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# Id3-mediated Enhancement of Cisplatin-induced Apoptosis in a Sarcoma Cell Line MG-63

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**Abstract.** *The molecular mechanism(s) underlying the resistance to cis-diamminedichloroplatinum (CDDP)-induced growth inhibition include DNA repair, apoptosis and cell cycle progression. Inhibitor of differentiation (Id) proteins, which belong to the group of helix-loop-helix proteins, regulate cell cycle progression, differentiation and apoptosis. We examined whether CDDP exposure modulates the expression pattern of Ids and whether ectopic expression of Ids influences CDDP-induced cell death. Cell growth was assessed by WST-8 assay kit. Reactive oxygen species (ROS) was evaluated by flow cytometry using dihydroethidium. MG-63 sarcoma cells were stimulated with CDDP for various times and Id expression was assessed by reverse transcription-polymerase chain reaction. CDDP induced a considerable transient up-regulation of Id3 mRNA, but not Id2, 1-2 h after stimulation. Enforced expression of Id3 caused the MG-63 sarcoma cells to be more sensitive to CDDP-induced growth inhibition, through generation of ROS and caspase-3 activation. Together, our results suggest that CDDP-induced cell death appears to involve Id3.*

*cis*-Diamminedichloroplatinum (CDDP) and its analogues have been widely used for treatment of tumors including sarcoma, squamous carcinoma and small cell lung cancer. However, their clinical use is limited by major problems, such as several side-effects (1) and inherent and/or acquired resistance (2, 3). CDDP enters the cells and reacts with DNA to generate a variety of covalent adducts including intrastrand and interstrand cross-links, initiating a cascade of events resulting in growth arrest and/or apoptosis in a variety of cells including sarcoma (2, 4). Recent analysis of CDDP action has contributed to the understanding of resistance to CDDP (2, 4). Increased repair of the CDDP-DNA adducts can cause resistance to CDDP. Altered expression of regulatory proteins in the signaling pathways that regulate apoptosis and/or cell growth can also modulate sensitivity to the drug.

Accumulating pieces of evidence indicate that inhibitor of differentiation or DNA binding (Id) proteins regulate cell cycle progression, apoptosis and differentiation in some cell types (5-8). Four Id genes (Id1 to Id4) have been identified in mammals (9). Id proteins – lacking a basic domain – function as dominant inhibitors of basic helix-loop-helix (bHLH) transcription factors through their sequestration by forming transcriptionally inactive hetrodimers (7). Id3 was originally cloned in mouse 3T3 fibroblasts based on immediate-early response to growth factor (10). Id proteins have been demonstrated to promote cell cycle progression and apoptosis, while preventing differentiation of cells.

Moreover, they antagonize the activities of several classes of transcriptional proteins including Ets domain proteins (11), Rb and related proteins (12), and Pax homeodomain-containing proteins (13). Deregulated expression of Id gene products have been reported in several tumor cell types such as breast cancer and squamous cell carcinoma (14-16). For example, deregulated expression of Id1 protein led to the malignant progression of a subset of human breast cancer cells. Enforced expression of Id genes resulted in immortalization of keratinocytes (16). In an apparent contrast, ectopic expression of Id into rat fibroblasts caused partial inhibition of colony formation (5). Similar to Ids, the growth promoting activity of oncogenes including c-myc is in some cases accompanied by induction of apoptosis (17).

Cell growth is determined by a relative balance among cell proliferation, survival and apoptosis. Induction of apoptosis is thought to play a crucial role in development, tissue homeostasis and elimination of potentially harmful virus-infected or transformed cells (18). CDDP-induced apoptosis is accompanied by mitochondrial changes such as generation of reactive oxygen species (ROS) and cytochrome c from

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mitochondria (19-22), which results in activation of effector caspases such as caspase-3 in combination with proteolytic activation of procaspase 9 and Apaf-1 in the presence of ATP (23). The activation of caspase-3 might be implicated in morphological changes characteristic of apoptosis, probably through the proteolytic cleavage of many substrates including poly(ADP-ribose)polymerase (PARP) and DNA fragmentation factor (23). To further improve our understanding of the molecular mechanisms underlying CDDP-induced cell death, we examined the Id mRNA levels following treatment of MG-63 sarcoma cells with cisplatin. Up-regulation of Id3 mRNA, but not Id2, was found after CDDP exposure. The enforced ectopic expression of Id3 in the sarcoma cells resulted in their being more sensitive to CDDP-induced apoptotic cell death. These findings provide valuable information for the design of treatment modalities for sarcoma cells.

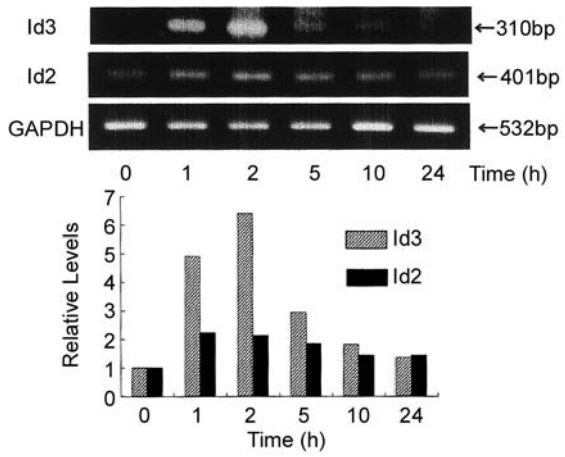


Figure 1. *Treatment with CDDP induces transient up-regulation of Id3 but not Id2 mRNA in sarcoma cell lines. MG63 cells were treated with 3 μg/ml CDDP for various times and the levels of Id2, Id3 and control GAPDH mRNA were measured by RT-PCR. Expression levels of Id2 and Id3 relative to GAPDH mRNA are shown. The results are representative of three independent experiments.*

## Materials and Methods

*Cell culture.* The human osteosarcoma cell line MG-63 (Cell Resource Center for Biomedical Research; Tohoku University, Sendai, Japan) was cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 mg kanamycin at 37˚C in humidified air with 5% $CO_2$. The cells were trypsinized once a week with trypsin/EDTA (Invitrogen Corp., Carlsbad, CA, USA) and split at 1:30 – 1:40 suspension.

*Reverse transcription (RT)-PCR.* The expression of human Id1, Id2, Id3 and Id4 was determined by RT-PCR method, as previously described (24). Briefly, total RNA was isolated from MG-63 cells exposed with or without CDDP using Tri Reagent (Sigma, St Louis, MO, USA) according to the manufacturer's protocol. RNA was reverse transcribed using RNA PCR Kit (AMV) (Takara, Tokyo, Japan). PCR reactions were performed with Taq polymerase (Toyobo, Tokyo, Japan) using the following primers: Id3 (forward, 5'-CTACGAGGCGGTGTGCTG-3'; reverse, 5'-GTCGTTGGAGATG ACAAGTTC-3'); Id2 (forward, 5'-GAAAGCCTTCAGTCCCGTG-3': reverse, 5'-AGCCACACAGTGCTTTGCTG -3'); Id1 (forward, 5'-AAGTCGCCAGTGGCAGCAC-3'; reverse, 5'-CAGCGACACAA GATGCGATC-3'); Id4 (forward, 5'-TGTGCCTGCAGTGCGA TATG-3'; reverse, 5'-TGTCGCCCTGCTTGTTCAC-3'); Flag-Id3 (forward, 5'-GGATCCATGGACTACAA-3'; reverse, 5'-GTCGTT GGAGATGACAAGTTC-3'); GAPDH (forward, 5'- AGGTGAA GGTCGGAGTCAAC-3'; reverse, 5'-GCATGGACTGTGGTCAT GAG-3'), generating fragments of 310 bp, 401 bp, 460 bp, 285 bp, 414bp and 532 bp, respectively. The PCR profile was 30 cycles at 94˚C 30 sec, 55˚C 30 sec, 72˚C 30 sec. The PCR products were resolved on 1.8% agarose gels and visualized with ethidium bromide.

*Construction of vectors.* The cDNA for mouse Id3 was generated by RT-PCR using primers (forward, 5'-CGCGAATTCATGAAGG CGCTGAGCCCGGTG -3'; reverse, 5'-CGCGAATTCTCAGT GGCAAAAGCTCCTCTTG -3') and the fidelity of the Id3 was confirmed by sequence analysis. The Id3 tagged with 3x Flag (3xFlag-Id3) was subcloned into the retrovirus vector pMX-IRES-GFP (25) (a kind gift from Prof. T. Kitamura; Institute of Medical Science, University of Tokyo, Japan), which contains an internal ribosomal entry site (IRES) and the cDNA of green fluorescent protein (GFP). The GFP was replaced by enhanced GFP (EGFP) (BD Biosciences). The fragment Flag-Id3-IRES-EGFP was subcloned into pEF1/V5-His vector (Invitrogen), resulting in 3xFlag-Id3-EGFP/pEF1 (Flag-Id3/EGFP).

*Transfections and establishment of stable MG-63 cells overexpressing Flag-Id3/EGFP.* MG-63 cells were transfected using FuGene-6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the recommended protocol. The Flag-Id3/EGFP or EGFP alone was transfected into cells, followed by selection with Geneticin (G418, 400 μg/ml) (Sigma, St. Louis, MO, USA). Individual clones were obtained by limiting dilution. The expression of Id3 was determined by RT-PCR.

*Assay for cell survival.* The cells transfected with or without vectors were seeded at a density of 1 x $10^4$ cells/well in 96-well microplates and preincubated overnight for cells to adhere to plates. Following exposure to CDDP (Nippon Kayaku, Tokyo, Japan) or medium alone (control) for various times, WST-8 reagents were added into the cultures. Absorbances at 450 nm and 655 nm were measured using a microplate reader (BioRad, Hercules, CA, USA) after 2-h incubation. Absorbances ($A_{450} - A_{655}$) are proportionally related to the number of living cells.

*Flow cytometric analysis of ROS.* To determine ROS, dihydroethidium (2, 7-diamino-10-ethyl-9-phenyl-9, 10-dihydrophenanthridine) (HE) (Sigma) was used as described by Zamzami *et al.* (21). Cells were incubated with 2 mM HE for 15 min at 37˚C and analyzed on a flow cytometer (FACSCalibur, Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Inside cells, HE is oxidized to convert into ethidium in the presence of ROS, in particular superoxide anions. A minimum of 10,000 events were counted for each sample.

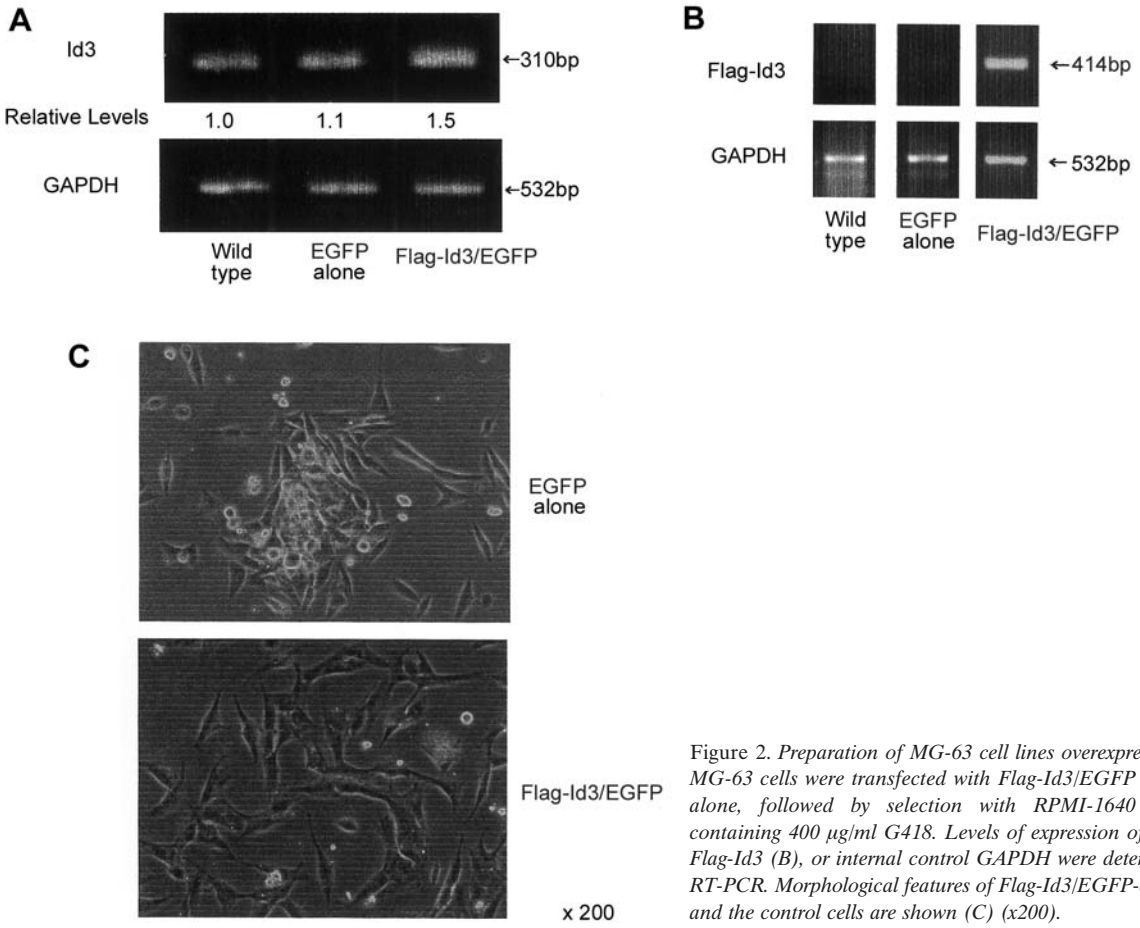


Figure 2. *Preparation of MG-63 cell lines overexpressing Id3. MG-63 cells were transfected with Flag-Id3/EGFP or EGFP alone, followed by selection with RPMI-1640 medium containing 400 μg/ml G418. Levels of expression of Id3 (A), Flag-Id3 (B), or internal control GAPDH were determined by RT-PCR. Morphological features of Flag-Id3/EGFP-expressing and the control cells are shown (C) (x200).*

*Assay for caspase-3 activation.* Activation of caspase-3 was assessed by enzymatic activity, as described previously (26). Enzymatic activity was assayed by the caspase-3 colorimetric protease assay kit according to the manufacturer's protocol (Bio Vision, Mountain View, CA, USA). Cells were lysed in lysis buffer on ice for 10 min, followed by centrifugation. The supernatants were used for the assay. An enzymatic reaction was performed in a mixture of the supernatant protein (200 mg) and caspase substrate DEVD-p-nitroanilide (*p*NA) at 37˚C for 2 h, and *p*NA light emission was measured with excitation at 405 nm using a microplate reader (Bio-Rad).

*Statistical analysis.* Data were expressed as means±SD for each group. Statistical significance was determined by Student's *t*-test and a difference of $p<0.01$ was considered to be significant.

## Results

*Transient substantial up-regulation of Id3, but not Id2, following stimulation with CDDP.* To assess the potential role for Ids in the sensitivity to CDDP, MG-63 cells were exposed to a substantially toxic concentration of 3 μg/ml CDDP (27) for various times, and the levels of Id expression were determined by RT-PCR. The Id3 expression was immediately induced, with its peak levels at 2 h, followed by a slight decline thereafter up to 24 h, whereas the Id2 expression was only marginally increased (Figure 1). The expression of both Id1 and Id4 mRNAs was undetected in the MG-63 cells (unpublished observation). These findings suggest that CDDP selectively up-regulates Id3mRNA expression in MG-63 sarcoma cells.

*Enforced expression of Id3 renders MG-63 cells to be more sensitive to CDDP-induced growth inhibition.* To explore the potential role of Id3 in CDDP-mediated growth inhibition, stable cell lines overexpressing Id3 were established. MG-63 cells were transfected with Flag-Id3/EGFP or control EGFP vector and incubated in selective medium containing G418. The levels of Id3 expression in the transfectants were moderately increased, approximately 1.5-fold, compared with those in control EGFP-transfected cells, as assessed by RT-PCR (Figure 2A). The exogenous Flag-Id3 expression in the transfectants were further verified by PCR using primers for 5'-Flag and 3'-Id3 (Figure 2B). The Flag-Id3/EGFP-

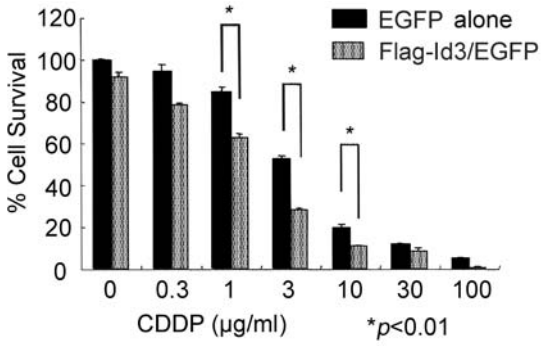


Figure 3. *Ectopic expression of Id3 causes the MG-63 sarcoma cells more sensitive to subsequent CDDP-induced growth inhibition. MG-63 cells overexpressing Flag-Id3/EGFP or control EGFP alone were stimulated with various concentrations of CDDP for 48 h and assessed by cell survival using WST-8 assay. The values shown are average±SD of three independent experiments. *, Statistically significant.*

expressing cells displayed elongated morphology and a tendency toward detachment from culture plates (Figure 2C). The growth rate between the Flag-Id3/EGFP and control cells was not statistically significant, as assessed by doubling-time (26.7 ± 4.7 d in control vector *versus* 25.9 ± 4.6 in Flag-Id3/EGFP). The experiments were done within 2 months after transformants were cloned by a limiting dilution.

The cell lines expressing Flag-Id3/EGFP or EGFP alone were cultured with various concentrations of CDDP for 48 h and assessed by cell growth using WST-8 assay. The Flag-Id3/EGFP-expressing cells were more sensitive to CDDP in a range of 1 to 10 mg/ml than the control cells expressing EGFP alone (Figure 3), suggesting that Id3/EGFP functions as a growth inhibitory molecule in combination with CDDP in the sarcoma cell line MG-63.

*Id3 renders MG-63 sarcoma cells more sensitive to CDDP-induced generation of reactive oxygen species and caspase-3 activation.* Cell growth reflects a relative balance among cell proliferation, survival and death. To examine whether Id3-mediated CDDP-induced growth inhibition involves cell death, MG-63 cells overexpressing Flag-Id3/EGFP or control EGFP alone were exposed to CDDP, followed by assay for ROS, since generation of ROS is accompanied by induction of apoptosis in some cell types (22). The cells were labeled with dihydroethidium for 15 min and analyzed on flow cytometer. CDDP induced ROS generation in a higher percentage of Flag-Id3/EGFP-MG-63 cells than of control EGFP-MG-63 cells (Figures 4A and 4B). The Flag-Id3/EGFP cells also were more sensitive than control cells in terms of caspase-3 activation following CDDP exposure (Figures 4C). These findings suggest that MG-63 cells expressing exogenous Id3 are more sensitive to ROS production and caspase-3 activation in response to CDDP than control cells.

## Discussion

CDDP and its analogs have been used in a variety of tumors including sarcoma (3, 28). However, their clinical use is sometimes limited by side-effects or inherent/acquired resistance to this drug (1, 2). To circumvent or overcome the drug resistance, an understanding of the molecular mechanisms underlying CDDP-induced cell death is urgently required. CDDP has been demonstrated to induce apoptosis in a variety of tumor cells through at least mitochondrial changes including the MG-63 sarcoma cell line (19, 20, 27, 29). It remains largely unknown what molecules activated by CDDP initiate mitochondrial change such as ROS generation. Id proteins, which belong to the HLH proteins, have been shown to promote cell cycle progression and/or apoptosis in several cell types (5, 6, 30). For example, the introduction of Id2 into 32D myeloid progenitors and U2OS sarcoma cells results in apoptosis (30). In the present study, we examined whether members of the Id family of proteins modulate CDDP-induced cell death.

CDDP induced an immediate and transient substantial up-regulation of Id3, but not Id2, followed by inhibition of cell growth in the MG-63 sarcoma cell line (Figure 1) (27). On introduction of the Id3 gene, the MG-63 cells were substantially tolerated in the culture medium containing 10% serum (Figure 3). The MG-63 cells expressing Id3 demonstrated an elongated morphology, compared with the control (Figure 2C). Consistent with our findings, Deed *et al.* (31) reported that Id3 induced a morphological transformed phenotype on ectopic transfection of the Id3 gene into mouse NIH3T3 cells, whereas Id3 induced apoptosis in rat embryonic fibroblasts upon transfection of the Id3 gene in serum-free conditions (5). These apparently contradictory observations might suggest that the function of Id3 varies depending on cell type or stage of activation.

Enforced expression of Id3 into sarcoma cells renders them more sensitive to subsequent CDDP-induced generation of ROS and caspase-3 activation, suggesting that CDDP-induced up-regulation of Id3 is somehow involved in the apoptotic events. Consistent with these findings, TGF-β induced a transient up-regulation of Id3 expression in B progenitor cells, accompanied by induction of apoptosis (32). Moreover, enforced expression of Id3 resulted in apoptosis of the B progenitor cells, suggesting that ectopic expression of Id3 mimics TGF-β signaling. Alternatively, it is possible that moderate Id3 expression, which did not cause growth inhibition in our culture condition, lowers the threshold to cell death stimuli including CDDP. In the present study, we could not clarify how the Id3 modifies the CDDP-induced cell death. However, it is conceivable that Id3 mediates the activation of a similar pathway to c-Myc, which functions as a promoter of cell death as well as cell cycle progression (17).

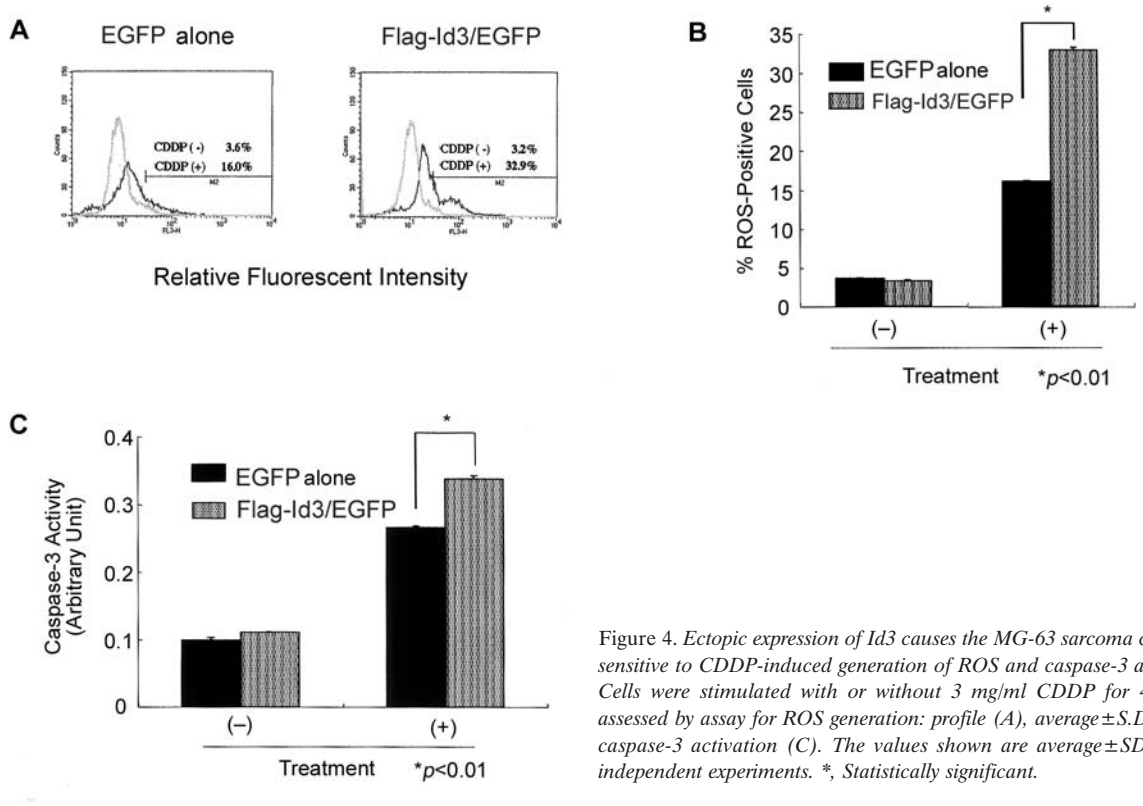


Figure 4. *Ectopic expression of Id3 causes the MG-63 sarcoma cells more sensitive to CDDP-induced generation of ROS and caspase-3 activation. Cells were stimulated with or without 3 mg/ml CDDP for 48 h and assessed by assay for ROS generation: profile (A), average±S.D (B) and caspase-3 activation (C). The values shown are average±SD of three independent experiments. *, Statistically significant.*

There is growing evidence for a role of mitochondria in the initiation of apoptosis upon stimulation with a variety of chemotherapeutic agents including CDDP (23, 29). Mitochondria are thought to be both a source and target of ROS. The Flag-Id3/EGFP cells displayed enhanced ROS production compared with the control (Figure 4C), which might contribute to the increased caspase-3 activation and apoptosis. Alternatively, it is also possible that caspase-3 activation initiates ROS generation, amplifying apoptotic responses.

Together, our present results might indicate that CDDP-induced up-regulation of Id3 is involved in CDDP-induced apoptosis, probably through mitochondria such as caspase-3 activation and ROS generation. These findings are valuable for an understanding of the mechanisms of resistance to CDDP-mediated apoptosis and also for the improved design of treatment modalities.

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