Breast Cancer Res Treat
DOI 10.1007/s10549-010-0956-2

PRECLINICAL STUDY

# Involvement of PKC delta (PKC$\delta$) in the resistance against different doxorubicin analogs

**María Ines Díaz Bessone · Damian E. Berardi · Paola B. Campodónico · Laura B. Todaro · Leonard Lothstein · Elisa D. Bal de Kier Joffé · Alejandro J. Urtreger**

Received: 6 March 2010 / Accepted: 14 May 2010


**Abstract** Doxorubicin is an anti-tumor antibiotic widely used in the management of cancer patients. Its main mechanism of action involves the generation of DNA damage and the inhibition of topoisomerase II, promoting apoptosis. AD 198 is a novel doxorubicin analog devoid of DNA binding and topoisomerase II inhibitory capacities. It has been proposed that AD 198 induces apoptosis by activating protein kinase C delta (PKC$\delta$); a PKC isoform described as growth inhibitory in a large number of cell types. We have previously demonstrated that PKC$\delta$ overexpression in NMuMG cells induced the opposite effect, promoting proliferation and cell survival. In this study, we found that PKC$\delta$ overexpression confers an enhanced cell death resistance against AD 198 cytotoxic effect and against AD 288, another doxorubicin analog that preserves its mechanism of action. These resistances involve PKC$\delta$-mediated activation of two well-known survival pathways: Akt and NF-$\kappa$B. While the resistance against AD 198 could be abrogated upon the inhibition of either Akt or NF-$\kappa$B pathways, only NF-$\kappa$B inhibition could revert the resistance to AD 288. Altogether, our results indicate that PKC$\delta$ increases cell death resistance against different apoptosis inductors, independently of their mechanism of action, through a differential modulation of Akt and NF-$\kappa$B pathways. Our study contributes to a better understanding of the mechanisms involved in PKC$\delta$-induced resistance and may greatly impact in the rationale design of isozyme-specific PKC modulators as therapeutic agents.

**Keywords** PKC$\delta$ · AD 198 · AD 288 · Cell death resistance

**Abbreviations**

| | |
|---|---|
| AD 198 | *N*-benzyladriamycin-14-valerate |
| AD 288 | *N*-benzyladriamycin |
| DAG | Diacylglycerol |
| Dox | Doxorubicin |
| FCS | Fetal calf serum |
| MEM | Minimum essential medium |
| Nuclear factor-$\kappa$B | NF-$\kappa$B |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PKC | Protein kinase C |
| PMA | Phorbol 12-myristate 13-acetate |
| Rott | Rottlerin |
| SDS | Sodium dodecyl sulfate |
| Topo II | Topoisomerase II |

## Introduction

Anthracyclines, as exemplified by doxorubicin (Dox), comprise a class of anti-tumor antibiotics widely used in the management of patients with cancer because of their broad spectrum of action [1]. The principal mechanism of Dox cytotoxicity involves the generation of DNA damage as well as the inhibition of topoisomerase II (topo II), causing double strand breaks and promoting apoptosis [2]. The curative potential of this drug is compromised by the

M. I. Díaz Bessone · D. E. Berardi · P. B. Campodónico · L. B. Todaro · E. D. Bal de Kier Joffé · A. J. Urtreger (✉)
Research Area, Institute of Oncology "Angel H. Roffo", University of Buenos Aires, Av. San Martín 5481, Buenos Aires C1417DTB, Argentina
e-mail: urtreger@fmed.uba.ar

L. Lothstein
The University of Tennessee Health Science Center and Center for Cancer Research, Memphis, TN, USA

Published online: 30 May 2010

cumulative dose-related cardiomyopathy and the development of drug resistance [3]. These considerations have generated the need of developing Dox derivatives with better therapeutic properties such as *N*-benzyladriamycin (AD 288) and *N*-benzyladriamicycin-14-valerate (AD 198). While AD 288 preserves Dox mechanism of action, AD 198 weakly binds DNA and is almost devoid of topo II inhibitory activity, but still retains its cytotoxic activity [1, 4].

AD 198 displays a higher antitumor activity than Dox since it is capable of circumventing multiple forms of Dox resistance, such as the overexpression of the P-glycoprotein and multidrug resistance protein transporters, as well as resistance caused by reduced topo II activity [3, 4]. Although Dox localizes in the nucleus, AD 198 rapidly localizes almost exclusively in the cytoplasm, and it has been proposed that it binds directly to the C1b domains of conventional and novel protein kinase C (PKC) isoforms. The AD 198-mediated PKC activation may then be the responsible for its cytotoxic effect [1, 3].

Protein kinase C is a family of lipid-dependent serine-threonine kinases that plays central roles in signal transduction pathways controlling proliferation, apoptosis, and malignant transformation [5, 6]. On the basis of their structural similarities and biochemical properties, PKC isoforms have been grouped into three families [7, 8]: *classical* ($\alpha$, $\beta$I, $\beta$II, and $\gamma$), which are activated by calcium and diacylglycerol (DAG); *novel* ($\delta$, $\varepsilon$, $\eta$, and $\theta$), which require DAG but are calcium-insensitive; and *atypical* ($\zeta$ and $\lambda/\iota$), which are not responsive to either DAG or calcium [5, 9].

A three-dimensional structure investigation showed a similar structural conformation between DAG and AD 198 thus indicating that classical and novel PKC isoforms might be AD 198 targets. Moreover, since PKC$\delta$ has been described as growth inhibitory in a large number of cell types, it was proposed that this kinase should be the main effector of AD 198 cytotoxicity [10]. In this sense, NIH3T3 cells overexpressing PKC$\delta$ have shown an important reduction in their proliferation rate [11, 12]. This effect has also been reported in other cell types, including glial [13], endothelial [14], and breast cancer [15] cells. Several reports have also shown that activation of PKC$\delta$ promotes apoptosis of colon [16] and prostate [17] tumor-derived cell lines.

On the other side, there are several studies showing that PKC$\delta$ could also act as a positive regulator of growth in mammary cells [6, 18], and moreover a pro-survival role for PKC$\delta$ in breast and lung cancer has also been reported [19, 20].

Cell death susceptibility depends on the expression and/or modulation of different molecules, transcription factors, and cell signaling pathways. PI3K/Akt is considered the most important pathway mediating cell survival against a high variety of pro-apoptotic stimuli and in a wide range of cell types [21]. Akt is a kinase that has the capacity to phosphorylate a wide variety of substrate proteins to perform its functions in the cell. These phosphorylations include procaspase-9 and Bad among others. Procaspase-9 phosphorylation by Akt turns it resistant to the activation process impairing the propagation of the caspase cascade. A similar effect occurs with Bad, a proapototic member of the Bcl-2 family. In the absence of Akt activity, Bad binds to the pro-survival member of the Bcl-2 family Bcl-XL and induces cell death by inhibiting its function. On the contrary, activated Akt phosphorylates Bad causing its dissociation from Bcl-XL thus, avoiding cell death induction [22].

Recent studies have shown that PKC$\delta$ is an important mediator involved in the regulation of nuclear factor-$\kappa$B (NF-$\kappa$B) dependent gene expression, and different signaling cascades have been involved in this process. Hsieh et al. have proposed the existence of a PKC$\delta$-dependent pathway linking ERK/MAPK with NF-$\kappa$B activation [23], while other authors consider that DAG mediates NF-$\kappa$B activation by promoting PKC$\delta$ translocation, favoring NF-$\kappa$B inhibitor degradation [24]. Therefore, there is cumulating evidence indicating that PKC$\delta$ and, in particular, its kinase activity are required for NF-$\kappa$B transactivation [25, 26]. NF-$\kappa$B is a transcription factor that regulates the expression of a wide variety of genes, especially those involved in survival, such as Bcl-2 and caspase inhibitors. The functionality of this transcription factor depends on the binding to its inhibitor (I$\kappa$B), which sequesters it into the cytoplasm. Upon phosphorylation, I$\kappa$B is degraded and NF-$\kappa$B can enter into the nucleus to induce the transcription of target genes [27].

In a previous study, we demonstrated that PKC$\delta$ overexpression promoted cell survival against different pro-apoptotic stimuli and induced a higher clonogenic capacity and the acquisition of the ability to grow in an anchorage-independent way, supporting the concept that this novel PKC was favoring the malignant transformation process in mammary cells [6]. In this study, we analyze mechanisms involved in PKC$\delta$-induced resistance against the cytotoxicity of different Dox-related drugs. We had a particular interest in the anthracycline AD 198, whose proposed mechanism of action involves PKC$\delta$ activation, as we mentioned above. As experimental approach, we overexpressed PKC$\delta$ in NMuMG cells, an established model of immortalized normal mouse mammary cells that has been widely used in mammary carcinogenesis studies [28–30]. We found that PKC$\delta$ overexpression confers an enhanced survival against both AD 198 and AD 288 independently of their mechanism of action. We also determined that PKC$\delta$ activates the two well-known survival pathways PI3K/Akt and NF-$\kappa$B in NMuMG-transfected cells. Interestingly, while PKC$\delta$-induced resistance to AD 198 could be

abrogated when either Akt or NF-κB pathways were inhibited, only NF-κB inhibition could revert the resistance to AD 288. The results presented in this article contribute to a better understanding of the mechanisms involved in PKCδ-induced resistance and may greatly impact the rationale design of isozyme-specific PKC modulators as therapeutic agents.

## Materials and methods

### Reagents and antibodies

Medium for cell culture, Geneticin (G418), and Lipofectamine Plus were obtained from Life Technologies Inc. (Rockville, MD). The transfection reagent Fugene was from Roche Applied Science (Indianapolis, IN). Fetal calf serum (FCS) was from Bioser (Buenos Aires, Argentina). Acrylamide and LY294002 were from Sigma (St. Louis, MO). All other reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). AD198 and AD 288 hydrochloride salts were synthesized by Dr. Mervyn Israel (The University of Tennessee Health Science Center and Center for Cancer Research, Memphis, TN). The PKCδ inhibitor rottlerin (Rott) was purchased from Merck Chemicals (Nottingham, UK). Monoclonal antibodies for PKCδ, Bcl-2, Bad, and phospho-Bad (pBad Ser 135) were purchased from BD Biosciences (San Diego, CA). Polyclonal antibodies for actin and monoclonal antibodies for NF-κB (p65) and IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies for Akt and phospho-Akt (pAkt, Ser 473) were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies and PMA were obtained from Sigma (St. Louis, MO). Hybond-P membranes for blotting and chemiluminescence reagents (ECL) were from Amersham (Aylesbury, UK).

### Cell line

NMuMG (Normal Murine Mammary Gland) cells, an immortalized mammary cell line derived from NAMRU mice [31], were cultured at 37°C in MEM supplemented with 10% FCS and 80 μg/ml gentamicin in a humidified air atmosphere with 5% $CO_2$.

### Expression vectors, transfection, and selection

NMuMG cells were stably transfected with 5 μg of pMTH-PKCδ, a mammalian expression vector encoding for murine PKCδ, using Lipofectamine Plus as previously reported [6]. NMuMG cells transfected with the empty vector (pMTH) were used as control. Forty-eight hours after transfection, cells were selected using G418 (500 μg/ml). After selection, ~40 resistant clones were pooled to avoid clonal variations. The overexpression of PKCδ in NMuMG cells promoted a mitogenic response leading to a significant increase in [$^3$H]-tymidine incorporation and cell proliferation, enhanced ERK-MAPK activation, and induced the acquisition of anchorage-independent growth capacity [6].

### Western blot

Semi-confluent monolayers were washed twice with ice-cold PBS and then lysed with 1% Triton X-100 in PBS by scrapping with a Teflon scraper. Samples were denatured by boiling in sample buffer with 5% β-mercaptoethanol and subjected to 10 or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fifty microgram of protein were loaded in each lane. Gels were blotted to Hybond-P membranes. After incubation for 1 h in PBS containing 5% skim milk with 0.1% Tween-20, membranes were incubated with the first antibody overnight at 4°C and then for 1 h with a secondary antibody coupled to horseradish peroxidase. Detection was performed by chemiluminescence. Bands were digitalized with a Foto/Analyst Express System (Fotodyne Inc. Hartland, WI), and signal intensity was quantified with Gel-Pro Analyzer software.

### PMA cell treatments and subcellular fractionation

NMuMG-PKCδ and NMuMG-vector cells were treated with or without PMA (50 nM) during 1 h. Monolayers were then washed twice with ice-cold PBS and lysed with 1% Triton X-100 in PBS. The cytosolic (soluble) fraction was obtained by collecting the supernatant after centrifugation of the cell lysate (1 h at 100,000×$g$). The remaining pellet represents the particulate fraction. Protein concentration of the total lysate and fractions was determined, and equal amounts of protein for each fraction (50 μg) were subjected to SDS-PAGE and transferred to Hybond-P membranes. Membranes were subjected to western blot using anti-PKCδ antibodies, as described above.

### Determination of NF-κB and IκB expression levels

NF-κB levels in cytoplasm and nucleus, and IκB levels in cytoplasm were analyzed by western blot, after separating nuclear and cytoplasmic proteins using NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford IL). Briefly, subconfluent monolayers growing in 100-mm Petri dishes were trypsinized and

centrifuged (500×$g$, 5 min). The supernatant was discarded, and the pellet was resuspended in the cytoplasmic extraction reagent 1 buffer (CERI). Then CERII buffer were added and the mixture was centrifuged (13,000×$g$, 5 min). Supernatant corresponds to the cytoplasmic protein fraction, and the remaining pellet was resuspended in nuclear extraction reagent buffer (NER) to obtain the nuclear protein fraction. Protein content of both fractions was determined, and samples were aliquoted and stored at −80°C and used only once after thawing.

### NF-κB-dependent reporter gene expression assay

NMuMG-PKCδ and NMuMG-vector cells were transiently co-transfected with 5 μg of NF-κB-Luc reporter plasmid and 0.5 μg of the Renilla Luciferase Control Reporter Vector (pRL-CMV; Promega, Madison, WI) using Fugene following manufacturer's instructions. Transfected cells were seeded onto 24-well plates, and after 48 h of culture cells were lysed and the luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) and normalized to the constitutive Renilla luciferase activity.

### AD 198 and AD 288 treatment and viability assay

AD 198 and AD 288 hydrochloride salts were prepared in the Department of Pharmacology of the University of Tennessee Health Science Center laboratory, according to the previously described procedures [4].

NMuMG-PKCδ and NMuMG-vector cells were seeded onto 96-well plates ($1 \times 10^4$ cell/well) and treated 24 h later with 0.1–20 μM of AD 198 or AD 288 for 1 h, washed twice with PBS, and subsequently incubated in MEM-10% FCS for 48 h.

In order to analyze the involvement of PKCδ in cell death induced by the AD compounds, cells were either depleted of PKCs by overnight exposure to PMA (200 nM) or treated for 1 h with the PKCδ inhibitor Rott (5 μM). After these treatments, cells were washed with PBS and incubated for 1 h in the presence of AD 198 or AD 288 as mentioned above. The modulation of PKCδ levels after both treatments was determined by western blot as described above.

In order to analyze the role of the PI3K/Akt signaling pathway in AD-mediated cell death induction, cells were pre-treated with the PI3K inhibitor LY294002 (30 μM) for 2 h, washed with PBS, and further incubated for 1 h in the presence of AD 198 or AD 288 as mentioned above. The decrease of pAkt levels after LY294002 exposure was determined by western blot as described above.

In order to analyze the role of NF-κB transcription factor in cell death induction by both ADs, cells were transfected with a construction containing an IκBα super-repressor (ssIκBα). This variant of IκB contains mutations at Ser 32 and Ser 36 which prevents its phosphorylation and proteolysis (kindly provided by Dr. MA. Costa, Institute Alfredo Lanari, Buenos Aires, Argentina). Cells were transiently transfected in suspension with 5 μg of ssIκBα using Fugene following manufacturer's instructions. Transfected cells were seeded onto 96-well plates and, after 24 h of culture, cells were treated for 1 h with different doses of AD 198 or AD 288. The increase of IκBα levels in IκB super-repressor transfected cells was determined by western blot as described above.

In all cases, cell viability was evaluated with the MTS assay (Celltiter 96™ Non Radioactive Proliferation Assay, Promega) as described by the manufacturer, and the lethal dose 50 (LD50) was determined.

## Results

### Expression and functionality of PKCδ in NMuMG-PKCδ cells

Using a stable transfection approach, we have previously developed a model of murine mammary cells useful to study the role of the novel PKCδ isozyme in the modulation of cell survival [6]. The level of PKCδ overexpression in transfected cells was ∼8-fold (Fig. 1a) and PKCδ overexpression did not alter the expression of other PKC isozymes present in NMuMG cells (data not shown). As

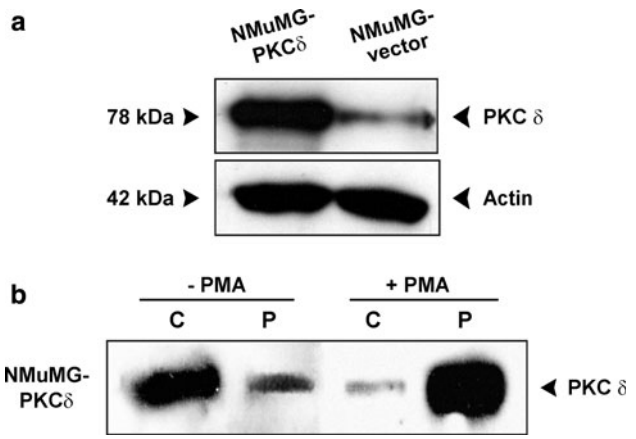


**Fig. 1** **a** PKCδ overexpression in NMuMG cells. Whole cell lysates prepared from NMuMG-vector or NMuMG-PKCδ cells were resolved on 10% SDS-PAGE and blotted with anti-PKCδ antibodies (50 μg/lane). Actin expression level was used as protein loading control. A representative experiment is shown. **b** Membrane translocation of PKCδ isozyme in NMuMG-transfected cells. NMuMG-PKCδ cells were incubated with PMA (50 nM) for 15 min. Cytoplasmic (C) and particulate (P) fractions were separated by ultracentrifugation and subjected to western blot analysis using anti-PKCδ antibodies

shown in Fig. 1b, similarly to wild type PKCs, overexpressed PKCδ was located mainly in the soluble (cytosolic) fraction and PMA induced its translocation to the particulate fraction. Thererfore, we have obtained an efficient expression of a phorbol ester-responsive PKCδ after the transfection assays.

## The overexpression of PKCδ enhances cell death resistance against anthracyclines

Previously, we had demonstrated that cells overexpressing PKCδ (NMuMG-PKCδ) were more resistant to cell death induced by Dox or serum starvation than control cells (NMuMG-vector) [6]. This observation suggested that this cellular model could be a useful tool to analyze the role of PKCδ as a drug target and as a survival-promoting molecule at the same time.

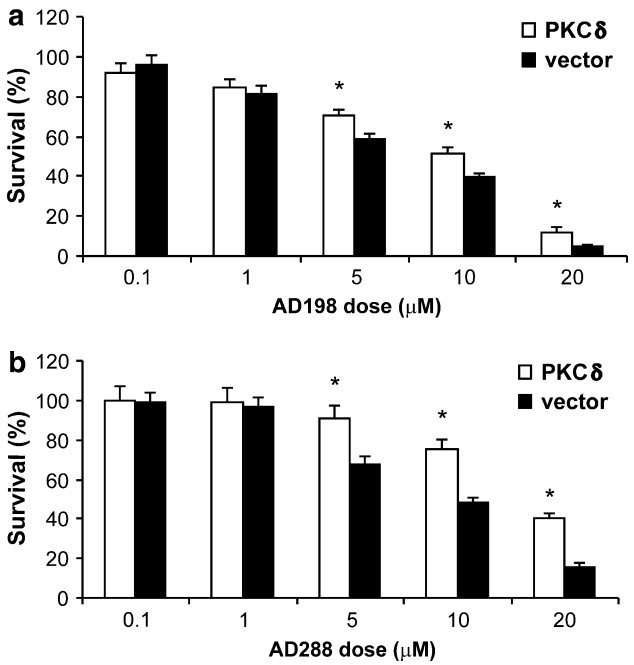


**Fig. 2** Involvement of PKCδ on cell death resistance against different antracyclins. **a** AD 198 treatment. Cells were treated with different doses of AD 198 (0.1–20 μM) for 1 h. Cell viability was evaluated 48 h later using the MTS assay. Values were compared to those obtained in both cell lines without treatment, which were set as 100% survival. Data expressed as the mean ± SD are representative of at least three independent experiments. * $P < 0.05$ versus NMuMG-vector cells treated with the same dose of AD 198 (Student's *t* test). **b** AD 288 treatment. Cells were treated with different doses of AD 288 (0.1–20 μM) for 1 h. Cell viability was evaluated 48 h later using the MTS assay. Values were compared to those obtained in both cell lines without treatment, which were set as 100% survival. Data expressed as mean ± SD ($n = 3$) are representative of at least three independent experiments. * $P < 0.05$ versus NMuMG-vector cells treated with the same dose of AD 288 (Student's *t* test)

**Table 1** Drug toxicity analysis of control and PKCδ overexpressing NMuMG cells

| | Lethal dose 50 (LD50) | |
|---|---|---|
| | AD 198 (μM) | AD 288 (μM) |
| NMuMG-PKCδ | $10.2 \pm 0.9^{*,\#}$ | $17.5 \pm 1.3^{*}$ |
| NMuMG-vector | $7.5 \pm 0.5^{\#}$ | $13.5 \pm 1.1$ |

* $P < 0.05$ versus NMuMG-vector cells treated with the same drug. Student's *t* test

# $P < 0.05$ versus the same cell line treated with AD 288. Student's *t* test

Data, expressed as the mean ± SD, are representative of three independent experiments

We found that AD 198 and AD 288 induced NMuMG cell death in a dose-dependent way with a higher cytotoxicity for AD 198 (Fig. 2a, b; Table 1). In addition, our results show that NMuMG-PKCδ cells were always about 30% more resistant to cell death than NMuMG-vector cells with any of the compounds (Fig. 2a, b; Table 1). Since both ADs have autofluorescence, we could analyze them by fluorescence microscopy. No differences between NMuMG-PKCδ and NMuMG-vector cells could be found either in the intensity or fluorescence pattern of any of the drugs, thus suggesting no differences in drug accumulation (data not shown).

## Involvement of PKCδ in cell death induced by AD 198 but not by AD 288

In order to confirm PKCδ involvement in cell death induced by AD 198, we applied two methodologies on NMuMG-PKCδ cells: PKC depletion by prolonged treatment with high doses of PMA (200 nM) and PKCδ inhibition by its pharmacological inhibitor Rott. Under PKC depletion conditions, cell death susceptibility to AD 288 was not altered, whereas a significant increase in cell survival was observed when cells were exposed to AD 198 (Fig. 3a). The extent of PKCδ inhibition is shown in Fig. 3b. This PKCδ dependency was still more evident when the enzyme was pharmacologically inhibited (Fig. 3c). As shown in Fig. 3d Rott also reduced PKCδ expression levels. These results confirm that PKCδ activation is mediating AD 198 effect, while AD 288 effect is independent of this kinase. The same degree of survival benefit to AD 198 was observed in NMuMG-vector cells treated with PMA or Rott (data not shown).

Western blot assays of NMuMG-PKCδ cells showed that only the treatment with AD 198 induced the appearance of the 40 kDa catalytic fragment of PKCδ, known to be involved in apoptosis promotion (Fig. 4, line 1). A significant increase in activated caspase 3 levels was also

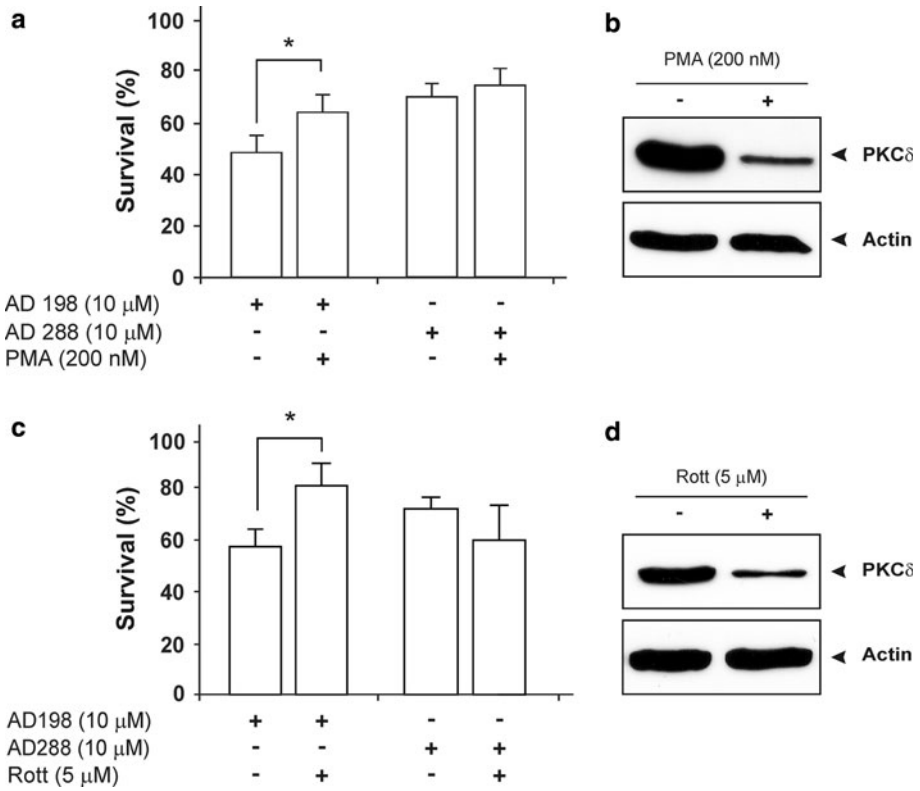


**Fig. 3** **a** Effect of PKC$\delta$ down-modulation on cell death induced by AD 198 and AD 288. NMuMG-PKC$\delta$ cells were treated with or without PMA (200 nM) to induce PKC depletion and then cells were incubated for 1 h with AD 198 or AD 288 (10 μM). Cell viability was evaluated 48 h later using the MTS assay. Values were compared to those obtained with cells treated with PMA but not with the AD compounds, which were set as 100% survival. PMA treatment alone did not alter cell survival. Data expressed as mean ± SD are representative of three independent experiments. * $P < 0.05$ versus NMuMG-PKC$\delta$ cells treated with PMA and AD 198 (Student's $t$ test). **b** Down-modulation of PKC$\delta$ levels by PMA treatment. Cell lysates prepared from NMuMG-PKC$\delta$ cells treated with or without PMA (200 nM) were subjected to western blot analysis using anti-PKC$\delta$ antibodies. Actin expression was used as protein loading control. A representative experiment is shown. **c** Effect of PKC$\delta$ inhibition on cell death induced by AD 198 and AD 288. NMuMG-PKC$\delta$ cells were treated with or without the pharmacological inhibitor of PKC$\delta$ Rott (5 μM) during 1 h and then cells were incubated for 1 h with AD 198 or AD 288 (10 μM). Cell viability was evaluated 48 h later using the MTS assay. Values were compared to those obtained with cells treated with Rott but not with the ADs, which were set as 100% survival. Data expressed as the mean ± SD are representative of three independent experiments. * $P < 0.05$ versus NMuMG-PKC$\delta$ cells treated with same dose of AD 198 (Student's $t$ test). **d** Down-modulation of PKC$\delta$ levels by Rott treatment. Cell lysates prepared from NMuMG-PKC$\delta$ cells, treated with or without rottlerin (5 μM), were subjected to western blot analysis using anti-PKC$\delta$ antibodies. Actin expression was used as protein loading control. A representative experiment is shown

found in these treatment conditions (Fig. 4, line 3). These results are consistent with the idea of the existence of a feedback loop between PKC$\delta$ and caspase 3, where caspase 3 cleaves PKC$\delta$ and the catalytic fragment generated may be acting upstream caspase 3 [32]. The inhibition of PKC$\delta$ with Rott prevented both the generation of the active PKC$\delta$ catalytic fragment and activation of caspase 3 induced by AD 198, confirming the involvement of PKC$\delta$ in these process (Fig. 4, lane 4, lines 1 and 3).

## Signaling pathways involved in NMuMG cell survival

Next, we determined which signaling pathways associated with cell survival were differentially modulated by PKC$\delta$ overexpressors and vector-transfected cells. As shown in Fig. 5a and b, we found that phosphorylated Akt (pAkt) levels were constitutively increased in NMuMG-PKC$\delta$ cells. In addition, nuclear NF-$\kappa$B levels were also increased in NMuMG-PKC$\delta$ cells concomitantly with a significant reduction in cytoplasmic I$\kappa$B$\alpha$ levels (Fig. 5c, lines 2 and 3). Using a gene reporter assay, we could also find a significant increase in NF-$\kappa$B activity in NMuMG-PKC$\delta$ cells (Fig. 5d).

Other molecules involved in survival/apoptotic pathways were also analyzed by western blot. As shown in Fig. 5c, an important increase in Bcl-2 and in pBad levels were detected in PKC$\delta$ overexpressors (lines 4 and 5, respectively). It is important to keep in mind that Bad phosphorylation prevents its apoptotic function. Other proteins such as Bad (Line 6), Bcl-XL, and Bax were not modulated by PKC$\delta$ overexpression (data not shown).

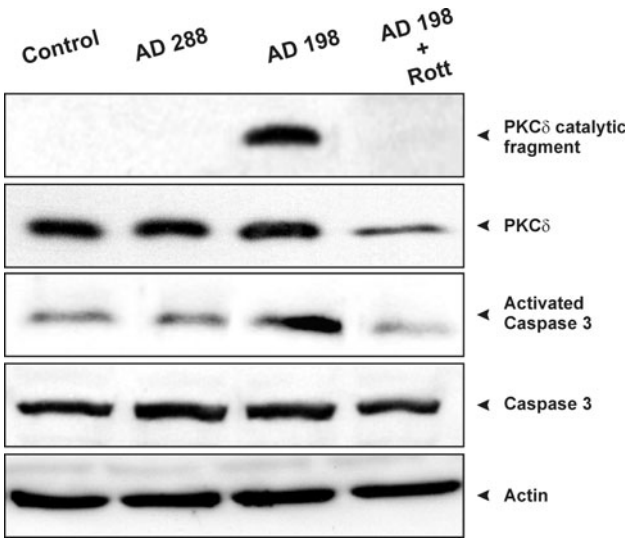


**Fig. 4** Induction of the 40 kDa PKC$\delta$ catalytic fragment and activated caspase 3 levels by AD 198 treatment. Cell lysates prepared from NMuMG-PKC$\delta$ cells, treated with or not 1 h with AD 198 (10 μM), AD 288 (10 μM), or AD 198 (10 μM) + Rott (5 μM) were resolved on 12% SDS-PAGE and blotted with anti-PKC$\delta$ and anti-caspase 3 antibodies (50 μg/lane). Actin expression level was used as protein loading control. A representative experiment is shown

### Role of PI3K/Akt and NF-$\kappa$B in the resistance against AD 198 and AD 288 cytotoxicity of NMuMG-PKC$\delta$ overexpressors

As PKC$\delta$ overexpression induces an increase in pAkt levels, next we evaluated whether the survival pathway PI3K/Akt was mediating the resistance of PKC$\delta$ expressing cells to both anthracyclines. For that NMuMG-PKC$\delta$ cells were treated with the PI3K inhibitor LY294002 before the treatment with the AD compounds. The inhibition of PI3K/Akt signaling pathway significantly enhanced the cytotoxic effect of AD 198 on NMuMG-PKC$\delta$ cells (Fig. 6a), making these cells looses the survival advantage over NMuMG-vector ones (LD50 AD 198 + LY294002: 3.1 ± 0.9 μM vs. 3.5 ± 0.5 μM in NMuMG-PKC$\delta$ and NMuMG-vector, respectively). On the other hand, the inhibition of this pathway did not enhance NMuMG-PKC$\delta$ cells susceptibility to AD 288 death induction (Fig. 6a), and NMuMG-PKC$\delta$ cells maintained their survival advantage over vector cells under this treatment (LD50 AD 288 + LY294002: 15.6 ± 1.3 μM vs. 11.4 ± 1.1 μM in NMuMG-PKC$\delta$ and NMuMG-vector, respectively, $P < 0.05$ Student's $t$ test). The effect of the PI3K inhibitor LY294002 on pAkt levels was confirmed by western blot analysis (Fig. 6b). No modulation of pAkt levels were observed upon ADs treatment (data not shown).

It was previously reported that AD 198 can override the anti-apoptotic effect of NF-$\kappa$B [2, 33]. The finding that the activity of this transcription factor is increased in PKC$\delta$ overexpressing cells prompted us to analyze the role of the NF-$\kappa$B on cell resistance against the different AD compounds. NF-$\kappa$B down-modulation was achieved by the transient expression of a super-repressor variant of I$\kappa$B$\alpha$, as described in "Materials and methods" section. The increased expression of this inhibitor (Fig. 6d) significantly reduced NF-$\kappa$B activity as determined by co-transfection assays with NF-$\kappa$B-Luc reporter gene (data not shown). Figure 6c shows that NF-$\kappa$B inhibition increased the susceptibility of PKC overexpressors to the cytotoxicity of both anthracyclines, suggesting that this transcription factor is probably mediating PKC$\delta$-induced cell death resistance. Moreover, NF-$\kappa$B seems to be a determinant factor for NMuMG cell survival independently of PKC$\delta$ expression levels, since its inhibition induced a major reduction in the LD50 for both drugs (LD50 AD 198: 1.24 ± 0.09 μM vs. 1.20 ± 0.2 μM in NMuMG-PKC$\delta$ and NMuMG-vector I$\kappa$B-transfected cells, respectively, and LD50 AD 288: 5.78 ± 0.6 μM vs. 5.01 ± 0.7 μM in NMuMG-PKC$\delta$ and NMuMG-vector I$\kappa$B-transfected cells, respectively). However, it is important to note that under any condition, cells were always more susceptible to AD 198 compound.

## Discussion

Most of the studies on PKC indicate that the $\delta$ isoform is involved in growth retardation or apoptosis induction in normal and transformed cell lines. There are only few studies, including our own, pointing out a pro-survival or anti-apoptotic role for PKC$\delta$ in different cell types, even conferring a more malignant phenotype and cytotoxic drug resistance [6, 10, 34, 35]. In this article, we test whether PKC$\delta$ overexpression could modulate cell death resistance to two antracyclines with different mechanism of action, given that we previously demonstrated that elevated PKC$\delta$ levels in NMuMG cells lead to an increased resistance to Dox and serum starvation induced cell death [6].

Using our model of NMuMG cells that overexpress PKC$\delta$, we analyzed the effect of AD 198, a Dox analog that binds to the C1b regulatory domain of classical and novel PKC isozymes (phorbol ester binding site) and induces cell death by activating these PKCs [2]. The effect of AD 198 treatment was compared to that obtained with AD 288, another anthracycline that induces cell death by generating DNA damage, similarly to Dox. Surprisingly, we found that while PKC$\delta$ overexpression induced a cell survival advantage, the treatment with a drug that activates this PKC isoform still induced evident cell death.

It is known that prolonged cell exposure to PKC activators can cause the degradation of the kinases. The treatment performed to induce this down-modulation significantly increased the resistance of PKC$\delta$ overexpressors

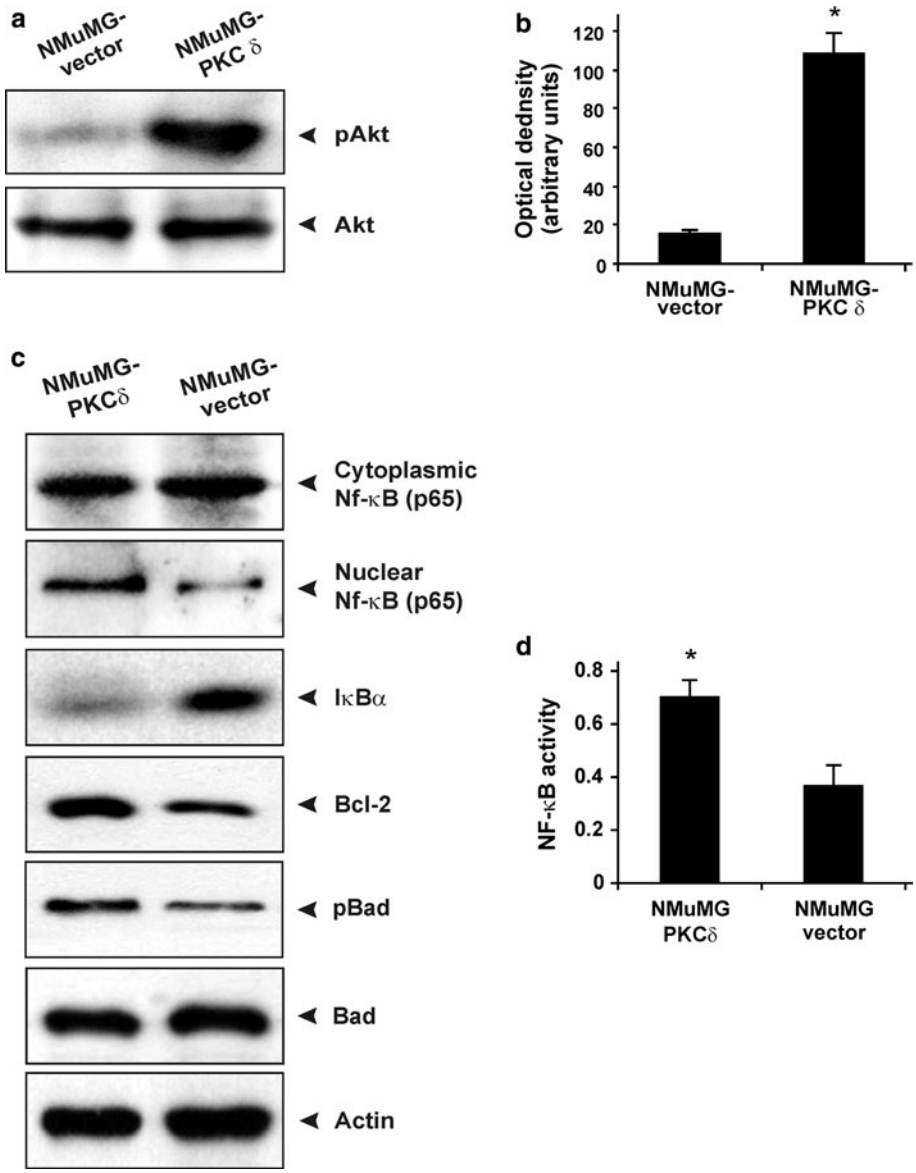


**Fig. 5** Signaling pathways involved in cell survival modulated by PKC$\delta$. **a** Elevated phospho-Akt levels in PKC$\delta$ overexpresors. Cell lysates prepared from control and PKC$\delta$ overexpressing cells were subjected to western blot analysis using anti-pAkt and anti-Akt antibodies. Total Akt level was used as protein loading control. Results are representative of three independent experiments. **b** Densitometric analysis of bands in panel **a**. * $P < 0.05$ versus NMuMG-vector cells without LY294002 treatment (Student's $t$ test). Data were expressed as the mean ± SD ($n = 3$). **c** Evaluation of apoptosis-associated proteins differentially expressed in PKC$\delta$ overexpressors by western blot. Nuclear and cytoplasmic protein fractions corresponding to NMuMG-vector and NMuMG-PKC$\delta$ cells were resolved on 10% SDS-PAGE and blotted with anti-NF-$\kappa$B (p65) and anti-I$\kappa$B$\alpha$ antibodies (only cytoplasmic fraction). Lanes were loaded with 50 μg protein for cytoplasmic determinations and 20 μg protein for nuclear determinations. Whole cell lysates were resolved on 12% SDS-PAGE (50 μg/lane) and blotted with anti-Bcl-2, anti-Bad, anti-pBad, and anti-actin antibodies. Actin expression was used as protein loading control. A representative experiment is shown. **d** NF-$\kappa$B gene reporter assay. NMuMG-PKC$\delta$ and NMuMG-vector cells were co-transfected with NF-$\kappa$B-Luc reporter plasmid and a Renilla luciferase vector as control. Luciferase activities were determined 48 h later as described in "Materials and methods" section and data were normalized to the constitutive Renilla luciferase activity. Data expressed as the mean ± SD are representative of three independent experiments. * $P < 0.05$ versus NMuMG-vector cells (Student's $t$ test)

to AD 198, probably due to the transient lack of the substrate necessary for the drug action. Similar results were obtained when NMuMG-PKC$\delta$ cells were pre-treated with the PKC$\delta$ inhibitor Rott, confirming that the mechanism of action of this drug involves PKC$\delta$. The finding that PMA or Rott treatments provided the same extent of survival benefit on vector-transfected cells confirms that this effect is not dependent on PKC$\delta$ expression levels. No effect on AD 288 cytotoxicity was observed when cells were either treated with Rott or PKC depleted.

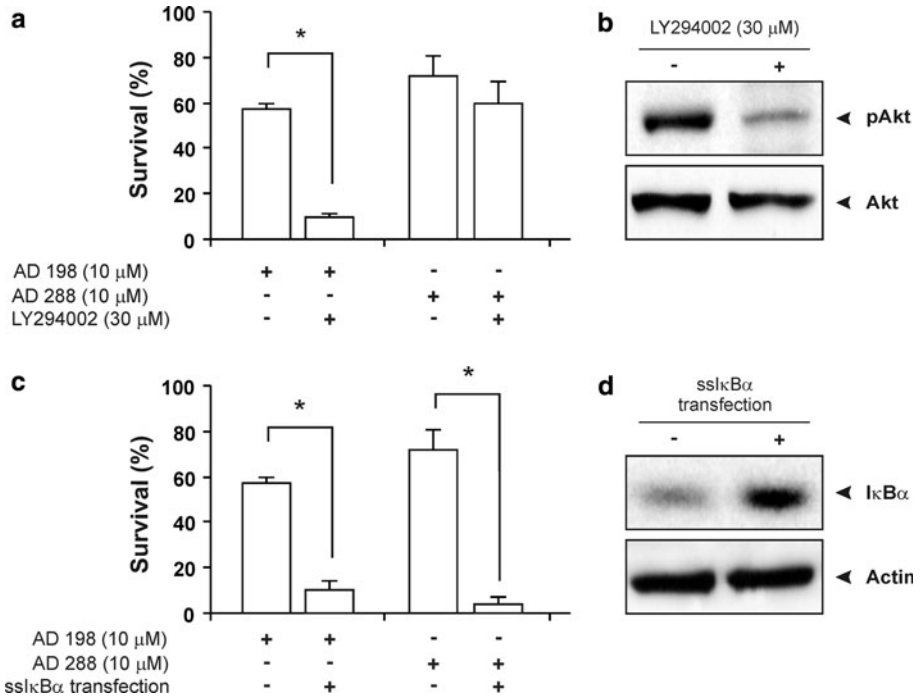


**Fig. 6** **a** Involvement of PI3K/Akt signaling pathway in the resistance against AD 198 and AD 288 cytotoxicity of PKC$\delta$ overexpressors. Cells were pretreated for or not for 2 h with the PI3K inhibitor LY294002 (30 μM) and then incubated with AD 198 or AD 288 (10 μM) for 1 h. Cell viability was evaluated 48 h later using the MTS assay. Values were compared to those obtained in NMuMG-PKC$\delta$ cells treated with LY294002, which were set as 100% survival. Data were expressed as the mean ± SD of three independent experiments. * $P < 0.05$ versus NMuMG-PKC$\delta$ cells treated with LY294002 (Student's $t$ test). **b** Reduction of p-Akt levels in PKC$\delta$ overexpresors. Cell lysates prepared from NMuMG-PKC$\delta$ cells pretreated with or without LY294002 (30 μM) were subjected to western blot analysis using anti-pAkt and anti-Akt antibodies. Total Akt level was used as protein loading control. A representative experiment is shown. **c** Role of the NF-$\kappa$B transcription factor in the resistance against AD 198 and AD 288 cytotoxicity of PKC$\delta$ overexpressing cells. NF-$\kappa$B down-modulation was achieved by the transient expression of a super-repressor variant of I$\kappa$B, as described in "Materials and methods" section. Control and I$\kappa$B transfectants were treated with AD 198 or AD 288 (10 μM) for 1 h. Cell viability was evaluated 48 h later using the MTS assay. Values were compared to those obtained in NMuMG-PKC$\delta$ cells transfected with I$\kappa$B repressor but not treated with ADs, which were set as 100% survival. Data expressed as the mean ± SD are representative of three independent experiments. * $P < 0.05$ versus the same cell line transfected with I$\kappa$B repressor (Student's $t$ test). **d** Transient expression of I$\kappa$B in NMuMG-PKC$\delta$ cells. Cell lysates prepared from NMuMG-PKC$\delta$ cells, transfected with or without a super-repressor variant of I$\kappa$B, were subjected to western blot analysis using anti-I$\kappa$B$\alpha$ antibodies. Actin expression was used as protein loading control. A representative experiment is shown

Although NMuMG cells were susceptible to the cytotoxicity of both anthracyclines, in all cases AD 198 LD50 was lower than AD 288 LD50. This result could be at least partially dependent on the increased activated caspase 3 levels observed in NMuMG-PKC$\delta$ cells treated with AD 198. It has been reported that PKC$\delta$ is a substrate of caspase 3; when caspase 3 is activated, PKC$\delta$ is cleaved, releasing a 40-kDa catalytic fragment. On the other hand, existence of a feedback loop between PKC$\delta$ and caspase 3 has also been proposed, thus indicating that PKC$\delta$ may regulate its own cleavage in response to apoptotic stimuli by activating caspase 3 [32]. Only AD 198 treatment was able to induce the PKC$\delta$ catalytic fragment, contributing to an increased level of activated caspase 3. This feedback loop involving PKC$\delta$ is probably more effective than DNA damage to induce caspase 3 activation; since in NMuMG-PKC$\delta$ cells treated with AD 288, no modulation of activated caspase 3 levels was observed.

Based in our previous results [6], it was surprising to find that activation of PKC$\delta$ by AD198 resulted in PKC$\delta$-mediated cell death. However, this could be partially explained by other authors' results that indicate that the PKC$\delta$ catalytic fragment has pro-apoptotic activity whereas the holoenzyme induces the opposite effect [32, 36]. Therefore, cell death induction by AD 198 could be explained as a balance between pro-survival signals induced by PKC$\delta$ crosstalk with different signaling pathways and pro-apoptotic signals induced by the generation of the PKC$\delta$ catalytic fragment.

Crosstalk between PKC$\delta$ and other signaling pathways, including ERK/MAPK, PI3K/Akt and NF-$\kappa$B, has been proposed by several authors [6, 35, 37, 38]. As our previous studies indicate, the MEK/ERK pathway is involved in PKC$\delta$-mediated proliferative behavior but not in cell survival [6]. We thus decided to investigate PI3K/Akt and NF-$\kappa$B, two of the main signaling pathways described as

modulated by PKC$\delta$ and also implicated in cell survival [22, 25, 26]. A substantial activation in both pathways was detected in PKC$\delta$ overexpressing cells. Akt hyperactivation returns to normal levels upon PI3K inhibition with LY940002, while NF-$\kappa$B activity could be reduced by I$\kappa$B transfection assays.

The role of Akt signaling in the response of mammary cells to anthracyclines was not analyzed previously by other authors since it is known that Akt is involved in the inactivating phosphorylation of Bax (member of the Bcl-2 family) and it is considered that AD 198 effect is independent of this gene family [39]. In this study, we demonstrate that the pharmacological inhibition of Akt pathway in NMuMG-PKC$\delta$ cells induces an important increase in AD 198 cytotoxicity, but has no effect on AD 288 activity. We still cannot explain why the inhibition of PI3K/Akt did not increase the cytotoxic effect of AD 288 on NMuMG-PKC$\delta$ cells. In this regard, the increased Bcl-2 levels displayed by PKC$\delta$ overexpressing cells could be involved in the augmented resistance to AD 288 cytotoxic affect. A similar effect could be consequence of increased pBad levels; since it has been described that Bad phosphorylation prevents its pro-apoptotic function. These last observations suggest that PKC$\delta$ overexpression may induce cell death resistance to AD 288 by a mechanism that involves other signaling pathways independent of PI3K/Akt.

In contrast to the results obtained with Akt inhibition, the down-modulation of NF-$\kappa$B induced a 10-fold increase in cell death induced by any of the AD compounds, with a concomitant loss of the survival advantage that PKC$\delta$ overexpressing cells displayed. These results strongly suggest that this transcription factor is highly involved in PKC$\delta$-mediated resistance to different apoptosis inducers. This was an unexpected result since NF-$\kappa$B has been reported as a transcription factor involved in the expression of Bcl-2, and this antiapoptotic protein should only be involved in the resistance against AD 288, since several reports indicate that AD 198 can override the anti-apoptotic effects of Bcl-2 and NF-$\kappa$B [2, 33, 39].

It was described that AD 198 induces apoptosis overriding multiple mechanisms of cell drug resistance including those mediated by overexpression of the multidrug transporters P-glycoprotein and multidrug resistance protein (MRP), Bcl-2, and p53 mediated resistance [2, 33, 39, 40]. In our model, PKC$\delta$ overexpression increased Bcl-2 levels and the modulation of this protein, together with the increased pBad levels, should be responsible for the reduction in AD 288-dependent apoptosis displayed by NMuMG-PKC$\delta$ cells related to vector ones, but does not explain results obtained with AD 198.

Although PKC$\delta$ activation is clearly involved in AD 198 cytotoxicity, the possibility that other proteins containing C1 domains play a role cannot be dismissed. In fact, AD 198 has also been shown to competitively inhibit phorbol ester binding to $\beta$2-chimaerin, a non-kinase phorbol ester receptor containing a C1 domain, and theoretically AD 198 could also bind to other chimaerins (Rac-GTPase-activating proteins), to ras guanyl-releasing protein (Ras-GRP), to Unc-13/Munc-13 protein family involved in exocytosis, and to protein kinase D [39]. Therefore, we cannot rule out an effect dependent on these signaling pathways involved in the resistance against AD 198.

Our findings support the idea that the study of the mechanisms involved in the pro- or anti-apoptotic responses mediated by PKC$\delta$ to different chemotherapeutic drugs could be of great impact in the design of PKC modulators as therapeutic agents for cancer treatment.

**Acknowledgments** The authors thank Dr. Martin Krasnapolski for his constant support and useful discussions. This work was supported by grants from ANPCyT (PICT 00417) and UBACyT (M003) awarded to Elisa Bal de Kier Joffé. Alejandro J. Urtreger, Laura B. Todaro and Elisa D. Bal de Kier Joffé are members of the National Council of Scientific and Technical Research (CONICET).

**Conflicts of interest statement** The authors declare that they have no conflict of interest.

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