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Induction of apoptosis in a leukemia cell line by triterpene saponins from *Albizia adianthifolia*

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Abstract—Triterpenoid saponins, which are present in plants and some marine animals, exert various important pharmacological effects. The present study examines the effects of adianthifoliosides A, B, and D (AdA, AdB, and AdD) together with two prosapogenins (Pro1 and Pro2) obtained from *Albizia adianthifolia* (Mimosaceae) on human leukemia T-cells (Jurkat cells) and on splenocytes. AdA, AdB, and AdD were found to exhibit a cytotoxic effect on Jurkat cells, whereas the prosapogenins were found to exert a lymphoproliferative effect on this cell type. Furthermore, all tested compounds were found to exert a synergistic lymphoproliferative activity with concanavalin A (ConA) on splenocytes.

The concentrations where the saponins were found to be cytotoxic on Jurkat cells are far below the concentration of hemolysis. These results indicate that another mechanism than membrane permeabilization formation is responsible of the cell cytotoxicity. Thus, we demonstrated that at 5 μM for AdA and at 1 μM for AdD, these compounds induce apoptosis in Jurkat cells. Early apoptotic events were detected by flow cytometry analysis by using a double annexin-V-FITC and propidium iodide staining. In addition, a disrupted mitochondrial membrane potential was observed in cells treated with AdA, AdB, and AdD. Furthermore, a DNA ladder was observed when Jurkat cells were incubated with 1 μM AdD for 24 h.

By comparison between the biological activities of the native compounds with the prosapogenins, we show in this work the important role of the acylation and esterification by different moieties at C-21 and C-28 of the aglycone (acacic acid) in the apoptosis-inducing capacity. Particularly, the monoterpene-quinovosyl moiety is shown to be important for the induction of apoptosis.

1. Introduction

Saponins are triterpene or steroid glycosides, which are present in plants and some marine animals, possess a broad range of biological and pharmacological properties such as molluscicidal, anti-viral, hypoglycemic, and immunomodulating activities.[1–3]

Recently, several saponins have been described to induce apoptosis in different cell types. Among these compounds, protopanaxadiol saponins such as ginsenoside Rh2 and Rg3 for example, have been found to inhibit tumor cell proliferation and tumor growth,[4,5] induce differentiation and apoptosis,[6,7] and inhibit tumor cell invasion and metastasis.[8,9] In addition, two triterpene saponins, securiosides A and B, isolated from the roots of *Securidaca inappendiculata*, induce apoptosis in macrophages,[10] as well as acylated triterpene saponins from *Silene fortunei*, which have been demonstrated to induce apoptosis on Jurkat cells.[11] Furthermore, the avicins, a family of saponins isolated from *Acacia victoriae* (Bentham) decrease tumor cell proliferation and induce apoptosis of Jurkat cells.[12,13]

Apoptosis is a selective, controlled, and genetically programmed cell death process that plays an important role in the balance between cell replication and cell death.[14] In contrast to necrosis, this tightly regulated and complex process exhibits some typical morphological changes, such as chromatin condensation, membrane blebbing, formation of apoptotic bodies, and in most cases, DNA fragmentation.[15]

Abbreviations: AdA, Adianthifolioside A; AdB, Adianthifolioside B; AdD, Adianthifolioside D; Pro1, Prosapogenin 1; Pro2, Prosapogenin 2; ConA, Concanavalin A; DiOC6, 3,3′-Dihexyloxacarbocyanine iodide; PI, Propidium iodide; $\Delta\Psi_m$, Mitochondrial membrane potential.
Keywords: Saponins; Prosapogenins; Lymphoproliferation; Apoptosis.
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We have earlier reported the isolation and structure elucidation of two new prosapogenins (Pro1 and Pro2)[16] from the mild alkaline hydrolysate of the crude saponins extract of the roots of *Albizia adianthifolia* (Mimosaceae). We have also reported the characterization and the cytotoxic activity on Jurkat cells of AdA and AdB, two new triterpene saponins isolated from the crude saponin extract of the roots.[17] The characterization of the new compound AdD from the roots of *A. adianthifolia*,[18] which share some structural identities with the apoptotic saponins avicins from *Acacia victoriae* (Mimosaceae)[19] leads us to explore the cytotoxic effects of our different molecules. The comparative dose effects of these compounds (AdA, AdB, AdD, Pro1, and Pro2) on the cell proliferation of splenocytes and on the viability of a tumor T-cell line and sheep erythrocytes were investigated to understand the mechanisms of action of the compounds. Thus, the present study leads us to examine, which structural part of a saponin is responsible of the apoptosis-inducing effect on Jurkat cells.

2. Results

2.1. Chemical analysis of AdA, AdB, AdD, Pro1, and Pro2

AdA, AdB, AdD, Pro1, and Pro2 were previously isolated and characterized from *A. adianthifolia* (Fig. 1).[16–18]

All these compounds have been shown to possess the same aglycone part (acacic acid). Furthermore, we demonstrated that the three native compounds (AdA, AdB, and AdD) possess the same tetrasaccharide moiety at C(28) of the aglycone, instead of a free C(28) in the case of the prosapogenins Pro1 and Pro2. The differences between these compounds are only due to the different chemical substituents at C(21) of the aglycone (salicylic acid or monoterpene-quinovosyl moiety) and the different oligosaccharidic moieties at C(3) (trisaccharide substituted by a N-acetamido unit or tetrasaccharide). These data will allow us to establish the influences of these chemical substituents on biological activities.

2.2. Lymphoproliferative and cytotoxic effects

In the following work, we compared the effect of saponins on normal lymphocytes and on leukemia cells (Fig. 2A), respectively. Furthermore, we wondered if we were able to observe a synergistic effect between saponins and a T lymphocyte mitogen (ConA) on splenocytes. Splenocytes were stimulated to proliferate with ConA, and proliferation capacity was estimated by [^{3}H]-thymidine incorporation (Fig. 2B).

As shown in Figure 2A, lymphoproliferative effects were observed on splenocytes with treatment with all compounds. The Figure 2B shows that splenocytes activated with ConA can proliferate by synergy with all compounds. For example, a SI of 1.99 ± 0.19 was observed for cells treated with 10^{-1} μM AdA and a SI of 15.33 ± 2.89 was observed in the case of the 1 μg/mL ConA-treated cells without saponins whereas a SI of 27.37 ± 2.37 was observed in the case of cells treated with both 10^{-1} μM AdA and 1 μg/mL ConA. A cytotoxic effect toward splenocytes was observed only following treatment with AdD at 1 μM and an inhibition of the proliferation of the ConA-treated splenocytes was observed following treatment with AdA and AdD at 1 μM.

By comparison, inhibition of Jurkat cells proliferation was observed followed treatment with AdA, AdB, and AdD, whereas the prosapogenins Pro1 and Pro2 were able to induce a weak lymphoproliferative effect on this cell type. AdD was found to be the most cytotoxic (from 10^{-1} μM), following by AdA and AdB, cytotoxic at 1 μM (Fig. 2A).

2.3. Hemolysis

Strong hemolytic activity is considered to be a typical characteristic of saponins, although some saponins show only weak or no hemolytic effect at all. As shown in Figure 3, although AdA and AdD curves are very close, AdD was shown to exhibit the strongest hemolytic activity with a HC_{50} of 12.5 μg/mL (=5.66 μM), followed by AdA with a HC_{50} of 17.7 μg/mL (=10.47 μM) and by AdB with a HC_{50} of 36.6 μg/mL (=20.20 μM), successively. The 100% hemolysis was obtained at 200 μg/mL for AdB (data not shown). Pro1 and Pro2 did not exhibit any hemolytic activity at all tested concentrations.

Cytotoxic activity on Jurkat cells was observed at 10^{-1} and 1 μM for AdD, and at 1 μM for AdA and AdB, but no hemolytic activity was observed at these concentrations for these compounds, so we evidenced at least one-log differential sensitivity.

2.4. Cell cycle analysis

The nuclei in apoptotic cell show reduction in DNA stainability with PI, indicated by the appearance of a sub-G1 peak on the DNA histogram.[20] Table 1 shows the flow cytometry analysis on fixed Jurkat cells stained with PI. In 24 h culture with 1 μM of saponin, the apparition of a sub-G_1 peak was observed by flow cytometry analysis in 14.8% of the AdD-treated cells, which is 1.85 fold more than untreated control cells. In 24 h with 5 μM of saponin, 35.9% of the AdD-treated cells and 39.6% of the AdA-treated cells exhibited a sub-G_1 peak, which is respectively 4.5 times and 4.9 times more than untreated control cells. Furthermore, lymphoproliferative effect of Pro1 and Pro2 is confirmed at 5 μM by observation of a reduction of the number of cells in sub-G1 phase.

2.5. Evaluation of apoptosis by annexin V-FITC staining

Apoptotic Jurkat cells were detected by double labeling with propidium iodide (PI) and annexin V-fluorescein isothiocyanate (FITC). Cells beginning to undergo apoptosis expose the phosphatidylserine groups to the outer cellular membrane and annexin V can bind it, but these early apoptotic cells conserved cytoplasmic membrane integrity and are not labeled by PI ($FITC^+PI^-$). The results were analyzed by flow cytometry.

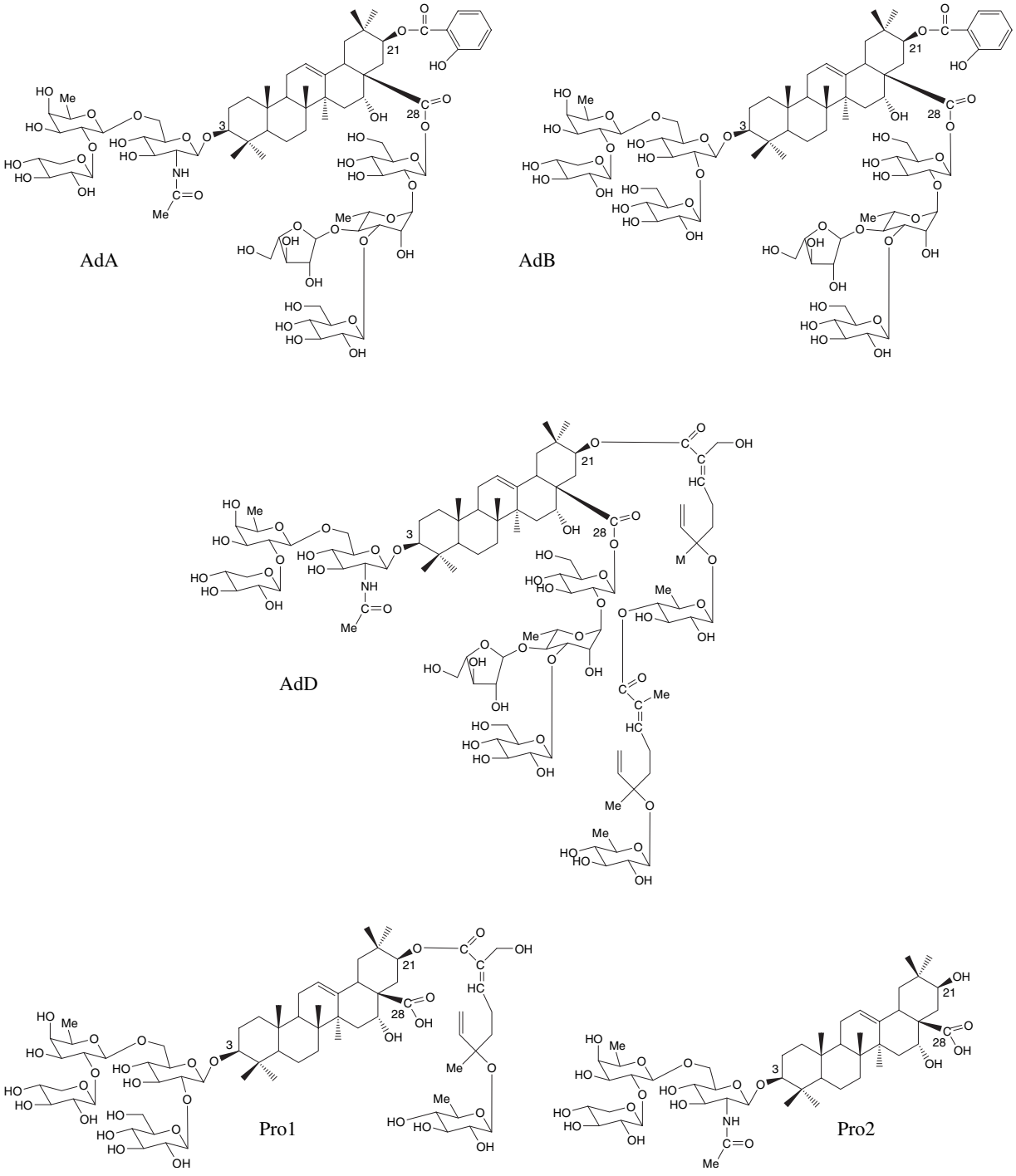

Figure 1. Chemical structures of AdA, AdB, AdD, Pro1, and Pro2.

As shown in Table 2, 26.8% of cells treated with 5 μM of AdA after 24 h bound Annexin V-FITC ($FITC^+PI^-$), suggesting that these cells were progressing through early stages of apoptosis, instead of a proportion of 4.9% after 24 h culture with 1 μM of AdA, and 3.4% for untreated control cells. In addition, effect is dose-dependent: 11.2% of the AdD-treated cells at 1 μM are $FITC^+PI^-$, instead of 28.9% at 5 μM. Moreover, the lymphoproliferative effect of Pro1 and Pro2 is confirmed at 5 μM by observation of an increasing number of $FITC^-PI^-$ cells, corresponding to the viable cells.

2.6. Effect of AdA, AdB, and AdD on mitochondrial membrane potential $\Delta\Psi_m$

The inner membrane of mitochondria is characterized by a transmembrane potential $\Delta\Psi_m$ generated through the activity of proton pump of the respiratory chain.[21] We determine here whether induction of apotosis by the saponins involves alteration of $\Delta\Psi_m$.

Figure 4 shows the effect of the saponins on mitochondrial membrane potential $\Delta\Psi_m$ by using DiOC6, a

mitotracker. A disrupted mitochondria membrane potential was observed in cells treated with 5 μM AdA, AdB or AdD for 4 h. This test allows us to confirm the relative effect of each component, with AdD the most active and then, AdA and AdB.

2.7. DNA fragmentation

One of the features of apoptotic cells is extensive degradation of the DNA at the linkers between the nucleosomes. The result is a series of oligomers of DNA with a unit length of about 180 bp, forming the typical 'DNA ladder'.[22] The observed breakage of cellular DNA detected by agarose gel electrophoresis shows the characteristics of apoptotic cell death. As shown in Figure 5, a 'DNA ladder' pattern appeared when Jurkat cells were treated with 1 μM of AdD for 24 h. No 'DNA ladder' appeared in the case of AdB, AdA, Pro2, and Pro1 at 1 or 5 μM (data not shown).

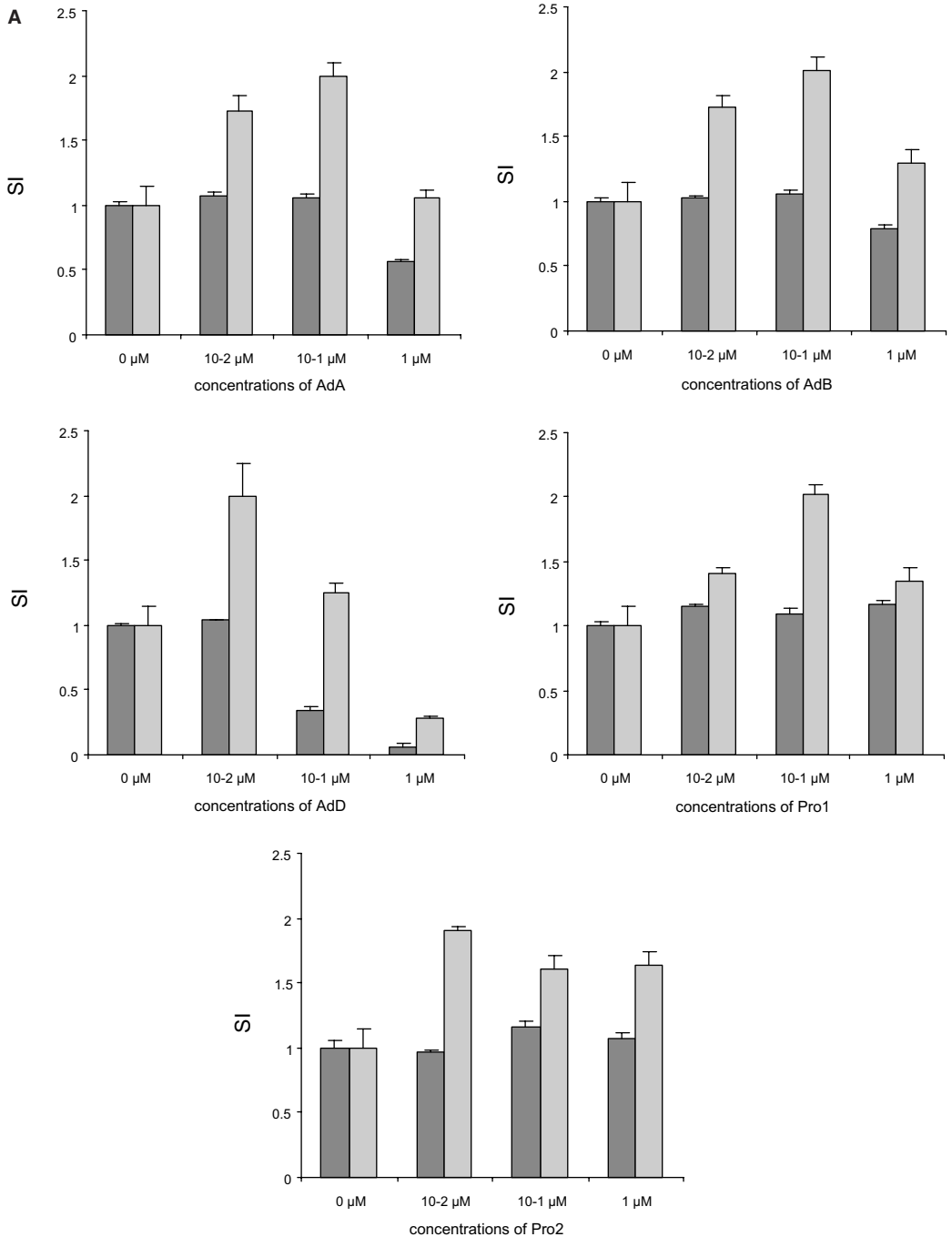

Figure 2. (A) Effect of compounds AdA, AdB, AdD, Pro1, and Pro2 on the cellular proliferation of Jurkat cells and of splenocytes measured by [^{3}H]-thymidine incorporation (see experimental section). SI = stimulation index. Each column represents the mean ±SE ($n = 4$). ▮ on Jurkat cells, ▮ on splenocytes. (B) Effect of compounds AdA, AdB, AdD, Pro1, and Pro2 on the cellular proliferation of splenocytes with concanavalin A measured by ^{3}H-thymidine incorporation (see experimental section). SI = stimulation index. Each column represents the mean ±SE ($n = 4$). Control: corresponds to cells stimulated with 1 μM ConA.

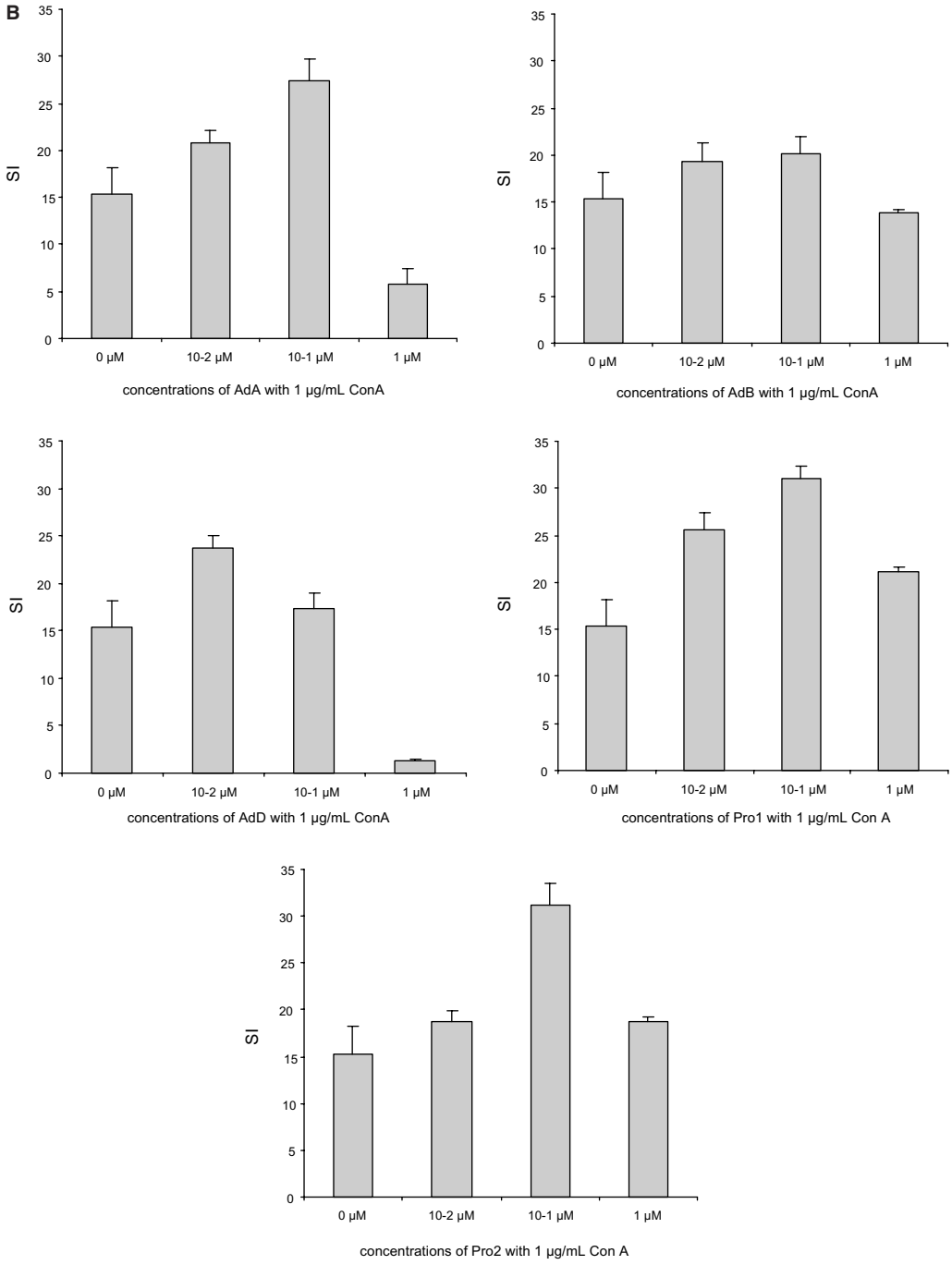

Figure 2. (*continued*)

3. Discussion

Natural products have been shown to be excellent and reliable sources for the development of new drugs. Triterpenoids are one family of natural compounds known for their medicinal value[1] and they form a large and a diverse class of organic compounds in plants.

We investigate here the cytotoxic and immunomodulatory effects of prosapogenins and triterpene saponins on Jurkat cell lines and on splenocytes. The aim of the present study was to compare the effects of AdA, AdB, AdD, Pro1, and Pro2, five compounds obtained from *A. adianthifolia*, on Jurkat cells and on splenocytes and to investigate their ability to induce apoptosis in a human T-cell leukemia cell line. We chose Jurkat cell line because recent studies have shown the ability for triterpene saponins to inhibit the growth of several tumor cell lines including Jurkat cells. We used splenocytes to compare selective biological activities of triterpene saponins between the two cell types, a tumor T lymphocyte cell line and normal lymphocytes.

The cellular proliferation was measured by ^{3}H-thymidine incorporation in the cellular DNA, and apoptotic cells were detected by double labeling with

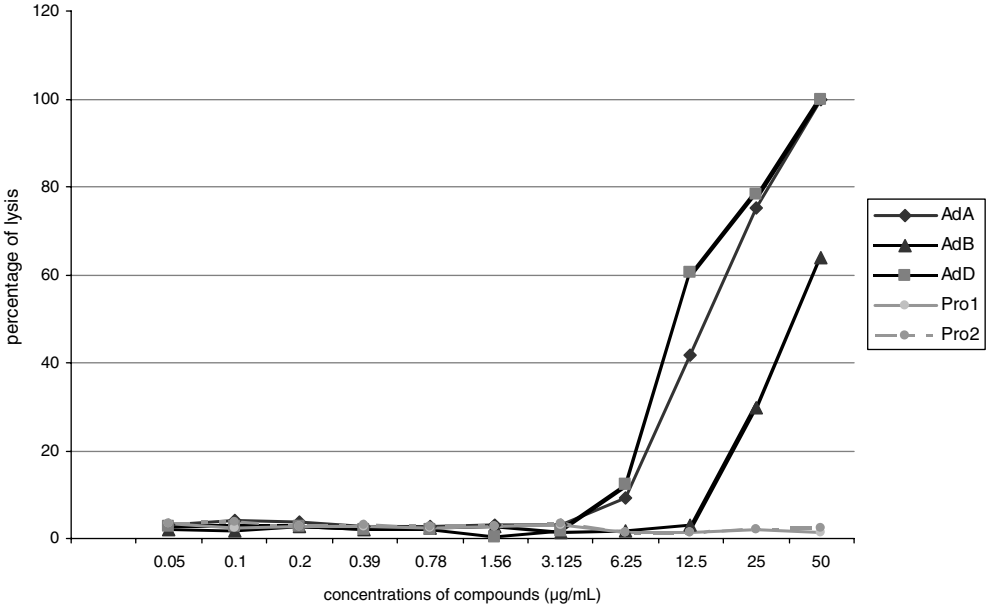

Figure 3. Evaluation of the hemolytic activities of AdA, AdB, AdD, Pro1, and Pro2 on sheep erythrocytes according to the procedure described in the experimental section.

Table 1. Percentage of the sub-G1 phase of Jurkat cells treated with AdA, AdB, AdD, Pro1, and Pro2

	Concentrations	Sub-G1 phase (% of cells)
Untreated cells		8.0
Anti-Fas antibody	50 ng/mL	34.2
AdA	1 μM	9.7
	5 μM	39.6
AdB	1 μM	8.9
	5 μM	12.3
AdD	1 μM	14.8
	5 μM	35.9
Pro1	1 μM	7.8
	5 μM	5.6
Pro2	1 μM	7.7
	5 μM	5.8

Positive control corresponds to cells incubated with 50 ng/mL anti-Fas antibody.

propidium iodide (PI) and annexin V. The mitochondrial membrane potential $\Delta\Psi_m$ was also investigated. These results were analyzed with flow cytometry technique. More-over, the integrity of DNA in cells was analyzed. In order to make a distinction between effects of these compounds on membrane cells and their effects on the cell activities, we evaluated the hemolytic properties of the triterpenoids on sheep erythrocytes.

In the present study, we show that AdD displays the strongest cytotoxic effect on Jurkat cells, followed by AdA and then by AdB, respectively, whereas some lymphoproliferative effects are observed on splenocytes for these saponins. These data suggest the selective effects of these compounds toward the two cell types. Splenocytes are more sensitive to a lymphoproliferative effect following treatment with saponins than leukemia T-cells, whereas these latter are more sensitive to a cytotoxic effect after treatment with these compounds than splenocytes. Nevertheless, the immunomodulation of these compounds is weak, with a stimulation index (SI) of 2. Thus, in order to study more precisely the immunomodulatory properties of the compounds, we investigate the effect of the saponins and prosapogenins on the proliferation of ConA-stimulated splenocytes. The tested compounds exert a synergistic lymphoproliferative activity with ConA on splenocytes at low concentrations whereas at high concentrations, no proliferative effect is observed. This result can be explained by the too high activation of the cells that lead to their death (by the AICD mechanism: Activation Induced Cell Death).[23] These immunomodulatory properties of the saponins are interesting for immunotherapy, particularly in the case of tumor specific T-cells. Furthermore, if we consider the mechanisms of action of the saponins, their hemolytic activity is explained by

Table 2. Effect of AdA, AdB, AdD, Pro1, and Pro2 on apoptosis of Jurkat cells

Compounds	Untreated cells	Anti-Fas	AdA		AdB		AdD		Pro1		Pro2	
Concentrations		50 ng/mL	1 μM	5 μM	1 μM	5 μM	1 μM	5 μM	1 μM	5 μM	1 μM	5 μM
[FITC$^-$PI$^-$]	91.8	5.7	88.0	60.8	89.8	86.9	68.8	20.6	93.0	92.6	92.2	93.6
[FITC$^+$PI$^-$]	3.4	18.8	4.9	26.8	3.2	6.3	11.2	28.9	2.0	2.2	2.1	2.6
[FITC$^+$PI$^+$]	3.5	73.3	5.5	8.0	4.7	4.3	15.4	46.4	3.1	3.1	3.3	1.9

Apoptosis was assayed by measuring Annexin V binding and PI staining as described in the experimental section.
[a] [FITC$^-$PI$^-$]: Viable cells; [FITC$^+$PI$^-$] cell undergoing apoptosis; [FITC$^+$PI$^+$] dead cells and those in late stage of apoptosis.

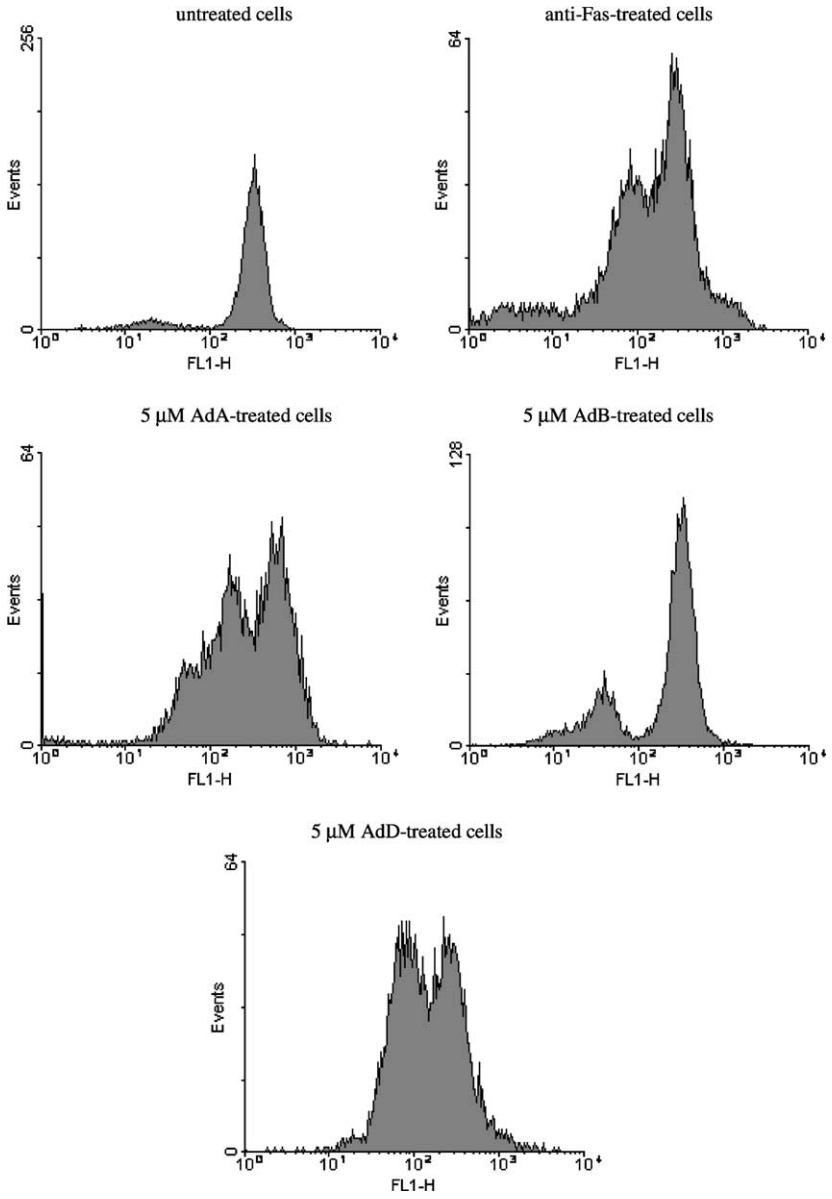

Figure 4. Effect of compounds AdA, AdB, and AdD on mitochondrial membrane potential. Jurkat cells were treated with 5 μM AdA, AdB, and AdD for 4 h. Cells were stained with DiOC6 and analyzed by flow cytometry as described in the experimental section. FL1-H corresponds to DiOC6 fluorescence. Events correspond to the relative cell number.

the interaction of the aglycone with the sterol of the plasma membrane followed by aggregation of the carbohydrate moieties, inducing the rearrangement of the phospholipids bilayer and the pore formation.[24] This provokes a leak of electrolytes and metabolites and increases the permeability of the plasma cell membrane. AdA, AdB, and AdD were not found to be hemolytic in the concentration range where these compounds were found to be cytotoxic on Jurkat cells. These data suggest that another mechanism than permeabilization formation is responsible of cell cytotoxicity and the induction of apoptosis on Jurkat cells observed following treatment with 5 μM AdA and 1 μM AdD after 24 h can explain the high cellular mortality. Furthermore, there is growing evidence that mitochondrial $\Delta\Psi_m$ is altered in cells undergoing apoptosis and our data show that AdD, AdA, and AdB, can provoke $\Delta\Psi_m$ loss in Jurkat cells. This apoptosis inducing property was then confirmed by observation of a DNA ladder pattern after treatment with 1 μM AdD for 24 h. Thus, we can wonder if apoptosis induction by triterpene saponins is due to interactions with cell membrane receptors, or to other mechanisms like direct interaction with plasma or mitochondrial membrane.

A comparison of structures and cytotoxic activities of the prosapogenins and of the native compounds indicates that both the tetrasaccharide at C(28) of the aglycone and the monoterpene-quinovosyl moieties at C(21) of the aglycone are important substituents required for apoptosis, since Pro1 and Pro2 are not cytotoxic. In addition, the structures of the compounds pairs AdA/AdB and of AdA/AdD are very similar. The only difference between AdA and AdB is due to the presence of

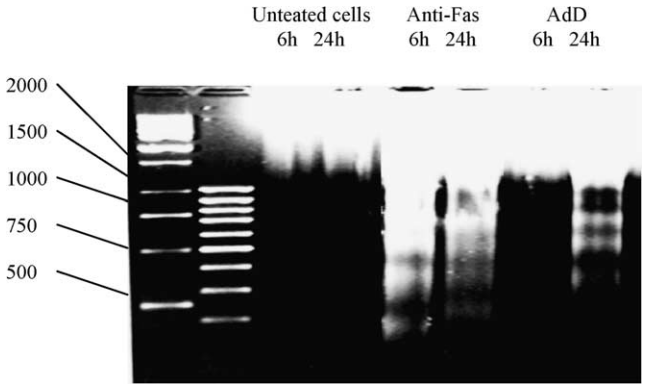

Figure 5. DNA fragmentation after exposure to AdD. Jurkat cells were exposed to 1 μM AdD for the indicated period. Genomic DNA was extracted as described in the experimental section, electrophoresed in a 1.8% agarose gel and visualized with ethidium bromide staining. Anti-Fas antibody was used as a positive control at a concentration of 100 ng/mL.

one N-acetamido group at C(2) of the glucose I substituted at C(3) of the aglycone in AdA, instead of a glucose at the same position in AdB. Thus, a comparison of the cytotoxicity of AdA and AdB suggests that the N-acetamido moiety at C(3) enhances activity, because AdA shows the strongest cytotoxicity. On the other hand, the only difference between AdA and AdD is due to the presence of one salicylic acid at C(21) in AdA whereas in the case of AdD there are two monoterpene-quinovosyl moieties substituted at this position. In the hemolysis assay, the curves obtained for AdA and AdD (when we use the μg/mL unit) are very close and this data show that both salicylic acid and monoterpene-quinovosyl moieties play an important role in the membrane permeabilization. Nevertheless, since AdD exhibits a highest proapoptotic activity comparing to AdA, this result indicates that the monoterpene-quinovosyl moieties increase the apoptosis induction potentiality of a saponin containing this kind of aglycone. If we compare the structures and the biological properties of AdD with those of the avicins, we can notice that all these compounds possess a structure including monoterpene-quinovosyl moieties and exhibited apoptotic activity.

Since these compounds are able at the same concentration to induce apoptosis of leukemia T-cells and activate the proliferation of ConA-stimulated splenocytes, these results should be useful in the search for new potential anti-tumor agents.

4. Experimental

4.1. Drugs

AdA, AdB, AdD, Pro1, and Pro2 were previously isolated by us from the roots of *A. adianthifolia* (Leguminoseae).[16–18] The 95% EtOH extract (20 g) obtained from the roots of *A. adianthifolia* (200 g) was suspended in H_2O and submitted to a partition against *n*-butanol saturated with H_2O. The *n*-butanol extract (7 g) was then purified by precipitation with diethyl ether, yielding a crude saponin mixture (2.5 g), which was then dialyzed for two days. An aliquot of this mixture (1.8 g) was submitted to column chromatography over Sephadex LH-20 and was separated by repeated medium-pressure liquid chromatography (MPLC) over normal silica gel and reversed phase, yielding AdA (13 mg), AdB (14 mg) and AdD (15 mg). The prosapogenins Pro1 (25 mg) and Pro2 (8 mg) were isolated by the same way from the mild alkaline hydrolysate of the roots of this plant. Each saponin was then prediluted in a Dulbecco's Modified Eagle's Medium (DMEM) solution (1 mg/mL), stored at −20 °C, and used within five months.

4.2. Cell culture

Human leukemia T-cells were cultured in DMEM supplemented with 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamin, and 6% FCS at 37 °C under humidified air with 5% CO_2. For proliferation tests, a medium without FCS was used.

Swiss mice were obtained from Depré, St. Doulchard (France). Mice were sacrificed by cervical dislocation and spleens were aseptically harvested and homogenized in a Petri dish. Splenocytes were washed and adjusted to 2.10^6 cells/mL in DMEM culture medium supplemented with 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamin.

4.3. Cell proliferation and viability assay

Jurkat cells resuspended at 2.10^5 cells/mL and splenocytes resuspended at 2.10^6 cells/mL in their respective medium were seeded in 24-well culture plates (NUNC Roskild, Denmark), and the volume of the appropriate concentration saponin sample solution was added. In the case of stimulated splenocytes, the saponin dilutions were added at the same time as ConA at a final concentration of 1 μg/mL (Sigma-Aldrich). The cells were then cultured at 37 °C under a 5% CO_2 atmosphere for 48 h. For the last 16 h, 200 μL aliquots of each well cell suspension were transferred in 96-well culture plates, and four replicates were tested for each dilution of saponin.

Lymphocyte proliferation was determined using ^{3}H-thymidine incorporation. The assay was performed by adding 0.5 μCi/well of commercial solution (Perkin–Elmer, Belgium), and the radioactivity incorporated into cells was measured using a liquid scintillation counter.

Results were calculated as follows:

$$\mathrm{SI(stimulation\ index)} = \frac{^3\text{H-thymidine uptake in cells + saponin}}{^3\text{H-thymidine uptake in cells alone}}.$$

4.4. Assay for hemolytic activity

Hundred microliters of 2.5% sheep erythrocytes suspension in PBS were incubated with 100 μL of saponin dilu-

tions (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.20, 0.10, and 0.05 μg/mL) in PBS at 37 °C for 30 min. After centrifugation (1500 rpm, 5 min), the hemoglobin content in the supernatant (duplicate of 75 μL) was measured at 450 nm using a multiwell spectrophotometer (Dynatech MR 500, Billinghurst, UK). Parallel measurements of saponin dilutions without erythrocytes, erythrocytes without saponin dilutions and buffer without both erythrocytes and saponin dilution were made. The results were then compared to a positive control sample containing erythrocytes in distilled water. The 50% hemolysing concentrations (HC_{50}) of each saponin were then inferred from the hemoglobin absorbance versus saponin concentration curve.

4.5. Cell cycle analysis

Jurkat cells resuspended at 2×10^6 cells/mL, in 1 mL of medium without FCS were seeded in 24-well culture plates with 0.2, 1, and 5 μM of each saponin sample solution, respectively. The cells were cultured at 37 °C under a 5% CO_2 atmosphere for 24 h. The cells were washed with cold PBS (pH 7.4), and then fixed with ice-cold 70% (v/v) ethanol at 4 °C for 1 h. The cells were washed twice with PBS, resuspended in 300 μL PBS, and stained with 40 μL of a solution containing 0.5 mg/mL of propidium iodide (PI). The cells were immediately analyzed by flow cytometry on a fluorescence-activated cell sorter (FACS) flow cytometer (Beckman-Coulter EPICS-ELITE/ESP cytometer).

4.6. Evaluation of apoptosis by annexin V staining

Jurkat cells were resuspended at 2×10^6 cells/mL, and 1 mL of cells without FCS was seeded in 24-well culture plates with 0.2, 1, and 5 μM of each saponin sample solution, respectively. The cells were cultured at 37 °C under a 5% CO_2 atmosphere for 24 h. After washing in cold PBS (pH 7.4), cells were incubated at 37 °C with annexin V-FITC conjugate (TEBU, CA) at 1 μg/mL resuspended in binding buffer (10 mM NaOH, 140 mM NaCl, 2 mM $CaCl_2$) for 15 min in the dark. Cells were washed with the binding buffer, stained with PI (2.5 μg/mL), and analyzed by flow cytometry.

4.7. Measurement of mitochondrial membrane potential

After treatment with AdA and AdD at 37 °C for 4 h, Jurkat cells were incubated with 40 ng/mL of DiOC6 (Molecular Probes, The Netherlands) for 15 min at room temperature before the flow cytometry analysis.

4.8. Evaluation of apoptosis by DNA fragmentation

Jurkat cells grown at a density of 1×10^6 cells/mL were exposed to compounds (AdA, AdB, AdD, Pro1, and Pro2) for different time periods (6 and 24 h) and concentrations (1 and 5 μM). Cells were centrifuged, lysed in 500 μL of 0.5% SDS, 20 mM Tris–HCl, and 5 mM EDTA and proteinase K was added at a final concentration 0.25 mg/mL. After 15 h of incubation at 42 °C, proteins were precipitated with 142 μL of 6 M NaCl solution. After two centrifugations at $2500\times g$ for 15 min at 4 °C, 4 μL of 20 mg/mL RNase A were added to the supernatant and then an incubation of 30 min at 37 °C was performed. DNA was precipitated with 2.5 vol absolute ethanol and stored over night at −20 °C. After centrifugation at $6000\times g$ for 30 min at 4 °C, the sample was washed with 70% ethanol, centrifuged again at $6000\times g$ for 15 min at 4 °C, dried, and resuspended in 30 μL H_2O. The extracted DNA was then applied to 1.8% agarose gel electrophoresis. The DNA fragmentation pattern was examined in photographs taken under UV trans-illumination. As a positive control, DNA isolated from apoptotic Jurkat cells induced by 50 ng/mL anti-Fas antibody (antibody anti-CD95, clone CH11, Immunotech, Marseille, France), was run in parallel.

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