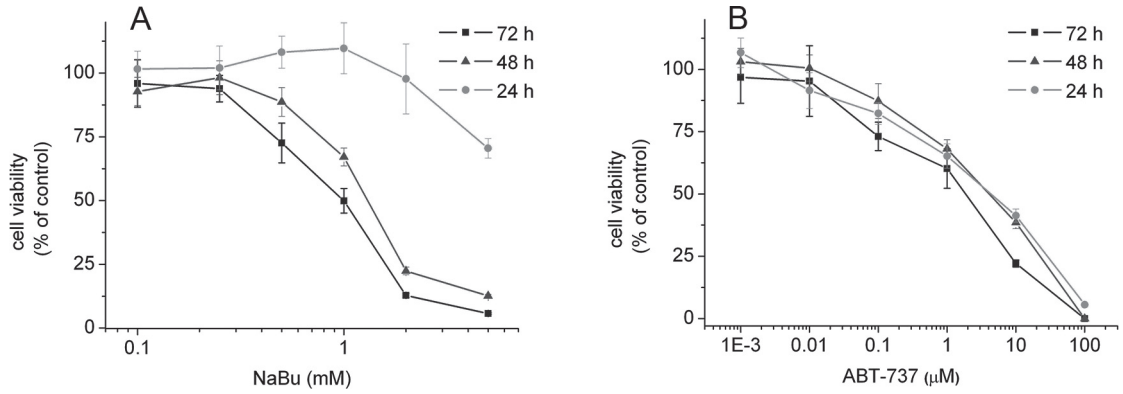

Figure 1. Effect of NaBu (**A**) and ABT-737 (**B**) on relative viability of HL-60 cells. HL-60 cells were incubated for 72 hours in the presence of various concentrations of either NaBu (0.1–5 mmol/l) or ABT-737 (0.001–100 μmol/l) and relative cell viability was determined by the MTT assay. Representative curve of three independent experiments results from one experiment performed in triplicate.

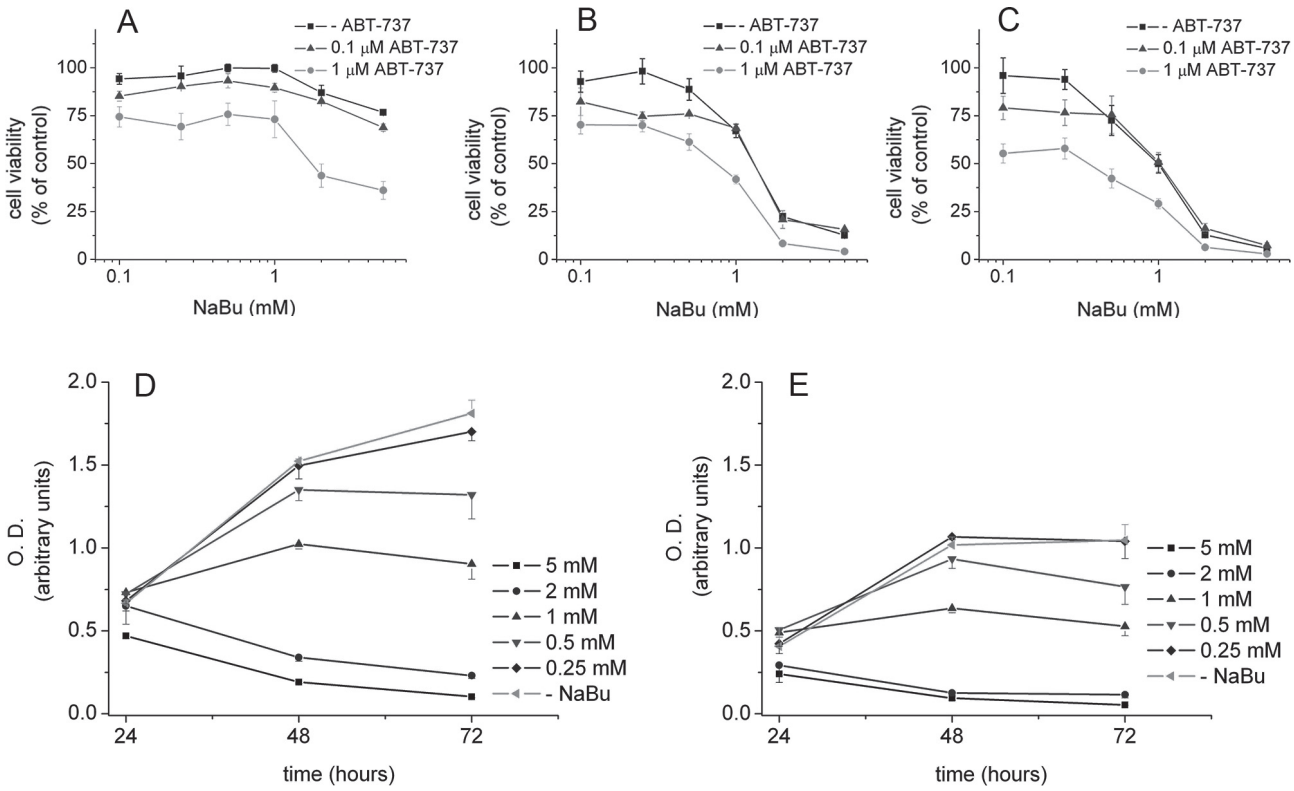

Figure 2. Effect of NaBu alone and in combination with of ABT-737 on relative viability of and growth of HL-60 cells. HL-60 cells were incubated for 24 (**A**), 48 (**B**) and 72 (**C**) hours in the presence of various concentrations of NaBu (0.1–5 mmol/l) alone and NaBu in combination with ABT-737 (either 0.1 or 1 μmol/l) and relative cell viability was determined by the MTT assay. Representative curve of three independent experiments results from one experiment performed in triplicate. HL-60 cells were grown for 72 hours in the presence of various concentrations of NaBu (0.1–5 mmol/l) alone (**D**) and NaBu in combination with 1 μmol/l ABT-737 (**E**). Cell growth was determined by the MTT assay and expressed as time course of optical density (O. D.) of formazan produced by particular cell population. Representative curve of three independent experiments results from one experiment performed in triplicate.

hours (Fig. 2B and 2C), the effect of NaBu in the presence of 1 μmol/l of ABT-737 on relative viability HL-60 cells was copying the effect observed when cells were incubated with NaBu only. The calculated combination indexes (Table 1) indicate that at concentrations of NaBu 2 and 5 mmol/l the effect of NaBu combination with 1 μmol/l ABT-737 is synergistic while NaBu in concentrations 1 mmol/l and less exhibit mainly antagonistic effect.

Table 1. Results of combined drug effect analysis

NaBu (mmol/l)	Combination index		
	24 hours	48 hours	72 hours
0.1	4.455	0.802	2.080
0.25	2.781	0.991	2.638
0.5	5.467	0.975	1.439
1	4.039	0.838	1.115
2	0.378	0.316	0.472
5	0.199	0.425	0.717

Combined drug effect analysis was performed as described in Materials and Methods using results presented on Figures 2A, 2B and 2C related to combination of 1 μmol/l ABT-737 with indicated concentrations of NaBu.

Incubation of HL-60 cells with different concentration of NaBu for 24, 48 and 72 hours has revealed that NaBu at concentration 1 mmol/l inhibits cell growth (Fig. 2D). NaBu in concentrations higher than 2 mmol/l induced death of HL-60 cells which was manifested by decreased optical density observed after 48 hours of incubation (Fig. 2D). Significant decrease of the cell viability was observed already after 24 hours of incubation of HL-60 cells either with 1 μmol/l ABT-737 or its combination with different concentrations of NaBu (Fig. 2E). Fast initial decrease of cell viability induced by ABT-737 alone was followed with inhibition of cell growth observed after 48 hours that culminated in cell growth arrest documented after 72 hours of incubation (Fig. 2E). The effect of NaBu in combination

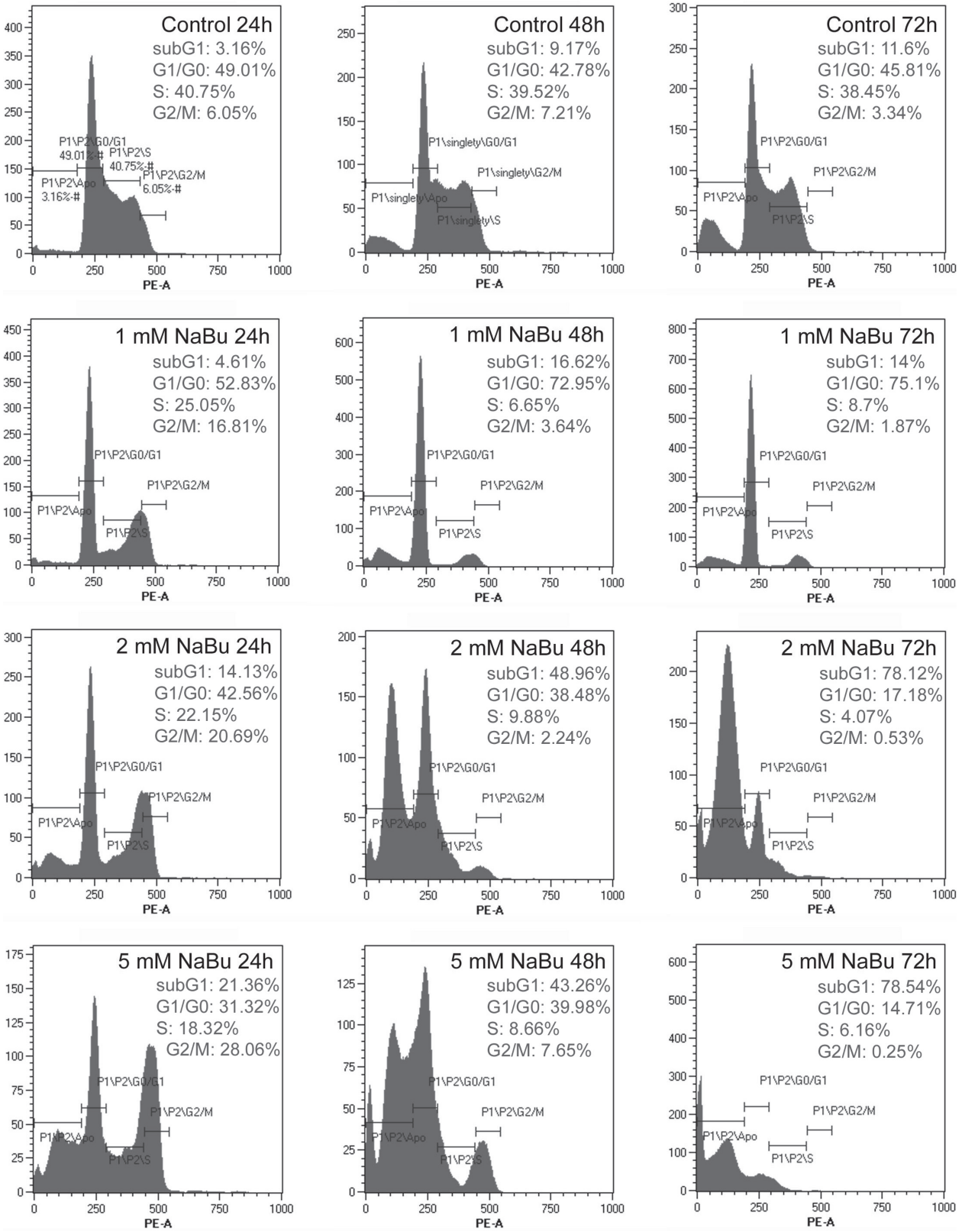

Figure 3. Effect of NaBu on cell cycle of HL-60 cells. HL-60 cells were incubated for 24, 48 and 72 hours in the presence of various concentrations of NaBu (1–5 mmol) and cell cycle was analysed by the propidium iodine staining. Representative curves of three independent experiments.

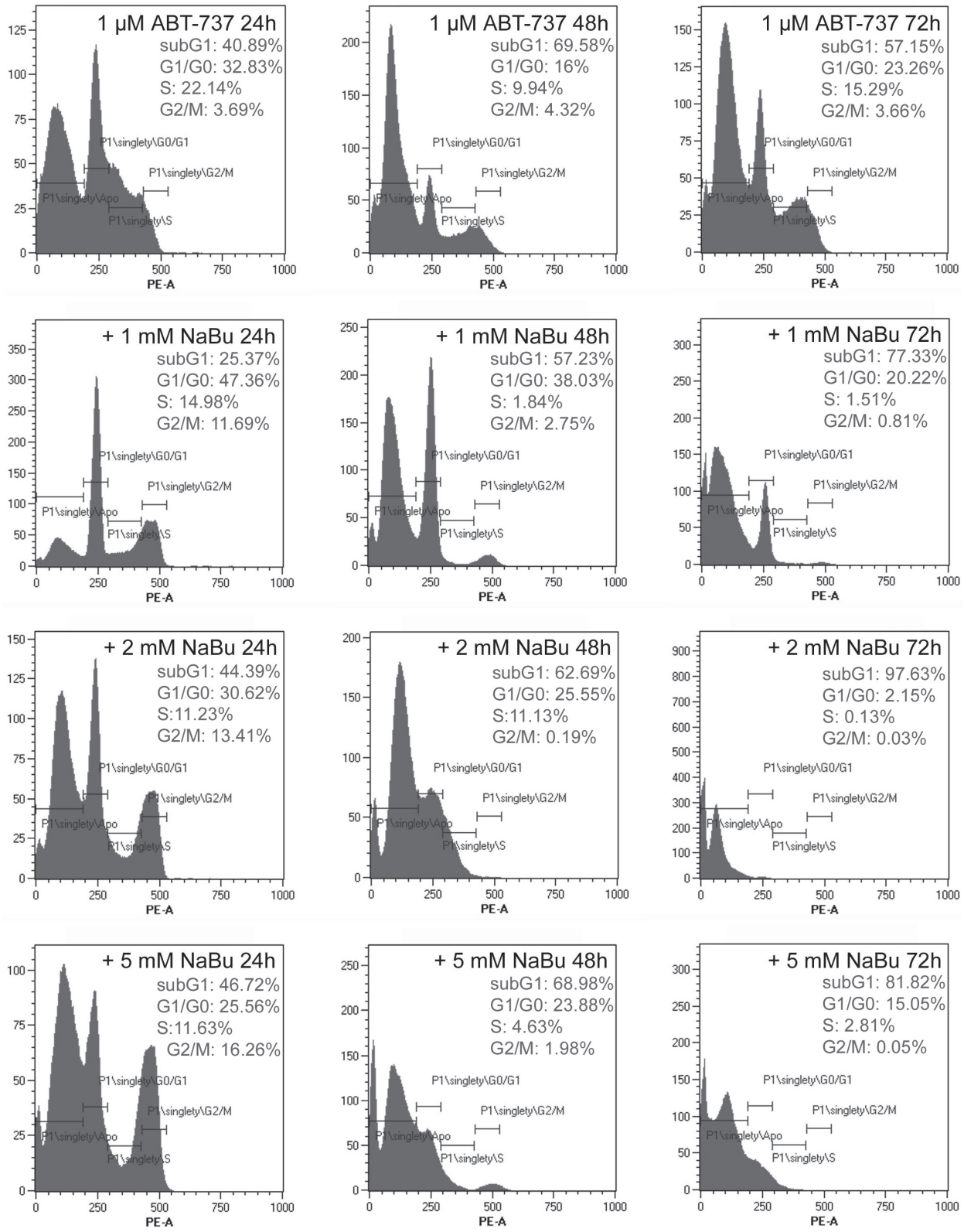

Figure 4. Effect of ABT-737 alone and in combination with NaBu on cell cycle of HL-60 cells. HL-60 cells were incubated for 24, 48 and 72 hours in the presence of 1 μmol/l ABT-737 alone and 1 μmol/l ABT-737 in combination with various concentrations of NaBu (1–5 mmol). Cell cycle was analysed by the propidium iodine staining. Representative curves of three independent experiments.

with 1 μmol/l ABT-737 was comparable to the effect of NaBu alone (Fig. 2E).

The study of the effect of NaBu and ABT-737 on the growth of HL-60 cells was paralleled with analysis of impact of NaBu alone and in combination with 1 μmol/l ABT-737 on cell cycle. As shown on Fig. 3, treatment of HL-60 cells with NaBu for 24 hours led to a concentration-dependent accumulation of G2-phase cells and decrease of S-phase and G1-phase cells, indicating block of both G1/S and G2/M cell cycle transition. After 48 and 72 hours of treatment with 1 mmol/l NaBu, accumulation of G1-phase cells and lack of S- and G2-phase cells was observed (Fig. 3) indicating permanent G1-phase cell cycle arrest and commitment of G2-phase cells to cell death. NaBu in concentrations 2 and 5 mmol/l led to the death of HL-60 cells. The kinetic analysis revealed that first the cells in G2-phase of cell cycle are dying later followed by cells in G1-phase of cell cycle. While after 48 hours of treatment, there were still some G1-phase cells present, mainly dead cells were observed at both concentrations after 72 hours of treatment (Fig. 3). ABT-737 in concentration 1 μmol/l led to decrease of number of cells in all three stages of cell cycle and to increase of the relative amount of dead cell that was observable already after 24 hours (Fig. 4). Combination of 1 μmol/l of ABT-737 with 1 mmol/l of NaBu inhibited cell growth, hampered cells in G1 phase and delayed cell death induced by ABT-737 (Fig. 4). Incubation of HL-60 cells with 1 μmol/l of ABT-737 in combination with 2 and 5 mmol/l of NaBu was associated with massive cell death that was observable already after 24 hours (Fig. 4).

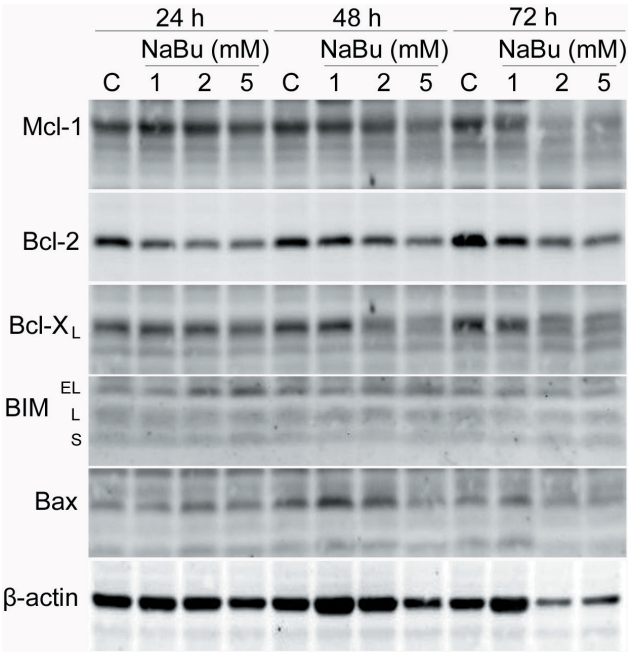

Figure 5. Effect of NaBu on expression of selected proteins of Bcl-2 family in HL-60 cells. Western blot analysis with the specific antibodies against selected proteins of Bcl-2 family of whole-cell extracts of HL-60 non-treated control cells (C) and cells treated with 1, 2 and 5 mmol/l NaBu for 24, 48, and 72 hours. Representative Western blots of three independent experiments.

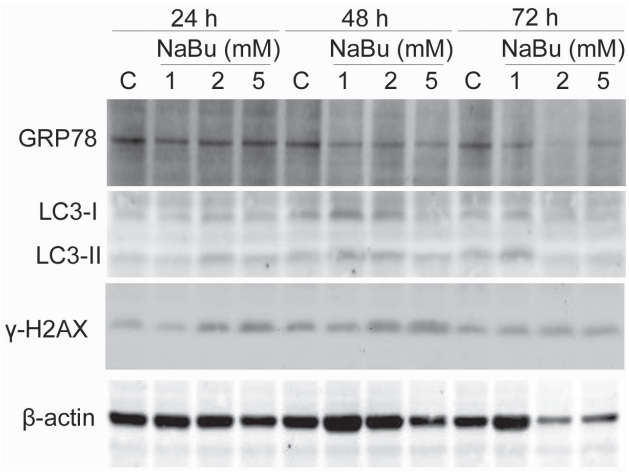

Figure 6. Effect of NaBu on expression of GRP78, LC3-I, LC3-II and γ-H2AX in HL-60 cells. Western blot analysis with the specific antibodies against GRP78, LC3β and γ-H2AX of whole-cell extracts of HL-60 non-treated control cells (C) and cells treated with 1, 2 and 5 mmol/l NaBu for 24, 48, and 72 hours. Representative Western blots of three independent experiments.

In order to explain accelerated cell death induced by combination of ABT-737 and NaBu, Western blot analysis of the effect of NaBu on selected proteins of Bcl-2 family was performed in time- and concentration-dependent manner. We have focused our interest on Bcl-2 and Bcl-X_L that are targets of ABT-737, Mcl-1 that might be responsible for resistance of cells to ABT-737 and BIM that might be responsible for synergistic effect of ABT-737 in combination with HDACi. As shown on Figure 5, incubation of HL-60 cells with NaBu in concentrations 2 and 5 mmol/l was associated with increase of extra-long isoform of BIM (BIM_{EL}) level that was observed only after 24 hours as well as decrease of anti-apoptotic proteins of Bcl-2 family (Bcl-2, Bcl-X_L and Mcl-1). However, the effect of NaBu on the level of anti-apoptotic proteins of Bcl-2 family was dominant after 48 hour of incubation. Finally, NaBu did not affect level of Bax protein (Fig. 5).

Since Bcl-2 family proteins might be involved in endoplasmic reticulum (ER) stress signalling that can be associated either with autophagy or unfolded protein response (UPR) (Rodriguez et al. 2011), we have focused our interest on proteins that are characteristic for both processes. After 48 hours, incubation of HL-60 cells with 1, 2 and 5 mmol/l of NaBu was associated with decreased level of master regulator of UPR, GRP78 (Fig. 6). In addition, increased level of LC3-II as a marker of autophagy (Kabeya et al. 2000) was observed after

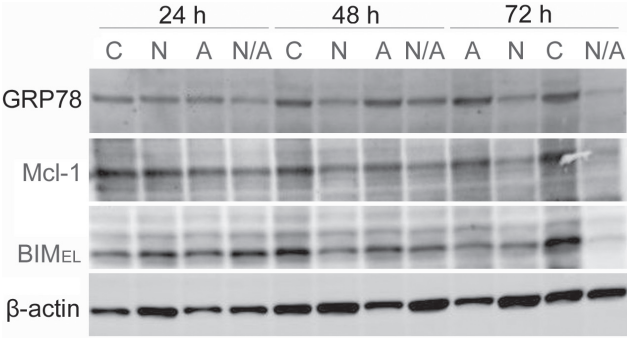

Figure 7. Effect of ABT-737 and ABT-737 in combination with NaBu on expression of GRP78, Mcl-1 and BIM_{EL} in HL-60 cells. Western blot analysis with the specific antibodies against GRP78, Mcl-1 and BIM_{EL} of whole-cell extracts of HL-60 non-treated control cells (C) and cells treated with 2 mmol/l NaBu (N), 1 μmol/l ABT-737 (A) and with combination of 2 mmol/l NaBu and 1 μmol/l ABT-737 (N/A) for 24, 48, and 72 hours. Representative Western blots of three independent experiments.

48 hour of incubation of HL-60 cells with 5 mmol/l of NaBu. Finally, level of phosphorylated histone H2AX (γ-H2AX) as a marker of DNA damage (Lukas et al. 2011) was analysed. Increased level of γ-H2AX was already observed after 24 hours of cell treatment with 2 and 5 mmol/l of NaBu (Fig. 6).

Finally, Western blot analysis of the effect of ABT-737 and ABT-737 in combination with NaBu on selected proteins of Bcl-2 family was performed. We have focused our interest on Mcl-1 that might be responsible for resistance of cells to ABT-737 and BIM_{EL} that might be responsible for synergistic effect of ABT-737 in combination with HDACi. The effect of ABT-737 and ABT-737 in combination with NaBu on the level of GRP78 was also investigated. As shown on Figure 7, incubation of HL-60 cells with 2 mmol/l of NaBu alone and 2 mmol/l NaBu in combination with 1 μmol/l ABT-737 was associated with increase of BIM_{EL} level that was observed only after 24 hours. The level of Mcl-1 was decreased after 48 and 72 hours of incubation of HL-60 cells with 2 mmol/l of NaBu alone and 2 mmol/l NaBu in combination with 1 μmol/l ABT-737. Interestingly, decreased level of GRP-78 was already observed after 24 hours of incubation of HL-60 cells with combination of 2 mmol/l NaBu and 1 μmol/l ABT-737. Decreased levels of GRP-78 were observed after longer incubations of HL-60 cell (for 48 and 72 hours) with both 2mmol/l of NaBu alone and 2 mmol/l NaBu in combination with 1 μmol/l ABT-737. ABT-737 alone did not exhibit impact on any of investigated proteins.

Discussion

The main finding of the presented study is that ABT-737 accelerates NaBu-induced death of cells of leukaemic cell line HL-60. The effect of combination of both drugs on survival of HL-60 cells seems to be synergistic at high concentrations of NaBu (2 and 5 mmol/l) while it is rather antagonistic at concentrations of NaBu less than 1 mmol/l. Our study has also revealed for the first time that incubation of HL-60 cells with NaBu is associated with decreased level of GRP78, in addition to decrease of Bcl-2, $Bcl\text{-}X_L$ and Mcl-1.

Our experiments have shown that both NaBu and ABT-737 at certain concentrations are inducing death of HL-60 cells, however, with significantly different kinetics. While ABT-737-induced cell death is fast, NaBu-induced cell death is rather slow and significant decrease of cell viability was observed after 48 hours of incubation. Fast kinetics of ABT-737-induced cell death is result of the ability of ABT-737 of direct binding to anti-apoptotic proteins of Bcl-2 family followed with consequent initiation and execution of mitochondrial apoptosis (Oltersdorf et al. 2005). It has been documented that ABT-737 induces mitochondrial apoptosis within 2 hours and apoptosis was completely inhibited in cells deficient for Bax/Bak or caspase 9 (Vogler et al. 2009). On the other hand, the slow kinetics of NaBu-induced death of HL-60 cells might be attributed to the fact that cell death is preceded by cell cycle arrest in G2 phase. This is evidenced by accumulation of G2-phase cells and consequent disappearance of these cells observed after 24 and 48 hours of incubation with NaBu, respectively. Disappearance of S-phase cells observed after 24 hours of incubation indicates that NaBu blocks also G1/S transition what is in hand with involvement of HDACs group I and II in G1-to-S progression (Yamaguchi et al. 2010). However, accumulation of G1-phase cells observed mainly after 48 hours of incubation with 1 mmol/l of NaBu indicates that G1 phase arrest is followed with cell death only at higher concentrations of NaBu. The effect of NaBu as well as other HDACi on cell growth and cell cycle arrest was documented using different cancer cell lines including leukaemic. It has been documented earlier that butyrate can induce cell cycle arrest in G1 phase by increasing the expression of $p21^{WAF/CIP1}$ (Rosato et al. 2002) and $p27^{Kip1}$ (Litvak et al. 1998) as well as by induction of Rb protein hypophosphorylation (Vaziri et al. 1998). HDACi can also mediate arrest of G2/M transition by activating a G2-phase checkpoint, although this is a much rarer event than HDACi-induced G1 arrest (Qiu et al. 2000; Burgess et al. 2001). The loss of the G2-phase checkpoint can determine sensitivity of cells to HDACi-induced apoptosis. The cells that retain a functional G2-phase checkpoint are resistant to the HDACi. After treatment with HDACi, the most of tumour cells that have a defective G2-phase checkpoint can initially accumulate in the G2 phase of the cell cycle, then move through this defective checkpoint and undergo apoptosis (Ruefli et al. 2001; Peart et al. 2003; Strait et al. 2005). This was also observed in our study since accumulation of G2-phase

cells documented after 24 hours of incubation with 2 and 5 mmol/l of NaBu was followed with disappearance of G2-phase cells and accumulation of dead cells after 48 hours of incubation. Unlike HDACi-mediated G1-phase arrest, the underlying mechanisms responsible for HDACi-mediated G2-phase arrest are poorly understood (New et al. 2012). It has been proposed that induction of an HDACi-associated G2 checkpoint might be related to hyperacetylation of pericentric heterochromatin and loss of this checkpoint can result in abnormal chromosomal segregation and nuclear fragmentation (Taddei et al. 2005). In hand with this, increased level of γ-H2AX as a marker of damage to DNA (Lukas et al. 2011) was observed in our experiments after 24 and 48 hours of treatment with 2 and 5 mmol/l of NaBu. Disappearance of G2-phase cells observed in our study correlated with changes at the level of Bcl-2 proteins. Down-regulation of anti-apoptotic proteins Bcl-2 and Bcl-X_L as well as activation of both caspase 9 and caspase 3 (Shao et al. 2004; Vrba et al. 2010) after incubation of cells with NaBu were observed in previous studies. In our study, we have documented decreased expression of antiapoptotic proteins Mcl-1, Bcl-2 and Bcl-X_L that was dominant after 48 hours of incubation. The decreased expression of antiapoptotic proteins of Bcl-2 family is associated with mitochondrial apoptosis initiation as it was documented by different experimental protocols aimed to find new treatment of acute leukaemia (Jurečeková et al. 2011). Previous studies have also documented that G2-phase arrest can be associated with consequent initiation of mitochondrial apoptosis (Strait et al. 2005; Xiao et al. 2007; Dai et al. 2011).

Up-regulation of pro-apoptotic proteins of Bcl-2 family represents another mechanism of mitochondrial apoptosis initiation. HDACi-induced up-regulation of BIM (Kuroda et al. 2006; Zhang et al. 2008) has been considered to be responsible for synergistic interaction between HDACi and ABT-737. In our experiments, increased level of BIM_{EL} has been observed after 24 hours of incubation of HL-60 cells with 2 and 5 mmol/l of NaBu. It seems that this up-regulation does not have significant impact on cell viability observed after 24 hours of incubation of HL-60 cells with 2 mmol/l of NaBu only. However, the up-regulation of BIM_{EL} correlated well with significantly decreased viability of HL-60 cells observed after 24 hour when 2 and 5 mmol/l of NaBu was combined with 1 μmol/l of ABT. Thus acceleration of cell death induction observed after incubation of HL-60 cells with combination of ABT-737 and NaBu might be attributable to the changes in BIM_{EL} level and more efficient killing of HL-60 cells with ABT-737. In addition, acceleration of death of HL-60 cells is most probably result of direct binding of ABT-737 to anti-apoptotic proteins of Bcl-2 family that exhibits faster kinetics than decrease of levels of Bcl-2 proteins observed after 48 hours incubation of cells with NaBu only.

Several members of Bcl-2 family also regulate physiological processes at the ER through dynamic interactions with different targets (Rodriguez et al. 2011). Therefore, changes in equilibrium between pro- and anti-apoptotic proteins of Bcl-2 family might be associated with ER stress signalling. Adaptation of cells to ER stress depends on the activation of unfolded protein response (UPR) or protein degradation pathways such as autophagy (Rodriguez et al. 2011; Gorman et al. 2012). Under chronic or irreversible ER stress, cells undergo apoptosis, where the Bcl-2 protein family plays a crucial role at the mitochondria to trigger cytochrome c release and formation of apoptosome (Rodriguez et al. 2011). The ability of ABT-737 (Malik et al. 2011) as well as HDACi (Shao et al. 2004; Tang et al. 2011) to induce autophagy has already been documented. Our results could be in favour of inducing autophagy by NaBu. However, changes at the level of LC3-II that is considered as molecular marker of autophagy induction (Kabeya et al. 2000) were observed only after 48 hours of incubation of HL-60 cells with 5 mmol/l of NaBu. Induction of ER stress signalling by ABT-737 through up-regulation of GRP78 expression (Risberg et al. 2011) as well as by class I HDACi associated with acetylation of GRP78 (Kahali et al. 2012) has been documented recently. GRP78 master regulator of ER stress signalling that is not member of Bcl-2 family proteins exhibits multiple anti-apoptotic properties (Reddy et al. 2003) and plays an important role in solid tumour progression and oncogenesis (Luo and Lee 2013). The function of GRP78 in the hematopoietic system is just emerging, however, recent study clearly revealed involvement of GRP78 in hematopoietic stem cell survival and lymphogenesis (Wey et al. 2012a). In addition, it has been shown that GRP78 expression is up-regulated in various forms of human leukaemia and implicated as causative factor for therapeutic resistance and early relapse (Tanimura et al. 2009; Rosati et al. 2010; Uckun et al. 2011; Wey et al. 2012b). In our experiments, we have detected GRP78 to be expressed in non-treated HL-60 cells and treatment of the cells with NaBu for 48 hours was associated with decrease of GRP78 level. In addition decreased level of GRP78 was documented after 24 hours of cell treatment with combination of ABT-737 and NaBu. Considering anti-apoptotic functions of GRP78, decrease of GRP78 observed after 48 hours of incubation of cells with NaBu might represent mechanism of NaBu-induced apoptosis initiation independent from that associated with changes in Bcl-2 protein levels or functions. Finally, decreased level of GRP78 after 24 hours of incubation of HL-60 cells with combination of NaBu and ABT-737 might represent another mechanism of acceleration of HL-60 cell death.

In conclusion, we have documented in this study that ABT-737 accelerates NaBu-induced death of HL-60 cells most probably due to common cell death mechanism that is mitochondrial apoptosis as well as due to accelerated de-

crease of GRP78 observed after the treatment of cells with combination of NaBu and ABT-737. The effect of combination of both drugs on survival of HL-60 cells seems to be synergistic at high concentrations of NaBu (2 and 5 mmol/) while it is rather antagonistic at concentrations of NaBu less than 1 mmol/l. Synergistic effect of NaBu and ABT-737 might be explained by the ability of NaBu to increase level of BIM_{EL} as well as by the impact of combination of NaBu and ABT-737 on the level of GRP78. The antagonistic effect of NaBu and ABT-737 might be probably attributed to the ability of low concentrations of NaBu to induce differentiation and thus to possible decrease of sensitivity of HL-60 to ABT-737. Finally, it might be assumed that NaBu is capable to induce cell death with mechanisms independent from mitochondrial apoptosis. In favour of this, our study has revealed that incubation of HL-60 cells with NaBu is also associated with decreased level of GRP78 that acts at the level of ER stress signalling and exhibits anti-apoptotic functions.

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