Downregulation of Bcl-xL and Mcl-1 is sufficient to induce cell death in mesothelioma cells highly refractory to conventional chemotherapy

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Malignant pleural mesothelioma (MPM) is an aggressive tumor with poor prognosis and limited response to platinum-based chemotherapy. Several lines of evidence support a role for the anti-apoptotic protein Bcl-xL in MPM chemoresistance. Since it has been recently suggested that Mcl-1 cooperates with Bcl-xL for protection against cell death, we investigated the response of mesothelioma cell lines to the downregulation of Bcl-xL (alone or in combination with cisplatin) and the potential interest of its concomitant inhibition with that of Mcl-1. Using RNA interference, we showed that Bcl-xL depletion sensitized two highly chemoresistant mesothelioma cell lines to cisplatin and that under this treatment, one cell line, MSTO-211H, displayed an apoptotic type of cell death, whereas the other, NCI-H28, evidenced mainly necrotic-type cell death. Otherwise, the inhibition of Mcl-1 by cisplatin may contribute to this induction of cell death observed after Bcl-xL downregulation. Strikingly, we observed that the simultaneous inhibition of Bcl-xL and Mcl-1 using small interfering RNA (siRNA) induced a massive cell death in the absence of chemotherapy and was sufficient to avoid escape to treatment in MSTD-211H cells. In NCI-H28, the addition of a low cisplatin concentration allowed to impede the long-term recovery observed after treatment by the siRNA combination. Together, these findings provide a strong molecular basis for the clinical evaluation of therapies targeting both Bcl-xL and Mcl-1, alone or in combination with conventional chemotherapy, for the treatment of MPM.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive and currently incurable malignancy arising from the mesothelial layer of pleural serosa. Chieflly associated to occupational asbestos exposure (1), MPM represents a worldwide public health problem since its incidence is expected to increase dramatically in the next three decades. For the majority of patients, disease is diagnosed at late stages when the tumor remains unresectable, the treatment thus relying on cisplatin-based chemotherapy in association with the antifolate drug pemetrexed (2). Nonetheless, the overall outcome of this disease remains very poor (median survival ranges between 9 and 17 months) and in the absence of an efficient management strategy for this cancer, there is an imperative need to understand the molecular mechanism(s) involved in the MPM chemoresistance that may subsequently help to develop new therapeutic strategies.

Abbreviations: CDDP, cisplatin; [cis-diamino-dichloro-platinum (II)]; MPM, malignant pleural mesothelioma; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; RT, room temperature; siRNA, small interfering RNA.

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Page purified siRNAs were synthesized and annealed by the Eurogentec Company (Liège, Belgium). Specific double-stranded 21 nt RNA oligonucleotides forming a 19 bp duplex core with 2 nt 3’ overhangs were used to silence Bcl-xL and Mcl-1 expression. The sequence of the double-stranded RNA used to inhibit Bcl-xL expression (noted siXL1, siRNA targeting Bcl-xL) is sense 5’-guacuuggcacucaaaagtt-3’ and antisense 5’-guacuuggcaagcaagtt-3’. siXL1 targets selectively the Bcl-xL messenger RNA but not the Bcl-xL messenger RNA. The sequence of the double-stranded RNA used to inhibit Mcl-1 expression (noted siMCL1, siRNA targeting Mcl-1) is sense 5’-guacuuggcuaacaa-3’ and antisense 5’-guacuuggcuaagca-3’. The sequence of the control siRNA siGFP is sense 5’-gacguaacggccacaagu-3’ and antisense 5’-acauagggcgaaauagac-3’. The control siRNA does not bear any homology with any relevant human genes. siRNA duplexes were transfected using the INTERFERin™ transfection reagent according to the manufacturer’s instructions (PolyPlus-Transfection, Strasbourg, France). Briefly, cells were seeded in 25 cm² flasks the day before to reach 30–50% confluency at the time of transfection. The transfection reagent and the siRNAs (at a final concentration of 10 nM) were mixed and complex formation was allowed to proceed for 15 min at room temperature (RT) before being applied to cells. The day after transfection, cells were changed to regular cell media. After indicated time, cells were trypsinized and washed with ice-cold phosphate-buffered saline (PBS) before to be analyzed. At least three independent experiments were carried out and typical results are shown.

Western blot analysis
Cells were rinsed with ice-cold PBS and lysed by a lysis buffer [50 mM Tris–HCl (pH 8.1), 150 mM NaCl, 1% Nonidet P-40, 5 mM ethylenediaminetetraacetic acid, 10 mM NaF, 4 mM phenylmethylsulfonyl fluoride, 2 mM aprotinin, 10 mM Na3VO4, 1 mM Na2VO4 and a complete mini mixture of protease inhibitors (Roche Applied Science, Meylan, France)] and incubated on ice for 30 min. Lysates were collected after centrifugation (13,200 g, 10 min, 4°C) and protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA). Equal amount of proteins (20 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 4–12% gradient polyacrylamide precast gel (Invitrogen, Cergy-Pontoise, France) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Saint-Quentin-Hercules, CA). Equal amount of proteins (20 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 4–12% gradient polyacrylamide precast gel (Invitrogen, Cergy-Pontoise, France) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Saint-Quentin-Hercules, France). After blocking non-specific binding sites for 1 h at RT by 5% (wt/vol) non-fat dry milk in tris-buffered saline with 0.1% (vol/vol) Tween20 (T-TBS), the membranes were incubated overnight at 4°C by 5% (wt/vol) non-fat dry milk in tris-buffered saline with 0.1% (vol/vol) Tween20 (T-TBS), the membranes were incubated overnight at 4°C or 2 h at RT with the following primary antibodies: Bcl-xL [28 kDa, 1:1000, 54H6 clone, Cell Signaling Technology (CST), Ozyme, Sain-Quentin-en-Yvelines, France], Mcl-1 [40 kDa, 1:750, S; Santa Cruz Biotechnology, Le Perray-en-Yvelines, France], poly (ADP-ribose) polymerase (PARP) [11 (689 kDa, 1:1000; CST), caspase-3 (351 719 kDa, 1:1000; CST), caspase-9 (473 735 kDa, 1:1000; CST), LC3B (1614 kDa, 1:1000; CST), Puma (23 kDa, 1:250; Calbiochem, VWR, Fontenay-sous-Bois, France) and Noxa (6 kDa, 1:250; Calbiochem). Membranes were then washed with T-TBS and incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:2000 or 1:5000, respectively; GE Healthcare, Orsay, France). Revelations were done by chemoluminescence (ECL Detection Reagents; GE Healthcare) using Kodak X-OMAT films. Blots were rehybridized with an actin monoclonal antibody (42 kDa, 1:10 000; Abscis, Paris, France) to control protein loading. Each immunoblot is representative of three distinct experiments.

Morphological characterization of apoptotic cells by nuclear staining with 4’,6-diamidino-2-phenylindole
After treatment, both detached and adherent cells were pooled after trypsinization, applied to a polylysine-coated glass slide by cytocentrifugation and fixed with a solution of ethanol:chloroform:acetic acid (6:3:1). The preparations were then incubated for 15 min at RT with a 1 µg/ml aqueous 4’,6-diamidino-2-phenylindole solution (Boehringer Mannheim-Roche, Mannheim, Germany), washed in distilled water, mounted under a coverslip in Mowiol (Calbiochem) and analyzed under a fluorescence microscope (BX51; Olympus, Rungis, France).

Cell cycle analysis
After treatment, detached cells were collected separately and adherent cells were dissociated by trypsin/ethylenediaminetetraacetic acid. Adherent and detached cells were then pooled and centrifuged at 2000 r.p.m. for 5 min before being fixed in 70% ethanol and stored at –20°C until analysis. Before flow cytometry analysis, the cells were centrifuged at 2000 r.p.m. for 5 min and incubated for 30 min at 37°C in PBS, to allow the release of low-molecular weight DNA (characteristic of apoptotic cells). After centrifugation at 2000 r.p.m. for 5 min, the cell pellets were resuspended and stained with propidium iodide using the DNA Prep Couter Reagent Kit (Beckman Coulter, Villepinte, France) at a final concentration of 10^6 cells/ml. Propidium-iodide-stained samples were analyzed using an EPICS XL flow cytometer (Beckman Coulter) after excitation at 488 nm by an argon laser. A 620 nm band pass filter was put on the red fluorescence of propidium iodide. Computerized gating was applied on the side and forward scatter to exclude very small debris and on pulse width and integral peak of red fluorescence to eliminate aggregates. EXPO 32 Acquisition Software (Beckman Coulter) was run for data acquisition.

Transmission electron microscopy
For transmission electronic microscopy, cells were fixed with 2.5% glutaraldehyde in PBS buffer, included in agar, rinsed in Sorenson’s buffer, post-fixed in osmium tetroxyde 1% in Sorenson’s buffer, dehydrated in ethanol and embedded in EPON resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined using a JEOL1011 transmission electron microscope.

Atomic absorption spectrometry
The DNA-linked concentration of platinum in cells was measured by atomic absorption spectrometry. After cisplatin ([cis-diamino-dichloro-platinum (II)] (CDDP) treatment, cells were trypsinized and rinsed with PBS. DNA extraction was performed with DNeasy® Blood and Tissue Kit (Qiagen, Courtabeuf, France). Platinum adducts was then measured, using a Zeeman atomic absorption spectrometer (Spectra-A; Varian, Les Ulis, France).

Results
Mesothelioma cell lines escape to cisplatin treatment in vitro
Cisplatin sensitivity was evaluated in vitro on a panel of mesothelioma cell lines (IST-Mes3, MSTO-211H, NCI-H28 and NCI-H2052). Although an inhibition of cell proliferation by prolongation of the S-phase and transient arrest in G1–M without associated cell death was noticed in all cell lines after treatment with 5 µg/ml CDDP (data not shown), most of them recovered a normal proliferation rate within ~5 to 7 days (Figure 1A). In contrast, two different responses were observed when these cell lines were treated with a higher cisplatin concentration. As monitored by cell viability, 20 µg/ml CDDP induced a highly cytotoxic effect on both IST-Mes3 and MSTO-211H cell lines, whereas only a strong cytostatic effect without associated apoptosis was observed in NCI-H28 and NCI-H2052 cells (Figure 1A and data not shown). We thus focused on MSTO-211H and NCI-H28 cell lines, which are representative of the different observed responses, and then characterized more precisely their response to CDDP. We found that 20 µg/ml CDDP elicited a G2–M phase arrest and apoptosis (as illustrated by the sub-G1 peak) in MSTO-211H. In contrast, the G2–M cell cycle arrest was maintained in NCI-H28 for ~2 weeks (Figure 1B). These observations were buttressed by the kinetic study of DNA platination showing that ~75% of the lesions have been eliminated 24 h after CDDP treatment in NCI-H28 cell line (in which apoptosis did not occurred), whereas a strong level of platination remained observable in MSTO-211H (in which cell death was observed) (Figure 1C). Of note, the levels of DNA adducts were quite similar in both cell lines after 6 h. Finally, after >3 weeks, MSTO-211H cells (where some proliferating clones were still observed), as well as NCI-H28 cells, had escaped to cisplatin treatment and recovered a normal proliferation as outlined by cell counting and cell cycle repartition analysis (Figure 1A and B). On the whole, these results demonstrated that although substantial different short-term response to cisplatin could be observed in these two cell lines, they still present in vitro the ability to escape to treatment and then recapitulate what is currently observed in clinic.

Bcl-xL downregulation sensitizes chemoresistant mesothelioma cells to cisplatin
Next, we investigated the effect of an siXL1 on the response to cisplatin in both mesothelioma cell lines. siXL1 efficiently reduced the Bcl-xL messenger RNA level up to 80% (data not shown) and started to reduce the level of Bcl-xL protein expression 48 h after transfection (data not shown), the optimal effect being observed after 72 h (Figure 2C and F). siXL1 exerted a partial cytotoxic effect (~20% of dead cells in MSTO-211H cells and 10% in NCI-H28 cells after 72 h), associated to a reduction of proliferation rate (unrelated to
transfection, as shown by the absence of such an effect in cells treated with control siRNA) on both MSTO-211H and NCI-H28 cell lines (Figure 2A, B, D and E). When siXL1 was associated with cisplatin, a drastic cytotoxic effect was observed both on MSTO-211H and on NCI-H28 cell lines. Indeed, 24 h after 5 or 20 μg/ml cisplatin exposure (i.e. 72 h post-transfection), whereas neither untransfected cells nor control siRNA-transfected cell presented major signs of cytotoxicity, siXL1-transfected cells were detached from the substratum and some morphological features of cell death could be observed (Figure 2A and D). The cell viability assessment by the trypan blue exclusion test indicated that only 11.8% (MSTO-211H) and 22.6% (NCI-H28) of cells remained viable in comparison with the control cells 24 h after exposure to 20 μg/ml CDDP (Figure 2B and E). In MSTO-211H, an increase of the sub-G1 population as well as the cleavage of PARP, caspase-3 and -9 confirmed that the combination of siXL1/CDDP induced a massive apoptotic cell death via the intrinsic mitochondrial pathway. These effects were obviously observable after siXL1/C20 (20 μg/ml CDDP) treatment (40.9% in sub-G1) in comparison with CDDP alone (15.1%) or associated with control siRNA (13.2%) (Figure 2A and C). Surprisingly, whereas the NCI-H28 siXL1-transfected cells exposed to 5 or 20 μg/ml cisplatin also detached from their substratum, presented altered morphology (Figure 2D) and were permeable to trypan blue (Figure 2E), no significant change in sub-G1 phase was noted (Figure 2D). Further, PARP was partially cleaved and none or weak caspase-3 and -9 cleavages were detected by western blot (Figure 2F). Finally, although Bcl-xL downregulation allowed cisplatin to exert a cytotoxic effect in MSTO-211H as well as in NCI-H28 mesothelioma cells, the nature of associated cell death seemed to be different between the two cell lines and was thereafter studied in more details.

Different types of cell death are observed in response to the siXL1/C20 association

The cellular morphology was dramatically altered in both cell lines treated by the siXL1/C20 combination, with detached and rounded cells (Figure 3A). In MSTO-211H cell line, the plasma membrane blebbing (typical of apoptosis) was frequently observed by phase contrast microscopy, along with both condensed and fragmented nuclei (Figure 3A and B) according to the sub-G1 peak described previously (Figure 2A). In contrast, NCI-H28 cells did not present that kind of alteration in the same conditions (Figure 3A). Indeed, blebbing was absent and nuclei condensation seemed to be incomplete (Figure 3B) and mainly unassociated to fragmentation. This is consistent with the absence of sub-G1 cell population (Figure 2D). Then, the ultrastructural appearance of cell death was analyzed by transmission electron microscopy to evaluate more precisely the
Fig. 2. Effect of siXL1 on response to cisplatin in mesothelioma cells. MSTO-211H and NCI-H28 cell lines were transfected with 10 nM siXL1 48 h before exposure to 5 or 20 μg/ml of CDDP for 2 h and cultured in drug-free medium for additional 24 h (i.e. 72 h post-transfection). Morphological features of MSTO-211H (A) and NCI-H28 (D) cell layers were observed by photon microscopy and DNA content was determined by flow cytometry after propidium iodide staining (for each condition, percentages of cells in the different phases of the cell cycle are indicated). Cell viability of MSTO-211H (B) and NCI-H28 (E) was assessed by trypan blue exclusion test 24 h after CDDP treatment. Results are expressed as the mean values of three independent experiments. Analysis of variance was used to determine the significance between each group. siXL1-transfected groups are significant compared with untrasfected- or control-siRNA-transfected groups for indicated cisplatin concentration (**P < 0.01). Protein expression levels of Bcl-xL and cleaved caspase-3, caspase-9 and PARP were monitored by western blot in MSTO-211H (C) and NCI-H28 (F). Actin was used to control protein loading.
nature of the cell death occurring after siXL1/C20 treatment in both cell lines. MSTO-211H cells exhibited important ultrastructural abnormalities, with chromatin condensation and nucleic fragmentation, typical of apoptosis, often associated with vesiculation and vacuolization of the cytoplasm, some of these vacuoles containing either debris-like dense materials or part of the cytosol (Figure 3Ca–d). This last observation incited us to verify if these debris-containing vacuoles (Figure 3Ca–e, magnification) may be related to an ‘autophagic cell death’ process. The processing of the LC3B-I protein into LC3B-II, which is a hallmark of autophagy (20), was clearly observed by western blot suggesting that this process occurred in MSTO-211H under siXL1/C20 treatment (Figure 3D), although these cells presented a constitutive processing of LC3B. In NCI-H28, even though sporadic cells displayed similar alterations (i.e. chromatin condensation and vacuolization linked with processing of LC3B) (Figure 3Ce and D), we observed that most of them exhibited dismantled cytoplasm with rupture of the plasma membrane (Figure 3Cf–h), which constitute typical features of necrotic cell death.

siXL1/C20 association avoids long-term escape to treatment

Wondering about the ability of the remaining cell population exposed to the siXL1/CDDP to escape to treatment and resume growth, we
therefore studied cell proliferation of both cell lines during the 3 weeks following CDDP exposure (Figure 4). First, it should be noticed that Bcl-x<sub>L</sub> downregulation was maintained until 6 days after siXL1 transfection in both NCI-H28 and MSTO-211H cell lines (supplementary Figure 1 is available at Carcinogenesis Online).

In MSTO-211H cells, we observed that the association of siXL1 with 5 µg/ml CDDP was transiently efficient, with moderate cytotoxicity between 24 and 96 h (as previously detailed) and subsequent recovering of normal proliferation, whereas siXL1/C20 treatment allowed a rapid (96 h) and complete annihilation of the cell population. In NCI-H28, the combination siXL1/C5 (5 µg/ml CDDP) induced a stronger cytotoxic effect than the one observed in MSTO-211H cells between 24 and 96 h, but the escape to treatment still occurred after 7–9 days. In response to the siXL1/C20 association, a massive cell death was observed during the few days following the exposure, and no proliferation recovering was observed, even after 3 weeks. The long-term effect of the association of control siRNA with CDDP did not substantially differ from the situation observed after exposure to cisplatin alone (data not shown). In both cell lines, the combination of siXL1 with 20 µg/ml CDDP led to a complete annihilation of the population, even in the highly resistant NCI-H28 cells in which neither siXL1 nor cisplatin alone induced cell death.

**Mcl-1 inhibition could be involved in siXL1-induced apoptosis in response to cisplatin**

Recently, it has been suggested that Bcl-x<sub>L</sub> and Mcl-1 cooperate to protect cells against apoptosis and that their concomitant inhibition is required for apoptosis induction via Bak (19). We thus investigated the potential role of cisplatin on the modulation of Mcl-1 expression or activity and the impact of such a modulation on the response to the siXL1/CDDP association, 24 and 96 h after cisplatin exposure. Indeed, a downregulation of Mcl-1 was observed in response to CDDP in both cell lines, although this effect appeared earlier in MSTO-211H (24 h post-CDDP) than in NCI-H28 (96 h post-CDDP) (Figure 5). Whatever the kinetics, a massive cell death was observed concomitantly to the disappearance of Mcl-1 (mainly observed after exposure to 20 µg/ml CDDP). However, we also noted apoptosis in response to the combination of siXL1/C5, whereas Mcl-1 protein expression was not markedly modified. We thus also studied the expression of the BH3-only proteins Noxa and Puma, which can impede the anti-apoptotic activity of Mcl-1 and which have been demonstrated to upregulate upon cisplatin treatment (21,22). Interestingly, Noxa and Puma protein levels were also increased in both mesothelioma cell lines after cisplatin treatment (even after exposure to 5 µg/ml CDDP) (Figure 5), with, however, a lower induction level of Puma in NCI-H28 cell line as compared with MSTO-211H.

**Downregulation of both Bcl-x<sub>L</sub> and Mcl-1 is sufficient to induce cell death in mesothelioma**

In order to demonstrate the cooperation between Bcl-x<sub>L</sub> and Mcl-1 to protect mesothelioma cells toward cell death, the association of siXL1 and siMCL1 siRNA was tested on the four chemoresistant mesothelioma cell lines initially described (Figure 1). We first checked the specificity and efficiency of each siRNA on its target (supplementary Figure 2 is available at Carcinogenesis Online) and that a correct silencing was obtained even if the siRNAs were used together (Figure 6B). Interestingly, the association induced a massive cell death after 48–72 h in all mesothelioma cell lines, in the absence of chemotherapy, as illustrated by cell detachment and shrinkage, by condensed or fragmented nuclei and by cell viability reduction (Figure 6A). After concomitant downregulation of Bcl-x<sub>L</sub> and Mcl-1, the proportion of viable cells was dramatically reduced to 2.2% in MSTO-211H, 24.4% in NCI-H28, 25.7% in NCI-H2052 and 38.3% in IST-Mes3 (Figure 6A). Moreover, PARP and caspase-3 cleavages were clearly observed in all different cell lines, with a lower extend in NCI-H28 and NCI-H2052 (Figure 6B). In contrast, none or little cell death was induced after transfection with single siRNA (Figure 6A) or with each of them associated with the control siRNA (data not shown). In NCI-H28, the long-term response study to the siXL1/siMCL1 association showed that the 25% viable cells remaining 72 h after treatment (Figure 6A), half died within 6 days but the rest of population still continued to proliferate and recolonized the flask after 2 weeks (Figure 6C). Of note, this condition of treatment recapitulates what we described previously (Figure 4) when cells were treated with siXL1/C5. We therefore studied the interest to combine both siXL1/ siMCL1 to a low cisplatin concentration (5 µg/ml) to eradicate the proliferating clones (Figure 6C): a nearly complete annihilation of tumor cells occurred under these conditions, allowing to consider that escape to treatment was not possible, even after 3 weeks. We also checked the effect of this association on normal mesothelial Met-5A cell line and did not observe such strong cytotoxicity, whereas these cells were highly sensitive to conventional chemotherapy (cisplatin) (supplementary Figure 3 is available at Carcinogenesis Online).

**Discussion**

Mesothelioma is one of the most chemoresistant cancer diseases and no curative treatment is currently available (23). Overcoming the
resistance to chemotherapy-induced cell death represents, therefore, a major challenge for the development of innovative therapies, to be associated or not to conventional chemotherapy. In mesothelioma, it has been suggested that the limited efficacy of chemotherapy implicates important functional defects in apoptosis signaling (24,25).

In this study, we used four mesothelioma cell lines, highly resistant to cisplatin and able to escape treatment even after exposure to a high concentration of cisplatin, recapitulating what it could be observed in patient with MPM. Interestingly, we observed that short-term responses are different between cell lines and then focused on two representative ones, MSTO-211H in which cisplatin induces a cytotoxic effect and NCI-H28 in which it exerts a cytostatic effect.

First, using RNA interference, we demonstrated that Bcl-xL down-regulation sensitizes different mesothelioma cell lines to cisplatin at low concentration (5 μg/ml), even the most chemoresistant NCI-H28 one. According to previous in vitro and in vivo studies targeting Bcl-xL (antisense or BH3-mimetics) to potentiate the effect of cytotoxic agents (13–18) in other mesothelioma cell lines, these results pinpoint the involvement of Bcl-xL in resistance to cisplatin in MPM. In the present study, siXL1 was used to get a nearly complete inhibition of Bcl-xL protein expression that is difficult to reach with other tools such as antisense oligonucleotides. For the first time, we showed that Bcl-xL downregulation was able to completely avoid escape to treatment in both cell lines in response to 20 μg/ml CDDP. However, even though siXL1 sensitized to lower cisplatin concentration (5 μg/ml), as illustrated by a substantial cell death in MSTO-211H and NCI-H28, this association was insufficient to impede the escape to treatment clearly observed after two weeks.

The massive cell death observed in NCI-H28 after exposure to the association siXL1/C20 without caspases activation and in the absence of sub-G1 peak incited us to characterize more precisely the nature of cell death. As it is evident that death may occur through different mechanisms leading to different morphologies and that individual cells will be at different stages of the dying process, the morphological ultrastructural appearance of cell death was also analyzed by transmission electron microscopy to precise the nature of the cell death in both cell lines. Whereas MSTO-211H displayed typical apoptotic features, the cell death observed in NCI-H28 was mainly related to necrosis. Of note, it has been previously reported, in gastric cancer, that different cell lines underwent either apoptosis or necrosis upon cisplatin treatment and that blockage of apoptosis can convert the mode of cell death from apoptosis to necrosis in the same cells (22). Otherwise, the different modalities of cell death could be explained by the different energetic status of the two cell lines since our previous results showed that NCI-H28 cell line presents a strong defect in mitochondrial respiration, as compared with MSTO-211H (26). Thus, due to insufficient adenosine triphosphate production, NCI-H28 cells could be unable to undergo apoptosis and then shifting to necrosis. This shift after adenosine triphosphate depletion or insufficient has been formerly described (27,28). Furthermore, in some cases, apoptosis can supersede necrosis after adenosine triphosphate supplementation (29). Finally, caspases inhibition, which could occurred via inhibitors of apoptosis proteins, frequently overexpressed in mesothelioma (30), may also determine the choice between the two death modalities, as previously suggested (31). We also observed some features of autophagic cell death, mainly in cells in which either apoptotic or necrotic features were concomitantly observable. This could suggest that autophagy could represent an early response to stress in both cell lines, eventually leading to apoptosis (MSTO-211H) or necrosis (NCI-H28).

In the second part of this study, we focused on Mcl-1, which has been shown to be overexpressed in MPM, and studied its possible implication in the resistance of MPM. In addition, because it was shown that Bcl-xL could cooperate with Mcl-1 (19) and that their concomitant inhibition could be needed for apoptosis induction, we hypothesized that the synergistic activity of cisplatin and siXL1 could be related, at least in part, to a cisplatin-mediated inhibition of Mcl-1 expression or activity. We thus analyzed Mcl-1 expression in response to cisplatin and showed that this drug was able to decrease Mcl-1 protein expression in a time- and concentration-dependent manner. It has been recently described as a downregulation of Mcl-1 in response to cisplatin treatment in renal tubular epithelial cancer cells that was related to a proteasome-dependent degradation mechanism (32). We then tried to figure out which mechanisms could explain the decrease of Mcl-1 observed in response to cisplatin in both cell lines. For instance, a variety of cellular stresses, including chemotherapy-induced DNA damages, usually activates the transcription of BH3-only proteins, Noxa and Puma, in a p53-dependent or -independent
manner, leading to Mcl-1 inactivation or degradation by proteasome 
(33,34). In this study, the expression of these two BH3-only proteins 
was induced after cisplatin exposure in a concentration-dependent 
manner in both cell lines. It should be noticed that Puma is able to 
stabilize and inactivate Mcl-1, whereas the interaction of Mcl-1 with 
Noxa will preferentially lead to its degradation. However, Noxa over-
expression does not always correlate with concomitant degradation of 
Mcl-1 (35), suggesting that both the threshold of Noxa expression

Fig. 6. Effect of siXL1/siMCL1 alone or associated to cisplatin in mesothelioma cells. Four mesothelioma cell lines were transfected with siXL1 and siMCL1 
(10 nM for each) and cultured for 48 h (MSTO-211H) or 72 h (IST-Mes3, NCI-H28 and NCI-H2052). (A) Morphological features of the cell layers, DNA content histograms and cell viability are presented for each cell line. Results are expressed as the percentage of viable cells in comparison with the control condition. Error bars represent standard deviation values. (B) Bcl-xL and Mcl-1 protein expression levels as well as PARP and caspase-3 cleavage were determined by western blot. Blots were probed with actin to confirm protein loading. (C) Long-term effect of siXL1/siMCL1 alone or associated with 5 μg/ml CDDP (noted C5) in the highly chemoresistant NCT-H28 cell line. The number of viable cells was assessed by the trypan blue exclusion test from 1 to 17 days after treatment (administration sequence is schematically represented). Results are mean of one representative experiment on the three performed. Error bars represent standard deviation values. Representative photographs of the cell layers 17 days after treatment (upper right corner) show the proliferation recovery of the cell population treated with siXL1/siMCL1 alone, whereas addition of 5 μg/ml CDDP prevents this phenomenon as showed by the black arrow on the graph.
and the expression levels of other Mcl-1 partners may be important. Otherwise, Mcl-1 could be inactivated after caspases cleavage, this mechanism constituting both a consequence of apoptotic cell death and a potential amplification system, since Mcl-1 cleavage fragment could present an intrinsic apoptotic activity (36). However, we did not observe caspase-cleaved Mcl-1 fragment (data not shown), and moreover, this hypothesis can be excluded in NCI-H28 cells since none or weak caspases activation has been detected. Further investigations remain thus necessary to completely define the molecular mechanisms involved in Mcl-1 inactivation in response to cisplatin.

Next, it has been suggested previously that Mcl-1 expression may protect cells from apoptosis induced by the loss of Bcl-2/Bcl-xL, such as that observed after downregulation by antisense oligonucleotides (37) or by BH3-mimetic molecules (38–40). However, in this work, it could also reciprocally be considered that Bcl-xL may protect cells from the loss of Mcl-1 since in the absence of Mcl-1 following cisplatin exposure or siRNA transfection, cell death occurred only if Bcl-xL was simultaneously downregulated.

This cooperation has been thereafter demonstrated by their concomitant siRNA-mediated downregulation, which induced a massive cell death in absence of chemotherapy in all studied mesothelioma cell lines. This suggests that a strong oncogenic stress pre-exists in these cells and that Bcl-xL and Mcl-1 may cooperate to protect cells toward this death signal. Moreover, it could be hypothesized that MSTO-211H is probably subjected to stronger oncogenic stress than other cell lines. Indeed, concomitant Bcl-xL and Mcl-1 downregulation led to a nearly complete annihilation of cell population whereas ~25% of the cells remained viable in other cell lines. However, transfection efficiency could also be incriminated since although a high transfection rate was reached in all cell lines (~90% after 24 h, data not shown), the downregulation of both proteins could vary from one cell to another, some of them being probably able to conserve a residual expression of Bcl-xL and/or Mcl-1 that could be sufficient to impede cell death induction.

Moreover, we further demonstrated that in NCI-H28 cells partially refractory to the siXLI/siMCL1 association, the strengthening of the death signal and/or the inhibition of Mcl-1 activity by addition of cisplatin avoid escape to treatment even at low concentration (5 μg/ml). Interestingly, these results are in agreement with our recent study, performed on two highly chemoresistant ovarian cancer cell lines, which demonstrated that the addition of cisplatin is necessary to impede the reconstitution of the flank (41). Nevertheless, in contrast to ovarian cancer cells, it should be noticed that siXLI/siMCL1 association in absence of chemotherapy is sufficient to avoid escape to treatment in MSTO-211H mesothelioma cell line.

In summary, we showed that Mcl-1 cooperates with Bcl-xL to protect mesothelioma cells against oncogenic stress and chemotherapy. Concomitant downregulation of these proteins is sufficient to induce massive cell death in these cells highly refractory to conventional chemotherapy and avoids escape to treatment, even in response to low cisplatin concentration. This work underlines that these two anti-apoptotic proteins are together indispensable for the survival of mesothelioma cells suggesting that they could constitute potential therapeutic targets for the treatment of this yet incurable disease. It may now be important to determine whether the cooperation between Bcl-xL and Mcl-1 to protect from cell death is a common phenomenon in other cancer cells types and whether their concomitant downregulation (in association or not with conventional chemotherapy) could be an interesting therapeutic strategy yielding the opportunity to overcome cancer chemoresistance. Incidentally, the progression of BH3-mimetic small-molecule inhibitors in preclinical and clinical development (42) together with recent progresses in the design and delivery of siRNA for therapeutic gene silencing in cancer (43,44), may offer novel and effective modalities to achieve such a targeted strategy within a few years.

### Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/
41. Brotn, E. et al. (2010) Bcl-x(L) and MCL-1 constitute pertinent targets in ovarian carcinoma and their concomitant inhibition is sufficient to induce apoptosis. Int J Cancer, 126(4), 885–895.

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