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Bcl-xL inhibits p53- but not apoptin-induced apoptosis in head and neck squamous cell carcinoma cell line

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Abstract

Non-functional p53 and especially up-regulation of Bcl-xL results in advanced disease and poor prognosis of patients suffering head and neck squamous cell carcinoma (HNSCC). Aberrancies of Bcl-xL and/or p53 in HNSCC lead to inability of anti-cancer drugs to induce apoptosis. Bcl-xL and/or mutated p53 inhibit the apoptotic process by preventing the mitochondrial release of cytochrome c and/or activation of execution caspases. Here, we report that expression of the avian-virus-derived apoptin protein resulted in induction of apoptosis in the HNSCC-derived cell line UMSSC-14B, despite the presence of non-functional p53. Apoptin activated the execution caspase-3 and induced the release of mitochondrial cytochrome c. Up-regulation of Bcl-xL in UMSSC-14B cells did not interfere with the apoptin-induced apoptosis, whereas it clearly negatively affected the p53-induced one. Bcl-xL significantly decreased the p53-induced cytochrome c release, but not the apoptin-triggered one. Our data demonstrate that apoptin induces apoptosis independent of Bcl-xL and p53, and may constitute a potential therapeutic agent for treatment of HNSCC.

Introduction

Head and Neck squamous cell carcinoma (HNSCC) accounts for more than 4% of all malignancies¹. Despite recent treatment advances with surgery, chemotherapy and radiation HNSCC is still among the most deadliest in the world² and rates of local recurrence and distant metastases are high³.

Impairment of apoptosis, the physiological process in which cells undergo self-destruction, plays an important role in the development of various cancers such as HNSCC⁴. Tumor cells overwhelmingly contain genetic lesions in the apoptotic decision pathway, such as mutated tumor-suppressor protein p53⁵ or over-express antiapoptotic proteins Bcl-2 and Bcl-xL⁶. In particular, Bcl-xL over-expression strongly contributes to the development of HNSCC⁷. Bcl-xL inhibits apoptosis via prevention of cytochrome c release from mitochondria into the cytosol, which is a crucial event during apoptosis for this step activates the downstream effector caspases resulting in irreversible execution of the apoptotic process^{6,8}.

HNSCC cells still can undergo apoptosis as soon as the right apoptotic signal is offered to them⁸. Unfortunately, many conventional therapies fail because they induce apoptosis via p53⁹ and/or are inhibited by antiapoptotic proteins such as Bcl-xL¹⁰. Furthermore, it has been shown that especially Bcl-xL is correlated with resistance to radiotherapy¹¹ and chemotherapeutic agents such as bleomycin, cisplatin, etoposide, vincristine, and doxorubicin¹². Moreover, over-expression of Bcl-xL plays a pivotal role in irradiation resistance independent of p53 status¹² and its over-expression increases the metastatic activity of cancer cells¹³.

Therefore, novel strategies for therapies treating HNSCC are needed to bypass the resistance for undergoing apoptosis due to high levels of Bcl-xL⁶. Previous *in-vitro* studies with the chicken anemia virus-derived protein apoptin demonstrated that this 121 amino acid protein can induce apoptosis independent of p53¹⁴ and is even stimulated by the antiapoptotic Bcl-2¹⁵. Furthermore, apoptin induces programmed cell death in immortalized

and transformed cells, but not in normal, diploid cells¹⁶. Apoptin-induced apoptosis does not require upstream caspases but activation of caspase-3 and possibly other downstream caspases are essential for a rapid apoptin-induced apoptosis¹⁷. Therefore, apoptin is a potential antitumor agent. In the present study, we explored apoptin's potency further for a possible treatment of HNSCC. We have examined whether apoptin can induce apoptosis in HNSCC-derived cells in the presence of high levels of Bcl-xL and independent of functional p53.

Materials and Methods

Cell culture.

The earlier described human head and neck squamous carcinoma cell line UMSCC-14B was a generous gift of Dr TE Carey, University of Michigan Medical Center, MI, USA. The cell line was established from a poorly differentiated squamous cell carcinoma of the floor of the mouth and has a mutated p53¹⁸. The cell line was routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin and streptomycin (Life Technologies, Rockville, Md.). Cultures were incubated at 37°C and passed twice a week by trypsinization. Eight hours after been passed in 6-cm-diameter dishes or 6-well plates, 20-40% confluent cultures of UMSCC-14B cells were transiently transfected with plasmid DNA.

Plasmids and transfection.

The plasmid pCMV-VP3 encoding apoptin and plasmid pCMV-p53 encoding p53 have been described before¹⁹. The plasmid pcDNA3.1/MycHis/LacZ (Invitrogen, Groningen, The Netherlands) encodes the negative apoptosis control LacZ with a Myc and His-tag attached to the C-terminus, regulated by a CMV promoter. The plasmid pCMV-Bcl-xL was constructed by cloning the complete Bcl-xL sequence into the EcoRI/

NotI site of the plasmid pcDNA3 (Invitrogen) downstream of T7 and CMV promoters²⁰, and was kindly provided by Dr S Korsmeyer, Harvard Medical School, Boston, MA, USA). The cells were transfected with Fugene 6 according the protocols of the manufacturer Roche Molecular Biochemicals, Almere, The Netherlands. Two µg DNA was transfected per dish.

Subcellular fractionation and Western Blot Analysis.

Subcellular fractionation was essentially performed as described by Juin et al.²¹. After transfection, the UMSSC-14B-cell monolayer was washed twice with ice-cold phosphate-buffered saline (PBS). The cell monolayer (10-6 cells/100 mm dish) was rinsed twice with ice-cold PBS, then scraped and centrifuged at 2000 g for 5 min. The cells were resuspended in cell extraction buffer (300 mM sucrose, 10 mM Hepes [pH 7.4], 50 mM KCl, 5 mM EGTA, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 100 µM cytochalasin B), left on ice for 30 min, and homogenized by means of vortexing the lysates. Unbroken cells and nuclei were pelleted by centrifugation for 5 min at 2000 g. Heavy membranes were removed from the resulting supernatant by centrifugation for 5 min at 14000 g. The resulting supernatant is the crude cytosolic fraction. All samples were frozen in liquid nitrogen and stored at -80° C until analysis by sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of cytosolic protein extract were boiled in reducing sample buffer for 5 min, loaded in a lane of a sodium dodecyl sulfide-15% polyacrylamide (SDS-PAA) gel, and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked overnight in PBST-buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat dried milk. Blots were incubated with the primary antibody. Bcl-xL was detected with the monoclonal antibody H-62 (dilution 1:200 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and cytochrome c with monoclonal antibody 7H8.2C12 (dilution 1:1000 Pharmingen, San Diego, CA, USA). Positive signals were visualized by enhanced chemiluminescence according

to the manufacturer's protocol (Amersham, Roosendaal, The Netherlands).

Immunofluorescence.

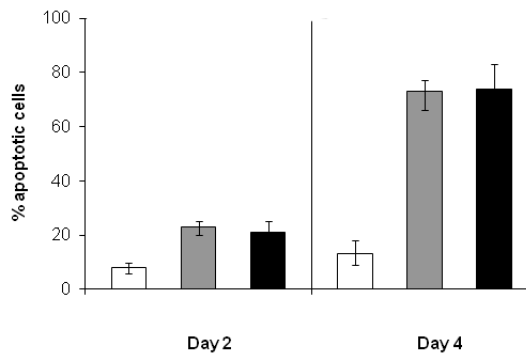
At different time points after transfection, UMSCC-14B cells were fixed with 80% acetone for 10 minutes. Indirect immunofluorescence assays were performed as described by Danen-van Oorschot et al.¹⁷. Prior to incubating the cells with antibodies, the cells were incubated for 30 minutes with PBS containing 0.05% Tween-20 and 5% normal goat serum. Apoptin was detected with mouse monoclonal antibody CVI-CAV-111.3 (supernatant, dilution 1:3)¹⁴, myc-tagged LacZ with anti-myc antibody 9E10 (dilution 1:2000)²², p53 with antibody DO-1 (dilution 1:100), and Bcl-xL with monoclonal antibody MAB4620 (dilution 1:200) (Chemicon International). A rabbit polyclonal antibody (dilution 1:200) was used to detect active caspase-3 (Pharmingen, San Diego, CA, USA). Secondary antibodies were either fluorescein isothiocyanate-labeled goat anti-mouse antibodies or rhodamine-labeled goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The chromatin was stained with 2,4 diamidino-2-phenylindole (DAPI). In apoptotic cells, the chromatin is condensed, the nucleus is often fragmented, and finally DAPI staining is lost due to fragmentation of the DNA²². The cells were analyzed by fluorescence microscopy (Olympus, Zoeterwoude, The Netherlands) for expression of the transfected protein, nuclear morphology or caspase-3 activity, indicating the apoptotic state of the cell. At least 100 cells per independent time point were counted to determine the percentage of apoptotic cells.

Results

Apoptin induces apoptosis in UMSSC-14B cells, which express non-functional p53.

To examine whether apoptin and wild-type p53 can induce apoptosis in HNSCC cells containing non-functional p53, the human HNSCC cell line UMSSC-14B was transiently transfected with plasmids encoding apoptin, wild-type p53 as a positive control, or LacZ as negative control. The cells were screened for production of the various transgenes by indirect immunofluorescence. Apoptosis was analyzed by staining with DAPI, which is known to stain intact nuclei strongly but apoptotic ones irregularly and weakly. Two days after transfection, approximately 20% of both the p53- and apoptin-positive cells became apoptotic, which increased after four days to approximately 80% for both p53- and apoptin-positive cells (Figure 1). The UMSSC-14B cells expressing non-apoptotic LacZ underwent up to 15% cell death, which is most likely due to the used transfection method¹⁵. These data indicate that apoptin can induce apoptosis in squamous cell carcinoma-derived cells lacking functional p53.

Figure 1



Apoptin induces apoptosis in HNSCC-derived UMSSC-14B cells lacking functional p53. The cells were transiently transfected with pCMV-VP3 encoding apoptin (black bars), pCMV-p53 (grey bars) or pCMV-LacZ (open bars). Cells were fixed 2 days (left panel) or 4 days (right panel) after transfection and analyzed by indirect immunofluorescence. The percentage of cells that stained abnormally with DAPI is given as a relative measure for apoptosis. Results are the means of at least three independent experiments. In each experiment at least 100 cells were examined that were positive for each transfected transgene product.

Apoptin induces cytochrome c release and caspase-3 activity.

Next, we investigated whether cytochrome c is released from mitochondria during apoptin-induced apoptosis. To that end, UMSSC-14B cells were transfected with plasmids encoding apoptin, p53 or LacZ. Three days later, cytosolic extracts were prepared by subcellular fractionation and analyzed by Western blotting. In cells transfected with apoptin or p53, the levels of cytochrome c were increased compared to cells transfected with the negative control LacZ (Figure 2), which indicates that cytochrome c is released from mitochondria during apoptin-induced apoptosis.

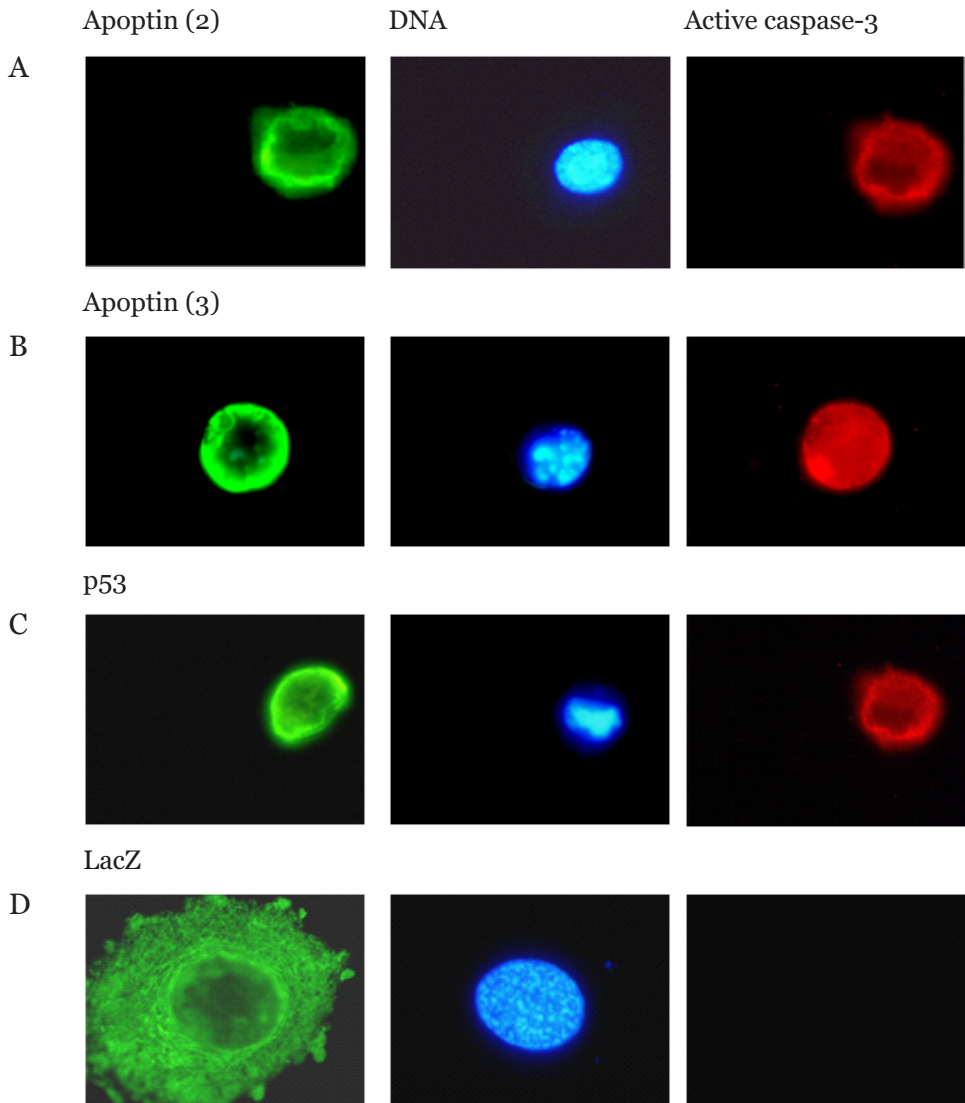
Figure 2



Cytochrome c release in apoptin-induced apoptosis. UMSSC-14B cells were transfected with plasmids encoding either LacZ (negative control), apoptin or p53 (positive control). Three days later, cytosolic extracts were prepared and analyzed by Western blotting for cytochrome c levels.

In parallel studies, we examined the possible activation of caspase-3 by apoptin in comparison to p53 and LacZ. UMSSC-14B cells were transfected with plasmids encoding apoptin, p53 or LacZ, fixed four days later and stained with an antibody specific for each transgene and with an antibody specific for active caspase-3. In the majority of the p53- as well as apoptin-expressing cells, active caspase-3 could be detected, but only in a few Lac-Z-positive UMSSC cells (Figure 3). The majority of apoptin-positive apoptotic UMSSC-14B cells appeared to have active caspase-3, and only some non-apoptotic apoptin-positive cells stained positive for active caspase-3. Our results indicate that upon apoptin induction of apoptosis in UMSSC-14B cells besides cytochrome c release, also caspase-3 becomes activated.

Figure 3

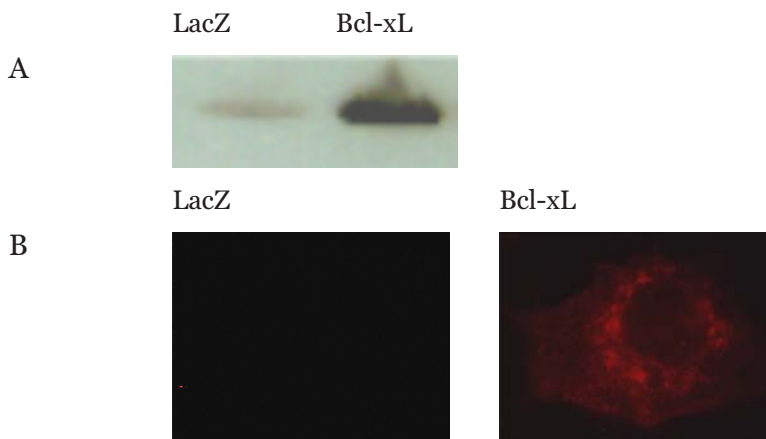


Active caspase-3 is present in UMSCC-14B cells undergoing apoptin-induced apoptosis. Cells were transfected with plasmids encoding LacZ (negative control), apoptin, or with pCMVp53 (positive control). At various time points after transfection, cells were fixed and stained with antibodies recognizing apoptin, p53, LacZ or active caspase-3, stained with DAPI and analyzed by fluorescence microscopy. The shown cells were transfected with apoptin and fixed two days (2) or 3 days (3) after transfection, or transfected with p53 or with LacZ and fixed after 3 days. Photographs were taken of representative cells at a magnification of 1000x.

Bcl-xL interferes with p53-induced apoptosis, but not with apoptin-induced apoptosis.

Finally, we examined the effect of over-expression of Bcl-xL on apoptin- and p53-induced apoptosis in UMSCC-14B cells. First, UMSCC-14B cells were transiently transfected with a plasmid encoding Bcl-xL or LacZ to examine the Bcl-xL-specific synthesis and its cellular localization. Western blot analysis revealed that only the Bcl-xL-transfected UMSCC-14B cells contain high levels of Bcl-xL protein with the expected MW of 28 kDa (Figure 4A). An indirect immunofluorescence assay with an antibody specifically directed against Bcl-xL showed that transfected UMSCC-14B cells contained detectable Bcl-xL protein in the cytoplasm (Figure 4B) and its distribution resembled that of mitochondria, as reported previously²³.

Figure 4

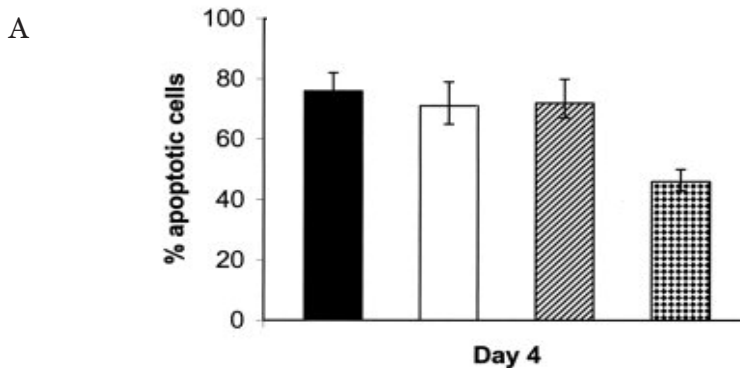


UMSCC-14B cells over-producing antiapoptotic Bcl-xL protein. Cells were transiently transfected with plasmid pCMV-Bcl-xL or with pCMV-LacZ. Western blot (panel A) or indirect immunofluorescence (panel B) of UMSCC-14B cells transfected with pCMV-Bcl-xL (+) or pCMV-LacZ (-) and stained for Bcl-xL protein with Bcl-xL-specific antibodies H-62.

Next, we studied whether Bcl-xL can inhibit apoptin and/or p53-induced apoptosis. To that end, UMSCC-14B cells were transfected with plasmids encoding apoptin or wild-type p53 alone or co-transfected with Bcl-xL and apoptin or with wild-type p53. Four days after transfection, cells were fixed and stained with antibodies recognizing apoptin, Bcl-xL and/or p53 and analyzed by fluorescence microscopy. Induction of apoptosis was scored by

analysis of nuclear morphology by DAPI staining. Four days post-transfection, co-expression of Bcl-xL did not inhibit apoptin activity, whereas p53-induced apoptosis was significantly reduced due to co-expression of Bcl-xL (Figure 5A).

Figure 5



Bcl-xL does not inhibit apoptin-induced apoptosis. Cells were transfected with plasmids encoding for apoptin (black bar), p53 (open bar), or co-transfected with Bcl-xL and apoptin (striped bar) or with p53 (dotted bar). Cells were fixed and analyzed by indirect immunofluorescence. The percentage of cells that stained abnormally with DAPI was used as a measure for apoptosis. Results given are the mean of 2 independent experiments. In each experiment at least 100 cells were examined that were positive for each transfected transgene product.

These results indicate that although Bcl-xL has an inhibiting effect on p53-induced apoptosis, it clearly does not interfere with the apoptin-induced apoptosis processes. Therefore, the next step was to determine whether cytochrome c was still released in the apoptin-expressing cells resulting in apoptosis but hampered in cells expressing wild-type p53. UMSCC-14B cells were transfected with plasmids encoding wild-type p53 or apoptin alone, or co-transfected with plasmids encoding Bcl-xL and apoptin or Bcl-xL and wild-type p53. Three days after transfection, cytosolic fractions were generated as described in the Material and Methods section. Western-blot analysis clearly showed that Bcl-xL reduces the release of cytochrome c in UMSCC-14B cells co-expressing wild-type p53 and Bcl-xL, but had no effect on the cytochrome c release when cells co-produced apoptin and Bcl-xL (Figure 5B). Therefore, Bcl-xL seems to be able to negatively interfere with p53-induced apoptosis by prevention of cytochrome c

release, which is not the case upon induction of apoptosis by apoptin.

Figure 5

B Apoptin & Bcl-xL LacZ & Bcl-xL p53 & Bcl-xL



Bcl-xL does not inhibit apoptin-induced cytochrome c release. UMSCC-14B cells were co-transfected with plasmids encoding for Bcl-xL and either LacZ, apoptin or p53. Three days later, cytosolic extracts were prepared, and analyzed by Western blotting for cytochrome c levels. Similar results were obtained in 2 independent experiments.

Discussion

Our report shows that apoptin-induced apoptosis in p53-non-functional UMSCC-14B cells is accompanied by release of cytochrome c and/or activation of caspase-3. Even of more importance is the observation that Bcl-xL did not inhibit apoptin-induced apoptosis in these HNSCC cells.

The observation that apoptin induces apoptosis in UMSCC-14B cells lacking functional p53 is of importance for e.g. Schmitz et al.²⁴ reported that disruptions in the p53-regulated apoptosis processes could lead to the rapid emergence of cellular drug resistance. Cells expressing some forms of mutant p53 show enhanced tumorigenic potential with increased resistance to chemotherapy and radiation²⁵.

Noutomi et al.¹⁰ generated squamous cell carcinoma cell lines with p53 mutations alone or combined with high levels of Bcl-xL. The cell lines with a p53 mutation belonged to the intermediate resistant group, whereas the cells with a p53 mutation and high levels of Bcl-xL were highly resistant to chemotherapy and radiation. We showed that UMSCC-14B cells co-expressing Bcl-xL and p53 had a significant lower rate of apoptosis when compared to expression of wild-type p53 alone, which correlates with the inhibition of cytochrome c. Therefore, it is surprising that co-expression of Bcl-xL and apoptin reached a similar apoptosis level

in comparison when apoptin was expressed alone. This result seems to be linked by the fact that Bcl-xL seems not to hamper the apoptin-induced release of cytochrome c. These features indicate that apoptin can induce apoptosis in the presence of high levels of Bcl-xL, which is known to prevent induction by a large variety of conventional anti-cancer therapies²⁶⁻²⁸.

The majority of apoptin-positive morphologically apoptotic UMSCC-14B cells appeared to have active caspase-3, whereas some morphologically non-apoptotic apoptin-positive cells stained positive for active caspase-3, indicating that caspase-3 activation precedes the apoptotic execution phase. Danen-van Oorschot and colleagues¹⁷ reported that only apoptin-expressing morphologically apoptotic human osteosarcoma Saos-2 cells contained active caspase-3. They concluded that caspase-3 is activated during apoptin-induced cell death, but this activation seems to occur at a late stage. The differential caspase-3 activation can also be explained by the fact that in Saos-2 cells caspase-3 activation results in immediate morphological cellular changes, whereas UMSCC-14B cells show a delayed execution of apoptosis after caspase activation.

Although Bcl-xL and Bcl-2 belong to the antiapoptotic members of the same protein family, they act in a different way on apoptin-induced apoptosis. Previously, it has been shown that Bcl-2 stimulates^{15,22} the effect of apoptin-induced apoptosis in human tumor cells, which is not observed for Bcl-xL. Similar differential effects were also seen in Hep3B and HeLa-S3 treated with honokiol, a phenolic compound purified from *Magnolia officinalis*. Hep3B and HeLa-S3 cells over-expressing Bcl-2 accelerated honokiol induced cell death, which could not be noticed for Bcl-xL²⁹. Nevertheless, both over-expression of Bcl-2 and Bcl-xL resulted in release of cytochrome c and subsequent activation of downstream caspase-3 upon apoptin-induced apoptosis. However, by which mechanism apoptin can induce the release of cytochrome c in the presence of the antiapoptotic proteins Bcl-2¹⁷ and Bcl-xL remains to be unraveled. The fact that apoptin induces apoptosis in a tumor-specific way²² in cases where therapeutic agents are known to fail

such as over-expression of Bcl-xL, makes it a potential anti-tumor agent.

In conclusion, we showed that apoptin induces apoptosis independent of functional p53 in HNSCC-derived cells, which cannot be inhibited by Bcl-xL. Therefore, apoptin forms a potential therapeutic agent for treatment of HNSCC.

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