



Involvement of protein kinase C- δ in DNA damage-induced apoptosis

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Received 17.11.00; revised 27.2.01; accepted 30.3.01
Edited by S Martin

Abstract

We have previously shown that the protein kinase C (PKC) signal transduction pathway regulates cell death by the DNA damaging agent *cis*-diamminedichloroplatinum(II) (cDDP). In the present study we have investigated how PKC influences the sequence of events that are triggered by cDDP-induced DNA damage. cDDP caused activation of caspases-8, -9, -3, -7 and cleavage of PKC δ . Rottlerin, a selective inhibitor of novel PKC δ , blocked activation of caspases, proteolytic activation of PKC δ and cell death induced by cDDP. In contrast, Gö 6976, an inhibitor of conventional PKC α and β 1, did not prevent cDDP-induced caspase activation and cDDP cytotoxicity. In HeLa cells, PKC δ was distributed both in the cytosol and heavy membrane (HM) fraction containing mitochondria. While caspase-8 was primarily cytosolic, a small amount of caspases-9, -7 and -3 could be detected in the HM fraction. cDDP caused a time-dependent increase in Cytochrome *c* release from the mitochondria and processing of both cytosolic and membrane-associated caspases, as well as proteolytic cleavage of PKC δ . Rottlerin attenuated late but not early release of Cytochrome *c* by cDDP. It, however, inhibited activation of caspases and proteolytic cleavage of PKC δ in both cytosolic and HM fractions. The antiapoptotic effect of rottlerin was evident when it was added together with or following cDDP addition but not when added after cDDP was removed from the medium. Thus, the PKC δ inhibitor acts at an early stage of the cDDP-induced cell death pathway that precedes caspase activation. *Cell Death and Differentiation* (2001) 8, 899–908.

Keywords: cisplatin; protein kinase C; caspases; apoptosis; Cytochrome *c*

Abbreviations: Ac-DEVD-AFC, Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarine; Apaf-1, apoptotic protease activating factors; BIM, bisindolylmaleimide; cDDP, *cis*-diamminedichloroplatinum(II); CF, catalytic fragment; Cox-II,

cytochrome oxidase subunit II; HM, heavy membrane; MBP, myelin basic protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; aPKC, atypical PKC; cPKC, conventional PKC; nPKC, novel PKC; PS, phosphatidylserine; Rot, rottlerin; TNF, tumor necrosis factor- α ; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Introduction

Caspases are essential for the execution of cell death by apoptotic stimuli.^{1–4} The pathway of cell death varies depending on the cell type as well as the apoptotic stimuli. It is generally believed that binding of Fas ligand or tumor necrosis factor- α (TNF) to their receptors causes activation of the initiator caspase-8 followed by the activation of a caspase cascade to execute cell death.^{2,5} In contrast, DNA damaging agents are known to induce release of mitochondrial Cytochrome *c*, which facilitates the interaction of apoptotic protease activating factor (Apaf-1) with procaspase-9 to initiate the activation of downstream effector caspases, such as caspases-3 or -7 to cause cell death.³ Both receptor-mediated and anticancer drug-induced apoptosis may, however, involve more than one pathway and there may be cross-talk between these two pathways.^{6–8}

cis-Diamminedichloroplatinum(II) (cDDP or cisplatin) is one of the most important anticancer agents used for the treatment of solid tumors.⁹ Although the antitumor activity of cDDP is believed to be due to its interaction with chromosomal DNA, only a small fraction of cDDP actually interacts with DNA and inhibition of DNA replication cannot solely account for its biological activity.¹⁰ The efficacy of chemotherapeutic drugs not only depends on their ability to induce DNA damage but also on the cell's ability to detect and respond to DNA damage.¹¹ cDDP, like other chemotherapeutic drugs, causes activation of caspases although the sequence of events that follow cDDP-induced DNA damage and lead to apoptosis remains to be unraveled.

We and others have shown that the PKC signal transduction pathway regulates cell death by cDDP.^{12–17} PKC is a family of 11 isozymes that are classified as the conventional PKCs (α , β 1, β 2 and γ), novel PKCs (δ , ϵ , η , and θ), atypical PKCs (ζ and λ) and novel/atypical PKC μ .¹⁸ PKC δ is a substrate for caspase-3 and the catalytic fragment of PKC δ has been directly associated with apoptotic cell death.^{19,20} We have, however, demonstrated that downregulation of PKC that decreased the abundance of PKC δ catalytic fragment was associated with increased cellular sensitivity to cDDP.¹⁷ These results raise the possibility that PKC acts upstream of caspases to regulate cell death by cDDP. It is not known which PKC

isozyme regulates activation of caspases and which step(s) of the cisplatin-induced cell death pathway is regulated by PKC.

Mitochondria play a pivotal role in the decision making process of a cell's life and death.²¹ It is believed that once Cytochrome *c* is released from mitochondria, cells are committed to die.²² An inability to induce release of Cytochrome *c* from mitochondria has been associated with cellular resistance to anticancer agents, including cDDP.²³ In the present study, we have investigated how PKC regulates release of Cytochrome *c* and activation of caspases that emanate from mitochondria. Our results show that in HeLa cells, PKC δ was localized not only in the cytosol but also in the mitochondrial fraction and cDDP induced processing of both cytosolic and membrane-associated PKC δ . Rottlerin, a specific inhibitor of PKC δ , blocked cDDP-induced activation of caspases and proteolytic cleavage of PKC δ in both cytosolic and HM fractions but inhibited only late but not early release of Cytochrome *c*. Furthermore, inhibition of nPKC δ , but not of cPKCs, protected cells against cDDP-induced cell death. Taken together, these results demonstrate that PKC δ influences cDDP-induced cell death by acting at an early step of the mitochondrial cell death pathway that precedes activation of caspases.

Results

Effects of PKC inhibitors on cDDP-induced cell death

We have previously shown that the PKC signal transduction pathway regulates cell death by cDDP.¹⁴⁻¹⁶ To examine the involvement of a specific PKC isozyme in cDDP-induced cell death, we compared the effects of several PKC inhibitors that

exhibit distinct specificities towards PKC isozymes on the cytotoxic effect of cDDP. Bisindolylmaleimide (BIM) inhibits all PKC isozymes with a rank order of potency of $\alpha > \beta I > \epsilon > \delta > \zeta$.²⁴ As shown in Figure 1, 1 μ M BIM did not influence cDDP sensitivity whereas 10 μ M BIM increased the IC₅₀ of cDDP by twofold. In contrast, Gö 6976, an inhibitor of cPKCs,²⁴ increased cDDP sensitivity by twofold, however it decreased HeLa cell proliferation by almost 50%. In addition, the effect of Gö 6976 on cDDP sensitivity was similar at both 1 and 10 μ M concentrations. Rottlerin, a specific inhibitor of nPKC δ ,²⁵ decreased cellular sensitivity to cDDP in a concentration-dependent manner; the IC₅₀ of cDDP was increased from 10.3 to 28.5 μ M by 10 μ M rottlerin and to greater than 40 μ M when cells were pretreated with 20 μ M rottlerin.

To examine if rottlerin specifically inhibits PKC δ activity in HeLa cells, we immunoprecipitated PKC δ from HeLa cells and determined its activity *in vitro* using myelin basic protein (MBP) as the substrate. Figure 2 shows that TPA caused significant phosphorylation of MBP and rottlerin blocked MBP phosphorylation by TPA, suggesting that rottlerin inhibits PKC δ activity. To check for the specificity of rottlerin, we examined the effect of rottlerin on cPKC α activity. As shown in Figure 2, 10 μ M rottlerin had only a slight effect on Ca²⁺-, PS/TPA-dependent phosphorylation of MBP by cPKC α although Gö 6976 completely inhibited cPKC α activity. Gö 6976 also appears to decrease MBP phosphorylation by PKC δ . The amount of PKC δ was, however, less in the reaction mixture as evident by the PKC δ immunoblot.

cDDP, like most chemotherapeutic drugs, is known to induce cell death by apoptosis.¹⁰ Figure 3 shows that when cells were treated with 10 μ M cDDP, cells rounded up and detached from the tissue culture dish; the number of floating cells increased further by 20 μ M cDDP. Rottlerin by

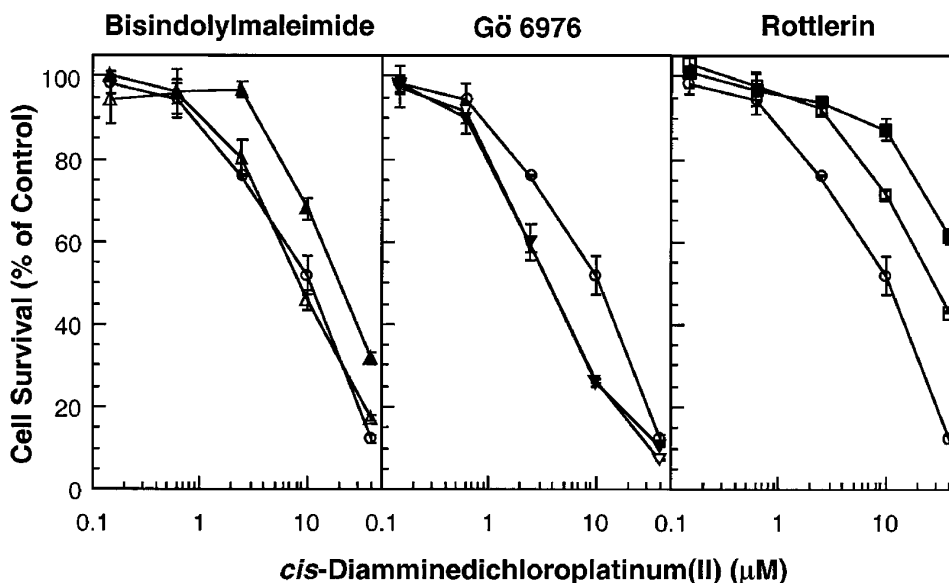


Figure 1 Effects of PKC inhibitors on cell death by cDDP. HeLa cells were pretreated without or with BIM, Gö 6976 or rottlerin for 1 h and then treated with different concentrations of cDDP for 2 h. The cell survival was determined after 24 h by an MTT assay as described in Materials and Methods. Qualitatively similar results were obtained when cells were treated continuously with cDDP. The results are representative of at least three experiments performed in quadruplicate. ○, control; ▲, 1 μ M BIM; ▴, 10 μ M BIM; ▽, 1 μ M Gö 6976; ▾, 10 μ M Gö 6976; □, 10 μ M rottlerin; ■, 20 μ M rottlerin

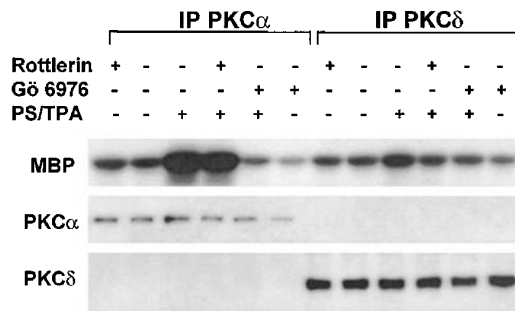


Figure 2 Effect of rottlerin and Gö 6976 on PKC activity. PKC α or δ was immunoprecipitated from HeLa cells using an antibody to PKC α or δ . Cells were treated with or without 10 μ M rottlerin or 5 μ M Gö 6976 and/or 0.2 mg/ml phosphatidylserine and 100 nM TPA. 0.5 mM CaCl₂ was also added with PKC activators in PKC α immunokinase assay. PKC activity was determined using MBP as the substrate and SDS-PAGE was performed as described under Materials and Methods. Autoradiography was performed with the dried gel and Western blot analyses were performed with PKC α and δ antibodies following transfer of proteins to PVDF membrane. Results are representative of 2–4 individual experiments

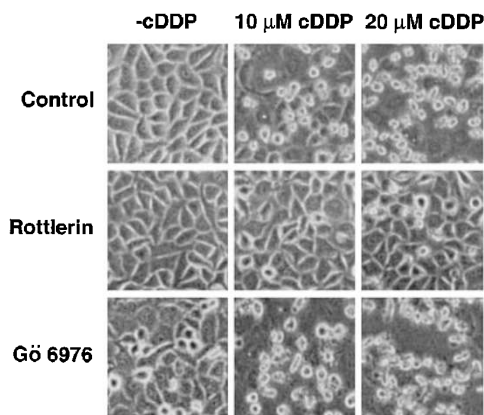


Figure 3 Effects of PKC inhibitors on cDDP-induced apoptotic morphology. HeLa cells were treated without or with 10 μ M rottlerin or Gö 6976 for 30 min and then with or without 10 or 20 μ M cDDP for 16 h. The cellular morphology was examined under a microscope

itself had no effect on cellular morphology but it reversed the effect of cDDP. In contrast, Gö 6976 was unable to block the effect of cDDP. These results suggest that inhibition of nPKC δ rather than cPKCs was responsible for the attenuation of cDDP-induced cell death.

Effects of PKC inhibitors on the proteolytic activation of PKC δ

Proteolytic cleavage of PKC δ by caspase-3 results in the generation of the catalytic fragment of PKC δ , thereby activating it.¹⁹ Figure 4 shows that cDDP caused cleavage of full-length PKC δ as evident by the decrease in 78-kDa full-length PKC δ with a concomitant increase in the 40-kDa catalytic fragment of PKC δ . Pretreatment of HeLa cells with 10 μ M rottlerin decreased proteolytic cleavage of PKC δ , whereas the cPKC inhibitor Gö 6976 was unable to block cDDP-induced proteolytic activation of PKC δ , suggesting that

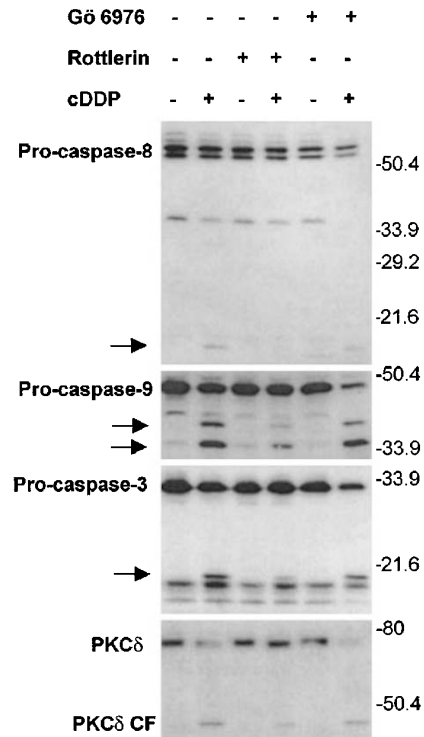


Figure 4 Effects of PKC inhibitors on cDDP-induced caspase activation and proteolytic cleavage of PKC δ . HeLa cells were pretreated with or without 10 μ M Gö 6976 or rottlerin for 30 min and then with 20 μ M cDDP for 8 h. Western blot analysis was performed with antibody to caspases-8, -9, -3 and PKC δ . The arrows indicate the processed forms. Results are representative of 3–7 experiments

cPKC inhibitor had no effect on the proteolytic activation of PKC δ in intact cells even at 10 μ M concentrations.

Since caspase-3 may be cleaved both by caspases-8 and -9, we examined the effect of rottlerin on cDDP-induced activation of caspases-8 and -9. As shown in Figure 4, an 8 h exposure of HeLa cells to cDDP had little effect on the activation of caspase-8 but did induce processing of caspases-9 and -3. Rottlerin but not Gö 6976 blocked cDDP-induced activation of these caspases. These results suggest that inhibition of caspase-9 by rottlerin blocks activation of caspase-3 and thus proteolytic cleavage of PKC δ .

Since cDDP caused proteolytic cleavage of PKC δ , we examined the effect of the PKC δ -specific inhibitor rottlerin on cDDP-induced activation of PKC δ . Figure 5 shows that a 12 h exposure to 20 μ M cDDP caused almost twofold stimulation of MBP phosphorylation in the absence of any lipid cofactors, suggesting that the catalytic fragment generated by cDDP was constitutively active. Rottlerin completely blocked activation of PKC by cDDP. These results suggest that rottlerin inhibits cDDP-induced activation of PKC δ .

Effect of cDDP and rottlerin on the activation of cDDP-induced caspase cascade

It is generally believed that caspases-8 and -9 are the apical caspases for receptor- and drug-induced apoptosis, respec-

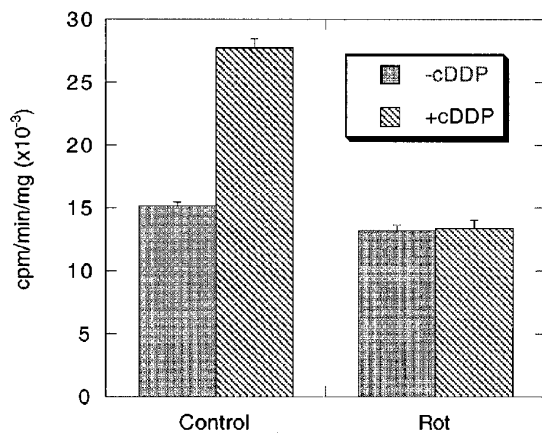


Figure 5 Effect of cDDP and rottlerin on the activation of PKC. Cells were pretreated with 10 μ M rottlerin for 1 h and then treated with or without 20 μ M cDDP for another 12 h. PKC activity was determined using MBP as the substrate as described under Materials and Methods. Results are representative of two experiments performed in duplicates

tively.^{3,5} It has recently been shown that both receptor and chemical-induced apoptosis can involve more than one pathway. For example, the anticancer agent etoposide caused activation of caspases-8, -9, -3 and -7 simultaneously.⁶ Furthermore, although the formation of the apoptosome complex and activation of caspase-9 and its downstream caspases are known to take place in the cytoplasm,²¹ little is known about compartmentalized activation of caspases in response to apoptotic stimuli. Since mitochondria play an important role in drug-induced apoptosis, we compared the effect of cDDP on the activation of caspases in both cytosolic and mitochondrial compartments. Although caspases are primarily localized in the cytoplasm, a small amount of caspase-9 could be detected in the crude mitochondrial fractions (Figure 6). A 6 h treatment with cDDP caused proteolytic cleavage of the 48-kDa procaspase-9 to 37- and 35-kDa processed forms in the cytosolic fraction. When duration of exposure to cDDP was increased, processing of procaspase-9 was evident in both cytosolic and membrane fractions.

Caspase-8 was primarily cytosolic and a 6 h exposure of HeLa cells to cDDP had no effect on the processing of procaspase-8. Processing of the 55-kDa procaspase-8 to 14- and 10-kDa fragments was apparent after 9 h but the active forms of caspase-8 were present exclusively in the cytosol of cDDP-treated cells. Since caspase-8 acts primarily through the receptor-mediated pathway and it has been reported that the membrane death receptor CD95 (Apo-1/Fas) is upregulated after cDDP exposure in HeLa cells,²⁶ we also examined the effect of neutralizing anti-FasL antibody on cDDP-induced cell death. A 16 h exposure to 20 μ M cDDP caused 70% cell death as determined by the appearance of a sub-G1 peak in FACS analysis. Preincubation with 5 μ g/ml of FasL neutralizing antibody for 30 min prior to cDDP addition resulted in 76% cell death. In addition, the neutralizing anti-FasL antibody had no effect on caspase activation (data not shown).

In HeLa cells, caspase-3 was present primarily in the cytosol. A 6 h exposure to cisplatin had little effect on the processing of procaspase-3 although faint bands could be detected near the 20-kDa region. The distribution and processing of caspase-7 was essentially similar to caspase-3 and with increasing duration of exposure to cDDP, the presence of active fragments could be detected in the HM fractions. We also determined the effect of rottlerin on cDDP-induced activation of caspases by measuring the cleavage of the fluorometric substrate Ac-DEVD-AFC. As indicated in Figure 6, the majority of DEVDase activity was present in the cytosolic fraction although a small DEVDase activity could be detected in the HM fraction. cDDP caused a time-dependent increase in DEVDase activity both in the cytosol and HM fractions and rottlerin inhibited DEVDase activity in both fractions. Thus, the caspase activity assay corroborated our results with Western blot analyses.

Since rottlerin blocked activation of the apical caspase-9, which was present both in the cytosol and HM fraction, we determined the subcellular distribution of PKC δ following treatment with cDDP. As shown in Figure 6, PKC δ was distributed both in the cytosol and HM fractions. A 6 h exposure of HeLa cells to cDDP caused cleavage of PKC δ in the cytosolic fraction, and by 12 h both cytosolic and mitochondrial PKC δ were processed almost completely. Rottlerin by itself had no effect on the subcellular distribution of PKC δ but it inhibited proteolytic cleavage of PKC δ in both fractions. These results suggest that rottlerin blocked proteolytic activation of PKC δ by inhibiting activation of caspase-9 and its downstream caspases.

Effect of cDDP and rottlerin on Cytochrome c release

Since release of mitochondrial Cytochrome c is believed to be one of the early events following DNA damage, we examined whether rottlerin inhibits the egress of Cytochrome c from the mitochondria. As shown in Figure 7A,B, cDDP caused a time-dependent increase in Cytochrome c release. Pretreatment of HeLa cells with rottlerin decreased release of Cytochrome c following an exposure to cDDP for ≥ 9 h but not when cells were treated with cDDP for less than 9 h. We consistently found that rottlerin by itself caused a small release of Cytochrome c. We probed the blot with anti-human Cytochrome oxidase subunit II (Cox-II) and Apaf-1 as markers for mitochondria and cytosol, respectively. The low abundance of Cox-II in the HM fractions following exposure to cDDP for 3 h could explain the decreased levels of Cytochrome c in those fractions. We also performed a parallel experiment to monitor cell death by FACS analysis. Without any cDDP treatment, 2–3% of cells appeared in the sub-G1 peak and treatment with cDDP for 3 and 6 h had little effect on cell death. The fraction of cells undergoing apoptosis increased to 10 and 26% following exposure to cDDP for 9 and 12 h, respectively, and rottlerin reduced the fraction of cells undergoing apoptosis to 4 and 6%, respectively. Since activation of caspase-9 appears to be important for cDDP induced cell death and Cytochrome c is believed to activate

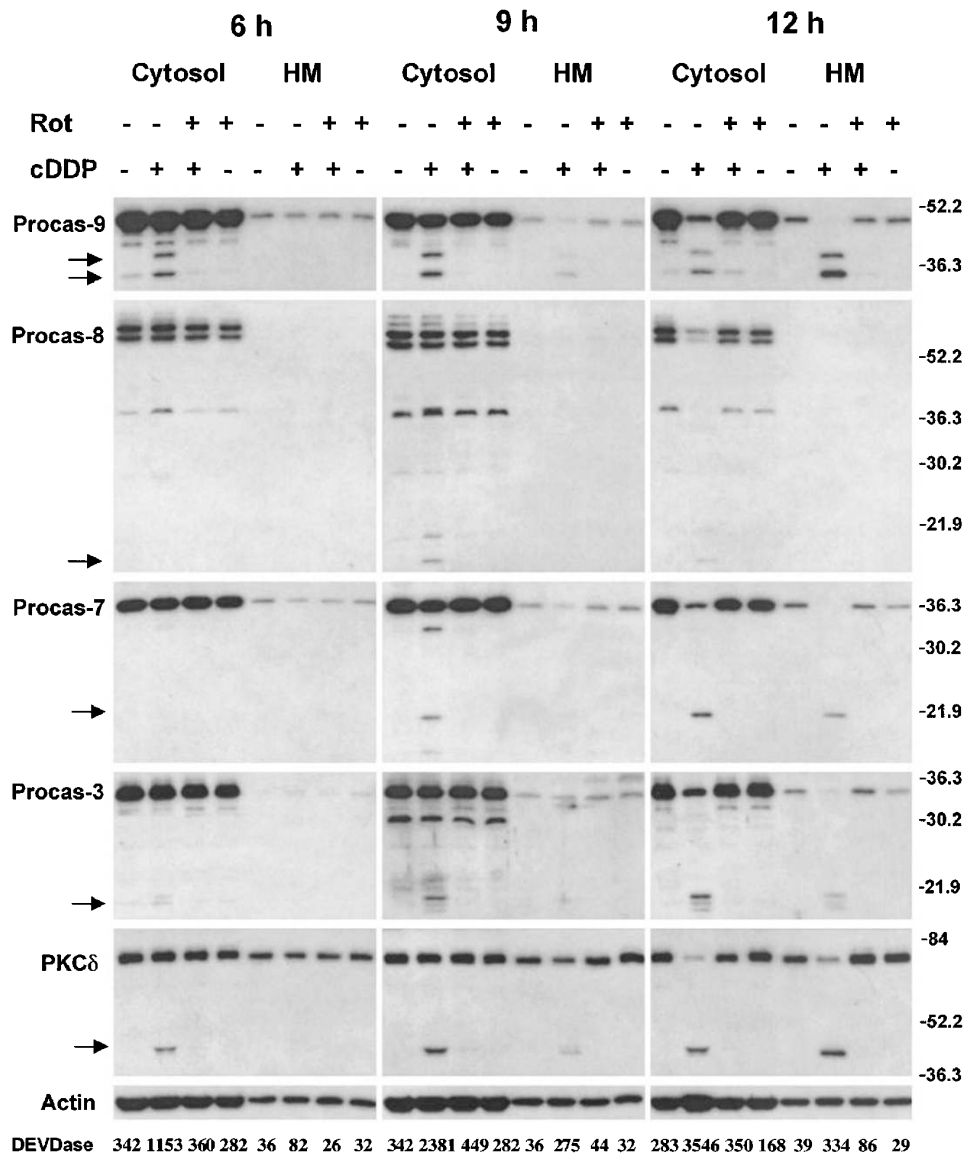


Figure 6 Effect of cDDP and rottlerin on the compartmentalized activation of caspases and PKC δ . HeLa cells were pretreated with or without 10 μ M rottlerin and then treated with or without 20 μ M cDDP for 6, 9 or 12 h. Cells were fractionated into cytosol and heavy membrane fraction containing mitochondria and immunoblot analyses were performed using indicated antibodies as described under Materials and Methods. The arrows indicate the processed forms. Results are representative of 3–6 experiments

caspase-9, we also monitored processing of procaspase-9 following exposure to cDDP. cDDP induced a time-dependent activation of caspase-9 and rottlerin inhibited cDDP-induced activation of caspase-9 in both cytosolic and mitochondrial fractions even though it did not prevent Cytochrome *c* release completely. Thus, the effect of rottlerin on cDDP-induced cell death correlated with its effect on caspase activation rather than on Cytochrome *c* release.

Effect of rottlerin incubation time on cDDP-induced cell death

To examine if rottlerin acts at an early or late stage of the cDDP-induced cell death pathway, we added rottlerin either

before, together with, or after cDDP addition. The apoptotic cells were monitored by their appearance in the subG0/G1 peak in a flow cytometer. When cells were treated with 15 μ M cDDP for 16 h, 54% cells appeared in the subG0/G1 phase (Figure 8A). Pretreatment of cells with rottlerin for 1 h prior to cDDP addition decreased the number of apoptotic cells to 12%. The effect of rottlerin on cDDP-induced apoptosis was similar when rottlerin was added together with or 1 h following cDDP treatment. When rottlerin was added 2 or 4 h subsequent to cDDP treatment, the fraction of cells in the subG0/G1 peak increased to 23 and 45%, respectively. There was a good correlation between the ability of rottlerin to inhibit caspase activation and to block cDDP-induced cell death (data not shown). Furthermore, when rottlerin was added after

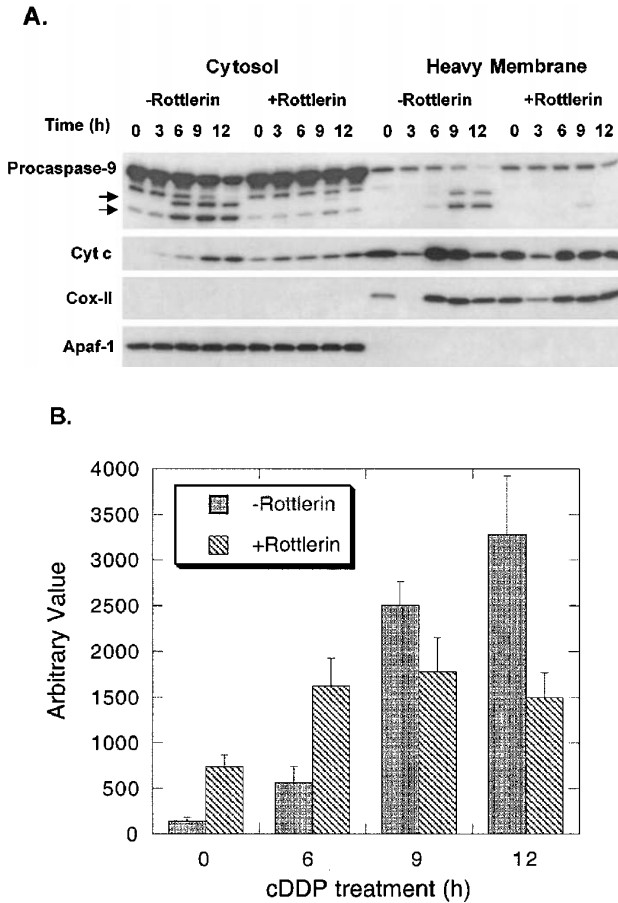


Figure 7 Effect of cDDP and rottlerin on the release of Cytochrome *c* from mitochondria. Cells were pretreated with or without 10 μ M rottlerin for 30 min and then treated with or without 20 μ M cDDP. (A) At indicated time intervals, cells were processed and immunoblot analyses were performed with cytosolic and HM fractions using indicated antibodies. (B) Cytochrome *c* released in the cytosol was quantified by scanning immunoblots with a laser densitometer and the values are the mean \pm standard error of 3–5 individual experiments

cDDP treatment was initiated, its ability to prevent Cytochrome *c* release decreased (Figure 8B). These results suggest that rottlerin acts at an early stage of the cDDP-induced cell death pathway.

Discussion

Although it is well-established that the PKC signal transduction pathway regulates cell death mediated by a variety of apoptotic stimuli, including the DNA damaging agent cDDP, it is not clear how PKC influences cell death by these agents. While activation of caspases is necessary to execute cell death, it is also important to inactivate the regulatory proteins that otherwise block caspase activation. Several intracellular signaling molecules, including Akt/PKB and MEK kinase, have been shown to regulate activation of caspases.³ It is believed that PKC acts at a late stage of the cell death pathway since PKC δ and θ are substrates for caspase-3^{19,27} and the catalytic fragment of these PKCs may induce apoptotic morphology.^{19,20} We have provided evidence that the PKC signal transduction

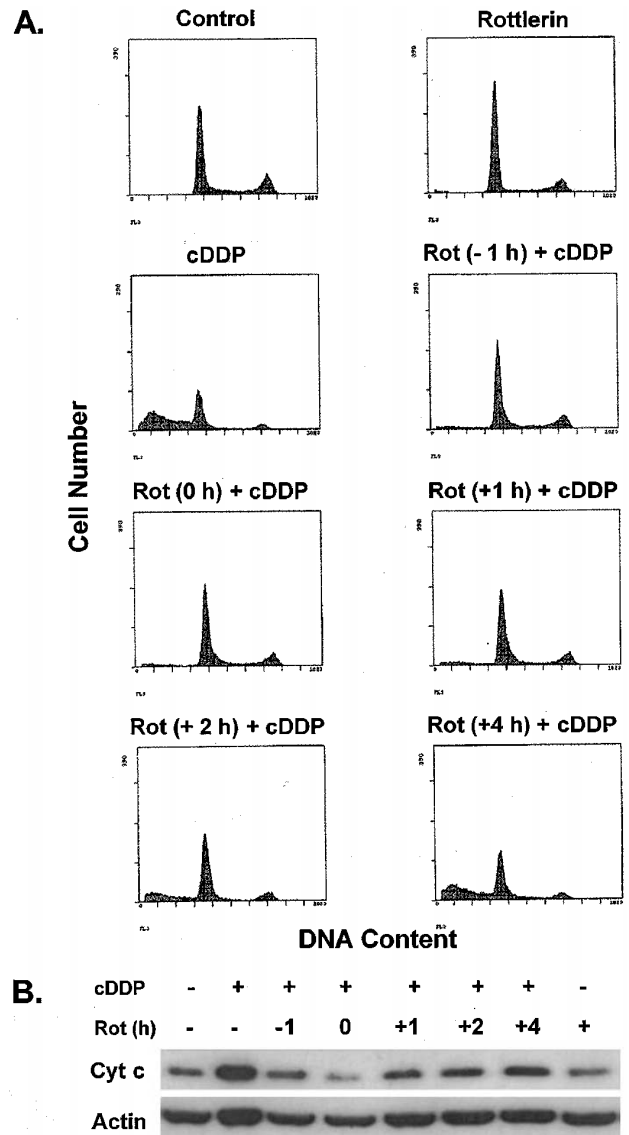


Figure 8 Effect of time of addition of rottlerin on cDDP induced cell death and Cytochrome *c* release. HeLa cells were treated with 15 μ M cDDP for 16 h. The time of cDDP addition was considered 0 h. Rottlerin (10 μ M) was added either 1 h prior to cDDP addition (–1 h), concomitantly with cDDP (0 h) or 1, 2 or 4 h after cDDP addition. (A) Cells were stained with propidium iodide and analyzed using a flow cytometer. The results are representative of 2–3 individual experiments. (B) Immunoblot analysis was performed with cytosolic extracts using antibody against Cytochrome *c* or actin as described under Materials and Methods

pathway in fact acts upstream of caspase-3.¹⁷ The results of the present study demonstrate that in HeLa cells PKC δ is constitutively associated with the HM fraction containing mitochondria and cisplatin induced proteolytic activation of PKC δ in both fractions. Furthermore, while inhibition of PKC δ only partially influences cDDP-induced Cytochrome *c* release, it inhibits cDDP-induced activation of caspases in both the cytosol and mitochondria. Thus, PKC δ acts at an early stage of the mitochondrial cell death pathway that precedes activation of caspase-9 to regulate cell death triggered by cDDP.

In the present study, we have used PKC inhibitors that exhibit distinct selectivity towards PKC isozymes to establish the involvement of a particular PKC isozyme in the regulation of cell death by cDDP. Bisindolylmaleimide inhibits all PKC isozymes, albeit with different potencies; cPKCs are the most sensitive and their IC_{50} values are in the nanomolar range in *in vitro* kinase assays, nPKCs can be inhibited in the submicromolar range whereas aPKCs are least sensitive to BIM.²⁴ We have shown that 1 μ M BIM that was sufficient to inhibit cPKCs in intact cells had no effect on cDDP-induced cell death whereas 10 μ M BIM decreased cDDP cytotoxicity, suggesting that inhibition of nPKCs or aPKCs was responsible for protection against cDDP cytotoxicity by BIM. Rottlerin inhibits PKC isozymes with IC_{50} values for PKC δ , 3–6 μ M, cPKCs, 30–40 μ M and PKC ϵ , - η and - ζ , 80–100 μ M.²⁵ We have shown that rottlerin at concentrations ≤ 20 μ M inhibited cDDP-induced cell death significantly, suggesting that inhibition of nPKC δ rather than inhibition of cPKCs, nPKC ϵ or aPKCs was important for the decrease in cellular sensitivity to cDDP by rottlerin. This is further corroborated by the observation that Gö 6976, which inhibits cPKC α and β 1 at nanomolar concentrations but has little effect on nPKCs or aPKCs,²⁴ failed to block cDDP-induced cell death even at 10 μ M concentrations. In fact, it increased cDDP cytotoxicity by twofold. We have found that even 1 μ M Gö 6976 by itself inhibited HeLa cell proliferation by almost 50%. Therefore, the increase in cellular sensitivity to cDDP by Gö 6976 could be secondary to its toxic side effect. In addition, rottlerin, but not Gö 6976, inhibited proteolytic cleavage of PKC δ by cDDP. These results suggest that nPKC δ rather than cPKCs influenced activation of caspase-3 and thus proteolytic activation of PKC δ by cDDP. This is consistent with the report that inhibition of nPKC δ by rottlerin partially or totally blocked all parameters of etoposide-induced apoptosis although inhibition of cPKC α and - β 1 by Gö 6976 had a slight effect on caspase-3 activation and apoptotic morphology in salivary gland acinar cells.²⁸

It is generally believed that caspase-8 is activated by the receptor-mediated pathway whereas caspase-9 is activated in response to DNA damage although there is cross-talk between these two pathways.^{6,29} We have found that cDDP caused significant activation of caspase-9 in the absence of any activation of caspase-8, suggesting that cDDP exerts its effect primarily through activation of the initiator caspase-9. Rottlerin blocked activation of caspase-9 whereas Gö 6976 was unable to prevent cDDP-induced activation of caspase-9 and its downstream target, caspase-3. These results suggest that rottlerin acts upstream of caspase-9.

We have detected a small amount of procaspases-9, -3 and -7 in the HM fraction. The presence of procaspase-3 in the mitochondria of HeLa cells has been demonstrated both by immunohistochemistry as well as by biochemical fractionations.³⁰ In addition, several investigators have reported that caspases-2, -3 and -9 zymogens may associate with heavy membrane fraction containing mitochondria.^{30–34} It is believed that these zymogens are released into the cytosol prior to their activation by Cytochrome *c* and Apaf-1.^{30–34} The regulation of the membrane-

associated caspase-3 was, however, distinct from that of cytosolic caspase-3; for example, membrane-associated caspase-3 was regulated by Bcl-2 but was insensitive to exogenous Cytochrome *c* whereas cytoplasmic caspase-3 was directly activated by Cytochrome *c*.³¹ We have also seen a decrease in procaspases-9, -7 and -3 in the HM membrane fraction following exposure to cDDP but a decrease in proform was associated with an increase in processed forms, suggesting that processing of caspases may in fact take place in the membrane fraction. This was substantiated by the increase in DEVD cleavage activity in the HM fraction as well as cleavage of the substrate PKC δ . It has been reported that during Fas-induced apoptosis, active caspase-7 associates with the mitochondrial and microsomal fractions and cleaves endoplasmic reticular-specific substrate.³⁵ Thus, if active fragments of caspases associate with mitochondria, it can explain the cleavage of these caspases and PKC δ in the membrane fraction. Alternatively, a caspase activation complex could form within the mitochondrial intermembrane space as has been speculated.³⁶ Another way to account for the presence of active caspases-9, -7 and -3 in the heavy membrane fraction is that the large apoptosome containing Apaf-1, caspases-9, -3 and -7 was precipitated with the mitochondria. Although we were unable to detect any Apaf-1 in the membrane fraction, we cannot rule out the possibility that a small amount of Apaf-1 that escaped our detection was in fact associated with the membrane fraction.

The observation that PKC δ is also localized and processed in the mitochondria is intriguing. It has been reported that PKC α may regulate Bcl-2 function in the mitochondria.³⁷ We have shown that inhibition of PKC δ prevents activation of the apical caspase-9. Since release of Cytochrome *c* is critical for the activation of the caspase cascade, one way to prevent caspase activation is to block Cytochrome *c* release. In fact, abrogation of mitochondrial Cytochrome *c* release has been associated with cellular resistance to multiple drugs, including cDDP.²³ In addition, mitochondrial translocation of PKC δ has been associated with apoptotic cell death in several cell types, including keratinocytes,³⁸ U-937 and MCF-7 cells.³⁹ Furthermore, PKC activator induced release of Cytochrome *c* and rottlerin attenuated Cytochrome *c* release by PKC activators.³⁹ Rottlerin by itself, however, contributed to a detectable release of Cytochrome *c*.³⁹ This is consistent with our observation that rottlerin caused a small release of Cytochrome *c* even without any cDDP treatment and that it inhibited Cytochrome *c* release when cells were exposed to cDDP for a prolonged period. It has been proposed that an initial small amount of Cytochrome *c* release induced by genotoxic stress is enough to trigger activation of caspase-9 but late Cytochrome *c* release is a consequence of activated caspases since caspase-3 can cause Cytochrome *c* release in isolated mitochondria.⁴⁰ Since activation of mitochondrial PKC δ has been shown to induce release of Cytochrome *c*,³⁹ we speculate that a small release of Cytochrome *c* induced by DNA damage causes activation of caspases in the cytosol followed by proteolytic activation of PKC δ in the mitochondria, resulting in more release of Cytochrome *c* from the mitochondria, thus forming a positive feedback loop. There-

fore, while initial release of Cytochrome *c* by cDDP may not be affected by rottlerin, subsequent release of Cytochrome *c* that results from activated PKC δ may be inhibited by rottlerin. Recently, however, it has been reported that release of Cytochrome *c* is kinetically invariant and is not affected by caspase inhibitors.²² Furthermore, it remains to be seen whether rottlerin influences the activity of other mitochondrial apoptogenic factors, such as apoptosis-inducing factor³² or second mitochondria-derived activator protein.⁴¹ Nevertheless, we have demonstrated that while release of only a small fraction of Cytochrome *c* by cDDP was enough to cause caspase activation, no caspase activation was noted when rottlerin was present, suggesting that rottlerin not only prevents Cytochrome *c* release but also inhibits caspase activation. As has been observed with etoposide-induced cell death,²⁸ we found that rottlerin blocked the entry of damaged cells to the apoptotic pathway rather than affecting cisplatin-induced DNA damage because when cells treated with rottlerin and cDDP were washed and incubated in drug-free media, cells underwent apoptosis. In addition, when cells were continuously exposed to cDDP and rottlerin was added at different times during the cDDP incubation period, the ability of rottlerin to prevent cell death correlated with its ability to block caspase activation and to inhibit late-stage Cytochrome *c* release. However, when rottlerin was added after cDDP was removed from the medium, it failed to block cDDP-induced cell death. Since DNA damage and caspase activation are coupled, rottlerin may have to be present prior to DNA damage in order to inhibit caspase activation. Thus, rottlerin acts at a step subsequent to DNA damage but prior to caspase-9 activation to regulate cell death by cDDP.

Materials and Methods

Materials

Bisindolylmaleimide, rottlerin and Gö 6976 were purchased from LC Service Corporation (Woburn, MA, USA). MTT and MBP were from Sigma (St. Louis, MO, USA). Polyclonal antibodies to PKC δ and PKC α were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and polyclonal antibody to caspase-8 was from R&D Systems (Minneapolis, MN, USA). Polyclonal antibodies to caspases-3, -9 and Apaf-1, monoclonal antibody to caspase-7 and FasL neutralizing antibody NOK-1 were from Pharmingen (San Diego, CA, USA). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmuno Research Lab. Inc. (West Grove, PA, USA). Poly(vinylidene difluoride) membrane was from Millipore (Bedford, MA, USA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL, USA). Caspase-3 fluorometric assay kit was obtained from BioVision (Palo Alto, CA, USA) and cDDP was a generous gift from Bristol-Myers Squibb Co. (Wallingford, CT, USA).

Cell culture

Human cervical carcinoma HeLa cells were maintained in Dulbecco's modified minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 37°C with 95% air and 5% CO₂.

Assessment of cell viability by MTT assay

Exponentially growing cells were plated in microtiter plates and incubated at 37°C in 5% CO₂. The following day, cells were pretreated without or with PKC inhibitors and then with different concentrations of cDDP as indicated in the text. The number of viable cells was determined using the dye MTT as previously described.¹⁴

Assessment of apoptosis by flow cytometric analysis

Cells were treated with PKC modulators and cDDP as described in the text. At the end of the incubation, cells were harvested and washed with phosphate-buffered saline. Nuclei were isolated, stained with propidium iodide and DNA content was analyzed by a flow cytometer (Coulter Epics).⁴²

PKC δ kinase assay

HeLa cells were lysed in 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.2 mM Sodium vanadate, 10 mM NaF and protease inhibitors. PKC α or - δ was immunoprecipitated with 1.0 μ g antibody and 50 μ l of protein A/G agarose. The immunocomplex was washed four times in ice-cold lysis buffer and then with kinase buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgAc, 0.5 mM EGTA, 0.1 mM DTT). An aliquot of the immunoprecipitate was incubated with assay buffer containing 20 μ M ATP, 5 μ Ci ATP, 0.2 mg/ml MBP in the presence and absence of 10 μ M rottlerin and/or 0.2 mg/ml phosphatidylserine and 100 nM TPA. In the PKC α immunokinase assay, 0.5 mM CaCl₂ was added with lipid activators to determine Ca²⁺-dependent cPKC α activity. The reaction mixture was incubated at 30°C for 15 min and the assay was terminated by the addition of SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and autoradiography was performed. A duplicate gel was run and Western blot analysis was performed with antibody to PKC δ to check for the amount of PKC δ in each assay. To determine the effect of cDDP on PKC δ activity, cells were pretreated with 10 μ M rottlerin for 1 h and then treated with or without 20 μ M cDDP for another 12 h. MBP phosphorylation was determined with cell extracts in the absence of any lipid cofactors as described before.¹⁹

Preparation of cytosolic and mitochondrial extracts

Following treatment with or without rottlerin and cDDP, cells were collected, washed twice with phosphate-buffered saline and subcellular fractionation was performed as described previously.⁴³ Briefly, the pellet was washed with PBS and then swelled in buffer containing 3 mM imidazole, pH 7.4, 0.25 mM sucrose and 10 μ g/ml each of leupeptin, aprotinin and soybean trypsin inhibitor for 30 min. Cells were homogenized with 20 strokes of a B-type pestle using a Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 300 \times g for 5 min and heavy membrane containing mitochondria were collected by centrifugation at 10 000 \times g for 15 min. The mitochondrial pellet was washed with isotonic lysis buffer, the supernatant was centrifuged at 150 000 \times g for 30 min and the resulting supernatant was used as the soluble cytosolic fraction.

Immunoblot analysis

Equivalent volumes of mitochondrial and cytosolic fractions were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to poly(vinylidene difluoride) membrane. Immunoblot analyses were performed as described before.¹⁷ Intensities of immunoreactive proteins were quantified by laser densitometry. In each experiment, the same blot was probed with several antibodies to account for any loading differences.

Caspase assay

DEVDase activity of mitochondrial and cytosolic fractions (equivalent volume) of cells treated with or without cDDP and rottlerin was determined at 37°C using an Ac-DEVD-AFC assay kit and the manufacturer's protocol. The fluorescence liberated from DEVD-AFC was measured using a SpectraMax GeminiXS fluorometer and SOFTmax PRO 3.1.1 software (Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 400-nm and emission wavelength of 505 nm.

Acknowledgements

This work was supported by grants CA71727 and CA85682 from the National Cancer Institute. We thank Dr Victoria L Rudick for her advice on subcellular distribution studies, Dr Richard Kitson for help with digital photography and Dr Baohua Sun for FACS analysis and for critical reading of the manuscript.

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