



Cytotoxic effect of potato aspartic proteases (*StAPs*) on Jurkat T cells

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ABSTRACT

StAPs are potato aspartic proteases with cytotoxic activity against plant pathogens and spermatozoa. *StAPs* cytotoxic activity is selective, since these proteins do not exert toxic effect on plant cells and erythrocytes. In this work, we investigated the capacity of *StAPs* to exert cytotoxicity on human leukaemia cells. Obtained results show that *StAPs* induce apoptosis on Jurkat T cells after a short time of incubation in a dose-dependent manner. However, no significant effect on the T lymphocytes viability was observed at all *StAPs* incubation times and concentrations tested. These results suggest that *StAPs* can be conceptually promising leads for cancer therapy.

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1. Introduction

Antimicrobial proteins and peptides (AMPPs) are important components of the natural defences of most living organisms against invading pathogens and are found in a wide range of eukaryotic organisms, from humans to plants [1–7]. The discovery of new groups of AMPPs as potential natural antibiotics represents a hit toward the discovery of a novel generation of drugs for the treatment of bacterial and fungal infections [8,9]. Moreover, the broad spectrum of antimicrobial activities reported for these molecules suggests their potential benefit in the treatment of viral or parasitic infections [10,11] and cancer [12–14]. In contrast to conventional antibiotics, they act by physical disturbance or destruction of the barrier function of the plasma membrane cell without involvement of a specific receptor [15,16]. Some have been successfully used to eliminate, e.g., prostate tumor cells *in vitro* [17], and others were also very effective *in vivo* in

the elimination of leukaemia, ascite and ovarian tumors [18,19]. In plants, *in vitro* antimicrobial activity has been demonstrated for the following peptides and proteins: (i) some of the so-called pathogenesis-related proteins, which were originally identified as pathogen-elicited proteins [20,21]; and (ii) a number of plant antimicrobial protein and peptide families [22]. Furthermore, plant proteins and peptides with cytotoxic activity and anticancer properties *in vitro* on human cancer cell lines have been reported [23–30].

Aspartic proteases (EC 3.4.23) (AP) are a class of widely distributed proteases present in animals, microbes, viruses and plants [31,32]. Biological functions of plant APs have not been characterized as those of their mammalian, microbial or viral counterparts [31–33]. Previously, we have studied the biochemical properties, the pattern of expression and the biological functions of potato aspartic proteases (*StAPs*: *Solanum tuberosum* aspartic proteases, Merops family A1) [34,35]. We have identified three *StAPs*: one from tuber (*StAP1*) and two from leaves (*StAP2* and *StAP3*). Two of these isoforms, *StAP1* and *StAP3*, have been purified and characterized [34,35]. Both proteins are induced by abiotic and biotic stresses, and have extracellular localization and antimicrobial activity towards potato pathogens [35–37]. The mechanism of action of *StAPs*, as well as other AMPPs,

Abbreviations: *StAP*, *Solanum tuberosum* aspartic protease; *AP*, Aspartic protease; *AMPPs*, Antimicrobial proteins and peptides.

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involved binding to the microbial cell surface, plasma membrane disruption and cell death [38]. Both StAPs act as spermicidal compound inducing plasma membrane destabilization and cell death on bovine and human spermatozoa, but no harmful effect was reported on human erythrocytes [39]. Based on these results and with results reported for other AMPPs, we speculate that StAPs could have antitumoral activity. In the present work, we therefore tested the cytotoxic effect of StAP1 and StAP3 on Jurkat T cells, a human leukaemia cell line, and on freshly isolated, non-transformed human peripheral T lymphocytes. Our results show that StAP1 and StAP3 cause both apoptotic and necrotic effects on Jurkat T cells; however, no significant effect was detected on non-transformed T lymphocytes.

2. Materials and methods

2.1. Cells and in vitro culture

Jurkat T-leukaemia cells were grown in suspension and propagated in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (all obtained from Sigma, St Louis, MO). To maintain exponential growth, the cultures were divided every third day by dilution to a concentration of 1×10^5 cells/ml.

Blood samples (20 ml) were obtained from AVIS (Italian Association of Voluntary Blood Donors); donors provide written, informed consent for the study and use of samples at the time of donation. Human mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Lymphocyte cells were cultured in RPMI 1640, containing 10% (v/v) heat-inactivated fetal bovine serum, and 5 µg/ml phytohemagglutinin (PHA; Sigma). Jurkat T cells and T lymphocyte cells were incubated at 37 °C, 5% CO₂. After PHA stimulation, >90% was activated T cells [40].

2.2. Protein purification

The Balcarce Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina, provided potato tubers (*S. tuberosum* L. cv. Pampeana). *S. tuberosum* L. cv. Pampeana INTA (MPI 59.789/12 × Huinkul MAG) is a cultivar from the Argentine Breeding Program (INTA-Balcarce).

Sterile disks (10 mm diameter, 2 mm thick) of potato tuber (*S. tuberosum* L. cv. Pampeana INTA) were prepared, washed extensively, suspended in sterile water (15 disks with 23 ml of water) and aerated for 24 h at 25 °C in an orbital shaker at 60 cycles/min. StAP1 was purified using a Q-Sepharose Fast Flow ion exchange chromatography column followed by a Sepharose-pepstatin A affinity column as previously described [34].

Potato leaves (*S. tuberosum* L. cv. Pampeana INTA) were detached and were placed at 18 °C in a moist chamber. StAP3 was purified using a Q-Sepharose Fast Flow ion exchange chromatography column, followed by a Sepharose-pepstatin A affinity column as previously described [35].

The purity of proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide gels [41]. Gels were stained with silver nitrate [42].

2.3. Cytotoxicity test

Viability was determined using Trypan blue dye-exclusion test, which distinguishes viable and non-viable cells [43]. Jurkat cells and T lymphocytes were treated with different concentrations of StAP1, StAP3 or bovine serum albumin (BSA) (0.25 µM, 1.5 µM, 1.8 µM, and 3.75 µM) for 24 and 48 h respectively (i.e. the exposure times correspond to the length of the cell-cycle for Jurkat T cells and T lymphocytes). Results were calculated as viable cells in StAPs-treated cultures relative to control. Concentration causing cell toxicity by 50% following one cell-cycle exposure (i.e. IC₅₀) was calculated by interpolation from dose–response curves.

2.4. Flow cytometry

Micro-flow cytometric analysis was performed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) with the cell assay extension. For cell assays, individual cell fluorescence intensities were measured at two wavelength channels (excitation wavelength 470 nm/emission wavelength 525 nm and excitation wavelength 635 nm/emission wavelength 680 nm). The loading of the microfluidics chip was performed according to the manufacturer's recommendations. Briefly, after pre-treatment of the chip with 10 µl of priming solution (Cell Fluorescence LabChip Kit, Agilent), a focusing dye solution (LabChip Kit) was applied to the appropriate well of the LabChip for adjustment of the Bioanalyzer optics to each individual chip. Prior to loading of samples, two aliquots (30 µl each) of Cell Buffer were pipetted into the appropriate wells. A total of 6 individual samples with a volume of 10 µl each were analyzed, on each chip.

2.5. Evaluation of apoptosis and necrosis by flow cytometry

Cells of exponentially growing cultures were collected at a time corresponding to one cell-cycle (24 h for Jurkat T cells and 48 h for T lymphocytes). In order to evaluate if the apoptotic and necrotic effects of StAPs require either acute (hours) or continuous (days) exposures to each compound, a shorter (6 h for Jurkat T cells and 24 h for T lymphocytes) and a longer (48 h for Jurkat T cells and 72 h for T lymphocytes) time-points were studied.

After treatment, cells were centrifuged (100 × g) for 10 min, resuspended at a density of 1×10^6 cells/ml in 500 µl of medium containing 10 µl of Media Binding Reagent (Merck KGaA, Darmstadt, Germany) and 1.25 µl of Annexin V–biotin (Merck KGaA) and incubated for 15 min. After washing, cells were resuspended in 500 µl of Binding Buffer (Merck KGaA) and treated with 1 µl of Cy-5-labelled streptavidine (1 mg/ml, GE Healthcare Biosciences, Uppsala, Sweden) and 0.5 µl of calcein 500 µM (Molecular Probes). After 30 min, the cells were centrifuged and resuspended in Cell Buffer (Agilent Technologies). The chip was prepared for the flow cytometric measurements as described above.

2.6. Statistical analysis

All data are the mean + S.E. of at least three experiments. Statistical significance of the results was evaluated using

analysis of variance followed by Student's *t*-test or Dunnett's *t*-test (SigmaStat 3.0). Differences between values were considered statistically significant if $P < 0.001$.

3. Results

The effect of StAPs on Jurkat T and T lymphocyte cells viability was analyzed by Trypan blue dye-exclusion test [43]. Fig. 1 shows the growth curves of Jurkat T cells and T lymphocytes after incubation with different amounts of StAPs. Both proteins were able to cause a dose-dependent decrease on the Jurkat T cells viability percentage. The IC_{50} values were determined by interpolation from the dose-response curves. For StAP1 and StAP3 IC_{50} values were i.e. 2.0 μ M and 2.5 μ M for StAP1 and StAP3 respectively. However the maximal cytotoxic effect of StAP3 was greater (80%) than the effect of StAP1 (60%) on Jurkat T cells viability, at the highest concentration assayed (4 μ M). The cytotoxicity of these proteins on T lymphocyte cells was lower than that observed on Jurkat T cells, at all concentrations assayed. StAP1 and StAP3 were able to produce a reduction of 20% and 30%, respectively, in the viability of T lymphocyte cells at the highest concentration assayed (4 μ M).

Changes in cell morphology were analyzed by microscopy. Only in Jurkat T cells at highest StAP concentrations, plasma membrane disruption and release of cytoplasm were observed (data not shown). No significant decreases in the cells viability were observed when Jurkat T cells and T lymphocytes were incubated with different amounts of BSA (data not shown).

Using flow cytometry we quantified the percentage of calcein^{positive} cells (i.e. necrotic cells), to determine the percentage of necrotic cells (Jurkat T cells and T lymphocytes) after incubation with StAPs. Cells were incubated with different amounts of StAPs for a time corresponding to one cell-cycle, i.e. 24 h for Jurkat T cells, 48 h for T lymphocytes. Additionally, a shorter (6 h for Jurkat T cells and 24 h for T lymphocytes) and a longer (48 h for Jurkat T cells and 72 h for T lymphocytes) times were analyzed. Results obtained demonstrate that StAPs (StAP1, Fig. 2; and StAP3, Fig. 3) were able to induce cell necrosis in Jurkat T cells, in a dose-dependent manner, at all times and concentrations assayed. No significant increases in the necrotic cells percentage with respect to the control were observed in T lymphocyte cells at all times and StAP concentrations assayed (Figs. 2D,E,F and 3D,E,F). At shortest incubation time, StAPs induce, in Jurkat T cells, a dose-dependent increase in the fraction of necrotic cells. Low concentrations of StAP1 (Fig. 2A) and StAP3 (Fig. 3A) were able to increase by 15% and 27% the percentage of necrotic cells, respectively. At the highest concentration of StAPs assayed (3.75 μ M), an increase in the necrotic cells of 55% approximately, was observed (Figs. 2A and 3A). After 24 h of incubation, the start of statistically significant increases in the percentage of necrotic cells was observed at 0.25 μ M to StAP1 and 1.5 μ M to StAP3. An increase in the percentages of necrotic cells was observed at 3.75 μ M of StAPs, with respect to these percentages at short time (60% and 82% to StAP1 and StAP3 respectively) (Figs. 2B and 3B). No significant increases in the percentages of necrotic cells

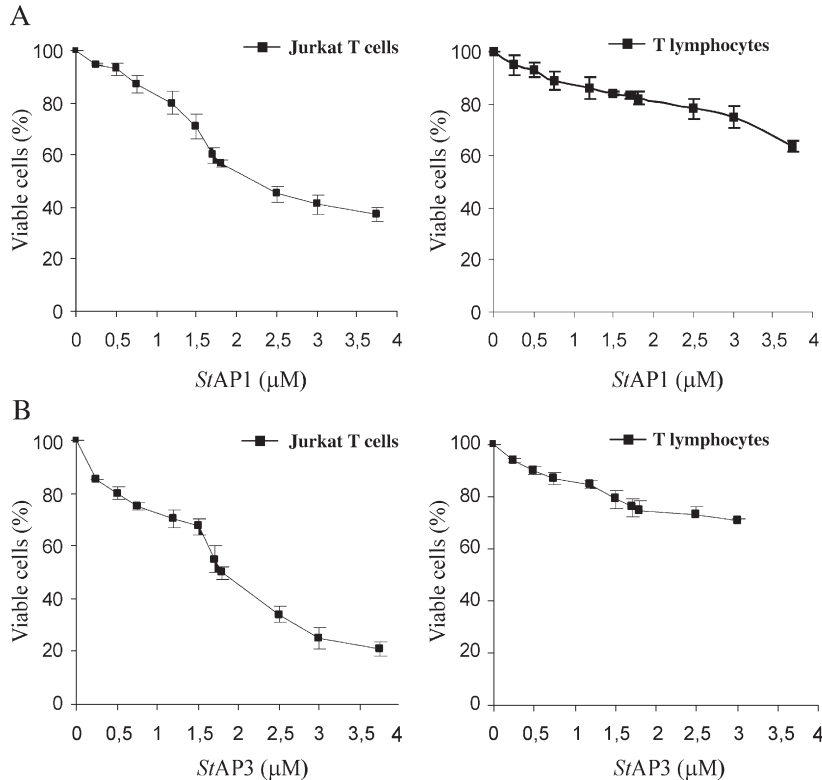


Fig. 1. The effects of StAP1 (A) and StAP3 (B) on viability of Jurkat T cells and T lymphocytes. Cells were removed from protein-treated and untreated (control) cultures after one cell-cycle. The data presented are average from three independent experiments, with error bars denoting standard errors.

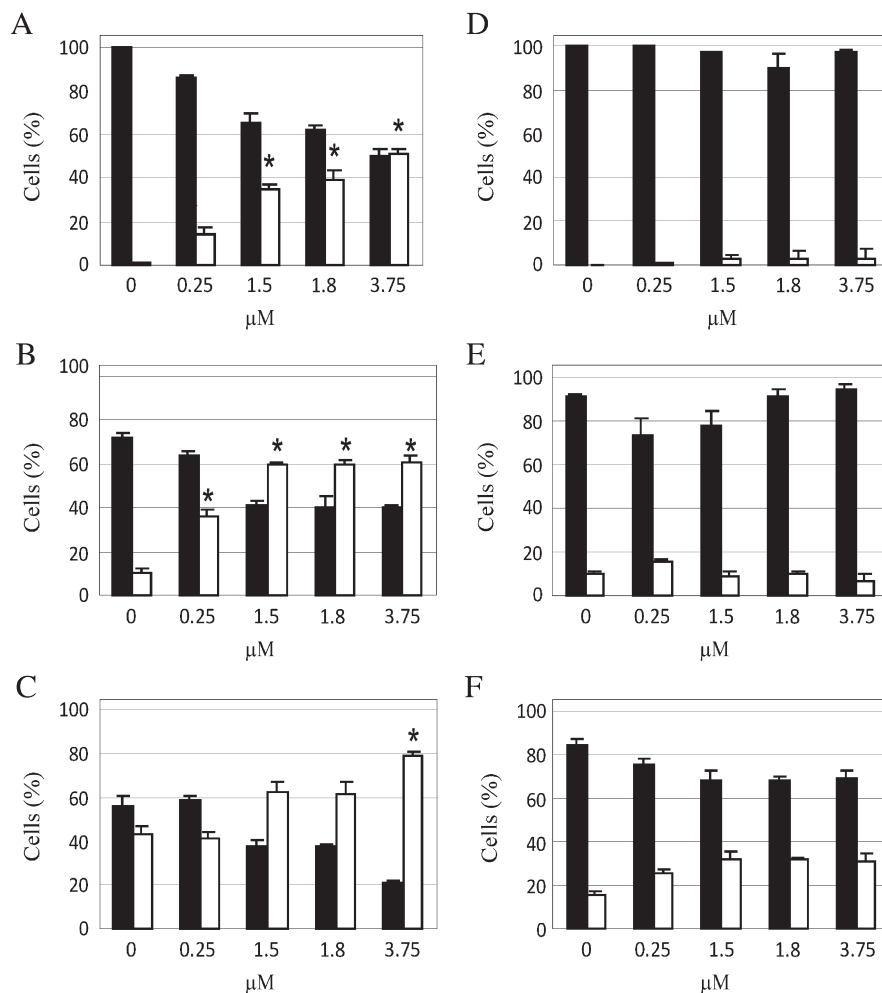


Fig. 2. Fraction of viable cells (filled bars) and necrotic cells (clear bars), as detected in Jurkat T cells (A, B, and C) and T lymphocytes (D, E, and F) treated with StAP1 at the indicated doses for 6, 24 and 48 h for Jurkat T cells (A, B and C respectively) and for 24, 48 and 72 h for T lymphocytes (D, E and F respectively). Cells were removed from treated and untreated cultures and stained with Annexin V–Cy-5 and calcein. The data presented are average from three independent experiments, with bars denoting standard error. * $P < 0.001$ with respect to the controls.

were observed, with respect to the control, in T lymphocytes treated with StAPs for 24 h (Figs. 2D and 3D); 48 h (Figs. 2E and 3E) and 72 h (Figs. 2F and 3F), at all concentrations assayed. No effects on the necrotic cell percentages were detected in Jurkat T cells and T lymphocytes treated with BSA (data not shown).

In order to analyze the induction of apoptosis of Jurkat T and T lymphocyte cells, the percentages of Annexin V^{positive}–calcein^{negative} cells (i.e. apoptotic cells) were measured by flow cytometry. Assays were performed incubating Jurkat T and T lymphocyte cells with different amounts of StAPs during 6, 24 and 48 h for Jurkat T cells and 24, 48 and 72 h for T lymphocytes. Table 1 shows that, StAPs induce apoptosis in a dose-dependent manner only in Jurkat T cells, at all times assayed, with respect to the percentage of apoptotic cells determined in the controls. Moreover, in Jurkat T cells, StAP1 caused apoptosis after 6 h of treatment, whereas StAP3 caused that effect at 6 h (Table 1) and 24 h (data not shown) of incubation. After 6 h of treatment at 3.75 μM of StAPs, the percentage of apoptotic Jurkat T cells was 26.6%

for StAP1 and 34.11% for StAP3 (versus 6.8% and 4.4% in the controls respectively). On the other hand, the incidence of apoptotic T lymphocytes at 24 h of incubation at all doses of StAPs assayed was not significantly different to the control (Table 1). No increases in the induction of apoptosis were detected in Jurkat T cell and T lymphocyte cultures treated with BSA (data not shown).

4. Discussion

Several studies on AMPPs have focused in their putative antitumoral activities on human and mammalian cancer cell lines *in vitro* [8–10,17–19,44–46]. In consequence, they have been characterized as alternative compounds to be employed in cancer treatment and/or prevention [8–10]. We have previously reported that StAPs have cytotoxic activity against potato pathogens and human and bovine spermatozoa [36,37]. We have demonstrated that cytotoxic activity of StAPs involves plasma membrane permeabilization and, subsequently, cell death [38,39]. In this work we show that

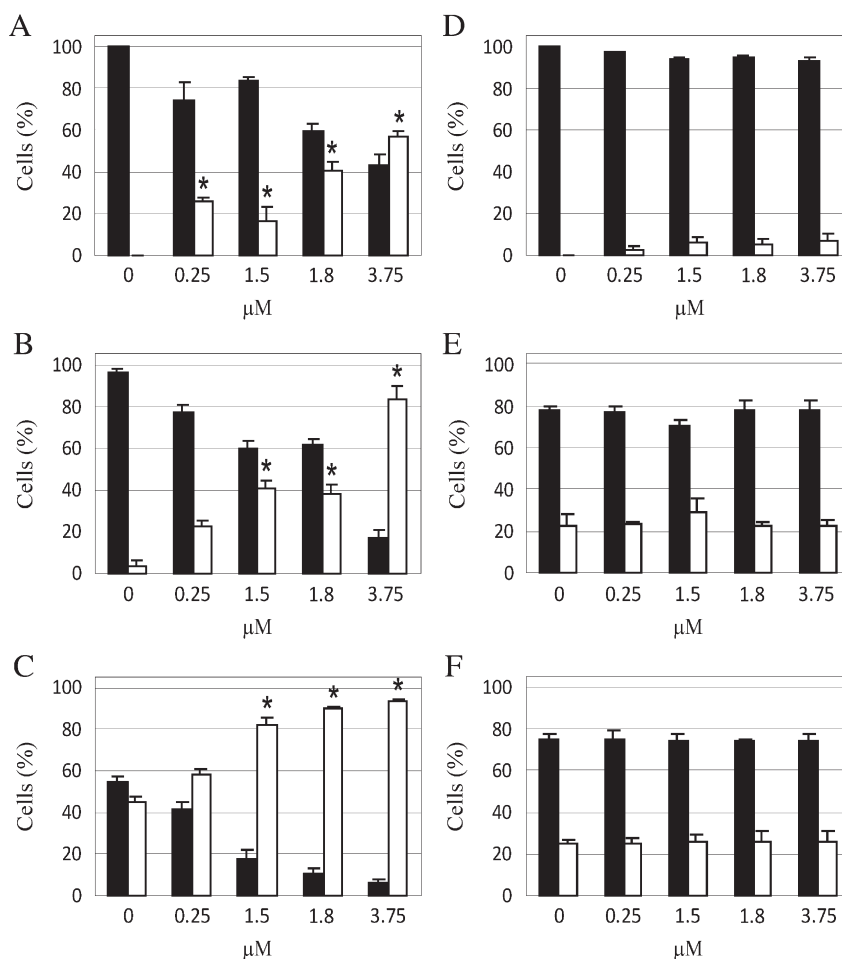


Fig. 3. Fraction of viable cells (filled bars) and necrotic cells (clear bars), as detected in Jurkat T cells (A, B, and C) and T lymphocytes (D, E, and F) treated with StAP3 at the indicated doses for 6, 24 and 48 h for Jurkat T cells (A, B and C respectively) and for 24, 48 and 72 h for T lymphocytes (D, E and F respectively). Cells were removed from treated and untreated cultures and stained with Annexin V–Cy-5 and calcein. The data presented are average from three independent experiments with bars denoting standard error. **P* < 0.001 with respect to the controls.

StAP1 and StAP3 exert cytotoxic effect on Jurkat T cell, a human leukaemia cell line. StAPs were able to produce significant dose-dependent decreases in the percentage of viable Jurkat T cells, whereas only a slight decrease was detected in the viability of non-transformed T cells. Also, cell

morphology alterations were also observed in Jurkat T cells incubated with different amounts of StAPs. These changes involve plasma membrane disruption and release of cytoplasm (data not shown). The StAP concentrations needed to reduce Jurkat T cell viability by 50% were in the same order of magnitude than those previously reported for plant peptides and proteins active against human tumor cells [23,47–49].

Table 1
Incidence of apoptosis in Jurkat T cells and T lymphocytes treated with different doses of StAP1 and StAP3.

StAP	Concentration of StAPs (μM)	% Apoptosis	
		Jurkat T (6 h)	Lymphocytes (24 h)
StAP1	Control	6.80 ± 0.80	4.30 ± 0.20
	0.25	8.03 ± 1.20	5.30 ± 0.30
	1.50	19.42 ± 6.20*	4.10 ± 0.80
	1.80	18.00 ± 7.80*	3.80 ± 0.70
	3.75	26.60 ± 7.40*	4.20 ± 0.40
StAP3	Control	4.40 ± 0.60	6.50 ± 0.80
	0.25	8.80 ± 1.20*	6.10 ± 1.50
	1.50	10.24 ± 1.50*	7.10 ± 2.30
	1.80	19.95 ± 1.40*	5.90 ± 3.40
	3.75	34.11 ± 0.30*	6.30 ± 6.50

* *P* < 0.001 with respect to the control.

The results herein obtained show that StAPs were able to produce necrosis in dose-dependent manner on Jurkat T cells, while this effect was not exerted on T lymphocytes. Additionally, we show that StAPs induce apoptosis only on Jurkat T cells after short times of incubation at the highest concentration assayed; however, no significant apoptosis induction was observed on T lymphocytes at any time and StAP concentrations assayed. These results are in accordance with previous reports showing that the capacity of many antitumoral compounds to cause necrosis or apoptosis *in vitro* could depend on the doses and/or the time of treatments. That is, some drugs could induce necrosis at high doses and prolonged times of treatment or alternatively, could induce apoptosis at a short time of treatment or subnecrotic doses [50–52]. The capacity of StAPs to induce

apoptosis on human leukaemia Jurkat T cells provides an interesting starting point for further investigations on the molecular mechanisms underlying that effect, as well as on the suitable conditions to induce programmed cell death without necrosis, which consequently produces an inflammatory process.

Because selective targeting for cancer cells is a fundamental requisite for potential chemotherapy agents, the lack of haemolytic activity of StAPs *in vitro* [39] and its no significant cell toxicity on non-transformed T lymphocytes, could be important features. However, more assays involving other cell lines should be performed.

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