ORIGINAL ARTICLE

BH3 inhibitor ABT-737, but not BH3I-1, triggers death of neuroblastoma SH-SY5Y and glioma T98G cells

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Abstract

OBJECTIVES: The role of pro- and anti-apoptotic Bcl-2 family proteins in tumor-pathogenesis is a frequent question of many scientific studies. Disturbances in the normal functions of Bcl2 family members are frequent feature of human malignant diseases and causal for therapy resistance.

DESIGN: We have studied the possible cytotoxic effects of two inhibitors of anti-apoptotic proteins of Bcl-2 family, BH3I-1 that binds to Bcl-xl and ABT-737 that binds to Bcl-2, Bcl-xl and Bcl-w. Our effort was focused on the effect of these inhibitors on survival and death of the cell lines derived from brain tumors, glioblastoma T98G and neuroblastoma SH-SY5Y cells. Finally, analysis of the expression of selected anti-apoptotic proteins of Bcl-2 family has been performed.

RESULTS: We have found that incubation of both SH-SY5Y and T98G cells with certain concentrations of ABT-737 is associated with significant decrease of relative viability of both SH-SY5Y and T98G cells, however, with significantly different kinetics of cellular response and sensitivity of these cells to ABT-737. SH-SY5Y cells are more sensitive to ABT-737 and the decrease of relative viability of SH-SY5Y cells is induced faster in comparison to T98G cells. Our experiments focused on morphology of treated cells have shown that decrease of relative viability of both SH-SY5Y and T98G cells was result of cell death induced by treatment of these cells with ABT-737. However, cells of both cell lines were insensitive to BH3I-1. Higher sensitivity of SH-SY5Y cells to ABT-737 could be attributed to significantly higher expression of Bcl-2 protein in SH-SY5Y than in T98G cells.

CONCLUSION: The results presented here support the rationale for development of inhibitors of anti-apoptotic proteins of Bcl-2 family for treatment of brain derived malignancies.

Introduction

In addition to other cellular functions (Hatok & Racay 2016), proteins of Bcl-2 family are crucial regulators of intrinsic (mitochondrial) pathway of apoptosis (Czabotar *et al* 2014). Initiation of mitochondrial apoptosis depends on changes of either equilibrium or protein-protein interactions between anti-apoptotic and pro-apoptotic proteins of Bcl-2 family as well as on translocation of pro-apoptotic proteins of Bcl-2 family to mitochondria (Youle & Strasser 2008). As documented in several previous studies, proteins of Bcl-2 family are often deregulated in malignant diseases (Juin *et al* 2013), including brain cancer (Kögel *et al* 2010; Racay *et al* 2011). This deregulation is not only associated with development of cancer, but is also responsible for resistance of cancer cells to the treatment (Labi & Erlacher 2015). Mitochondrial apoptosis plays important role in the development of central nervous system (Yuan & Yankner 2000), where two proteins of Bcl-2 family, apoptotic Bax and anti-apoptotic Bcl-xl, play essential role as documented by Bax or Bcl-x knockout mice (reviewed in Youle & Strasser 2008). However, initiation of mitochondrial apoptosis in mature brain induced mainly by accumulation of toxic protein aggregates is associated with development of neurodegenerative diseases (Muchowski & Wacker 2005), like Alzheimer or Parkinson diseases. In addition, deregulation of mitochondrial apoptosis in mature brain is associated with malignant transformation of the cells of nervous system (Kögel *et al* 2010). Over expression of anti-apoptotic Bcl-2 was shown to be associated with development of neuroblastoma (Castle *et al* 1993) and anti-apoptotic Bcl-xl was shown to be over-expressed in gliomas (Krajewski *et al* 1997). It has been recognized that small molecules with ability to bind to BH3 domain of anti-apoptotic proteins of Bcl-2 family are capable of inducing very fast apoptotic response that is associated with release of cytochrome c from mitochondria, activation of caspases and that culminates in death of malignant cells (Vogler *et al* 2009). Although they were developed with the main aim to treat hematologic malignancies (Kang & Reynolds 2009; Cassier *et al* 2017) they are also tested as potential treatment of other types of cancer (Juin *et al* 2013) including gliomas (Tagscherer *et al* 2008) and other malignancies affecting central nervous system (Kögel *et al* 2010). Current treatment of glioblastoma based on combination of surgery, radiation therapy and consequent chemotherapy using alkylating agent temozolomide is associated with low response rate and relative low increase of overall survival of patients (Stupp *et al* 2005). Therefore several other molecules targeting different cellular targets, including anti-apoptotic proteins of Bcl-2 family (Kögel *et al* 2010), are tested in preclinical and clinical studies in order to increase response rate, to prolong overall survival patients or to cure patient in terms of complete remission of brain tumours.

The aim of this work was to study the effect of BH3 domain inhibitors, ABT-737 targeting anti-apoptotic proteins Bcl-2, Bcl-xl and Bcl-w and BH3I-1 that binds to Bcl-xl, on survival and death of the cell lines derived from brain tumours. We have used glioblastoma T98G and neuroblastoma SH-SY5Y cells representing two main types of brain tumours, glioblastoma derived from glial cells and neuroblastoma that are of neuronal origin. Finally, analysis of the expression of selected anti-apoptotic proteins of Bcl-2 family has been performed.

Materials and methods

Sodium dodecylsulphate (SDS), Bovine serum albumin (BSA) and (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), BH3I-1 (all Sigma-Aldrich), 3-[(3-Cholamidopropyl)dimethylamonio]-1-propanesulfonate hydrate (CHAPS) (ApliChem), ABT-737 (Santa Cruz Biotechnology), HALT™ protease inhibitor cocktail (ThermoFisher Scientific), prestained protein standards (BioRad, cat. no. 1610373). Mouse monoclonal antibodies against Bcl-2 (SC-7382) and β-actin (SC-47778) (all Santa Cruz Biotechnology). Rabbit polyclonal antibody against Bcl-xl (SC-7195) and MCL1 (sc-20679) (all Santa Cruz Biotechnology). Goat anti-rabbit (A0545) and goat anti-mouse (A0168) (all Sigma-Aldrich) secondary antibodies conjugated with horse radish peroxidase.

Cell culture and treatment

SH-SY5Y cells (ATCC) were maintained in DMEM:F12 (1:1) medium supplemented with 10% 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. T98G cells (ATCC) were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (all PAA) at an optimal cell density of 0.2×10^6 cells/mL at 37 °C and 5% CO_2 humidified atmosphere. The media were changed every 3 days.

Cell viability

Cells were seeded in 96-well plates at concentrations of 0.4×10^6 SH-SY5Y cells per mL and 0.2×10^6 T98G cells per ml. Control cells and cells treated with either ABT-737 or BH3I-1 were further incubated 24, 48 and 72 hours at 37 °C in 5% CO_2 humidified atmosphere. At the end of incubation, 0.01 mL of MTT solution (5 mg/mL) were added to each well and the cells were further incubated for 4 hours at 37 °C and 5% CO_2 humidified atmosphere. The insoluble formazan, which resulted from oxidation of added MTT by vital cells, was dissolved by addition of 0.1 ml of SDS solution (0.1 g/mL) and overnight incubation at 37 °C and 5% CO_2 humidified atmosphere. The absorbance of formazan was determined spectrophotometrically using microplate

reader Bio-Rad 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 per cent of control. For each treatment time, the optical density value of non-treated control cells was considered as 100% of viable cells.

Western blotting

Both SH-SY5Y and T98G cells were incubated 24 hours at 37 °C and 5% CO_2 humidified atmosphere. At the end of incubation, the cells were washed 3 times with ice cold PBS and then re-suspended in a lysis buffer (30 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% CHAPS, 1× protease inhibitor cocktail, pH = 7.6) for total protein extraction. Protein concentrations were determined by protein Dc assay kit (Bio-Rad) using BSA as standard.

Isolated proteins (30 µg of proteins loaded per lane) were separated on 12% SDS-polyacrylamide gels (PAGE) under reducing conditions. Separated proteins were transferred to nitrocellulose membranes using semidry transfer and membranes were probed with antibodies specific to Bcl-2 (1:200), Bcl-xl (1:200), MCL1 (1:200), and β-actin (1:2000). Further incubation of membranes with particular secondary antibodies (all 1:5000) was followed by visualization of immunopositive bands using the chemiluminiscent substrate Super-Signal West Pico (Thermo Scientific) and Chemidoc XRS system (Bio-Rad). Intensities of specific bands were quantified by Quantity One software (Bio-Rad). The intensities of bands of interest were normalized to corresponding intensities of bands of β-actin and expressed as intensity of the band of particular protein in treated cells relative to intensity of band in control non-treated cells.

Statistical analysis

For the comparison of differences in protein expression between either cell types or ABT-737-induced changes among all groups, one-way ANOVA (GraphPad InStat V2.04a, GraphPad Software) was first carried out to test for differences among all experimental groups. Additionally, the Tukey's test was used to determine differences between individual groups. $p<0.05$ was considered as being significant.

Results

Incubation of SH-SY5Y cells with different concentrations of ABT-737 for 24, 48 and 72 hours has revealed time- and concentration-dependent impact of ABT-737 on relative viability of both SH-SY5Y (Figure 1A). The estimated lethal concentrations of ABT-737 leading effectively to decrease of SH-SY5Y cell population to 50% of control (LC_{50}) were 4.0±0.5, 0.56±0.08 and 1.26±0.14 µmol/l after 24, 48 and 72 hours, respectively.

Incubation of T98G cells with different concentrations of ABT-737 for 24, 48 and 72 hours has also revealed time- and concentration-dependent impact of ABT-737 on relative viability of both T98G (Figure 1B), however, the significant differences in kinetics and sensitivity were observed in comparison to impact of ABT-737 on SH-SY5Y cells. The LC_{50} values for ABT-737 were estimated to be 56.0±5.4, 6.3±0.82 and 2.0±0.24 µmol/L after 24, 48 and 72 hours, respectively.

Incubation of both SH-SY5Y and T98G cells with different concentrations of BH3I-1 for 24, 48 and 72 hours showed no significant time- and concentration-dependent impact of BH3I-1 on relative viability of both SH-SY5Y and T98G cells (Figure 2).

In order to distinguish whether reduced viability is a result of cell growth inhibition or induction of

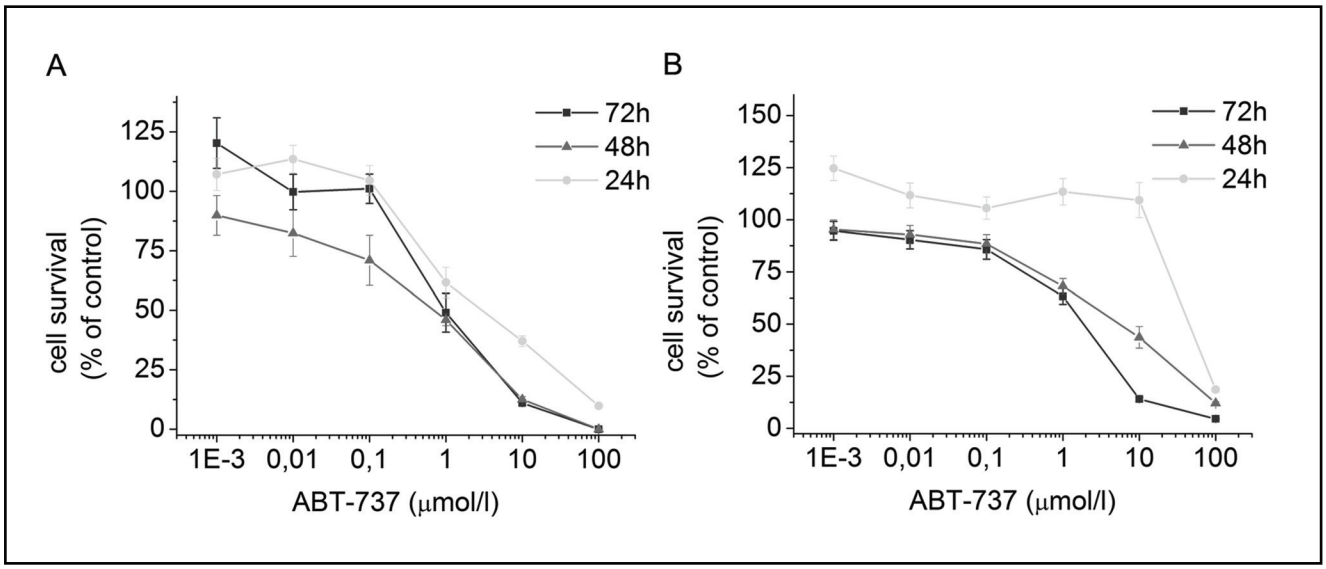

Fig. 1. Effect of ABT-737 on relative viability of SH-SY5Y (A) and T98G (B) cells. SH-SY5Y and T98G cells were incubated for 24, 48 and 72 hours in the presence of various concentrations of ABT-737 and relative cell viability was determined by the MTT assay as described in Material and Methods. Data are shown as mean ± SD (three independent experiments performed in triplicate per each cell line).

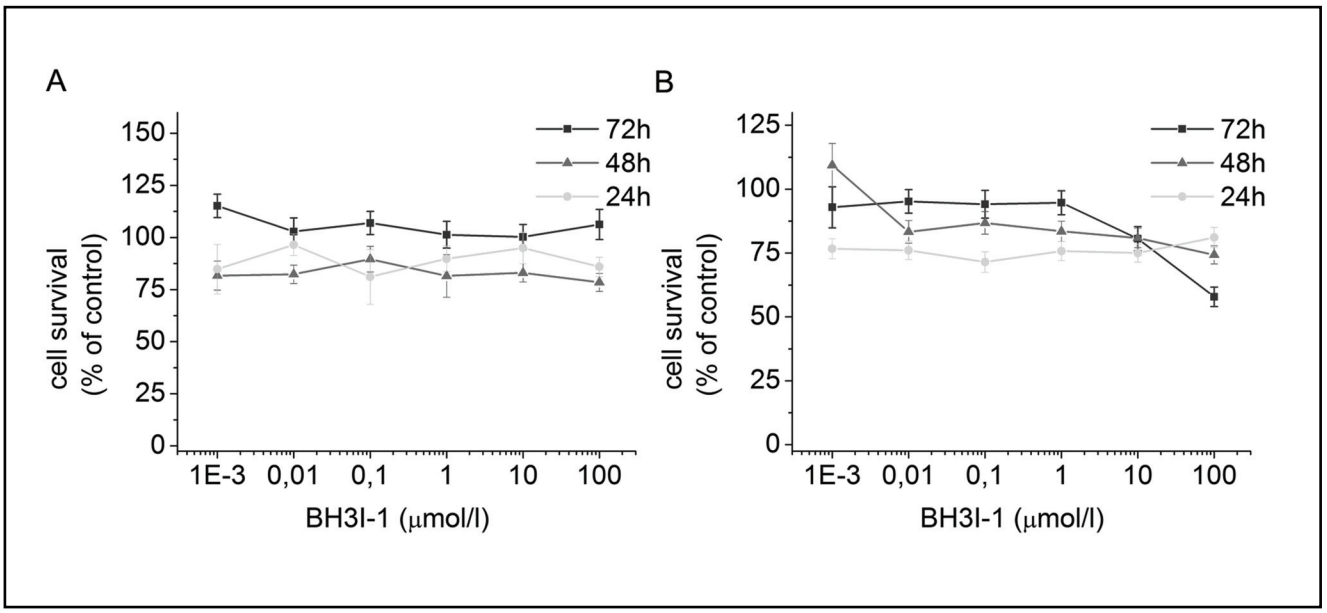

Fig. 2. Effect of BH3I-1 on relative viability of SH-SY5Y (A) and T98G (B) cells. SH-SY5Y and T98G cells were incubated for 24, 48 and 72 hours in the presence of various concentrations of BH3I-1 and relative cell viability was determined by the MTT assay as described in Material and Methods. Data are shown as mean ± SD (three independent experiments performed in triplicate per each cell line).

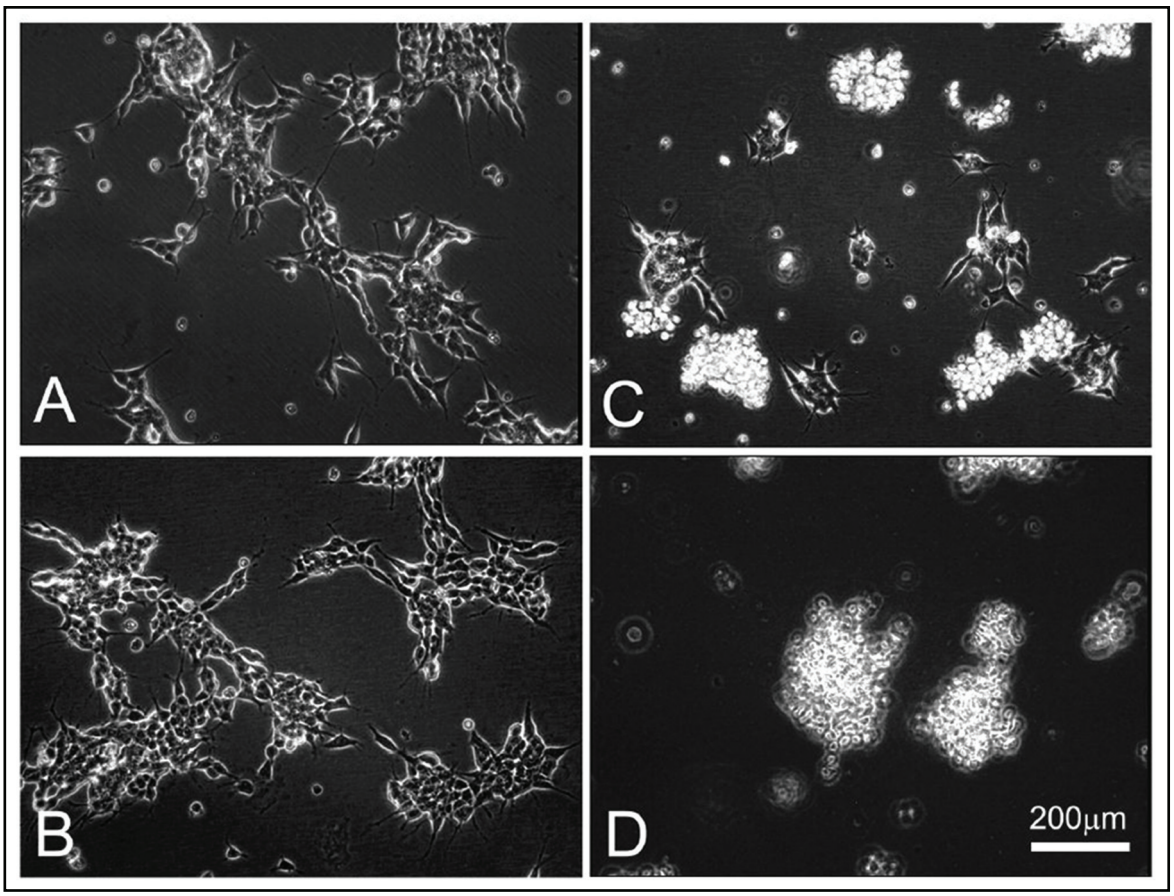

Fig. 3. Phase contrast microscopy of the effect of ABT-737 and BH3I1 on SH-SY5Y cells. SH-SY5Y cells were incubated for 24 hours with ABT-737 and BH3I1 and then the pictures were done. A – control untreated cells, B – cells incubated with 10 μmol/L BH3I1, C – cells incubated with 10 μmol/L ABT-737, D – cells incubated with 100 μmol/L ABT-737

cell death, we have examined treated cells using phase contrast microscopy. Figure 3 shows SH-SY5Y cells incubated for 24 hours with ABT-737 and BH3I-1, demonstrated by phase contrast microscopy. Figure 3A demonstrates untreated SHSY5Y cells. Figure 3B demonstrates SH-SY5Y cells incubated with BH3I-1, show-

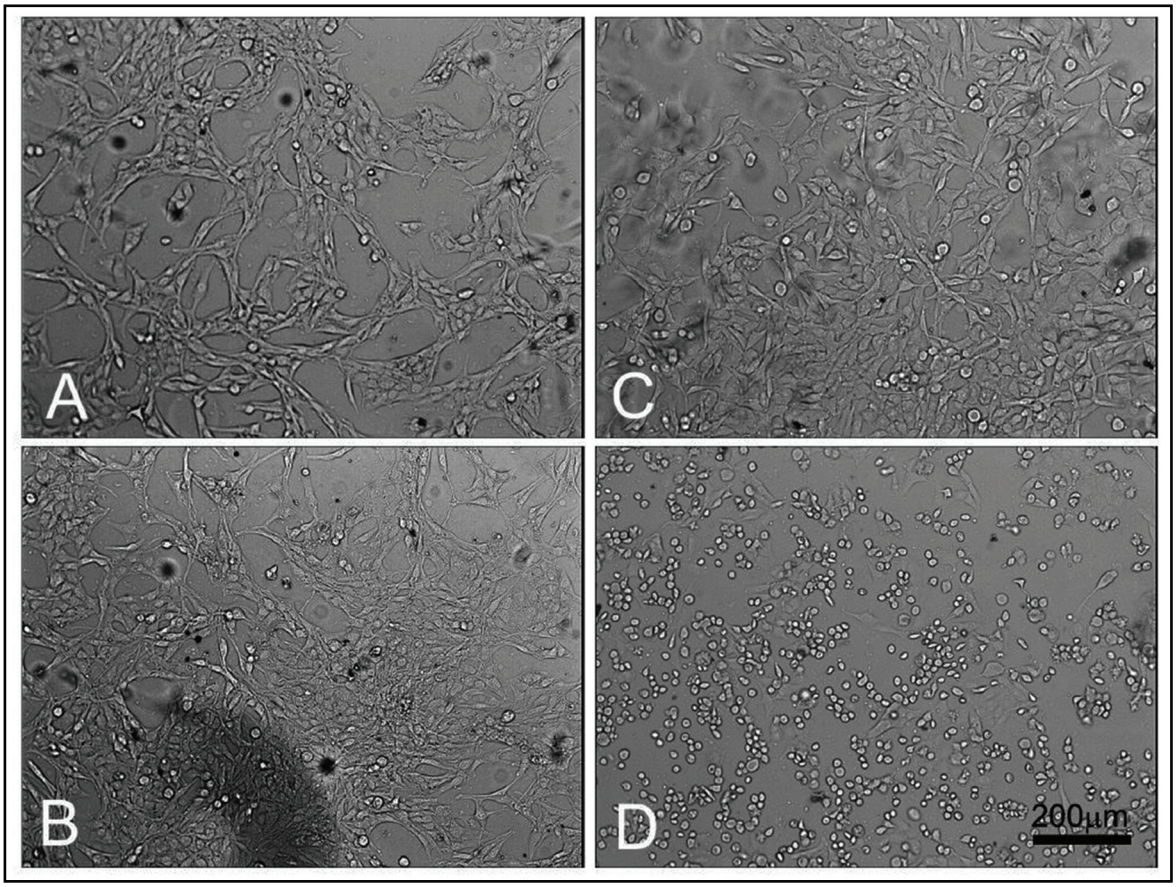

Fig. 4. Phase contrast microscopy of the effect of ABT-737 and BH3I1 on T98G cells. T98G cells were incubated for 24 hours with ABT-737 and BH3I1 and then the pictures were done. A – control untreated cells, B – cells incubated with 10 µmol/L BH3I1, C – cells incubated with 10 µmol/L ABT-737, D – cells incubated with 100 µmol/L ABT-737

ing an unaltered cell morphology like that seen in Figure 3A. Figure 3C and 3D demonstrate SH-SY5Y cells after incubation with ABT-737, showing a dose-dependent change of cellular morphology with loss of normal cell structure and possible formation of apoptotic bodies. Figure 4 shows T98G cells incubated for 24 hours with ABT-737 and BH3I-1, demonstrated by phase contrast microscopy. Figure 4A demonstrates untreated T98G cells. Figure 4B demonstrates T98G cells incubated with BH3I-1, showing an unaltered cell morphology like that seen in Figure 4A. Figure 4C and 4D demonstrate T98G cells after incubation with ABT-737, showing a dose-dependent change of cellular morphology with loss of normal cell structure and possible formation of apoptotic bodies.

In order to explain differences in sensitivity of SH-SY5Y and T98G cells to ABT-737 we have performed Western blot analysis of the levels of selected anti-apoptotic proteins of Bcl-2 family that might be responsible for sensitivity of malignant cells to inhibitors of Bcl-2 proteins and are often over-expressed in cancer cells. We have focused our interest to Bcl-2, Bcl-xl and MCL1. We have found significantly higher protein level of Bcl-2 in SH-SY5Ycells (2.69 fold, $p<0.001$) compared to T98G cells (Figure 5) while the differences in Bcl-xl and MCL1 protein levels between analysed cell lines was not significant.

Discussion

In this study, we have shown that ABT-737 targeting anti-apoptotic proteins Bcl-2, Bcl-xl and Bcl-w was effective in killing of both neuroblastoma SH-SY5Y cells and glioblastoma T98G cells, whereas BH3I-1 that binds to Bcl-xl was ineffective and did not induce death of either SH-SY5Y or T98G cells. In addition, we have shown that neuroblastoma SH-SY5Y cells are more sensitive to ABT-737 than glioblastoma T98G cells.

Higher sensitivity of SH-SY5Y cells to ABT-737 might be a result of the significantly higher expression of Bcl-2 protein in these cells since Bcl-2 was shown to be a better ABT-737 target than Bcl-X_L or Bcl-w (Roo-swinkel *et al* 2012). Bcl-2 protein is also expressed in cells of nervous system but similarly to expression of MCL1, the levels of Bcl-2 in adult brain are rather low (Krajewski *et al* 1995). However, significantly higher expression of Bcl-2 was documented in neuroblastoma (Castle *et al* 1993) and in the tumour tissue biopsy samples from patients diagnosed with glioblastoma multiforme compared with Bcl-2 expression in human

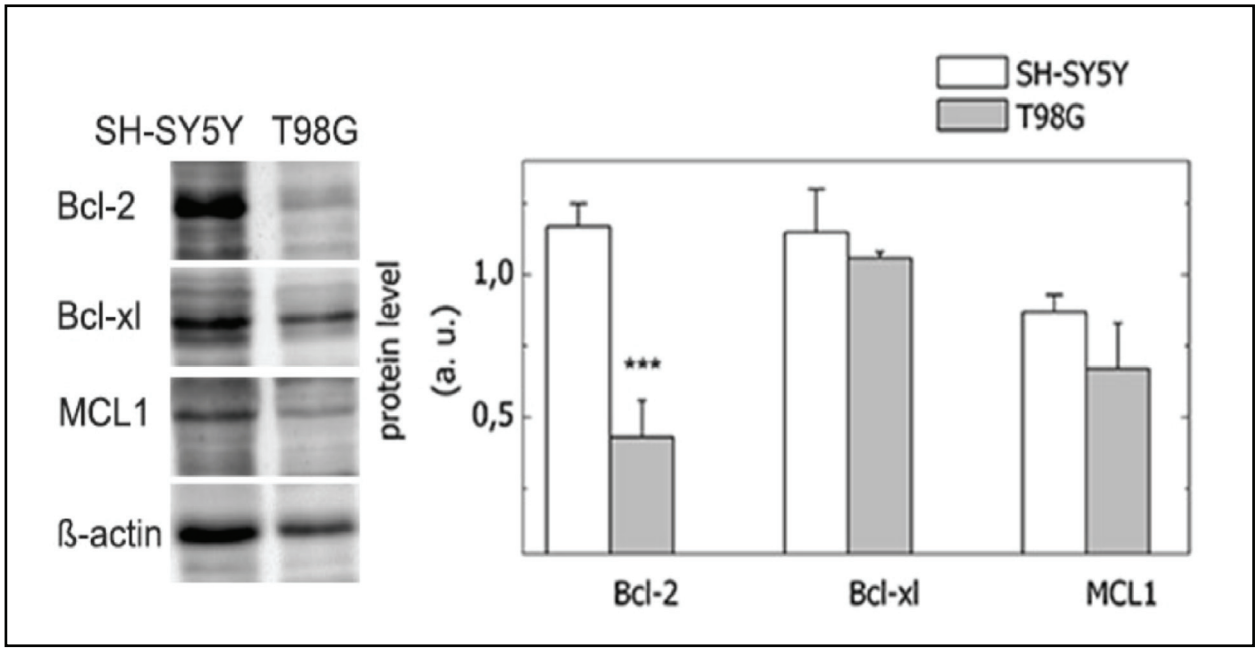

Fig. 5. Levels of Bcl-2, Bcl-xl and MCL1 proteins in SH-SY5Y and T98G cells. After incubation of T98G and SH-SY5Y cells in standard growth media, cellular proteins were isolated, separated by PAGE and analysed by Western blotting as described in Material and Methods. The data were normalised to β-actin level and expressed as intensity of band of particular Bcl-2 family protein relative to intensity of band of β-actin in particular cell type. Data are presented as means ± SD (n=3 per each cell line). ***p<0.001 (ANOVA, followed by Tukey's test to determine differences between particular Bcl-2 protein in both types of cells).

astrocyte cell line used as a reference (Blahovcova *et al* 2015). In addition, our Western blot analysis revealed that the level of Bcl-2 protein was comparable to that of Bcl-xl and was significantly higher in SH-SY5Y cells than in T98G cells. The results of Western blot analysis was in accord with qRT-PCR analysis that have revealed significantly higher expression of Bcl2 mRNA, but not Bcl-xl and MCL1, in SH-SY5Y cells as compared to T98G cells (Pilchova *et al* 2017). The significantly higher level of Bcl-2 in SH-SY5Y cells than in T98G cells correlate well with sensitivity of these cells to ABT-737 since SH-SY5Y cells exhibited approximately 2 times higher sensitivity to ABT-737 in comparison to T98G cells. Sensitivity of malignant cells to ABT-737 is also determined by expression of MCL1 protein that does not bind to ABT-737 and plays an essential role in antagonizing of cytotoxic effect of ABT-737 (Lin *et al* 2007). Downregulation of MCL1 increased sensitivity of different types of glioma cells to ABT-737 (Kiprianova *et al* 2015). Since our Western blot analysis did not reveal significant differences in expression of MCL1 between SH-SY5Y and T98G cells we assume that expression of MCL1 protein is not a reason of differential response of SH-SY5Y and T98G cells to the treatment with ABT-737. Finally, the results of recent studies support the importance of Bcl-2 in survival and growth of glioma cells (Yang *et al* 2014; Duan *et al* 2016).

Bcl-xl is the major anti-apoptotic protein of Bcl-2 family expressed in brain (Krajewski *et al* 1994) having an important impact on proper development of central nervous systems (Motoyama *et al* 1995). Therefore, ineffectiveness of BH3I-1 to kill T98G and SH-SY5Y cells represents an apparent discrepancy. It is well known that BH3I-l requires higher concentrations (more than 20 μmol/L) to exhibit cytotoxic effects in cancer cells (Degterev *et al* 2001), which may limit its clinical application. In order to answer question about significant differences in cytotoxic responses elicited by ABT-733 and BH3I-1 it is important to compare affinities of both inhibitors to target proteins. The affinity of ABT-737 to target proteins is around 1 nmol/L (Kang and Reynolds, 2009) whereas affinity of BH3I-1 to Bcl-xl is 2.4 μmol/L (Degterev *et al* 2001). Thus, ABT-737 exhibits more than 1000 times higher affinity to target proteins than BH3I-1. If we consider that values of LC_{50} for ABT-737 were in order of 1 μmol/L for both SH-SY5Y and T98G cells, it is not surprising that we did not observe cytotoxic effect of BH3I-1 since the highest concentration of BH3I-1 used in our experiments was 100 μmol/l. The higher concentrations of BH3I-1 are irrelevant with respect of its clinical use. With respect of Bcl-xl it is also important to note that inhibition of Bxl-xl is associated with serious adverse effects. Since Bcl-xl is important for survival of platelets (Mason *et al* 2007), treatment of different types of malignant diseases with inhibitors of Bcl-xl protein was associated with thrombocytopenia (Souers *et al* 2013). This fact represents rationality why the water soluble analogue of ABT-737, ABT-263, was in clinical trials focused on acute or chronic leukaemia replaced with ABT-199 that binds only to Bcl-2 (Souers *et al* 2013).

In conclusion, we have shown in this study that both neuroblastoma SH-SY5Y and glioblastoma T98G cells are sensitive to ABT-737 and that the sensitivity depends on expression of anti-apoptotic proteins of Bcl-2 family. The results presented here support the rationale for development of inhibitors of anti-apoptotic proteins of Bcl-2 family for treatment of brain derived malignancies.

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Disclosures

The authors declare no conflicts of interests.

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