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Differential response of normal, dedifferentiated and transformed thyroid cell lines to cisplatin treatment

Antonella Muscella [a], *Loredana Urso* [b], *Nadia Calabriso* [d], *Antonella Ciccarese* [c], *Danilo Migoni* [c], *Francesco Paolo Fanizzi* [c], *Bruno Di Jeso* [a], *Carlo Storelli* [b], *Santo Marsigliante* [b,*]

[a] *General Pathology Laboratory, Dipartimento di Scienze e Tecnologie Biologiche e Ambientali (Di.S.Te.B.A.), Università di Lecce, Italy*
[b] *Cell Physiology Laboratory, Dipartimento di Scienze e Tecnologie Biologiche e Ambientali (Di.S.Te.B.A.), Università di Lecce, Italy*
[c] *General and Inorganic Chemistry Laboratory, Dipartimento di Scienze e Tecnologie Biologiche e Ambientali (Di.S.Te.B.A.), Università di Lecce, Italy*
[d] *C.I.R.C.M.S.B. Unità di Ricerca di Lecce*

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ABSTRACT

The effects of cisplatin (*cis*Pt) on the extra cellular signal-regulated kinase (ERK) and the protein kinase B (PKB/Akt), known to play important roles in promoting cell survival and in down regulating apoptosis, were investigated in thyroid cell lines. The cytotoxic effect of *cis*Pt was highest in normal PC-Cl3 cells, intermediate in dedifferentiated PC-E1A and PC-raf cells and lowest in fully transformed and tumorigenic PC-E1Araf cells. *Cis*Pt provoked ERK phosphorylation; such phosphorylation was unaltered by Gö6976, a conventional PKC inhibitor, whilst 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, but not in PC-Cl3 cells, the *cis*Pt-provoked ERK phosphorylation was also blocked by a myristoylated PKC-ζ pseudo substrate peptide (PS-ζ). The cytotoxic effects of *cis*Pt increased when cells were pre-incubated with the mitogen-activated protein kinase (MEK) inhibitor PD98059. *Cis*Pt provoked the phosphorylation of PKB/Akt and this effect was blocked by LY294002, a PI3K inhibitor. In PC-Cl3 cells pre-incubated with LY294002 the effects of *cis*Pt on ERK phosphorylation and cell mortality resulted unaffected; conversely, LY294002 reduced the ERK phosphorylation and increased *cis*Pt cytotoxity of in PC-E1Araf cells. Furthermore, in PC-E1Araf cells pre-incubated with LY294002 and PS-ζ ERK phosphorylation was abolished and *cis*Pt cytotoxicity was highest. Altogether results highlight a role for PKCs in the upstream regulation of ERK pathway facing the cell response to *cis*Pt treatments. Understanding the mechanisms by which cells process *cis*Pt provides important insights for designing more efficient platinum-based drugs.

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum; *cis*Pt) is a potent inducer of growth arrest and/or apoptosis in most cell types and is among the most effective and widely used chemotherapeutic agents employed for treatment of human cancers. A major limitation of *cis*Pt chemotherapy is serious drug resistance. Multiple mechanisms have been implicated in the development of *cis*Pt resistance including reduced accumulation of the drug, increased levels of glutathione (GSH), enhanced expression of metallothionein, enhanced DNA repair, increased levels of Bcl-2-related anti-apoptotic genes,

* *Corresponding author*. Tel.: +39 0832 298 711; fax: +39 0832 324 220.
E-mail address: santo.marsigliante@unile.it (S. Marsigliante).
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and alterations in signal transduction pathways involved in apoptosis [1–3]. Apoptosis induced by *cis*Pt is generally considered to be the result of its ability to damage DNA [4], but the detailed mechanisms by which such DNA damage triggers cell death remain unclear. Understanding the molecular basis of *cis*Pt-mediated apoptosis could lead to strategies resulting in improved therapeutic benefits.

*Cis*Pt has been shown to induce activation of Ras and its downstream effector kinases, Raf/MEK/ERK [5]: 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 [6–8]. ERK is activated by some conditions of stress, particularly oxidant injury, and in such circumstances is believed to confer a survival advantage to cells [8–10]. 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 [11,12]. However, elevated expression of Ras, an upstream component of the ERK signaling pathway, has been connected with enhanced sensitivity to *cis*Pt [13,14]. Continued investigation into the mechanism by which the ERK pathway and other signal transduction pathways modulate the response to *cis*Pt may be helpful in the development of new strategies for improving the therapeutic use of platinum drugs.

Current chemotherapy for anaplastic thyroid carcinoma (ATC) is based primarily on doxorubicin [15] and *cis*Pt [16]. 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 [17,18], 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 [17,18]. These cells have been successfully employed to reproduce in vitro a multi-step model of cancerogenesis and to dissect the sensitivity to neoplastic transformation of the differentiation program of thyroid cells [17,18].

In this study, we sought to determine whether ERK plays a role in the cellular stress response to the chemotherapeutic agent *cis*Pt in normal PC-Cl3, dedifferentiated PC-E1A and PC-raf and in completely transformed PC-E1Araf cells, transformed, respectively, by the adenovirus E1A gene, the *raf* oncogene, or by a combination of these oncogenes [17,18].

2. Materials and methods

2.1. Reagents

Glutamine, gentamicin, the MEK inhibitor PD098059, the PI3K inhibitors LY294002 and wortmannin, the PKC inhibitors GF109203X and Gö6976 were obtained from Sigma Chemical Co. (Milan, Italy). PKC isoforms, phospho-specific ERK1 and ERK2 antibody, goat anti-rabbit IgG conjugated with peroxidase, as well as control antibodies, were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The phospho-specific Akt antibody and myristoylated PKC-ζ pseudo substrate peptide (Myr-SIYRRGARRWRKL) was obtained from Calbiochem-Novabiochem (Schwalbach, Germany).

2.2. 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) [17,18]. PC-E1A, PC-raf and PC-E1Araf are PC-Cl3 cells transformed, respectively, by the adenovirus E1A gene, the *raf* oncogene, or by a combination of these oncogenes [17,18]. They were grown in the same medium as PC-Cl3 cells, but lacking the mixture of hormones and growth factors.

2.3. Cytotoxicity assay

Cells at 70–80% confluency were trypsinized (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 24–72 h. The cell number was determined using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide) assay, as described by Mosmann [19], with some modification as described previously [20].

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

2.4. Cell count

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

2.5. Apoptosis analysis

Cellular DNA was extracted from PC-Cl3 and PC-E1Araf cells using the DNeasy Tissue kit (Quiagen, Milan, Italy) according to the manufacturer's instructions. One microgram of DNA was electrophoresed on 1% agarose gel in Tris–borate at 30 V for approximately 4 h. DNA was stained with ethidium bromide visualised with UV and photographed.

2.6. 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 dry milk in buffered saline. PKC isozyme proteins were detected using antibodies specific for different PKC isoforms (Santa Cruz Biotechnology). 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. Phosphorylated Akt/PKB, was detected by a specific antibody (Calbiochem-Novabiochem), then the blots were stripped and reprobed with another antibody (Santa Cruz Biotechnology) which recognises both active and basal forms of the Akt proteins.

The proteins were detected with goat anti-rabbit IgG conjugated with peroxidase (Santa Cruz Biotechnology), using the ECL (Amersham Life Sciences Inc., Amerham, UK). The intensity of the bands was quantified by scanning densitometry using the NIH Image 1.62 software (NIH, USA).

3. Results

3.1. *Correlation of viable cell number with MTT test absorbance*

The conversion of MTT by cells was used as an indicator of cell number. This method measures the reduction of MTT by active mitochondria, which results in a colorimetric 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.

3.2. *Correlation between the level of phosphorylated ERK and the sensitivity to cisPt*

To investigate the correlation between the level of ERK activation and the sensitivity to *cis*Pt toxicity during neoplastic transformation, we employed normal and transformed PC-Cl3 thyroid cells presenting various degrees of malignancy. 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 higher in PC-raf, PC-E1A and 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 all cell lines (Fig. 1A).

One hundred micromolar *cis*Pt provoked a phosphorylation of ERK1/2 in all thyroid cell lines. Evaluation of viable cell number, determined after 24 h *cis*Pt treatment, revealed a strong correlation between the level of phosphorylated ERK and the *cis*Pt sensitivity ($p < 0.01$). Fig. 1B shows that after *cis*Pt exposure approximately 50% of PC-Cl3, 65% of PC-raf, 70% of PC-E1A and 86% of PC-E1Araf had survived, suggesting that neoplastic transformation desensitize cells to this drug.

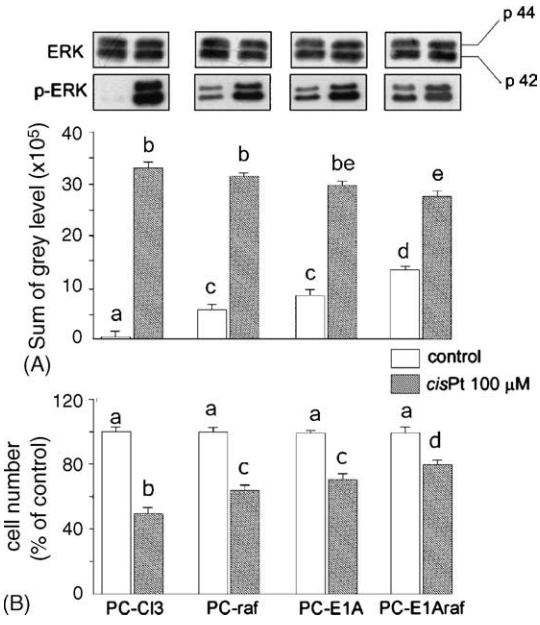

Fig. 1 – Relationship between the level of ERK activation and the sensitivity to *cis*Pt in thyroid cell lines. Thyroid cells were treated without or with 100 μM *cis*Pt for 24 h. (A) Cell lysates were electrophoretised through 10% SDS-PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 or the anti-total ERK antibody. Representative autoradiographs are shown and results from densitometry are expressed as mean ± S.D. ($n = 3$) of sum of the grey level values. (B) Viable cell numbers assessed by a MTT assay performed as described in Section 2. The data are means ± S.D. 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.

3.3. *The sensitivity of thyroid cells to cisPt*

Cells were treated with various concentrations of *cis*Pt, and viable cell number was determined 12, 24, 48 and 72 h later by MTT colorimetrical assay (Fig. 2). As shown in Fig. 2, the IC_{50} values (*cis*Pt concentration required for 50% growth inhibition) were highest in transformed PC-E1Araf and lowest in normal PC-Cl3 cells (53.3 and 5.2 μM, respectively, after 72 h of *cis*Pt exposure; $p < 0.001$). The IC_{50} for PC-raf and PC-E1A were 9.9 and 20.5, respectively (Fig. 2).

Fig. 2E shows that *cis*Pt induced more DNA fragmentation in PC-Cl3 cells compared with PC-E1Araf cells at all incubation times.

3.4. *The effects of cisPt on ERK1/2phosphorylation*

The dose–response and time course of ERK1/2 to *cis*Pt is illustrated in Fig. 3A and B. In PC-Cl3 cells, an increase in

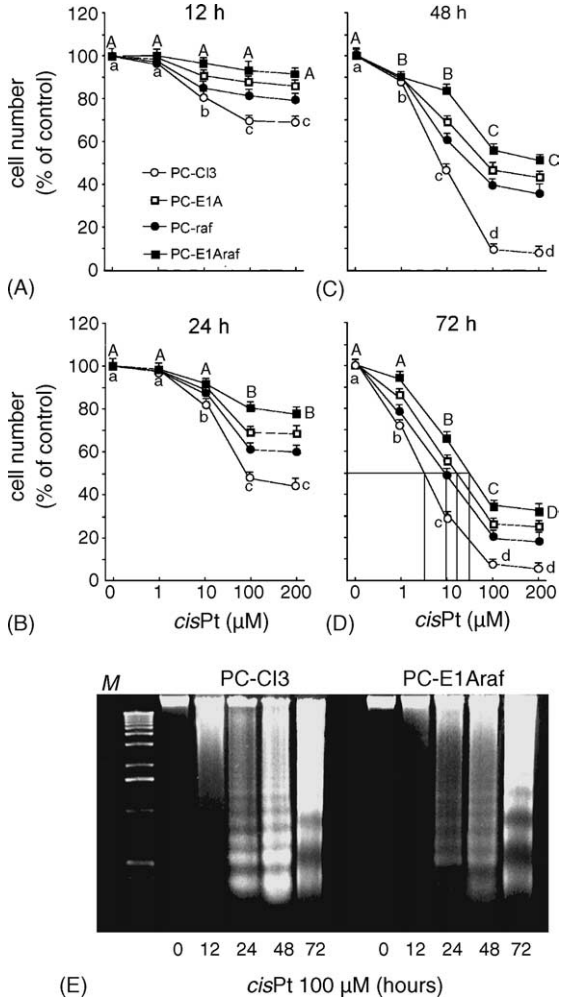

Fig. 2 – The sensitivity of thyroid cell lines to *cis*Pt. Cells were treated without or with various concentrations of *cis*Pt, and viable cell number was determined 12 h (A), 24 h (B), 48 h (C) and 72 h (D) later by MTT assay. The IC_{50} values (*cis*Pt concentration required for 50% growth inhibition) after 72 h *cis*Pt exposure are indicated. The data are means ± S.D. of four different experiments run in eight replicate and are presented as percent of control. For PC-Cl3 and PC-E1Araf cell lines only, values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests. (E) DNA fragmentation assay of PC-Cl3 and PC-E1Araf cells. Total DNA was isolated and separated on a 1% agarose gel. A 100-bp DNA size marker was used (*lane* M). A representative example of three independent experiments is shown.

phosphorylation of ERK1/2 (15-fold above basal level) was observed with 100 μM *cis*Pt (Fig. 3A, left panel). 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 (2.1-fold above basal levels) (Fig. 3A, right panel). Therefore, subsequent experiments were carried out using 100 μM *cis*Pt. The effect of 100 μM *cis*Pt on the phosphorylation state of ERK1/2 was time-dependent. There was a threshold increase at 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 (Fig. 3B for the effects in PC-Cl3 and PC-E1Araf cell lines). *Cis*Pt did not have effects on the total ERK1/2 levels in both cell lines (Fig. 3).

PD98059, a specific inhibitor of MAPK/ERK kinases 1 (MEK1) [21], 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, whilst significantly decreased it in PC-E1A and in PC-E1Araf cells (Fig. 4A). All thyroid cell lines pre-treated with PD98059 showed a dose-dependent inhibition of the *cis*Pt-induced phosphorylation of ERK1/2 (Fig. 4B).

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 (Fig. 4B) inasmuch as a significant decrease in cell survival after *cis*Pt treatment was observed in both cell lines (ANOVA: $p < 0.05$). At concentration of 30 μM PD98059, the percentage of surviving cells was about 30% in all cell lines, significantly less than in the absence of PD98059 (50% and 86% in PC-Cl3 and PC-E1Araf cells, respectively) (Fig. 4B). Thus, the cytotoxicity of *cis*Pt appeared to depend upon the phosphorylation state of ERK1/2.

Since the effects of *cis*Pt on ERK1/2 appeared similar in all cell lines and because differences in desensitization to *cis*Pt were highest between normal PC-Cl3 and fully transformed PC-E1Araf cells the subsequent experiments were performed on PC-Cl3 and PC-E1Araf cells only.

3.5. *The mechanism of cisPt-induced ERK phosphorylation*

The PKC isozymes expression in PC-Cl3 cells was previously determined [22]. PC-Cl3 expressed PKC-α, -β1, -δ, -ε, -ι and -ζ but not -β2 and -γ. PC-E1Araf cells expressed the same pattern of PKC isozymes (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).

Pre-treatment with Gö6976 (0.1, 1 and 10 μM), a conventional PKC inhibitor, did not have any effect on the phosphorylation of ERK1/2 provoked by *cis*Pt (Fig. 5A). GF109203X (0.1, 1 and 10 μM) was also used; when PC-Cl3 cells were pre-incubated for 30 min with GF109203X, the effects of *cis*Pt on ERK1/2 were completely inhibited at the lowest concentration used (Fig. 5C); conversely, in PC-E1Araf cells the complete inhibition of the *cis*Pt effects was obtained only at the highest concentration of 10 μM.

Ten micromolar GF109203X also completely inhibited the basal phosphorylation state of ERK1/2 in PC-E1Araf cells (Fig. 5C). Since GF109203X has a half-maximal inhibitory constant (IC_{50}) for atypical PKC isozymes greater than 5 μM whereas of 210 nM or lower for all the other PKC isoforms [23], these results suggest that different PKC isoforms could be involved in the cell response to *cis*Pt. Thus, both PC-Cl3 and PC-E1Araf cells were pre-incubated with 10, 50 and 100 μM myristoylated PKC-ζ pseudo substrate peptide (ζ-PS) for 60 min [24,25] and then with *cis*Pt. The cell-permeable ζ-PS inhibited the *cis*Pt-provoked ERK1/2 phosphorylation in PC-E1Araf cells, but not in PC-Cl3 cells (Fig. 6C and D). ζ-PS

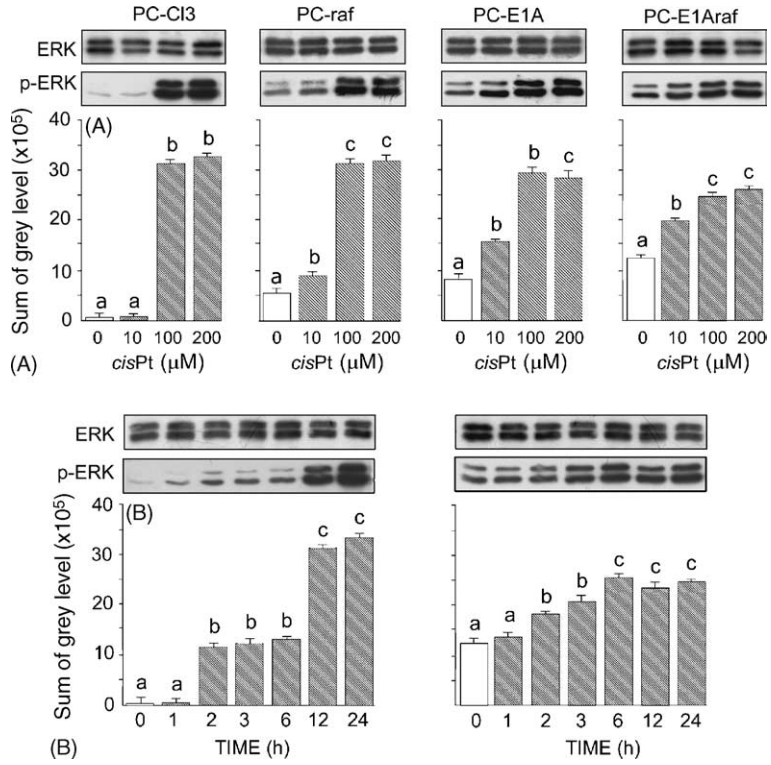

Fig. 3 – Dose- and time-dependent activation of ERK by *cis*Pt in thyroid cell lines. Thyroid cells were treated without or with various concentrations of *cis*Pt, for 24 h (A), or with 100 μM *cis*Pt, for the indicated times (B). Lysates from thyroid cell lines were electrophoretised through 10% SDS-PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 or the anti-total ERK antibody. Representative autoradiographs are shown and results from densitometry are expressed as mean ± S.D. ($n = 3$) of sum of the grey level values. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

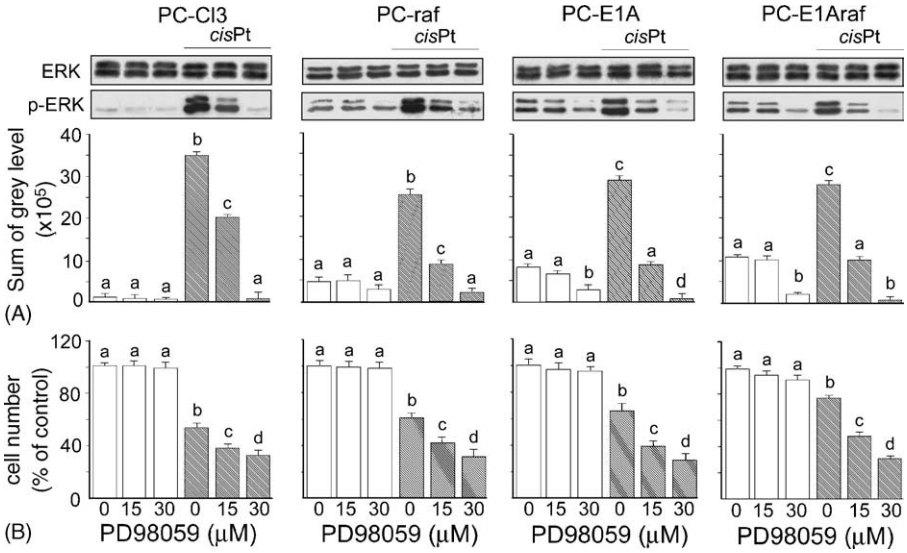

Fig. 4 – PD98059 sensitizes thyroid cell lines to *cis*Pt. Cells were pre-treated without or with various concentrations of PD98059 for 30 min and then without or with 100 μM *cis*Pt, for 24 h. (A) Lysates from thyroid cell lines were electrophoretised through 10% SDS-PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 or the anti-total ERK antibody. Representative autoradiographs are shown and results from densitometry are expressed as mean ± S.D. ($n = 3$) of sum of the grey level values. (B) Viable cell numbers assessed by a MTT assay as described in Section 2. The data are means ± S.D. 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.

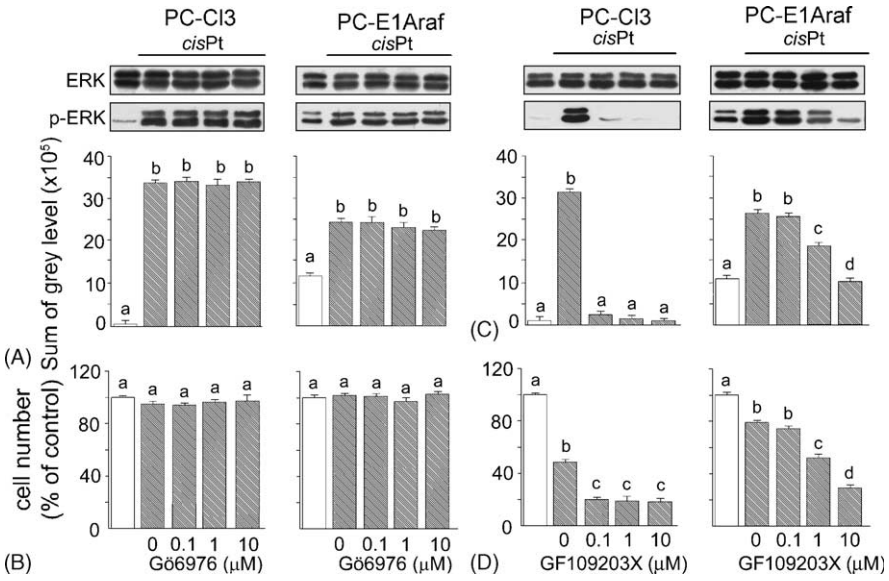

Fig. 5 – Role of PKC in *cis*Pt-induced ERK phosphorylation and in *cis*Pt cytotoxicity in thyroid cell lines. (A) PC-Cl3 and PC-E1Araf cells were pre-treated without or with various concentrations of Gö6976 for 30 min, and then without or with 100 μM *cis*Pt, for 24 h. (C) PC-Cl3 and PC-E1Araf cells were pre-treated without or with various concentrations of GF109203X for 30 min and then without or with to 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 or the anti-total ERK antibody. Representative autoradiographs are shown and results from densitometry are expressed as mean ± S.D. ($n = 3$) of sum of the grey level values. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests. (B and D) Viable cell numbers of cells pre-incubated with Gö6976 or with GF109203X as in (A) and (C) assessed by a MTT assay as described in Section 2. The data are means ± S.D. 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.

also inhibited the basal phosphorylation state of ERK1/2 in PC-E1Araf cells (Fig. 6B). On the whole these results suggest that in PC-Cl3 cells novel PKCs are required for the phosphorylation of ERK1/2 after *cis*Pt treatment, whilst in PC-E1Araf cells the operativity of atypical PKC-ζ is required.

Pre-incubation 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. 5D, left) 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. 5D, right). Gö6976 (0.1, 1 and 10 μM) did not have any effect on cells sensitivity to *cis*Pt (Fig. 5B).

3.6. *The role of PI3K/Akt in cisPt cytotoxicity in thyroid cell lines*

Western analysis performed with an antibody recognising the phosphorylated form of PKB/Akt showed that its basal phosphorylation state was higher in PC-Cl3 than in PC-E1Araf cells; after 24 h exposure to *cis*Pt the levels of PKB/Akt phosphorylation was significantly increased only in PC-Cl3 and PC-raf cells; conversely, *cis*Pt provoked a transient phosphorylation of PKB/Akt in PC-E1Araf cells, which was maximal at 2 h, and declined to the basal level at 3 h (Fig. 7A and C).

The pre-incubation of PC-Cl3 and PC-E1Araf cells with LY294002 (1–50 μM), a PI3K inhibitor, inhibited the phosphorylation of PKB/Akt and did not have any effect on the *cis*Pt-provoked ERK1/2 phosphorylation at 24 h in PC-Cl3 cells (Fig. 8A) suggesting that the ERK pathway activated by *cis*Pt was not related to the activity of PI3K. Conversely, in PC-E1Araf cells LY294002 reduced significantly both the *cis*Pt-stimulated and the basal level of phosphorylated ERK1/2 (Fig. 8B).

When cells were pre-incubated with LY294002 (1–50 μM) for 30 min and then exposed to *cis*Pt, the number of surviving PC-Cl3 cells did not change (Fig. 8C), whilst it decreased significantly in PC-E1Araf cells (Fig. 8D). When PC-E1Araf cells were pre-incubated with both LY294002 (10 and 50 μM) and ζ-PS (100 μM) for 60 min, the phosphorylated ERK1/2 forms were barely visible (Fig. 8B) and the sensitivity to *cis*Pt was drastically enhanced, with surviving cells less than 15% after 24 h (Fig. 8D). ζ-PS alone did not influence the effects of phoshorylation state of PKB/Akt (Fig. 8B); Fig. 8D also shows that the combination of LY294002 and ζ-PS had an additional effect on PC-E1Araf cell survival compared to ζ-PS alone.

4. Discussion

Recent data suggest that ERK is activated in response to cellular stress induced by DNA-damaging agents, including UV

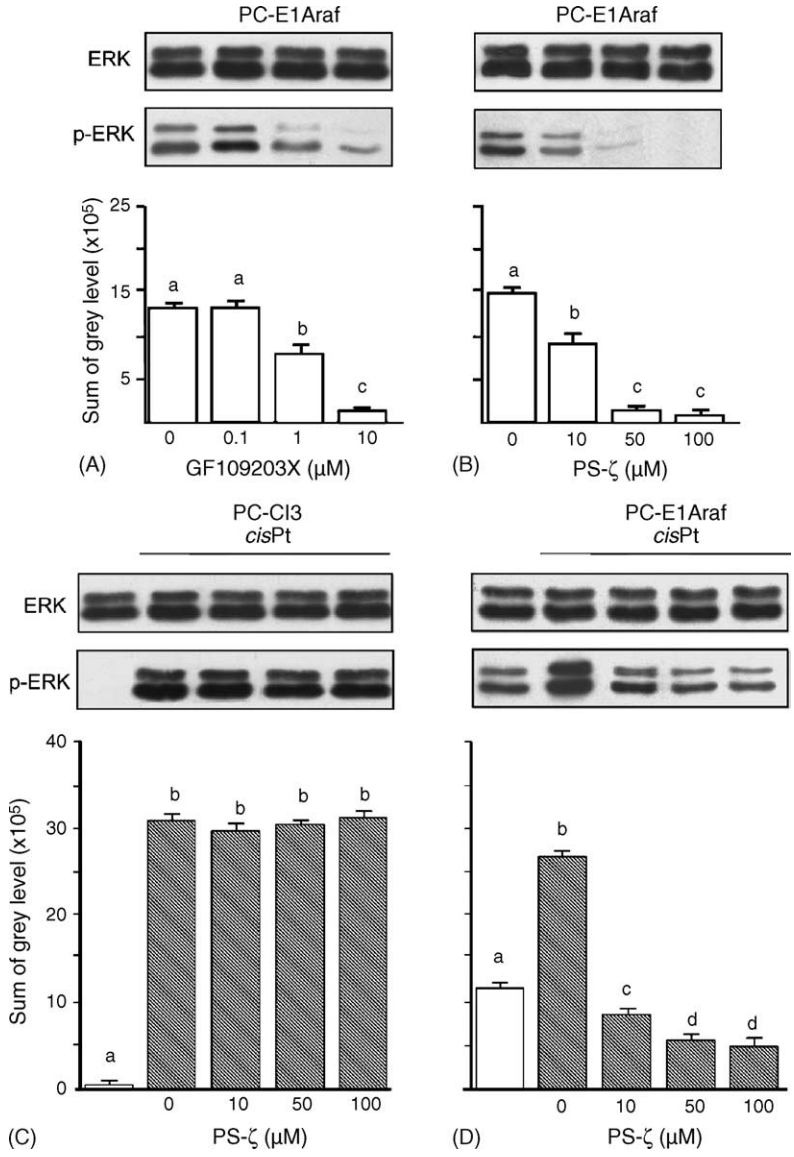

Fig. 6 – Role of PKC in both basal and *cis*Pt-induced ERK phosphorylation. PC-E1Araf cells were pre-treated without or with various concentrations of GF109203X for 30 min (A) or with myristoylated PKC-ζ pseudo substrate peptide (ζ-PS) for 1 h (B) without further incubation with *cis*Pt. PC-Cl3 (C) and PC-E1Araf cells (D) were pre-treated without or with various concentrations of myristoylated PKC-ζ pseudo substrate peptide (ζ-PS) 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 or the anti-total ERK antibody. Representative autoradiographs are shown and results from densitometry are expressed as mean ± S.D. ($n = 3$) of sum of the grey level values. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

[26], ionizing radiation [27], hydrogen peroxide [9] and *cis*Pt [28–30]. Thus, ERK cascade may mediate a physiological response to DNA damage, such as induction of one or more DNA repair enzymes [31,32]. In this study, we sought to determine whether ERK plays a role in the cellular stress response to *cis*Pt in a thyroid multi-step carcinogenesis system, which is made of cells transformed by the adenovirus E1A gene and the *raf* oncogene which display a basal higher phosphorylated ERK1/2 than parental normal PC-Cl3 cells. 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.

In this paper we show that the fully transformed and tumorigenic PC-E1Araf cells were consistently more resistant to the cytotoxic effect of *cis*Pt than dedifferentiated PC-raf and PC-E1A cell lines. Normal and differentiated PC-Cl3 cells were more sensible to the cytotoxic effects of *cis*Pt. The examination of the *cis*Pt resistance displayed by the different thyroid cell lines revealed a negative correlation between the level of basal ERK1/2 phosphorylation and *cis*Pt toxicity. In addition, in both

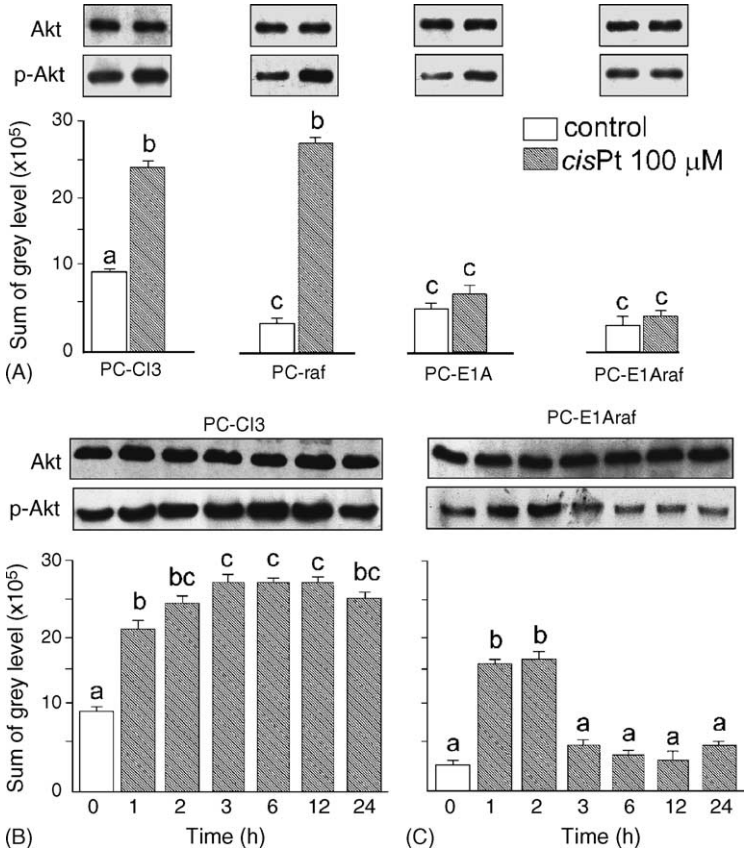

Fig. 7 – Basal phospho-Akt, and activation of Akt by *cis*Pt in thyroid cells. (A) Thyroid cells were treated without or with 100 μM *cis*Pt for 24 h. (B) PC-Cl3 and PC-E1Araf cells were treated without or with 100 μM *cis*Pt, for the indicated times. Lysates from PC-Cl3 (left) and PC-E1Araf (right) cells were electrophoretised through 10% SDS-PAGE and analysed by Western blotting using the antibody against the active (phosphorylated) Akt or the anti-total Akt antibody. Representative autoradiographs are shown and results from densitometry are expressed as mean ± S.D. ($n = 3$) of sum of the grey level values. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

thyroid 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 treatment with PD98059, have been described in various cell lines [12,33]. Opposing effects of ERK pathways have been demonstrated in human melanoma cell line AA [33] in PC12 pheochromocytoma cells [6] and in HeLa endometrial carcinoma cells [29]. PD98059 protects against *cis*Pt-induced cytotoxicity, partially by enhancing *cis*Pt induced NF-κB activation [33,34]. 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.

Multiple signalling pathways involving PKCs and PI3K seemed to lead to accentuated *cis*Pt sensitivity. In PC-Cl3 cells, PKCs are well known to stimulate ERK activity [22,35]; in the present report, results indicated that non-calcium-dependent PKCs are crucial elements in the pathway linking *cis*Pt to the ERK/MAPK cascade inasmuch as inhibition (with GF109203X, but not with the conventional PKCs inhibitor Gö6976) of PKCs had significant effects on the *cis*Pt-evoked ERK phosphorylation. In PC-E1Araf cells the effect of *cis*Pt on the phosphorylation of ERK was blocked by the cell-permeable myristoylated PKC-ζ pseudo substrate peptide (ζ-PS), and by μmol concentrations of GF109203X, a molecule known to inhibit conventional and novel PKC isoforms in the nM range, except atypical isozymes that require μmol concentrations [23]. 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 appear to be due to the activity of such isoform. In normal PC-Cl3 cells the GF109203X concentration required to completely block the effect of *cis*Pt on ERK is much lower, and ζ-PS has no effect. Accordingly, it would be reasonable to assume that whilst in PC-Cl3 cells novel PKC isoforms have a role in the upstream regulation of ERK, in tumorigenic PC-E1Araf cells atypical PKC-ζ is instead responsible for the *cis*Pt-induced ERK activation. Activation of PKC-ζ has been associated with cell survival [36,37]. In this regard, it is known that PKC-ζ is subjected to modulation by protein regulators and physically interacts with Ras [38,39]. Recent evidence indicates that the activation of the MAPK pathway by classical and novel PKCs involves Raf activation [40], whereas the PKC-ζ actions are Raf-independent but mediated by MEK [40]. In other words, PKC-ζ may constitute a pathway parallel to Raf for ERK activation.

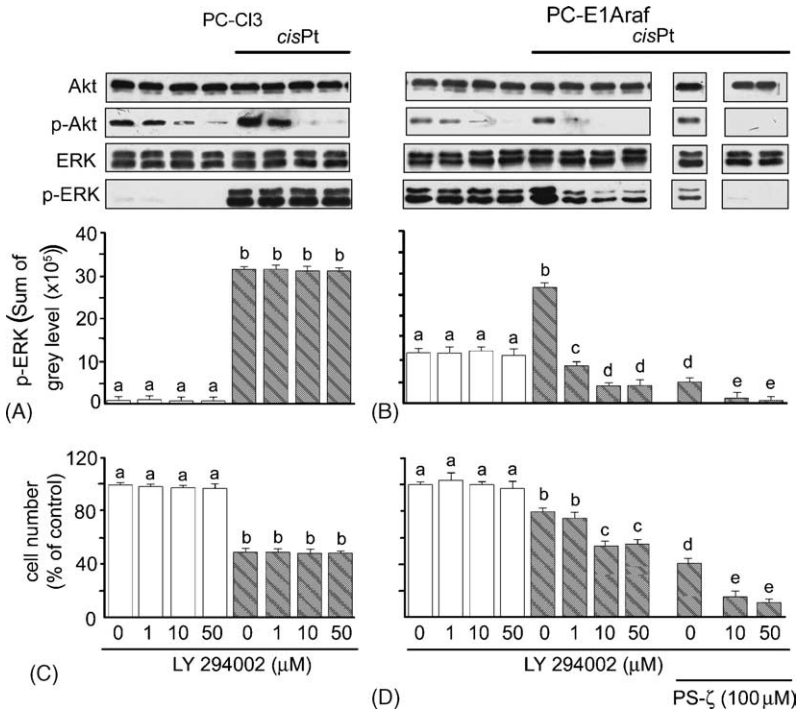

Fig. 8 – The role of PI3K/Akt in *cis*Pt-induced ERK phosphorylation and in *cis*Pt cytotoxicity in thyroid cell lines. (A) PC-Cl3 cells were pre-treated without or with various concentrations of the PI3K inhibitor LY294002 for 30 min and then without or with 100 μM *cis*Pt, for 24 h. (B) PC-E1Araf cells were pre-treated without or with various concentrations of the PI3K inhibitor LY294002 alone or with 100 μM myristoylated PKC-ζ pseudo substrate peptide (ζ-PS) together with LY294002 (10 or 50 μM) before treatment 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 ERK1/2 (or Akt) or the anti-total ERK antibody. Representative autoradiographs are shown and results from densitometry are expressed as mean ± S.D. ($n = 3$) of sum of the grey level values. (C and D) Viable cell numbers assessed by a MTT assay as described in Section 2 in experiments performed as in (A) and (B). The data are means ± S.D. 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.

Oncogenes, such as Raf, modulate the activity of PI3K and vice versa [41,42]. Active PI3K leads to recruitment to the plasma membrane of the 67-kDa ubiquitously expressed kinase PDK1 (PtdIns (3)P-dependent kinase 1) [43], that phosphorylates protein kinase B (PKB, also known as Akt [44]), a key serine/threonine kinase that mediates PI3K actions. Active PKB/Akt interferes with the apoptotic machinery and activate the transcription factor nuclear factor KB (NF-κB), leading to expression of anti-apoptotic genes, and the activation of PI3K/Akt pathway is associated with chemoresistance in human cancers [45]. We have found that Akt was constitutively phosphorylated in all thyroid cell lines and it was not correlated to *cis*Pt resistance in PC-Cl3 cells. Furthermore, cells transfected with E1A show a decrease in the basal phosphorylation status of PKB/Akt; in these cells *cis*Pt did not increase the phosphorylation of Akt, as previously reported in other cells [46]. The inhibition of PI3K by the use of LY294002 lead to the decrease of constitutively phosphorylated Akt, without affecting the phosphorylation of ERK, in PC-Cl3 but not in PC-E1Araf cells, where it was also noticed a significant decrement of the surviving cell number. In PC-E1Araf cells ERK phosphorylation and cell survival decreased greatly in the presence of both LY294002 and ζ-PS. These results suggest that PI3K/Akt signalling may play a role in *cis*Pt resistance in PC-E1Araf cells, and that it depends upon ERK phosphorylation and atypical PKC-ζ activity. In ovarian cancer cells exposed to *cis*Pt, the induced DNA damage provoked the phosphorylation of Bad (Bcl-2-associated death protein), which suppressed its apoptotic effect, via both ERK and PI3K/Akt cascades, and the inhibition of either of these pathways sensitized cells [47].

With this study we demonstrated that ERK cascade was differentially activated by *cis*Pt; ERK basal phosphorylation state also appeared to be important in maintaining the cell vitality after *cis*Pt treatment in thyroid cells. Moreover, *cis*Pt brings about a signalling pathway mediated by atypical PKC-ζ in tumoural PC-E1Araf cells and possibly by novel PKC isozymes in normal PC-Cl3 cells. The in vitro multi-step model of cancerogenesis used here appears to be 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. Hence, this multi-step model could also help to dissect the sensitivity of tumoural thyroid cells to novel therapeutic approaches.

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