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Increased Cytotoxicity of Cisplatin in SK-MEL 28 Melanoma Cells upon Down-Regulation of Melanoma Inhibitor of Apoptosis Protein

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ABSTRACT

Background: Malignant melanoma is a highly metastatic cutaneous cancer and typically refractory to chemotherapy. Deregulated apoptosis has been identified as a major cause of cancer drug resistance, and upregulated expression of the inhibitor of apoptosis protein melanom, an inhibitor of apoptosis (ML-IAP) is frequent in melanoma. **Methods:** Based on the conclusion that ML-IAP expression contributes to a malignant phenotype, we down-regulated the ML-IAP mRNA using sequence optimized antisense oligonucleotides. **Results:** As measured by real-time PCR, oligonucleotides M706 and M711 inhibited ML-IAP mRNA expression by 47% and 52%, respectively in the highly metastatic and drug resistant SK-MEL28 cell line. Oligonucleotide M706, which was previously evaluated in G361 cells as the most efficient inhibitor of ML-IAP expression, was chosen to compare cell viability and drug sensitivity of these two melanoma cell lines with different p53 functionality. Protein expression was reduced by oligonucleotide M706 to 49% of the normal level and resulted in a dose-dependent specific reduction of cell viability with a maximum of 39% at 600 nM. Typical morphological changes showed that loss of viability was mainly due to cell death. In combination experiments, the use of oligonucleotide M706 resulted in a two-fold increase of cisplatin cytotoxicity at different concentrations of oligonucleotide and cisplatin ($P<0.05$). This is in line with our previous findings in G361 melanoma cell line, in which oligonucleotide M706 caused a 3-fold increase in cisplatin cytotoxicity. **Conclusion:** Our data suggest the use of ML-IAP antisense oligonucleotides to overcome drug resistance in metastatic melanoma, in spite of its p53 status. *Iran. Biomed. J. 13 (1): 27-34, 2009*

Keywords: Melanoma, Melanoma inhibitor of apoptosis (ML-IAP, Livin), Cytotoxicity, Antisense, Cisplatin

INTRODUCTION

Malignant melanoma is a typical example of treatment refractory tumor with rapidly increasing incidence [1]. In comparison to localized melanoma, which is often curable by surgery, metastasized disease has a poor prognosis with no durable response to conventional therapies [2]. Moreover, the use of chemotherapy is often associated with adverse side effects and the induction of drug resistance [3]. This situation warrants the development of novel more efficient treatments for this disease.

The accumulation of neoplastic cells can occur through enhanced proliferation, diminished cell turnover, or a combination of both. Recent studies have elucidated the mechanisms by which alterations in the apoptotic machinery contribute to the process of carcinogenesis and drug resistance [4]. Over the last decades, various studies demonstrated that melanoma chemoresistance is partly due to deregulated apoptosis through the intrinsic and extrinsic pathways [5]. In this context, multiple defects at different levels have been shown including up regulation of anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl-xL, and Mcl-1 [6], loss of phosphatase and tensin homologue as a regulator of protein kinase B activity [7], inactivation of the apoptosis effector Apaf-1[8], and overexpression of members of the IAP (inhibitor of apoptosis proteins) family such as survivin [9,10] and melanoma, an inhibitor of apoptosis proteins

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(ML-IAP) [11].

Members of the IAP family of cell death inhibitors are characterized by at least one zinc-binding motif, termed baculovirus IAP repeat, which suppresses apoptosis, basically by binding to activated caspases [12]. ML-IAP (synonymously named Livin/KIAP) is a relatively novel human IAP which is significantly upregulated in malignant melanoma cell lines [11, 13-15] and in tumor tissues including melanoma, leukemia, bladder and lung carcinoma [11, 16-19]. It is of interest that the basal expression levels of ML-IAP, similar as survivin, is low and almost undetectable in most normal adult tissues [11, 13-15]. Furthermore, there is increasing evidence that endogenous expression of ML-IAP contributes to a drug resistant phenotype in melanoma [20-22], due to inhibition of caspases 3 and 9 [11, 13, 23]. Similarly, ectopic expression of ML-IAP in MCF7 breast cancer cells and HeLa cells resulted in protection against various apoptotic stimuli including tumor necrosis factor α (TNF-α), UV irradiation and chemotherapy [13, 21, 22]. All these findings together identify ML-IAP as a candidate mediator of melanoma drug resistance.

Antisense oligonucleotides and small interfering RNA (siRNA) have emerged as valid tools to specifically modulate gene expression and validate new targets for cancer therapy. Along the same lines, it has been demonstrated that antisense-mediated simultaneous down-regulation of Bcl-2 and Bcl-xL facilitates melanoma cells apoptosis and sensitizes cells to chemotherapy [24, 25]. Moreover, down-regulation of Bcl-xL with specific antisense oligonucleotides significantly increased the sensitivity of melanoma cells to cisplatin [26]. Similarly, survivin targeting using a dominant negative mutant also inhibited melanoma growth *in vivo* [27].

It has been recently demonstrated by Crnkovic-Mertens *et al.* [21] that livin targeting with a vector-borne siRNA sensitized HeLa cells to apoptosis induced by various stimuli, such as UV irradiation and TNF-α. More recently, the authors showed that the livin β isoform plays the dominant role in the antiapoptotic activity of the protein [28]. In contrast to siRNA, the antisense approach to targeted down-regulation of antiapoptotic proteins has already been exploited in numerous clinical trials; several of them have started to unveil positive results [29].

In a continuation of our previous study with G361 melanoma cells [22], we chose the SK-MEL28 cell line [30] as a further clinically relevant *in vitro* model with high expression of ML-IAP to assess the potential of ML-IAP antisense to overcome melanoma drug resistance. Moreover, p53 is mutated in this cell line and also its extrinsic apoptotic pathway is not functional. Therefore, this model could give us information about efficiency of ML-IAP down-regulation in those tumors with same characteristics as SK-MEL28 including p53 mutation and aberrant extrinsic apoptotic pathway.

MATERIALS AND METHODS

Tumor cell lines. The melanoma cell line SK-MEL28 was obtained from American Type Culture Collection (ATCC) and was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

Antisense oligonucleotides. The secondary structure of the 1260 bp ML-IAP mRNA (GenBank, accession number AF311388) was predicted by the RNA structure program to identify putative single stranded sequences as hybridization sites for antisense oligonucleotides, as described previously [31]. A total of five 20-mer phosphorothiolate antisense oligonucleotides were synthesized by Microsynth Inc. (Balgach, Switzerland). Two scrambled and one 3-base mismatch (bold letters) controls to the most potent antisense oligonucleotides were also provided [22].
Sequences were as follows:

Antisense M184	5`-TTAGGTCCCATGGAGGGAAC-3`
Antisense M250	5`-GTGGGACCATCACCGGCTGC-3`
Antisense M678	5`-TCTCCTGCACACTGTGGACA-3`
Antisense M706	5`-TTCCGGTTCTTCCCACGGGT-3`
Antisense M711	5`-GCGTCTTCCGGTTCTTCCCA-3`
Mismatch M706a	5`-TT**TT**GGTTCTTCCCAC**A**GGT-3`
Scrambled M706b	5`-TGTGCTCCCTCGTAGCCTTG-3`
Scrambled M706c	5`-GTTCGCCTCTCCTGGATCGT-3`

Treatment of cells with oligonucleotides and cisplatin. Oligonucleotides were delivered in the form of complexes with Lipofectin (Invitrogen, Basel, Switzerland) as described previously [22]. Briefly, Lipofectin was allowed to complex with oligonucleotides in serum- and antibiotic-free medium before dilution and addition to cells, which had been plated the previous day in supplemented RPMI-1640 with 10% FBS without antibiotic. Cells were incubated with oligonucleotides and Lipofectin for the indicated time periods and either harvested for further experiments or the transfection mixture was replaced by fresh standard medium. In

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combination experiments cisplatin (Platinol, Bristol-Myers Squibb, Baar, Switzerland) was added following a 20-h transfection with oligonucleotide in various concentrations of 2.5 and 5 μM, and cells were incubated another 72 h for viability analysis.

Real-time PCR. Total RNA was extracted from cells using the RNeasy Mini kit (QIAGEN Inc., Basel, Switzerland). For cDNA synthesis, 0.5 μg extracted RNA and Taqman reverse transcription reagents (including random hexamer) were used as described in the user's manual of the ABI PRISM 7700 sequence detection system (Perkin Elmer Applied Biosystems, Foster City, CA, USA) for real-time monitoring of cDNA amplification. Previously described primer and probe were used for the amplification of ML-IAP cDNA and relative quantification of gene expression was performed using rRNA as internal standard [22].

Determination of cell viability. Cell viability was measured in colorimetric MTT assays as described elsewhere [22]. For each experiment, 10^4 cells per well were seeded in 96-well plates and incubated for 24 h before a 20-h transfection with serial dilution of oligonucleotides and lipofectin alone. At the indicated time points, the MTT reagent (Sigma, St. Louis, MO, USA) was added (10 μl/well of 10 mg/ml in PBS) and allowed to react for another hour prior to the addition of 100 μl/well of solubilizing reagent [22]. After overnight solubilization, substrate cleavage was monitored at 570 nm using an ASYS microplate reader (HITECH Inc., Austria) and analyzed using MikroWin 2000 software (HITECH Inc., Austria). Viability of SK-MEL28 cells was further assessed by morphological examination using an inverted phase contrast microscope (Leitz, Axiovert 25, Wetzler, Germany).

Western-blotting. Cells were extracted in RIPA lysis buffer (Upstate Biotech Inc., NY, USA) on ice for 30 min and cell debris was removed by centrifugation at 14,000 ×g at 4°C for 10 min. Protein concentration was determined using Bradford assay [32]. For Western-blotting, 50 μg proteins was resolved by SDS-PAGE and then transferred to nitrocellulose filter paper (BIO-RAD Laboratories, CA, USA) by semidry blotting for 1 h. Recombinant human Livin α protein (R and D system, Inc., Minneapolis, MN, USA) was loaded as positive control. Blocking was performed in Tris-buffered saline containing 5% BSA and followed by overnight incubation with biotinylated goat antihuman ML-IAP (livin) antibody (R and D system, Inc., Minneapolis, MN, USA) and β-actin antibody (ICN Biomedicals, Inc., Aurora, OH, USA). As secondary antibodies, horseradish-peroxidase-conjugated anti-biotin (Cell Signaling, Beverly, MA, USA), rabbit anti-mouse (Sigma, Germany) immunoglobulin peroxidase conjugates were added for 1 h at room temperature. Visualization was performed using the ECL western blotting detection kit (Amersham, CA, USA) according to the manufacturer`s instructions. Relative protein levels were measured upon scanning the x-ray films using a Uvitec scanner (ASYS Hitech GmbH, Eugendorf, Austria) and total lab program (Version 1.1, U.K.). The signal strength of each band was normalized for the respective β-actin control.

Statistical analysis. All data are presented as the mean ± SD of three independent determinations. *P* values ≤ 0.05 calculated by unpaired student's *t*-test were considered significant.

RESULTS

Down-regulations of ML-IAP mRNA in SK-MEL 28 melanoma cells by antisense treatment. Down-regulation of ML-IAP expression was achieved using phosphorothioate antisense oligonucleotides [22] hybridizing to various regions of the ML-IAP mRNA. SK-MEL 28 cells were transfected with 600 nM antisense in the presence of lipofectin. ML-IAP mRNA levels relative to rRNA were analyzed 20 h after transfection using real-time PCR. Treatment with the most potent antisense sequences M706 and M711 reduced ML-IAP mRNA expression by 47% and 52%, respectively (Fig. 1A). Since the activity of these two antisense sequences was not significantly different, we chose oligonucleotide M706 to see whether it is effective in SK-MEL28 melanoma cells as it was reported for G361 cells. To control for unspecific effects of M706, two scrambled and one 3-base mismatch sequence control of M706 were used, which only marginally reduced ML-IAP expression (Fig. 1B). The scrambled oligonucleotide M706c was chosen as control in all subsequent experiments. Similar to the real-time PCR results, upon a 20h transfection M706 effectively inhibited ML-IAP protein expression in SK-MEL28 cells by 51%. The M706c control only slightly decreased the level of ML-IAP protein (Fig. 1C). These results identified oligonucleotide M706 as a specific and effective inhibitor of ML-IAP expression in melanoma cells.

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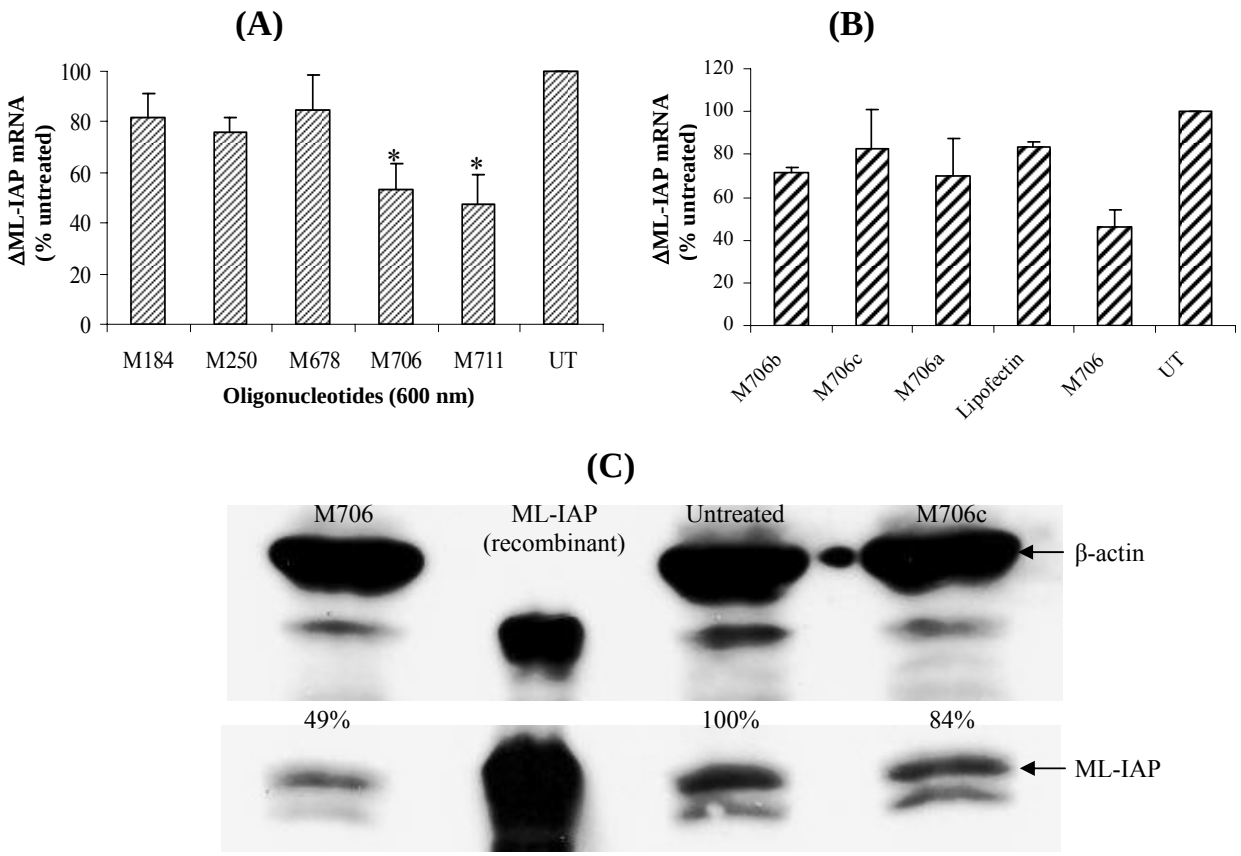

Fig. 1. Down-regulation of ML-IAP expression by antisense oligonucleotides. **(A)** SK-MEL28 melanoma cells were treated with 600 nM antisense M184, M250, M678, M706 and M711, and harvested after a 20-h incubation. Quantification of ML-IAP mRNA relative to rRNA as internal standard was performed using real-time PCR. Data are presented as the mean ± SD of three independent experiments performed in quadruplicates. $P<0.005$ for both M706 and M711, compared to untreated cells, was shown with *. ML-IAP mRNA level relative to rRNA in untreated cells was taken as 100%. **(B)** Melanoma cells were treated either with lipofectin or with 600 nM antisense M706, M706a (mismatch), M706b and M706c (both scrambled controls), and harvested after a 20-h incubation. Quantification of ML-IAP mRNA relative to rRNA as internal standard was performed using real-time PCR. **(C)** Whole cell extracts prepared from SK-MEL28 cells transfected with M706 or M706c for 20 h were immunoblotted with antibodies against ML-IAP. Recombinant ML-IAP protein was used as positive control. β-actin staining was used to control for equal protein loading. Numerical values represent the relative levels of ML-IAP protein corrected for β-actin as untreated cell's integrated volume, which was measured by Totalab software after scanning the films as described in Materials and Methods.

Oligonucleotide M706 decreases melanoma cell viability. To analyze the effect of ML-IAP down-regulation on SK-MEL28 cell viability, cells were treated with different concentrations of M706 or M706c control and analyzed in MTT assays. As shown in Figure 2A, oligonucleotide M706 reduced cell viability dose-dependently, and at a concentration of 600 nM decreased the viability of SK-MEL28 cells by 39% compared to untreated cells. Oligonucleotide (1200 nM) is non-specifically toxic for the cells. The cytotoxicity of M706c was negligible over the whole dose range tested (Fig. 2A). Cell death induction upon antisense M706 treatment at 400 nM dose was confirmed by morphology analysis. Whereas most cells treated with oligonucleotide M706 detached from the culture surface, cells treated with the control sequence M706c remained mainly adherent (Fig. 2B-D).

Oligonucleotide M706 increased cisplatin cytotoxicity. To evaluate whether ML-IAP inhibition has the potential to sensitize SK-MEL28 to cisplatin, a combination treatment was performed. Two concentrations of oligonucleotide M706 were combined with cisplatin and cytotoxicity was measured in MTT assays. Figure 3A and 3B show a significant increase in cisplatin cytotoxicity upon

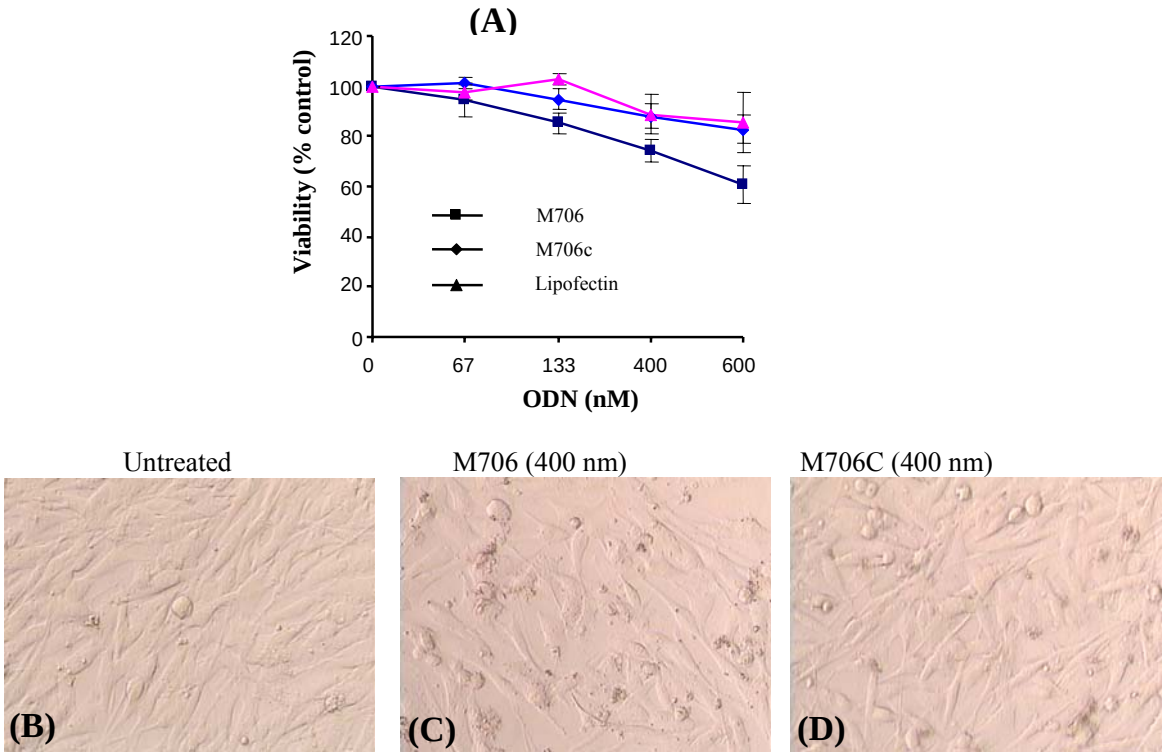

Fig. 2. Viability of SK-MEL28 cells upon treatment with ML-IAP antisense. **(A)** Cells were treated with serial dilutions (600 nM-67 nM) of lipofectin, antisense M706 or M706c for 20 h. Three days after transfection, cell growth was determined in MTT assays as described in Materials and Methods. Values represent the mean ± SD of three independent experiments performed in quadruplicates. $P<0.05$ when comparing the effect of M706 and M706c at 600 nM. **(B-D)** Photomicrographs of SK-MEL28 cells treated with 400 nM antisense M706 and M706c for 20 h followed by 72 h growth in normal medium. Cells treated with M706 antisense rounded up and detached, whereas M706c treated cells remained mainly attached to the surface.

treatment with two concentrations of M706. At a dose of 800 and 400 nM, oligonucleotide M706 alone reduced cell growth by 43% and 29% compared with 30% and 20% of the M706C control sequence. The combination of 800 nM antisense and 5μM cisplatin enhanced cytotoxicity by 56%, 72 h post transfection, which reflects an approximately 2-fold increase in cisplatin cytotoxicity. Combined treatment with cisplatin (5 μM) and M706 (800 nM) induced extensive cell death in SK-MEL28 melanoma cells compared with cisplatin alone or with the combination of cisplatin and M706C as shown by morphology analysis (Fig. 3C-F). Live cells remained attached to the dish while dead cells detached and rounded up. Similar observations were made with 400 nM oligonucleotide M706 and 2.5 μM cisplatin (data not shown).

DISCUSSION

ML-IAP is a novel member of the IAP family and frequently upregulated in cancer, including melanoma, breast cancer, leukemia, lung cancer and renal cell carcinoma [11, 19, 33]. It has emerged as an interesting target for cancer therapy due to its crucial role in antagonizing both death receptor and mitochondrial-based apoptotic pathways [13]. Over the past two decades, antisense technology has emerged as a reliable tool for selective gene modulation [29], and ongoing *in vitro* studies with antisense oligonucleotides have constituted an important part of target validation for cancer therapy [34]. Several of these basic *in vitro* experiments resulted in promising clinical trials [29]. Similar to other apoptosis-related proteins, the role of ML-IAP in cancer drug resistance has been elucidated using nucleotide-based approaches in different tumor cells including HeLa, melanoma cells, non-small cell lung cancer and renal cell carcinoma [13, 19, 21, 22, 35]. We previously identified oligonucleotide M706 among five 20-mer phosphorothioate oligo-nucleotides as the most effective antisense sequence ML-IAP in G361 melanoma cells that would decrease ML-IAP mRNA and protein expression by 68% and 78%, respectively [22].

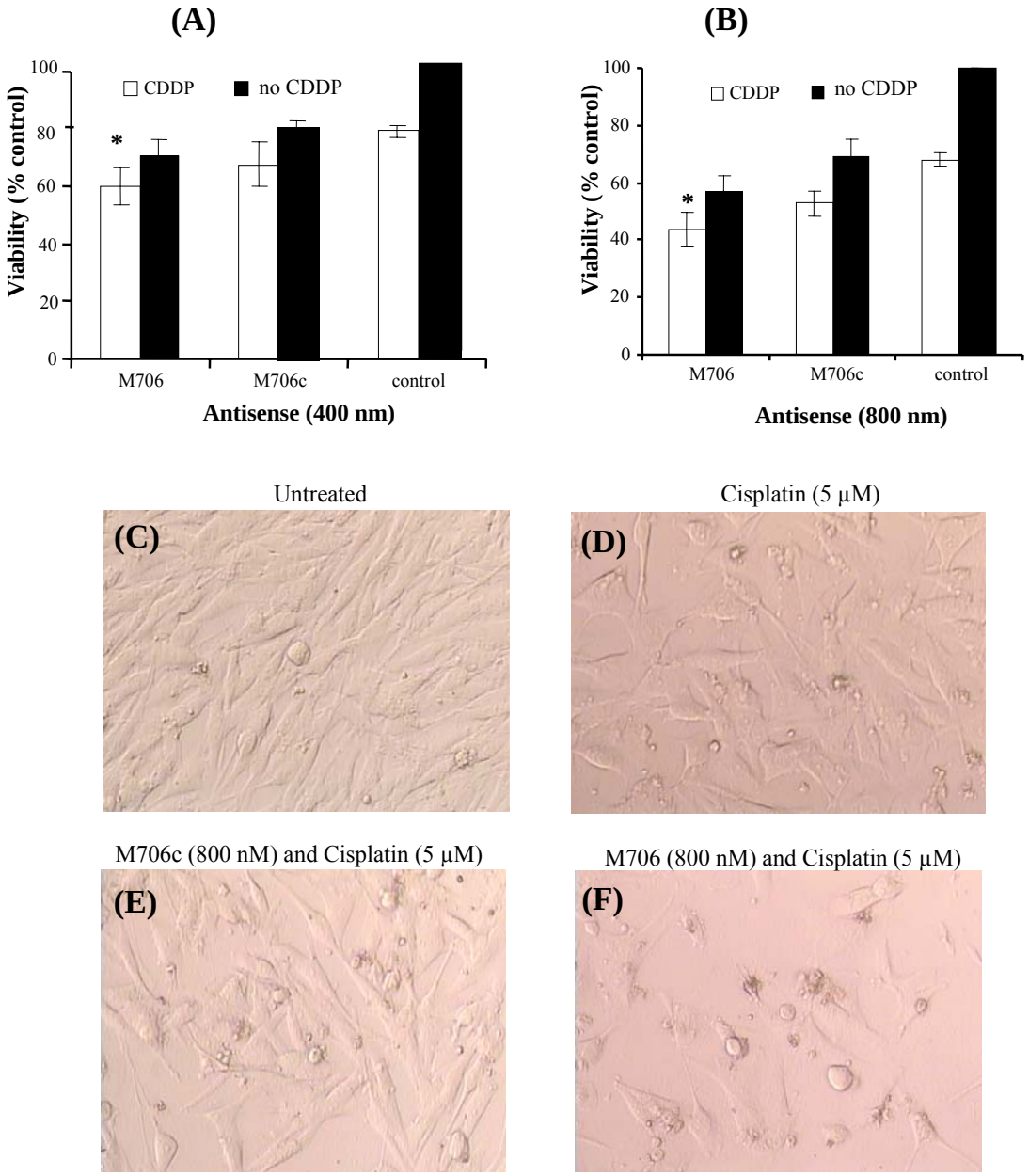

Fig. 3. Cisplatin cytotoxicity upon antisense treatment in SK-MEL28 cells. (**A** and **B**) 1×10^4 cells were transfected with 800 nM and 400 nM M706 or M706C for 24 h. Cell viability was determined in triplicate cultures 72 h after addition of either 5 or 2.5 µM cisplatin, or of fresh medium to the transfected cells, using the MTT reagent as described in Material and Methods. Each value represents the mean ± SD of three independent experiments. The cytotoxicity of cisplatin combined with M706 was significantly higher than combination of cisplatin and M706C control or cisplatin alone in both combinations ($P<0.05$ when comparing the effect of M706 and M706c as indicated by *). **(C-F)** Morphology of the Sk-MEL28 cells treated with 5 µM cisplatin and 800 nM M706 were observed by phase contrast microscopy (magnification, 40×).

In the present study, oligonucleotide M706, which demonstrated very similar efficacy as oligonucleotide M711 in SK-MEL28 cells, was chosen to evaluate its cytotoxicity and ability to restore drug sensitivity in SK-MEL28 cells. These cells were chosen based on their highly drug resistant phenotype and the lack of functional p53, which is typical for melanoma chemo-resistant phenotype [30]. We could demonstrate that oligonucleotide M706 is slightly less, but still considerably effective in targeting ML-IAP in SK-MEL28 cells compared to G361 cells (53% versus

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68% mRNA down-regulation, respectively), under identical experimental conditions. It is well known that the efficacy of antisense to inhibit expression of a target gene can vary from one cell line to another, even if the cells are of the same histological origin. This may be attributed, at least in part, to either lower transfection efficiency or higher ML-IAP mRNA level in SK-MEL28 cells compared with G361 cells.

Next, we examined whether oligonucleotide M706 alone is able to decrease SK-MEL28 cell viability, and found that it inhibited cell growth in a dose-dependent and specific manner. Maximum specific inhibition was achieved with at a 600 nM dose in serial dilutions starting from 1200 nM, whereas a 39% decrease in viability of SK-MEL28 cells was measured. This indicates that ML-IAP down-regulation alone is less efficient in inducing cell death and suggests that it does not act as a crucial survival factor in the absence of additional stimuli. In line with this conclusion is that the antiapoptotic proteins XIAP and Bcl-2 are also over expressed in SK-MEL28 cells (data not shown) and may thus compensate for the loss of ML-IAP upon antisense treatment. This is also in good agreement with the findings of others showing that down-regulation of Bcl-2 and survivin *per se* in melanoma cells does not result in significant cell death in the absence of cytotoxic stress [25, 27]. On the other hand, our previous finding showed a 30% reduction in G361 cell viability upon treatment with the same dose of M706 (600 nM) [22]. This indicates that M706 decreases viability in both cell lines without significant difference (P>0.05). Morphological changes in SK-MEL28 upon treatment with antisense M706 confirmed that ML-IAP down-regulation induced cell death characterized by detachment of cells from the culture surface.

Finally, we wanted to investigate whether antisense mediated down-regulation of ML-IAP expression has potential to sensitize SK-MEL28 cells to the chemotherapeutic agent cisplatin. The cytotoxicity of antisense M706 combined with cisplatin was higher than each reagent alone (56% versus 43% and 30%, respectively), and oligonucleotide M706 induced a 2-fold increase in cisplatin toxicity in SK-MEL28 cells. This observation is in line with our previous finding showing a 3-fold increase in cisplatin cytotoxicity upon ML-IAP down- regulation in G361 cells [22]. Lower efficiency of combination treatment in SK-MEL28 cells could be due to the p53 mutation in this cell line that makes it more drug resistant. Similarly, combinations of survivin or Bcl-2 down-regulation with cisplatin could also cause supra-additive effects on cell viability [25, 27].

In conclusion, the here reported findings in SK-MEL28 cells as a highly metastatic melanoma model confirm our previous observation in G361 melanoma cells that targeted ML-IAP inhibition has potential to sensitize melanoma cells to chemotherapy. Further preclinical investigations of ML-IAP antisense and siRNA are warranted to assess the full potential of this intervention approach for melanoma therapy, before extending experiments toward *in vivo* evaluations.

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