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Cisplatin Cytotoxicity in Organ of Corti-Derived Immortalized Cells

Maurizio Previati,[1]* Irene Lanzoni,[2] Laura Astolfi,[1] Francesco Fagioli,[3] Giorgio Vecchiati,[4] Antonella Pagnoni,[3] Alessandro Martini,[5] and Silvano Capitani[1]

[1]Department of Morphology and Embryology, Human Anatomy Division, University of Ferrara, Ferrara, Italy
[2]Center of Bioacustics, University of Ferrara, Ferrara, Italy
[3]Department of Chemistry, University of Ferrara, Ferrara, Italy
[4]Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy
[5]Department of Audiology, University of Ferrara, Ferrara, Italy

Abstract Cisplatin is an anticancer drug currently used in the treatment of genital and head and neck tumors. Its use in these and other types of tumors is narrowed by onset of chemoresistance and severe undesired side effects, like as nephro- and ototoxicity, whose mechanisms of action are only partially understood. In the present study we investigated the effects of cisplatin (cis-dichlorodiaminoplatin, CDDP) on a cell line (OC-k3) developed from organs of Corti of transgenic mice. We observed at 48 h that cell death due to cisplatin was time and concentration-dependent. The cell death displayed some morphological hallmarks of apoptosis, including nuclear fragmentation into several large nuclear fragments, surrounded by a rearranged and thickened actin cytoskeleton. No DNA laddering was detected, suggesting absence of endonuclease activity, nor annexin V positivity, suggesting absence of phosphatidylserine externalization. Several molecules protected the cells against CDDP induced cytotoxicity, including methionine, suramin and PD98059. Methionine reduced CDDP-uptake, while suramin, a polycathionic compound a specifically binding external proteins, did not. This finding suggested that suramin could exert its protective effect by acting on an intracellular transduction pathway. We tested this hypothesis by studying the effect of suramin and PD98059, a MEK inhibitor, on the mitogen activated protein kinase (MAPK) cascade. After CDDP treatment, we found an increase of phosphorylation of extracellular regulated kinases (ERK)1/2, that could be inhibited by PD98059 and suramin. These data suggest that ERK pathways can play a role in mediating the cell death induction in presence of a CDDP challenge. J. Cell. Biochem. 101: 1185–1197, 2007.

Key words: OC-k3; cisplatin; ERK 1/2; apoptosis; suramin; PD98059

Cisplatin (cis-diamminedichloroplatinum; CDDP), a potent inducer of growth arrest and/or apoptosis in most cell types, has been clinically used for decades against a variety of human malignancies, including ovarian, testicular, bladder, head and neck, esophageal, and small-cell lung cancers. In addition to effects on cancer cells, in vivo CDDP induces some adverse drug reactions, including ototoxicity, renal, peripheral sensory and autonomic nervous system toxicity. The presence of these dangerous side effects has prompted research to achieve a better understanding of the biochemical mechanisms underlying the cytotoxicity.

Cisplatin-induced cell death has been associated with both necrosis and apoptosis. Apoptosis is an active form of cell death characterized by energy dependence, DNA and nuclear condensation and fragmentation, cell shrinkage and formation of apoptotic bodies in presence of an intact cell membrane and expression of several biochemical hallmarks. On the other hand, necrosis usually occurs as a

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*Correspondence to: Dr. Maurizio Previati, Department of Morphology and Embryology, University of Ferrara, Via Fossato di Mortara 66, 44100 Ferrara, Italy.
E-mail: prm@unife.it

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fast event when cells are physically or chemically stressed beyond the possibility of self-repair and is characterized by cell membrane damage with protein loss, massive cross membrane ion exchange followed by organelle swelling.

Although DNA platinum binding [Jameson and Lippard, 1999] and cell cycle arrest have been widely recognized as major determinants in cisplatin toxicity, other mechanisms have been claimed to be involved, including oxidative stress and activation of specific intracellular pathways that mediate the action of membrane receptors [Zanke et al., 1996; Chen et al., 1999]. The importance of these mechanisms is highlighted by the fact that their modulation allows the control of cisplatin-dependent apoptosis. Actually, increased tolerance to DNA damage as well as antioxidant administration or altered expression of key proteins in signal transduction can increase resistance to cisplatin [Fink et al., 1997; Gabaizadeh et al., 1997; Lanzi et al., 1998; Sanchez-Pérez and Peroma, 1999; Mase et al., 2000].

The mitogen-activated protein kinase (MAPK) family is a multigene protein-family that represents as a whole a relevant member of the signal transduction machinery coordinating cell response to a wide number of extracellular signals. MAPK classification is based on the sequence of the phosphorylation motif threonine-X-tyrosine (TXY) that is phosphorylated by MEK (MAPK kinase, an immediate upstream regulator of MAPK). This motif represents the specific factor that discriminates the MAPK family into three subfamilies, usually termed ERK 1/2, JNK/SAPK and p38, which respectively include a large number of isoforms. These three subclasses can undergo specific activation by different stimuli, including hyperosmotic stress, electromagnetic radiation, growth factors, cytokines and exposure to phorbol esters and chemical stress [Duff et al., 1992; Han et al., 1994; Bogoyevitch et al., 1995]. Although MAPKs play a pivotal role in regulating cell growth or survival response to damaging agents, more recently they have been linked to apoptosis induction in response to several agents, including cisplatin [Sanchez-Pérez et al., 1998; Wang et al., 2000; Bacus et al., 2001; Park et al., 2001].

The molecular links that join CDDP-induced DNA damage, signal transduction and apoptosis remain unclear. In an attempt to focus on cytotoxicity occurring at inner ear level, we employed the OC-k3 cell line, an immortalized cell line from the organ of Corti of transgenic mice [Kalinec et al., 1999]. These cells, constitutively expressing the SV40 large T antigen, are driven to proliferate indefinitely by the oncogenic temperature—sensitive protein product. This cell line is positive for the neuroepithelial precursor nestin, for specific auditory cell markers including myosin VIIa and acetylcholine receptor α-9 and for the inner hair cell (IHC) specific marker OCP2; On the other hand, it does not express glial or neuronal markers [Kalinec et al., 1999], and was previously shown to represent a suitable model for an in vitro study of CDDP ototoxicity [Bertolaso et al., 2001]. On this cell line we studied cell death due to cisplatin administration by morphological and biochemical criteria, especially examining the involvement of the ERK protein family as well as the protective role of MAPK family pharmacological inhibitors.

MATERIALS AND METHODS

Materials

2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059), D,L-methionine, rotenone, propidium iodide (PI), phalloidine-FITC and all other common laboratory reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Suramin was obtained from ICN (Irvine, CA) and pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-FMK (Z-VAD-FMK) from Calbiochem (Calbiochem, Darmstaadt, Germany). Anti-actin and anti p-ERK antibody were obtained from Santa Cruz Biotechnology (San Diego, CA)

Cell Culture

OC-k3 cells, obtained from the organ of Corti of transgenic mice (Immortomouse^{TM} H-2Kb-tsA58, Charles River Laboratories, Wilmington, MA; Kalinec et al., 1999), were cultured in the presence of 10% CO_2 at 33°C, in Dulbecco's Modified Eagle Medium (DMEM Gibco BRL, Grand Island, NY) supplemented with 10% foetal bovine serum (Gibco BRL) and 50 U/ml of recombinant mouse γ-interferon (Gibco BRL, Grand Island, NY, USA) without antibiotics.

All drugs used to challenge cells were added directly to the culture medium, provided that the input volume did not exceed 5% for water soluble agents and 0.5% for water insoluble

agents, such as PD98059, that was dissolved in DMSO. The final medium concentrations were: 50 μM CDDP, 300 μM suramin, 60 μM PD98059, 1 mg/ml DL-methionine, 100 μM rotenone, 50 μM Z-VAD-FMK.

Cellular Cisplatin Determination

To determinate the time course of cisplatin uptake, cells were exposed to 50 μM CDDP for the indicated times, quickly washed with phosphate buffered saline (PBS) three time to remove unloaded CDDP and detached by trypsinization: cell monolayer was incubated at 37°C for 3 min in the presence of 500 mg/L trypsine, 200 mg/L EDTA; then trypsine was blocked by adding an equal amount of medium plus 10% foetal serum; aliquots were sampled to determine cell number in a Burker counting chamber. Cells were then sedimented 10 min at 200 g and the pellet immediately redissolved in 200 μl 65% nitric acid and stored at −20°C. Atomic absorption spectroscopy was performed using a Perkin Elmer model Analyst 800 spectrometer equipped with a THGA graphite furnace. The detection limit was 5.5 μg Pt/L. The accuracy of the determination was estimated by evaluation of the recovery percentage, which accounted for 102%. The precision of PT determination, expressed as relative standard deviation, was 12%. In inhibition experiments, cells were preincubated with 1 mg/ml methionine or 300 μM suramin for 30 min before the addition of CDDP, in order to determinate the effect of these drug on CDDP uptake.

Viability Assay

Before treatment, cells were seeded into six-well plates at densities ranging from 100 to 200×10^3/well, left overnight to attach to the substratum and acquire the normal flattened morphology, and finally challenged with CDDP 50 μM or vehicle. Cell viability was determined by a PI exclusion assay. Briefly, the cells were treated for 6 h (pulse treatment) or 48 h with CDDP. All samples were processed at 48 h after initiation of the CDDP exposure: cells were detached by trypsin (see above) and proteolysis blocked with medium containing 2 μg/ml PI. Incubation was allowed for 15 min and then samples were immediately analyzed by flow cytometry (FACStar Becton Dickinson, St. José, CA). Untreated cells were used to identify the PI-negative (PI-neg) population, while to identify PI-bright population, OC-k3 cells were permeabilized by adding nonidet P40 NP40 (0.05% final concentration) and then incubated with PI. Consequently, PI-dim cells represent the cells showing a fluorescence intensity intermediate between PI-neg and bright cells evaluated by subtracting the number of PI-neg and PI-bright cells from the whole cell number.

In Situ Immunocytochemistry

OC-k3 cells were grown on uncoated glass coverslips, pulse treated with 50 μM CDDP (see above) and, at 48 h, washed twice with PBS and then fixed with 4% paraformaldehyde for 30 min at room temperature. Fixed cells were washed with PBS and permeabilized with 0.1% Tween/PBS for 10 min, then the surfactant was removed by one PBS wash. Non-specific binding was blocked by a 30 min incubation with 0.1% Tween/PBS containing 1.4% bovine serum albumin (BSA) and 1.4% milk for 60 min at 37°. For F-actin visualization, coverslips were stained with FITC-conjugated phalloidin 1:200 for 90 min at 37°, washed three times with 0.1% Tween/PBS and subsequently counterstained with 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma), which selectively stains DNA and allows for the evaluation of nuclear morphology. Slides were mounted with DABCO glycerol and examined under a fluorescence microscope equipped with the appropriate filters (Axiophot Zeiss, Oberkochen, Germany).

Protein Analysis

OC-k3 cells (3×10^6 cells) were treated with 50 μM CDDP for different times, detached by trypsin enzymatic digestion, counted and sedimented as described above, resuspended in lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP40, plus 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 25 μg/ml leupeptin) and lysed for 30 min on ice. Samples were centrifuged at 10,000*g* for 15 min at 4°C in a microfuge and analyzed by polyacrylamide-SDS electrophoresis on 10% denaturating gels according to Laemmli, [1970]. After blotting, the nitrocellulose mebranes were stained for 5 min with the protein sain Ponceau Red (0.5% in 1% acetic acid), and the pattern of protein separation was obtained by nitrocellulose scanning. After extensive washing, conventional immunodetection was performed.

DNA Analysis

OC-k3 cells were treated with 50 μM CDDP for 24 or 48 h. As positive control for DNA degradation we used 50 μM rotenone for 24 h. Genomic DNA was analyzed by migration on an agarose gel (0.8%) at 100 V for 1 h, and visualized with ethidium bromide staining. As molecular weight markers we used 1 Kb from Fermentas (St. Leon-Rot, Germany).

Protein Determination

Protein concentration was determined using the bicinchoninic acid assay (BCA), according the producer instruction, using a 2 mg/ml BSA solution as standard (Pierce, Rockford, IL).

Statistical Analysis

Statistical analyses were performed using the Kruskal–Wallis test, followed by a multiple comparison test. A $P<0.05$ was considered significant.

RESULTS

Cisplatin Treatment Induces Apoptosis in OC-k3 Cells

In a previous report we have demonstrated that cell death induced by 13–200 μM CDDP was not significant at 24 h of treatment, whereas it resulted highly significant and CDDP-concentration-dependent at 48 h [Bertolaso et al., 2001].

A morphological approach was used to firmly establish if cell death occurred in an active and organized pattern, and not by necrosis. DAPI staining allowed to observe that during CDDP treatment the cell nuclei underwent drastic changes, including fragmentation into several large ovoidal nuclear fragments, but without evident signs of chromatin packaging and margination. Examination of cell morphology by phase contrast microscopy revealed that this fragmentation occurred in presence of cell membrane and cytoplasmic integrity. This initial step was followed by cell shrinkage and cytoplasm vacuolization, that produced small cell fragments, still attached to the flask surface, often containing single well-defined large nuclear fragments (Fig. 1A–N).

Furthermore, this process of partitioning nuclear bodies into cell fragments was sustained by cytoskeletal reorganization. After 48 h of 50 μM CDDP treatment, the actin cytoskeleton, examined by phalloidin staining on adherent cells, appeared to reorganize showing an increased stainability and thickening around and among apoptotic large nuclear fragments, apparently contributing to separate them from each other (Fig. 1O,P).

In addition, both DNA fragmentation and annexin V were analyzed. Data showed that cell death occurred without simultaneous exposure of PS on the external leaflet of cell membrane (data not shown), and without DNA degradation at the internucleosomal level (Fig. 2).

Quantitative Evaluation of Cell Death

We quantitatively evaluated the CDDP cytotoxic effects using PI supravital labeling, an assay based on the different fluorescence showed by viable and dead cells in presence of PI. Viable cells were capable of actively excluding PI, so only a low fluorescence could be detected during flow cytometry (these cells were referred to as PI-neg in Fig. 3A). On the contrary, detergent-lysed cells displayed a mean fluorescence, roughly 100-fold higher (PI-bright, Fig. 3A). To better evidence the effects of chemical stress, OC-k3 cells were treated both with a pulse (6 h) or continuous (48 h) treatment.

Exponentially growing OC-k3 cells were treated with 50 μM CDDP, either during the entire incubation period of 48 h or for only 6 h (pulse-treatment), after which the medium was replaced, and incubation prolonged up to 48 h. We have found that 6 and 48 h exposure to cisplatin showed a different fluorescence distribution. Indeed, in addition to PI-neg and PI-bright cells, an additional population was detected, showing an intermediate fluorescence intensity, which was referred to as PI-dim (Fig. 3B). In other cell lines this group of PI dim cells previously appeared to express some of the classical apoptosis markers [Zamai et al., 2001]. The percentage of cells present in PI-dim and PI-bright cells depended on CDDP time exposure (Fig. 3B).

Cytoprotective Drugs

When we incubated cells with CDDP plus putative cytoprotectants, we found a highly significant protective effect: 1 mg/ml methionine decreased cell death to few percent, not significantly different from that in control cells, both during 6 h pulse and 48 h treatment. In addition, 300 μM suramin or 60 μM PD98059, a

flavonoid which is a potent inhibitor of mitogen-activated protein kinase kinase (MEK), were also effective as cytoprotectants. Both drugs significantly decreased the total cell death percentage, with both 6 or 48 h treatment, with $P < 0.005$ (Fig. 3C).

In more detail, during the pulse treatment both suramin and PD98059 remarkably

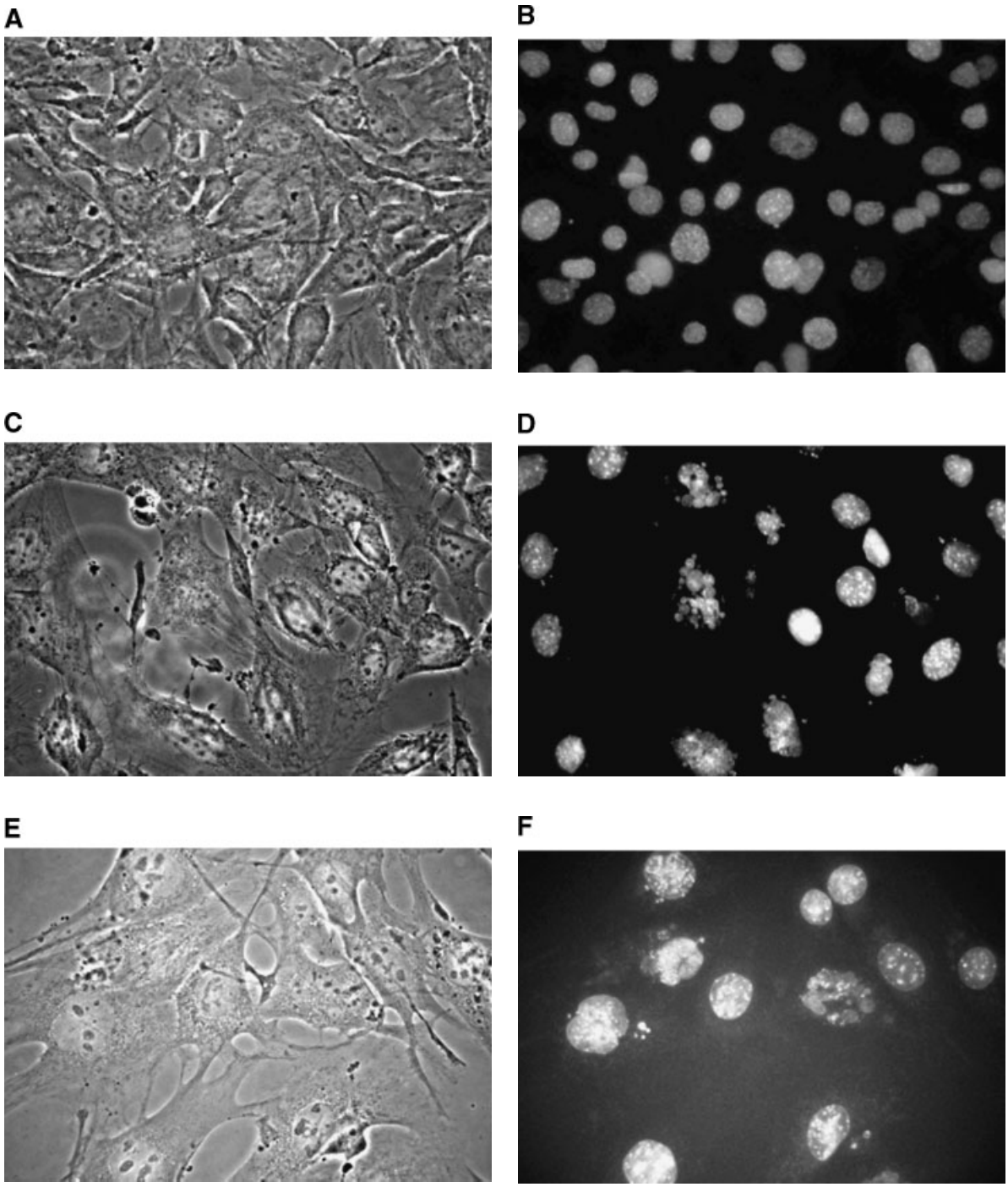
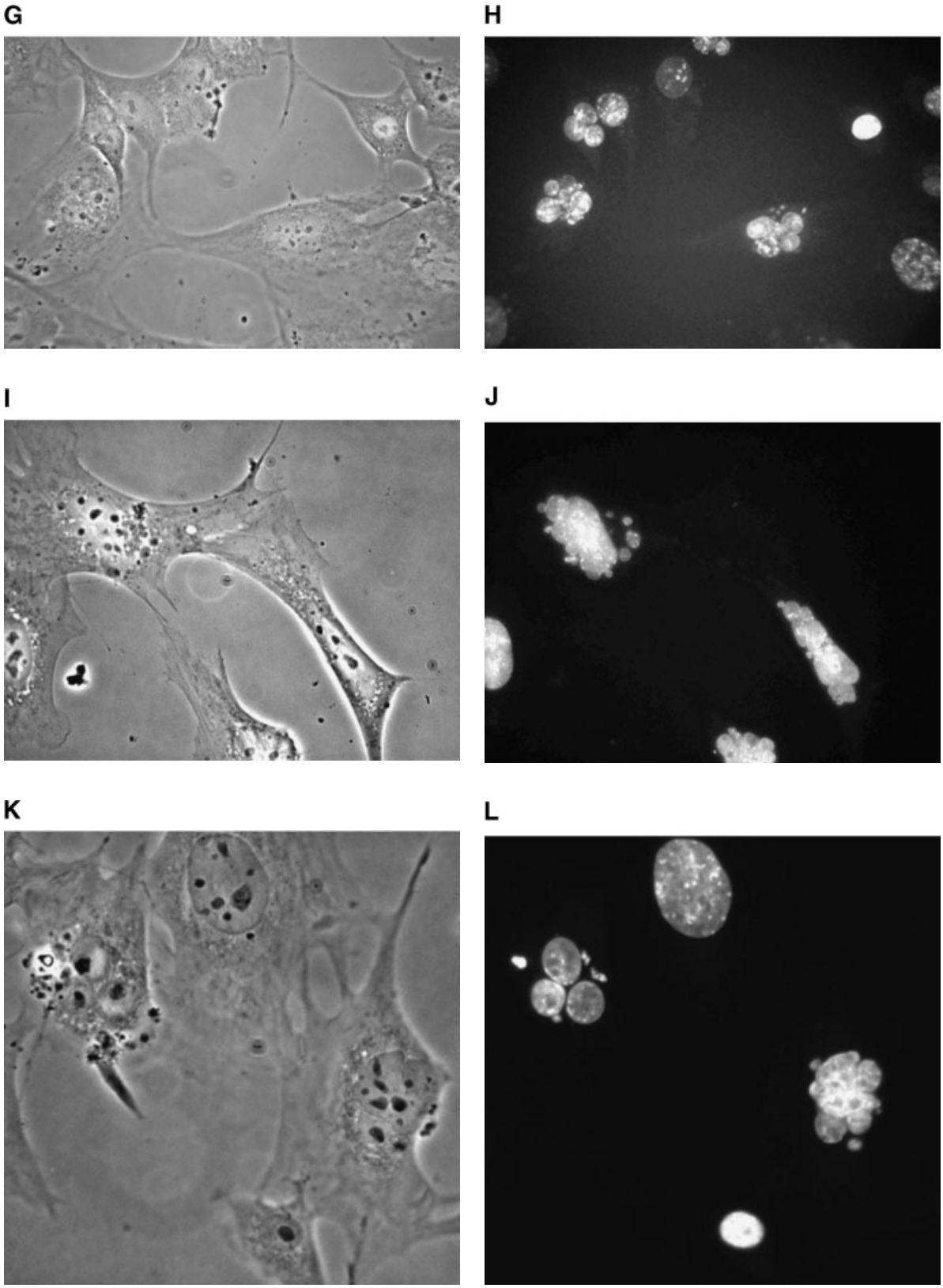


Fig. 1. Morphological evaluation of cell death. Cells were grown on a glass coverslip and treated respectively with vehicle (**A**, **B**), 10 μM CDDP (C–F), 20 μM CDDP (G–J) or 50 μM CDDP (K–N) for 24 h (**C**,**D**,**G**,**H**,**K**,**L**) or 48 h (**E**,**F**,**I**,**J**,**M**,**N**) fixed in paraformaldehyde and stained with DAPI. **O** and **P**: Microphotograps of phalloidin-FITC staining on control cells (O) or cells treated with 50 μM CDDP for 48 h (P); Magnification is 800×.



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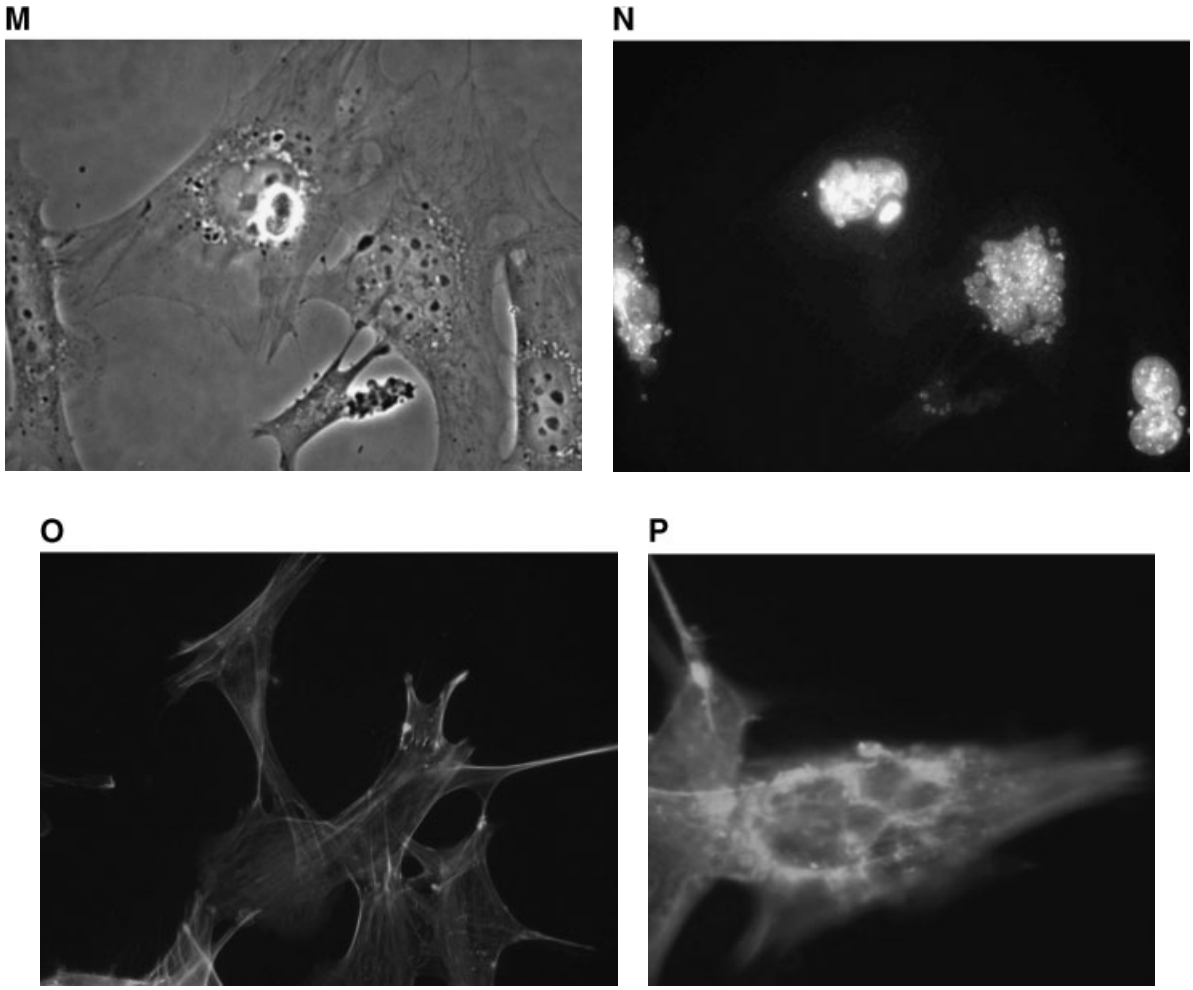

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reduced the PI-dim population ($P < 0.005$), while only suramin significantly reduced the PI-bright population ($P < 0.005$). During a 48 h treatment, suramin deeply changed the reciprocal ratio between PI-dim and PI-bright subpopulations: indeed, in the CDDP-suramin co-treated samples we observed a marked reduction of PI-bright cells, while the PI-dim cells, significantly increased in comparison to controls. On the contrary, treatment with PD98059 reduced the two subpopulations (dim and bright) to a lower extent in comparison to suramin, roughly maintaining the reciprocal ratio (Fig. 3C).

When we treated OC-k3 cells with Z-VAD-FMK, a pan-caspase inhibitor, we did not see any significant cytoprotective effect in presence of 50 μM CDDP (Fig. 3C), and consistently, caspase 3 was not subjected to proteolytic activation after 48 h in presence of different CDDP external concentrations (Fig. 4).

Effect of Methionine and Suramin on CDDP Uptake

Suramin is a polysulfonated compound that can interact with different membrane proteins and is known to non-specifically reduce growth factor binding to cell membrane receptors [Coffey et al., 1987]. Thus suramin could exert its protective action by reducing the rate of CDDP binding and uptake. In order to investigate the putative mechanism of suramin action, we wanted to assess if suramin could interfere with platinum loading.

First, we measured the amount of cisplatin present in OC-k3 cells at different treatment

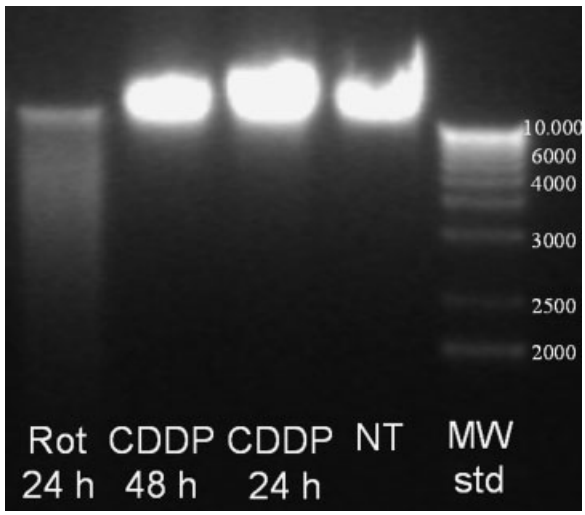


Fig. 2. DNA separation on agarose gel. Cells were treated with 50 μM CDDP or 50 μM rotenone for the indicated times, and DNA extracted and electrophoresed on 0.8% agarose gel as described in Materials and Methods.

times. Figure 5A shows that 100 μM cisplatin treatment did not allow to reach saturation in the time evaluated. Once we determined that cisplatin uptake apparently followed first order kinetics, we investigated the effect of suramin and methionine on 50 μM CDDP uptake. We found a platinum load of 0.15 ± 0.04 μg/10^6 cell. Methionine, that has a sulfydryl group able to covalently react with CDDP [Lempers and Reedijk, 1990; Ishikawa and Ali-Osman, 1993] was able to significantly decrease the amount of cellular platinum to 0.11 ± 0.02 μg/10^6, while suramin treatment did not significantly reduce the platinum loading (Fig. 5B).

ERK Extracellular Signal-Regulated Kinase 1/2 (ERK1/2)

Because the total intracellular amount of platinum was unaffected by suramin-cisplatin coincubation, and because PD98059 is a MEK inhibitor, we investigated the possibility that CDDP could activate the ERK signaling pathway, and in turn suramin and PD98059 could

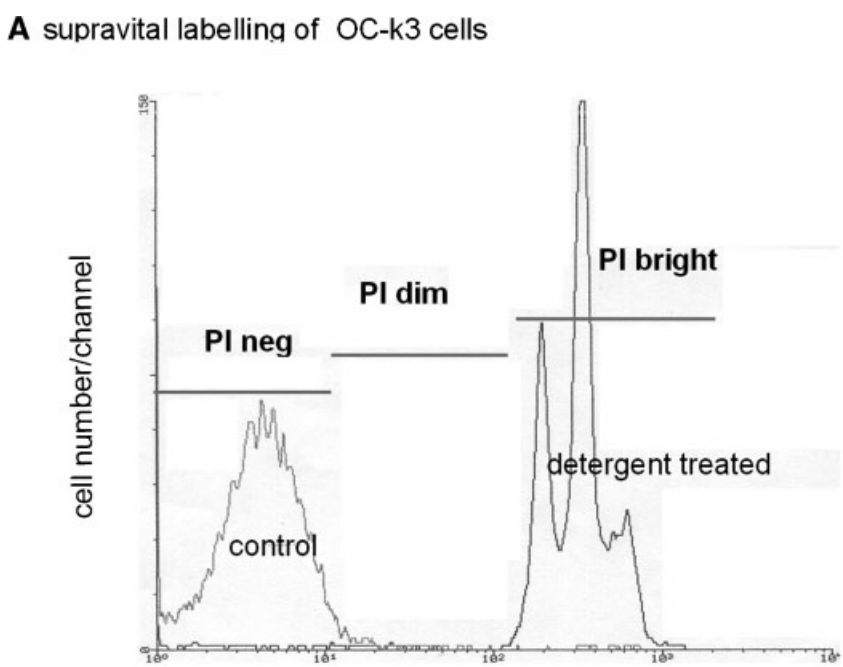
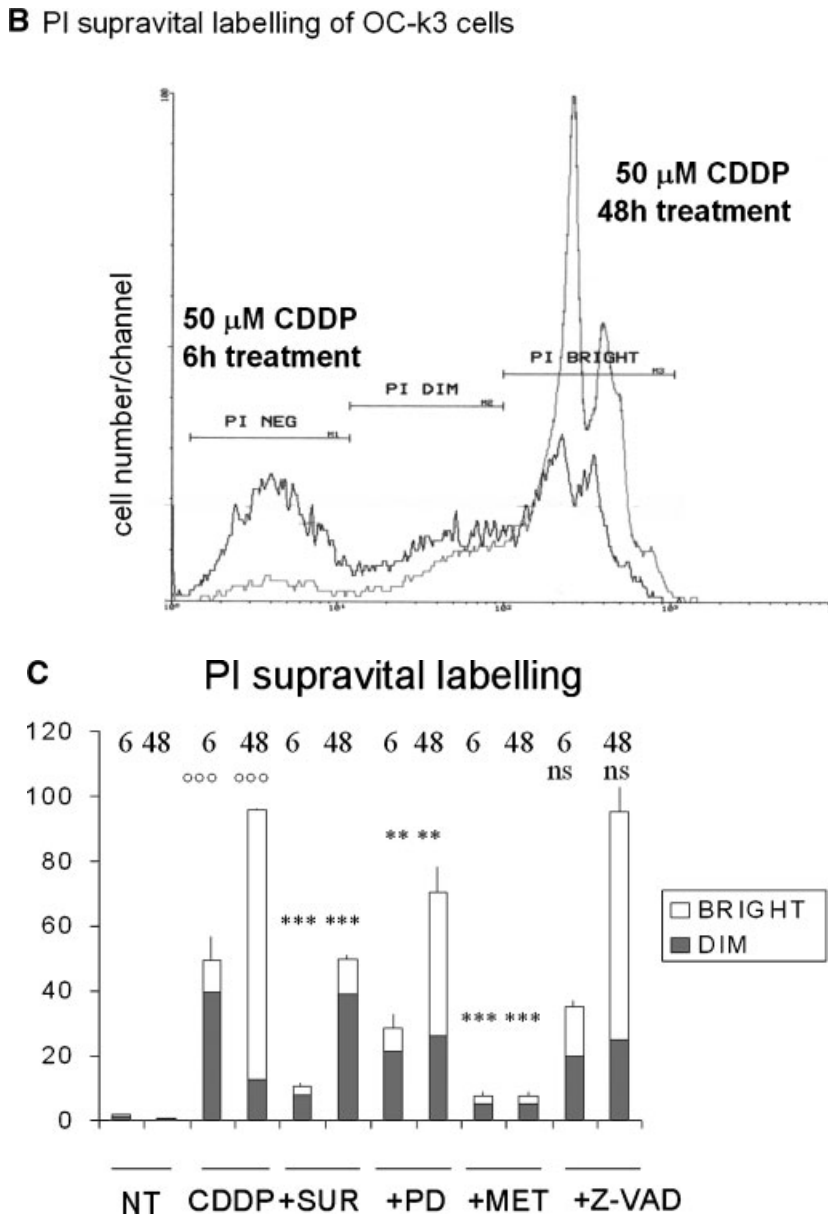


Fig. 3. PI supravital labeling of detached OC-k3 cells. Cells were treated as adherent cells for the indicated times with 50 μM CDDP, detached by proteolytic treatment, immediately labeled with 2 μg/ml PI and analyzed by flow cytometry. In **A** and **B**, values on abscissa represent the intensity of PI fluorescence signal. Values on ordinate represent the number of cells in a total number of 10,000 analyzed events; in (**C**) values on ordinate represent percent of PI labeling. A: Control and 0.1% NP40 permeabilized cells. B: Cells treated with 50 μM CDDP for 6 or 48 h. C: Cells treated with 50 μM CDDP for 6 or 48 h and, where indicated, co-treated with 1 mg/ml methionine, 300 μM suramin, 60 μM PD98059, or 50 μM Z-VAD-FMK. Data represent the mean ± SD of four replicates from one experiment representative of a group of three. $^{**}P<0.01$, $^{***,\circ\circ\circ}P<0.005$; ns = not significantly different. Empty symbols indicate significance toward non-treated samples, asterisks indicate significance toward CDDP-treated samples.



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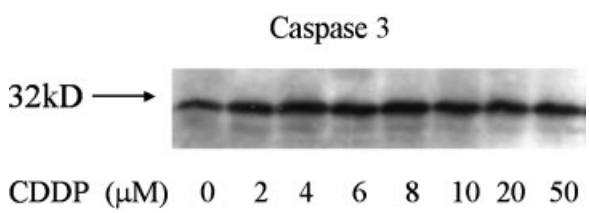

Fig. 4. Analysis of caspase 3 activation. Cells were treated with different cisplatin concentrations for 48 h, afterwards the samples were processed as in Materials and Methods, analyzed by SDS–PAGE electrophoresis, transferred to nitrocellulose and immunolabeled for caspase 3.

protect cells by inhibiting this cascade. We found that CDDP increased ERK 1/2 phosphorylation, starting at 10 h up to 24 h (Fig. 6). PD98059 exerted a slight inhibition, especially visible at 16 h. On the contrary, suramin reduced phosphorylation to basal levels at all times explored (Fig. 6).

DISCUSSION

In this work we employed OC-k3 cells to investigate the molecular mechanism of cisplatin toxicity on inner ear-derived cells.

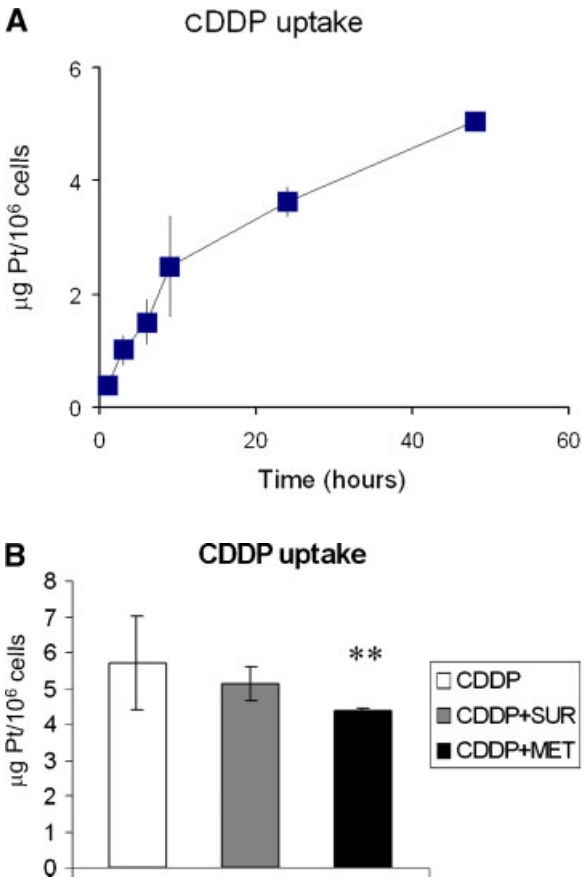

Fig. 5. CDDP uptake in adherent OC-k3 cells. **A**: Time course of CDDP uptake by OC-k3 cells incubated with 50 μM CDDP for the indicated times. After the incubation, cells were detached by proteolytic treatment and counted; 0.5×10^6 cells were sedimented and the pellet dissolved in nitric acid and analyzed by atomic absorption, as described in Materials and Methods. **B**: CDDP uptake after 48 h of treatment with 50 μM CDDP plus or minus 1 mg/ml methionine or 300 μM suramin. Data are given as ng CDDP/10^6 cells. The mean ± SD of five replicates from one experiment representative of a group of two. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Initially, we investigated the uptake of cisplatin in this cell line, and found it followed a first order kinetic, without clear signs of saturation before the onset of cell death. These data suggest that uptake occurs by simple diffusion, probably through the non-aquated, uncharged form of cisplatin, which can freely diffuse through the cell membrane.

Afterwards we quantitatively evaluated cell death using a PI exclusion assay, which discriminate among three cell subpopulations: the living cells, only weakly labeled by PI; the necrotic post-apoptotic cells, strongly labeled by PI; and a subpopulation which displayed an intermediate fluorescence. This subpopulation was previously reported to undergo the initial apoptotic phases, both in attached and in suspension grown cells [Zamai et al., 2001]. The results showed that OC-k3 cells are significantly sensitive to 50 μM cisplatin both with 6 h pulse treatment and with continuous 48 h CDDP treatment. Cell labeling depended upon cisplatin load: we found an increase of post-apoptotic necrosis in presence of higher cisplatin uptake, and, consistently, a lower cell death percentage and a greatest number of cells in the initial phases of apoptosis in presence of lower cisplatin incorporation.

At the concentration tested, cisplatin-induced cell death did not occur by necrosis, but involved complex and well structured processes, such as cell and nuclear fragmentation, indicating the occurrence of apoptosis. When we examined morphological changes in DAPI-stained cells, we found a deep rearrangement of both cell and nuclear shape after 48 h treatment with 50 μM CDDP. We observed an extensive nuclear disassembly, with production of several large nuclear fragments, and rearrangement of cell cytoskeleton. The actin network typically invaded the original nuclear space, reorganizing around each large nuclear fragment and separating them from each other. Frequently, post-apoptotic cell fragments containing single large nuclear fragments could be observed attached to the substratum.

Although the occurrence of an organized pattern of nuclear fragmentation was firmly assessed, on the other hand we did not find expression of some typical apoptosis markers, such as internucleosomal fragmentation and PS exposure on the outer leaflet of cell membrane. Conflicting data have been reported in the literature on internucleosomal fragmentation after cisplatin challenge. Several authors have described TUNEL positivity in cochlear tissues after cisplatin treatment [Alam et al., 2000; Teranishi et al., 2001]. In other cells, other authors have shown that cisplatin cytotoxicity can occur without DNA breakage [Ormerod et al., 1994]. Furthermore, cisplatin addition can lead to caspase inactivation, followed by cell death stop and by paradoxical rescue of mice from TNF and lipopolysaccharide-induced liver shock [Shin et al., 2005]. Consistently, in OC-k3 cells, caspases did not seem to be the major effector of cell death, as evidenced by the failure of pan-caspase inhibitor Z-VAD-FMK to reduce cell death. This can explain the lack

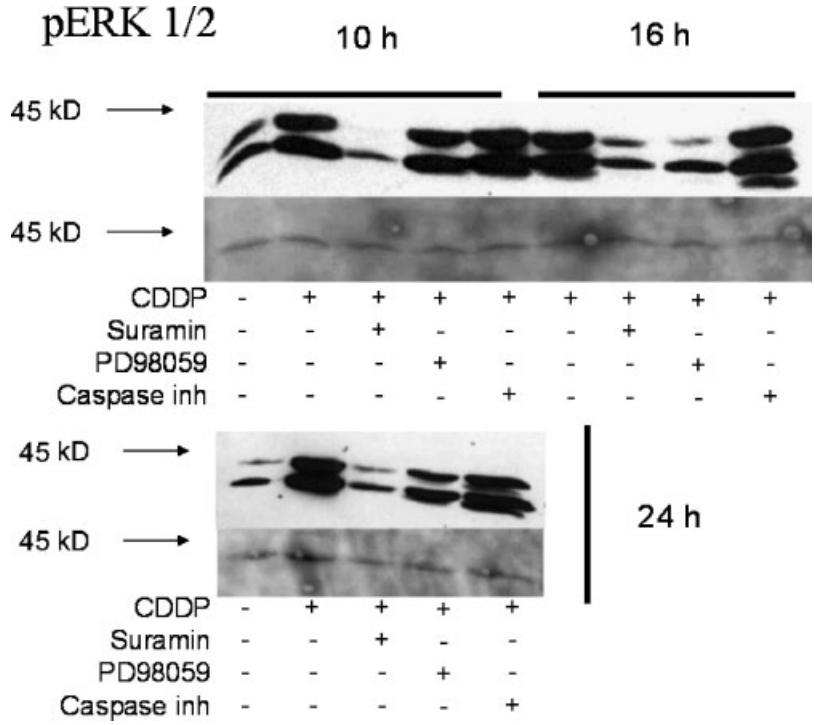

Fig. 6. Analysis of ERK 1/2 phosphorylation by Western Blot. OC-k3 cells were treated for 6 h with 50 μM CDDP plus or minus 300 μM suramin or 60 μM PD98059. Afterwards, the medium was replaced and the incubation allowed to continue in fresh medium plus suramin or PD98059, where indicated. At 10, 16, and 24 h the samples were processed as in Materials and Methods, analyzed by SDS–PAGE electrophoresis, transferred to nitrocellulose and immunolabeled by an antibody that specifically recognizes phosphorylated Tyr 204 on ERK1 and ERK2.

of internucleosomal fragmentation, because it is well known that DFF45, the protein that inhibits the nuclease effector DFF40, is removed by caspase proteolysis. In absence of proteolytical activation, DFF40 cannot trigger internucleosomal DNA degradation.

In addition, OC-k3 cells express SV40 large T antigen, whose mechanism used to immortalize eukariotic cells implies p53 binding, formation of large nuclear complexes and inhibition of its DNA transactivating properties. This originates cell lines functionally depleted of p53, inhibits p53-dependent apoptosis triggering [Lane and Crawford, 1979] and could originate an atypical apoptotic pathway, with a unsolicited part of the executionary machinery. Thus it is reasonable to speculate that the lack of an extensive proteolytic activation, which represents a relevant part of the executionary phase of apoptosis, originates in HL60 an incomplete apoptotic phenotype.

Several data from literature suggest that, beside the addition to guanine residues with formation of intra- and interstrand bridges, other mechanisms, including intracellular signal transduction, can be involved in cisplatin toxicity. Consistently, our data show a recruitment of MAPK signaling cascade, through an upstream mechanism not yet clarified, potentially involving receptor stimulation.

In detail, we demonstrated that cisplatin increased ERK 1/2 phosphorylation from 10 to 24 h. This activation occurs later than what reported by Wang et al. [2000] who showed that cisplatin-induced ERK 1/2 activation occurs 6 h after treatment. Consistently with involvement of ERK 1/2 in cisplatin toxicity, we found that PD98059, is a MEK inhibitor and suramin, a polysulphonated compound previously used as inhibitor of extracellular receptors for growth factors [Schrell et al., 1995; Li et al., 1999; Laubinger et al., 2003], not only inhibited ERK 1/2 activation but also reduced the extent of CDDP-induced cell death. These compounds, which can both block the upstream signaling to ERK 1/2, are also able to significantly reduce the

percentage of dead cells proportionally to lowering ERK phosphorylation. Indeed, PD98059 is less effective than suramin in both lowering ERK phosphorylation and PI labeling over long term treatments. Suramin is able to significantly reduce total cell death, both in presence of lower and higher cisplatin load. In addition, it decreases the number of post-apoptotic necrotic events at both treatment times, simultaneously increasing the PI-dim population. This suggests that suramin, with an efficiency depending on the cisplatin load, can not only reduce the number of cells entering into apoptosis but also slow down the passage from the intermediate to the terminal phases of cell death.

Methionine also provides a fairly complete protection against cisplatin. However, we should note that methionine has been reported to covalently react with cisplatin, giving raise to several different linear or closed ring adducts. This can affect cisplatin uptake, which seems to be based on its uncharged, unreacted form, and consequently limit the extent of cell damage by reducing the extent of DNA and protein cross-linking [Eastman, 1987]. Theoretically, suramin could also act by reducing cisplatin loading, because it is able to bind its positive charges to the negatively charged proteins on the external cell surface. To investigate the effects of the above drugs on CDDP entry, we measured platinum uptake in presence of methionine or suramin, and found that methionine, but not suramin, significantly reduced the cisplatin uptake in OC-k3 cells. The efficacy of suramin in protecting cells without affect platinum load may suggest several interpretations: first it could indicate a non-receptor mediated cisplatin uptake; secondarily, cisplatin toxicity could be mediated by recruitment of extracellular receptors, in turn able to induce ERK activation and a caspase-independent, p53-independent apoptosis. This can be supported by the finding that EGF treatment [Kroning et al., 1995; Dixit et al., 1997] and ras activation [Gao et al., 1995; Fokstuen et al., 1997] increase tumor sensitivity to cisplatin, suggesting the requirement of extracellular activation for apoptosis induction. These extracellular receptors can activate ATM and ras [Damu et al., 2002; Woessmann et al., 2002] and, finally, the MAPK cascade [Wang, 2000]. ATM, and ERK can, in turn, induce both p53-dependent and independent cell cycle block and apoptosis [Petrache et al., 1999; Pavlovic et al., 2000].

As a whole, our data indicate that in OC-k3, an inner ear-derived p53-lacking cell line, cisplatin induces nuclear fragmentation, actin cytoskeleton rearrangement and cell death in a p53- and caspase-independent manner. The activation of ERK 1/2 is a possible main effector of cell death, linking receptor recruiting and induction of toxicity.

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