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Induction of apoptosis by the plant alkaloid sampangine in human HL-60 leukemia cells is mediated by reactive oxygen species

Jérôme Kluza, Romain Mazinghien, Klara Degardin, Amélie Lansiaux, Christian Bailly *

INSERM U-524 and Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, IRCL, 1 Place de Verdun, 59045 Lille, France

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Abstract

Sampangine is a plant-derived copyrine alkaloid extracted from the stem bark of *Cananga odorata*. This azaoxoaporphine alkaloid primarily exhibits antifungal and antimycobacterial activities but also displays in vitro antimalarial activity against *Plasmodium falciparum* and it is cytotoxic to human malignant melanoma cells. Recently, sampangine was described as a pro-apoptotic agent, but the biochemical pathway leading to cell death remained unclear. Considering that sampangine possesses an iminoquinone moiety, potentially functioning as an oxidizing agent, we have investigated the implication of an oxidant stress on sampangine-induced cytotoxicity. We show that the treatment of human HL-60 leukemia cells for 48 h with sampangine induced an important oxidative burst. Real time flow cytometry measurements indicated that the production of oxidative species is very rapid, within minutes following the drug addition. Quenching of reactive oxygen species by the antioxidants *N*-acetyl cystein, vitamin C and vitamin E abolishes the pro-apoptotic activity of sampangine. The drug-induced production of reactive oxygen species is associated with cell cycle perturbations and mitochondrial alterations. This study shed light on the mechanism of action of sampangine and provides novel opportunities to use azaoxoaporphine alkaloids as lead compounds for the design of pro-apoptotic anticancer agents.

Keywords: Sampangine; Apoptosis; Reactive oxygen species; Plant alkaloid; Mitochondria; Caspase

1. Introduction

Sampangine (Fig. 1) is a plant-derived copyrine alkaloid extracted from the stem bark of *Cananga odorata.* It has been also isolated from the tree *Duguetia hadrantha*. This azaoxoaporphine alkaloid primarily exhibits antifungal and antimycobacterial activities but also displays in vitro antimalarial activity against *Plasmodium falciparum* and it is cytotoxic to human malignant melanoma cells (Orabi et al., 1999). This alkaloid is known to inhibit cell aggregation but no molecular target has been yet identified. Sampangine is structurally close to the marine alkaloid ascididemin (Fig. 1), isolated from various sponges (Dias et al., 2004). Ascididemin is highly cytotoxic to various types of tumor cells and exhibits potent pro-apoptotic activities (Dassonneville et al., 2000; Dirsch et al., 2004). This pyridoacridine derivative functions as a topoisomerase II poison but recent studies suggested that its DNA cleaving properties do not rely on topoisomerase II inhibition but rather implicate the production of reactive oxygen species responsible for direct DNA damages independently of any enzymatic catalyst (Matsumoto et al., 2003). Considering the structural analogy between sampangine and ascididemin, we considered that the two compounds could exhibit a common mechanism of action and therefore, we investigated the implication of reactive oxygen species in the cellular pathway through which sampangine induces apoptosis. The work reported here is focused on the cell cycle modification, the mitochondrial membrane potential variation and a particular interest was paid to on the drug-induced oxidative stress. Two key points have been demonstrated: (i) the capacity of sampangine to rapidly produce reactive oxygen species in cells and (ii) a link between the production of reactive oxygen species and the induction of apoptosis through mitochondrial perturbation, caspase activation and nuclear degradation.

* Corresponding author. Present address: Centre de Recherche en Oncologie Expérimentale, Institut de Recherche Pierre Fabre, 3 rue des satellites, 31140 Toulouse, France.
E-mail addresses: christian.bailly@lille.inserm.fr, christian.bailly@pierre-fabre.com (C. Bailly).

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2. Materials and methods

2.1. Drug and chemicals

The isolation of sampangine from *Duguetia hadrantha* tree has been previously described (Peterson et al., 1992). A pure sample of sampangine was kindly provided by Pr AM Clark (National Center for Natural Products Research, University of Mississippi). The drug was dissolved in dimethylsulfoxide (DMSO) to obtain a 10 mM stock solution and fresh working solutions were prepared extemporaneously by dilution with deionized water. RNase A, propidium iodide, carbonyl cyanide *p*-chlorophenylhydrazone (ClCCP), *N*-acetyl cysteine, vitamin C (L-ascorbic acid sodium salt), vitamin E (α-tocopherol acetate), cyclosporin A were purchased from Sigma Chemical Co. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1), 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein diacetate, acetyl ester (CM-H_2DCFDA), 3,8-phenanthridinediamine, 5-ethyl-5,6-dihydro-6-phenyl (hydroethidine) were obtained from Molecular Probes (Eugene, OR) and the fluorescent probe Phiphilux G1D2 was from OncoImmunin Inc (College Park, MD). Benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (z-VAD.fmk) was obtained from Bachem (Basel, Switzerland).

2.2. Cells and culture conditions

The leukemia cell line HL-60 was obtained from the American Type Culture Collection (CCL 240). Cells were grown at 37 °C under 5% CO_2/air in RPMI medium containing 100 U/ml penicillin, and 0.1 ng/ml streptomycin and supplemented with 10% heat-inactivated FCS. Prior to the drug treatment, 25×10^4 exponentially growing cells (25×10^4 in 1 ml) were seeded in 24-well plates for 2 h prior to incubation the drug. Drug concentrations and exposure times (generally 48 h) are indicated in the figure legends.

2.3. Cell cycle variations and nuclear apoptosis

Hypodiploid cells (subG1 cells) were characterized by cell cycle measurements. Cells were fixed overnight at 4 °C with 70% ice-cold ethanol/phosphate-buffered saline (PBS) and then stained with a solution containing propidium iodide (50 μg/ml) and RNase A (0.5 mg/ml) and analyzed with a Becton Dickinson FACScan cytofluorometer. Data were analyzed with the WinMDI and CyclRed softwares.

2.4. Cytofluorometric analysis of reactive oxygen species

For the fixed-time analysis, the production of peroxides was determined using the non-fluorescent substances hydroethidine or CM-H_2DCFDA (DCF) which are oxidized by hydroxyl radicals to give products emitting a red or green fluorescence, respectively. Cells (25×10^4/ml) were exposed for 30 min at 37 °C to 2.5 μM hydroethidine prior to the cytofluorometric analysis (excitation, 355 nm; emission, 420 nm). A real time analysis of peroxides production was also performed. HL-60 cells (25×10^4/ml) were exposed for 30 min at 37 °C to 2.5 μM hydroethidine or 5 μM CM-H_2DCFDA. A solution (1 μl) of sampangine or H_2O_2 was added to the cell medium, 5 min after the analysis was started and cell fluorescence was measured over a period of 20 min every 5 s.

2.5. Cytofluorometric analysis of caspase-3 activity

On whole cells, caspase 3-like activities in living cells were assayed using the fluorescent substrate Phi-PhiLux DEVD-fluorescein G1D2 (10 μM), according to the manufacturer's recommendations. After the incubation, cells (5×10^5/ml) were immediately analyzed on the cytofluorometer and the mean fluorescence intensity (MFI) was measured (excitation, 488 nm; emission, 575 nm). On cell extracts, caspase 3-like activities were determined similarly using the EnzChek Caspase-3 Assay Kit #2, according to the manufacturer's recommendations (Molecular Probes). After the incubation with the rhodamine 110-derived substrate Z-DEVD-R110, cell extracts (from 2×10^6 cells) were immediately analyzed by fluorescence (λ^{exc}: 496 nm; λ^{em}: 520 nm).

2.6. Cytofluorometric analysis of mitochondrial changes

To evaluate the mitochondrial transmembrane potential ($\Delta\psi_m$), a procedure described previously was followed (Kluza et al., 2004). Briefly, cells (5×10^5/ml) were incubated for 30 min at 37 °C with the fluorescent probed JC-1 (1 μM in PBS) or with tetramethylrhodamine methyl ester (TMRM, 125 nM). Samples were stored in ice prior to the cytofluorometric analysis. JC-1 exists as a monomer at low values of $\Delta\psi_m$ (green fluorescence; emission, 527 nm) while it forms aggregates at high $\Delta\psi_m$ (orange fluorescence; emission, 590 nm). TMRM was incorporated in mitochondria matrix proportionally to $\Delta\psi_m$ (orange fluorescence; emission, 549 nm).

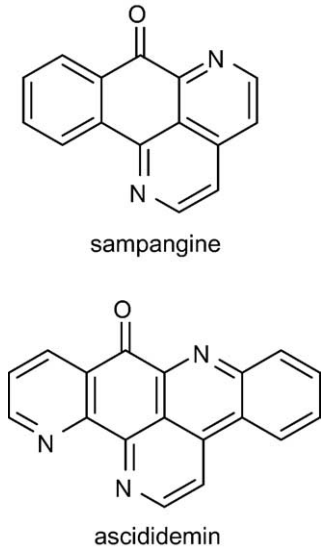

Fig. 1. Structures of sampangine and ascididemine.

3. Results

3.1. Cell cycle variation and apoptosis induction

To evaluate drug-induced apoptosis, we measured the percentage of hypodiploid cells and caspase-3 activities in HL-60 cells treated with 1 to 20 μM sampangine for 48 h. Caspase-3 activity was measured with the probe phiphilux G1D2, a tetrapeptide (DEVD) associated with two fluorescein residues at the Nt and Ct termini. Quenching, due to the proximity of the two fluorescein moieties, prevents the fluorescence emission. Activation of caspase-3 results in the cleavage of the DEVD sequence so as to release the two fluorescein residues which can then emit a green fluorescence detected by flow cytometry. As shown in Fig. 2A, the activation of caspase-3 is directly proportional to the drug concentration. Low drug concentrations (1–4 μM) show little activation of caspase-3 whereas at higher concentrations (8–20 μM), cells become highly fluorescent and at 20 μM sampangine, 56% of the cell population stained positively for caspase-3 activation (Fig. 2A). This test was performed on whole cells. To support these results, additional experiments were undertaken using HL-60 cell extracts. Cytosolic extracts were incubated with the Z-DEVD-R110 substrate for 30 min at room temperature prior the fluorescence measurement (Fig. 2B). A very low fluorescence emission was recorded with the cytosolic fraction from untreated cells or cells exposed to 50 μM Z-VAD or 4 μM sampangine for 48 h. In the opposite, extracts from cells treated with 20 μM sampangine emitted a high fluorescence intensity, suggesting a high level of caspase 3 activity. Moreover, this high fluorescence decreased drastically when cells were treated by 50 μM Z-VAD prior to the sampangine treatment. This large spectrum inhibitor is able to fully inhibit caspase 3-like activity in sampangine-treated HL-60 cells. The two data set using the phiphilux (whole cells) and Z-DEVD-R110 (cell extracts) tend to validate each other.

Apoptotic nuclear alterations (hypodiploid cells, subG1 peak) were observed concomitantly on the cytofluorometric profiles (Fig. 2C). A link may be established between the extent of caspase-3 activation and the appearance of sub-G1 cells in the cell cycle measurements. In fact, both types of effects started to appear for drug concentrations >4 μM. Moreover, subG1 alterations induced by 20 μM sampangine were fully inhibited by 50 μM Z-VAD (Fig. 2C). This result confirms the existence of a direct link between caspase activation and nuclear alterations. This first set of data validates the induction of apoptosis by sampangine. Then we went on investigating the possible mechanism of apoptosis induction. We have prioritized the oxidant stress hypothesis, because sampangine possesses an iminoquinone moiety potentially implicated in drug-induced oxidant stress activation in cells, as reported for the structurally related compound ascididemin (Matsumoto et al., 2003). To evaluate the redox state, HL-60 cells were treated with sampangine at a pro-apoptotic concentration (20 μM) and labelled with specific fluorescent probes.

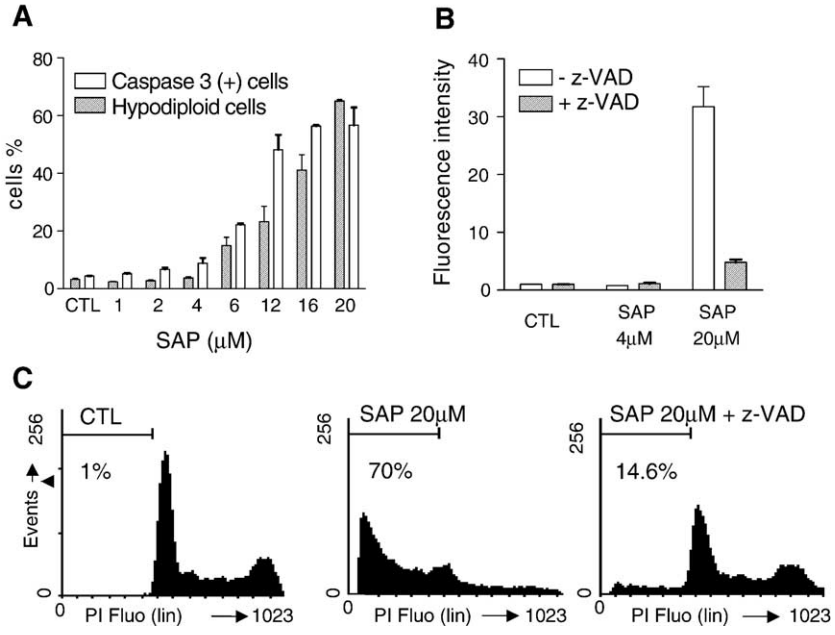

Fig. 2. Apoptotic features in sampangine-treated HL-60 cells. (A) Cells were exposed to graded concentrations of sampangine for 48 h and the percentage of cells with an active caspase-3 enzyme was determined using the PhiPhiLux-G1D2 probe. In parallel, the percentage of hypodiploid (sub-G1) cells was determined (from the cell cycle profiles after fixation by ethanol and IP-labeling). (B) Cells were treated with 50 μM Z-VAD or DMSO for 2 h and incubated with 4 or 20 μM for 48 h. The caspase 3 activity was determined by fluorescence emission in cell extracts incubated with the ZDEVD-R110 substrate. (C) Cells were pretreated by 50 μM Z-VAD or DMSO and next incubated for 48 h with 20 μM sampangine prior to the permeabilization and fixation with 70% ethanol overnight. After IP labeling, fluorometric profiles were monitored by flow cytometry and the percentage of cells in the subG1 phase was determined. Results are representative of three independent experiments.

3.2. Production of reactive oxygen species

This issue was addressed by flow cytometry through the use of hydroethidine which is converted into a red fluorescent dye (ethidium) after oxidation by reactive oxygen species. As shown in Fig. 3A, the treatment of hydroethidine-containing HL-60 cells with graded concentrations of sampangine shows a dose-dependent increase of the percentage of cells emitting an intense red fluorescence. Oxidative stress appeared significantly with 4 μM sampangine for 48 h (in 23% of treated cells) and was maximal with 20 μM sampangine (69%). To confirm the implication of reactive oxygen species, we used various scavengers: the thiol compound *N*-acetyl cysteine, L-ascorbate (vitamin C) or the α-tocopherol acetate (vitamin E). In agreement with a previous study (Huang et al., 2002), we have optimized the concentration of each scavenger to quench the reactive oxygen species. HL-60 cells were co-incubated with sampangine (4 and 20 μM) and *N*-acetyl cysteine (0.1 to 10 mM), vitamin C (100 μM) or vitamin E (100 μM) for 48 h prior to the hydroethidine fluorescence measurements. The oxidative burst induced by 4 or 20 μM sampangine was fully decreased in the presence of 10 mM *N*-acetyl cysteine and was partially decreased with vitamin C or vitamin E (Fig. 3B). We have also measured the production of reactive oxygen species induced by sampangine in Z-VAD treated-cells. Z-VAD treatment did not modify reactive oxygen species production, suggesting thus that caspase activation was not implicated in the oxidative stress.

To evaluate the implication of reactive oxygen species on cytotoxicity, we measured the capacity of sampangine to induce apoptosis in the presence of a scavenger. Hypodiploid cell contents were determined under these conditions (Fig. 3C). At 10 mM, *N*-acetyl cysteine fully inhibits the appearance of the subG1 cell population and similar results were obtained with vitamins C and E (100 μM each) but with a reduced efficacy. These experiments indicated that reactive oxygen species play an important role in sampangine-mediated apoptosis and for this reason, a time course study of reactive oxygen species production was carried out.

We set up a real time method to measure the production of reactive oxygen species by cytometry using hydroethidine and CM-H_2DCFDA. The two probes react with reactive oxygen species to produce compounds emitting red fluorescence for oxidized hydroethidine and a green fluorescence for oxidized DCF. In contrast to the above experiments, in this case cells were stained with the dye before the drug treatment. Every 5 s over a period of 20 min, a fraction of treated cells was collected and analyzed by cytometry to measure the mean fluorescence intensity (MFI). Five minutes after the start of the experiment, sampangine (20 μM), H_2O_2 or DMSO were added to the cell suspension (plain white arrow in Fig. 4A and B). A time-dependent increase of the oxidant cell population was measured.

To validate this novel experimental method, HL-60 cells were incubated with hydrogen peroxide, which is a potent oxidative agent. In the cell samples exposed to H_20_2 (0.1 mM), the fluorescence of the oxidized products ethidium and

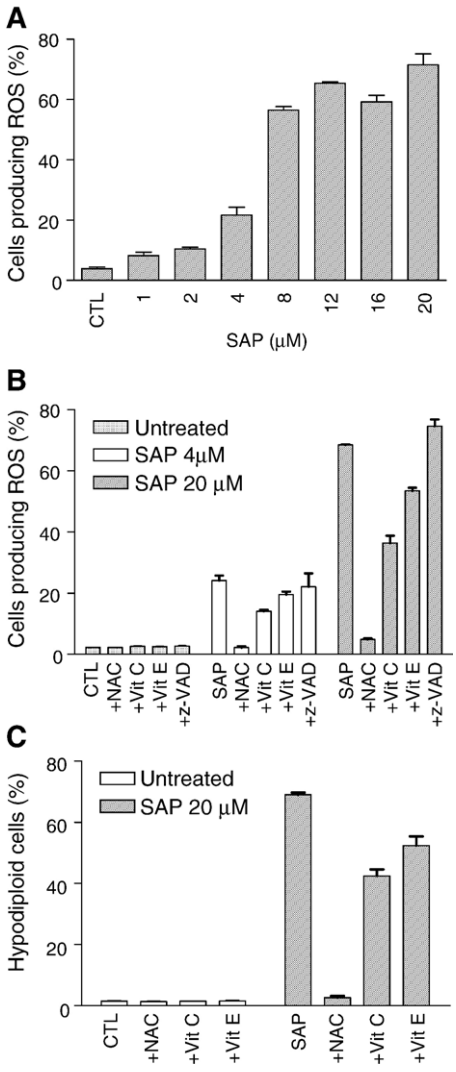

Fig. 3. Production of reactive oxygen species and their quenching by radical scavengers in sampangine-treated HL-60 cells. (A) Cells were treated with sampangine for 48 h at the indicated concentration and then labeled with hydroethidine which emits a red fluorescence upon oxidation by reactive oxygen species. The percentage of cells producing reactive oxygen species was determined after cytometric analysis with the software Cellquest. (B) Cells were incubated with 10 mM *N*-acetyl cysteine for 30 min, 100 μM L-ascorbate (Vit C) or 100 μM α-tocopherol acetate (Vit E) for 2 h and then treated with 0, 4 or 20 μM sampangine prior to the labeling with hydroethidine. The percentage of cells producing reactive oxygen species was determined after cytometric analysis with the software Cellquest. (C) Inhibition of apoptosis by scavengers of reactive oxygen species in sampangine-treated HL-60 cells. Cells were incubated with 10 mM *N*-acetyl cysteine for 30 min, 100 μM L-ascorbate (Vit C) or 100 μM α-tocopherol acetate (Vit E) for 2 h and then treated with 20 μM sampangine prior to permeabilization and fixation with 70% ethanol overnight. After IP labeling, fluorometric profiles were monitored by flow cytometry and subG1 cell populations were determined.

DCF increases after the injection (as indicated by a plain white arrow in Fig. 4A and B). The effect is more pronounced with the high amount of H_2O_2 whereas almost no variation occurs with the control samples receiving a drug-free 0.4% DMSO solution. With the same method, we show that 20 μM sampangine has also a noticeable positive effect on hydroethidine fluorescence. The effect is much more pronounced with the

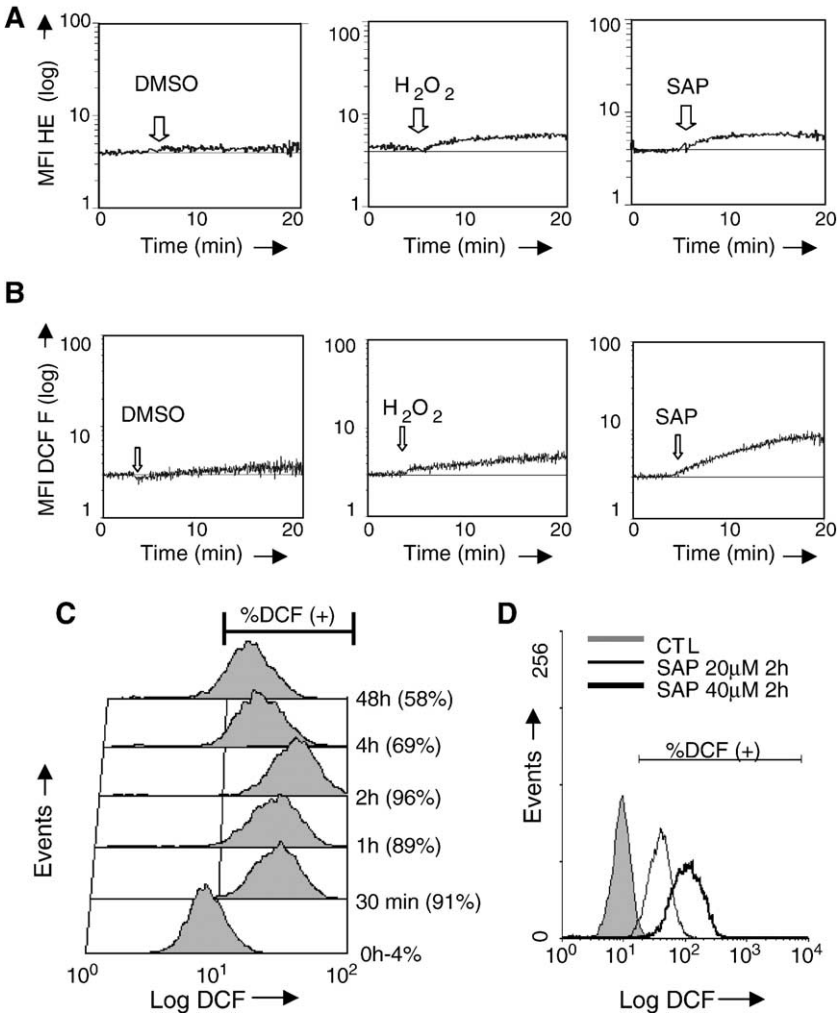

Fig. 4. Real time production of reactive oxygen species in HL-60 cells. Cells were labeled with (A) hydroethidine or (B) CM-H_2DCFDA probes and the level of red (from oxidized hydroethidine) or green (from oxidized DCF) fluorescence was measured. The curves show the mean fluorescence at the indicated time, and the black horizontal line indicates the fluorescence level at the beginning of the experiment. After 5 min (vertical arrow), a solution of DMSO (0.1%), hydrogen peroxide (0.1 mM) or sampangine (20 μM) was added to the cell suspension and the signal was monitored during 15 min. (C) Cells were treated with sampangine for 30 min, 1, 2, 4 and 48 h at 20 μM then labeled with CM-H_2DCFDA which emits a green fluorescence upon oxidation by reactive oxygen species. The percentage of cells producing reactive oxygen species (% DCF (+)) was determined from the cytometry analysis with the software Cellquest. (D) Cells were treated with sampangine for 2 h at 20 and 40 μM then labeled with CM-H_2DCFDA. Fluorescence intensity were monitored by flow cytometer. Results are representative of three independent experiments.

H_2DCFDA labeling. The drug massively promotes the fluorescence of DCF. This approach is most useful to characterizing the early oxidative burst induced by the alkaloid.

To evaluate the time course of reactive oxygen species production, we used the probe CM-H2DCFDA which we considered as a highly sensitive probe in our experimental conditions. After 30 min of treatment, 91% of cells emitted a high DCF fluorescence, suggesting that they nearly all produced reactive oxygen species. This percentage decreased quickly for the first 4 h and remained constant around 60% until 48 h (Fig. 4C). These data are compatible with cells producing reactive oxygen species at 48 h as detected with the hydroethidine probe (Fig. 3A). Using the DCF probe, we have also shown that a higher sampangine concentration (40 μM) after 2 h of treatment of HL-60 cells can induce a high percentage (98%) of cells producing reactive oxygen species but with a more extensive oxidative burst as seen by the increase of the DCF fluorescence (Fig. 4D).

3.3. Drug-induced variation of the mitochondrial membrane potential

Mitochondria are often active partners of the apoptotic process. To evaluate the mitochondrial alterations during sampangine treatment, we have measured the mitochondrial membrane potential ($\Delta\Psi_m$) by cytometry using the fluorescent probe JC-1 which is considered as one of the most mitochondria-specific probe (Salvioli et al., 1997). Mitochondria with normal $\Delta\Psi_m$ concentrate JC-1 into aggregates (orange fluorescence) while in depolarized mitochondria JC-1 forms monomers (green fluorescence). The treatment for 48 h with 20 μM sampangine leads to the detection of a massive

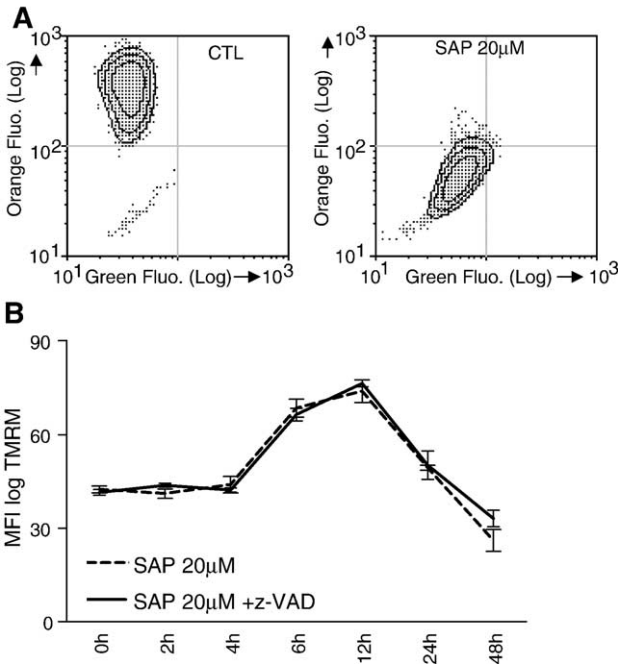

Fig. 5. Mitochondrial changes observed in sampangine-treated HL-60 cells. (A) Cytofluorometric analysis of JC-1 fluorescence in HL-60 cells treated with sampangine (20 μM) for 48 h. Data represent typical results of one out of three independent experiments. (B) Cells were treated with 50 μM Z-VAD or DMSO for 2 h and incubated with sampangine (20 μM) at the time indicated and finally labeled with TMRM. The orange fluorescence, proportional to the mitochondrial membrane potential ($\Delta\psi_m$), of the cells was recorded by flow cytometry.

population of cells with depolarized mitochondria (92%), characterized by a low level of JC-1 aggregates and a high level of JC-1 monomers (Fig. 5A).

Another fluorescent probe, the rhodamine derivative TMRM, was used to confirm the drug-induced variations of $\Delta\psi_m$. Moreover, kinetic experimentations were performed to evaluate the delay necessary to observe mitochondrial alteration. HL-60 cells were treated with 20 μM sampangine and $\Delta\psi_m$ was measured by cytometry. We confirmed that treated cells were depolarized at 48 h, as indicated by a decrease of the TMRM mean fluorescence. This event began after 24 h of treatment and was preceded by a transient fluorescence increase, corresponding to mitochondria hyperpolarization, after 6 h of drug treatment (Fig. 5B). In parallel, we performed the same experiment with cells treated with 50 μM Z-VAD for 2 h before the incubation with sampangine. This treatment had no effect on the mitochondrial alteration induced by sampangine (Fig. 5B).

To establish a link between the mitochondrial events and the oxidative stress, we tried to inhibit the mitochondrial alteration using cyclosporin A, a well-known inhibitor of the permeability transition. Cyclosporine A prevents the apoptosis-associated drop of $\Delta\psi_m$ (Costantini et al., 2000). Cells were incubated with 1 μM cyclosporin A for 30 min prior to the treatment with sampangine or dithiodipyridine (DTDP, 50 μM) for 48 h and then labeled with the TMRM probe. DTDP is a well-known inducer of the permeability transition (Costantini et al., 2000) and was used here as a positive control. This molecule induces a drop of $\Delta\psi_m$ associated with the production of reactive oxygen species, which is inhibited by co-incubation with cyclosporin A (Fig. 6A). But under these experimental conditions, cyclosporin A did not inhibit the mitochondrial depolarization induced by sampangine (Fig. 6A). These results suggest that the mitochondria alterations observed in the presence of sampangine are not associated with a permeability transition. Next, we tried to prevent mitochondria modification using the protonophore ClCCP. It has been shown previously that the treatment of cells with nanomolar doses of the uncoupling agent ClCCP inhibits mitochondrial alterations, blocking the hyperpolarization and the ensuing depolarization phase (Giovannini et al., 2002). This observation prompted us to adopt this strategy to evaluate further the effect of the alkaloid. HL-60 cells were co-incubated with sampangine (20 μM) and ClCCP (500 nM) for 48 h and the mean fluorescence of TMRM-labeled cells was measured (Fig. 6A). The extent of hyperpolarization induced by 20 μM sampangine at 6 h was markedly reduced in the presence of ClCCP (data not shown). Moreover, the

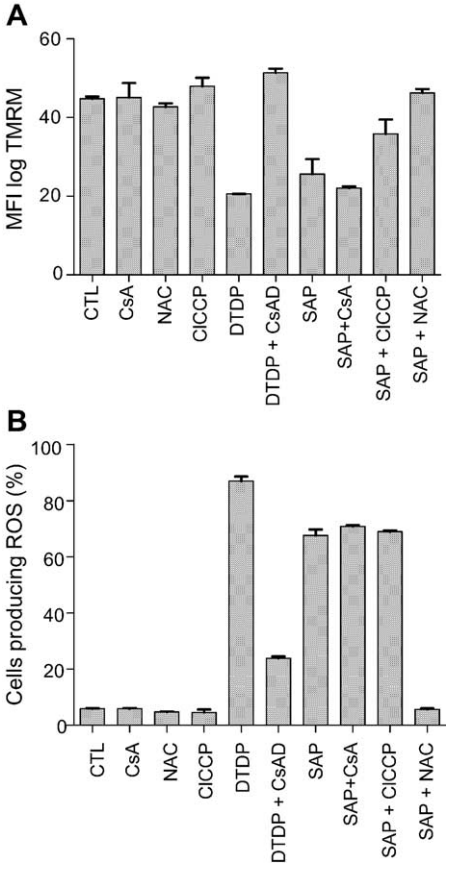

Fig. 6. Mitochondrial changes and production of reactive oxygen species after co-treatment with scavengers, cyclosporin A or ClCCP and sampangine. For each experiment, HL-60 cells were incubated with vitamin C (100 μM), vitamin E (100 μM) for 2 h, *N*-acetyl cysteine (10 mM), cyclosporin A (1 μM) or ClCCP (500 nM) for 30 min. Cells were then treated with 20 μM sampangine for 48 h and labeled with the TMRM or hydroethidine probe. (A) The (orange) mean fluorescence of TMRM-labeled cells was recorded by flow cytometry to determine the variations of the mitochondrial membrane potential ($\Delta\psi_m$). (B) The percentage of DCF-labeled cells producing reactive oxygen species was determined by flow cytometry with the software Cellquest.

depolarization effect induced in the presence of 20 μM sampangine after a 48 h treatment was significantly diminished when cells were co-treated with 500 nM ClCCP (Fig. 6A). In parallel, the extent of oxidative stress was measured. The pre-incubation of HL-60 cells with ClCCP did not prevent reactive oxygen species production suggesting therefore that this production did not originate from mitochondria (Fig. 6B).

The production of reactive oxygen species in drug-treated HL-60 cells was totally quenched in the presence of 10 mM *N*-acetyl cysteine (Figs. 3B and 6B). Cells were treated with sampangine for 48 h in the presence of *N*-acetyl cysteine and the mitochondrial membrane potential was measured with the TMRM probe. No difference was observed between drug-treated and untreated cells. The quenching of reactive oxygen species by *N*-acetyl cysteine totally prevents the mitochondrial effects of sampangine (Fig. 6A). These results suggest that reactive oxygen species are connected with, if not responsible for, the mitochondria alterations induced by sampangine.

4. Discussion

In a recent study, we showed that sampangine reduces the proliferation of HL-60 cells and preliminary evidences for a pro-apoptotic action were also reported (Kluza et al., 2003). The drug was shown to induce nuclear alterations (hypodiploid cells), caspase-3 activation and mitochondrial depolarization of HL-60 cells. To define the molecular mechanism underlying these effects, we extended the study using complementary cytometry approaches. The goal was to identify the molecular circuits activated by the alkaloid during the apoptotic program. The key observation reported here is that an oxidative stress is responsible for the induction of apoptosis by sampangine in HL-60 cells. The production of oxygen-based free radicals by sampangine appears as an early event which can be linked to the subsequent mitochondrial alterations and cell cycle perturbations characterizing the apoptotic process. The cell cycle changes induced by high concentrations of sampangine reflect the activation of apoptosis. The hypodiploid cell population with a reduced DNA content is particularly high when using 8 to 20 μM sampangine. At such high doses, caspase-3 is markedly activated. In contrast, at low doses (<4 μM) the drug essentially induces an arrest in the G1 phase of the cell cycle (Kluza et al., 2003) and the caspase-3 activity is not enhanced.

Two independent considerations prompted us to focus on the oxidative stress that accompanies apoptosis in drug-treated HL-60 cells. On the one hand, it has been shown that hydrogen peroxide provokes a G1 arrest in cancer cell lines and this effect is often associated with the production of free radicals during the initial phase of the oxidative stress (Lee et al., 2000; Wang et al., 2004). On the other hand, sampangine possesses the same iminoquinone moiety as the structurally closed compound ascididemine which has revealed pro-oxidant activities (Matsumoto et al., 2003). The two products may act through a common mechanism implicating an oxidizing system. For these reasons, we oriented our sampangine study toward a mechanism of action implicating reactive oxygen species. The experimental data reported here fully validate this hypothesis. In particular, we show that the dose-dependent generation of reactive oxygen species in HL-60 cells can be totally blocked by the antioxidant *N*-acetyl cysteine and partially by vitamin E and vitamin C. The percentage of hypodiploid cells induced by sampangine is markedly reduced in the presence of these scavengers, suggesting that reactive oxygen species play an important role in the cytotoxic action of the alkaloid.

Real time measurements of cytosolic reactive oxygen species were performed using the two different probes hydroethidine and CM-H_2DCFDA which display complementary properties. The two probes can be oxidized by reactive oxygen species but the oxidation products emit a red fluorescence for oxidized hydroethidine and a green fluorescence for oxidized DCF. Interestingly, the two probes have different sensitivities to reactive oxygen species. Hydroethidine is essentially oxidized by $O_2^{-\bullet}$ species whereas CM-H_2DCFDA can be efficiently oxidized by a larger range of species including $HO^{\bullet}$, H_2O_2, $HOO^{\bullet}$ and ONOO. Using these probes, we observed that the treatment of HL-60 cells with sampangine increases the fluorescence of oxidized hydroethidine and DCF. The effect occurs only a few minutes after the addition of the drug to cells. The fluorescence increase is more pronounced with CM-H_2DCFDA compared to hydroethidine, possibly due to the wider range of reactive oxygen species reacting with the former agent. Altogether, these experiments validate the implication of an oxidative stress in the cellular response to the sampangine treatment. We have also seen that during the first 2 h of treatment with sampangine, initially almost all cells produce reactive oxygen species (96%) and this percentage decreases to about 70% during the first 4 h and then remained constant at 60% for the next 48 h. This observation could be explain in several ways. Some cells may used a detoxification system, such as the P-glycoprotein export to extract drug from cells or metabolism enzymes to modify/inactivate the drug. Alternatively, it is plausible that some cells increase their cytosolic content in natural antioxidants, such as glutathione. At a higher concentration (40 μM), sampangine induces a more pronounced oxidative stress. In this condition, the cell death process began more quickly but provokes preferentially necrosis in comparison to apoptosis (as shown by the blue trypan exclusion test, not shown).

Different hypotheses can be invoked to explain the cytosolic production of reactive oxygen species after the sampangine treatment. By analogy with ascididemin (Matsumoto et al., 2003), it can be proposed that the bioreduction of the iminoquinone function of sampangine facilitates the production of H_2O_2 in cells and that this oxidative stress contributes to the cytotoxicity of the plant alkaloid. But alternative effects can also be proposed to explain the production of reactive oxygen species. Some compounds produce reactive oxygen species after interaction with their cellular targets. This is the case for example for capsaicin, a vanilloid quinone analogue, which inhibits the plasma membrane electron transport system (Macho et al., 1999). By this process, capsaicin induces a rapid increase of reactive oxygen species followed by mitochondrial alterations in mitogen activated human T cells.

Similarly cinnamaldehyde induces depletion of intracellular thiol proteins, thereby modifying the redox status of the cells and facilitating the production of reactive oxygen species to mediate apoptosis (Ka et al., 2003). An effect of sampangine on the plasma membrane electron transport, thiol proteins or related systems may also be considered.

During apoptosis the mitochondrial alterations are often associated with the release of reactive oxygen species in the cytosol (Marchetti et al., 1996). This consideration prompted us to evaluate the mitochondrial alterations during apoptosis induced by sampangine. We initially considered that reactive oxygen species could originate from mitochondria but this hypothesis was subsequently invalidated. Nevertheless, considerable alterations of the mitochondrial functions were detected in the presence of sampangine. The drug induces important perturbations of the mitochondrial membrane potential ($\Delta\psi_m$). With 20 μM sampangine, we observed a collapse of $\Delta\psi_m$ which began after 12 h of drug treatment. The depolarization is preceded by a transient increase of $\Delta\psi_m$. This hyperpolarization effect has been commonly observed with HL-60 cells and various pro-apoptotic drugs (Kluza et al., 2000; Facompré et al., 2000), as well as in other tumor cell models (Poppe et al., 2001; Scarlett et al., 2000; Ly et al., 2003). The mitochondrial alterations induced by sampangine are not affected by cyclosporin A and for this reason we concluded that the permeability transition is not implicated in the action of sampangine.

Giovannini et al. (2002) have described an elegant method to inhibit selectively mitochondrial alteration using very low concentrations (the so called “baby doses”) of the uncoupling agent ClCCP. This original procedure was applied here to investigate further the effects of sampangine on mitochondria. Upon the pre-treatment of HL-60 cells with 500 nM ClCCP, the early hyperpolarization (after 6 h of treatment) of mitochondria induced by sampangine (20 μM) is abolished and as a consequence, the depolarization effect induced by sampangine at 48 h significantly decreases. It is worth mentioning that such a low concentration ClCCP is not cytotoxic to HL-60 cells; cytotoxic effects were observed for concentrations >10 μM (data not shown). There are frequent cases in the literature showing a link between the production of reactive oxygen species and perturbations of the mitochondrial membrane potential. For example, Ouyang et al. (2002) demonstrated that an increase in reactive oxygen species induced by serum deprivation was responsible of mitochondria dysfunctions. The authors observed a marked increase of $\Delta\psi_m$ after 2–3 h followed by a decrease of $\Delta\psi_m$ in primary astrocytes cells. Similarly, Li et al. (1999) showed that a production of reactive oxygen species in HeLa cells caused a transient increase of $\Delta\psi_m$ followed by a decrease. A link has been established between the mitochondrial hyperpolarization and the production of reactive oxygen species. Oxidative damages to mitochondria alter the adenine nucleotide translocator (ANT) and/or the voltage-dependent anion channel (VDAC), which are proteins regulating the ADP/ATP exchange (Ouyang et al., 2002). The inaccessibility of ADP to the mitochondrial matrix prevents the F1F0-ATPase from utilizing the H+ ion gradient created by electron transport, which causes a hyperpolarization of the inner mitochondrial membrane and a swelling of the matrix. This effect provokes the rupture of the mitochondrial outer membrane with depolarization-like consequences. This hypothesis could explain why the permeability transition inhibitor cyclosporine A was ineffective to block the mitochondrial alterations induced by sampangine.

To conclude, we have demonstrated that sampangine, an alkaloid isolated from stem bark of *C. odorata*, induces apoptosis in HL-60 leukemia cells and that reactive oxygen species play a significant role in the pro-apoptotic activity of the molecule. The oxidative stress induced by the drug precedes the mitochondrial alterations. Our work supports the hypothesis that the oxidative burst induced by sampangine is a key step of the apoptotic pathway and a central event of the mechanism of action of the drug. The drug-induced production of reactive oxygen species reported here with human leukemia cells may also contribute to the antifungal, antibacterial and/or antiparasitic activities of the alkaloid.

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