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# Conditional expression of exogenous Bcl-$X_S$ triggers apoptosis in human melanoma cells in vitro and delays growth of melanoma xenografts

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**Abstract The Bcl-2-related proteins Bcl-$X_L$ and Bcl-$X_S$ represent alternative splice products and exert opposite activities in the control of apoptosis, but their significance for melanoma is not yet clear. Applying the tetracycline-inducible expression system Tet-On, we found overexpression of Bcl-$X_S$ by itself sufficient to induce apoptosis in vitro in stably transfected human melanoma cell lines. Combination with proapoptotic agents such as etoposide, pamidronate, and ceramide resulted in additive proapoptotic effects, whereas Bcl-$X_L$ protected from apoptosis caused via CD95/Fas stimulation. In nude mice growth of melanoma xenotransplants derived from stably transfected cells was significantly reduced after induction of Bcl-$X_S$ by doxycycline. Our results indicate that Bcl-X proteins are of major importance for control of apoptosis in malignant melanoma.**


*Key words:* Bcl-X; Melanoma; Apoptosis; Transfection; Chemotherapeutics

## 1. Introduction

Melanoma is a highly malignant tumor developing from transformed melanocytes or nevus cells. Since apoptosis deficiency is one mechanism of chemotherapeutic resistance as shown for several human tumors [1], further understanding of apoptotic signaling pathways appears helpful also for developing new therapeutic strategies. Important factors in regulation of apoptosis at the mitochondrial level are Bcl-2 proteins which form a large family and segregate into two functional groups of antiapoptotic proteins, such as Bcl-2, A1, Mcl-1, Bcl-W, and Bcl-$X_L$, as well as proapoptotic proteins such as Bax, Bak, Bad, Bik/NBK, Bid, Bim, and Bcl-$X_S$ [2,3]. The activity of several Bcl-2 proteins is considered to be regulated through homo- and heterodimerization, suggesting that their relative concentrations may act as a rheostat for cell suicide programs [4,5]. Recently, it was reported that oligomerization of Bax and Bak to at least tetramers was required for cytochrome *c* release from mitochondria [6,7]. The activity of BH-3-only proteins, a large subgroup of proapoptotic Bcl-2 proteins, seems to be dependent on either Bax or Bak. Thus, exogenous overexpression of Bik/NBK (Bcl-2 interacting killer/natural born killer) triggers apoptosis only in Bax-positive, but not in Bax-deficient cells as shown for colorectal cancer cells [8].

The Bcl-X gene is expressed in several alternative splice products including mRNAs for Bcl-$X_L$ and Bcl-$X_S$ [9,10]. Whereas Bcl-$X_L$ exerts antiapoptotic activity in the outer mitochondrial membrane, Bcl-$X_S$ serves as a dominant negative inhibitor of both Bcl-$X_L$ and Bcl-2 [11]. Another splice variant, Bcl-$X_\beta$, has been shown to interact with Bax but its physiological role with respect to apoptosis control is still unclear [12]. While it was shown to exert a proapoptotic activity in rat promyeloid cells [9] it was antiapoptotic in mouse neurons [13].

Recent investigations have presented evidence that in melanoma cells pro- and antiapoptotic signaling cascades are at least partially active [14], therefore, combination of chemotherapeutics and proapoptotic factors may represent a promising therapeutic strategy for melanoma. In previous studies, we have shown that melanoma cell lines exhibit varying sensitivities to agonistic activation of the CD95/Fas pathway as well as to the sphingolipid ceramide [15]. Resistant melanoma cells were generally characterized by a high Bcl-2/Bax ratio, and the important role of this ratio was confirmed by stably Bcl-2-transfected melanoma cell lines which became resistant [4]. In the present study, we investigated the role of Bcl-$X_S$ and of Bcl-$X_L$ for the regulation of apoptosis in melanoma cells using tetracycline-regulated expression systems (Tet-On and Tet-Off) in vitro and in animal experiments in vivo.

## 2. Materials and methods

### 2.1. Cell culture

Normal human melanocytes (NHM) were isolated from human foreskins after trypsin digestion, and were cultivated under serum-free conditions as previously described [16]. Ten human melanoma cell lines were investigated: A-375 [17]; Bro [18]; Mel-2a [19]; M-5 [20]; Mel-HO [21]; MeWo [22]; SK-Mel-13; SK-Mel-19; SK-Mel-23; SK-Mel-28 [23]. They were maintained in Dulbecco's modified Eagle's medium (4.5 g/l glucose; Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum and antibiotics (Biochrom, Berlin, Germany).

For induction of apoptosis, cells were treated for 10 h with 0.5 μg/ml of the agonistic anti-CD95 antibody CH-11 (IgM mouse monoclonal; Immunotech, Marseille, France) or with 20 μM $C_2$-ceramide in ethanol (Alexis, Gruenberg, Germany). Other cultures were treated for 24 h with 100 μg/ml pamidronate (3-amino-1-hydroxy-propylidene-1,1-bisphosphonate; Novartis Pharmaceuticals, Basel, Switzer-

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*Abbreviations:* NHM, normal human melanocytes; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

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land) or 2 μM etoposide in dimethyl sulfoxide (VP-16; Sigma, Taufkirchen, Germany).

### 2.2. cDNA cloning and subcloning

Full-length cDNA for Bcl-$X_L$ and Bcl-$X_S$ was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) starting with total RNA of the melanoma cell line M-5 using primers corresponding to the untranslated regions at the 5′ end (TTGGACAATGGACTGGTTGA) and at the 3′ end (GTAGAGTGGATGGTCAGTG) of Bcl-X mRNA. The resulting cDNA fragments of Bcl-$X_L$ (765 bp) and Bcl-$X_S$ (576 bp) were subcloned into the TOPO-TA plasmid (Invitrogen, Groningen, The Netherlands), and identity was confirmed by restriction and sequence analysis. The cDNA fragments were further subcloned into the *Eco*RI site of the pTRE-1 plasmid (Clontech, Heidelberg, Germany) downstream of a tetracycline-regulatable promoter resulting in pTRE-Bcl-$X_S$ and pTRE-Bcl-$X_L$.

### 2.3. Cellular transfection

For inducible gene expression, tetracycline-regulatable gene expression systems (Tet-On and Tet-Off; Clontech) described by Gossen and Bujard [24] were applied for melanoma cells. Tetracycline-controllable regulation of apoptosis was investigated after transfection of Tet-On and Tet-Off melanoma cell lines (SKM13-Tet-On, Bro-Tet-On and Mel2a-Tet-Off) with pTRE-Bcl-$X_L$ and pTRE-Bcl-$X_S$. Tetracycline-regulatable cell lines established by stable transfection of pTet-On and pTet-Off plasmids (Clontech) into melanoma cell lines SK-Mel-13, Bro, and Mel-2a, respectively, have been described previously [25]. In vitro promoter induction of Tet-On cell lines or repression of the Tet-Off cell line Mel-2a was achieved by addition of doxycycline (2 μg/ml; ICN, Aurora, OH, USA) to the growth medium.

Transfections of melanoma cells were performed at a cellular confluence of 50% using 0.5% Pfx-2 (Invitrogen) and 1.5 μg/ml plasmid DNA as described previously [26]. Stably transfected cell clones were selected with 100 μg/ml hygromycin (Life Technologies) and/or 400 μg/ml geneticin (Boehringer, Mannheim, Germany). Individual cell clones were obtained by limited dilution in microtiter plates and were continuously cultured under antibiotic pressure. Stable SKM13-Tet-On cell clones were established by transfection of pTRE-Bcl-$X_L$ and pTRE-Bcl-$X_S$. Clones were screened after transfection for mRNA overexpression by Northern blot analysis, and two cell clones each were further investigated (SKM13-Bcl-$X_S$-3, -5 and SKM13-Bcl-$X_L$-11, -15). As controls, pTRE-1-transfected cell clones were generated from SKM13-Tet-On (mock).

### 2.4. Quantification of apoptosis and cytotoxicity

For promoter induction, cells were treated with doxycycline for 48 h immediately following transient transfection. Stably transfected cell clones were induced with doxycycline also for 48 h at a cellular confluence of 50%. Apoptosis was subsequently quantified by using a cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics, Mannheim, Germany), which detects mono- and oligonucleosomes formed in apoptotic cells according to a protocol described previously [26]. Relative apoptotic rates were calculated as the ratio of ELISA values between doxycycline-induced and non-induced cells or by comparison to vector-transfected cells (pTRE-1). Each assay consisted of triple values, and at least three independent experiments were performed.

Cytotoxicity was determined in parallel by a lactate dehydrogenase assay (Roche Diagnostics) as described previously [27].

### 2.5. Hoechst-33258 staining

Cells were harvested by trypsinization, fixed in 4% formaldehyde (methanol-free) for 30 min at 4°C and washed once with phosphate-buffered saline (PBS). 1 μg/ml Hoechst-33258 dye (Sigma) was added for 20 min at room temperature after which cells were washed again with PBS. Cells were mounted (MoBiTec, Göttingen, Germany) and examined by fluorescence microscopy. Apoptotic cells were identified by condensed and fragmented nuclei. For quantification, a minimum of 500 cells were counted, and the ratio of apoptotic cells was calculated as percent of total cells counted.

### 2.6. Expression analyses

Expression of mRNA was examined by Northern blot analysis and by RT-PCR. The primers used for RT-PCR (for sequences see above) enclosed the reading frames for both Bcl-$X_S$ and Bcl-$X_L$ and enabled simultaneous mRNA amplification.

For protein extraction, cells were harvested 48 h after doxycycline treatment of transient transfectants or stable cell clones, with lysis buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10% glycerine, 0.2% sodium dodecyl sulfate (SDS), 10 μg/ml trasylol (Bayer, Leverkusen, Germany), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 5 μg/ml pepstatin. Extracts were homogenized and centrifuged at 10 000 × g for 10 min. SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis of total proteins were performed as previously described [26]. Western blots were incubated with anti-Bcl-X polyclonal antibody (M-125, sc-1690; Santa Cruz, Heidelberg, Germany; 1:200). Following incubation with a peroxidase-labelled anti-rabbit secondary antibody (Dako, Hamburg, Germany; 1:5000), antigen–antibody complexes were detected by ECL Western blotting detection reagents on Hyperfilm ECL (Amersham Pharmacia Biotech, Freiburg, Germany).

### 2.7. Animal studies

For inoculation, melanoma cells were harvested by brief incubation with trypsin/EDTA, and subsequently trypsin was inactivated by washing cells twice with growth medium containing 10% fetal calf serum. Briefly before inoculation, cells were washed again with PBS and were finally resuspended in PBS in a concentration of $10^6$ cells/200 μl. Into both flanks of female BALB/c nu/nu mice at an age of 6–8 weeks (M&B, Ry, Denmark) each $10^6$ cells (200 μl) were injected subcutaneously. For in vivo induction of the tetracycline-responsive promoter, animals received doxycycline (2 mg/ml) in the drinking water further enriched with 50 mg/ml sucrose beginning 1 day after inoculation, whereas control mice received only sucrose-enriched water. After tumors became visible, body weight and tumor size were measured twice a week. For animal experiments, the guidelines of the Federation of European Laboratory Animal Sciences Associations (FELASA) were applied.

## 3. Results

### 3.1. Bcl-$X_L$ is strongly expressed in melanoma cell lines in vitro in contrast to Bcl-$X_S$

Expression of Bcl-X proteins was investigated by Western blot analysis in a series of eight human melanoma cell lines and seven primary cultures of NHM. Strong expression levels of Bcl-$X_L$ with a size of 29 kDa was seen in four melanoma

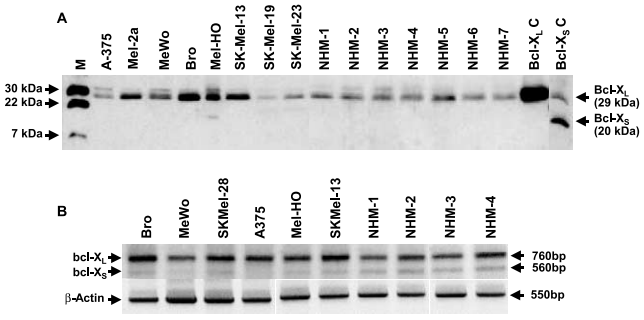


Fig. 1. Weak expression of Bcl-$X_S$ in human melanoma cell lines. A: Protein expression was analyzed in eight melanoma cell lines and in seven cultures of NHM. Each 60 μg of protein lysates were separated by SDS–PAGE (12%). The anti-human Bcl-X antibody used recognizes both Bcl-$X_L$ and Bcl-$X_S$, as shown by positive controls of stably transfected melanoma cells (Bcl-$X_L$-C and Bcl-$X_S$-C), however, Bcl-$X_S$ protein was not detectable in untransfected melanocytic cells. A molecular weight marker is given on the left (M). An extra band of 34 kDa obtained in some cell lines in this blot was not reproducible in other Western blots. B: mRNA expression of Bcl-$X_L$ and Bcl-$X_S$ was analyzed in six melanoma cell lines and four cultures of NHM by means of RT-PCR. The PCR primers included both coding regions to allow simultaneous amplification of Bcl-$X_L$ (760 bp) and Bcl-$X_S$ (560 bp); β-actin was used as PCR control (550 bp). As can be seen, melanoma cell lines showed a tendency to stronger expression of Bcl-$X_L$ and weaker expression of Bcl-$X_S$ as compared to NHM.

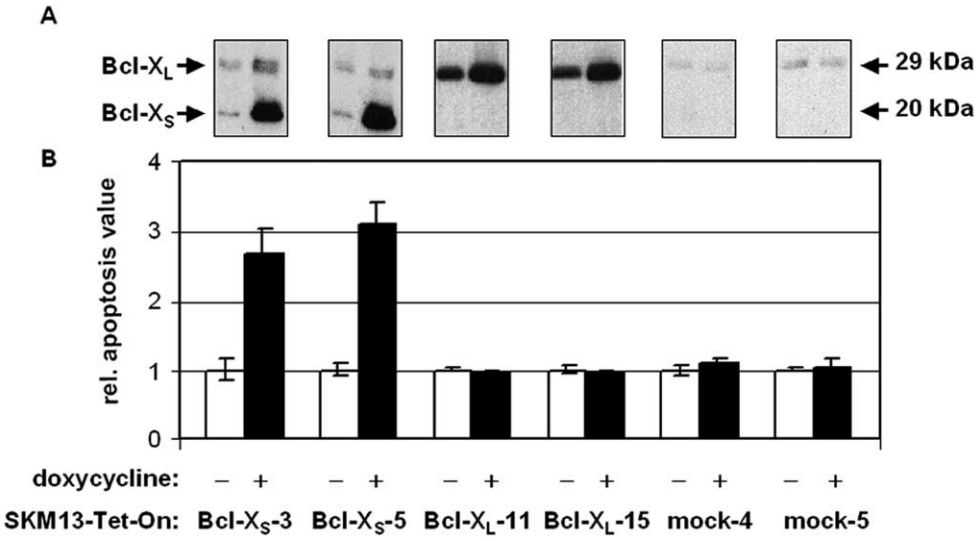


Fig. 2. Exogenous expression of Bcl-$X_S$ induces apoptosis in stably transfected melanoma cells. Cell clones resulted from stable transfection of SKM13-Tet-On with pTRE-Bcl-$X_L$ (SKM13-Bcl-$X_L$-11, -15), with pTRE-Bcl-$X_S$ (SKM13-Bcl-$X_S$-3, -5), as well as with pTRE-1 plasmid (mock-4 and mock-5). A: For Western blot analysis of total proteins, an antiserum specific for both Bcl-$X_S$ and Bcl-$X_L$ was used. Equal amounts of proteins (60 μg) were loaded in each lane. Strong induction of Bcl-$X_S$ and Bcl-$X_L$ can be seen after doxycycline induction, whereas mock-transfected cells were unaffected. B: Apoptosis was quantified by determination of DNA fragmentation, and relative apoptotic rates were calculated as the ratio of cells treated with doxycyline versus untreated cells. Relative apoptotic rates of untreated cells were separately set at 1. Values represent the mean of a triple experiment ± S.D., and each experiment was performed at least three times giving comparable results.

cell lines (SK-Mel-13, Mel-HO, Bro, and A-375), whereas all in vitro cultures of normal melanocytes investigated as well as the remaining four melanoma cell lines revealed only moderate expression. In contrast, Bcl-$X_S$ protein with a size of 20 kDa was not detectable in any of the melanocytic cell cultures studied. Specificity of the used antibody for both Bcl-$X_L$ and Bcl-$X_S$ was proven by using stably transfected cell clones as positive controls (Fig. 1A). An extra band of 34 kDa obtained in the blot shown in Fig. 1 was not reproducible in other Western blots and may therefore result from unspecific binding of the antibody used.

Northern blot analysis revealed a largely parallel expression

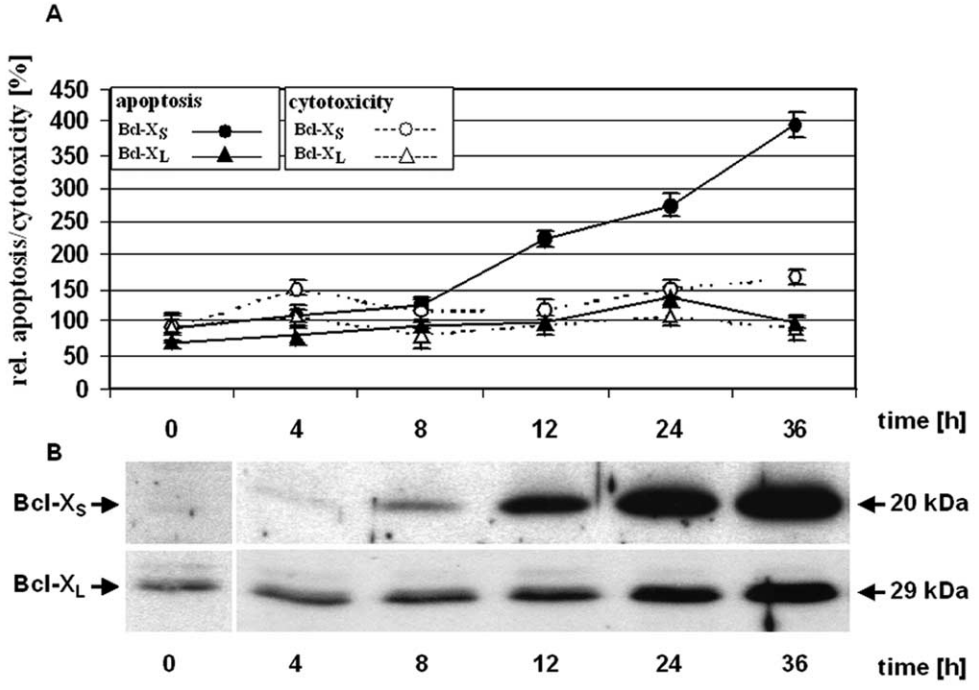


Fig. 3. Time-dependent induction of apoptosis by Bcl-$X_S$. A: SKM13-Tet-On melanoma cells stably transfected with pTRE-Bcl-$X_S$ (Bcl-$X_S$-5, circles) and pTRE-Bcl-$X_L$ (Bcl-$X_L$-15, triangles) were treated with doxycycline beginning at $t = 0$, and relative apoptotic rates (filled symbols) and cytotoxicity (open symbols) were determined at different times after induction. B: Parallel cultures were used for isolation of total cellular protein and for subsequent Western blot analysis. Note that high expression of the Bcl-$X_L$ protein did not cause apoptosis and that Bcl-$X_S$ expression correlated with increased apoptosis but not with increased cytotoxicity.

pattern for Bcl-$X_L$ mRNA, whereas Bcl-$X_S$ mRNA was also not detectable in melanocytic cultures by Northern blot analysis (data not shown). Simultaneous RT-PCR amplification of Bcl-$X_L$ and Bcl-$X_S$ mRNA proved expression of Bcl-$X_S$ in all melanocytic cell cultures, however, its expression was significantly lower than that of Bcl-$X_L$. Apparent weaker signals for Bcl-$X_S$ were found in three melanoma cell lines (MeWo, A-375, Mel-HO), as compared to normal melanocytes (Fig. 1B).

### 3.2. *Inducible overexpression of Bcl-$X_S$ triggers apoptosis in melanoma cell lines*

Full-length cDNAs of Bcl-$X_L$ and Bcl-$X_S$ were cloned and inserted into a tetracycline-regulatable expression plasmid resulting in pTRE-Bcl-$X_L$ and pTRE-Bcl-$X_S$. After transient transfection of pTRE-Bcl-$X_S$ in the tetracycline-regulatable melanoma cell lines SKM13-Tet-On and Mel2a-Tet-Off, apoptosis was significantly elevated by doxycycline induction in SKM13-Tet-On (132 ± 4%) and respectively elevated by doxycycline withdrawal in Mel2a-Tet-Off (167 ± 17%), as compared to transfected non-induced cells. On the other hand, transient transfection of Bcl-$X_L$ resulted in decreased basic apoptotic rates in Mel2a-Tet-Off (62 ± 6%) and in Bro-Tet-On (68 ± 5%) after induction. Western blot analysis performed in parallel showed inducible overexpression of Bcl-$X_L$ and Bcl-$X_S$ proteins after transient transfection, however, the expression level of both proteins was relatively low after transient transfection (data not shown).

After stable transfection of SKM13-Tet-On with pTRE-Bcl-$X_L$ and pTRE-Bcl-$X_S$, expression of Bcl-$X_S$ was induced 30–40-fold in SKM13-Bcl-$X_S$-3 and SKM13-Bcl-$X_S$-5 after doxycycline induction, and expression of Bcl-$X_L$ was induced three-fold in SKM13-Bcl-$X_L$-11 and SKM13-Bcl-$X_L$-15 as shown by Western blot analysis. Also, elevated expression of Bcl-X proteins was seen without doxycycline treatment (leakiness), which was more pronounced in Bcl-$X_L$ clones (Fig. 2A). In parallel to Bcl-$X_S$ expression, apoptosis was strongly enhanced by 2.7-fold and three-fold in Bcl-$X_S$-3 and Bcl-$X_S$-5 after doxycycline treatment (Fig. 2B).

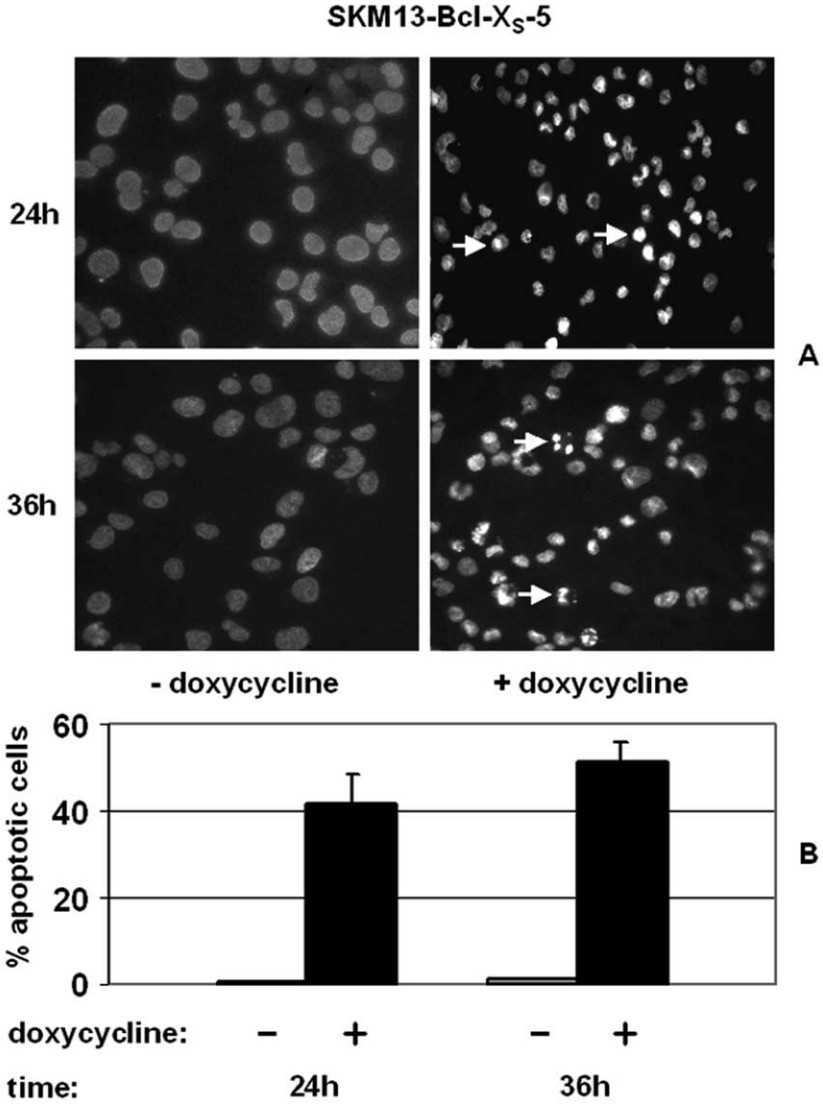


Fig. 4. Induction of Bcl-$X_S$ in melanoma cells causes nuclear condensation and fragmentation. Cell clones resulted from stable transfection of SKM13-Tet-On with pTRE-Bcl-$X_S$ (SKM13-Bcl-$X_S$-5). A: Nuclear condensation and fragmentation were visualized by Hoechst-33258 staining. 24 h and 36 h after induction of Bcl-$X_S$ by doxycycline, a high proportion of cells showed clear indications of apoptosis (examples indicated by arrows). B: The quantitative data represent mean values of triplicate experiments ± S.D.

Time kinetics revealed induced expression of Bcl-X proteins in Bcl-$X_S$-5 and Bcl-$X_L$-15 as early as 8 h after addition of doxycycline, and expression levels further increased up to 36 h. This was paralleled by stepwise induced apoptosis in Bcl-$X_S$-5, whereas induction of Bcl-$X_L$ did not trigger apoptosis. Cytotoxicity determined in parallel was generally low for both subclones at all times (Fig. 3).

Typical morphological signs of apoptosis (nuclear condensation and fragmentation) were observed after labeling with Hoechst-33258 in the cell clone Bcl-$X_S$-5 24 h and 36 h after induction of Bcl-$X_S$ (Fig. 4A). Cell counting revealed a high number of apoptotic cells of 40 ± 7% (24 h) and 50 ± 5% (36 h) after Bcl-$X_S$ induction as compared to only 1–2% found in non-induced cells (Fig. 4B).

### 3.3. *Bcl-$X_S$ enhances the effect of additional proapoptotic stimuli, whereas Bcl-$X_L$ blocks CD95-induced apoptosis*

When Bcl-$X_L$ expression was induced in two stably transfected subclones of SKM13-Tet-On (Bcl-$X_L$-11 and Bcl-$X_L$-15) by pre-incubation with doxycycline for 38 h, the proapoptotic effect of the CD95 agonistic antibody CH-11 seen in mock-transfected cells was strongly antagonized. Also without induction, a reduced apoptotic effect of CH-11 was seen in Bcl-$X_L$-transfected cells, likely due to leaky expression from the tetracycline promoter (Fig. 5A).

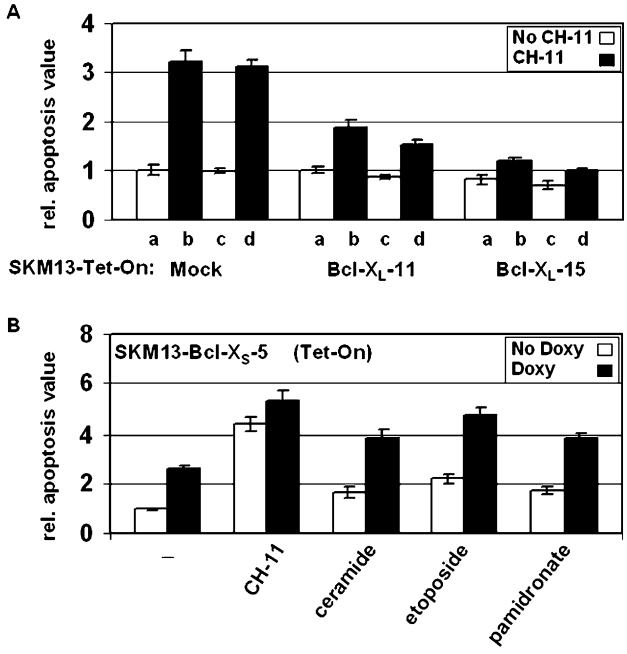


Fig. 5. Bcl-$X_S$ increases the effect of proapoptotic stimuli, whereas Bcl-$X_L$ inhibits CD95-dependent apoptosis. A: Two SKM13-Tet-On subclones expressing Bcl-$X_L$ after doxycycline treatment (Bcl-$X_L$-11 and Bcl-$X_L$-15) and a vector-transfected control clone (mock) were grown without doxycycline (a,b) or were preincubated with doxycycline for 38 h (c,d). For induction of apoptosis, cells were treated in addition with the agonistic CD95 antibody CH-11 for another 10 h (b,d). Relative apoptotic rates were calculated in relation to untreated mock cells, which were set at 1. B: The SKM13-Tet-On subclone Bcl-$X_S$-5 expressing Bcl-$X_S$ after doxycycline treatment was preincubated for 24 h or 38 h with doxycycline (black columns) or left without (white columns). After preincubation, cells were treated in addition with different proapoptotic stimuli for a further 10 h (CH-11, ceramide) or for 24 h (etoposide, pamidronate). Relative apoptotic rates were calculated with respect to untreated cells, which were set at 1. Values of A and B represent the mean of triple experiments ± S.D.; each experiment was performed at least three times giving comparable results.

On the other hand, effects of various proapoptotic stimuli were significantly enhanced by concomitant expression of Bcl-$X_S$. Apoptosis induction showed an additive relation for the combination with etoposide, pamidronate and ceramide, however, CH-11-induced apoptosis was only moderately enhanced after Bcl-$X_S$ induction (Fig. 5B). This may result from the fact that SKM13-Bcl-$X_S$ cells were already highly sensitive to CH-11 without induction (Fig. 5B), as also shown for mock-transfected cells (Fig. 5A).

### 3.4. *Exogenous Bcl-$X_S$ expression inhibits melanoma growth in mice*

In animal experiments using subcutaneous injections of the stably transfected Bcl-$X_S$-5 melanoma cell clone, reduced tumor growth was found when mice received doxycycline in the drinking water for induction of Bcl-$X_S$ expression. In five control mice large tumors had developed after 30 days, whereas the average tumor size was seven times smaller in five animals treated with doxycycline. Furthermore, melanoma growth was further retarded up to day 51, when tumors were still four times smaller in doxycycline-treated animals, as compared to untreated controls (Fig. 6A). In contrast, when control mice were inoculated with mock-transfected cells, doxycycline treatment had no significant effect on tumor growth (data not shown). Interestingly, 7 weeks after inoculation rapid tumor growth also appeared in doxycycline-treated animals (Fig. 6B).

## 4. Discussion

In the present study we were able to show the relevance of Bcl-X proteins for the regulation of apoptosis in human melanoma cells. Bcl-$X_L$ is structurally and functionally closely related to Bcl-2 [28], and its antiapoptotic function for certain tumor cell types has been reported [29,30]. In our study we detected high levels of Bcl-$X_L$ in human melanoma cell lines at both the protein and the mRNA levels as compared to in vitro cultures of NHM. Relatively high expression levels of Bcl-$X_L$ and Bcl-2 have been reported in several tumor cell entities such as in adenocarcinoma cells and in mammary carcinoma cells [30–34]. In melanoma cells, expression of Bcl-$X_L$ was investigated earlier at the mRNA level by RT-PCR, and the degree of its expression seemed to be correlated with tumor progression [35].

Our present data indicate that exogenous overexpression of Bcl-$X_L$ leads to significant reduction of basic apoptotic rates in melanoma cells and can confer partial resistance to CD95-induced apoptosis. Also in mammary carcinoma cells Bcl-$X_L$ overexpression neutralized the proapoptotic effect of CD95 ligand and tumor necrosis factor-α [36], and in neuroblastoma cells, high expression of Bcl-$X_L$ was shown to be responsible for chemoresistance and tumorigenicity [29,32]. Finally, increased chemoresistance against cisplatin had been found after exogenous overexpression of Bcl-$X_L$ in a melanoma cell line [37].

The mechanism of apoptosis induction by Bcl-$X_S$ is largely unknown and may partially depend on dominant negative inhibition of Bcl-$X_L$ and Bcl-2 [11,38]. According to our investigations, Bcl-$X_S$ was only weakly expressed in melanocytic cells and even showed a tendency to lower expression in mel-

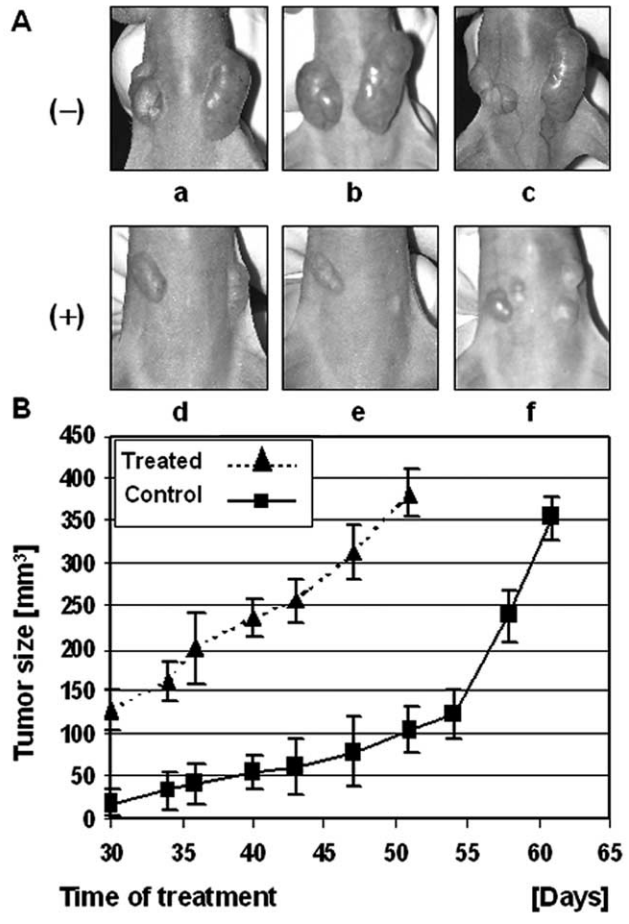


Fig. 6. Overexpression of Bcl-$X_S$ inhibits melanoma growth in vivo. A: Photographs of living nude mice were taken 7 weeks after subcutaneous injection of SKM13-Bcl-$X_S$-5 cells into both flanks. Large tumors developed when mice were left untreated (a–c), whereas treatment with doxycycline from the beginning of the experiment resulted in significantly smaller tumors (d–f; each 3/5 animals shown). B: Tumor development is shown between day 30 and day 60 after inoculation (each means for five animals/10 tumors). Triangles, interrupted line: untreated mice, experiment stopped at day 51. Squares, continuous line: mice received doxycycline for Bcl-$X_S$ induction.

anoma cell lines, as compared to cultures of normal melanocytes. Weak expression of Bcl-$X_S$ was also found for other tumor cells such as mammary carcinoma and neuroblastoma [39,40], and no Bcl-$X_S$ expression had been reported for another panel of four melanoma cell lines [41].

Tetracycline-regulatable gene expression offers several advantages such as high inducibility and fast response times [42]. Possible pleiotropic effects of doxycycline on melanoma cells were ruled out in our experimental system by utilizing both Tet-Off and Tet-On melanoma cell lines in transient transfections. The stable transfections were performed with a Tet-On cell line derived from SK-Mel-13 which is characterized by an average melanocytic differentiation status [43]. Leakiness of the tetracycline-regulatable promoters as seen here in melanoma cells had been reported previously [44], and was explained by absence of chromatin repression, high copy numbers, and possibly nearby enhancers [45].

By using the Tet-On gene expression system, we showed that apoptosis can be triggered in stably transfected melanoma cells by induction of Bcl-$X_S$ expression, and the combination of Bcl-$X_S$ expression with additional apoptotic stimuli such as etoposide [46], ceramide [47], pamidronate [48], and agonistic CD95 activation [15] resulted in additive proapoptotic effects.

The induction of apoptosis resulted from a high proportion of apoptotic cells as shown by chromatin staining which is a generally accepted technique for identification of apoptotic cells [49,50]. In accordance with these data, adenovirus-mediated expression of Bcl-$X_S$ had been reported to induce apoptosis in mammary carcinoma and neuroblastoma cells [39,40,51]. A sensitization to chemotherapeutics by overexpression of Bcl-$X_S$ as shown here for melanoma has only been described for MCF-7 mammary carcinoma cells when treated with either taxol or etoposide. In MCF-7 Bcl-$X_S$ overexpression, however, was not able to trigger apoptosis by itself after stable transfection [46].

The significance of apoptosis induction by Bcl-$X_S$ for reduced melanoma tumorigenicity was demonstrated in vivo in human xenotransplants on nude mice. Induction of Bcl-$X_S$ resulted in reduced tumor size and delayed tumor development. Recovered tumor growth after 7 weeks may be driven by acquired resistance of the tumor cells to Bcl-$X_S$-induced apoptosis, but the basis for developing resistance still remains unclear. Inhibition of tumor growth due to Bcl-$X_S$ expression in vivo has been reported only for MCF-7 mammary carcinoma cells after repeated intratumoral injection of Bcl-$X_S$ adenovirus [40].

Our data underline the decisive role of Bcl-X proteins for the regulation of apoptosis in melanoma. The strong proapoptotic effect of exogenous Bcl-$X_S$ expression in melanoma cells both in vitro and in vivo, shown here for the first time, may be of further value for therapeutic approaches against melanoma. Particularly the additive proapoptotic effect of Bcl-$X_S$ in conjunction with various chemotherapeutics appears promising for combination therapies.

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