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Impact of targeting the AU-rich element of *bcl-2* mRNA with oligoribonucleotides on apoptosis, cell cycle and neuronal differentiation in SHSY-5Y cells

Laura Papucci, Ewa Witort, Anna Maria Bevilacqua, Martino Donnini, Matteo Lulli, Elisabetta Borchi, Khalid S. A. Khabar, Alessio Tempestini, Andrea Lapucci, Nicola Schiavone, Angelo Nicolin and Sergio Capaccioli

Department of Experimental Pathology and Oncology, University of Florence, Italy (L.P., E.W., M.D., M.L., E.B., A.T., A.L., N.S., S.C.)

Program in Biomolecular Research, King Faisal Specialist Hospital and Research Center, Riyadh 11211, Saudi Arabia (K.A.K.)

Department of Pharmacology, University of Milan, Italy (A.M.B, A.N.)

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Correspondence should be addressed to Nicola Schiavone, Department of Experimental Pathology and Oncology, University of Florence, Viale G.B. Morgagni 50, 50134 - Florence, Italy, e-mail: nicola@unifi.it and Sergio Capaccioli, Department of Experimental Pathology and Oncology, University of Florence, Viale G.B. Morgagni 50, 50134 - Florence, Italy, e-mail sergio@unifi.it

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ABSTRACT

We have previously identified a destabilizing AU-rich element (ARE) in the 3′UTR of *bcl-2* mRNA that interacted with ARE-binding proteins (AUBPs) to down-regulate *bcl-2* gene expression in response to apoptotic stimuli. We have also described three contiguous 2′-O-methyl oligoribonucleotides both in sense and in antisense orientation with respect to the *bcl-2* ARE that are able to regulate the *bcl-2* mRNA half-life and Bcl-2 protein level in two different cell lines. Here we show that treatment of neuronal cell line (SHSY-5Y) with antisense ORNs targeting the *bcl-2* ARE (*bcl-2* ARE asORNs) prevents *bcl-2* down-regulation in response to apoptotic stimuli with glucose/growth factor starvation (Locke medium) or oxygen deprivation, and enhances the apoptotic threshold as evaluated by Time lapse videomicroscopy, FACS analysis and caspase-3 activation. Additional effects of *bcl-2* ARE asORNs included inhibition of cell cycle entry and a marked increase of cellular neurite number and length, a hallmark of neuronal differentiation resulting from *bcl-2* up-regulation. The ability of *bcl-2* ARE asORNs to enhance the apoptotic threshold and to induce neuronal differentiation implies their potential application as a novel informational tool to protect cells from ischemic damage and to prevent neuronal degeneration.

INTRODUCTION

Regulation of apoptosis is of vital importance for adult organisms to maintain the numeric homeostasis of cell populations or to eliminate damaged cells. Consequently, pathogenesis of a wide variety of human diseases is frequently related to apoptosis impairment. For instance, tumors and autoimmune diseases result very often from defective apoptosis. Similarly, excessive apoptosis plays a key role in the pathogenesis of numerous diseases including neurodegenerative disorders, such as multiple sclerosis, Alzheimer's, Parkinson's, or Huntington's disease, or pathological conditions, in particular ischemia, affecting different tissues. *Bcl-2* expression is a general mechanism of resistance to apoptosis that acts at various locations inside the cell (Annis, 2004). In order to undergo apoptosis cells need to switch-off the *bcl-2* gene expression (Haldar, 1994). Therefore, preventing *bcl-2* down-regulation could be, at least in principle, a very suitable strategy to treat diseases characterized by excessive apoptosis and, particularly in the neurodegenerative pathologies, to promote conditions that could slow down neuron loss (Yi, 2006, Belcredito, 2001). Furthermore, stabilizing *bcl-2* expression could rescue cells committed to apoptosis by hypoxia/ischemia related stress (Cao, 2002). Recently simvastatin has been found to protect neurons from exicitotoxicity by upregulating *bcl-2* mRNA and protein by a still to clarify mechanism (Johnson-Anuna LN., 2007).

Differentiation and cell cycle are other cellular programs in which *bcl-2* plays a role. Evidence indicates that *bcl-2* expression is involved in promoting and accelerating neuronal differentiation, (Abe-Dohmae, 1993; Suzuki and Tsutomi 1998; Eom, 2004). On the other hand, *bcl-2* up-regulation is also known to have inhibitory effect on cell cycle entry independently from its antiapoptotic activity (Mazel, 1996; Vairo, 1996; Huang, 1997).

Increased knowledge of molecular determinants of apoptosis regulation and execution is now offering new molecular targets suitable to repair apoptosis dysfunctions (Nicholson, 2000; Hersey, 2006). Several antiapoptotic molecules have been proposed as therapeutics for neurodegenerative

diseases (Garber, 2005). Post-transcriptional control of gene expression based on mRNA half-life or translation regulation has been known for many years for a variety of genes. A number of cis-acting elements are known to stabilize or destabilize the relevant mRNA, among which adenine + uracyl rich elements (AU-rich elements, AREs) located in the 3′UTR of many mRNAs modulate mRNA stability via interaction with stabilizing or destabilizing ARE-binding proteins (AUBPs) (Bevilacqua, 2003; Barreau, 2006). The ability of AREs to interact with endogenous AUBPs has suggested that these sequences could be also accessible to exogenously vehiculated molecules (Luzi, 2003; Bevilacqua, 2003). An ARE-based mechanism of post-transcriptional control of *bcl-2* expression accounting for mRNA half-life regulation and its reduction during apoptosis has been previously described by us (Schiavone 2000; Lapucci 2002; Donnini, 2006).

In a previous work, in an attempt to stabilize the *bcl-2* mRNA by hampering its degradation machinery, we have targeted the ARE of *bcl-2* mRNA with peculiar 2′-O-methyl oligoribonucleotides (ORNs) in antisense orientation (Ghisolfi, 2005). Unlike standard DNA oligonucleotides, which form DNA/RNA heteroduplexes that can be cleaved by the RNase H (Schiavone, 2006), RNA oligonucleotides form stable RNA/RNA homoduplexes that are not cleaved by the RNase H (Schiavone, 2006). These *bcl-2* ARE targeting antisense ORNs (*bcl-2* ARE asORNs), vehiculated by cationic lipids into neuroblastoma and hematopoietic cell lines (i.e. SHSY-5Y and HL60, respectively), stabilized *bcl-2* mRNA and increased the level of Bcl-2 protein in a dose dependent manner. The effect was confirmed in cell free experiments evaluating mRNA decay. In a second work, the same effects have been obtained with oligoribonucleotides homologous to the *bcl-2* ARE, and therefore acting as decoy-aptamers (Bevilacqua, 2005).

Here we demonstrate that *bcl-2* ARE asORNs were able to inhibit apoptosis by partially preventing the degradation of *bcl-2* mRNA and consequently reduction of Bcl-2 protein levels in response to apoptotic stimuli. Apoptosis inhibition was accompanied by neuronal differentiation, as evaluated by counts of neurites and cell morphology, and inhibition of cell proliferation. These effects render *bcl-2* ARE asORNs potential candidate for pharmacological interventions, in

particular in the field of ischemic stress and neurodegenerative diseases.

MATERIALS AND METHODS

Cell cultures and transfection. SHSY-5Y cell line, a neuronal sub-line of bone marrow biopsy-derived line SK-N-SH of human neuroblastoma, was purchased from ECACC and maintained in Ham's F12:MEM (1:1) medium supplemented with 5% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 1% glutamine in 100% humidity, 37°C, 5% CO_2 atmosphere. Transient transfections of SHSY-5Y cells with plasmid pCΔJ-bcl-2 or pCΔJ-SV2 empty vector (Tsujimoto, 1989) were carried out with lipofectamine reagent (Invitrogen, USA) according to manufacturer instructions 48 hours prior to treatment with oligonucleotides described below.

2'-O-methyl antisense oligonucleotides. Three synthetic 26-27mer chemically modified (2'-O-methyl derivatives) antisense oligoribonucleotides (asORNs) sequentially overlapping the *bcl-2* ARE asORN1 from 1020 to 994 nt, asORN2 from 993 to 967 nt and asORN3 from 966 to 943 nt of b-RNA, GenBank number M14745) were synthesized and PAGE purified by Dharmacon (USA) as previously described (Ghisolfi, 2005). A degenerated 25mer oligoribonucleotide (degORN) was used as control (Ghisolfi, 2005). The sequences of the asORNs are the following: asORN1 (27mer) 5'-UGUCUUAAAUAAAUAAAUCUUUUUUC -3'; asORN2 (26mer) 5'-UUAAUAAUGUAAAAAAUAAAUGAUAU-3'; asORN3 (26mer) 5'-UUCCCUUUGGCAGUAAAUAGCUGAUU-3'; degORN (26mer) 5'-NNNNNNNNNNNNNNNNNNNNNNNNNN-3' where N is any nucleotide. ORNs were 2'-O-methyl-modified, which increased their stability with respect to the natural RNA derivatives (Yoo, 2004). ORN cellular uptake and stability were increased by their vehiculation with the cationic lipid DOTAP (Boehringer Mannheim), used as lipofection reagent, as previously reported (Luzi, 2003; Ghisolfi, 2005).

Treatments. SHSY-5Y cells were seeded at a density of $5x10^5$ cells/60 mm dish one day before

lipofection with ORNs. The lipofection mixtures were prepared by mixing 1 mM DOTAP (Roche Diagnostics) with 1 mM of either the three *bcl-2* ARE asORNs or the degORN and incubated at room temperature for 20 minutes before adding to cells as previously described (Capaccioli, 1993). The final concentration of each ORN was 0.5 µM. Apoptosis was induced three days after ORN lipofection either by replacing the culture medium without serum and glucose, namely Locke medium (Martinez de la Escalera, 1992), or by culturing cells in a hypoxic atmosphere, i.e. containing 1% oxygen. The transcriptional block was obtained by treating cells with the transcription blocker DRB (5,5-dichloro-1-β-D-ribofuranosylbenzimidazole, Sigma-Aldrich, Saint Louis, MO) at 20 µg/ml added at the 3rd hour after application of apoptotic stimuli.

Total RNA extraction and Real Time RT-PCR. Following treatments, total cellular RNA was extracted from about 10^6 cells collected at 0, 30, 60, 120, 180, 240 and 360 minutes after induction of apoptosis. The RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions. Briefly, total RNA isolated from SHSY-5Y cells lipofected with asORNs or degORN was treated with RNase-free DNase (Invitrogen) and analyzed spectroscopically and by gel electrophoresis for the purity and integrity, respectively. Total RNA (0.1 µg/µl) was reverse-transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using standard manufacturer's conditions in a total volume of 50 µl. Levels of *bcl-2* and GAPDH cDNAs of each sample were determined by quantitative Real Time PCR applying TaqMan Universal MasterMix (Applied Biosystems) standard manufacturer's conditions: 5 µl of total cDNA were amplified with 2x TaqMan Universal Master Mix Buffer, 20x PDAR System target bcl-2, 20x PDAR System control GAPDH, and Nuclease Free Water (Promega) to a total volume of 25 µl. Each reaction was triplicated for a better statistical reliability of results. The PCR reactions were carried out in an ABI PRISM 7000 Sequence Detection System under the following conditions: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 1 min at 60°C (40 cycles). Amplification plot and CT data were elaborated with ABI PRISM 7000 SDS Software 1.1 updated version. GAPDH data were used to

normalize *bcl-2* cDNA values.

Western blotting. The assays were performed according to standard conditions. Briefly, following treatments, cells were washed twice in ice-cold PBS and resuspended in 100 µl of ice-cold RIPA lysis buffer, vortexed for 3 sec, and incubated on ice for 30 min. The protein lysates were obtained by centrifugation at high speed for 20 min at 4°C to separate non soluble cell debris. Proteins (20 µg/lane) were analyzed by 12% SDS-PAGE, blotted onto nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, UK) in a Bio-Rad Trans-blot apparatus at 100 V for 90 min. Blots were processed by an enhanced chemoluminescence (ECL Plus) detection kit according to supplier's instructions (Amersham Biosciences). The blots were probed with a mouse monoclonal anti-Bcl-2 antibody (Upstate, Lake Placid, NY, USA), rabbit polyclonal anti-Bax antibody (Santa Cruz, USA), mouse monoclonal anti-caspase-3 antibody (Santa Cruz, USA). A mouse monoclonal anti-α-Tubulin antibody (Sigma-Aldrich) was used as protein loading control.

Quantitative evaluation of differentiated neurons. SHSY-5Y cells were lipofected with *bcl-2* ARE asORNs or the degORN as described above and cultured in 60 mm Petri dishes in growth medium. Three days after treatments the cells were analyzed by an inverted phase contrast microscope equipped with a 10x objective and photographed by a Nikon digital camera. The number of neurites was obtained as total number of neurites per 50 cells, counted independently by three researchers.. The counts were performed independently for each digital image corresponding with specific treatment. The neurite length was obtained by measuring the distance between cell nucleus and the distal part of the neurite (Aruga, 2003)

Evaluation of cell growth. SHSY-5Y cells were lipofected with *bcl-2* ARE asORNs or degORN control as described above and cultured in 100 mm Petri dishes in growth medium. Each day, during a 5 days period, cells were detached and evaluated by MTT (3-(4,5-Dimethylthiazol-2-yl) -

2,5 diphenyltetrazolium bromide) colorimetric assay (van de Loosdrecht, 1994).

Evaluation of cell proliferation by FACS analysis. Number of cell divisions were assessed with the carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon, USA) assay and evaluated by FACS analysis (Becton Dickinson, San Jose, CA). CFSE covalently binds cellular components yielding a fluorescence (measured by flow cytometry) that is divided equally between daughter cells at each division (Lyons, 2001) and allows calculating successive rounds of replication. Confluent cultures of SHSY-5Y cells were starved for 14 hours in the growth medium described above but containing only 0,5 % FBS. Cells were then harvested and labeled for 10 min with 10 µM CFSE at 37°C and washed twice with culture medium. Labeled cells were plated ($6x10^4$/60 mm dish) and lipofected with asORNs or degORN the following day as described above. An aliquot of freshly-labeled cells was used to measure the starting fluorescence level (day 0). Cells were harvested on day 5 to determine decrease in fluorescence. These values were used to calculate the number of replicative rounds elapsed from day 0 in response to treatments (ModFit LT for Macintosh, Proliferation Protocol, Verity Software House Inc., Topsham, ME, USA)

Evaluation of apoptotic events. Following cell transfer to Locke medium, apoptotic events were counted by the Time-lapse videomicroscopy using a Zeiss inverted phase contrast microscope equipped with a 10x objective, Panasonic CCD cameras and JVC BR9030 time-lapse video recorders, as previously reported (Luzi, 2003). An apoptotic event was scored at the moment the cell became fully shrank and splitted in apoptotic bodies. Apoptotic cells were also evaluated by flow cytometry with GUAVA Personal Cell Analysis System with Guava Nexin[TM] Assay (GUAVA Technologies, Hayward, CA) that utilizes Annexin V-PE to detect phosphatidyl serine on the external membrane of apoptotic cells. The cell impermeant dye 7-AAD (7-aminoactinomycin D) is included in the kit as an indicator of membrane structural integrity and for the differential

assessment of the Annexin V-reactive cells into early and late stage of apoptosis. 7-AAD is

excluded from live, healthy cells and early apoptotic cells, but permeates late stage apoptotic and

dead cells. The assay was performed according to manufacturer's instructions.

Statistical Analysis. The statistical evaluation of the data was performed with the two-tailed

Student's *t* test for unpaired values. Differences were considered statistically significant when $p \leq$

0.05. The data are reported as percentage of the maximal value.

RESULTS

The molecular and phenotypic effects of *bcl-2* ARE-targeting asORNs in response to apoptotic stimuli were analyzed in SHSY-5Y neuronal cells lipotransfected with asORNs or control degenerated ORN (degORN) at the concentration of 0.5 µM established previously to be the most effective (Ghisolfi et al., 2005), three days before application of apoptotic stimuli. Apoptosis was induced by culturing cells either in Locke minimal medium, mimicking condition of glucose/growth factor deprivation, or in condition of hypoxia.

Bcl-2 ARE asORNs markedly attenuate the decrease of *bcl-2* mRNA half-life and Bcl-2 protein level in response to growth factor deprivation.

Transfection of the SHSY-5Y cell line with *bcl-2* specific asORNs resulted in Bcl-2 protein increase as shown in **Fig. 1**, while no effect was observed on expression of Bax, a pro-apoptotic member of the bcl-2 family.

The effect of *bcl-2* ARE asORNs on *bcl-2* mRNA stability following glucose/growth factor deprivation as an apoptotic stimulus (Locke medium) in SHSY-5Y is shown in the **Fig. 2**. *Bcl-2* mRNA stability was evaluated by quantification of *bcl-2* mRNA levels at various times after application of a transcriptional block (20 µM DRB added 3 hours after transfer to Locke medium). The *bcl-2* mRNA half-life decreased from 3 hours in conventional medium to 30 min in the Locke medium. The addition of asORNs to the Locke medium counteracted the decrease, raising the *bcl-2* mRNA half-life from 30 min to 2 hours. The degORN used as control did not elicit any effect on the mRNA levels. This indicates that, asORNs are endowed with strong stabilizing activity toward *bcl-2* mRNA also in condition of apoptotic stress.

We have then evaluated the effect of asORNs on Bcl-2 protein levels by Western blot analysis in response to Locke medium, **(Fig. 3)**. On the third day of culturing in the Locke medium the Bcl-2 protein levels underwent a marked decrease in SHSY-5Y cells with respect to controls cultured in

normal medium (**Fig. 3A**). The addition of asORNs to the Locke medium markedly attenuated this decrease, whilst the addition of degORN did not have significant effects. Histograms in **Fig. 3B** were obtained by the densitometric analysis of bands in **Fig. 3A** and are means of three independent experiments.

Bcl-2 ARE asORNs dramatically lower the number of apoptotic cells in response to Locke medium or to hypoxic condition.

The possibility that inhibition of _bcl-2_ down-regulation in response to apoptotic stimuli induced by the _bcl-2_ ARE asORNs could lead to enhancement of the apoptotic threshold has been evaluated. The number of apoptotic events occurring in SHSY-5Y cells cultured either in the Locke medium or in the condition of hypoxia, and lipofected either with asORNs or with degORN have been quantified by the Time-lapse Videomicroscopy (**Fig. 4**). An apoptotic event was scored at the moment the cell detached from the substrate, shrank and blebbed. The SHSY-5Y cells cultured in normal growth medium served as untreated control. As shown in the **Fig. 4A**, _bcl-2_ ARE asORNs but not the degORN, dramatically reduced the number of apoptotic events occurring in SHSY-5Y cells cultured in the Locke medium.

The protective effect of _bcl-2_ ARE asORNs against apoptosis of SHSY-5Y cells induced by hypoxia was evaluated by flow cytometry with Annexin V-PE assay. The cells were maintained in the hypoxic condition for up to 48 hours. As shown in the **Fig. 4B**, already at the 12th hour the hypoxia induced massive mortality (less than ~5% of viable cells) as compared to controls cultured in standard atmosphere. Treatment with _bcl-2_ ARE asORNs markedly counteracted this effect, protecting cells from apoptosis: the percentage of viable cells in hypoxic conditions was substantially at the level of untreated controls at the 12th hour and decreased to ~80% and ~50% of viable cells at the 24th and 48th hours, respectively. Treatment with the degORN did not have any significant effect on the viability as compared to the untreated control.

Bcl-2 ARE asORNs inhibit caspase-3 activation in response to Locke medium

Caspase-3 has been reported to activate death effector molecules resulting in the fragmentation of genomic DNA in association with structural and morphological changes characteristic of apoptosis. Caspase-3 is initially present as a pro-enzyme of 32 kDa that is cleaved in the cell undergoing apoptosis into its enzymatically active form, composed of two subunits of 17 kDa (p17) and 11 kDa (p11). Activity of the caspase-3 in SHSY-5Y cells lipofected with *bcl-2* ARE asORNs or with degORN was evaluated by Western blot analysis following 0, 24, 48 and 72 hours in Locke medium (**Fig. 5**). The activity of caspase-3 in SHSY-5Y cells maintained in normal growth medium provided untreated control. As expected, the cleaved 17 kDa active caspase-3 subunit, undetectable in untreated controls, was evident in SHSY-5Y cells cultured in Locke medium, but was markedly lowered when *bcl-2* ARE asORNs were added. The degORN, did not elicit any effect. The SHSY-5Y cells transiently transfected with a *bcl-2* harboring plasmid did not show significant caspase activation in response to Locke medium with respect to mock transfected, untransfected cells, and degORNs transfected cells.

Bcl-2 ARE asORNs inhibit cell proliferation

Bcl-2 expression has been previously reported to markedly diminish cell proliferation by preventing quiescent cells from re-entering the cell cycle (Borner, 1996; Huang 1997; Vairo, 1996). We have evaluated the possibility that *bcl-2* ARE asORNs affect cell proliferation by MTT assay and by flow cytometry (**Fig. 6**). The *bcl-2* ARE asORNs markedly reduced the rate of SHSY-5Y cell proliferation, evaluated by analysis of cell viability every day for 5 days with the MTT assay, as compared to untreated or degORN treated controls (**Fig. 6A**). The distribution of cell divisions in *bcl-2* ARE asORN-treated SHSY-5Y cells compared to untreated or degORN-treated controls was determined by flow cytometry on the basis of the CFSE dye dilution (**Fig. 6B**). This assay allows to count the subsequent rounds of cell divisions (generations) that start from fully labeled SHSY-5Y cells on the day 0. On the 5th day, approximately 70% of untreated cells approached the 9th

generation, 20% of these reached the 10th generation and only the 17% was still at the 8th generation. Similar results were obtained in cells lipofected with the degORN. Instead, only 8% of *bcl-2* ARE asORN-treated cells reached the 10th generation, 50% was at 9th generation and 30% of cells were at the 8th generation. Furthermore, 10% of cells treated with *bcl-2* ARE asORNs arrested at 7th generation, demonstrating significant inhibition of SHSY-5Y proliferation rate.

Bcl-2 ARE asORNs induce neuronal differentiation

In addition to its antiapoptotic function, *bcl-2* has been reported to have differentiative and neuroprotective properties, promoting dendrite branching and regeneration of damaged neurons. We have evaluated the ability of *bcl-2* ARE asORNs to induce neuronal differentiation in SHSY-5Y cells, assuming neuron length and number as differentiation index. The morphology of SHSY-5Y cells treated for 5 days with asORNs or the degORN or untreated controls is shown in the **Fig. 7.** Most asORN-treated cells have assumed the differentiated phenotype, characterized by the presence of numerous, relatively long and interconnected neurites (**Fig. 7A**). Instead, untreated cells and degORN-treated cells maintained the undifferentiated phenotype, characterized by very small number or even total absence of neurites (**Fig. 7A**). As expected, transient transfection of a *bcl-2* harboring plasmid, but not of an empty vector, was able to promote neurite formation in SHSY-5Y cells (**Fig. 7A**). The median length of neurites and the median number of neurites per cell are reported in the **Fig. 7B**, upper panel, and in the **Fig. 7B**, lower panel, respectively.

DISCUSSION

The antisense strategy, aimed to specifically down-regulate gene expression, flourished in the last decades and culminated with the burst of pharmacogenomics and RNA interference. On the contrary, little attention has been given so far to strategies aimed to up-regulate the expression of target genes and/or to prevent their switching off. This is happening despite the wide number of human diseases involving inadequate expression of specific genes and the suitability of deterministic up-regulation of a given gene product to study its function, overcoming the drawbacks of gene transfection. In particular, excessive apoptosis resulting from down-regulation of *bcl-2* expression plays often a key role in the pathogenesis of several human diseases, ranging from neurodegenerations to AIDS, from atherosclerosis to ophthalmologic diseases, which suggests the breadth of potential therapeutic opportunities offered by *bcl-2* up-regulating molecular tools.

In a previous work, we have shown that three contiguous synthetic antisense 2'-O-methyl oligoribonucleotides (asORNs) targeting the *bcl-2* mRNA regulative ARE stabilize *bcl-2* mRNA and enhance Bcl-2 protein levels in a dose-dependent fashion (Ghisolfi, 2005). The effectiveness of this strategy relies on the accessibility of any mRNA cis-acting element by the relevant trans-acting factors modulating the RNA decay machinery that assembles on it (Bevilacqua, 2003). Therefore, up-regulation of *bcl-2* gene expression by targeting its destabilizing *bcl-2* ARE with synthetic modified single strand RNAs could be a paradigm for any gene regulated by AREs or to any other gene carrying cis-acting elements in their RNA.

Here we show that this innovative approach, stabilizing *bcl-2* mRNA and leading to increased Bcl-2 protein level without affecting the *bcl-2* family member Bax, in SHSY-5Y cells, inhibits *bcl-2* gene down-regulation in response to apoptotic stimuli. Consequently, this approach prevents apoptosis and modifies fundamental cellular programs. Indeed, the three synthetic *bcl-2* ARE asORNs protected *bcl-2* mRNA from the fast degradation triggered by the deprivation of growth factors and glucose or by hypoxic stress, and maintained the Bcl-2 protein at relatively high levels

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in SHSY-5Y cells as compared to degORN-treated or untreated controls. The relatively high steady-state level of Bcl-2 protein in asORN-treated cells, in line with the relatively high *bcl-2* mRNA level (Ghisolfi, 2005), counteracted activation of apoptotic programme. Counteraction of apoptosis was maximal in the initial phases of the apoptotic program, but was still apparent at 48 hours.

Although the main function of Bcl-2 protein is to increase the apoptotic threshold of the cells, Bcl-2 has also another fundamental activity that is inhibition of cell proliferation. Moreover, in proper settings, the above biological property of Bcl-2 can be associated to the activation of differentiation program (Grossmann, 2000). Both effects have been obtained in the SHSY-5Y neuronal cell line consequently to Bcl-2 overproduction induced by treatment with *bcl-2* ARE asORNs or by transient transfection of a *bcl-2* harboring plasmid. Clearly, *bcl-2* ARE asORNs reduced cell number without affecting cell viability, and lowered proliferative kinetics as compared to untreated controls. Most relevantly, *bcl-2* ARE asORNs induced neuronal cell differentiation evaluated on the basis of the length of neurites and their number per cell, albeit at a lower degree than exogenously expressed *bcl-2*. Impact of synthetic single strand RNAs complementing the cis-acting AU-rich element of *bcl-2* mRNA on proliferation and differentiation of human cells suggests the high pharmacological potential of an innovative strategy able to modify cellular programs by acting at the post-transcriptional level of the gene expression.

The hypothesis that short *bcl-2* ARE-targeting single strand RNAs could modulate *bcl-2* expression was in part inspired by our discovery in t(14;18) lymphoma cells that a hybrid *bcl-2*/IgH antisense transcript caused *bcl-2* over-expression by overlapping the *bcl-2* ARE of the *bcl-2*/IgH mRNA (Capaccioli,1996). This hypothesis is strongly supported by evidences obtained by Meisner et al. (Meisner, 2004), analyzing the effects of synthetic ORNs on the secondary structure and stability of ARE-controlled transcripts. They demonstrated in peripheral blood mononuclear cells (PBMC) that ARE accessibility of synthetic ARE-controlled transcripts (TNFα and IL-2) by the stabilizing AUPB HuR can be either opened or closed by computationally designed ORNs. This

property decides the fate (stabilization or decay) of the transcripts, indicating the possibility to manipulate ARE-controlled gene expression by exogenous ARE openers or closers. Conceivably, pairing of the *bcl-2* ARE with asORNs in our cellular model could have opened the *bcl-2* ARE accessibility to stabilizing AUBPs or closed the *bcl-2* ARE accessibility to destabilizing AUBPs, or could have done both.

Short 2-O'-methyl-oligonucleotides designed to target a complementary region within an endogenous messenger RNA are not supposed to activate ribonuclease activities, nor they are known to induce RNA degradation by heteroduplex ribonucleases (Schmitz, 2001). Actually, 2'-O-methyl ORNs have been used to temporary interfere with translation or other mRNA-involving processes, like enforced exon skipping (Schmitz, 2001). Also, 2'-O-methyl modified small interfering RNA duplexes shown reduced efficacy in down-regulating gene expression, even when the modification is restricted to as few as three positions out of twenty on the antisense strand (Prakash, 2005). We previously shown that our *bcl-2* ARE asORNs mask the *bcl-2* mRNA ARE with respect to the relevant trans-acting AUBPs and inhibit their functions in a reversible fashion (Ghisolfi, 2005). Furthermore, their application to SHSY-5Y cells did not induce obvious toxic effects other than the specific action at the level of the target gene.

Despite the success of the *bcl-2* ARE asORNs in up-regulating *bcl-2* expression in a neuronal cell line, the pharmacological potential of these tools requires some general considerations. Indeed, the molecular and phenotypic effects of modified *bcl-2* expression are far from being obvious. Despite *bcl-2* over-expression is commonly associated to tumor onset and progression, the role of Bcl-2 protein in tumors needs to be further clarified. Indeed, rapid tumor progression and bad prognosis are often paradoxically associated with loss of Bcl-2 function, an effect that could be in part explained by the ability of Bcl-2 to inhibit the cell cycle. Similarly, *bcl-2* over-expression is usually associated to unspecific drug resistance in most tumors (Kiechle, 2002).

Furthermore, studies and even clinical trials conducted to evaluate the anticancer effectiveness of combined application of *bcl-2* down-regulating antisense molecules with conventional

chemotherapeutics gave controversial results. Numerous evidences indicated a synergism between antisense oligodeoxyribonucleotide-mediated *bcl-2* down-regulation and classic anticancer compounds (Milella, 2004). Whatever is the role of Bcl-2 in tumors, maintaining the basal level of Bcl-2 above the threshold that triggers apoptosis execution might represent a new therapeutic strategy to treat those humans diseases in which deficient *bcl-2* expression plays a key role and to prevent tissue damages in case of ischemic and stress conditions.

Particularly attractive is the potential application of *bcl-2* ARE asORNs to neurodegenerative conditions, in which induction of high level of Bcl-2 protein has already shown therapeutic values (Lawrence, 1996). The recent finding that statins are implied in neuroprotection by a bcl-2 dependent mechanism (Johnson-Anuna LN., 2007) further supports our approach. Authors suggest that transcriptional as well post-transcriptional mechanisms can be involved in simvastatin-induced upregulation of bcl-2. In particular statins have already been demonstated to positively modulate mRNA stability (Bonetti, 2003) and can be thus considered for synergic therapeutic effects.

Besides asORN-mediated up-regulation of *bcl-2* expression by the specific targeting of its ARE, this work might open a new avenue for the pharmacological enhancement of any gene expression, provided a destabilizing element is harbored on its mRNA. Besides enhancement of oncosuppressor functions in cancer gene therapy, the restoration of depressed activity of specific genes is a common requirement for the therapy of human diseases. Although oligonucleotides are still facing substantial difficulties entering into cells and explicating activity in experimental animals, the reversibility of their action and their apparent low toxicity could encourage developing a general modality to apply this molecules to regulate positively gene expression in a very specific fashion.

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FIGURE LEGENDS

Figure 1 Molecular effect of *bcl-2* ARE asORNs. Cells were lipofected with 0.5 μM asORNs or the degORN and 10 μM DOTAP. At the indicated times cells were collected, and protein extracts were prepared for the Western blot analysis of Bcl-2 and Bax expression. α-Tubulin provided the loading control.

Figure 2. Determination of *bcl-2* RNA half-life in glucose/growth factor deprived cells (Locke medium) treated with *bcl-2* ARE asORNs.

Cells were maintained as in **Fig. 1** 3 days before application of the apoptotic stimulus (Locke medium). Transcriptional block was obtained by adding 20 μM DRB3 hours after transfer to Locke medium. At the indicated times after DRB transcriptional block, cells were collected, total RNA was extracted, and *bcl-2* mRNA was analyzed by quantitative Real-Time RT-PCR. Results were normalized to β-actin cDNA. Each point is the mean ± SE of three independent experiments. ***$p \leq 0.001$ asORNs versus degORN or untreated at 30, 60, 120, 240 min

Figure 3. Determination of Bcl-2 protein level in cells maintained in Locke medium treated with *bcl-2* ARE asORNs.

(a) Cells were maintained and treated as in **Fig. 2** except for omission of DRB. At the indicated times, cells were collected, and protein were extracted for the analysis of Bcl-2 by Western blot. α-Tubulin provided the loading control. Result is the representative of three independent experiments. (b) Densitometric histograms of relative band intensities are shown. Data are means ± SE of three independent experiments. **$p \leq 0.01$ asORNs compared to degORN. ***$p \leq 0.001$ asORNs compared to degORN

Figure 4. Evaluation of apoptosis in cells maintained in Locke medium or in hypoxic conditions and treated with *bcl-2* ARE asORNs or degORN.

(a) Apoptotic events in SHSY-5Y cells maintained in Locke medium following a three day pretreatment with asORNs (as in **Fig. 3**) were counted by Time-lapse videomicroscopy. Apoptotic events were scored at the moment the cells were fully shrunk and apoptotic bodies appeared. Each point is the mean number of cumulative apoptotic events ± SE of three independent experiments. *$p \leq 0.005$ Locke+asORNs versus Locke or Locke+degORN from 40 to 72 hours. (b) Cells, maintained in hypoxic conditions for 12, 24 and 48 hours and treated as above, were analyzed by flow cytometry for expression of Annexin V. Bar graphs show the compiled mean values ± SE of four independent experiments.

Figure 5. Caspase-3 activation in cells maintained in Locke medium and treated with *bcl-2* ARE asORNs.

Protein extracts from cells maintained and treated as in **Fig. 3** and from cells transiently transfected as indicated, were analyzed for the expression of the non-activated and activated caspase-3 by Western Blot. Caspase-3 activation was evaluated as decrease of the 32 kDa pro-enzyme band and/or increase of bands corresponding to the enzymatically active subunits of 17 kDa (p17) and 11 kDa (p11) at the indicated times. α-Tubulin provided the loading control. Data are representative of three independent experiments**.**

Figure 6. Viability and proliferation rate in growth factor deprived cells treated with *bcl-2* ARE asORNs

(a) Viability of cells, maintained and treated as in **Fig. 2**, was evaluated by the MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5 diphenyltetrazolium bromide) colorimetric assay. Data are compiled means ± SE of five independent experiments. **$p \leq 0.01$ asORNs compared to degORN or untreated at 4 days. ***$p \leq 0.001$ asORNs compared to degORN o untreated at 5 days. (b) The rate

of proliferation was analyzed for CFSE fluorescence by flow cytometry on day 0 and day 5

following lipofection. Fluorescence peaks decrease according to the loss of CFSE fluorescence over

time as cells divide. Peak numbers correspond to cell division/generation's number. Values are

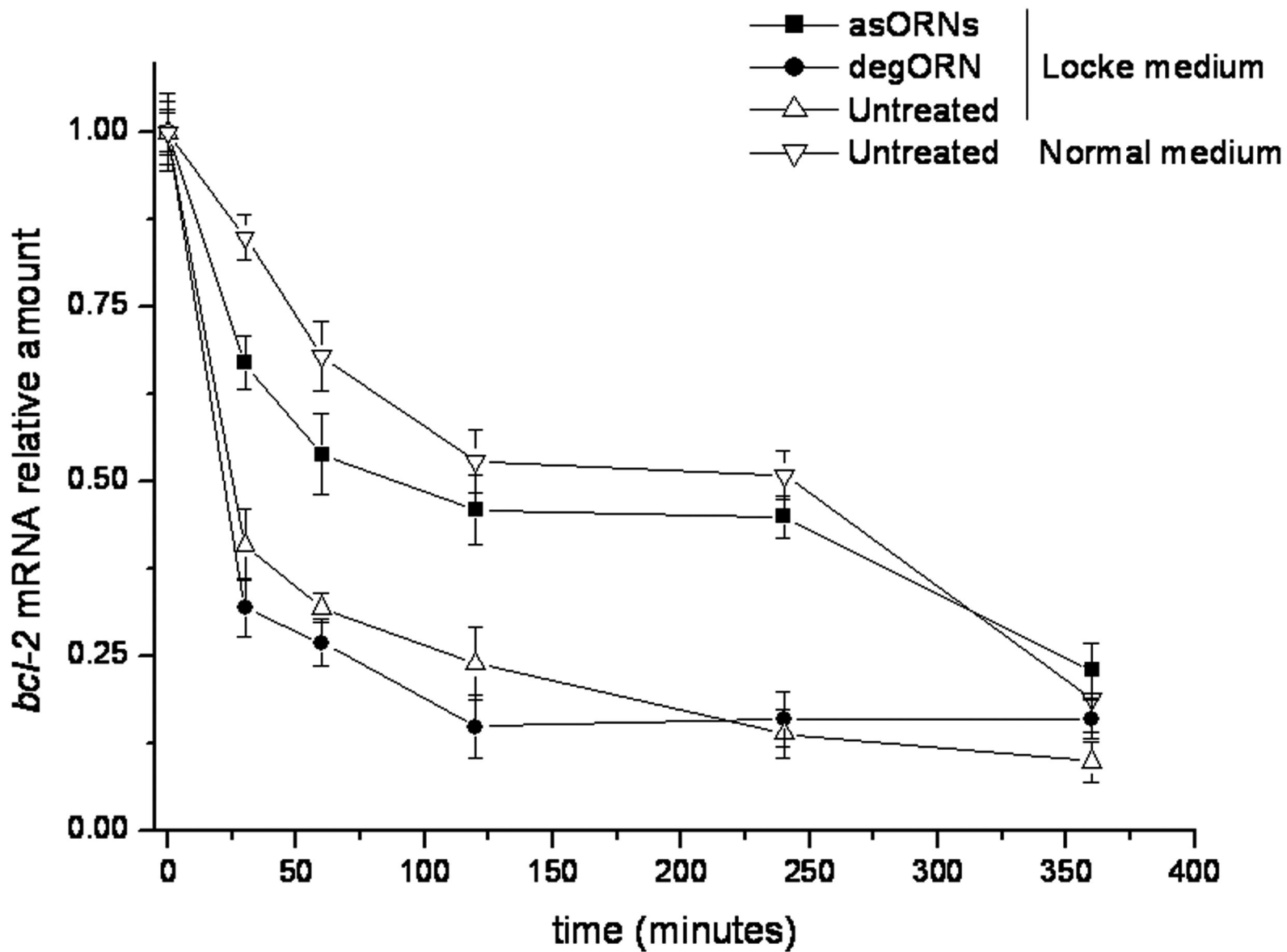representative of five independent experiments.

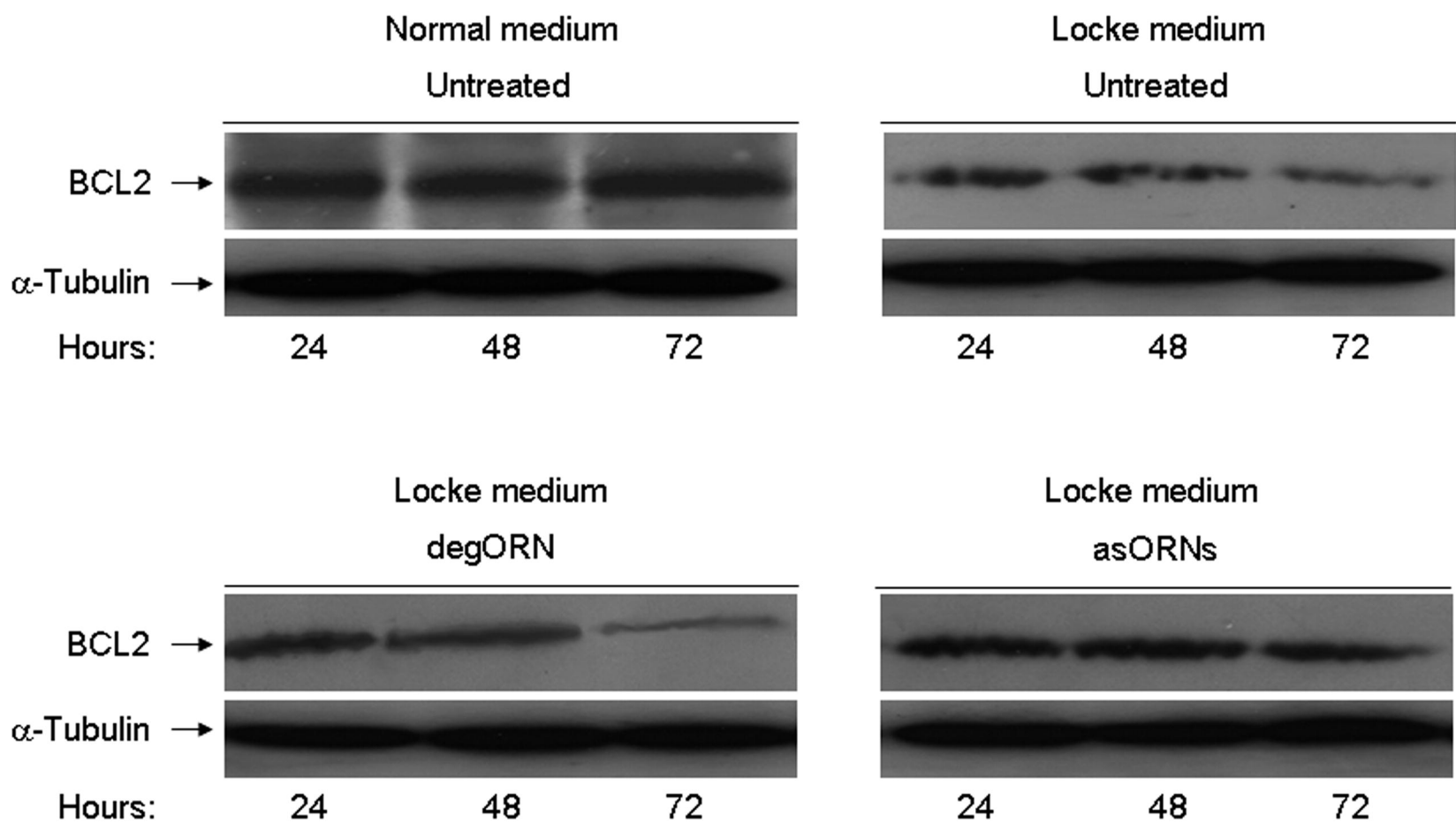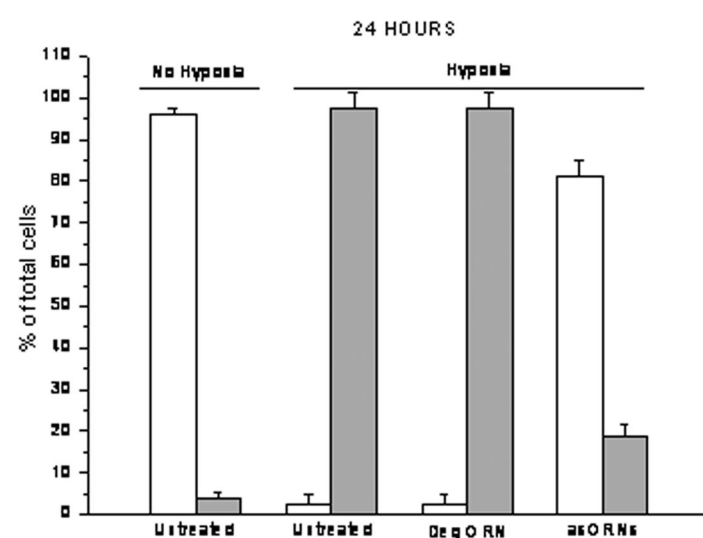**Figure 7. Neuronal differentiation induced by *bcl-2* ARE asORNs**

(a) Cells were treated with asORNs, degORN, transiently transfected as indicated or untreated,

respectively, and analyzed microscopically for sprouting of neurites. Scale bar, 10 µm. (b) The

number of neurites/cell and their length were calculated as indicated in Materials and Methods.

Data are the means ± SE of 5 independent experiments. **$p \leq 0.001$ asORNs compared to degORN

or untreated.

Bcl-2   **BAX**

asORNs →

degORN →

α-Tubulin →

Days: 1   3   5   1   3   5

1

2

Normal medium — Untreated; Locke medium — Untreated; Locke medium — degORN; Locke medium — asORNs. BCL2 and α-Tubulin blots at 24, 48, 72 hours.

3a

3b

4a

4b

Normal medium
Untreated

32 kDa →
17 kDa →
α-Tubulin →

Hours: 0 24 48 72

Locke medium
Untreated

Hours: 0 24 48 72

Locke medium
degORN

32 kDa →
17 kDa →
α-Tubulin →

Hours: 0 24 48 72

Locke medium
asORNs

Hours: 0 24 48 72

Locke medium
Bcl-2 Transfected

32 kDa →
17 kDa →
α-Tubulin →

Hours: 0 24 48 72

Locke medium
Mock Transfected

Hours: 0 24 48 72

5

6a

6b

asORNs

degORN

Untreated
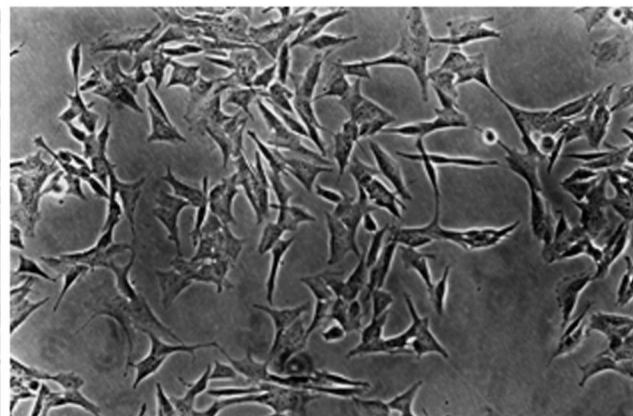
bcl-2 transfected

Mock transfected

7a
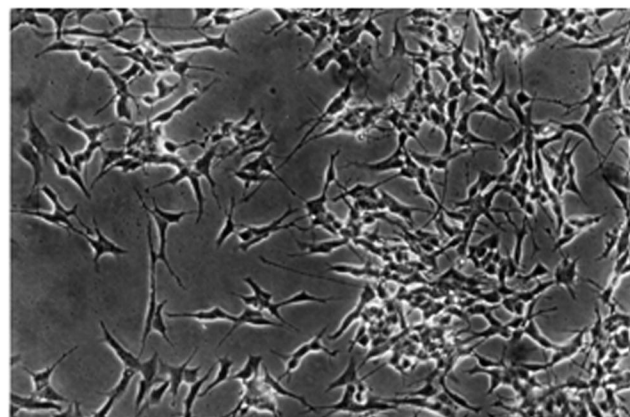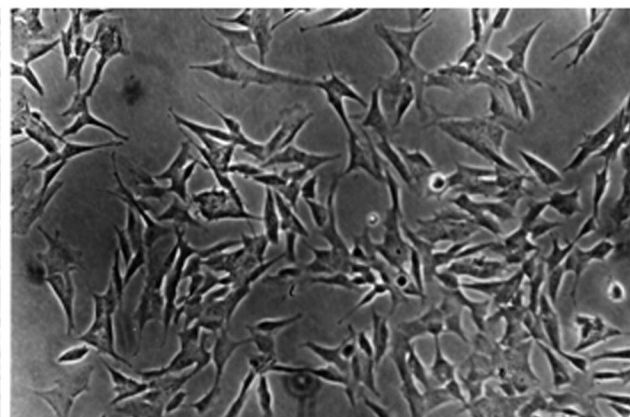
Samples	n° of neurites/50 cells	Neurites/cell
Untreated	13	0.26
degORN	16	0.31
asORNs	48	0.96
bcl-2 transf.	60	1.10
Mock	20	0.49

7b