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Differential functions of PKC-δ and PKC-ζ in cisplatin response of normal and transformed thyroid cells

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Abstract

We investigated the effects of cisplatin (*cis*Pt) in normal PC Cl3 and in transformed and tumourigenic PC E1Araf cells. *cis*Pt cytotoxicity was higher in PC Cl3 than in PC E1Araf cells. In both cell lines, *cis*Pt provoked the ERK1/2 phosphorylation; this was unaltered by Gö6976, a conventional PKC inhibitor, whilst it was blocked by low doses (0.1 μM) or high doses (10 μM) of GF109203X, an inhibitor of all PKC isozymes, in PC Cl3 and in PC E1Araf cells, respectively. In PC E1Araf, the *cis*Pt-provoked ERK phosphorylation was also blocked by the use of a myristoylated PKC-ζ pseudosubstrate peptide. Conversely, in PC Cl3 the *cis*Pt-provoked ERK phosphorylation was blocked by the use of rottlerin, a PKC-δ inhibitor. Results show that *cis*Pt activates both PKC (the -δ and the -ζ isozymes in PC Cl3 and in PC E1Araf cells, respectively) and ERK in association with prolonged survival of thyroid cell lines.

Keywords: PC Cl3; Cisplatin; ERK; PKC-ζ; PKC-δ

The chemo-therapeutic agent cisplatin (*cis*-diamminedichloroplatinum; *cis*Pt) is widely employed for treatment of human cancer, and is a potent inducer of growth arrest and/or apoptosis in most cell types. A major limitation of *cis*Pt chemotherapy is serious drug resistance. Alterations in signal transduction pathways involved in apoptosis and/or cell survival have been implicated in the development of *cis*Pt resistance [1–3]. *cis*Pt has been shown to induce activation of Ras and its downstream effector kinases, Raf/MEK/ERK, important mediators of signal transduction processes, that serve to coordinate the cellular response to a variety of extracellular stimuli. The ERK pathway also plays a major role in the apoptotic processes [4–6]. ERK is activated by some conditions of stress, particularly oxidant injury, and in such circumstances is believed to confer a survival advantage to cells [6–8]. There is conflicting evidence for the role of ERK in influencing cell survival of *cis*Pt-treated cells. For example, studies have suggested that ERK activation is associated with enhanced survival of *cis*Pt-treated cells [9,10]. However, elevated expression of Ras has been connected with enhanced sensitivity to *cis*Pt [11,12]. It has also been shown that the protein kinase C (PKC) signal transduction pathway plays an important role in influencing *cis*Pt-induced apoptosis [13]. Novel PKC-δ is a substrate for caspase-3 and proteolytic activation of PKC-δ has been directly associated with DNA damage-induced apoptosis [14]. PKC can interact with other signalling pathways to elicit its biological responses. There have been several reports which suggest that several PKC isozymes, including PKC-δ, activate ERK via activation of Raf [15–17]. Furthermore, *cis*Pt-induced DNA damage results in the activation of ERK1/2 via PKC-δ [18].

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Anaplastic thyroid carcinoma (ATC) is usually associated with a poor prognosis, with most patients dying within a few months. The mechanism of its carcinogenesis is unclear, and its rapid growth and spread often prevent effective surgical therapy. Thus, chemotherapy is necessary. However, ATC is often resistant to anticancer drugs, so that prediction of chemosensitivity appears important in selecting appropriate treatment. Current chemotherapy for ATC is based primarily on doxorubicin [19] and *cis*Pt [20]. Although several factors implicated in *cis*Pt resistance have been identified, the resistance mechanisms in detail are not fully understood yet.

PC Cl3 cells are fully differentiated thyroid cells [21,22], which express the typical markers of thyroid differentiation, such as thyroglobulin (Tg), thyroperoxidase (TPO), thyrotropin receptor (TSHR), and sodium iodide symporter (NIS); they are sensitive to thyrotropin (TSH) stimulation for their growth [21,22]. In this study, we sought to determine whether ERK and PKCs play a role in the cellular stress response to *cis*Pt in normal PC Cl3 and in PC Cl3-derived cells (tumourigenic PC E1Araf cells) transformed by a combination of the adenovirus E1A gene and the *raf* oncogene [21,22]. We show a central role of PKC-δ and PKC-ζ in PC Cl3 and PC E1Araf cells, respectively, in the upstream activation of ERK pathway after *cis*Pt treatments of thyroid cell lines.

Materials and methods

Reagents. Glutamine, gentamicin, the MEK inhibitor PD098059, the PKC inhibitors GF109203X and Gö6976 were obtained from Sigma Chemical (Milan, Italy). Rottlerin was purchased from EMD Biosciences (La Jolla, CA). PKC isoforms, phospho-specific ERK1 and ERK2 antibody, goat anti-rabbit IgG conjugated with peroxidase, polyclonal caspase-3, as well as control antibodies, were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Myristoylated PKC-ζ pseudosubstrate peptide (Myr-SIYRRGARRWRKL) was obtained from Calbiochem–Novabiochem (Schwalbach, Germany).

Cell lines. PC Cl3, a rat differentiated thyroid cell line, was grown in Coon's modified Ham's F-12 medium (Celbio, Pero Milan, Italy) supplemented with 5% calf serum (Sigma, Milan, Italy) and a mixture of hormones and growth factors (insulin 1 μg/ml; TSH 1 mIU/ml; glycyl-histidyl-L-lysine 10 ng/ml; human transferring 5 μg/ml; cortisone 10 nM; somatostatin 10 ng/ml) (Sigma). PC E1Araf are PC Cl3 cells transformed by the adenovirus E1A gene and the *raf* oncogene [21,22]. They were grown in the same medium as PC Cl3 cells, but lacking the mixture of hormones and growth factors.

Cytotoxicity assay. Cells at 70–80% confluency were trypsinised (0.25% trypsin with 1 mM EDTA), washed, resuspended in growth medium, and plated in 96-well plates with 0.1 ml of the 10^4 cell/ml cell suspension seeded in each well. After overnight incubation, cells were treated with specific reagents for 24 h to 72 h.

The conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide) by cells was used as an indicator of cell number as described by Mosmann [23], with some modification as described previously [24]. This method measures the reduction of MTT by active mitochondria, which results in a colourimetric change measured at OD_{550}. In experiments done to correlate cell numbers with absorbance obtained by spectrophotometric assay of viable cells and to define the linear range of the assay, the number of viable cells formed a tight correlation up to about 50,000 cells per well (data not shown). Increasing number of heat-killed cells per well (killed by incubating at 70 °C for 15 min) caused no significant change in the absorbance; thus, this spectrophotometric method was a valid technique for measuring the number of viable cells. All of the experiments performed were within the linear range of the assay.

The percentage of survival was calculated as the absorbance ratio of treated to untreated cells. The data presented are means ± standard deviation (SD) from eight replicate wells per microtitre plate and replicate for four times.

Cell count. Thyroid cells were seeded at 5×10^4 cells/well on 24-well plates, and cells were counted in a Thomas cell chamber after treatments as described above.

Apoptosis analysis. For 4,6-diammine-2-phenylindol (DAPI) staining, cells were fixed with 3% formalin and stained with 1 mg/ml DAPI in PBS for 10 min. Cells were mounted on glass slides, covered, and analysed using fluorescence microscopy. For statistical analysis of each experiment, 5–10 fields (magnification, 400×) were counted per stimulation and cell type (between 400 and 700 cells in total). The mean ± SD was calculated and displayed as bar graph.

Western blot analysis. To obtain whole protein cell extracts for Western blot analysis, thyroid cells were scraped in the following buffer (mM): 20 Tris–HCl, pH 8, containing 420 NaCl, 2 EDTA, 2 Na_3VO_4, and 2% Nonidet P-40. Cells were then passed several times through a 20 gauge syringe and centrifuged at 16,000*g* for 20 min at 4 °C.

Proteins were determined with the Bio-Rad protein assay kit 1 (Milan, Italy), using lyophilised bovine serum albumin as a standard.

Equal amounts of proteins (25 μg) from homogenates were loaded and separated on 10% SDS gels by electrophoresis and transferred to a nitrocellulose membrane. The sheet was blocked with 3% non-fat dried milk in buffered saline. PKC isozyme proteins were detected using antibodies specific for different PKC isoforms (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Dually phosphorylated ERK1 and ERK2, corresponding to the active forms of the enzymes, were detected by a specific antibody (Santa Cruz Biotechnology). The blots used for active ERK1/2 detection were then stripped and reprobed with another antibody (Santa Cruz Biotechnology). which recognises both active and basal forms of the ERK enzymes.

The proteins were detected with goat anti-rabbit IgG conjugated with peroxidase (Santa Cruz Biotechnology), using the ECL (Amersham Life Sciences, Amersham, UK).

Statistical analysis. Experimental points represent means ± standard deviation (SD) of 3–6 replicates. Statistical analysis was carried out using the ANOVA. When indicated, post hoc tests (Bonferroni/Dunn) were also performed. A P value less than 0.05 was considered to achieve statistical significance.

Results

Level of phosphorylated ERK and sensitivity to cisPt

By the antibody recognising the dually phosphorylated forms of ERK1 and ERK2, we found that the unstimulated level of phosphorylated ERK1/2 was scarcely detectable in PC Cl3, and significantly high in PC E1Araf cells (Fig. 1A). Total ERK levels, as detected using an antibody which recognises active and basal forms of the ERK enzymes, were approximately the same in both cell lines (Fig. 1A). One hundred micromolar of *cis*Pt provoked a phosphorylation of ERK1/2 in both cell lines (Fig. 1A).

Cells were treated with various concentrations of *cis*Pt, and viable cell number was determined 24 and 48 h later by MTT colourimetric assay. As shown in Fig. 1B, after 24 h *cis*Pt treatment, approximately 50% of PC Cl3 and 86% of PC E1Araf had survived, suggesting that neoplastic

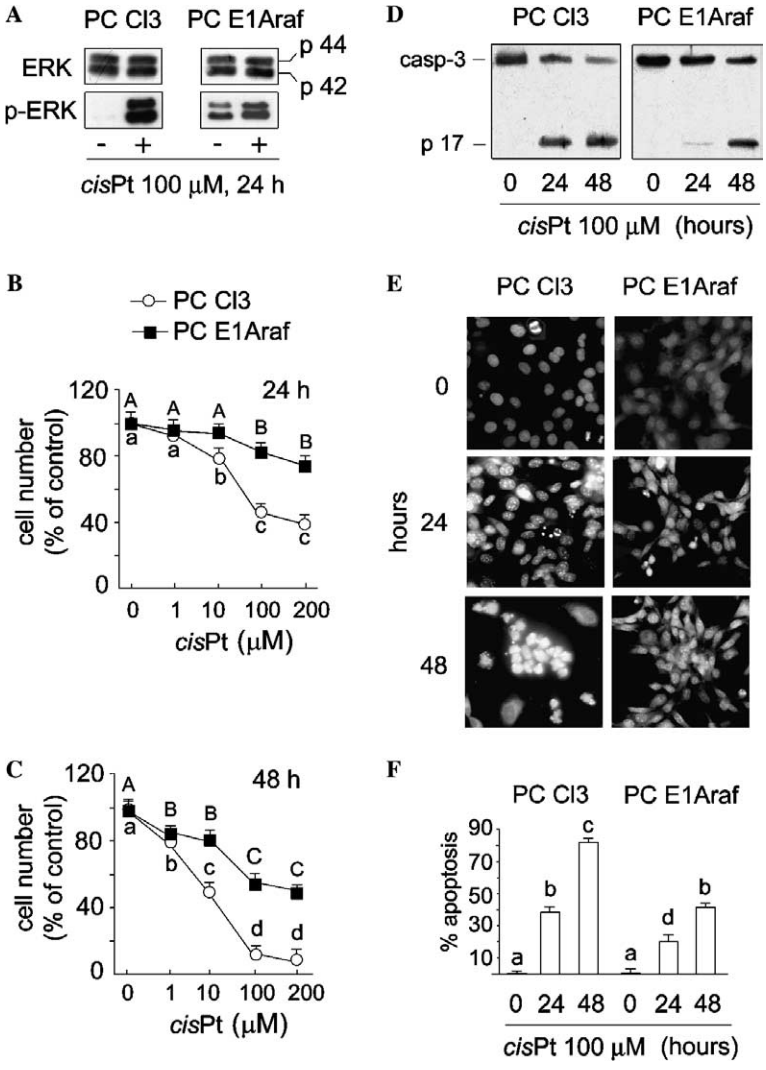

Fig. 1. (A) Thyroid cells were treated without or with 100 µM *cis*Pt for 24 h. Cell lysates were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK) or the anti-total ERK antibody. The figure is representative of four independent experiments. (B,C) Cells were treated without or with various concentrations of *cis*Pt, and viable cell number was determined 24 (B) and 48 (C) h later by MTT assay. The data are means ± SD of four different experiments run in eight replicate and are presented as percent of control. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests. (D) The processing of caspase-3 after incubation of cells with 100 µM *cis*Pt was analysed by Western blot and a representative autoradiograph of four independent experiments is shown. (E,F) PC Cl3 and PC E1Araf cells were treated or not with *cis*Pt for 24 and 48 h. (E) Cells were stained with DAPI, and representative fields of one of four independent experiments are shown. (F) Quantification of the percentage of apoptotic nuclei obtained from cells stained with DAPI (mean ± SD; $n = 4$). Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

transformation desensitises cells to this drug. The same trend was obtained after 48 h of *cis*Pt treatment (Fig. 1C).

The cleavage pattern of caspase-3 was analysed by Western methods. The results, shown in Fig. 1D, indicate that the activation of caspase-3, as evidenced by the appearance of caspase-active forms, occurred 12 and 48 h after *cis*Pt treatment in PC Cl3 and PC E1Araf cells, respectively. Fig. 1E shows the results of a DAPI staining, visualising the extent of nuclear fragmentation before stimulation and 24 or 48 h after *cis*Pt treatment in both cell lines. Quantification of nuclear changes revealed that PC Cl3 and PC E1Araf cells differently reacted to *cis*Pt-evoked apoptosis (Fig. 1F).

Dose–response and time course of cisPt phosphorylation of ERK1/2

The dose–response and time course of ERK1/2 to *cis*Pt are illustrated in Figs. 2A-D. In PC Cl3 cells, an increase in phosphorylation of ERK1/2 was observed with 100 µM *cis*Pt (Fig. 2A). Conversely, in PC E1Araf cells a threshold increase of phosphorylated ERK1/2 was observed at 10 µM *cis*Pt, with a maximum at 100 µM *cis*Pt (Fig. 2B). Therefore, subsequent experiments were carried out using 100 µM *cis*Pt in both thyroid cell lines. The effect of 100 µM *cis*Pt on the phosphorylation state of ERK1/2 was time-dependent. There was a threshold increase at

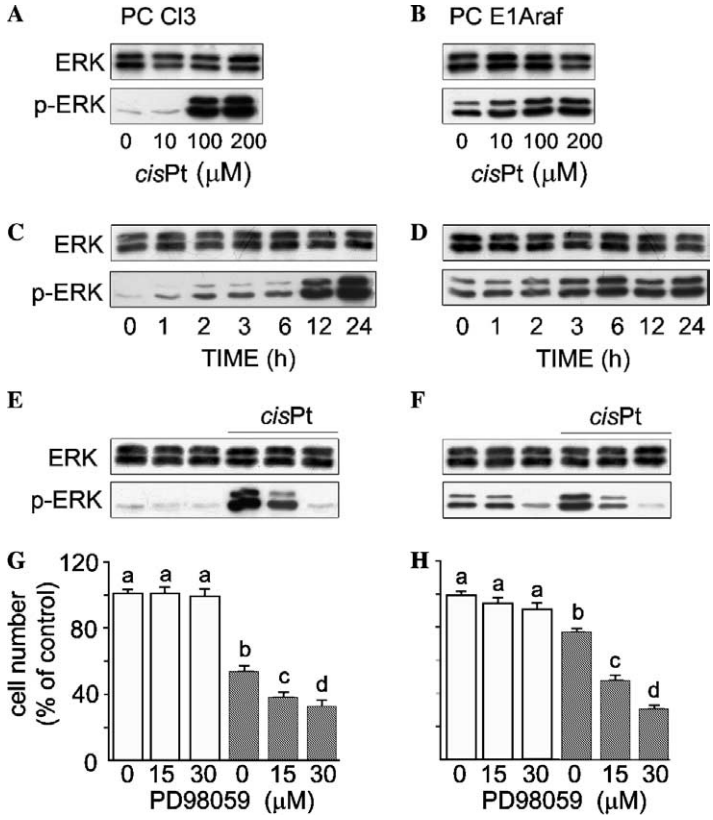

Fig. 2. PC Cl3 (A) and PC E1Araf (B) cells were treated without or with various concentrations of *cis*Pt, for 24 h, or with 100 μM *cis*Pt, for the indicated times (C,D). Cell lysates were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK) or the anti-total ERK antibody. The figure is representative of four independent experiments. (E–H) Cells were pretreated without or with various concentrations of PD98059 for 30 min and then without or with 100 μM *cis*Pt, for 24 h. Lysates from PC Cl3 (E) and PC E1Araf (F) cells were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK) or the anti-total ERK antibody. PC Cl3 (G) and PC E1Araf (H) viable cell numbers assessed by a MTT assay as described under Materials and methods. The data are means ± SD of four different experiments run in eight replicate and are presented as percent of control. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

2 h in both cell lines, a maximal effect at 12 and 6 h in PC Cl3 and PC E1Araf, respectively; no further effects with longer incubation times were observed (Figs. 2C and D). *cis*Pt did not have effects on the total ERK1/2 levels in both cell lines (Fig. 2).

PD98059, a specific inhibitor of MAPK/ERK kinases 1 (MEK1) [25], was used in order to determine whether MEK was involved in *cis*Pt-induced phosphorylation of ERK1/2. The pre-treatment of cells with 15 and 30 μM PD98059 for 15 min did not alter the basal phosphorylation state of ERK1/2 in PC Cl3 (Fig. 2E), whilst it significantly decreased it in PC E1Araf cells (Fig. 2F). PC Cl3 and PC E1Araf cells pre-treated with PD98059 showed a dose-dependent inhibition of the *cis*Pt-induced phosphorylation of ERK1/2 (Figs. 2E and F).

We examined whether a phosphorylated ERK is required for the *cis*Pt cytotoxicity in thyroid cell lines. Pre-treatment with PD98059 resulted in enhanced sensitivity to *cis*Pt (Figs. 2G and F) in as much as a significant decrease in cell survival after *cis*Pt treatment was observed in both cell lines (ANOVA: $P < 0.05$). At a concentration of 30 μM PD98059, the percentage of surviving cells was about 30% in both cell lines, significantly less than in the absence of PD98059 (50% and 86% in PC Cl3 and PC E1Araf cells, respectively). Thus, the cytotoxicity of *cis*Pt appeared to depend upon the phosphorylation state of ERK1/2.

Role of PKC in the cisPt-induced ERK phosphorylation

The PKC isozyme expression in PC Cl3 cells was previously determined [26]. PC Cl3 expressed PKC-α, -β1, -δ, -ε, -ι, and -ζ but not -β2 and -γ. We here showed that PC E1Araf cells expressed the same pattern of PKC isozymes (Fig. 3A). The relative distribution of the PKC isozymes among the cytosolic and membrane fractions was determined; all PKC isozymes were mostly in the cytosolic fraction, with various amounts in the membrane fraction in both cell lines (data not shown). The specificity of the immunoreactivity of PKCs was verified by absorption of antibodies with isozyme-specific peptide antigen at 10 ng ml (data not shown).

The PKC inhibitor GF109203X (0.1, 1, and 10 μM) was used; when PC Cl3 cells were pre-incubated for 30 min with

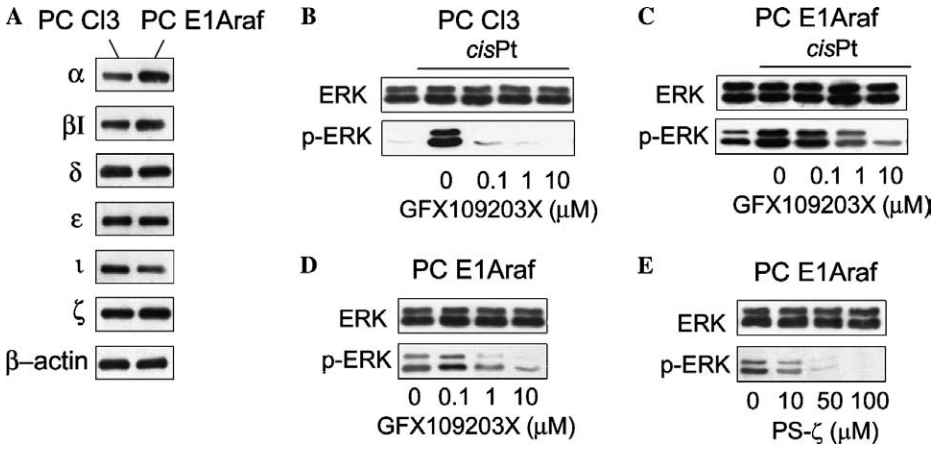

Fig. 3. (A) Immunoblots of protein kinase C isoforms in PC Cl3 and PC E1Araf cells. The figure is representative of four independent experiments. (B,C) Cells were pretreated without or with various concentrations of GF109203X for 30 min and then without or with 100 μM *cis*Pt, for 24 h. Lysates from PC Cl3 (B) and PC E1Araf (C) cells were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK) or the anti-total ERK antibody. The figures are representative of four independent experiments. (D,E) Role of PKCs in the basal phosphorylation state of ERK in PC E1Araf cells. PC E1Araf cells were pretreated without or with various concentrations of GF109203X for 30 min (D) or with myristoylated PKC-ζ pseudosubstrate peptide (ζ-PS) for 1 h (E) without further incubation with *cis*Pt. Cell lysates were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK) or the anti-total ERK antibody. The figures are representative of four independent experiments.

GF109203X, the effects of *cis*Pt on ERK1/2 were completely inhibited at the lowest concentration used (Fig. 3B); conversely, in PC E1Araf cells the complete inhibition of the *cis*Pt effects was obtained only at the highest concentration of 10 μM (Fig. 3C).

Ten micromolar of GF109203X also completely inhibited the basal phosphorylation state of ERK1/2 in PC E1Araf cells (Fig. 3D). Since GF109203X has a half-maximal inhibitory constant (IC_{50}) for atypical PKC isozymes greater than 5 μM whereas it was 210 nM or lower for all the other PKC isoforms [27], these results suggest that different PKC isoforms could be involved in the cell response to *cis*Pt. Thus, PC E1Araf cells were pre-incubated with 10, 50, and 100 μM myristoylated PKC-ζ pseudosubstrate peptide (ζ-PS) for 60 min [28,29]. The cell-permeable ζ-PS inhibited the basal phosphorylation state of ERK1/2 in PC E1Araf cells (Fig. 3E).

Both PC Cl3 and PC E1Araf cells were pre-incubated with ζ-PS for 60 min and then with 100 μM *cis*Pt; ζ-PS inhibited the *cis*Pt-provoked ERK1/2 phosphorylation in PC E1Araf cells, but not in PC Cl3 cells (Figs. 4C and D). Pre-treatment of both PC Cl3 and PC E1Araf cells with Gö6976 (0.1 and 10 μM), a conventional PKC inhibitor, did not have any effect on the phosphorylation of ERK1/2 provoked by *cis*Pt (Figs. 4C and D). On the whole these results suggest that in PC E1Araf cells atypical PKC-ζ is required for the phosphorylation of ERK1/2 after *cis*Pt treatment, whilst in PC Cl3 cells the operativity of novel PKCs is required.

Since it is known that PKC-δ plays an important role in DNA damage-induced apoptosis, we studied if the PKC-δ signalling pathway is affected in cells facing the genotoxic effects of *cis*Pt. As shown in Figs. 5A and B, the expression of novel PKC-δ increased significantly after 6 h treatment

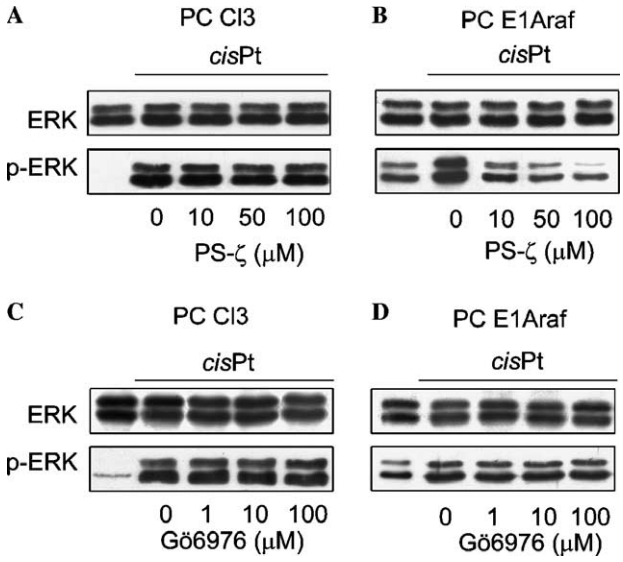

Fig. 4. PC Cl3 (A) and PC E1Araf cells (B) were pretreated without or with various concentrations of myristoylated PKC-ζ pseudosubstrate peptide (ζ-PS) for 1 h and then incubated with 100 μM *cis*Pt, for 24 h. PC Cl3 (C) and PC E1Araf cells (D) were pretreated without or with various concentrations of Gö6976 for 30 min, and then without or with 100 μM *cis*Pt, for 24 h. Cell lysates were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK) or the anti-total ERK antibody. The figures are representative of four independent experiments.

with *cis*Pt in both cell lines. Moreover, *cis*Pt induced a proteolytic activation of PKC-δ starting after 12 h in PC Cl3, but detectable only after 48 h in PC E1Araf cells. No variations in the level of PKC-ζ were found in both cell lines (Figs. 5A and B). Both PC Cl3 and PC E1Araf cells were pre-incubated with rottlerin (1–20 μM) for 60 min and then with 100 μM *cis*Pt; rottlerin inhibited the *cis*Pt-provoked

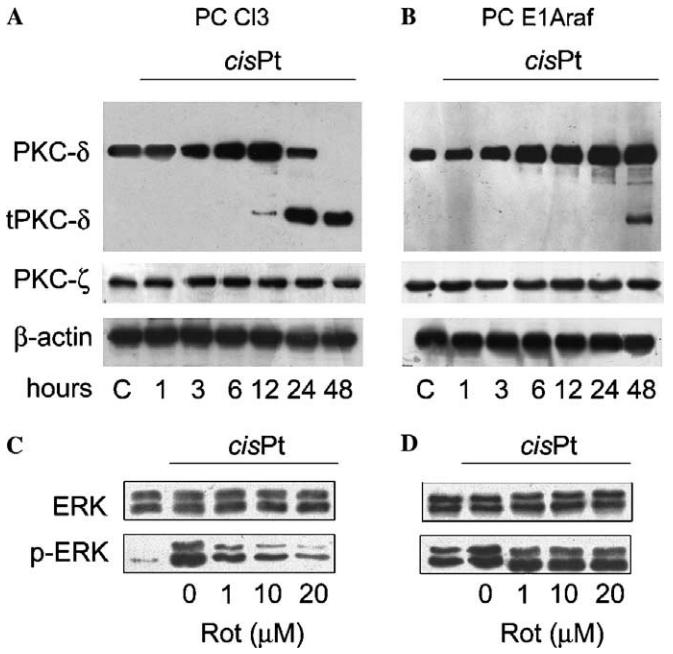

Fig. 5. PC Cl3 (A) and PC E1Araf cells (B) were pretreated without or with 100 μM *cis*Pt for the indicated times. Cell lysates were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the PKC-δ or against the PKC-ζ isozymes. The figures are representative of four independent experiments. PC Cl3 (C) and PC E1Araf cells (D) were pretreated without or with various concentrations of rottlerin for 1 h and then incubated with 100 μM *cis*Pt, for 24 h. Cell lysates were electrophoretised through 10% SDS–PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK) or the anti-total ERK antibody. Representative autoradiographs are shown. β-Actin was used as a loading control.

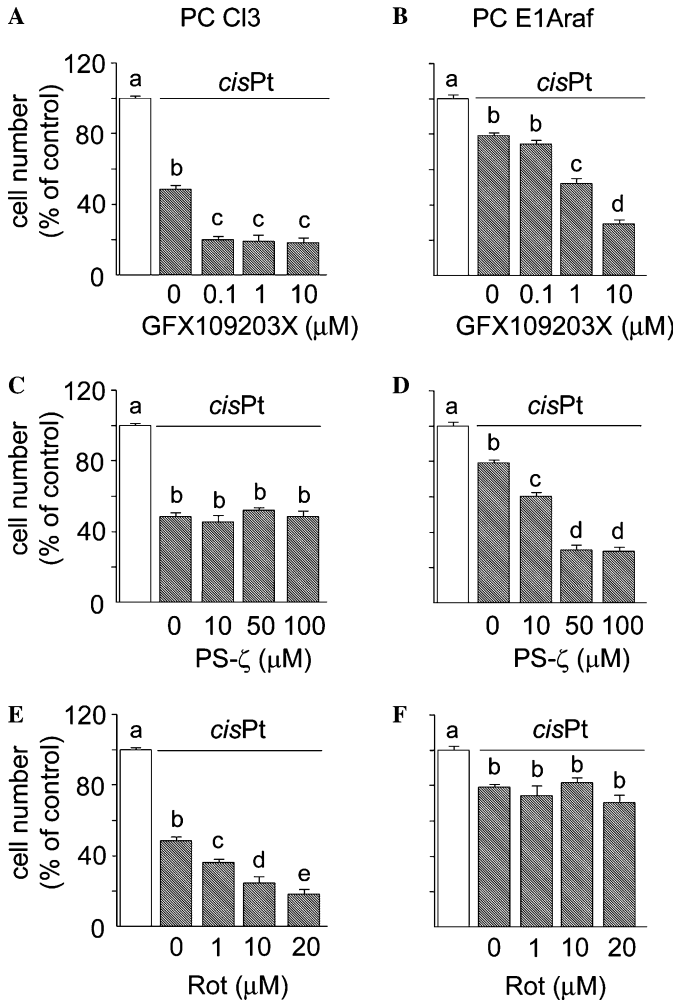

Fig. 6. PC Cl3 (left panels) and PC E1Araf (right panels) cells were pretreated without or with various concentrations of the indicated inhibitors and then without or with 100 μM *cis*Pt, for 24 h. Viable cell numbers assessed by a MTT assay as described under Materials and methods. The data are means ± SD of five different experiments run in eight replicates and are presented as percent of control. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

ERK1/2 phosphorylation in PC Cl3 cells, but not in PC E1Araf cells (Figs. 5C and D).

The role of PKC-δ/ζ in cisPt cytotoxicity in thyroid cells

Preincubation of cells with GF109203X (0.1–10 μM) for 30 min before *cis*Pt treatment (100 μM for 24 h) strongly enhanced the PC Cl3 cells' sensitivity to *cis*Pt (Fig. 6A) in a dose-dependent way, whereas the effects in PC E1Araf cells were less pronounced and highly significant only at the highest GF109203X dose (Fig. 6B). When cells were preincubated with ζ-PS (10–100 μM) for 60 min and then exposed to *cis*Pt, the number of surviving PC Cl3 cells did not change (Fig. 6C), while it decreased significantly in PC E1Araf cells (Fig. 6D). Conversely, the pre-incubation with rottlerin (1–20 μM) for 60 min drastically enhanced the sensitivity to *cis*Pt in PC Cl3 (Fig. 6E) but not in PC E1Araf cells (Fig. 6F).

Discussion

In this study, we sought to determine whether ERK plays a role in the cellular stress response to *cis*Pt in two thyroid cell lines, the parental, normal PC Cl3 cells and the PC Cl3-derived PC E1Araf cells which are cells transformed by the adenovirus E1A gene and the *raf* oncogene. PC E1Araf cells display a basal higher phosphorylated ERK than parental normal PC Cl3 cells. In addition to many other cell functions, ERK is activated in response to cellular stress induced by DNA-damaging agents, such as UV [30], ionizing radiation [31], hydrogen peroxide [7], and *cis*Pt [32–34]. Thus, ERK cascade may mediate a physiological response to DNA damage such as induction of one or more DNA repair enzymes [35,36]. Consistent with a prosurvival function of ERK, we here provided evidence that the activation of ERK is important for the induction of *cis*Pt resistance also in thyroid cells. In fact, *cis*Pt treatment resulted in high and sustained activation of ERK, and by the use of strategies ending to the inhibition of ERK activity, an accentuated *cis*Pt-induced cell death was found. We show that the fully transformed and tumourigenic PC

E1Araf cells were consistently more resistant to the cytotoxic effect of *cis*Pt than normal and differentiated PC Cl3 cells. In both cell lines, the *cis*Pt-provoked activation of ERK depended upon the activity of MEK1, since its inhibitor, PD98059, markedly decreased both the phosphorylation of ERK1/2 and the cell survival. Similar enhanced cytotoxic effects of *cis*Pt, following inhibition of ERK pathway, have been described in human melanoma cell line C8161 [37], in the UCI 101 [10], and SK-OV-3 ovarian carcinoma cell lines [10]. Opposing effects of ERK pathways have been demonstrated in human melanoma cell line AA [37], in PC12 pheochromocytoma cells [4], and in HeLa endometrial carcinoma cell line [18,34]. PD98059 protects against *cis*Pt-induced cytotoxicity, partially by enhancing *cis*Pt-induced NF-κB activation [37,38]. This discrepancy indicates that the relationship between the activity of ERKs and the cellular response to *cis*Pt might depend on the individual cellular context and levels of stress.

It is known that the establishment of cross-linking *cis*Pt-DNA adducts involves at least two stages [39]. The initial formation of a mono-adduct is followed by a slower step, in which an intrastrand or interstrand cross-link is formed. The second reaction may require hours to complete. However, in the present study the phosphorylation of ERK induced by *cis*Pt was detectable in approximately 2 h, consistently with an activation occurring before the formation of DNA adduct. ERK phosphorylation remained elevated for a prolonged period of time (24 h after exposure).

With regard to upstream mediators implicated in the *cis*Pt-induced ERK activation, inhibition of PKCs could attenuate *cis*Pt-induced activation of ERK. The PKCs involved in the DNA damage-induced apoptosis are diverse in the two cell lines, and also different could be the mechanisms by which they are differentially activated. Results indicated that non calciumdependent PKCs are crucial elements in the pathway linking *cis*Pt to the ERK cascade inasmuch as inhibition (with GF109203X, but not with the conventional PKCs inhibitor Gö6976) of PKCs had significant effect on the *cis*Pt-evoked ERK phosphorylation. In PC E1Araf cells, the effect of cisPt on the phosphorylation of ERK was blocked by the cell-permeable myristoylated PKC-ζ pseudosubstrate peptide (ζ-PS), and by micromole concentrations of GF109203X, a molecule known to inhibit conventional and novel PKC isoforms in the nanomolar range, except atypical isozymes that require micromole concentrations [27]. This effect suggests the actions of *cis*Pt in PC E1Araf be mediated by atypical PKC-ζ isoform; noteworthy, the basal phosphorylation state of ERK also appears to be due, to some extent, to the activity of such an isoform since ζ-PS had a modest, though significant, effect. In normal PC Cl3, cells the GF109203X concentration required to completely block the effect of *cis*Pt on ERK was much lower, and ζ-PS had no effect. Accordingly, it would be reasonable to assume that whilst in PC Cl3 cells novel PKC isoforms (only -δ and -ε isozymes are present in these cell lines) have a role in the upstream regulation of ERK, in tumourigenic PC E1araf cells atypical PKC-ζ is instead responsible for the *cis*Pt-induced ERK activation. Consistent with these data, activation of PKC-ζ has been associated with cell survival [40–42]. It is also known that most apoptotic stimuli, including *cis*Pt, induce proteolytic activation of PKC-δ [14,43,44]. Activation of caspase-3 during apoptosis cleaves the full-length PKC-δ at the hinge region and separates the inhibitory regulatory domain from the catalytic domain. This happens fast in PC Cl3 cells and only later on in PC E1Araf cells where after 48 h of *cis*Pt treatment the mortality reaches the 45%, and both caspase-3 and PKC-δ appeared activated. Regarding PKC-ζ, no proteolisis was detectable, but it is known that it is subjected to modulation by protein regulators and physically interacts with Ras [45,46]. Recent evidence indicates that the activation of the MAPK pathway by PKCs involves Raf activation [16], whereas the PKC-ζ actions are Raf-independent but mediated by MEK [16]. In other words, PKC-ζ may constitute a pathway parallel to Raf for ERK activation.

In conclusion, this study showed that *cis*Pt differentially activated ERK cascade, which is important for maintaining the cell vitality after *cis*Pt treatment in both PC Cl3 sensitive and PC E1Araf resistant cells. *cis*Pt brings about a signalling pathway mediated by PKC-δ in normal PC Cl3 cells and by atypical PKC-ζ in tumoral PC E1Araf cells. Thyroid cell lines may represent a helpful model in the investigation of the mechanisms by which ERK and other signal transduction pathways modulate the response to *cis*Pt and promote cell survival in response to *cis*Pt treatment. Finally, a better understanding of the signal transduction pathways that modulate the *cis*Pt response may be beneficial in the development of novel therapeutic approaches for improvement of *cis*Pt efficacy and of *cis*Pt resistance.

Acknowledgment

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References

[1] A. Eastman, Activation of programmed cell death by anticancer agents: cisplatin as a model system, Cancer Cells 2 (1990) 275–280.
[2] B. Koberle, J.R. Masters, J.A. Hartley, R.D. Wood, Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours, Curr. Biol. 9 (1999) 273–276.
[3] X. Yang, F. Zheng, H. Xing, Q. Gao, W. Wei, Y. Lu, S. Wang, J. Zhou, W. Hu, D. Ma, Resistance to chemotherapy-induced apoptosis via decreased caspase-3 activity and overexpression of antiapoptotic proteins in ovarian cancer, J. Cancer Res. Clin. Oncol. 130 (2004) 423–428.
[4] Z. Xia, M. Dickens, J. Raingeaud, R.J. Davis, M.E. Greenberg, Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis, Science 270 (1995) 1326–1331.
[5] B. Brenner, U. Koppenhoefer, C. Weinstock, O. Linderkamp, F. Lang, E. Gulbins, Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153, J. Biol. Chem. 272 (1997) 22173–22181.

[6] X. Wang, J.L. Martindale, Y. Liu, N.J. Holbrook, The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival, Biochem. J. 333 (1998) 291–300.
[7] K.Z. Guyton, Y. Liu, M. Gorospe, Q. Xu, N.J. Holbrook, Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury, J. Biol. Chem. 271 (1996) 4138–4142.
[8] R. Aikawa, I. Komuro, T. Yamazaki, Y. Zou, S. Kudoh, M. Tanaka, I. Shiojima, Y. Hiroi, Y. Yazaki, Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats, J. Clin. Invest. 100 (1997) 1813–1821.
[9] J. Hayakawa, M. Ohmichi, H. Kurachi, H. Ikegami, A. Kimura, T. Matsuoka, et al., Inhibition of extracellular signal-regulated protein kinase or c-Jun N-terminal protein kinase cascade, differentially activated by cisplatin, sensitizes human ovarian cancer cell line, J. Biol. Chem. 274 (1999) 31648–31654.
[10] D.L. Persons, E.M. Yazlovitskaya, W. Cui, J.C. Pelling, Cisplatin-induced activation of mitogen-activated protein kinases in ovarian carcinoma cells: inhibition of extracellular signal-regulated kinase activity increases sensitivity to cisplatin, Clin. Cancer Res. 5 (1999) 1007–1014.
[11] X.S. Gao, J. Asaumi, S. Kawasaki, K. Nishikawa, M. Kuroda, Y. Takeda, Y. Hiraki, M. Ihara, T. Ohnishi, Sensitivity of anticancer drugs in NIH3T3 cells transfected with oncogenes accompanied by pSV2neo vector, Anticancer Res. 15 (1995) 1911–1914.
[12] T. Fokstuen, Y.B. Rabo, J.N. Zhou, J. Karlson, A. Platz, M.C. Shoshan, J. Hansson, S. Linder, The Ras farnesylation inhibitor BZA-5B increases the resistance to cisplatin in a human melanoma cell line, Anticancer Res. 17 (1997) 2347–2352.
[13] A. Basu, Involvement of PKC-δ in DNA damage-induced apoptosis, J. Cell Mol. Med. 7 (2003) 341–350.
[14] Y. Emoto, Y. Manome, G. Meinhardt, H. Kisaki, S. Kharbanda, M. Robertson, T. Ghayur, W.W. Wong, R. Kamen, R. Weichselbaum, D. Kufe, Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells, EMBO J. 14 (1995) 6148–6156.
[15] Y. Ueda, S. Hirai, S. Osada, A. Suzuki, K. Mizuno, S. Ohno, Protein kinase C d activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf, J. Biol. Chem. 271 (1996) 23512–23519.
[16] D.C. Schönwasser, R.M. Marais, C.J. Marshall, P.J. Parker, Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes, Mol. Cell. Biol. 18 (1998) 790–798.
[17] J.A. Clark, A.R. Black, O.V. Leontieva, M.R. Frey, M.A. Pysz, L. Kunneva, A. Woloszynska-Read, D. Roy, J.D. Black, Involvement of the ERK signaling cascade in protein kinase C-mediated cell cycle arrest in intestinal epithelial cells, J. Biol. Chem. 279 (2004) 9233–9247.
[18] A. Basu, H. Tu, Activation of ERK during DNA damage-induced apoptosis involves protein kinase C-δ, Biochem. Biophys. Res. Commun. 334 (2005) 1068–11073.
[19] G. Wallin, G. Lundell, J. Tennvall, Anaplastic giant cell thyroid carcinoma, Scand. J. Surg. 93 (2004) 272–277.
[20] W. Voigt, A. Bulankin, T. Muller, C. Schoeber, A. Grothey, C. Hoang-Vu, H.J. Schmoll, Schedule-dependent antagonism of gemcitabine and cisplatin in human anaplastic thyroid cancer cell lines, Clin. Cancer Res. 6 (2000) 2087–2093.
[21] A. Fusco, M.T. Berlingieri, P.P. Di Fiore, G. Portella, M. Grieco, G. Vecchio, One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes, Mol. Cell. Biol. 7 (1987) 3365–3370.
[22] M.T. Berlingieri, M. Santoro, C. Battaglia, M. Grieco, A. Fusco, The adenovirus E1A gene blocks the differentiation of a thyroid epithelial cell line, however the neoplastic phenotype is achieved only after cooperation with other oncogenes, Oncogene 8 (1993) 249–255.
[23] T. Mosmann, Rapid colourimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay, J. Immunol. Methods 65 (1983) 55–63.
[24] A. Muscella, S. Greco, M.G. Elia, C. Storelli, S. Marsigliante, Angiotensin II stimulation of Na+/K+ATPase activity and cell growth by calcium-independent pathway in MCF-7 breast cancer cells, J. Endocrinol. 173 (2002) 315–323.
[25] D.R. Alessi, A. Cuenda, P. Cohen, D.T. Dudley, A.R. Saltiel, PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo, J. Biol. Chem. 270 (1995) 27489–27494.
[26] S. Marsigliante, A. Muscella, M.G. Elia, S. Greco, C. Storelli, Angiotensin II AT1 receptor stimulates Na^+/K^+ATPase activity through a pathway involving PKC-zeta in rat thyroid cells, J. Physiol. 546 (2003) 461–470.
[27] G. Martiny-Baron, M.G. Kazanietz, H. Mischak, P.M. Blumberg, G. Kochs, H. Hug, D. Marme, C. Schachtele, Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö6976, J. Biol. Chem. 268 (1993) 9194–9197.
[28] M.L. Standaert, L. Galloway, P. Karnam, G. Bandyopadhyay, J. Moscat, R.V. Farese, Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport, J. Biol. Chem. 272 (1997) 30075–30082.
[29] M.P. Sajan, M.L. Standaert, G. Bandyopadhyay, M.J. Quon, T.R. Burke, R.V. Farese, PKC-ζ and phosphoinositide-dependent protein kinase-1 are required for insulin-induced activation of ERK in rat adipocytes, J. Biol. Chem. 274 (1999) 30495–30500.
[30] K. Merienne, S. Jacquot, M. Zeniou, S. Pannetier, P. Sassone-Corsi, A. Hanauer, Activation of RSK by UV-light: phosphorylation dynamics and involvement of the MAPK pathway, Oncogene 19 (2000) 4221–4229.
[31] T. Wang, Y.C. Hu, S. Dong, M. Fan, D. Tamae, M. Ozeki, Q. Gao, D. Gius, J.J. Li, Co-activation of ERK, NF-kappaB, and GADD45beta in response to ionizing radiation, J. Biol. Chem. 280 (2005) 12593–12601.
[32] W. Cui, E.M. Yazlovitskaya, M.S. Mayo, J.C. Pellings, E.L. Persons, Cisplatin-induced response of c-jun Nterminal kinase 1 and extracellular signal-regulated protein kinases 1 and 2 in a series of cisplatin-resistant ovarian carcinoma cell lines, Mol. Carcinogen. 29 (2000) 219–228.
[33] D.L. Persons, E.M. Yazlovitskaya, J.C. Pelling, Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin, J. Biol. Chem. 275 (2000) 35778–35785.
[34] X. Wang, J.L. Martindale, N.J. Holbrook, Requirement for ERK activation in cisplatin-induced apoptosis, J. Biol. Chem. 275 (2000) 39435–39443.
[35] J.F. Sweeney, P.K. Nguyen, G. Omann, D.B. Hinshaw, Granulocyte-macrophage colony-stimulating factor rescues human polymorphonuclear leukocytes from ultraviolet irradiation-accelerated apoptosis, J. Surg. Res. 81 (1999) 108–112.
[36] D. Upadhyay, V. Panduri, D.W. Kamp, Fibroblast growth factor-10 prevents asbestos-induced alveolar epithelial cell apoptosis by a mitogen-activated protein kinase-dependent mechanism, Am. J. Respir. Cell. Mol. Biol. 32 (2005) 232–238.
[37] A. Mandic, K. Viktorsson, T. Heiden, J. Hansson, M.C. Shoshan, The MEK1 inhibitor PD98059 sensitizes C8161 melanoma cells to cisplatin-induced apoptosis, Melanoma Res. 11 (2001) 11–19.
[38] P.Y. Yeh, S.E. Chuang, K.H. Yeh, Y.C. Song, C.K. Ea, A.L. Cheng, Increase of the resistance of human cervical carcinoma cells to cisplatin by inhibition of the MEK to ERK signaling pathway partly via enhancement of anticancer drug-induced NF-κB activation, Biochem. Pharmacol. 63 (2002) 1423–1430.
[39] K.C. Micetich, D. Barnes, L.C. Erickson, A comparative study of the cytotoxicity and DNA damaging effects of cis-(diammino)(1,1 cyclobutanedicarboxylato)-platinum (II), Cancer Res. 45 (1985) 4043–4047.
[40] L. Smith, L. Chen, M.E. Reyland, T.A. DeVries, R.V. Talanian, S. Omura, J.B. Smith, Activation of atypical protein kinase C zeta by

caspase processing and degradation by the ubiquitin-proteasome system, J. Biol. Chem. 275 (2000) 40620–40627.
[41] C. Bezombes, A. de Thonel, A. Apostolou, T. Louat, J.P. Jaffrezou, G. Laurent, A. Quillet-Mary, Overexpression of protein kinase C zeta confers protection against antileukemic drugs by inhibiting the redox-dependent sphingomyelinase activation, Mol. Pharmacol. 62 (2002) 1446–1455.
[42] G. Wang, J. Silva, K. Krishnamurthy, E. Tran, B.G. Condie, E. Bieberich, Direct binding to ceramide activates protein kinase C-ζ before the formation of a pro-apoptotic complex with PAR-4 in differentiating stem cells, J. Biol. Chem. 280 (2005) 26415–26424.
[43] P.K. Majumder, P. Pandey, X. Sun, K. Cheng, R. Datta, S. Saxena, S. Kharbanda, D. Kufe, Mitochondrial translocation of protein kinase C delta in phorbol ester-induced cytochrome c release and apoptosis, J. Biol. Chem. 275 (2000) 21793–21796.
[44] M. Blass, I. Kronfeld, G. Kazimirsky, P.M. Blumberg, C. Brodie, Tyrosine phosphorylation of protein kinase C delta is essential for its apoptotic effect in response to etoposide, Mol. Cell. Biol. 22 (2002) 182–195.
[45] M.T. Diaz-Meco, J. Lozano, M.M. Municio, E. Berra, S. Frutos, L. Sanz, J. Moscat, Evidence for the in vitro and in vivo interaction of Ras with protein kinase C zeta, J. Biol. Chem. 269 (1994) 31706–31710.
[46] M.W. Wooten, M.L. Seibenhener, L.H. Matthews, G. Zhou, E.S. Coleman, Modulation of zeta-protein kinase C by cyclic AMP in PC12 cells occurs through phosphorylation by protein kinase A, J. Neurochem. 67 (1996) 1023–1031.