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Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells

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Abstract We previously reported that paclitaxel acted directly on mitochondria isolated from human neuroblastoma SK-N-SH cells. Here, we demonstrate that the direct mitochondrial effect of paclitaxel observed in vitro is relevant in intact SK-N-SH cells. After a 2 h incubation with 1 μM paclitaxel, the mitochondria were less condensed. Paclitaxel (1 μM, 1–4 h) also induced a 20% increase in respiration rate and a caspase-independent production of reactive oxygen species by mitochondria. The paclitaxel-induced release of cytochrome *c* was detected only after 24 h of incubation, was caspase-independent and permeability transition pore-dependent. Thus, paclitaxel targets mitochondria upstream of caspase activation, early during the apoptotic process in intact human neuroblastoma cells.

Key words: Apoptosis; Mitochondrion; Paclitaxel; Cytochrome *c*; Reactive oxygen species; Respiration

1. Introduction

Paclitaxel (Taxol®) is an anticancer drug highly efficient in the treatment of several malignancies [1], currently under evaluation for childhood tumors [2]. It is an anti-tubulin agent that stabilizes the microtubule network and inhibits the dynamics of microtubules [3]. Paclitaxel induces apoptosis after mitotic block in proliferating cells [4], but the mechanism of paclitaxel-induced apoptosis remains unclear [5,6]. Caspases are involved in this process [7,8] but their role has not been fully elucidated yet [9]. Mitochondria play a key role in apoptosis and mitochondrial outer membrane permeabilization is a critical event during apoptosis. Several apoptogenic factors, including cytochrome *c* (cyt *c*), apoptosis inducing factor, endonuclease G, SMAC/Diablo, procaspases, and calcium, can be released into cytosol upon various apoptotic stimuli [10]. The molecular mechanisms by which cyt *c* is released remain controversial and several models are currently proposed [10–13]. We have previously shown that paclitaxel could act directly on isolated mitochondria from human neuroblastoma cells to induce the permeability transition pore (PTP)-dependent release of mitochondrial cyt *c* [14], and that this effect was probably mediated by the presence of mitochondrial tubulin that interacts with the voltage-dependent anion channel (VDAC) [15]. Varbiro et al. [16] have confirmed our results on the release of cyt *c* triggered by paclitaxel and added that paclitaxel could induce the production of reactive oxygen species (ROS) or the decrease of mitochondrial membrane potential on isolated mitochondria from the HepG-2 hepatocellular carcinoma cell line, the BRL3A rat liver cell line, and kidney, heart and liver from rat. Recently, Kidd et al. [17] have shown that paclitaxel could directly target PTP to modify calcium signaling in mouse pancreatic acinar cells. We have also demonstrated that the in vitro mitochondrial effect of paclitaxel mediates the antagonism in cytotoxicity observed between arsenic trioxide and paclitaxel on the SK-N-SH cell line [18] indicating that the in vitro mitochondrial effect of paclitaxel could be relevant in intact cells. In the current study, we investigate the effects of clinically relevant concentrations of paclitaxel on mitochondria in intact SK-N-SH human neuroblastoma cells and we demonstrate that paclitaxel targets mitochondria upstream of caspase activation, early during the apoptotic process.

2. Materials and methods

2.1. Drugs and reagents

Stock solutions of paclitaxel (Sigma), betulinic acid (Calbiochem), rotenone (Sigma), bongkrekic acid (Calbiochem) and the general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-promethyl ketone (Z-VAD-fmk) (Eurobiol) were prepared in dimethylsulfoxide (DMSO). Stock solutions of doxorubicin (Dakota) and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (Sigma) were prepared in distilled water and ethanol respectively.

2.2. Cell culture and drug treatment

Human neuroblastoma SK-N-SH cells were cultured as described previously [7]. Drug treatment started when cell confluence reached 70%. Bongkrekic acid, rotenone, KCN, or Z-VAD-fmk were added 5 min before anticancer agents (paclitaxel, doxorubicin, betulinic acid).

2.3. Analysis of mitochondrial morphology by transmission electron microscopy

Cells were treated with 1 μM paclitaxel or DMSO (control) for 2 h at 37°C, fixed, dehydrated in ethanol, embedded in Epon, and cut into thin sections [19]. The samples were imaged by a transmission electron

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Abbreviations: cyt *c*, cytochrome *c*; PTP, permeability transition pore; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; RR, respiration rate; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-promethyl ketone

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microscope (JEOL 1220). At least 100 mitochondria were analyzed and the cristae/matrix surface ratio was calculated using image analysis software (IPS Samba Technologies, Grenoble, France).

2.4. Respiration rate (RR) measurement

SK-N-SH cells were treated with paclitaxel or doxorubicin for 1–4 h. Then, cells (15×10^6) were harvested and suspended in supplemented growth culture medium at 37°C. The uncoupling agent (25 μM FCCP) and the inhibitor of mitochondrial respiration (1.2 mM KCN) were added directly to the cell suspension during the measurement of the respiration rate by using a Clark oxygen electrode [14].

2.5. ROS measurement

ROS measurement was performed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) as described [16]. SK-N-SH cells were cultured into 96 well plates, incubated with 1 μM paclitaxel, 1.5 μM rotenone, 1.2 mM KCN, 50 μM Z-VAD-fmk alone or in combination for 1–4 h. Formazan blue dye formed by the effect of ROS on MTT was solubilized in DMSO. Optical densities were read with a Metertech S960 ELISA plate reader at 550 nm. Experiments were performed three times in quadruplicate. To confirm these results, a lucigenin assay based on the chemiluminescence properties of lucigenin was used as previously described [20].

2.6. Western blot analysis of cytosolic cyt c

Cytosolic fraction was prepared as described [21]. The detection of cyt *c* was preformed by Western blot analysis as described [17].

2.7. Immunofluorescence visualization of cyt c

Cells were grown on 8 well plates (Labtek), incubated with different anticancer agents alone or in combination with Z-VAD-fmk or bongkrekic acid for up to 48 h. Cells were then permeabilized with 1% saponin, fixed with 3.7% paraformaldehyde, successively incubated with anti-cyt *c* antibody (Pharmingen), and secondary antibody coupled with fluorescein isothiocyanate (Amersham). Cells were observed using a Leica DM-IRBE microscope coupled with a digital camera (CCD camera coolsnapFX; Princeton Instruments). Two hundred cells were analyzed with Metamorph software for each experiment which was performed in triplicate.

3. Results

3.1. Changes in mitochondrial morphology

Apoptosis is often associated with modifications of mitochondrial morphology [22]. Thus, we studied the effect of a 2 h

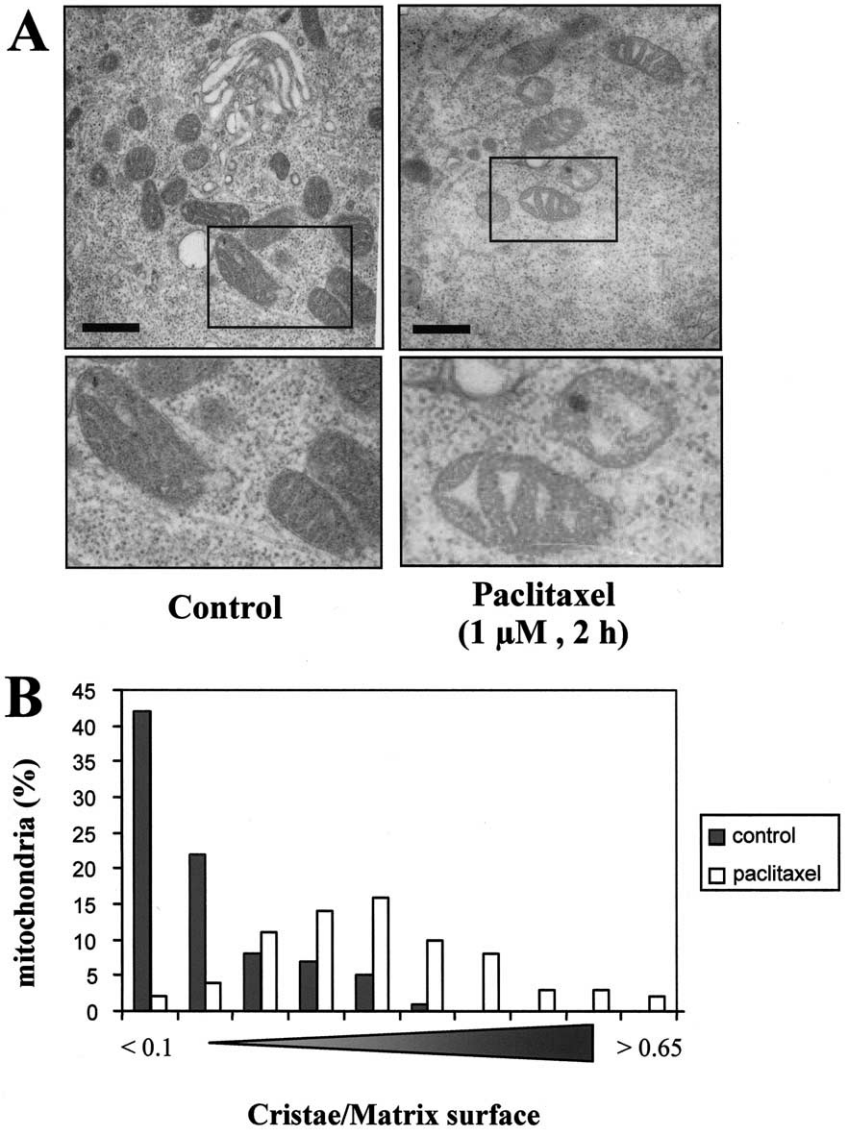

Fig. 1. Mitochondrial morphology in intact SK-N-SH cells after paclitaxel incubation. A: Morphology of mitochondria in control and paclitaxel-treated (1 μM, 2 h) SK-N-SH cells. Bar represents 500 nm. Higher magnification of the boxed parts (2×) has been added. B: Cristae/matrix surface ratio of mitochondrial population before and after paclitaxel treatment.

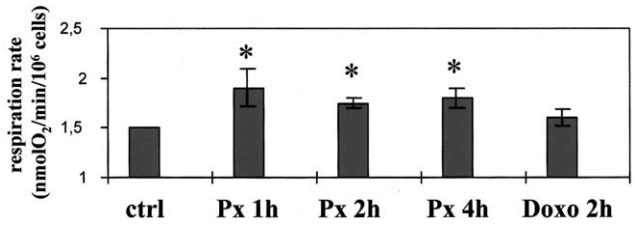

Fig. 2. Increase in RR in paclitaxel-treated SK-N-SH cells. Means ± S.D. of three independent experiments studying the increase in RR after 1 μM paclitaxel for 1–4 h, 500 nM doxorubicin for 2 h. *$P < 0.05$ vs. control (ctrl).

paclitaxel treatment on mitochondrial morphology in intact cells. As shown by electron microscopy, mitochondria are less condensed in paclitaxel-treated cells (Fig. 1A). Paclitaxel induces a statistically significant increase of the cristae/matrix surface ratio of mitochondria compared to control cells (0.22 ± 0.1 vs. 0.07 ± 0.05, $P < 0.0001$) (Fig. 1B). In contrast, no difference was observed in maximum/minimum diameter ratio between mitochondria from treated and control cells (1.70 ± 0.8 vs. 1.74 ± 0.7). These results show that paclitaxel early affects mitochondrial morphology without inducing swelling. These changes in mitochondrial morphology very likely represent an early involvement of mitochondria in paclitaxel-induced apoptosis.

3.2. Increase in respiration rate

We checked whether the increase in RR induced by paclitaxel that we described in a cell-free system [14] could also be observed in living cells. As shown in Fig. 2, paclitaxel induces a statistically significant increase in RR ($20 \pm 11\%$, $P < 0.005$). This effect started after a 1 h treatment and remained stable for at least 4 h. When the mitochondrial uncoupler FCCP was added to the cell suspension, an equivalent increase in RR was observed in paclitaxel-treated and control cells (data not shown). The total inhibition of RR induced by KCN confirms the mitochondrial origin of the oxygen consumption (data not shown). Interestingly, doxorubicin, a drug with a mechanism of action different from that of paclitaxel, used at a concentration that induces massive apoptosis [18], did not change RR after 1–4 h treatment. These results indicate that paclitaxel specifically acts on mitochondria in living cells to induce an increase in RR.

3.3. Increase in ROS production

As ROS production is definitely implicated in apoptosis and can be closely related to oxidative phosphorylation [23], we then investigated the effect of paclitaxel on ROS production. Paclitaxel (1 μM, 1–4 h) induced a statistically significant time-dependent increase in ROS production (10–40%, $P < 0.05$) (Fig. 3). Inhibition of ROS production by 1.5 μM rotenone or 1.2 mM KCN confirms that the ROS production came from the respiration of mitochondria. Similar results were obtained using the lucigenin assay: paclitaxel (1 μM, 4 h) induced a $44 \pm 13\%$ ($P < 0.05$) increase in ROS production that was inhibited by rotenone ($19 \pm 6\%$; $P < 0.05$). Furthermore, the increase in ROS production was caspase-independent as Z-VAD-fmk did not decrease paclitaxel-induced ROS production (Fig. 3). Thus, paclitaxel induces an early increase in mitochondrial ROS production upstream of caspase activation.

3.4. Release of cyt c

Western blot analysis of cytosolic cyt *c* show that no release of cyt *c* was observed until after 24 h of treatment with paclitaxel (Fig. 4A). These results were confirmed using immunofluorescence experiments (Fig. 4B). Control cells have a punctiform cyt *c* staining whereas cells that have released cyt *c* display a diffuse staining as previously described [24]. To further investigate the mechanism of cyt *c* release, we pretreated cells with 25 μM bongkrekic acid, a PTP inhibitor, or with 50 μM Z-VAD-fmk, a caspase inhibitor (Fig. 4C). We observed a 63% reduction of cyt *c* release induced by paclitaxel after pretreatment with bongkrekic acid whereas pretreatment with Z-VAD-fmk did not modulate the release of cyt *c*. On the other hand, doxorubicin-induced release of cyt *c*, which occurs with the same kinetics as paclitaxel, was caspase-

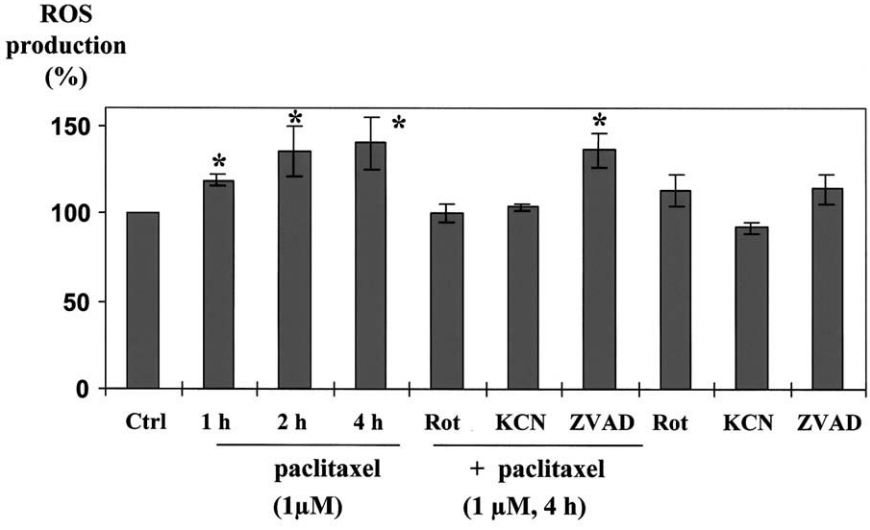

Fig. 3. Early increase in ROS production after paclitaxel treatment. ROS were measured as described in Section 2. Cells were treated with paclitaxel alone for 1–4 h, or for 4 h in combination with 1.5 μM rotenone (Rot) or 1.2 mM KCN, or 50 μM Z-VAD-fmk. Data shown are the mean ± S.D. of at least three experiments performed in quadruplicate. $P < 0.05$ vs. control (ctrl).

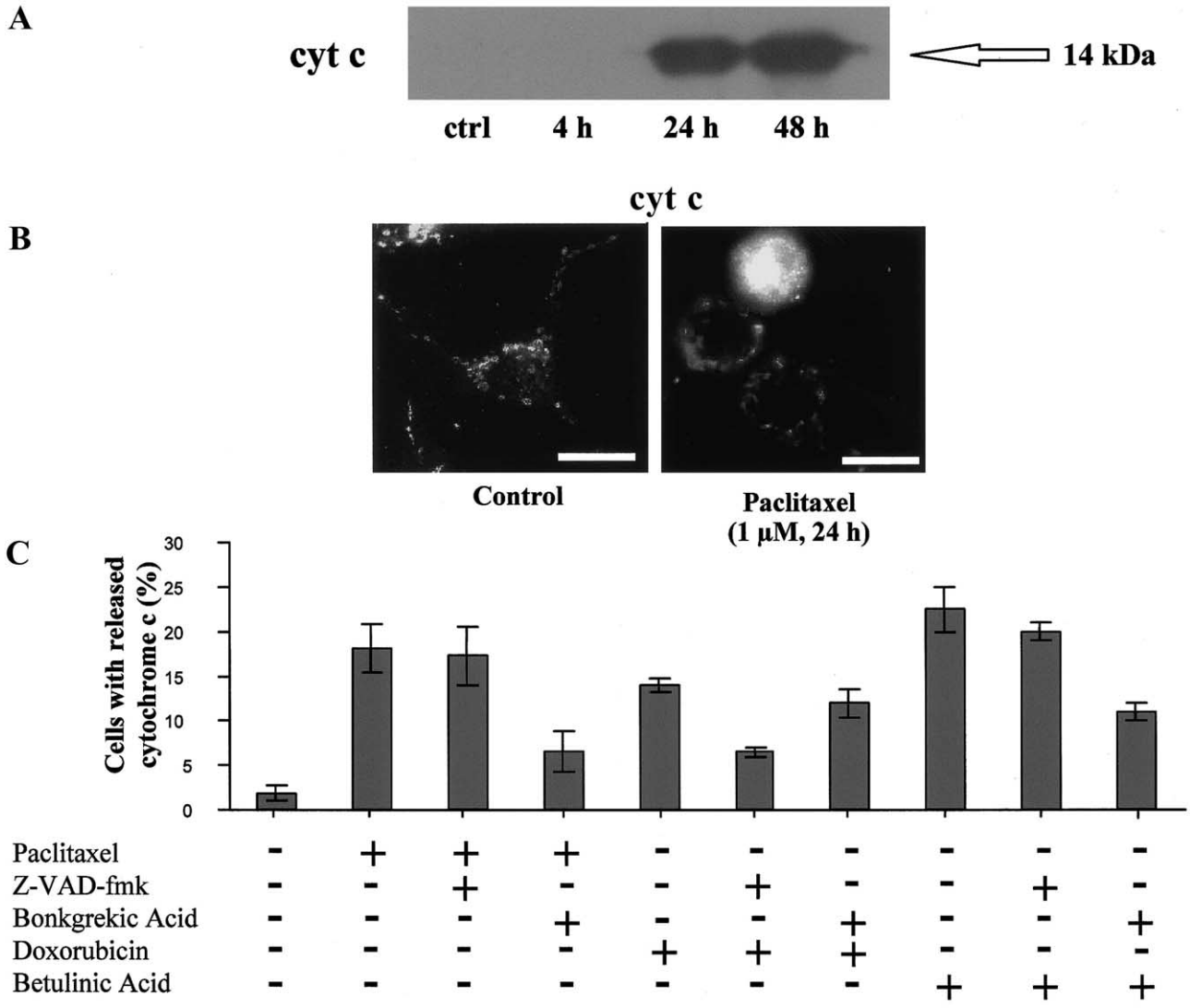

Fig. 4. Analysis of cytosolic release of mitochondrial cyt *c*. A: Western blot analysis of cytosolic cyt *c* after treatment with 1 μM paclitaxel for 4–48 h. B: Immunofluorescent analysis of the release of cyt *c*. C: Percentage of cells with cyt *c* released from mitochondria after a 24 h incubation with different anticancer agents alone or in combination with the PTP inhibitor bongkrekic acid or with the caspase inhibitor Z-VAD-fmk. $^{*}P < 0.05$ vs. control (ctrl).

dependent and PTP-independent. Finally, as additional control to support the mitochondrial effect of paclitaxel, we tested the antimitochondrial agent betulinic acid [25], and observed that it induced the release of cyt *c* in a caspase-independent and PTP-dependent manner. Thus, paclitaxel induces a PTP-dependent cyt *c* release which occurs upstream of caspase activation in the same manner as an antimitochondrial agent does.

4. Discussion

In this study, we demonstrate that the direct mitochondrial effect of paclitaxel observed in vitro [14,16,17] also occurred in intact cells. Paclitaxel induces early changes in the morphology of mitochondria associated with an increase in both RR and mitochondrial ROS production. Paclitaxel also triggers the release of cyt *c* from mitochondria, upstream of caspase activation. Involvement of mitochondria starts very early during paclitaxel-induced apoptosis (1 h after the beginning of the treatment) as massive apoptosis of SK-N-SH cells is only observed after 48 h of treatment with paclitaxel.

The 20% increase in RR observed in paclitaxel-treated cells is the same as that measured in our cell-free system [14]. This property is not shared by doxorubicin which does not target mitochondria or tubulin. The increase in RR could be secondary to partial specific uncoupling capacities of paclitaxel. The increase in ROS production can be related to the increase in RR [23]. The increase of mitochondrial ROS production that we measure in SK-N-SH cells is consistent with data from Su et al. [26] or from Varbiro et al. [16] who showed a 10% increase in ROS production after paclitaxel treatment (1 μM, 1 h) of the BRA-3A cell line. The role of both the early increase in ROS production and the increase in RR is still unclear but analysis of cell viability after 72 h of cotreatment with paclitaxel and rotenone or KCN (our unpublished data) indicates that inhibition of mitochondrial ROS production and of RR decreases paclitaxel-induced cytotoxicity. Moreover, recent reports suggest that changes in RR define an

early pathway for the induction of apoptosis [27] and that deficiency in respiration could lead to a marked resistance to cyt *c* release and apoptosis [28]. Thus, the increase in RR and the early increase in ROS production triggered by paclitaxel are very likely to be early pro-apoptotic effects. Moreover, these mitochondrial effects occur for paclitaxel concentrations and treatment durations that induce bundling of microtubules.

Our results clearly indicate that paclitaxel induces cyt *c* release in a caspase-independent and PTP-dependent manner after a 24 h treatment, as betulinic acid, an antimitochondrial agent, does [25]. In contrast, doxorubicin-induced release of cyt *c* is caspase-dependent and PTP-independent [25]. Shimizu et al. [29] also demonstrated that inhibition of the opening of the PTP with a VDAC (PTP main component) monoclonal antibody could almost totally inhibit paclitaxel-induced apoptosis, stressing the importance of the PTP in paclitaxel-induced cell death.

The paclitaxel-induced cyt *c* release is compatible with a direct mitochondrial effect of paclitaxel. Nevertheless, there is a 24 h delay between the release of cyt *c* in a cell-free system and in intact cells. This difference has already been observed with other PTP inducers [25,30]. Several hypotheses can be proposed to explain this phenomenon. First, in intact cells, drugs may have several targets and thus a smaller amount of drug may be effectively available for mitochondria. Second, the mitochondrial isolation procedure may also induce changes in mitochondria, such as translocation of pro-apoptotic molecules like Bax or modification of mitochondrial lipids. Individually, these changes are not sufficient to induce cyt *c* release but they may represent the first step of cyt *c* release [31–33]. Lastly, in intact cells, the mitochondrial effect of drugs may be delayed upon Bcl-2 phosphorylation [34] or inhibited by cytosolic factors such as p27 [35] or HSP27 [36]. Paclitaxel-induced release of cyt *c* may also be inhibited by integrin signaling [37]. These factors could represent resistance factors to chemotherapy. The mechanism of this intriguing delay is currently under investigation.

Together with our previous work [14,15,18], these results strongly suggest that paclitaxel induces apoptosis at least partially via its direct effect on mitochondria, probably upon its binding to mitochondrial tubulin that is specifically associated with VDAC.

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