Apoptosis by [Pt(O,O′-acac)(γ-acac)(DMS)] requires PKC-δ mediated p53 activation in malignant pleural mesothelioma

Antonella Muscella[1]*, **Carla Vetrugno**[1], **Luca Giulio Cossa**[2], **Giovanna Antonaci**[2], **Amilcare Barca**[3], **Sandra Angelica De Pascali**[4], **Francesco Paolo Fanizzi**[4], **Santo Marsigliante**[2]

1 Laboratory of Cell Pathology, Department of Biological and Environmental Sciences and Technologies (Di. S.Te.B.A.), University of Salento, Lecce, Italy, 2 Laboratory of Cell Physiology Di.S.Te.B.A., University of Salento, Lecce, Italy, 3 Laboratory of Physiology Di.S.Te.B.A., University of Salento, Lecce, Italy, 4 Laboratory of Inorganic Chemistry, Di.S.Te.B.A., University of Salento, Lecce, Italy

* antonella.muscella@unisalento.it

Abstract

Mesothelioma cancer cells have epithelioid or sarcomatoid morphology. The worst prognosis is associated with sarcomatoid phenotype and resistance to therapy is affected by cells heterogeneity. We recently showed that in ZL55 mesothelioma cell line of epithelioid origin [Pt(O,O′-acac)(γ-acac)(DMS)] (Ptac2S) has an antiproliferative effect *in vitro* and *in vivo*. Aim of this work was to extend the study on the effects of Ptac2S on ZL34 cell line, representative of sarcomatoid mesothelioma. ZL34 cells were used to assay the antitumor activity of Ptac2S in a mouse xenograft model *in vivo*. Then, both ZL34 and ZL55 cells were used in order to assess the involvement of p53 protein in (a) the processes underlying the sensitivity to chemotherapy and (b) the activation of various transduction proteins involved in apoptosis/survival processes. Ptac2S increases ZL34 cell death *in vivo* compared with cisplatin and, *in vitro*, Ptac2S was more efficacious than cisplatin in inducing apoptosis. In Ptac2S-treated ZL34 and ZL55 cells, p53 regulated gene products of apoptotic BAX and anti-apoptotic Bcl-2 proteins via transcriptional activation. Ptac2S activated PKC-δ and PKC-ε; their inhibition by PKC–siRNA decreased the apoptotic death of cells. PKC-δ was responsible for JNK1/2 activation that has a role in p53 activation. In addition, PKC-ε activation provoked phosphorylation of p38MAPK, concurring to apoptosis. In ZL34 cells, Ptac2S also activated PKC-α thus provoking ERK1/2 activation; inhibition of PKC-α, or ERK1/2, increased Ptac2S cytotoxicity. Results confirm that Ptac2S is a promising therapeutic agent for malignant mesothelioma, giving a substantial starting point for its further validation.

Introduction

The incidence of malignant pleural mesothelioma (MPM) is growing due to wide asbestos usage in various developing countries [1]. The most efficacious MPM treatment able to lengthen sufferers' life is the combination of pemetrexed or raltitrexed, multi-folate inhibitors

bromide; PK, pharmacokinetics; Ptac2S, [Pt(*O,O′*-acac)(γ-acac)(DMS)]; SRB, sulforhodamine B.

and cisplatin; nevertheless, the median survival is 12 months, with response rates of about 40% [2, 3]. Biologic agents targeting oncogenetic pathways, such as histone deacetylases, phosphatidylinositol 3-kinase /mammalian target of rapamycin, nuclear factor kB and neoangiogenesis have also been tested [4]. However, none of these treatments showed to impact significantly on this neoplasm; thus, there is an urgent need for new drugs. Histologically, MPM can be classified in the following three subtypes: epithelioid (50%), sarcomatoid (16%), and mixed type or biphasic (34%). Sarcomatoid mesotheliomas are characterized by aggressive biological behaviour, resistance to systemic treatments, more frequent distant spread and poor prognosis. Great care has been given on designing new platinum-based compounds having fewer toxicity and more favourable therapeutic indices than *cisplatin*. In regard to this, it was synthesized the Pt(II)-derived drug [Pt(*O,O′*-acac)(γ-acac)(DMS)] (Ptac2S) having non-genomic targets [5]. Ptac2S achieved increasing heed as potential anticancer drug as its high and selective cancer cell cytotoxicity observed in immortalized cell lines and in breast cancer cells in primary culture [6–10] and *in vivo* [11–13]. Notably, in a preclinical model made of hypodermic injection of breast cancer cells, Ptac2S shows up for an anticancer activity higher than cisplatin; in Wistar rats it as well showed increased pharmacokinetics, bio distribution and tolerability in comparison to cisplatin. Pharmacokinetics studies with Ptac2S uncovered lengthened systemic blood persistence of Pt and diminished nephrotoxicity and hepatotoxicity. In principle, this Pt-compound would yield a wider use, since Ptac2S also exerts specific antimetastatic responses in vitro [13–14]. As said, it seems notable to understand whether Ptac2S has also cytotoxic effects on MPM. Previously, we used the epithelioid ZL55 cells and showed that cisplatin provoked apoptosis together with the activation of PKC-α and ERK1/2 pro-survival pathways by the synthesis of ROS [15]. In the same ZL55 cells we also tested the effects of Ptac2S and observed a greater cytotoxicity than cisplatin. Ptac2S was able to activate different transduction pathways with strong pro-apoptotic activity (p38 and PKC-δ), while the PKC-α pro-survival pathway activated by cisplatin was not observed. Therefore, the higher cytotoxicity of Ptac2S in these cells may be due to the fact that it does not activate PKC-α [12]. In the current investigation, we assess the cytotoxicity of Ptac2S also on mesothelioma cells of sarcomatoid origin that are generally more aggressive and less susceptible to chemotherapy. Therefore, this study was conducted using the ZL34 cells both *in vitro* and *in vivo* with the technique of the xenograft on nude mice. Furthermore, we also looked for the differences between responses to Ptac2S and cisplatin and the molecular mechanisms that determine the ZL34 cell death/survival fate.

Materials and methods

Cell culture

The human mesothelioma cell lines ZL34 and ZL55 [15] were grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were maintained at 37˚C in the presence of 5% CO_2 in air. Cells were grown to 70–80% confluence and then treated with Pt-compounds at various concentrations and for different incubation periods.

In vivo xenograft experiments

Athymic nude mice (6 wks. old, female, 20 to 30 g body weight) were purchased from Harlan Laboratories (San Pietro al Natisone UD, Italy) and maintained under pathogen-free conditions. They were given free access to standard food and water, with a 12 h light-dark cycle at a temperature of 22+/−2˚C. Approximately 6×10^6 ZL34 cells (8 mice) were injected subcutaneously into the flank. Animals were monitored daily for general health and body weights were

measured twice weekly. Tumour size was measured with slide callipers and volumes were calculated as $(LxW^2)/2$, where L and W are the major and minor diameters, respectively. Once tumour volumes reached ~50 mm^3, mice were randomly divided into three groups and treated by a single intravenous of saline as a control, or 10 mg/kg of Ptac2S or 10 mg/kg cisplatin. The mice were sacrificed after 35 days of treatment and the tumours were excised. As described previously [11], all animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985), as well as in accordance with the Italian laws on animal experimentation (art. 4 and 5 of D.L. 116/92). Ethical Committee on Animal Research (Ministero della Salute D.M. 109/2014-B) approved the protocols. All efforts were made to minimize suffering to animals; thus, the experimental procedures used in the work described in this article were in compliance with the guidelines for reporting experiments involving animals [16].

Cytotoxicity assay

We evaluated the IC$_{50}$ in ZL34 cells with SRB and MTT assays. The SRB (sulforhodamine B) assay and the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide) by mesothelioma cells were used as indicator of cell number as described previously [7]. Viable cells were also counted by the trypan blue exclusion assay and light microscopy. The data presented are means ± standard deviation (S.D.) from eight replicate wells per microtitre plate.

Clonogenic survival assay

ZL34 cells were seeded in 100 mm Petri dishes at low density (~3X10^4 per dish) and left to adhere for 24 h in a standard medium. Crescent concentrations of Ptac2S or cisplatin were added and clonogenic survival assay was performed as described previously [8].

Preparation of subcellular fraction and western blots

Preparation of sub cellular fraction, western blotting analysis and immunodetection were performed as previously reported [17]. Western blotting and immunodetection analyses were performed as previously described [18].

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from ZL34 and ZL55 cells using an SV Total RNA isolation kit and performed according to the manufacturer's protocols (Promega, Madison, WI, USA) as previously described [8]. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. For each gene, relative expression was determined using the $2^{-\Delta\Delta CT}$ methods and normalized to β-actin expression.

Design and preparation of small interfering RNA (siRNA)

PKC-α, PKC-δ and PKC-ε siRNAs were prepared by an *in vitro* transcription method, according to the manufacturer's protocol (Promega, Madison, WI, USA) as previously described [8].

siRNA transfection

The cells (50–70% confluence) were transfected with siRNA duplexes using the protocol supplied with the CodeBreaker siRNA transfection reagent (Promega, Madison, WI, USA) as

described previously [15]. Quantitative analysis of protein expression, as measured by intensity of immunoreactivity in siRNA-transfected cells, revealed a very high reduction in PKC-α, PKC-δ and PKC-ε expression.

Statistical analysis

The experimenter measuring the tumours and the data analyst were unaware of the treatments given to the animals. Data, presented as means ± SD, were collected in blinded fashion and analysed using GRAPHPAD PRISM 5 software (GraphPad Software, La Jolla, CA, USA). Unpaired Student's t-test or one-way ANOVA, and when this returned P < 0.05, post hoc analysis using Bonferroni test, were performed; we used the Bonferroni-Dunn post hoc test in the ANOVA after a significant omnibus F-test. P < 0.05 was accepted as a level of statistical significance.

Materials. Ptac2S was prepared as previously reported [5, 19]. Cisplatin was purchased from Sigma (Milan, Italy). RPMI 1640 medium, antibiotics, glutamine and foetal bovine serum were purchased from Celbio (Milan, Italy). Caspase -9 and -7, BAX, PARP-1, phospho p38MAPK, phospho JNK1/2 antibodies were obtained from Cell Signalling (Celbio, Milan, Italy). PKC isoforms and phospho ERK1/2 antibodies, goat anti-rabbit conjugated with peroxidase, as well as control antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All others reagents were from Sigma (Milan, Italy).

Results

Anticancer activity of Ptac2S in a MPM preclinical model

ZL34 and ZL55 cell lines represent sarcomatoid and epithelioid MPM, respectively. The *in vivo* efficacy of Ptac2S in preclinical model of epithelioid MPM was already determined [12]. Here, sarcomatoid model was assessed by the hypodermic injection of ZL34 cells in the flank of BALB/c nude mice. When cancers reached the size of ~50 mm^3, in order to reduce weight and tumour size odds, mice were randomized in three groups. 10 mg/kg of Ptac2S was found before to be effective without notable side effects in animal studies with xenografts of human breast cancerous cells [11]. Hence, after that an only intravenous of saline (control) or 10 mg/kg of Ptac2S or 10 mg/kg of cisplatin was dispensed, tumour volumes were evaluated by vernier calliper every 3 days for 5 weeks. The mean volumes of the tumours in each group were assessed, and we drew the related tumour growth curves. Ptac2S displayed higher anticancer activity than cisplatin toward ZL34 tumours examined, inducing up to 50% growth inhibition. In mice inoculated with ZL34, during 5 weeks mean tumour volume augmented from 46.6 ±6.78 to 285.11±38.69 mm^3 for the saline group, to 251.87±49.36 mm^3 for the cisplatin group (10mg/kg; p>0.05) and to 133.72±41.22 mm^3 for the Ptac2S group (Fig 1). Mice displayed a significant decrease of tumour mass for each experimental time considered in the Ptac2S groups compared with both control and cisplatin-treated mice (p < 0.05). In addition, during observation time, no health problems were observed and the overall behaviour was similar to that of the control animals.

Cytotoxicity of Ptac2S

In vitro cytotoxicity data were achieved by MTT and validated by SRB assays to exclude consequences of Ptac2S on enzymes of mitochondria. Furthermore, similar results are attained when cell number is defined through their counting (data not shown); thus, SRB assay was used for all the experiments shown herein. A dose-dependent decrement of cell survival was obtained when MPM cells were incubated with cisplatin or Ptac2S (from 1 μM to 200 μM,
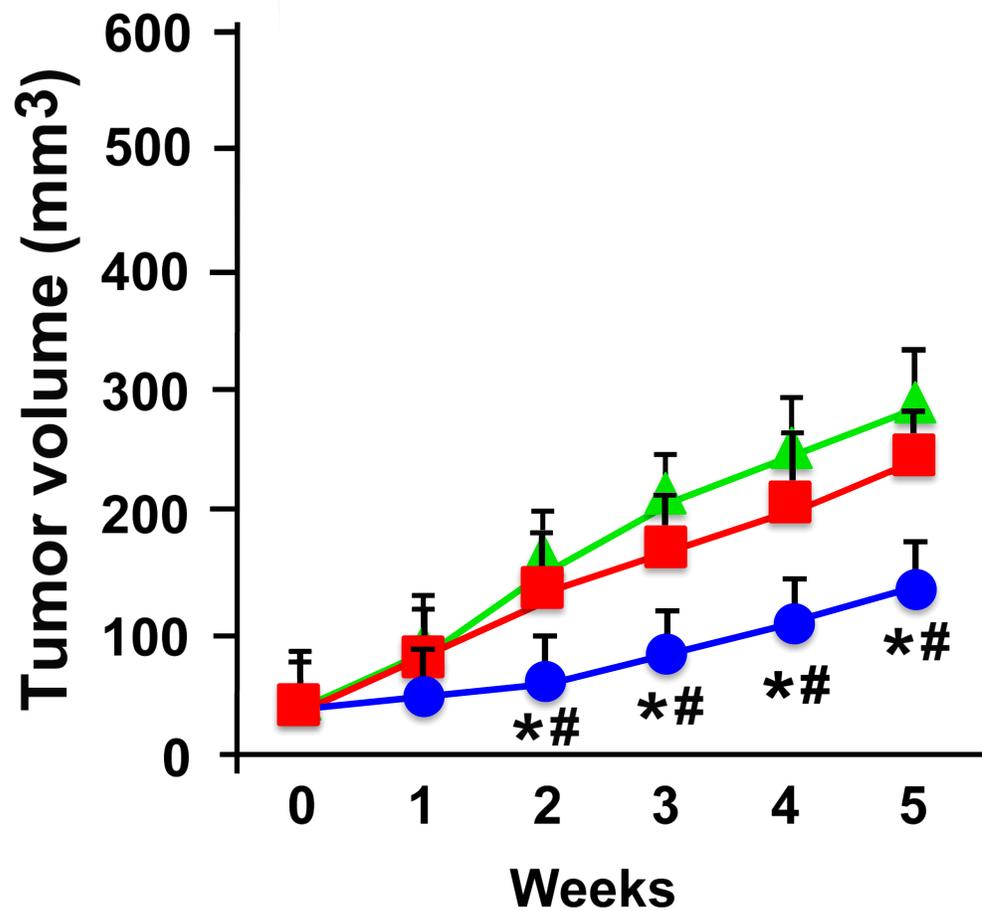
	Average tumor size (mm^3)	% of control
▲ Saline	285.11 ± 38.69	100
■ Cisplatin 10 mg/Kg	251.87 ± 49.36	88
● Ptac2S 10 mg/Kg	133.72 ± 41.22	47

Fig 1. Growth inhibitory effect of Ptac2S and cisplatin in a xenograft model of mesothelioma. Balb/c nude mice carrying sarcomatoid or epithelioid mesothelioma developed by injection of ZL34 (around 50 mm^3) received intravenous Ptac2S (10 mg/kg) or cisplatin (10 mg/kg). Tumour volume was measured every 3 days for a total of 35 days. Results are showed as mean ± S.D. (animals per group n = 8). *P < 0.05, significantly different from saline control; #P < 0.05, significantly different between Ptac2S and cisplatin. **(Table)** After killing, tumours were collected and measured.

https://doi.org/10.1371/journal.pone.0181114.g001
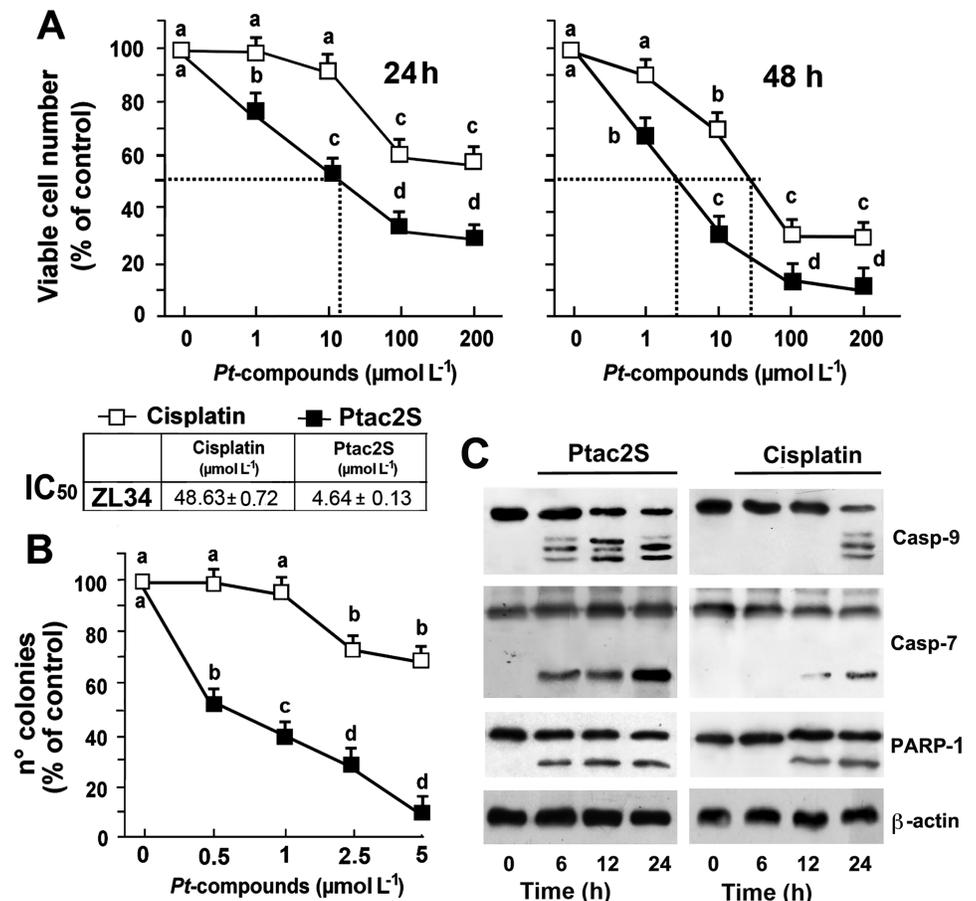
Fig 2. Sensitivity of MPM cells to Ptac2S and cisplatin. (A) ZL34 cells were treated or not with increasing concentrations of Ptac2S or cisplatin for 24 and 48 h, or continuously exposed to 50 μM cisplatin or 5 μM Ptac2S. Cell viability was obtained by SRB assay and data are means ± S.D. of 6 independent experiments with 8 replicates in each, and are presented as per cent of control. For both cisplatin and Ptac2S, P < 0.0001 by one-way ANOVA (n = 6); values with shared letters are not significantly different according to Bonferroni/ Dunn post hoc tests. **(B)** Clonogenic survival assay in ZL34 cells treated with the indicated amounts of Ptac2S or cisplatin for 2 h, and after 15 days of growth; only colonies consisting of more than 50 cells were scored. The percentage of number colonies represents the means ± S.D. of six-independent experiments. For cisplatin and Ptac2S, P < 0.001 and P<0.0001 by one-way ANOVA (n = 6), respectively; values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests. **(C)** Cytosolic and nuclear proteins were obtained from ZL34 cells treated or not with 5 μM Ptac2S or 50 μM cisplatin. Samples were dissolved in SDS buffer and separated on SDS gel. Immunoblotting was performed using monoclonal antibodies specific to PARP (from nuclear fractions) and to caspases-9, and -7 (cytosolic fractions). Sequential incubation with anti-β-actin confirmed the equal protein loading. Figures are representative of 6 independent experiments. **Inset**: The IC_{50} values to cisplatin and Ptac2S calculated after 48 h.

Fig 2A). In sarcomatoid cells cisplatin was significantly less cytotoxic than Ptac2S (IC_{50} 4.64 ±0.13 and 48.63±0.72 μM n = 6, for Ptac2S and cisplatin, respectively; p<0.001, see table in Fig 2). Clonogenic assay performed on ZL34 showed that Ptac2S was more cytotoxic than cisplatin (Fig 2B).

Ptac2S causes caspases proteolysis

Fig 2C shows western blotting of caspase-9 and -7 activation and proteolysis of PARP in ZL34 and ZL55 cells. PARP was cleaved in cells treated with 5 μM Ptac2S or with 50 μM cisplatin;

however, proteolysis was faster with Ptac2S. Subsequent incubation of blots with an antibody against β-actin validated that loaded protein amount was the same.

Ptac2S induces p53 activation

Since drugs may stabilize p53, that is not mutated in many MPM specimens [20], we assessed Ptac2S effects on p53 and its related genes Bcl-2 and BAX. Ptac2S treatment increased p53 protein levels (Fig 3A), increased BAX and decreased Bcl-2 proteins (Fig 3A) in both ZL34 and ZL55 cells. By RT-PCR we found that Ptac2S up-regulated p53 and BAX mRNA expression, and decreased Bcl-2 mRNA in a time-dependent way (ANOVA p < 0.01, Fig 3B, 3C and 3D). We next used an inhibitor of p53 transcriptional targets, PFT-α [21]. Fig 4 shows that 30 μM PFT-α inhibited p53 and BAX up-regulation and Bcl-2 down-regulation due to Ptac2S in both cell lines, indicating that apoptosis induced by Ptac2S was mediated by p53 and BAX.

Ptac2S-induces MAPKs phosphorylation

We demonstrated previously that Ptac2S activates the MAPKs signalling pathways in several tumour cell lines [6, 8, 9, 22], ZL55 cells included [12] and that cisplatin activates ERK1/2 in ZL55 cells [15]. We showed that whereas Ptac2S activated all three MAPKs in ZL34 cells, in ZL55 cells it activated p38MAPK and JNK1/2 but not ERK1/2 (Fig 4A and ref. [12]). Pre-incubation with JNK1/2 inhibitor SP600125, or with the p38MAPK inhibitor SB203580, significantly reduced Ptac2S-induced cytotoxicity in both cell lines (Fig 4). Furthermore, SP600125 markedly inhibited Ptac2S-induced activation of p53 (Fig 4), thus suggesting that JNK1/2 mediates p53 induction. PD98059, an inhibitor of MEK1/2, the ERK1/2 upstream kinase, significantly increased Ptac2S-induced cytotoxicity, in ZL34 cells (Fig 4). Jointly these results indicated that JNK1/2 and p38MAPK are pro-apoptotic pathways whereas ERK1/2, in ZL34 cells, behaves like an anti-apoptotic survival pathway.

Role of PKCs in Ptac2S-induced apoptosis in MPM cells

As the cellular effects of Ptac2S go together with the activation of various PKC isoforms, we here have studied their activation. In the previous study [12] in ZL55 cells, we showed that PKC-ε and PKC-δ translocated from the cytosol to the membranes; similarly to what happened with cisplatin [15], the cells treated with Ptac2S also show the proteolytic activation of PKC-δ. While the full-length PKC-δ moved to the membrane and nuclei, its fragment was located to the mitochondria. In contrast to cisplatin, the PKC-α was not activated (data not shown).

Here, the cytosol-to-membrane translocation of PKCs was followed by immunoblotting in ZL34 cells incubated with Ptac2S (0–20 min). Of the various isoforms expressed, PKC-ε, PKC-δ and PKC-α were activated by translocation: PKC-ε translocated to the plasma membrane and PKC-δ to plasma membrane and nucleus (Fig 5A). The roles of PKCs were evaluated using siRNA technique to inhibit PKC-ε, PKC-δ or PKC-α. After seeing by western blotting that PKC-siRNAs decreased PKC-ε, PKC-δ and PKC-α expressions (Fig 5B) it was shown that PKC-δ inhibition increased survival (Fig 5C and 5D) and decreased caspase-9 activation and PARP cleavage in ZL34 cells treated with Ptac2S (Fig 5C). Thus, in ZL34 cells the role of activated PKC-δ appears the same as the role it plays when the ZL55 cells are incubated with cisplatin [15] or with Ptac2S [12]. In addition, PKC-δ–siRNA (10 nM) inhibited Ptac2S-induced JNK1/2 phosphorylation, p53 and BAX induction as well as Bcl-2 down-regulation (Fig 5C and 5D), in both cell lines. PKC-ε–siRNA (10 nM) inhibited p38MAPK phosphorylation, caspase-9 activation and PARP cleavage but also increased the survival of Ptac2S-treated ZL34
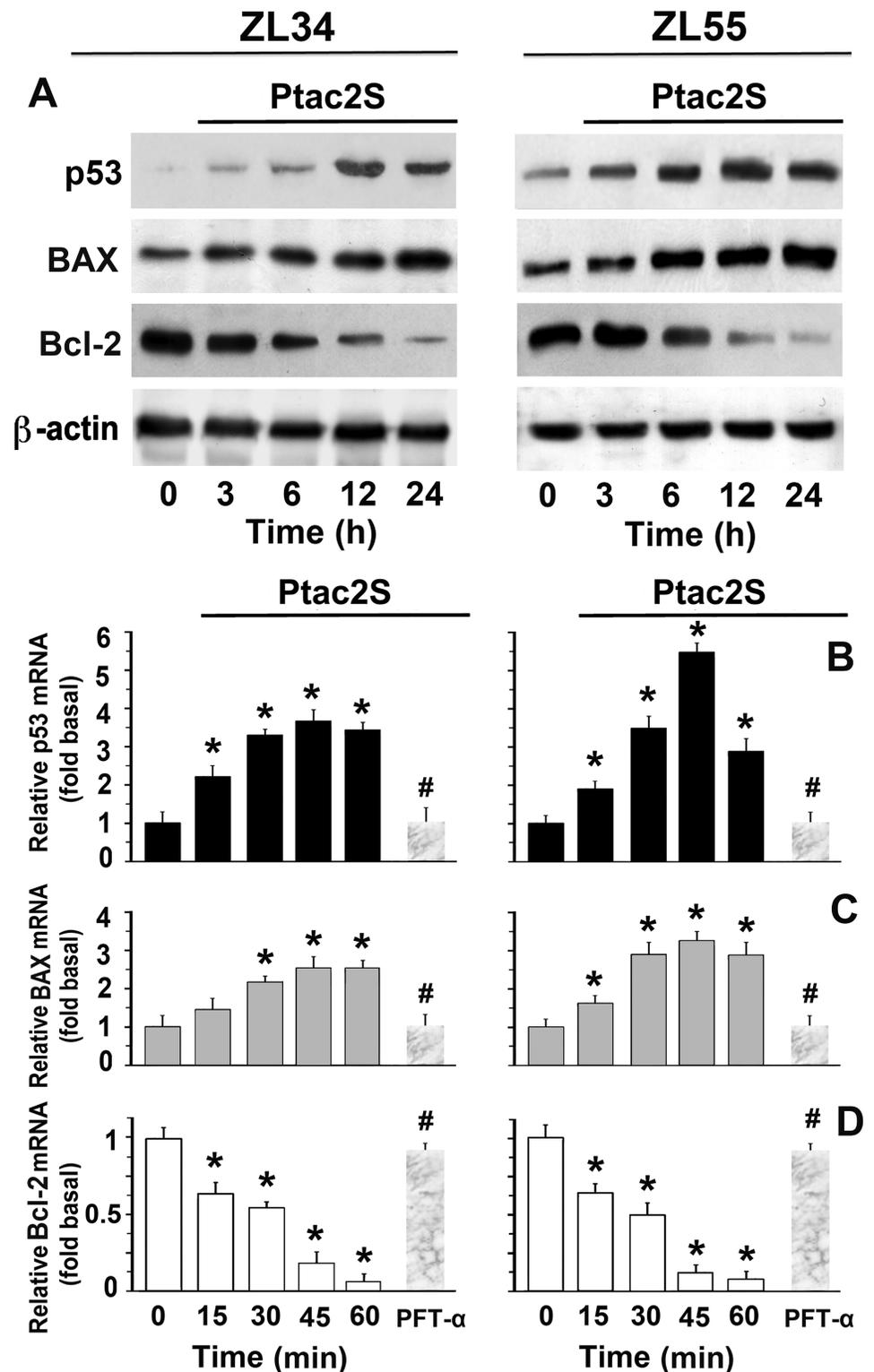
Fig 3. Ptac2S induces p53 in MPM cells. (A) Cytosolic proteins were obtained from ZL34 or ZL55 cells, treated or not with 5 μM Ptac2S. Immunoblotting was performed using monoclonal antibodies specific to p53, Bax and Bcl2. Sequential incubation with anti-β-actin confirmed the equal protein loading. These figures are representative of six independent experiments. **(B-D)** Cells, pre-treated or not with 30 μM PFT-α, were treated or not with 5 μM Ptac2S for different times and then RNA was extracted. RNA was reverse-transcribed and

analysed by real-time PCR, with specific primers for p53 (**B**), BAX (**C**) and Bcl-2 (**D**) and for the housekeeping gene β-actin. mRNA levels were presented as fold change values relative to control. Data were expressed as the mean ± S.D. six different experiments. *P < 0.05, significantly different from saline control; #P < 0.05, significantly different between Ptac2S and PFT-α.

https://doi.org/10.1371/journal.pone.0181114.g003

cells ([Fig 5C](#)). PKC-α-siRNA (10 nM) inhibited the phosphorylation of ERK1/2 ([Fig 5C](#)) indicating that PKC-α activation is crucial for the survival of Ptac2S-treated ZL34 cells. The same results were obtained when 1 μM Go6976 (inhibitor of conventional PKCs) was used (data not shown).
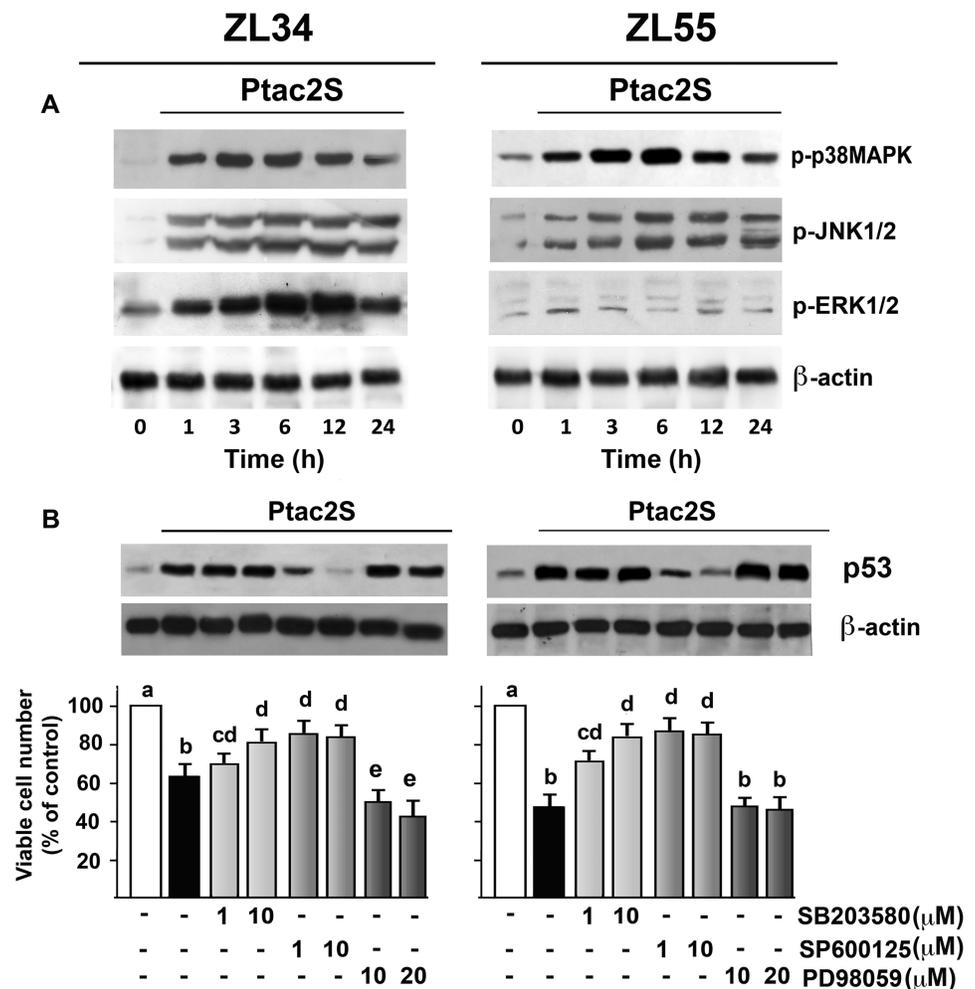
Fig 4. Ptac2S induces MAPKs phosphorylation in MPM cells. (**A**) ZL34 and ZL55 cells were treated or not with 5 μM Ptac2S for indicated time. Cell lysates were analysed by western blotting with anti-phosphorylated p38MAPK, JNK1/2 and ERK1/2 antibodies. (**B**) Cells, pre-treated or not with the p38MAPK inhibitor SB203580 (1 and 10 μM), the JNK inhibitor SP600125 (1 and 10 μM) or MEK inhibitor (10 and 20 μM), were then incubated with Ptac2S. Cell lysates were analysed by western blotting using monoclonal antibody specific to p53. Sequential incubation with anti-β-actin confirmed the equal protein loading. These figures are representative of six independent experiments. Viable cell number was determined 24 h later by SRB assay. The data are means ± S.D. of five different experiments run in eight replicates and are presented as percent of control. P < 0.0001 by one-way ANOVA (n = 5); values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

https://doi.org/10.1371/journal.pone.0181114.g004
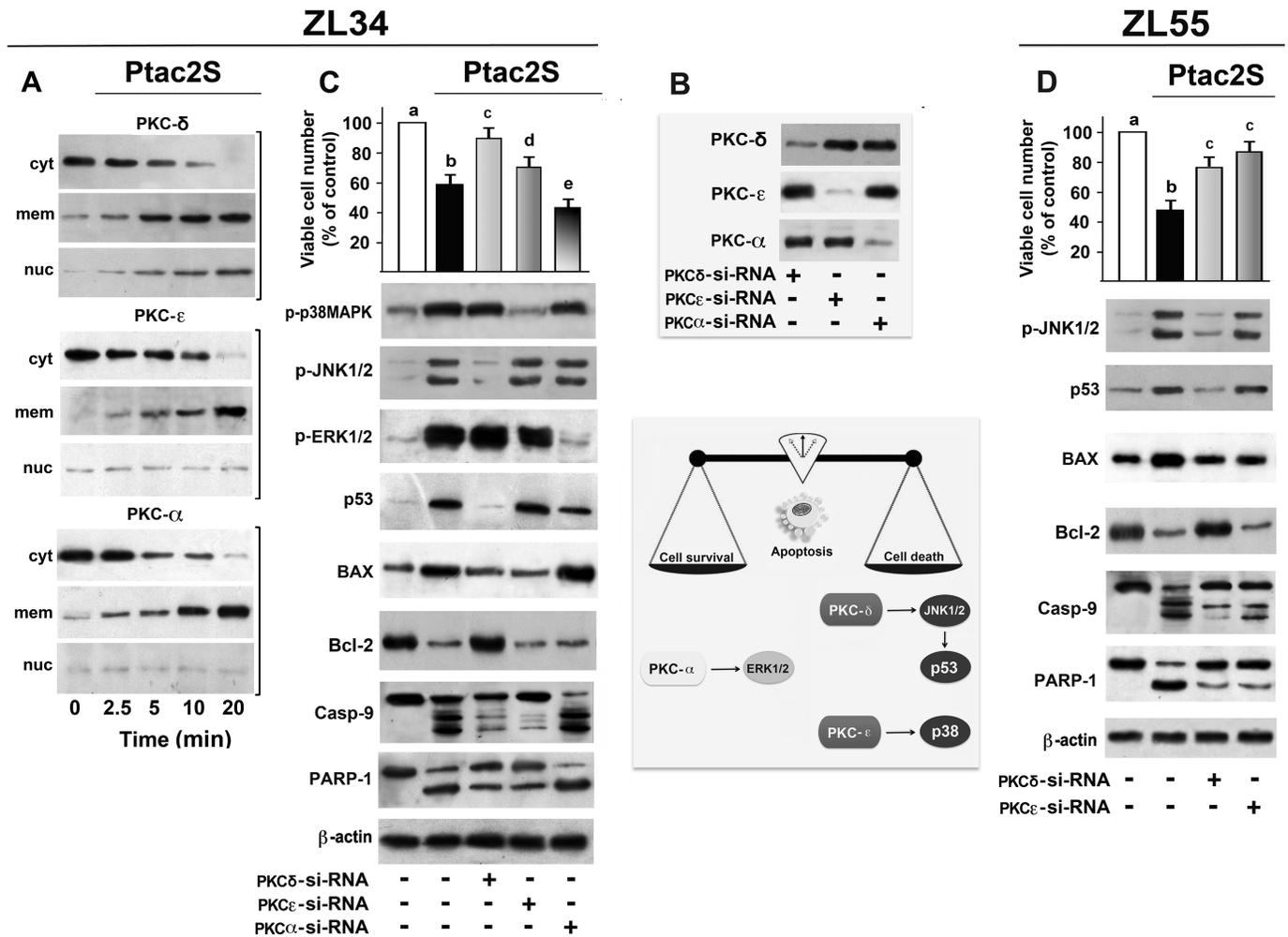
Fig 5. Role of PKCs in Ptac2S-induced apoptosis in MPM cells. (A) ZL34 cells were treated without or with 5 µM Ptac2S for the indicated times. For PKCs translocation studies, cytosol (cyt), membrane (mem), nuclei (nuc) fractions were analysed by western blotting with specific antibodies. The figures are representative of six independent experiments. (**B**) MPM cells were transfected with siRNA–PKC-δ or siRNA–PKC-ε or siRNA–PKC-α and then were incubated with 5 µM Ptac2S; western blotting of total lysates was then performed with specific anti-PKCs antibodies in order to show the decrement of PKC-δ, PKC-ε or PKC-α expressions. The figures are representative of six independent experiments. (**C**) ZL34 cells were transfected with siRNA–PKC-δ or siRNA–PKC-ε or siRNA–PKC-α whilst (**D**) ZL55 cells were transfected with siRNA–PKC-δ or siRNA–PKC-ε only and then incubated with Ptac2S. Viable cell number was determined 24 h later by SRB assay. The data are means ± S.D. of five different experiments run in eight replicates and are presented as percent of control. P < 0.0001 by one-way ANOVA (n = 5); values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests. Cytosolic or nuclear (for PARP-1) fractions were analysed by western blotting with antibodies against PKC-δ, PKC-ε, PKC-α phosphorylated p38MAPK, phosphorylated JNK1/2, phosphorylated ERK1/2, p53, BAX, Bcl2, caspase-9 (Casp-9) and PARP-1; ß-actin was used as a control for protein loading. Representative immunoblots of five experiments are depicted. **Inset**: crosstalk between MAPKs, p53 and PKCs pathways, key factors affecting cell death and survival in Ptac2S-treated MPM cells.

Discussion

MPM originates from mesothelium cells that form a specialised monolayer that line serous cavities of pleura, pericardium or peritoneum [23]. MPM cells can be either epithelioid or sarcomatoid [24] and several studies demonstrated a responsiveness to chemotherapeutics dependent from the phenotypes [25–26]. Therapies for MPM associates pemetrexed to cisplatin getting so a 40% response rate, and 3 months and 1 year average survival and median survival times, respectively [3, 27, 28]; the same happens associating carboplatin, liposomized doxorubicin and gemcitabine [29]. There is an urgent need for effective therapy since about 50% of

MPM patients are primarily resistant and all eventually evolve resistance [30]. Ptac2S is a platinum complex holding 2 acetylacetonate ligands and dimethyl sulphide in the Pt coordination sphere with compelling biological activities [6–14, 21]. Unlike cisplatin, that has genomic (formation of DNA adducts) and non-genomic activities [6, 7], Ptac2S reacts poorly with nucleobases and has characteristic reactivity with sulphur ligands, indicating that cell targets could be protein aminoacid residues. This can make it inherently less capable of evoking chemo resistance [6]. In previous studies comparing Ptac2S and cisplatin activities, Ptac2S has shown a great anti-tumour activity *in vivo*, and a reduced nephrotoxicity and acute toxicity [11]; these results urged the present preclinical study in order to evaluate the therapeutic potential of Ptac2S also in mesothelioma. In addition, we showed that Ptac2S was more efficacious than cisplatin in inducing apoptosis in epithelioid ZL55 cells and, in a preclinical model based on injection of ZL55 cells, Ptac2S shows up an anticancer activity higher than that of cisplatin [12]. To investigate the difference in sensitivity between epithelioid and sarcomatoid mesothelioma cell lines to treatment with Ptac2S, we prepared xenograft models of sarcomatoid mesothelioma by injection of ZL34 cells. In Ptac2S group mice displayed an important curtailment of tumour volume at each time point compared with both not treated and cisplatin-treated mice (Fig 1). The *in vivo* effects of Ptac2S (53% reduction of tumour mass) were considerably greater than those of cisplatin (12% reduction).

In vitro antitumor activity was consistent with the *in vivo* sensitivity, since the cytotoxic effects of Ptac2S were grater than those elicited by cisplatin (Fig 2). In our experiments, the cleavage of PARP happens very quickly (though, already after just 3 hours in ZL55 cells, Ref. [12]) suggesting that Ptac2S causes rapid apoptosis onset. The caspase-7 cleavage pattern was detected earlier in cells treated with Ptac2S compared to cisplatin, and is similar to PARP proteolysis time course, hence supporting the study that shows that the proteolysis of PARP is due to caspase-7 [31]. Consistently with previous results, in Ptac2S-treated MPM cells the activation of caspase-9 occurred along with the activation of caspase-7, denoting the implication of the intrinsic pathway. Furthermore, after few hours of Ptac2S treatment Bcl-2 and BAX expression levels decreased and increased, respectively. In epithelioid ZL55 cells Ptac2S was approximately 12-fold more cytotoxic than cisplatin (IC_{50} were 0.98±0.14 μM for Ptac2S and 11.26±0.41 μM for cisplatin, see Ref [12]); such cell type was also significantly more sensitive to both cisplatin and Ptac2S than sarcomatoid ZL34 cells. *In vivo* we also noted a phenotype-dependent sensitivity to Ptac2S (ZL55 tumour size decreased to 38% [12], whilst ZL34 tumour size decreased to 47%), a result in agreement with the conclusion that patients with sarcomatoid tumours have a poor prognosis [23]. This result points out the necessity of a greater understanding of the relationship between MPM phenotype and the sensitivity of the cancerous cells such as the correlation to predictive markers, in order to increase clinical Pt derivatives efficacy. However, in both ZL34 and ZL55, Ptac2S induced apoptosis by up-regulating p53 protein and mRNA levels (Fig 3). Many pathways mediate the apoptosis due to p53, and among these there is one mediated by BAX proteins and by the pro apoptotic components of Bcl-2 protein family [32, 33]. BAX gene promoter contains several consensus sequences for p53 binding and is heavily trans activated by p53 [34]. BAX is able to foster cytochrome c release in the cytosol, thus activating caspase 9, which leads to apoptosis [35, 36]. In addition, BAX may bind to Bcl-2 inhibiting its apoptosis suppression function. P53 may suppress Bcl-2 expression [37]. Such down-regulation, noted in various models of apoptosis, diminishes Bcl-2 ability to heterodimerise with BAX [38]. In contrast, in mesothelioma cell lines and MPM cancer specimens, the defensive character of Bcl-2 is fewer evident [39, 40]. Albeit in other MPM cell lines are found small levels of Bcl-2 mRNA /protein, in ZL34 MPM cells, treatment with Bcl-2 antisense oligonucleotides reduced apoptosis threshold [41]. However, the transcriptional activation of p53 target genes and *de novo* synthesis of their products are not obligatory

for p53 to induce apoptosis in certain experimental models. In the presence of actinomycin D or cycloheximides, which block RNA synthesis, p53 mediated apoptosis still occurred [42]. In Ptac2S-treated ZL34 and ZL55 cells, p53 regulated proapoptotic BAX and antiapoptotic Bcl-2 proteins *via* transcriptional regulation, as demonstrated by p53 inhibition. As stated above, activated MAPKs are involved in apoptosis [43] also when apoptosis is due to Ptac2S, as shown in human neuroblastoma and breast cancer cells [8, 9]. We have show that Ptac2S causes activation of p38MAPK in ZL55 cells [12], and in the present paper we also show that Ptac2S causes activation of MAPKs and that p38MAPK and JNK1/2 have a pro-apoptotic role in both ZL34 and ZL55. The activation of PKC-ε in Ptac2S treated cells is accountable for the sustained p38MAPK phosphorylation. The role of p38MAPK is underscored by the fact that its inhibition by PKC-ε-siRNA significantly decreased Ptac2S-induced cytotoxicity (Fig 5). Similarly, active p38MAPK is necessary for apoptosis in leukaemia cells [44], in CdCl$_2$-treated promonocytic cells [45] and in cortical neuronal cells treated with calyculin A [46]. The blockage of p53 activation due to JNK1/2 inhibition may suggest that the activation of p53 signalling happens downstream of JNK1/2 (Fig 5). This is in disagreement with precedent studies showing that JNK1/2 is downstream of p53 [47, 48]. On the other hand, various studies demonstrated that JNK1/2 might modulate p53 and its targets and can positively affect apoptosis [49, 50]. Activation and stabilization of p53 by JNK1/2 signalling has been reported in mouse fibroblast [51] and in human multiple myeloma cells [52]. PKC-δ is a key element in apoptosis, as reported in HeLa cells [53], in acinar cells of the salivary glands [54] and in ZL55 cells [12,15].

In this work, we show that JNK1/2 activation is mediated by PKC-δ, similarly to what observed during apoptosis in acinar cells of the salivary glands [54] since JNK1/2 inhibition revert the apoptotic effects of Ptac2S. With regard to PKC-α, it is able to protect from apoptosis [55]. PKC-α phosphorylates Bcl-2 *in vitro*, and over expression of PKC-α increases phosphorylation of Bcl-2 and suppresses apoptosis of pre-B REH cells [56]. Contrariwise, apoptosis of prostatic cancerous cells is provoked by PKC-α over expression or activation through phorbol-12-myristate-13-acetate [57].

In this report we show that PKC-α was activated by Ptac2S in ZL34 cells, starting an antiapoptotic program able to activate a signalling pathway comprehending ERK1/2 (ERK1/2 phosphorylation was blocked by siRNA-PKC-α, Fig 5). ERK1/2 phosphorylation may wield either an anti- [58] or a pro- apoptotic [59] result relying on cellular context and/or as yet unclear regulatory mechanisms. Coherent with a pro-survival action of ERK1/2 [60,61] we here supplied evidences that ERK1/2 is involved in drug resistance induction. Indeed, the cytotoxic effects of Ptac2S increased when the Ptac2S-provoked ERK1/2 phosphorylation was inhibited (Fig 5).

We conclude by pointing out that Ptac2S is effective in pleural mesothelioma and that these new acquisitions enhance the knowledge of the anti tumour activity of this compound. Finally, it appears facilitated the ability to translate this information into clinical practice, in order to improve the response to chemotherapy of resistant tumours.

Author Contributions

Conceptualization: AM SM.

Data curation: AM.

Formal analysis: AM.

Funding acquisition: FPF.

Investigation: CV GA LGC AB.

Methodology: CV AB SM SADP.

Project administration: AM SM.

Resources: FPF.

Software: AM CV.

Supervision: AM.

Validation: AM.

Visualization: AM CV.

Writing – original draft: AM.

Writing – review & editing: AM SM.

References

1. Delgermaa V, Takahashi K, Park EK, Le GV, Hara T Sorahan T (2011) Global mesothelioma deaths reported to the World Health Organization between 1994 and 2008. Bull World Health Organ 89: 716–724, 724A–724C. https://doi.org/10.2471/BLT.11.086678 PMID: 22084509

2. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P et al (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 21: 2636–2644. https://doi.org/10.1200/JCO.2003.11.136 PMID: 12860938

3. Kindler HL. (2008) Systemic treatments for mesothelioma: standard and novel. Curr Treat Options Oncol. 9:171–9. https://doi.org/10.1007/s11864-008-0071-3 PMID: 18770046

4. Pasello G, Favaretto A. (2009) Molecular targets in malignant pleural mesothelioma treatment. Current Drug Targets. 10: 1235–44. PMID: 19909234

5. De Pascali SA, Papadia P, Ciccarese A, Pacifico C, Fanizzi FP (2005) First examples of b–diketonate platinum(II) complexes with sulfoxide ligands. Eur J Inorg Chem 5: 788–796.

6. Muscella A, Calabriso N, De Pascali SA, Urso L, Ciccarese A, Fanizzi FP et al (2007) New platinum(II) complexes containing both an O,O'–chelated acetylacetonate ligand and a sulfur ligand in the platinum coordination sphere induce apoptosis in HeLa cervical carcinoma cells. Biochem Pharmacol 74: 28–40. https://doi.org/10.1016/j.bcp.2007.03.027 PMID: 17481588

7. Muscella A, Calabriso N, Fanizzi FP, De Pascali SA, Urso L, Ciccarese A et al (2008) [Pt(O,O'–acac)(γ–acac)(DMS)], a new Pt compound exerting fast cytotoxicity in MCF–7 breast cancer cells via the mitochondrial apoptotic pathway. Br J Pharmacol 153: 34–49. https://doi.org/10.1038/sj.bjp.0707576 PMID: 18026127

8. Muscella A, Calabriso N, Vetrugno C, Fanizzi FP, De Pascali SA, Marsigliante S (2011) The signalling axis mediating neuronal apoptosis in response to [Pt(O,O'–acac)(γ–acac)(DMS)]. Biochem Pharmacol 81: 1271–1285. https://doi.org/10.1016/j.bcp.2011.03.007 PMID: 21420390

9. Muscella A, Vetrugno C, Fanizzi FP, Manca C, De Pascali SA, Marsigliante S (2013) A new platinum(II) compound anticancer drug candidate with selective cytotoxicity for breast cancer cells. Cell Death Dis 4: e796. https://doi.org/10.1038/cddis.2013.315 PMID: 24030148

10. Vetrugno C, Muscella A, Fanizzi FP, Cossa LG, Migoni D, De Pascali SA et al (2014) Different apoptotic effects of [Pt(O,O'–acac)(γ–acac)(DMS)] and cisplatin on normal and cancerous human epithelial breast cells in primary culture. Br J Pharmacol 171: 5139–5153. https://doi.org/10.1111/bph.12831 PMID: 24990093

11. Muscella A, Vetrugno C, Migoni D, Biagioni F, Fanizzi FP, Marsigliante S (2014) Antitumor activity of [Pt(O,O'–acac)(γ–acac)(DMS)] in mouse xenograft model of breast cancer. Cell Death Dis. 5:e1014. https://doi.org/10.1038/cddis.2013.554 PMID: 24457958

12. Muscella A, Vetrugno C, Cossa LG, Antonaci G, De Nuccio F, Fanizzi FP.et al., (2016) In Vitro and In Vivo Antitumor Activity of [Pt(O,O'-acac)(γ-acac)(DMS)] in Malignant Pleural Mesothelioma. PLoS ONE 11:e0165154. https://doi.org/10.1371/journal.pone.0165154 PMID: 27806086

13. Muscella A, Vetrugno C, Migoni D, Biagioni F,Calabriso N, Calierno MT et al. (2016) Antitumor and Anti-angiogenic Activities of [Pt(O,O'-acac)(γ-acac)(DMS)] in a xenograft model of human renal cell carcinoma. Br J Pharmacol 173 (2016) 2633–2644.

14. Muscella A, Calabriso N, Vetrugno C, Urso L, Fanizzi FP De Pascali SA (2010). Sublethal concentrations of the platinum(II) complex [Pt(O,O'–acac)(γ–acac)(DMS)] alter the motility and induce anoikis in MCF–7 cells. Br J Pharmacol 160: 1362–1377. https://doi.org/10.1111/j.1476-5381.2010.00782.x PMID: 20590627

15. Muscella A, Vetrugno C, Antonaci G, Cossa LG, Marsigliante S (2015). PKC-δ/PKC-α activity balance regulates the lethal effects of cisplatin. Biochem Pharmacol 98: 29–40. https://doi.org/10.1016/j.bcp.2015.08.103 PMID: 26300055

16. Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ et al. (2010). Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer 102:1555–1577. https://doi.org/10.1038/sj.bjc.6605642 PMID: 20502460

17. Marsigliante S, Vetrugno C, Muscella A (2013) CCL20 induces migration and proliferation on breast epithelial cells. J Cell Physiol 228: 1873–1883. https://doi.org/10.1002/jcp.24349 PMID: 23460117

18. Muscella A, Urso L, Calabriso N, Vetrugno C, Storelli C, Marsigliante S (2009). Anti-apoptotic effects of protein kinase C-delta and c-fos in cisplatin-treated thyroid cells. Br J Pharmacol 156(5):751–63. https://doi.org/10.1111/j.1476-5381.2008.00049.x PMID: 19254279

19. De Pascali SA, Papadia P, Capoccia S, Marchiò L, Lanfranchi M, Ciccarese A et al (2009) Hard/soft selectivity in ligand substitution reactions of b–diketonate platinum(II) complexes. Dalton Trans 37: 7786–7795.

20. Mor O, Yaron P, Huszar M, Yellin A, Jakobovitz O, Brok-Simoni F et al., (1997) Absence of p53 mutations in malignant mesotheliomas. Am J Respir Cell Mol Biol. 16:9–13. https://doi.org/10.1165/ajrcmb.16.1.8998073 PMID: 8998073

21. Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science.1999; 285:1733–7. PMID: 10481009

22. Muscella A, Vetrugno C, Calabriso N, Cossa LG, De Pascali SA, Fanizzi FP, et al (2014b) [Pt(O,O'-acac)(γ-acac)(DMS)] alters SH-SY5Y cell migration and invasion by the inhibition of Na+/H+ exchanger isoform 1 occurring through a PKC-ε/ERK/mTOR Pathway. PLoS One 9(11): e112186.

23. Fennell DA, Gaudino G, O'Byrne KJ, Mutti L, van Meerbeeck J. (2008) Advances in the systemic therapy of malignant pleural mesothelioma. Nat Clin Prac Oncol. 5:136–47.

24. Ceresoli GL, Locati LD, Ferreri AJ, Cozzarini C, Passoni P, Melloni G et al. (2001) Therapeutic outcome according to histologic subtype in 121 patients with malignant pleural mesothelioma. Lung Cancer. 34:279–87. PMID: 11679187

25. Sun X, Gulyás M, Hjerpe A, Dobra K. (2006) Proteasome inhibitor PSI induces apoptosis in human mesothelioma cells. Cancer Letters. 232:161–9. https://doi.org/10.1016/j.canlet.2005.02.022 PMID: 16458112

26. Nilsonne G, Olm E, Szulkin A, Mundt F, Stein A, Kocic B, et al. (2009) Phenotype-dependent apoptosis signalling in mesothelioma cells after selenite exposure. J Exp Cli Cancer Res. 28:92–2.

27. Ceresoli GL, Favaretto AG, Grossi F, Bidoli P, Del Conte G, Ceribelli A, et al. (2006) Phase II study of pemetrexed plus carboplatin in malignant pleural mesothelioma. J Clin Oncol. 24:1443–8. https://doi.org/10.1200/JCO.2005.04.3190 PMID: 16549838

28. Favoni RE, Florio T. (2011) Combined chemotherapy with cytotoxic and targeted compounds for the management of human malignant pleural mesothelioma. Trends Pharmacol Sciences. 32:463–79.

29. Hillerdal G, Sorensen JB, Sundström S, Riska H, Vikström A, Hjer (2008) Treatment of Malignant Pleural Mesothelioma with Carboplatin, Liposomized Doxorubicin, and Gemcitabine: A Phase II Study. J Thorac Oncol. 3:1325–31. https://doi.org/10.1097/JTO.0b013e31818b174d PMID: 18978569

30. Mujoomdar AA, Tilleman TR, Richards WG, Bueno R, Sugarbaker DJ. (2010) Prevalence of in vitro chemotherapeutic drug resistance in primary malignant pleural mesothelioma: Result in a cohort of 203 resection specimens. J Thorac Cardiovasc Surg. 140:352–5. https://doi.org/10.1016/j.jtcvs.2009.11.072 PMID: 20653100

31. Germain M, Affar EB, D'Amours D, Dixit VM, Salvesen GS, Poirier GG (1999) Cleavage of automodified poly(ADP-ribose) polymerase during apoptosis. Evidence for involvement of caspase-7. J Biol Chem 274: 28379–28384. PMID: 10497198

32. Streffer JR, Rieger J, Naumann U, Rodemann HP, Weller M. (2002) BCL-2 family proteins modulate radiosensitivity in human malignant glioma cells. J Neurooncol. 56:43–9. PMID: 11949826

33. Bandoh N, Kishibe K, Takahara M, Imada M, Nonaka S, Harabuchi Y (2002) Prognostic value of p53 mutations, bax, and spontaneous apoptosis in maxillary sinus squamous cell carcinoma. Cancer. 94:1968–80. PMID: 11932899

34. Beckerman R, Prives C. Transcriptional Regulation by P53. (2010) Cold Spring Harbor Perspectives in Biology, 2:a000935. https://doi.org/10.1101/cshperspect.a000935 PMID: 20679336

35. Lowe SW, Lin AW. (2000) Apoptosis in cancer. Carcinogenesis. 21:485–95 PMID: 10688869

36. Communal C, Sumandea M, de Tombe P, Narula J, Solaro RJ, Hajjar RJ. (2002) Functional consequences of caspase activation in cardiac myocytes. PNAS. 99:6252–6. https://doi.org/10.1073/pnas.092022999 PMID: 11972044

37. Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, et al. (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene. 9:1799–805. PMID: 8183579

38. Allen RT, Cluck MW, Agrawal DK (1998) Mechanisms controlling cellular suicide: role of Bcl-2 and caspases. Cell Mol Life Sci 54: 427–445. https://doi.org/10.1007/s000180050171 PMID: 9645223

39. Narasimhan SR, Yang L, Gerwin BI, Broaddus VC (1998) Resistance of pleural mesothelioma cell lines to apoptosis: relation to expression of Bcl-2 and Bax. Am J Physiol 275: 165–171.

40. Soini Y, Kinnula V, Kaarteenaho-Wiik R, Kurttila E, Linnainmaa K, Pääkkö P (1999) Apoptosis and expression of apoptosis regulating proteins bcl-2, mcl-1, bcl-X, and bax in malignant mesothelioma. Clin Cancer Res 5: 3508–3515. PMID: 10589765

41. Hopkins-Donaldson S, Cathomas R, Simões-Wüst AP, Kurtz S, Belyanskaya L, Stahel RA et al (2003) Induction of apoptosis and chemosensitization of mesothelioma cells by Bcl-2 and Bcl-xL antisense treatment. Int J Cancer 106: 1660–1666.

42. Caelles C, Helmberg A, Karin M. (1994) p53-Dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature. 370:220–3. https://doi.org/10.1038/370220a0 PMID: 8028670

43. Fan M, Chambers TC (2001) Role of mitogen-activated protein kinases in the response of tumour cells to chemotherapy. Drug Res Updat 4: 253–267.

44. Zhuang S, Demirs JT, Kochevar IE (2000) p38 mitogen-activated protein kinase mediates Bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen, but not by hydrogen peroxide. J Biol Chem 275: 25935–25948.

45. Galan A, García-Bermejo M L, Troyano A, Vilaboa NE, de Blas E, Kazanietz MG, et al (2000) Stimulation of p38 mitogen-activated protein kinase is an early regulatory event for the cadmium-induced apoptosis in human promonocytic cells. J Biol Chem 275: 11418–11424. PMID: 10753958

46. Ko HW, Han KS, Kim EY, Ryu BR, Yoon WJ, Jung YK et al (2000) Synergetic activation of p38 mitogen-activated protein kinase and caspase-3-like proteases for execution of calyculin A-induced apoptosis but not N-methyl-d-aspartate-induced necrosis in mouse cortical neurons. J Neurochem 74: 2455–2461. PMID: 10820206

47. Chen L, Wang S, Zhou Y, Wu X, Entin I, Epstein J. et al. (2010) Identification of early growth response protein 1 (EGR-1) as a novel target for JUN-induced apoptosis in multiple myeloma. Blood. 115:61–70. https://doi.org/10.1182/blood-2009-03-210526 PMID: 19837979

48. Raab MS, Breitkreutz I, Tonon G, Zhang J, Hayden PJ, Nguyen T, et al. (2009) Targeting PKC: a novel role for beta-catenin in ER stress and apoptotic signaling. Blood. 113:1513–21. https://doi.org/10.1182/blood-2008-05-157040 PMID: 19018094

49. Fuchs SY, Adler V, Buschmann T, Yin Z, Wu X, Jones SN et al. (1998) JNK targets p53 ubiquitination and degradation in nonstressed cells. Genes Dev. 12:2658–63. PMID: 9732264

50. Fan M, Birrer MJ, Chambers TC. (2001) The c-Jun NH(2)-terminal protein kinase/AP-1 pathway is required for efficient apoptosis induced by vinblastine. Cancer Res. 61:4450–8. PMID: 11389075

51. Fuchs SY, Adler V, Pincus MR, Ronai Z. (1998) MEKK1/JNK signaling stabilizes and activates p53. PNAS. 95:10541–6. PMID: 9724739

52. Saha MN, Jiang H, Yang Y, Zhu X, Wang X, Schimmer AD, et al. (2012) Targeting p53 via JNK Pathway: A Novel Role of RITA for Apoptotic Signaling in Multiple Myeloma. PLoS ONE. 7:e30215. https://doi.org/10.1371/journal.pone.0030215 PMID: 22276160

53. Basu A, Woolard MD, Johnson CL (2001) Involvement of protein kinase C delta in DNA damage-induced apoptosis. Cell Death Differ 8: 899–908. https://doi.org/10.1038/sj.cdd.4400885 PMID: 11526445

54. Reyland ME, Matassa AA, Barzen KA, Quissell DO. (1999) Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. J Biol Chem 274:19115–23. PMID: 10383415

55. Berra E, Sanz L, Frutos S, Diaz-Meco MT, Moscat J. (1997) Positioning atypical protein kinase C isoforms in the UV-induced apoptotic signaling cascade. Mol Cell Biol. 17:4346–54. PMID: 9234692

56. Ruvolo PP, Deng X, Carr BK, May WS. (1998) A Functional Role for Mitochondrial Protein Kinase Cα in Bcl2 Phosphorylation and Suppression of Apoptosis. J Biol Chem. 273:25436–42. PMID: 9738012

57. Powell CT, Brittis NJ, Stec D, Hug H, Heston WDW, Fair WR. (1996) Persistent membrane translocation of protein kinase C α during 12-O- tetradecanoylphorbol-13-acetate-induced apoptosis of LNCaP human prostate cancer cells. Cell Growth Differ. 7:419–28. PMID: 9052983

58. Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, et al. (2000) The MEK1–ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO J. 19:6341. https://doi.org/10.1093/emboj/19.23.6341 PMID: 11101507

59. Persons DL, Yazlovitskaya EM, Pelling JC. (2000) Effect of extracellular signal regulated kinase on p53 accumulation in response to cisplatin. J Biol Chem. 275:35778–85. https://doi.org/10.1074/jbc.M004267200 PMID: 10958792

60. Muscella A, Urso L, Calabriso N, Ciccarese A, Migoni D, Fanizzi FP et al. (2005) Differential response of normal, dedifferentiated and transformed thyroid cell lines to cisplatin treatment. Biochem Pharmacol. 71:50–60. https://doi.org/10.1016/j.bcp.2005.10.022 PMID: 16297374

61. Wang X, Martindale JL, Liu Y, Holbrook NJ. (1998) The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochemical J. 333:291–297.