Mcl-1 Antisense Therapy Chemosensitizes Human Melanoma in a SCID Mouse Xenotransplantation Model

Christiane Thallinger,[*†] Markus F. Wolschek,[*‡] Volker Wacheck,[*] Helmut Maierhofer,[*] Patrick Günsberg,[*] Peter Polterauer,[§] Hubert Pehamberger,[†¶**] Brett P. Monia,[††] Edgar Selzer,[**‡‡] Klaus Wolff,[†] and Burkhard Jansen[†1*]

[*]Department of Clinical Pharmacology, Section of Experimental Oncology, †Department of Dermatology, Division of General Dermatology, ‡Department of Internal Medicine IV, Division of Gastroenterology and Hepatology, §Department of Surgery, Division of Vascular Surgery, ¶Ludwig Boltzmann Institute of Clinical Experimental Oncology, and [**]Centre of Excellence for Clinical and Experimental Oncology, University of Vienna, Austria; ††ISIS Pharmaceuticals Inc., Carlsbad, California, USA; ‡‡Department of Radiotherapy and Radiobiology, University of Vienna, Austria

It is well established that high expression of the antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL can significantly contribute to chemoresistance in a number of human malignancies. Much less is known about the role the more recently described Bcl-2 family member Mcl-1 might play in tumor biology and resistance to chemotherapy. Using an antisense strategy, we here address this issue in melanoma, a paradigm of a treatment-resistant malignancy. After *in vitro* proof of principle supporting an antisense mechanism of action with specific reduction of Mcl-1 protein as a consequence of nuclear uptake of the Mcl-1 antisense oligonucleotides employed, antisense and universal control oligonucleotides were administered systemically in combination with dacarbazine in a human melanoma SCID mouse xenotransplantation model. Dacarbazine, available now for more than three decades, still remains the most active single agent for treatment of advanced melanoma. Mcl-1 antisense oligonucleotides specifically reduced target protein expression as well as the apoptotic threshold of melanoma xenotransplants. Combined Mcl-1 antisense oligonucleotide plus dacarbazine treatment resulted in enhanced tumor cell apoptosis and led to a significantly reduced mean tumor weight (mean 0.16 g, 95% confidence interval 0.08–0.26) compared to the tumor weight in universal control oligonucleotide plus dacarbazine treated animals (mean 0.35 g, 95% confidence interval 0.2–0.44) or saline plus dacarbazine treated animals (mean 0.39 g, 95% confidence interval 0.25–0.53). We thus show that Mcl-1 is an important factor contributing to the chemoresistance of human melanoma *in vivo*. Antisense therapy against the Mcl-1 gene product, possibly in combination with antisense strategies targeting other antiapoptotic Bcl-2 family members, appears to be a rational and promising approach to help overcome treatment resistance of malignant melanoma. ***Key words: antisense oligonucleotide/apoptosis/chemoresistance/human melanoma/Mcl-1. J Invest Dermatol 120:1081–1086, 2003***

Apoptosis is important for the physiologic removal of unwanted cells during development, for tissue homeostasis, and in host defense mechanisms (Thompson, 1995; Strasser *et al*, 1997; Vaux and Korsmeyer, 1999). It provides an irreversible mechanism for the elimination of excess and damaged cells, and induction of apoptosis is also the mechanism of action of various chemotherapeutic agents (Lowe *et al*, 1993; 1994; Brown and Wouters, 1999; Solary *et al*, 2001; Makin, 2002).

One key group of intracellular factors regulating apoptosis is the Bcl-2 family of proteins. Bcl-2 was the founding member of what is now a multigene family that consists of partially interacting proteins that can be found in the evolution ladder from mammals down to the nematodes (Hockenbery *et al*, 1990; Korsmeyer, 1992; Cohen, 1993). A delicate balance between antiapoptotic and proapoptotic Bcl-2 family members exists in each cell and determines whether the cell survives or undergoes apoptosis.

Mcl-1 (*m*yeloid *c*ell *l*eukemia-*1*) was first discovered as an early induction gene during the differentiation of a human myeloid leukemia cell line (Kozopas *et al*, 1993). The Mcl-1 protein contains structural motifs that characterize it as a member of the Bcl-2 protein family. Like other Bcl-2 family members, the Mcl-1 protein possesses Bcl-2 homology domains BH 1–3 (Akgul *et al*, 2000b; Bae *et al*, 2000). In contrast to Bcl-2 and Bcl-xL, Mcl-1 contains PEST (proline, glutamate, serine, and threonine) sequences (Rogers *et al*, 1986), which are commonly found in proteins with a high turnover rate. Surprisingly, deletion of 104 amino acids (residues 79–183) that contain putative PEST sequences and other stability regulating motifs did not affect protein stability (Akgul *et al*, 2000a). Moreover, the intracellular distribution of Mcl-1 appears to be more widespread than Bcl-2 because, in addition to its predominant localization at the mitochondrial membranes, Mcl-1 is also distributed over a variety of

Manuscript received August 1, 2002; revised December 23, 2002; accepted for publication January 14, 2003

[1]Present address: Prostate Center, University of British Columbia D9, 2733 Heather Street, Vancouver, BC, Canada V5Z 3J5.

Reprint requests to: Burkhard Jansen, Department of Clinical Pharmacology, University of Vienna, Waehringer Guertel 18–20, A-1090 Vienna, Austria; Email: Burkhard.Jansen@univie.ac.at

Abbreviations: ASO, antisense oligonucleotide; DTIC, dacarbazine; SCID, severe combined immunodeficient; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

0022-202X/03/$15.00 ·

nonmitochondrial compartments (Yang *et al*, 1995; Leuenroth *et al*, 2000; Murphy *et al*, 2000). Mcl-1 has a short half-life (1–3 h) (Rogers *et al*, 1986; Akgul *et al*, 2000b; Schubert and Duronio, 2001) and is rapidly induced in a variety of cell types (Kozopas *et al*, 1993; Akgul *et al*, 2000b).

The role of Mcl-1 expression in supporting cell survival has been studied in various cell systems. Human neutrophils express neither Bcl-2 nor Bcl-xL, but they do express Mcl-1. Upon shutdown of Mcl-1 expression, this pro-survival gene product is rapidly depleted, resulting in induction of apoptosis (Moulding *et al*, 2001). Similarly, downregulation of Mcl-1 by antisense oligonucleotides causes a rapid entry into apoptosis in differentiating human myeloblastic leukemia cells (U937) (Moulding *et al*, 2000). Overexpression of Mcl-1 in Chinese hamster ovary cells (Reynolds *et al*, 1994; 1996) and murine myeloid progenitor cells (FDC-P1) (Zhou *et al*, 1997), on the other hand, has been shown to delay apoptosis induced by miscellaneous stimuli. Taken together, Mcl-1 prolongs cell viability under various cytotoxic conditions (etoposide, calcium ionophore, ultraviolet irradiation, growth factor withdrawal) that cause apoptotic cell death, as has been also shown in murine myeloid progenitor cells (Zhou *et al*, 1997).

The mechanisms through which Mcl-1 promotes cell survival, however, are not clearly understood. Although reports on the interaction of Apaf-1 and antiapoptotic Bcl-2 family members are controversial, one possible mechanism is the inhibition of caspase activation by retaining the adapter molecule Apaf-1 in an inactive state (Moriishi *et al*, 1999; Conus *et al*, 2000). In HL-60 cells, an increase of Mcl-1 has been associated with inhibition of apoptosis by downregulating cell-damage-induced mitochondrial cytochrome c release (Wang and Studzinski, 1997). Bcl-xL and Mcl-1 have in common the ability to heterodimerize with Bax, which has been reported to translocate from cytosol to mitochondria following exposure to apoptotic stresses and neutralize its cell death-inducing activity (Akgul *et al*, 2000b; Pedersen *et al*, 2002).

Recently, it was shown that Mcl-1 is also widely expressed in normal and malignant human melanocytic cell lines and in melanoma metastases (Selzer *et al*, 1998). Increased Mcl-1 and Bcl-xL levels were observed in thin primary melanomas as well as in metastatic malignant melanomas but not in benign nevi, suggesting that upregulation of these proteins represents an early event associated with malignant transformation (Tang *et al*, 1998). Several clinical studies have provided evidence for the hypothesis that high level expression of antiapoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL, and Mcl-1, confers a clinically important chemoresistant phenotype to cancer cells (Reed, 1995; 1998; Strasser *et al*, 1997; Kaufmann *et al*, 1998). In human melanoma it was recently reported that Bcl-2 (Jansen *et al*, 1998; 2000) expression is linked to chemoresistance. Apoptosis and its regulation may play an important pathogenic role in the development and chemoresistance of human melanoma (Serrone and Hersey, 1999).

Antisense oligonucleotides (ASO), chemically modified stretches of single-stranded DNA, are pharmacologically potent inhibitors of disease-related protein expression (Webb *et al*, 1997; Jansen *et al*, 2000; Monia *et al*, 2000; Nicholson, 2000; Waters *et al*, 2000; Jansen and Zangemeister-Wittke, 2002). They are designed to bind to their complementary mRNA sequence once they get inside the cell, thereby inhibiting expression of the encoded protein (Myers and Dean, 2000).

Based on the available evidence, Mcl-1 seems to be a suitable molecular target to enhance chemosensitivity in human melanoma. In this study we used Mcl-1 ASO to downregulate Mcl-1 protein expression in human melanoma cells *in vitro* and *in vivo*. This downregulation chemosensitized human melanoma to subsequent treatment with dacarbazine (DTIC) in a severe combined immunodeficient (SCID) mouse xenotransplantation model.

MATERIALS AND METHODS

Cell lines and culture The human melanoma cell line 518A2 was a kind gift of Peter Schrier, University of Leiden (The Netherlands), which has been described recently (Jansen *et al*, 1998). The human melanoma cell line Mel Juso (Lehmann *et al*, 1989) was kindly provided by Dr. Judith Johnson (University of Munich, Germany). All cell lines were maintained in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Paisley, U.K.) and an antibiotic mixture containing 100 units per ml penicillin, 100 μg per ml streptomycin, and 0.25 μg per ml amphotericin B (all Gibco) in a fully humidified 5% CO_2, 95% ambient air atmosphere at 37°C.

Oligonucleotides and transfection 2′-O-Methoxyethyl/2′-deoxynucleotide chimeric phosphorothioate antisense oligonucleotides were kindly provided by ISIS Pharmaceuticals (Carlsbad, CA). The sequence of Mcl-1 antisense oligonucleotide (ISIS 20408) is 5′-TTGGCTTTGTG TCCTTGGCG-3′. A universal control oligonucleotide pool (ISIS 29848) was used, which is synthesized as a mixture of A (adenine), G (guanine), T (thymine), and C (cytosine) bases so that the resulting preparation contains an equimolar mixture of all possible oligonucleotides. The oligonucleotide chemistry of ISIS 29848 is identical to that of ISIS 20408. For cellular uptake studies Mcl-1 ASO is fluorescein-labeled with a 5′-oligonucleotide fluorescein labeling kit (Amersham, Buckinghamshire, U.K.) according to the manufacturer's instructions.

For transfection, 300,000 cells were seeded in a 75 cm^2 plate 24 h prior to the oligonucleotide treatment. Oligonucleotides were complexed with lipofectin (Gibco) in antibiotic-free medium without serum as described by the supplier. Subsequently, cells were incubated for 4 h with 250 nM complexed oligonucleotides (10 μg per ml lipofectin) in antibiotic-free medium without serum. After washing with DMEM, cells were further cultured in complete medium containing serum. Alternatively, cells were incubated with oligonucleotides (1 μM) for 24 h in medium supplemented with 10% fetal bovine serum.

Oligonucleotide uptake Following incubation with fluorescein-labeled oligonucleotides with and without uptake-enhancing lipids cells were fixed with paraformaldehyde (Sigma, St. Louis, MO) (4% in phosphate-buffered saline). Slides were evaluated under an Axioplan 2 (Zeiss, Vienna, Austria) fluorescence microscope equipped with a Zeiss Axiocam using Axiovision 3.0 (Zeiss) software.

Western blot analysis Cell extracts were prepared in lysis buffer containing 0.14 M NaCl, 0.4 M triethanolamine, 0.2% Na deoxycholate, and 0.5% Nonidet P-40, supplemented with 1 mM phenylmethylsulfonyl fluoride, 4.0 μg per ml aprotinin, and 4.0 μg per ml leupeptin. The amount of soluble proteins was quantified by means of a modified Bradford analysis (Bio-Rad, Richmond, CA). Total lysates (15 μg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA), and probed with anti-Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-tubulin (Sigma) antibody. Alkaline-phosphatase-conjugated goat antimouse and goat antirabbit immunoglobulins (Tropix, Bedford, MA) were used for secondary incubations. Reactive bands were visualized by chemiluminescence using CSPD® as substrate (Tropix).

Protein expression levels were quantified by densitometry of autoradiograms with an Ultrascan XL densitometer (Pharmacia, Uppsala, Sweden).

Evaluation of oligonucleotide effects *in vivo* Pathogen-free female C.B.-17 *scid/scid* (SCID) mice (4–6 wk, Harlan Winkelmann, Borchen, Germany) were randomly assigned to experimental groups of six animals each. SCID mice were injected subcutaneously with 2×10^7 518A2 human melanoma cells into the lower left flank. Seven days after injection, animals had developed melanomas of similar size (nodules of about 5 mm in diameter) and treatment was initiated. Animals were anesthetized and miniosmotic pumps (Alzet 2002, Alzet, Palo Alto, CA) prefilled with saline, antisense, or universal control oligonucleotides were inserted subcutaneously into a paraspinal pocket. Implanted pumps released their contents by continuous infusion at a rate of 20 mg per kg per d over a period of 2 wk. Mice were treated with DTIC (80 mg per kg per d) administered intravenously for 5 d (days 12–16). During the experiments, tumor weight was assessed twice a week by caliper measurement as previously described (Boehm *et al*, 1997). On day 21 all animals were sacrificed, tumors were weighed, and tumor xenotransplants were fixed in formalin for immunohistochimestry and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) analysis.

Apoptosis assay and immunohistochemistry Histology of human melanoma xenotransplants was evaluated by hematoxylin and eosin staining. The histochemical detection of apoptosis in formalin-fixed sections was performed by TUNEL using fluorescein-dUTP (Boehringer Mannheim, Germany). Tissue sections were counterstained using DAPI

(4′,6-diamidine-2′-phenylindole dihydrochloride, Roche Diagnostics, Vienna, Austria).

The expression pattern of Mcl-1 *in vivo* was evaluated by immunohistochemistry with an anti-Mcl-1 antibody (1:50, Santa Cruz Biotechnology) and a DAKO LSAB 2 System using diaminobenzidine as chromogen.

Statistical analysis Statistical significance of differences in tumor weight among treatment groups was calculated by using one-way ANOVA and the Scheffe test for *post hoc* testing (SPSS 10.0.7, SPSS, Chicago, IL). p-values less than 0.05 were considered to be of statistical significance.

RESULTS

Uptake of Mcl-1 antisense oligonucleotides in human melanoma cells *in vitro* To assess the efficiency of oligonucleotide uptake, 518A2 human melanoma cells were incubated with 250 nM or 1 μM fluorescein-labeled Mcl-1 ASO in the presence or absence of uptake-enhancing lipids, respectively. Twenty-four hours later cells were fixed with paraformaldehyde and evaluated by fluorescence microscopy. Fluorescein-labeled oligonucleotides were found to be localized to the nucleus but also to the cytosol as well as to vesicular compartments (**Fig 1**). These findings were also demonstrated for another human melanoma cell line (Mel JUSO; data not shown).

Reduction of Mcl-1 protein by Mcl-1 antisense oligonucleotides in human melanoma cells *in vitro* The two melanoma cell lines used in this work were shown to express the antiapoptotic protein Mcl-1 (**Fig 2**). Using Mcl-1 ASO at nanomolar concentrations in the presence of the uptake enhancer lipofectin, we observed a pronounced reduction in Mcl-1 levels in our cell lines *in vitro* (**Fig 2**). Normalization to β-tubulin demonstrated that ASO treatment caused a significant reduction of Mcl-1 protein levels compared to saline-control-treated cell lines. In 518A2 cells, ASO treatment reduced Mcl-1 levels by 84% [95% confidence interval (CI) 79.03–88.97], whereas in Mel Juso cells a reduction of 83% (CI 95% 74.00–93.68) was detected (data are given as the range of three independent experiments and are expressed relative to lipofectin-treated control cells). In contrast, universal control treatment decreased Mcl-1 levels by 5% (95% CI 4.70–15.37) in 518A2 cells and 11% (95% CI 3.35–19.32) in Mel Juso cells. Similarly, effects were also observed at a higher oligonucleotide concentration in the absence of uptake-enhancing lipids (data not shown). Treatment of melanoma cells with Mcl-1 ASO did not change Bcl-2 or Bcl-xL protein levels (data not shown).

Immunohistochemical analysis of Mcl-1 protein levels in 518A2 human melanomas *in vivo* To test whether Mcl-1 ASO is capable of downregulating Mcl-1 expression *in vivo*, Mcl-1 ASO, universal control oligonucleotides, or saline as a vehicle control were administered to SCID mice bearing subcutaneously implanted 518A2 tumors. After 14 d of treatment the expression pattern of Mcl-1 was evaluated by immunohistochemistry (**Fig 3**). Mcl-1-specific staining was found in all specimens evaluated; however, levels were markedly reduced in Mcl-1 ASO treated tumors compared to mice that received either saline or control oligonucleotides.

Mcl-1 ASO treatment chemosensitizes human melanoma *in vivo* Based on these findings we next tested whether reduction of Mcl-1 expression has the potential to enhance the chemosensitivity of melanoma xenotransplants. Tumor size measurements with a caliper during the course of the experiment already indicated that tumor growth is reduced in mice treated with Mcl-1 ASO/DTIC compared to mice treated with either universal control oligonucleotide-DTIC or saline/DTIC (**Fig 4*a***). This observation was confirmed by assessing the tumor weight at the end of the experiment (**Fig 4*b***). The

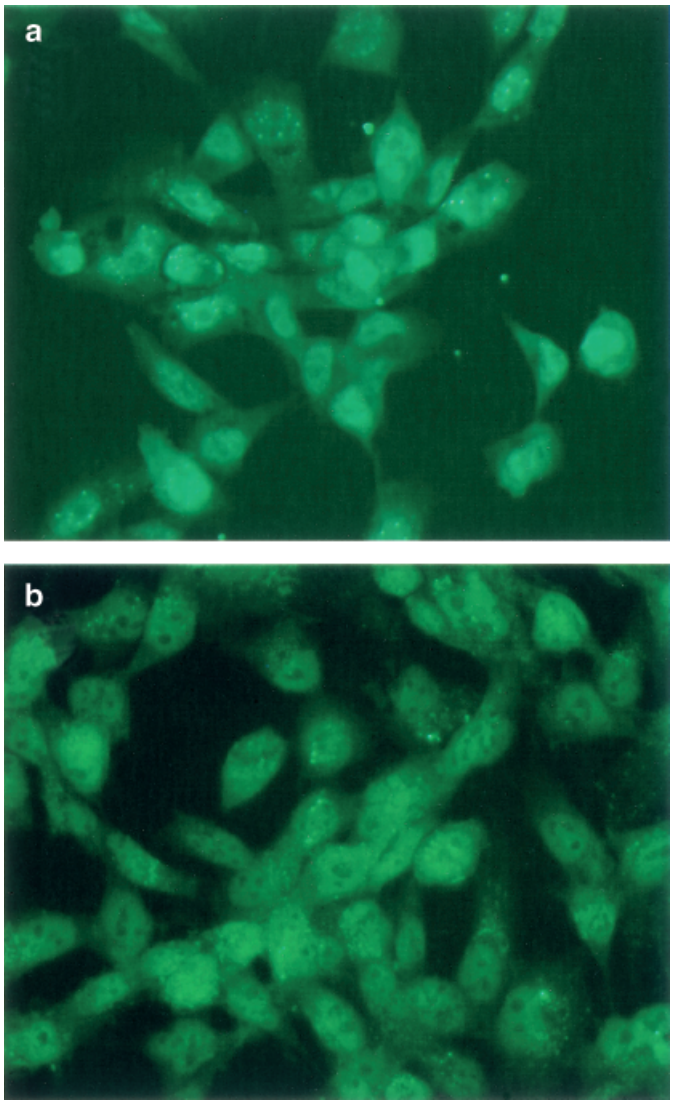

Figure 1. *In vitro* oligonucleotide uptake by human melanoma cells. 518A2 cells were incubated with fluorescein-labeled Mcl-1 ASO (*a*) 250 nM, in the presence of uptake-enhancing lipids (lipofectin); (*b*) 1 μM, without uptake-enhancing lipids. Twenty-four hours later, cells were fixed with paraformaldehyde and evaluated under a fluorescence microscope. *Original magnification*: 400 × .

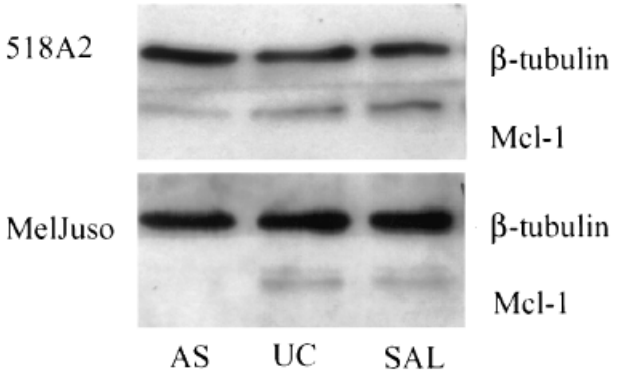

Figure 2. *In vitro* Mcl-1 downregulation in human melanoma cells. 518A2 and Mel Juso cells were incubated for 4 h with the indicated oligonucleotides or vehicle control (AS, antisense; UC, universal control, SAL, saline control) in the presence of lipofectin. Twenty-four hours later, protein was extracted and analyzed for Mcl-1 and β-tubulin expression by Western blotting. Representative data from one of three independent experiments are shown.

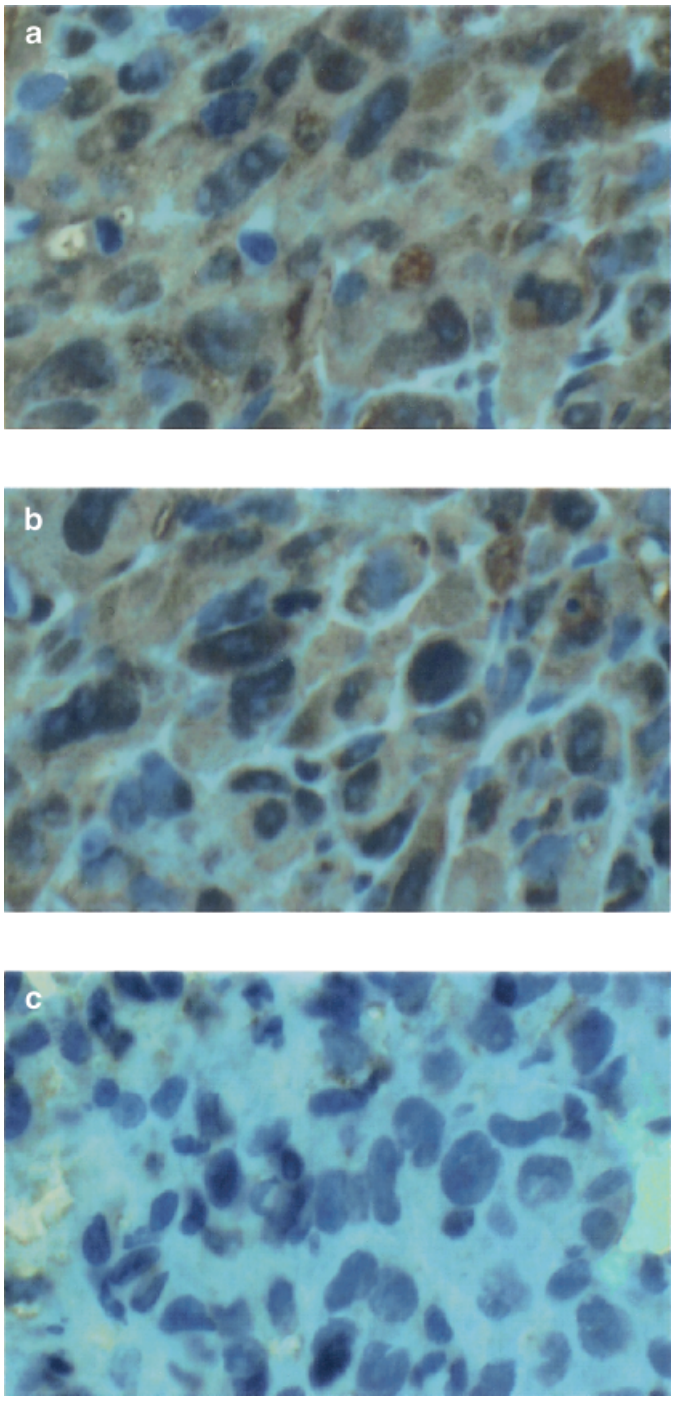

Figure 3. Downregulation of Mcl-1 expression in human melanomas grown in SCID mice. Immunohistochemical detection of Mcl-1 in tumor xenotransplants of mice with established 518A2 tumors were treated for 14 d with the indicated oligonucleotide (*a*, SAL; *b*, UC; *c*, AS) delivered by miniosmotic pumps.

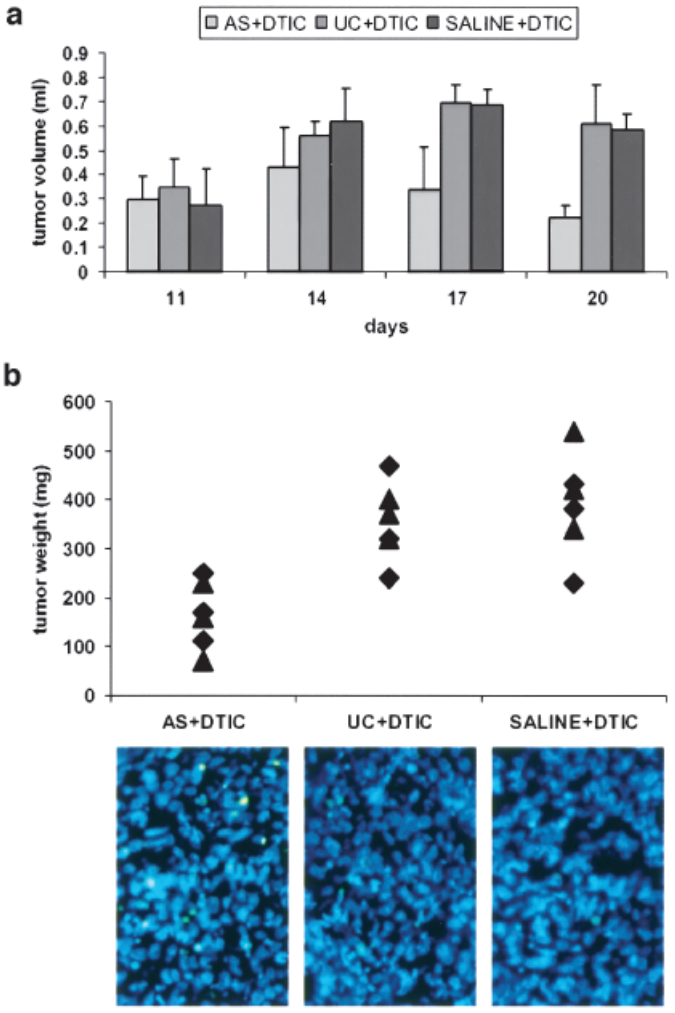

Figure 4. Mcl-1 ASO chemosensitizes human melanoma *in vivo*. SCID mice with established 518A2 tumors received a 14 d infusion by miniosmotic pumps of Mcl-1 ASO (AS), universal control oligonucleotide (UC), or saline ($n = 6$ animals per group). In addition, animals received DTIC treatment on days 12–16. (*a*) During the course of the experiment tumor size was assessed by caliper measurement and is given in mL as mean ± SD. (*b*) At the end of the experiment (day 21) tumors were resected and weighed. Tumor weight is given in mg as mean ± SD. Furthermore, melanoma xenografts were analyzed by TUNEL assay (apoptotic cells appear green) and counterstained with DAPI. Representative photographs of tumors are shown. *Original magnification*: 200 × .

combination of Mcl-1 ASO and DTIC resulted in a significantly lower mean tumor weight (mean 0.16 g, 95% CI 0.08–0.26) compared to saline plus DTIC (mean 0.39 g, 95% CI 0.25–0.53) or universal control oligonucleotides plus DTIC (mean 0.35 g, 95% CI 0.27–0.44) (**Fig 4*b***). DTIC alone exerted an antineoplastic response without eradicating the tumors and reduced the mean tumor weight compared with that of untreated animals by approximately two-thirds. Assessing the influence of oligonucleotide monotreatment in an independent pilot experiment, no significant differences were found in the mean tumor weight between mice treated with ASO alone (mean 1.85 g, 95% CI 1.53–2.17) or universal control oligonucleotides (mean 1.85 g, CI 1.35–2.35) compared to those treated with saline (mean 2.00 g, 95% CI 1.27–2.90).

Detection of apoptosis in human melanomas grown in scid mice by TUNEL To investigate whether this chemosensitization effect by Mcl-1 ASO was linked to a higher rate of apoptotic cell death in tumor xenografts, tumors were examined for apoptotic cell death by TUNEL staining (**Fig 4*b***). Mcl-1 ASO plus DTIC treatment increased the number of apoptotic cells approximately 2-fold (7% apoptotic cells) within 518A2 tumors compared to the saline plus DTIC or the universal control plus DTIC control group (2% and 3% apoptotic cells, respectively).

DISCUSSION

The expansion of a tumor is directly related to the difference in the rates of proliferation and cell death (apoptotic and necrotic

cell death). Apoptosis plays an indispensable role in the maintenance of homeostasis within tissue. Moreover, impaired apoptosis may promote metastasis by enabling tumor cells to survive in the circulation and to grow at distant sites even in the absence of an otherwise required fine-tuned growth factor milieu.

Apoptosis is a highly conserved cellular process activated in response to a variety of stimuli, which culminates in the activation of nucleases and cysteine aspartic acid specific proteases (caspases) responsible of degradation of cellular proteins that ultimately lead to a decay of affected cells (Reed, 1998). The Bcl-2 family of proteins is of special relevance, as its members act at a checkpoint upstream of effector caspases and other apoptosis regulators (Chao and Korsmeyer, 1998).

Given the widespread expression of the antiapoptotic Bcl-2 family member Mcl-1 in human melanoma (Selzer *et al*, 1998; Tang *et al*, 1998) and experimental evidence from other systems, we hypothesized that Mcl-1 may be one key factor influencing the chemosensitivity of this malignancy. In contrast to Bcl-2 (Jansen *et al*, 1998; 2000) and Bcl-xL (Heere-Ress *et al*, 2002) there is no direct experimental information regarding the role that Mcl-1 might play in the control of apoptosis and/or chemoresistance of human melanoma.

The aim of this study was to address this issue and to restore the apoptosis signaling pathway in human melanoma by downregulation of Mcl-1 protein expression employing ASO. ASO are useful tools for biologic research and hold great promise as a new class of therapeutic agents. They provide an interesting approach for the development of target-selective drugs, which can be modified and optimized with relative ease. In this study, a Mcl-1 ASO previously shown to be capable of downregulating Mcl-1 expression in endothelial cells were used (Bannerman *et al*, 2001). *In vitro* experiments demonstrated a strong and specific downregulation of the Mcl-1 target protein in human melanoma cells. The observation that expression of Bcl-2 and Bcl-xL, two other antiapoptotic members of the Bcl-2 family with homology to the Mcl-1 protein (Kozopas *et al*, 1993; Johnson, 1999), was not altered by Mcl-1 ASO treatment (data not shown) further supports a specific antisense mechanism of action of the Mcl-1 ASO used. Furthermore, in a recent report an Mcl-1 ASO with the identical sequence was found to cause a pronounced Mcl-1 downregulation in endothelial cells, whereas a control oligonucleotide that contained four mismatches was without any effect on Mcl-1 expression levels (Bannerman *et al*, 2001). Notably, no reciprocal upregulation of Bcl-2 or Bcl-xL was observed in our studies. This phenomenon had been reported as a compensatory mechanism after downregulation of other Bcl-2 family members in certain malignancies (Hockenbery *et al*, 1990; Chao and Korsmeyer, 1998).

By employing immunohistochemical analysis, we also demonstrated a strong Mcl-1 downregulation in 518A2 human melanoma grown in SCID mice following systemic administration of Mcl-1 ASO. Assessing antitumor response, we observed a clear chemosensitizing effect of Mcl-1 ASO to DTIC for human melanoma. In contrast to previous reports on Bcl-2 antisense strategies (Jansen *et al*, 1998; Wacheck *et al*, 2001), single agent ASO treatment did not significantly alter tumor growth compared to untreated mice. As previously observed (Jansen *et al*, 1998) DTIC exerted an antineoplastic response; however, Mcl-1 ASO plus DTIC further reduced tumor weight by approximately 60%.

Several clinical correlative studies have provided support for the hypothesis that high Bcl-2, Bcl-xL, and Mcl-1 expression confers a clinically relevant chemoresistant phenotype to cancer cells (Reed, 1995; 1998; Strasser *et al*, 1997).

The data presented here clearly provide first direct evidence that Mcl-1 contributes to chemoresistance in human malignant melanoma that is independent of Bcl-2 and Bcl-xL. We further show for the first time that Mcl-1 downregulation by ASO has the potential to enhance chemosensitivity in melanoma *in vivo*. Although the exact mechanism remains elusive, Mcl-1 may exert its antiapoptotic function by heterodimerization with Bax, thereby neutralizing its death inducing activity (Murphy *et al*, 2000; Pedersen *et al*, 2002), or by interacting with Apaf-1. Reports on the interaction of antiapoptotic Bcl-2 family members with this adapter molecule remain somewhat controversial, however (Moriishi *et al*, 1999; Conus *et al*, 2000).

Previously, our group reported a chemosensitizing effect of Bcl-2 ASO – also without effects on Bcl-xL levels – in human melanoma both preclinically and in a recently completed phase I/II trial (Jansen *et al*, 1998; 2000). Preclinical antisense strategies using monospecific or even bispecific ASO against Bcl-2 and/or Bcl-xL have shown promise as treatment options for malignant melanoma (Heere-Ress *et al*, 2002; Olie *et al*, 2002). The balance between antiapoptotic and pro-apoptotic Bcl-2 family members determines the threshold of susceptibility to chemotherapy. Therefore, it is reasonable to speculate that antisense strategies may prove even more promising if ultimately more than one of the antiapoptotic family members that exhibit a certain degree of redundancy in inhibiting apoptosis is targeted in combination with chemotherapy. As our group previously reported a chemosensitizing effect of Bcl-2 ASO in human melanoma (Jansen *et al*, 1998; 2000), a combination of ASO targeted against Bcl-2 as well as against Mcl-1 and/or Bcl-xL may prove even more promising.

We thank the members of the Center for Biomedical Research, University of Vienna, Vienna, Austria (in particular Dr. Helga Bergmeister and Helga Pischinger), for outstanding animal care. The work was supported by the Austrian National Bank, the Austrian Science Fund, the Hochschuljubiläumsstiftung, the Niarchos Foundation, the Hygiene Fund, the Virology Fund, The Bürgermeister Fonds der Stadt Wien, and the Kamillo Eisner Stiftung.

REFERENCES

Akgul C, Moulding DA, White MR, Edwards SW: *In vivo* localisation and stability of human Mcl-1 using green fluorescent protein (GFP) fusion proteins. *FEBS Lett* 478:72–76, 2000a

Akgul C, Turner PC, White MR, Edwards SW: Functional analysis of the human MCL-1 gene. *Cell Mol Life Sci* 57:684–691, 2000b

Bae J, Leo CP, Hsu SY, Hsueh AJ: Mcl-1S, a splicing variant of the antiapoptotic Bcl-2 family member Mcl-1, encodes a proapoptotic protein possessing only the BH3 domain. *J Biol Chem* 275:25255–25261, 2000

Bannerman DD, Tupper JC, Ricketts WA, Bennett CF, Winn RK, Harlan JM: A constitutive cytoprotective pathway protects endothelial cells from lipopolysaccharide-induced apoptosis. *J Biol Chem* 276:14924–14932, 2001

Boehm T, Folkman J, Browder T, O'Reilly MS: Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* 390:404–407, 1997

Brown JM, Wouters BG: Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* 59:1391–1399, 1999

Chao DT, Korsmeyer SJ: BCL-2 family: regulators of cell death. *Annu Rev Immunol* 16:395–419, 1998

Cohen JJ: Apoptosis. *Immunol Today* 14:126–130, 1993

Conus S, Rosse T, Borner C: Failure of Bcl-2 family members to interact with Apaf-1 in normal and apoptotic cells. *Cell Death Differ* 7:947–954, 2000

Heere-Ress E, Thallinger C, Lucas T, *et al*: Bcl-X (L) is a chemoresistance factor in human melanoma cells that can be inhibited by antisense therapy. *Int J Cancer* 99:29–34, 2002

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334–336, 1990

Jansen B, Zangemeister-Wittke U: Antisense therapy for cancer – the time of truth. *Lancet Oncol* 3:672–683, 2002

Jansen B, Schlagbauer-Wadl H, Brown BD, *et al*: Bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat Med* 4:232–234, 1998

Jansen B, Wacheck V, Heere-Ress E, *et al*: Chemosensitisation of malignant melanoma by Bcl2 antisense therapy. *Lancet* 356:1728–1733, 2000

Johnson AL: Mcl-1 – just another antiapoptotic Bcl-2 homolog? *Endocrinology* 140:5465–5468, 1999

Kaufmann SH, Karp JE, Svingen PA, Krajewski S, Burke PJ, Gore SD, Reed JC: Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. *Blood* 91:991–1000, 1998

Korsmeyer SJ: Bcl-2 initiates a new category of oncogenes: Regulators of cell death. *Blood* 80:879–886, 1992

Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW: Mcl1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to Bcl2. *Proc Natl Acad Sci USA* 90:3516–3520, 1993

Lehmann JM, Riethmuller G, Johnson JP: MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion

molecules of the immunoglobulin superfamily. *Proc Natl Acad Sci USA* 86:9891–9895, 1989

Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH: The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis. *J Leukoc Biol* 68:158–166, 2000

Lowe SW, Ruley HE, Jacks T, Housman DE: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 74:957–967, 1993

Lowe SW, Bodis S, McClatchey A, *et al*: p53 status and the efficacy of cancer therapy *in vivo*. *Science* 266:807–810, 1994

Makin G: Targeting apoptosis in cancer chemotherapy. *Expert Opin Ther Targets* 6: 73–84, 2002

Monia BP, Holmlund J, Dorr FA: Antisense approaches for the treatment of cancer. *Cancer Invest* 18:635–650, 2000

Moriishi K, Huang DC, Cory S, Adams JM: Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. *Proc Natl Acad Sci USA* 96:9683–9688, 1999

Moulding DA, Giles RV, Spiller DG, White MR, Tidd DM, Edwards SW: Apoptosis is rapidly triggered by antisense depletion of Mcl-1 in differentiating U937 cells. *Blood* 96:1756–1763, 2000

Moulding DA, Akgul C, Derouet M, White MR, Edwards SW: Bcl-2 family expression in human neutrophils during delayed and accelerated apoptosis. *J Leukoc Biol* 70:783–792, 2001

Murphy KM, Ranganathan V, Farnsworth ML, Kavallaris M, Lock RB: Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. *Cell Death Differ* 7:102–111, 2000

Myers KJ, Dean NM: Sensible use of antisense: How to use oligonucleotides as research tools. *Trends Pharmacol Sci* 21:19–23, 2000

Nicholson DW: From bench to clinic with apoptosis-based therapeutic agents. *Nature* 407:810–816, 2000

Olie RA, Hafner C, Kuttel R, *et al*: Bcl-2 and bcl-xL antisense oligonucleotides induce apoptosis in melanoma cells of different clinical stages. *J Invest Dermatol* 118:505–512, 2002

Pedersen IM, Kitada S, Leoni LM, *et al*: Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. *Blood* 100:1795–1801, 2002

Reed JC: Bcl-2: prevention of apoptosis as a mechanism of drug resistance. *Hematol Oncol Clin North Am* 9:451–473, 1995

Reed JC: Dysregulation of apoptosis in cancer. *Cancer J Sci Am* 4:S8–S14, 1998

Reynolds JE, Yang T, Qian L, Jenkinson JD, Zhou P, Eastman A, Craig RW: Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. *Cancer Res* 54:6348–6352, 1994

Reynolds JE, Li J, Craig RW, Eastman A: Bcl-2 and Mcl-1 expression in Chinese hamster ovary cells inhibits intracellular acidification and apoptosis induced by staurosporine. *Exp Cell Res* 225:430–436, 1996

Rogers S, Wells R, Rechsteiner M: Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* 234:364–368, 1986

Schubert KM, Duronio V: Distinct roles for extracellular-signal-regulated protein kinase (ERK) mitogen-activated protein kinases and phosphatidylinositol 3-kinase in the regulation of Mcl-1 synthesis. *Biochem J* 356:473–480, 2001

Selzer E, Schlagbauer-Wadl H, Okamoto I, Pehamberger H, Potter R, Jansen B: Expression of Bcl-2 family members in human melanocytes, in melanoma metastases and in melanoma cell lines. *Melanoma Res* 8:197–203, 1998

Serrone L, Hersey P: The chemoresistance of human malignant melanoma: An update. *Melanoma Res* 9:51–58, 1999

Solary E, Plenchette S, Sordet O, *et al*: Modulation of apoptotic pathways triggered by cytotoxic agents. *Therapie* 56:511–518, 2001

Strasser A, Huang DC, Vaux DL: The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumourigenesis and resistance to chemotherapy. *Biochim Biophys Acta* 1333:F151–F178, 1997

Tang L, Tron VA, Reed JC, *et al*: Expression of apoptosis regulators in cutaneous malignant melanoma. *Clin Cancer Res* 4:1865–1871, 1998

Thompson CB: Apoptosis in the pathogenesis and treatment of disease. *Science* 267:1456–1462, 1995

Vaux DL, Korsmeyer SJ: Cell death in development. *Cell* 96:245–254, 1999

Wacheck V, Heere-Ress E, Halaschek-Wiener J, Lucas T, Meyer H, Eichler HG, Jansen B: Bcl-2 antisense oligonucleotides chemosensitize human gastric cancer in a SCID mouse xenotransplantation model. *J Mol Med* 79:587–593, 2001

Wang X, Studzinski GP: Antiapoptotic action of 1,25-dihydroxyvitamin D3 is associated with increased mitochondrial Mcl-1 and RAF-1 proteins and reduced release of cytochrome c. *Exp Cell Res* 235:210–217, 1997

Waters JS, Webb A, Cunningham D, Clarke PA, Raynaud F, Di Stefano F, Cotter FE: Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 18:1812–1823, 2000

Webb A, Cunningham D, Cotter F, *et al*: Bcl-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet* 349:1137–1141, 1997

Yang T, Kozopas KM, Craig RW: The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol* 128:1173–1184, 1995

Zhou P, Qian L, Kozopas KM, Craig RW: Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. *Blood* 89:630–643, 1997