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C-Myc Down-Regulation Increases Susceptibility to Cisplatin through Reactive Oxygen Species-Mediated Apoptosis in M14 Human Melanoma Cells

ANNAMARIA BIROCCIO, BARBARA BENASSI, SARAH AMODEI, CHIARA GABELLINI, DONATELLA DEL BUFALO, and GABRIELLA ZUPI

Experimental Chemotherapy Laboratory, Experimental Research Center, Regina Elena Cancer Institute, Rome, Italy

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ABSTRACT

Our aim in this work was to define the role of c-Myc in the susceptibility to cisplatin [*cis*-diamminedichloroplatinum(II) (CDDP)] in human melanoma cells. Two M14 melanoma cell clones obtained by transfection and expressing six to ten times lower c-Myc protein levels than the parental cells and the control clone were employed. Analysis of survival curves demonstrates an increase in CDDP sensitivity in c-Myc low-expressing clones if compared with the control clone and the parental line. The enhanced sensitivity is unrelated to the impairment in enzymatic DNA repair activity. Cell cycle analysis demonstrates that although the control clone is able to completely recover from the CDDP-induced S-G_2/M block, this arrest is prolonged in c-Myc low-expressing clones and a fraction of cells undergoes apoptosis. Although no changes in P53, Bax, Bcl-2, and Bcl-$x_{L/S}$ protein levels are observed, apoptosis is associated with the formation of reactive oxygen species (ROS), activation of caspase-1, caspase-3 and cleavage of the specific caspase substrate poly-ADP-ribose polymerase. The use of the antioxidant *N*-acetyl cysteine and caspase inhibitors prevents CDDP-induced apoptosis in c-Myc low-expressing clones, demonstrating that ROS, caspase-1, and caspase-3 are required for apoptotic cell death. Moreover, ROS generation depends on caspase-1-like activation because the Ac-YVAD-cho inhibitor abrogates CDDP-induced ROS in the c-Myc low-expressing clones.

c-myc is a member of the *myc* gene family, which has been found to be overexpressed in a large subset of tumors (Nesbit et al., 1999). c-Myc protein has recently been shown to represent a prognostic marker in melanoma tumors, where it is frequently associated with Clark's level and poor prognosis (Lazaris et al., 1995).

c-Myc oncoprotein is a transcriptional factor implicated in many cellular processes such as proliferation, differentiation, and transformation (Marcu et al., 1992; Desbarats et al., 1996). c-Myc has also been found to induce apoptotic cell death under certain conditions such as deprivation of survival factors, viral infection, and treatment with tumor necrosis factors and chemotherapeutic agents (Desbarats et al., 1996). The molecular mechanisms underlying c-Myc-mediated apoptosis in response to different stimuli are yet to be understood. Much effort has been dedicated to determining how c-Myc causes apoptosis. Some data demonstrate that wild-type P53 is required for c-Myc-induced apoptosis (Hermeking and Eick, 1994), even though P53-independent mechanisms have also been reported (Sakamuro et al., 1995). Furthermore, it has been found that the c-Myc-induced apoptosis can require the CD95 receptor-ligand pathway (Hueber et al., 1997) and can also be prevented by overexpression of the Bcl-2 oncoprotein (Bissonnette et al., 1992). Biochemical analyses have recently implicated proteases, including caspase-3 (CPP32), in the c-Myc-induced apoptosis (Kangas et al., 1998).

Although c-Myc expression has been found to implement cells with programs for both proliferation and cell death, the role of c-Myc protein in cellular susceptibility to anticancer drugs is controversial. In fact, overexpression of c-Myc protein has been reported to enhance tumor cell sensitivity (Lotem and Sachs, 1993; Dong et al., 1997; Nesbit et al., 1998) and to induce resistance in response to antineoplastic agents (Sklar and Prochownik, 1991; Kinashi et al., 1998). In this context, several in vitro studies suggest that elevated c-Myc expression can confer resistance to CDDP (Sklar and

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ABBREVIATIONS: CPP32, caspase-3; CDDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; ODN, oligodeoxynucleotide; ICE, caspase-1; ROS, reactive oxygen species; ADR, doxorubicin (Adriamycin); CPT, camptothecin; Z-VAD-fmk, Z-Val-Ala-Asp (OMe)-fluoromethylketone; Ac-YVAD-cho, *N*-acetyl-Tyr-Val-Ala-Asp-CHO; Ac-DEVD-cho, *N*-acetyl-Asp-Glu-Val-Asp-CHO; β-gal, β-galactosidase; BrdU, bromodeoxyuridine; NAC, *N*-acetyl-L-cysteine; LUC, luciferase; PARP, poly-ADP-ribose polymerase; PI, propidium iodide; CHO, Chinese hamster ovary.

Prochownik, 1991), an antineoplastic agent with demonstrated clinical effectiveness against several tumors, including melanoma. Resistance to CDDP and other chemotherapeutic agents represents a major obstacle to effective cancer therapy because clinically significant levels of resistance quickly emerge after treatment. Mechanisms for acquired CDDP resistance include induction of DNA repair enzymes, decreased drug accumulation, and increased levels of glutathione and glutathione transferase (Gately and Howell, 1993). Another mechanism of drug resistance is the inability of tumor cells to activate the apoptotic program. In fact, it is widely demonstrated that cytotoxic drugs, irrespective of their intracellular target, act by inducing apoptosis in susceptible cells (Fisher, 1994). Several genes have been implicated in the modulation of drug-induced apoptosis including *c-myc* (Sklar and Prochownik, 1991; Lotem and Sachs, 1993; Dong et al., 1997; Fearnhead et al., 1997; Kinashi et al., 1998).

The involvement of c-Myc protein expression in CDDP sensitivity and activation of the apoptotic program has been largely documented by our group. In particular, we previously demonstrated in some human melanoma lines that the treatment with *c-myc* antisense oligodeoxynucleotides (ODNs) is an effective inhibitor of in vitro and in vivo cell growth (Leonetti et al., 1996) and enhances CDDP antitumoral efficacy, both in vitro and in nude mice (Citro et al., 1998; Leonetti et al., 1999).

Our aim here was 2-fold: 1) to define the role of c-Myc in the susceptibility to CDDP in melanoma cells by using cellular stable c-Myc low-expressing transfectants, and 2) to clarify the mechanism(s) by which c-Myc influences CDDP sensitivity. We found that two cellular clones, expressing lower levels of c-Myc protein than the parental M14 cell line, are more sensitive to CDDP than the parental line or the control transfectant. The CDDP-induced cytotoxicity in the c-Myc low-expressing clones is closely related to the induction of apoptosis, which results from ROS production and caspase-1 and -3 activation. The use of specific ROS and caspase inhibitors demonstrate that CDDP-induced apoptosis in the c-Myc low-expressing clones requires reactive oxygen species, which in their turn depend on caspase-1-like activation.

Materials and Methods

Culture Conditions, Transfection, and Cell Growth. The M14 human melanoma cell line was grown at 37°C, in a 5% CO_2/95% air atmosphere, in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM L-glutamin and antibiotics.

M14 cells (1×10^6/200 μl) were transfected by electroporation (960 μF, 200 V; Gene Pulser; Bio-Rad, Milan, Italy) with the expression vector pcDNAI neo carrying 1.3 kilobase pairs of c-myc (exon 2 + exon 3) cloned in antisense orientation and the gene for the resistance to neomycin. To obtain individual clones, transfected cells were grown in neomycin-containing medium (0.8 mg/ml; Invitrogen). Two weeks later, clones were expanded and screened for c-Myc expression by Western and Northern blot analysis.

The growth of the M14 parental line, the MN2 control clone and two c-Myc low-expressing clones (MAS51 and MAS53) was assessed by seeding 5×10^4 cells in 60-mm Petri dishes (Nunc, Mascia Brunelli, Milan, Italy). Cell counts (Coulter Counter model ZM; Kontron Instruments, Watford, Herts, UK) and viability (trypan blue dye exclusion) were determined daily, from day 1 to day 7 of culture.

Western Blotting. Western blot was performed as reported previously (Citro et al., 1998). Total proteins (40 μg) were loaded from each sample on denaturing SDS-polyacrylamide gel electrophoresis. Immunodetection of c-Myc, CPP32, PARP, Bcl-2, P53, Bax, Bcl-$x_{L/S}$, and ICE was done using anti-c-myc (1:1000, clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CPP32 (1:500, E-8; Santa Cruz Biotechnology), anti-PARP (1:2000, VIC5; Roche Molecular Biochemicals, Mannheim, Germany), anti-Bcl-2 (clone 124; DAKO SA, Glostrup, Denmark), anti-P53 (1:500, clone Pab 1801, Santa Cruz Biotechnology) monoclonal antibodies, and anti-Bax (1:500; N-20, Santa Cruz Biotechnology); anti-Bcl-x (1:500, S-18, Santa Cruz Biotechnology), and anti-ICE (1:200; Oncogene Research, Cambridge MA) polyclonal antibodies. Enhanced chemiluminescence was used for detection. To check the amount of proteins transferred to nitrocellulose membrane, β-actin was used as control and detected by an anti-human β-actin monoclonal antibody (1:500; Santa Cruz Biotechnology). The relative amounts of the transferred proteins were quantified by scanning the autoradiographic films with a gel densitometer scanner (Bio-Rad) and normalized to the related β-actin amounts.

Northern Blotting. Total RNA was isolated by TRIzol (Invitrogen) following standard protocols and quantified spectrophotometrically. Total RNA (30 μg) was size-fractionated on denaturing formaldehyde agarose gel, blotted onto nylon filter, and hybridized with the c-myc cDNA. Filter was exposed to autoradiographic film for 2 days. Sedimentation values of 28S and 18S were used as an internal standard for RNA integrity/loading.

Treatments. Clinical grade CDDP (Pronto Platamine) and doxorubicin (ADR; Adriblastina) were obtained from Pharmacia (Milan, Italy). Camptothecin (CPT) was purchased from Sigma (Milan, Italy). Drug dilutions were freshly prepared before each experiment. Cells were seeded in 60-mm Petri dishes (Nunc; Mascia Brunelli, Milan, Italy) at a density of 2×10^5cells per dish. After 24 h, cells were exposed for 2 h to different doses of CDDP (ranging from 0.1 to 5 μg/ml), ADR (ranging from 0.1 to 0.6 μg/ml), and CPT (ranging from 0.1 to 3.5 μg/ml). To evaluate cell colony-forming ability, aliquots of cell suspension from each sample were seeded into 60-mm Petri dishes with complete medium and incubated for 10 to 12 days. Colonies were stained with 2% methylene blue in 95% ethanol and counted (1-colony $\geq$ 50 cells). Surviving fractions were calculated as the ratio of absolute survival of the treated sample/absolute survival of the control sample. All the experiments were repeated four times and each experimental sample was seeded in triplicate.

In the experiments with NAC antioxidant (Sigma), cells were preincubated with 5 mM NAC (dose with no toxic effect on the survival) for 6 h. Then cells were washed three times and treated with 2 μg/ml CDDP for 2 h. NAC was readded to the cells at the end of CDDP treatment and left in the culture medium until the time of analysis.

Caspase inhibitors (Biomol, Plymouth Meeting, PA) Z-VAD-fmk (pan caspase inhibitor), Ac-DEVD-cho (caspase-3 inhibitor), and Ac-YVAD-cho (caspase-1 inhibitor) were used at 50 μM concentration (dose with no toxic effect on the survival) and added to the culture medium after the end of CDDP treatment (2 μg/ml CDDP, 2 h) and left in the culture medium until the time of analysis.

Host Cell (β-Gal) Reactivation Assay. The host cell reactivation assay was performed as described by Fan et al. (1997). Briefly, the PEQ-176 plasmid (Promega Corporation, Madison, WI), encoding β-gal enzyme, was treated with 10 μM CDDP in 1 mM Tris-HCl, pH 7.8, 10 mM NaCl, and 1 mM EDTA for 1 h at 37°C. Cells were transfected with 5 μg of the CDDP-damaged or -undamaged PEQ-176 plasmid using calcium phosphate method (Profection mammalian transfection system calcium phosphate; Promega). To normalize for transfection efficiency, PGL-3 luciferase (Promega) plasmid was included in the transfections. Transient β-gal gene expression was assayed 48 h after transfection. Values were normalized to luciferase (LUC) internal control.

Cell Cycle, ROS, and Apoptosis. The progression through the different cell cycle phases was analyzed by bromodeoxyuridine

(BrdU; Becton-Dickinson, San Jose, CA) incorporation. Cells were pulsed with BrdU (24 h after the end of CDDP treatment) at a final concentration of 10 μM for 15 min, washed, and incubated in medium without BrdU. After the appropriate intervals, cells were harvested, resuspended in phosphate-buffered saline, fixed with ice-cold 70% ethanol, and stored overnight at 4°C. After this fixation step, DNA was denatured with 0.1 N HCl containing 0.5% Triton X-100 on ice for 10 min followed by heating in boiling water for 10 min. Cells were incubated with 2 μg/ml of mouse anti-BrdU (clone BMC 9318; Roche Diagnostics, Indianapolis, IN) for 30 min at room temperature, washed in phosphate-buffered saline and revealed with FITC-conjugated anti-mouse for 30 min (1:20, DAKO) after the addition of 0.5 μg/ml propidium iodide (PI). Cell percentages in the different phases of the cell cycle were measured by flow cytometric analysis of PI-stained nuclei as described previously (Citro et al., 1998) using CELLQuest software (Becton-Dickinson).

For the ROS content analysis, untreated and CDDP-treated adherent cells were first assayed for viability by trypan blue dye exclusion and then incubated with 4 μM dihydroethidium (Molecular Probes, Eugene, OR) for 45 min at 37°C. After incubation, the cells were analyzed by flow cytometry. ROS production was evaluated 24, 48, 72, and 96 h after the end of CDDP treatment (2 μg/ml for 2 h). As internal ROS control, the MN2 cells were treated with 15 mM H_2O_2 for 1 h. Untreated and H_2O_2-treated viable cells were analyzed for the ROS production as described above.

Apoptosis was evaluated by morphological examination. Cytocentrifuge preparations were stained with Hoechst 33258 dye (Sigma) and cover-slipped. Cell morphology was evaluated by fluorescence microscopy. Two independent observatories, taking into account three separate experiments, calculated the percentage of apoptotic cells. Apoptosis was evaluated 48, 72, 96, and 120 h after the end of CDDP treatment (2 μg/ml for 2 h).

Results

c-Myc Down-Regulation Increases the Sensitivity to CDDP, But Not to ADR and CPT. To define the role of c-Myc on melanoma susceptibility to antineoplastic drugs, c-Myc protein expression was decreased in the M14 human melanoma line by transfecting an expression vector carrying exon 2 and 3 of the *c-myc* gene cloned in antisense orientation. M14 line, characterized previously, shows gene amplification accompanied by about 9-fold higher c-myc mRNA levels and about 15-fold higher protein expression compared with normal cells (Leonetti et al., 1996; data not shown). Figure 1A shows Western blot analysis of c-Myc protein in the M14 parental line, the MN2 control clone (transfected with the empty vector), and two c-Myc low-expressing clones (MAS51 and MAS53) chosen for the experiments. The amount of c-Myc protein in the MAS51 and MAS53 transfectants are 6.5 and 10 times lower than the MN2 control clone. The MN2 control clone shows the same level of c-Myc protein expression observed in the M14 parental line. Northern blot analysis was also performed to evaluate *c-myc* expression at the transcription level (Fig. 1B). The MAS51 and MAS53 transfectants display lower c-myc mRNA levels than the control clone, whereas c-myc mRNA levels in the M14 line and MN2 clone are similar when normalized to 28s rRNA.

As expected, the decreased c-Myc expression alters the in vitro growth behavior of the M14 line. Figure 2 reports the growth curves of the M14 parental line, the control clone and the two c-Myc low-expressing clones. It is evident that after day 2 of growth the c-Myc transfectants show a reduced cell proliferation with a saturation density of about 5.5×10^5 cells compared with about 2.5×10^6 for the control cells. Moreover, after day 5 of culture, a reduction in cell number is observed in the c-Myc transfectants, whereas the M14 and the MN2 control cells are in the plateau phase.

To evaluate drug sensitivity, the two c-Myc low-expressing clones, the parental cells, and the control clone were treated with CDDP, ADR, and CPT 24 h after plating (day 1 of culture), when the doubling time of the different lines is similar (about 24 h). Figure 3 shows the survival curves of the M14 parental line, the MN2 control clone, and the two c-Myc low-expressing clones exposed for 2 h to increasing doses of CDDP, ADR, and CPT. c-Myc low-expressing clones clearly display a greater sensitivity to CDDP compared with the parental line and the control clone (Fig. 3A). On the contrary, no difference between the parental, control, and c-Myc low-expressing cells in sensitivity to ADR (Fig. 3B) and

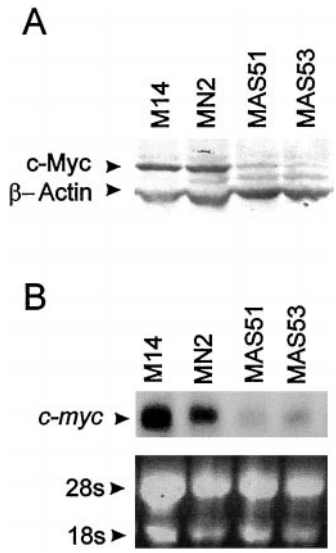


Fig. 1. A, Western blot analysis of c-Myc protein in the cell lysates of the M14 parental line, the MN2 control clone, and the MAS51 and MAS53 c-Myc low-expressing clones. The relative amount of the transferred c-Myc proteins were quantified and normalized to the corresponding β-Actin protein amount. B, Northern blot analysis of *c-myc* gene expression in the M14 parental line, the MN2 control clone, and the MAS51 and MAS53 c-Myc low-expressing clones. The bottom of the figure shows the ethidium bromide staining of the gel.

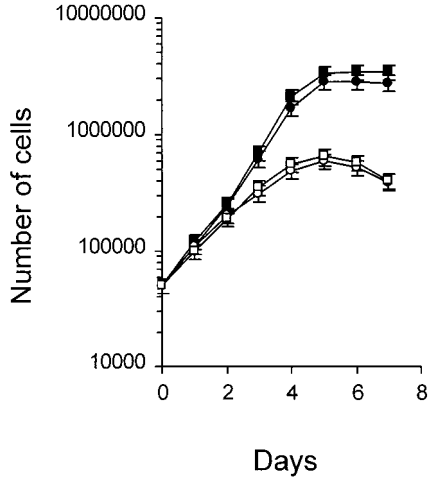


Fig. 2. In vitro growth curves of the M14 parental line (■), the MN2 control clone (●), and the MAS51 (□) and MAS53 (○) c-Myc low-expressing clones. The figure shows a representative experiment performed in quintuplicate with S.D..

CPT (Fig. 3C) is observed. The survival curves to CDDP of both control cell lines show a biphasic exponential trend with a positive shoulder, indicating that a fraction of the cell population is able to recover from the sublethal damage. On the contrary, the survival curves of the c-Myc low-expressing clones are simple exponential types, indicating the inability of the clones to recover from the CDDP damage, even at low doses of the drug. The different sensitivity among MN2, M14 cells, and the c-Myc low-expressing clones is also evident at the highest CDDP dose (5 μg/ml), the surviving fraction being about 5% and 0.01%, respectively.

To evaluate whether the different sensitivity to CDDP of the c-Myc low-expressing clones and the parental cells was related to different cell cycle perturbation induced by the drug, we followed the progression of S-phase cells through the different phases of cell cycle in untreated and CDDP-treated cells using a 15-min pulse of BrdU (Fig. 4). Moreover, the relative number of cells in each phase of the cell cycle was estimated from DNA content (PI staining) by CELLQuest software analysis. CDDP treatment induces a similar (about 90%) block in the S-G_2/M phases of cell cycle 24 h after the end of treatment (0 h, Fig. 4) in both the MN2 control clone and the MAS51 c-Myc low-expressing clone. However, a significant difference between the two clones is observed during the progression through the cell cycle phases. In fact, at 16 h after BrdU pulse labeling, although the MN2 control clone is able to recover the CDDP-induced block, the MAS51 c-Myc low-expressing clone is still arrested in the S-G_2/M phases. The inability of the MAS51 c-Myc low-expressing clone to recover S-G_2/M block induced by CDDP is caused mainly by cell cycle alteration caused by c-Myc down-regulation. In fact, even though both control and c-Myc low-expressing clones incorporate BrdU at approximately the same extent (about 20%) during the pulse (0 h), the progression of cell lines out of the S phase, first into G_2/M and then into G_0/G_1, is slower in the c-Myc low-expressing clone than in the control cells. In agreement with our previous data obtained by using c-myc antisense ODNs (Citro et al., 1998), these results suggest that the prolonged S-G_2/M block in the c-Myc low-expressing clones might be responsible for the increased CDDP sensitivity. The data obtained using the MN2 control clone and the MAS51 c-Myc low-expressing clone are similar to those in the M14 parental line and the MAS53 clone, respectively (data not shown).

To study whether the inability of the c-Myc low-expressing clones to recover from CDDP damage to DNA was caused by an alteration in the nucleotide excision repair activity, the host cell reactivation assay was performed in the different cell lines. We transfected the different cells with CDDP-damaged β-gal reporter plasmid and then measured the ability to reactivate the damaged plasmid. Figure 5 shows the relative β-gal activity in the CHO-UV47/cl3 cells, the MN2 control clone, and the two c-Myc low-expressing clones. The CHO-UV47/cl3 cell line (Chinese hamster ovary cells, UV-sensitive excision repair-defective mutant) was employed, as control, to confirm that CDDP damage was sufficient to completely inactivate the β-gal gene in the plasmid. We found that the MN2 control clone and the two c-Myc low-expressing clones are able to restore β-gal activity to the same extent, indicating that the different sensitivities to CDDP are not caused by different enzymatic DNA repair activity. The results obtained using the MN2 control clone are similar to those in the M14 parental line (data not shown).

c-Myc Down-Regulation Cooperates with CDDP Treatment to Induce Apoptosis with No Changes in the Expression of P53, Bax, Bcl-2, and Bcl-$x_{L/S}$ Proteins. We demonstrated previously that c-Myc down-regulation, obtained by treatment with c-myc antisense ODNs, is able to activate the apoptotic program by inhibiting cell progression into the cell cycle after CDDP treatment (Citro et al. 1998, Leonetti et al., 1999). On the basis of these previous results, cytofluorimetric analysis was performed from 48 to 120 h after the end of CDDP treatment, to evaluate the presence of cells with a hypodiploid DNA content. Because the analysis revealed the presence of a sub-G_1 peak only in c-Myc low-expressing clones (data not shown), morphological evaluation was performed to confirm the nature of the observed cellular death. Figure 6 shows the percentage of apoptotic cells in the MN2 control clone, the MAS51, and MAS53 c-Myc low-expressing clones untreated and treated with

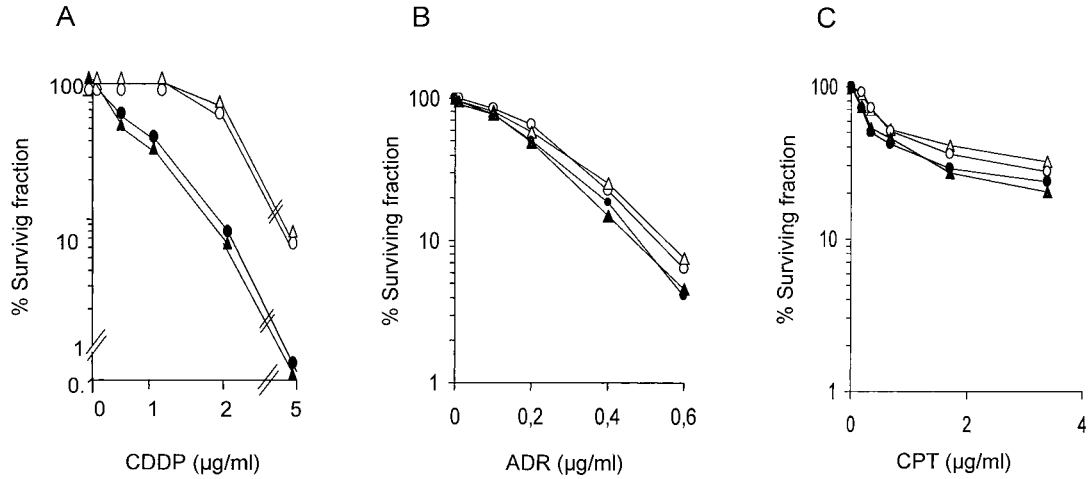


Fig. 3. Survival curves of M14 parental line (○), MN2 control clone (△), and MAS51 (▲) and MAS53 (●) c-Myc low-expressing clones exposed for 2 h to different doses of CDDP ranging from 0.1 to 5 μg/ml (A), ADR ranging from 0.1 to 0.6 μg/ml (B), and CPT ranging from 0.1 to 3.5 μg/ml (C). Surviving fractions were calculated as the ratio of absolute survival of the treated sample/absolute survival of the control sample. The data represent the mean of four independent experiments with S.D. values less than 10%.

CDDP (2 μg/ml for 2 h), evaluated 48, 72, 96, and 120 h after the end of the treatment. It is evident that CDDP does not activate the apoptotic program in the MN2 control cells, the percentage of apoptotic cells being less than 5% until 120 h after the end of CDDP treatment. On the contrary, c-Myc down-regulation is able per se to induce apoptosis in a fraction of the cells (about 20% at 120 h) and in concert with CDDP efficiently enhances the death process. In fact, about 40 and 80% of apoptotic cells are evident in c-Myc low-expressing clones 96 and 120 h after the end of CDDP treatment, respectively. Increasing doses of CDDP (5 and 10 μg/ml for 2 h) induce apoptosis in MN2 control clone (the percentage of apoptotic cells being about 8 and 25%, respectively, 48 h after the end of CDDP treatment) and speed up the apoptotic process in c-Myc low-expressing clones (the percentage of apoptotic cells being about 20 and 50%, respectively, 48 h after the end of CDDP treatment) (data not shown). However, the doses of 5 and 10 μg/ml of CDDP were not chosen for the experiments because they are not clinically achievable. The results obtained using the MN2 control clone were similar to those of the M14 parental line (data not shown).

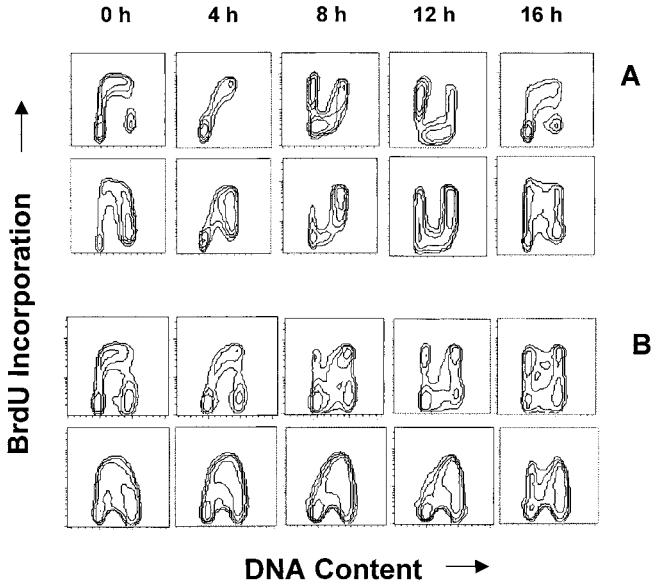


Fig. 4. Kinetics of progression through the stages of cell cycle after BrdU labeling in the MN2 control (A) and the MAS51 c-Myc low-expressing clone (B) untreated (top) and 2 μg/ml CDDP treated for 2 h (bottom). BrdU was added 24 h after the end of CDDP treatment and cytofluorometric analysis was performed at the end of the 15 min-pulse with BrdU (0 h) and from 4 to 16 h after the end of the pulse.

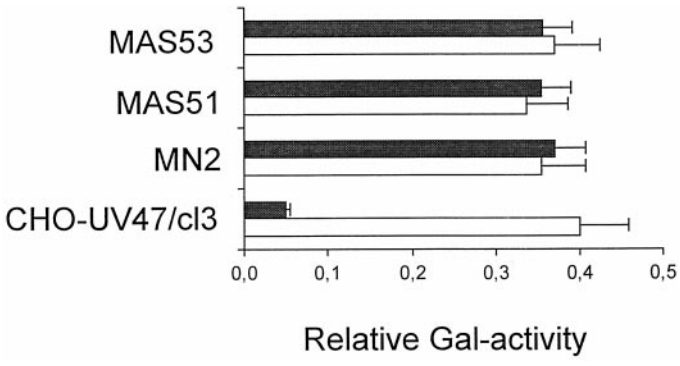


Fig. 5. Host cell (β-gal) reactivation assay performed in the MN2 control clone and the MAS51 and MAS53 c-Myc low-expressing clones. Studies were also conducted on the CHO-UV47/cl3 cells, which have defective nucleotide excision repair activity. The different cells were transfected with the CDDP-damaged (■) or undamaged (□) PEQ-176 plasmid using the calcium phosphate method. Transient β-gal gene expression was assayed 48 h after transfection. Values were normalized to LUC internal control. Results shown are the mean of three independent experiments ± S.D.

To elucidate the mechanism(s) by which c-Myc down-regulation cooperates with CDDP to induce apoptosis, we tested the involvement of P53, Bax, Bcl-2, and Bcl-$x_{L/S}$ proteins in the death process. Western blot analysis was performed from 0 to 96 h after the end of CDDP treatment. Figure 7 shows Western blot analysis of P53, Bax, Bcl-2, and Bcl-x_L in the MN2 control clone and the MAS51 c-Myc low-expressing clone at three representative times (24, 48, and 72 h) after the end of CDDP exposure. No appreciable changes are observed in the expression of these proteins both in the MN2 and in the MAS51 cells after CDDP treatment at times analyzed. Bcl-x_S was not detectable in our experimental conditions. The results obtained using the MN2 control clone and the MAS51 c-Myc low-expressing clone are similar to those in the M14 parental line and the MAS53 clone, respectively (data not shown).

c-Myc Down-Regulation Cooperates with CDDP to Induce ROS Production. Several studies have demonstrated that biochemical modifications represent an alternative apoptotic pathway (Kroemer et al., 1997). Reactive oxygen species are per se inducers of apoptosis and several drugs are able to induce the apoptotic program through generation of ROS (Kroemer et al., 1997, Miyajima et al., 1997). To test whether the inability of the c-Myc low-expressing transfectants to progress through the cell cycle activates the apoptotic program by generating ROS, reactive oxygen species were measured by flow cytometry in the still viable adherent cells during CDDP recovery. Figure 8 shows the ROS production in the MN2 control clone and the MAS51 c-Myc low-expressing clone, evaluated 24, 48, 72, and 96 h after the end of CDDP treatment. As control of the relative fluorescence shift,

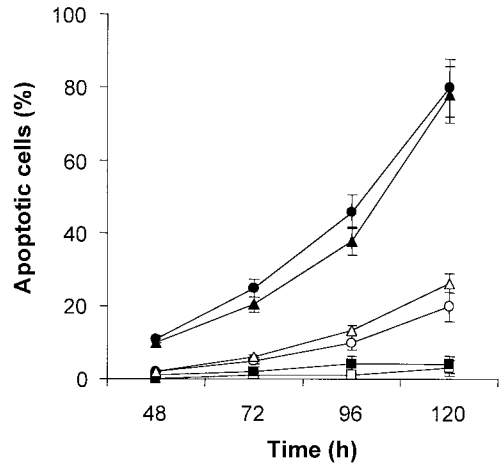


Fig. 6. Percentage of apoptotic cells determined by morphological examination in the MN2 control clone (□), the MAS51 (○), and the MAS53 (△) c-Myc low-expressing clones untreated (open symbols) or treated (filled symbols) with CDDP (2 μg/ml for 2 h). The analysis was performed 48, 72, 96, and 120 h after the end of treatment. Cytocentrifuge preparations were stained with Hoechst 33258 dye and cover-slipped. Cell morphology was evaluated by fluorescence microscopy. Results shown are the mean of five independent experiments ± S.D.

the MN2 cells treated with H_2O_2 were also assayed for the ROS production. Gating the viable cells by means of assessing the forward- and side-scatter values, no changes in the ROS content are evident in the relative fluorescence in the untreated or CDDP treated MN2 clone. On the contrary, a slight increase in the ROS production is observed in the untreated MAS51 c-Myc low-expressing clone (only about 15% at 96 h). c-Myc down-regulation, by inhibiting the recovery from CDDP damage, induces ROS production already 24 h after the end of CDDP treatment and the percentage of ROS progressively increases from 24 (about 15%) to 96 h (about 50%) after the end of the treatment. The results obtained using the MN2 control clone and the MAS51 c-Myc low-expressing clone are similar to those in the M14 parental line and the MAS53 clone, respectively (data not shown).

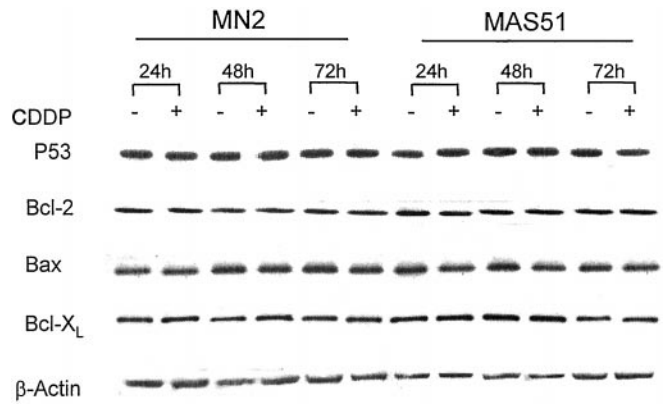


Fig. 7. Western blot analysis of P53, Bcl-2, Bax, and Bcl-x_L proteins in the cell lysates of the MN2 control clone and the MAS51 c-Myc low-expressing clone analyzed 24, 48, and 72 h after CDDP treatment (2 μg/ml, for 2 h). The Western blot analysis was repeated three times using three different protein lysates.

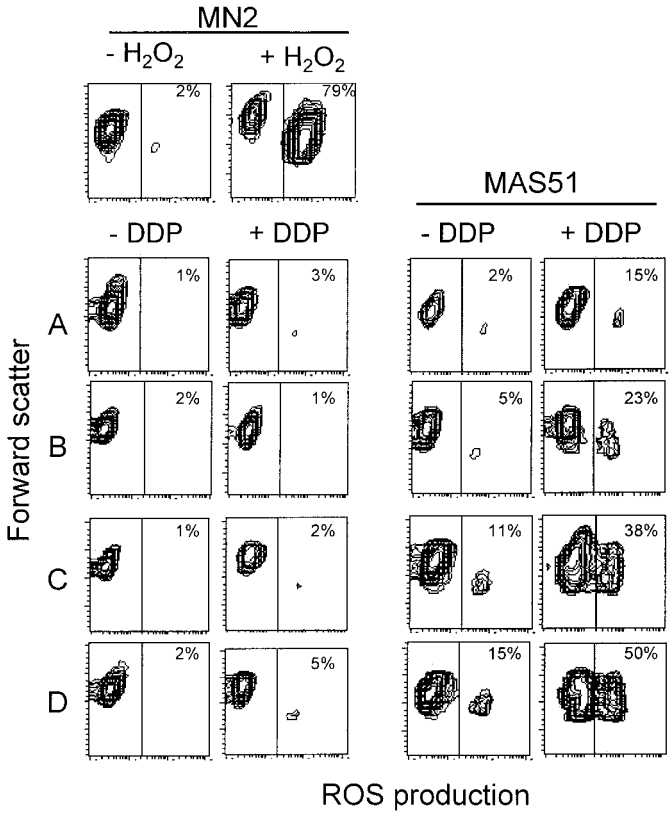


Fig. 8. Flow cytometric analysis of ROS content in the MN2 control clone and the MAS51 c-Myc low-expressing clone, untreated or treated with CDDP. The analysis was performed 24 (A), 48 (B), 72 (C), and 96 h (D) after the end of CDDP treatment (2 μg/ml for 2 h) by incubating the cells with 4 μM dihydroethidium. As internal ROS control, the MN2 cells were treated with 15 mM H_2O_2 for 1 h (top). DDP, CDDP.

c-Myc Down-Regulation Cooperates with CDDP to Induce Caspase-1 and -3 Activity and Proteolytic PARP Cleavage. To determine whether CDDP-induced apoptosis in the c-Myc low-expressing clones involves caspase(s) activation, we monitored caspase-1, -3, and PARP, a prototype CPP32 substrate. Figure 9 shows the protein expression levels of ICE, CPP32 proteases, and the processing of PARP in the MN2 control clone and the MAS51 c-Myc low-expressing clone, untreated or treated with CDDP. The analysis was performed from 24 to 96 h after the end of CDDP treatment. The results demonstrate that both ICE and CPP32 are cleaved in the MAS51 c-Myc low-expressing clone after CDDP treatment at different times. In fact, although the 45-kDa inactive prepro-ICE already decreases at 24 h after the end of CDDP treatment, the reduction of 32-kDa inactive precursor of CPP32 is evident only 72 and 96 h after the end of CDDP treatment. On the contrary, no change in their expression are observed in the MN2 control clone under the same conditions. The increase in the caspase-1 (about 4-fold at 24 h) and caspase-3 activity (about 3-fold at 72 h) in CDDP-treated c-Myc low-expressing clones confirms the involvement of these proteases in the apoptotic cell death (data not shown). Because the proteolytic cleavage of the 116 kDa PARP to an 89-kDa product is a marker of apoptosis, we also looked at the behavior of PARP in the MN2 control clone and the MAS51 c-Myc low-expressing clone after CDDP treatment. Immunoblotting experiments reveal that the 116-kDa protein is the only form evident in the MAS51 clone treated with CDDP up to 48 h after the end of treatment, whereas the 89-kDa cleavage fragment begins to appear after 72 h and is well-evident 96 h after the end of CDDP exposure. On the contrary, no processing of the PARP substrate is observed in the MN2 control clone treated with CDDP at any times examined. All the results obtained using the MN2 control clone and the MAS51 c-Myc low-expressing clone are similar to those in the M14 parental line and the MAS53 clone, respectively (data not shown).

ROS Production and Caspases Activation Are Essential for CDDP-Induced Apoptosis in the c-Myc Low-Expressing Clones. To evaluate the role of ROS production and caspase(s) in CDDP-induced apoptosis of the c-Myc low-expressing clones, the effect of NAC antioxidant and caspase inhibitors on apoptosis, ROS production, and caspase activation, was assessed. Fig. 10A shows the percentage of apoptotic cells, analyzed by morphological examination, in the two c-Myc low-expressing clones evaluated 120 h after the end of CDDP treatment, in the absence or presence of the NAC antioxidant, Z-VAD-fmk (pan-caspase inhibitor), Ac-YVAD-cho (caspase-1 inhibitor), and Ac-DEVD-cho (caspase-3 inhibitor) caspase inhibitors. The percentage of apoptotic cells is about 80% in the absence of inhibitors, whereas it is comparable with that of the untreated cells in the presence of NAC, Z-VAD-fmk, and Ac-YVAD-cho. The addition of the Ac-DEVD-cho in CDDP-treated c-Myc low-expressing clones inhibits apoptosis less efficiently (about 40%) than other inhibitors used (about 20%). Taking into account that the NAC

antioxidant may interact with CDDP, analysis of cell cycle of CDDP-treated c-Myc transfectants, performed with or without NAC, was assessed. The results reveal a similar block in S-G_2/M phases of cell cycle (about 90% at 24 h after the end of treatment) demonstrating that the NAC protective effect occurs after CDDP-induced DNA damage (data not shown). Moreover, pretreatment with the NAC overcomes CDDP cytotoxicity, the survival curves of CDDP-treated c-Myc low-expressing clones being similar to those of the M14 parental line (data not shown). The inhibition of apoptosis by the NAC antioxidant is specifically caused by the effect of the antioxidant on ROS production (Fig. 10B). In fact, the percentage of ROS evaluated 96 h after the end of CDDP treatment is reduced from about 53% to about 11% for the MAS51 and from 47 to 12% for the MAS53 c-Myc low-expressing clones. These data demonstrate that NAC scavenges the CDDP-induced ROS and inhibits apoptosis, indicating that ROS production plays a key role in CDDP-induced cytotoxicity in c-Myc low-expressing clones.

To investigate whether ROS generation constitutes the primary event in the apoptosis induction or depends on caspase activation, ROS production was evaluated by exposing the cells to caspase inhibitors. As reported in Fig. 10B, the universal caspase inhibitor Z-VAD-fmk abrogates CDDP-induced ROS, demonstrating a production of reactive oxygen species mediated by caspase(s). To further evaluate which

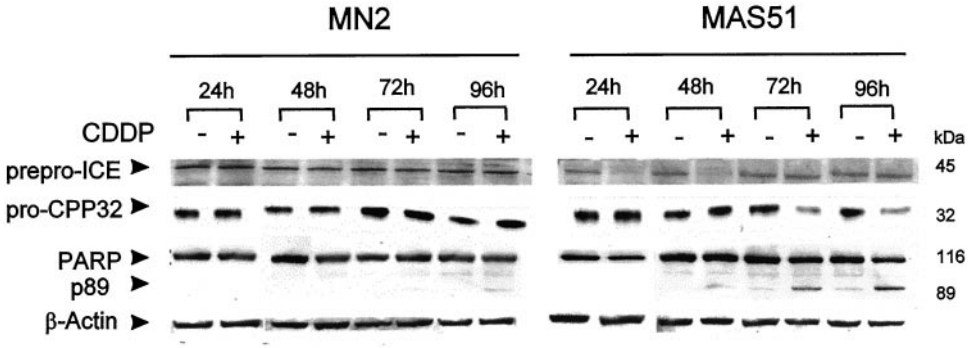
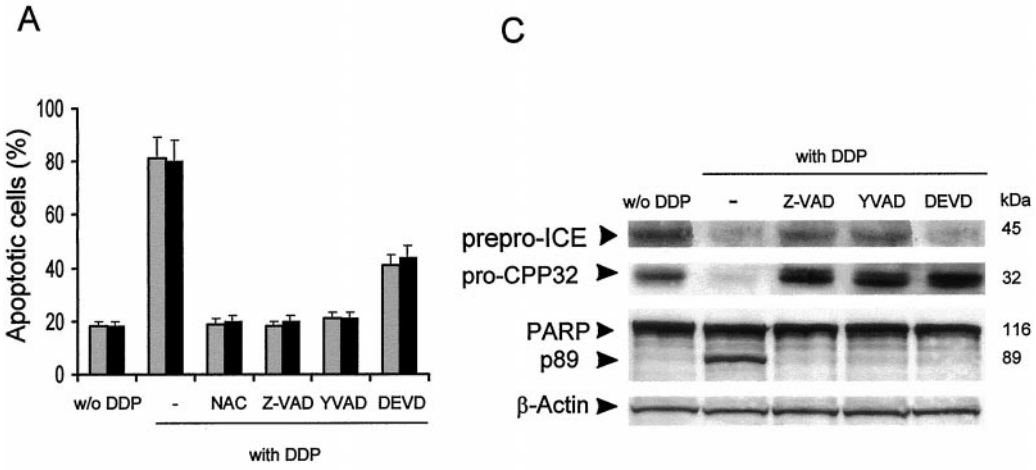


Fig. 9. Western blot analysis of prepro-ICE, pro-CPP32, and PARP proteins in the cell lysates of the MN2 control clone and the MAS51 c-Myc low-expressing clone performed 24, 48, 72, and 96 h after CDDP treatment (2 μg/ml for 2 h). The relative amounts of the transferred proteins were quantified and normalized to the corresponding β-Actin protein amounts.



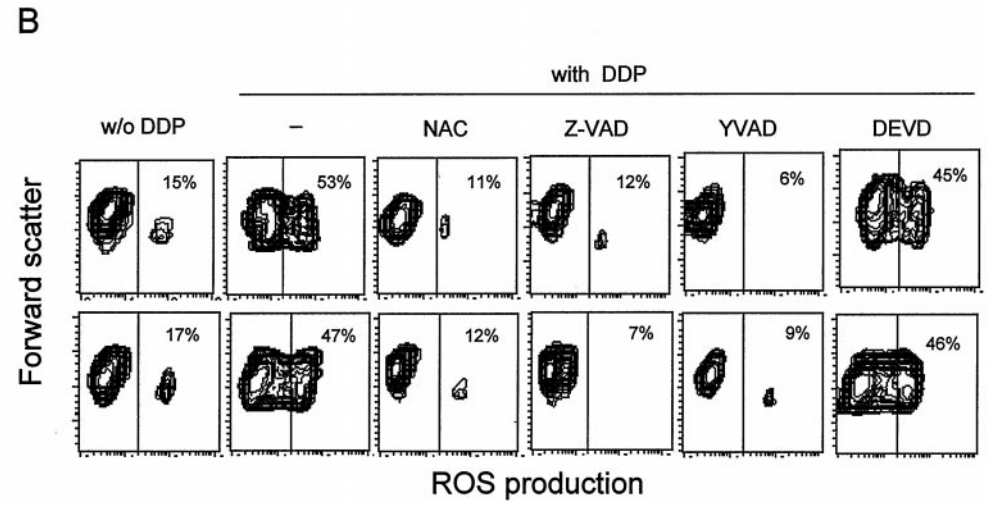


Fig. 10. A, effect of the NAC antioxidant, Z-VAD-fmk, Ac-YVAD-cho, and Ac-DEVD-cho caspase inhibitors on apoptosis evaluated 120 h after the end of CDDP treatment (2 μg/ml for 2 h) in the MAS51 (gray bars) and the MAS53 (black bars) c-Myc low-expressing clones. Means ± S.D. of three experiments are shown. B, effect of the NAC antioxidant, Z-VAD-fmk, Ac-YVAD-cho, and Ac-DEVD-cho caspase inhibitors on ROS production evaluated 96 h after the end of CDDP treatment (2 μg/ml for 2 h) in the MAS51 (top) and the MAS53 (bottom) c-Myc low-expressing clones. C, effect of the Z-VAD-fmk, Ac-YVAD-cho, and Ac-DEVD-cho on prepro-ICE (evaluated 24 h after the end of CDDP treatment), pro-CPP32 and PARP cleavage (evaluated 96 h after the end of CDDP treatment) in the MAS51 c-Myc low-expressing clone. DDP, CDDP.

caspases are involved in the production of ROS, the Ac-YVAD-cho and Ac-DEVD-cho inhibitors were used. The results demonstrate that although the caspase-1 inhibitor completely blocks ROS production, no effect is found using the caspase-3 inhibitor. The effect of caspase inhibitors on caspase-1 and caspase-3 expression and PARP cleavage was also analyzed (Fig. 10C). Z-VAD-fmk and Ac-YVAD-cho inhibitors, added to the culture medium at the end of CDDP treatment, block caspase-1, -3, and PARP cleavage, demonstrating a sequential activation from ICE to CPP32. The Ac-DEVD-cho, in the same conditions, is able to block caspase-3 cleavage and processing of the PARP substrate. Experiments, performed by adding the caspase-1 inhibitor after the activation of capsase-3 (72 h after the end of CDDP treatment), demonstrate that the Ac-YVAD-cho inhibitor does not directly interfere with the caspase-3 (data not shown).

Discussion

We demonstrated previously the involvement of c-Myc protein in the in vitro and in vivo growth and CDDP sensitivity of several melanoma lines (Leonetti et al., 1996; Citro et al., 1998; Leonetti et al., 1999). Our aim was to define the role of c-Myc in the cellular sensitivity to CDDP in melanoma cells by using the M14 human melanoma cell line in which c-Myc expression has been decreased by an expression vector carrying *c-myc* gene cloned in antisense orientation. We found that down-regulation of c-Myc expression increases CDDP sensitivity. Our results are in agreement with several other groups that have suggested that elevated c-Myc expression can confer resistance to CDDP (Sklar and Prochownik, 1991; Kinashi et al., 1998). The differences in CDDP sensitivity observed in c-Myc low-expressing clones are related to the drugs used. In fact, c-Myc down-regulation does not change ADR and CPT sensitivity.

The enhanced cytotoxic effect elicited by CDDP in the c-Myc low-expressing clones is unrelated to a different effect of the drug on the cell cycle phase distribution because a similar block of cells in S-G_2/M phases is observed 24 h after the end of treatment both in the control cells and in the c-Myc low-expressing clones. However, although the control clone is able to recover CDDP-induced block, this accumulation is persistent in the c-Myc low-expressing clones. This result is consistent with the delay of the c-Myc low-expressing clones in the progression through the cell cycle. Moreover, we found that the different cell lines are able to repair CDDP damage to the same extent, indicating that the c-Myc does not influence the DNA repair activity. We also demonstrate that down-regulation of c-Myc increases susceptibility to CDDP by activating the apoptotic program. In fact, although CDDP is unable to induce apoptosis in the parental line, c-Myc down-regulation cooperates with CDDP to efficiently induce the programmed cell death. When the c-Myc low-expressing clones are treated with CDDP, they are already committed to apoptosis, which is probably the cause of this cooperating effect. A fraction of cells undergoes apoptosis without CDDP treatment at a later stage of culture. The induction of apoptosis by c-Myc down-regulation is not caused by an increased growth factor requirement because the addition of serum is able to increase proliferation rate of cells but does not change the percentage of apoptotic cells (data not shown). These data are consistent with our previous data obtained in different melanoma cell lines by using *c-myc* antisense ODN treatment plus CDDP exposure (Citro et al., 1998; Leonetti et al., 1999).

Our experiments also provide information about the mechanism(s) by which c-Myc down-regulation and CDDP treatment induce apoptosis. Even though in some cell lines DNA damage is reported to induce P53, which can promote apoptosis by increasing Bax levels (Miyashita and Reed, 1995; Ding et al., 1998), we found that CDDP-induced apoptosis occurs in the c-Myc low-expressing clones without changes in the expression of these two proteins. Instead, our data provide evidence that the massive apoptotic cell death induced by CDDP in the c-Myc low-expressing clones is associated to ROS generation. In fact, a significant percentage of ROS production is exclusively observed in the c-Myc low-expressing clones after CDDP treatment. This is in agreement with other findings demonstrating that ROS per se are potent inducers of apoptosis (Kroemer et al., 1997). Because ROS do not appear either in CDDP-treated control cells or immediately at the end of CDDP treatment in the c-Myc low-expressing clones, it would suggest that 1) CDDP alone is not able to induce ROS at the dose employed in our experimental model and 2) ROS production occurs only in cells unable to recover CDDP damage by c-Myc down-regulation. Apoptosis occurs when the amount of ROS produced cannot be handled by radical scavenging cellular antioxidant (Lennon et al., 1991; Kroemer et al., 1997). Bcl-2 protein, which is one of the most common proteins with antioxidant function, has been described to increase cell resistance to ROS or block ROS production by regulating the opening of permeability transition pore (Meijer et al., 1987; Hockenbery et al., 1993; Jacobsen et al., 1993). In our model, the ROS production induced by CDDP in c-Myc low-expressing clones is not caused by a decrease in the levels of Bcl-2 or other Bcl-2 family members, such as Bcl-$x_{L/S}$, because no changes in their expression were observed. Even though the precise mechanism(s) by which c-Myc down-regulation cooperates with CDDP treatment to induce ROS production needs clarification, ROS generation is a key event in the death process. In fact, the use of NAC antioxidant, by inhibiting ROS production, clearly protects c-Myc transfectants from the CDDP-induced apoptosis. Our results are in agreement with data indicating that the CDDP-induced apoptosis is blocked by antioxidant treatment (Park et al., 2000). Apoptosis induced by CDDP treatment in the c-Myc low-expressing clones occurs through caspase-1 and -3 activation. In fact, ICE and CPP32 proteases and PARP substrate are cleaved after CDDP treatment exclusively in the c-Myc low-expressing clones, suggesting a different regulation of these proteases after CDDP treatment in c-Myc low-expressing clones compared with the parental cells. Moreover, time course analysis reveals that activation of caspase-1 precedes caspase-3 because it is evident at 24 h after the end of treatment, whereas caspase-3 is activated at 72 h. Data from several experimental systems are consistent with this model. Apoptosis induced by several stimuli has been proposed to proceed through a process involving ROS and caspase activation (Johnson et al., 1996; Green and Reed, 1998). ICE protease has been reported to mediate CDDP-induced apoptosis in ovarian and glioma cancer cells (Kondo et al., 1995; Chen et al., 1996). Recently, caspase-3 has emerged as one of the key proteases in spontaneous,

anti-Fas-, and TNF-mediated apoptosis (Tewari et al., 1995). A specific cleavage of the 116-KDa PARP to a 89-kDa proteolytic fragment seems to occur in apoptosis in several model systems (Kaufmann et al., 1993; Darmon et al., 1995).

Moreover, ROS generation does not constitute the primary event in apoptosis induction, but depends on caspase 1-like protease activation. In fact, ROS production is abrogated by the Ac-YVAD-cho and not by Ac-DEVD-cho. Our results are in agreement with several studies demonstrating an involvement of caspases, including caspase-1, on ROS generation (Tan et al., 1998).

We also demonstrate that caspases activation is essential for CDDP-induced apoptosis in the c-Myc low-expressing clones. In fact, both ICE and CPP32 inhibitors abrogate apoptosis, although the caspase-3 inhibitor is less efficient than the caspase-1 inhibitor. These results suggest that 1) a sequential activation model, where proteolytically activated caspase-1 generates ROS production that would cleave and activate CPP32, thus inducing the programmed cells death; 2) more executors of the programmed cell death could be activated during CDDP-induced apoptosis in the c-Myc low-expressing clones.

In conclusion, our results demonstrate that c-Myc down-regulation increases cellular susceptibility to CDDP in melanoma cells. The CDDP-induced cytotoxicity in the c-Myc low-expressing clones is closely related to induction of apoptosis, a result of ROS production and caspase-1 and -3 activation. The use of specific inhibitors demonstrates that CDDP-induced apoptosis in the c-Myc low-expressing clones requires reactive oxygen species, which in turn depend on caspase-1-like activation.

Taken together, these results clearly demonstrate that c-Myc plays an important role in the sensitivity to CDDP in melanoma tumors. Moreover, our findings suggest that melanoma carrying low levels of c-Myc protein could be responsive to CDDP treatment and that the down-regulation of oncogenes might represent a useful goal to improve the efficacy of antineoplastic drugs.

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Address correspondence to: Dr. G. Zupi, Experimental Chemotherapy Laboratory, Experimental Research Center, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy. E-mail: zupi@ifo.it