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

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ABSTRACT

*St*APs are potato aspartic proteases with cytotoxic activity against plant pathogens and spermatozoa. *St*APs 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 *St*APs to exert cytotoxicity on human leukaemia cells. Obtained results show that *St*APs induce apoptosis on Jurkat T cells after a short time of incubation in a dose-dependent manner. However, no significative effect on the T lymphocytes viability was observed at all *St*APs incubation times and concentrations tested. These results suggest that *St*APs can be conceptually promising leads for cancer therapy.

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 (*St*APs: *Solanum tuberosum* aspartic proteases, Merops family A1) [34,35]. We have identified three *St*APs: one from tuber (*St*AP1) and two from leaves (*St*AP2 and *St*AP3). Two of these isoforms, *St*AP1 and *St*AP3, 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 *St*APs, as well as other AMPPs,

Abbreviations: *St*AP, *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 *St*APs 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 *St*APs could have antitumoral activity. In the present work, we therefore tested the cytotoxic effect of *St*AP1 and *St*AP3 on Jurkat T cells, a human leukaemia cell line, and on freshly isolated, non-transformed human peripheral T lymphocytes. Our results show that *St*AP1 and *St*AP3 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% CO_2. After PHA stimulation, >90% was activated T cells [40].

2.2. Protein purification

The Balcarce Experimental Station of the Instituto National 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. *St*AP1 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. *St*AP3 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 *St*AP1, *St*AP3 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 *St*APs-treated cultures relative to control. Concentration causing cell toxicity by 50% following one cell-cycle exposure (i.e. IC_{50}) 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 *St*APs 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 ($100\times g$) 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 resupended 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 Dunnet's *t*-test (SigmaStat 3.0). Differences between values were considered statistically significant if $P<0.001$.

3. Results

The effect of *St*APs 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 *St*APs. 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 *St*AP1 and *St*AP3 IC_{50} values were i.e. 2.0 μM and 2.5 μM for *St*AP1 and *St*AP3 respectively. However the maximal cytotoxic effect of *St*AP3 was greater (80%) than the effect of *St*AP1 (60%) on Jurkat T cells viability, at the highest concentration assayed (4 μM). The cytotoxicity of these proteins on T lymphocyte cells was lower than that observed on Jurkat T cells, at all concentrations assayed. *St*AP1 and *St*AP3 were able to produce a reduction of 20% and 30%, respectively, in the viability of T lymphocyte cells at the highest concentration assayed (4 μM).

Changes in cell morphology were analyzed by microscopy. Only in Jurkat T cells at highest *St*AP 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 *St*APs. Cells were incubated with different amounts of *St*APs 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 *St*APs (*St*AP1, Fig. 2; and *St*AP3, 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 *St*AP concentrations assayed (Figs. 2D,E,F and 3D,E,F). At shortest incubation time, *St*APs induce, in Jurkat T cells, a dose-dependent increase in the fraction of necrotic cells. Low concentrations of *St*AP1 (Fig. 2A) and *St*AP3 (Fig. 3A) were able to increase by 15% and 27% the percentage of necrotic cells, respectively. At the highest concentration of *St*APs assayed (3.75 μM), 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 μM to *St*AP1 and 1.5 μM to *St*AP3. An increase in the percentages of necrotic cells was observed at 3.75 μM of *St*APs, with respect to these percentages at short time (60% and 82% to *St*AP1 and *St*AP3 respectively) (Figs. 2B and 3B). No significant increases in the percentages of necrotic cells

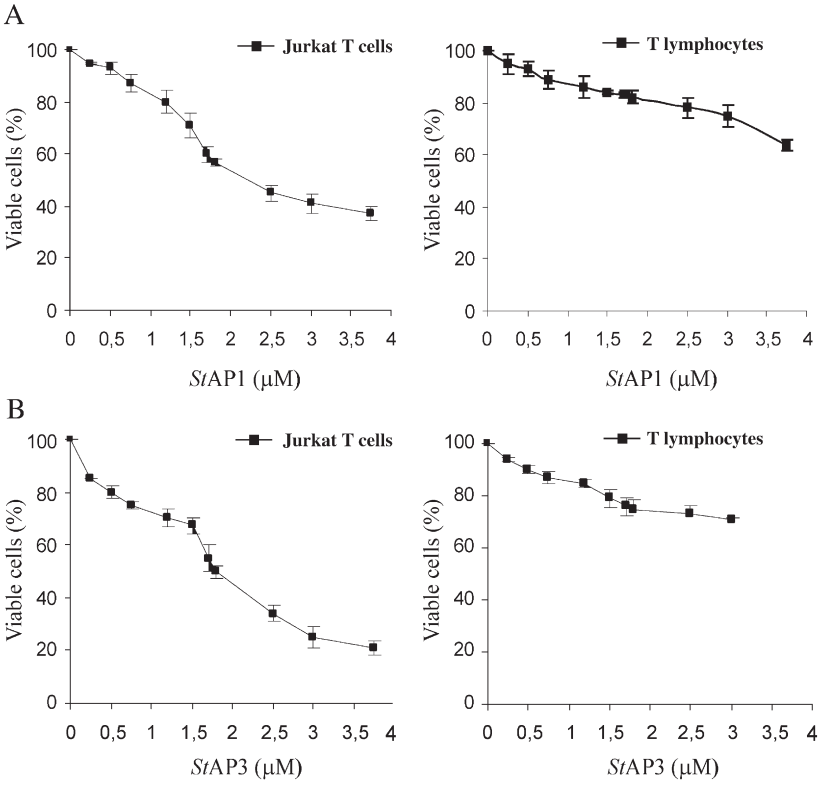

Fig. 1. The effects of *St*AP1 (A) and *St*AP3 (B) on viability of Jurkat T cells and T lymphocytes. Cells were removed from protein-treated and untreated (control) cultures after one cell-cycle. The data presented are average from three independent experiments, with error bars denoting standard errors.

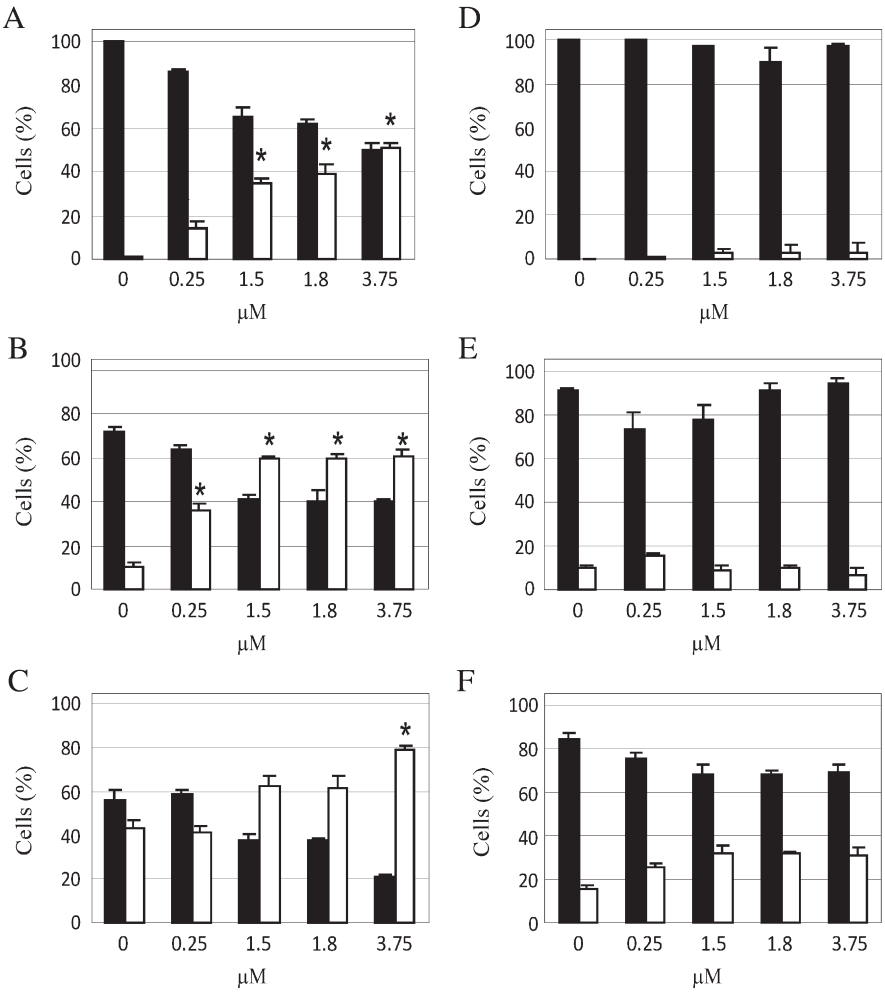

Fig. 2. Fraction of viable cells (filled bars) and necrotic cells (clear bars), as detected in Jurkat T cells (A, B, and C) and T lymphocytes (D, E, and F) treated with *St*AP1 at the indicated doses for 6, 24 and 48 h for Jurkat T cells (A, B and C respectively) and for 24, 48 and 72 h for T lymphocytes (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 *St*APs 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 *St*APs during 6, 24 and 48 h for Jurkat T cells and 24, 48 and 72 h for T lymphocytes. Table 1 shows that, *St*APs 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, *St*AP1 caused apoptosis after 6 h of treatment, whereas *St*AP3 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 *St*APs, the percentage of apoptotic Jurkat T cells was 26.6% for *St*AP1 and 34.11% for *St*AP3 (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 *St*APs 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 *St*APs have cytotoxic activity against potato pathogens and human and bovine spermatozoa [36,37]. We have demonstrated that cytotoxic activity of *St*APs involves plasma membrane permeabilization and, subsequently, cell death [38,39]. In this work we show that

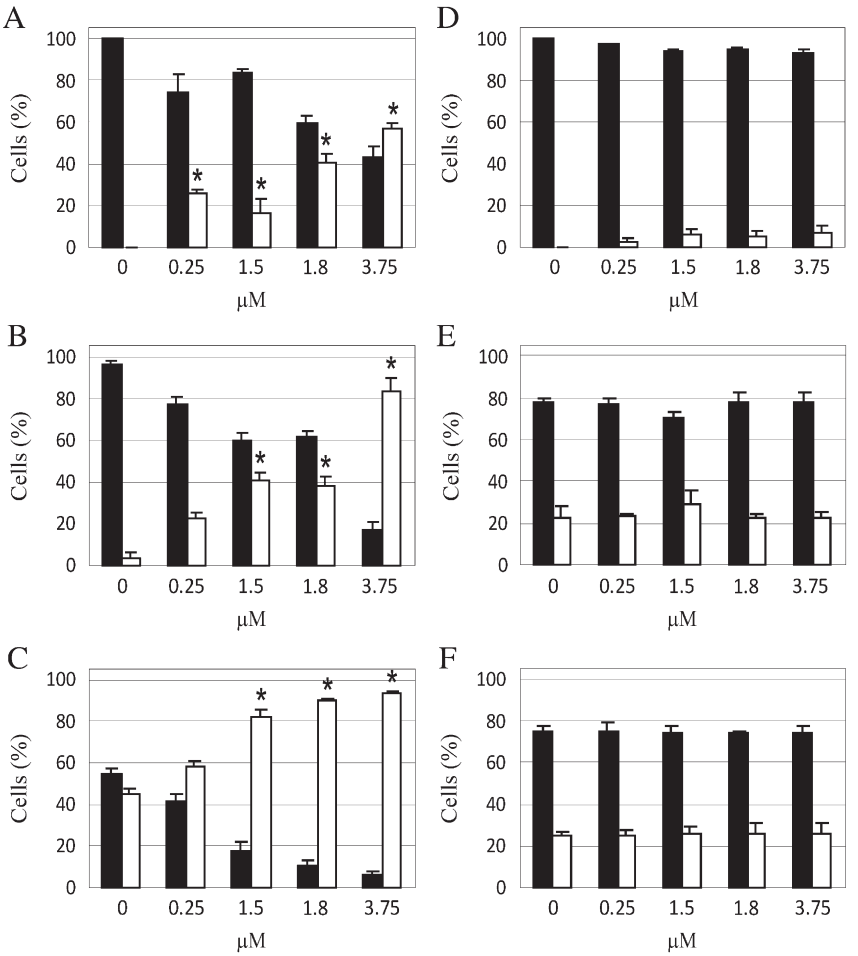

Fig. 3. Fraction of viable cells (filled bars) and necrotic cells (clear bars), as detected in Jurkat T cells (A, B, and C) and T lymphocytes (D, E, and F) treated with *St*AP3 at the indicated doses for 6, 24 and 48 h for Jurkat T cells (A, B and C respectively) and for 24, 48 and 72 h for T lymphocytes (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.

*St*AP1 and *St*AP3 exert cytotoxic effect on Jurkat T cell, a human leukaemia cell line. *St*APs 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 *St*APs. These changes involve plasma membrane disruption and release of cytoplasm (data not shown). The *St*AP 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 *St*AP1 and *St*AP3.

*St*AP	Concentration of *St*APs (μM)	% Apoptosis	
		Jurkat T (6 h)	Lymphocytes (24 h)
*St*AP1	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
*St*AP3	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 *St*APs 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 *St*APs induce apoptosis only on Jurkat T cells after short times of incubation at the highest concentration assayed; however, no significative apoptosis induction was observed on T lymphocytes at any time and *St*AP 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 *St*APs 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 *St*APs *in vitro* [39] and its no significative 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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References

[1] Hancock RE, Chapple D. Peptides antibiotics. Antimicrob Agents Chemother 1999;43:1317–23.
[2] Marshall SH, Arenas G. Antimicrobial peptides: a natural alternative to chemical antibiotics and a potential for applied biotechnology. Electron J Biotechnol 2003;6:271–84.
[3] Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564–82.
[4] Bell G, Gouyon PH. Arming the enemy: the evolution of resistance to self-proteins. Microbiology 2003;149:3343–4.
[5] Hancock RE, Scott MG. The role of antimicrobial peptides in defenses. Proc Natl Acad Sci USA 2000;97:8856–61.
[6] Levy O. Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. J Leukoc Biol 2004;76:1–16.
[7] Redy KV, Yedery RD, Aranha C. Antimicrobial peptides: premises and promises. Int J Antimicrob Agents 2004;25:448–9.
[8] De Lucca AJ. Antifungal peptides: potential candidates for the treatment of fungal infection. Expert Opin Investig Drugs 2000;9:17796–9.
[9] Hancock REW. The role of antimicrobial peptides in defences. Expert Opin Investig Drugs 2000;9:1723–9.
[10] Chinchar VG, Wang J, Murti G, Carey C, Rollins-Smith L. Inactivation of frog virus 3 and channel catfish virus by esculentin-2P and ranatuerin-2P, two antimicrobial peptides isolated from frog skin. Virology 2001;288:351–7.
[11] Chernysh S, Kim SI, Bekker G, Pleskach VA, Filatova NA, Anikin VB, et al. Antiviral and antitumor peptides from insects. Proc Natl Acad Sci U S A 2002;99:12628–32.
[12] Cruciani RA, Barker JL, Zasloff M, Chen HC, Colamonici O. Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. Proc Natl Acad Sci U S A 1991;88:3792–6.
[13] Leuschner C, Hansel W. Membrane disrupting lytic peptides for cancer treatments. Curr Pharm Des 2004;10:2299–310.
[14] Papo N, Shai Y. Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 2005;13:61–92.
[15] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2001;415:389–95.
[16] Zhang L, Rozek A, Hancock REW. Interaction of cationic antimicrobial peptides with model membranes. J Biol Chem 2001;276:35714–22.
[17] Warren P, Li L, Song W, Holle E, Wei Y, Wagner T, et al. *In vitro* targeted killing of prostate tumor cells by a synthetic amoebapore helix 3 peptide modified with two gamma-linked glutamate residues at the COOH terminus. Cancer Res 2001;61:6783–877.
[18] Baker MA, Maloy WL, Zasloff M, Jacob LS. Anticancer efficacy of magainin 2 and analogue peptides. Cancer Res 1993;53:3052–7.
[19] Hansel W, Leuschner C, Gawronska B, Enright F. Targeted destruction of prostate cancer cells and xenografts by lytic peptide-betaLH conjugates. Reprod Biol 2001;1:20–32.
[20] Van Loon LC, van Kammen A. Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. “Samsun” and “Samsun NN”. II. Changes in protein constitution after infection with tobacco mosaic virus. Virology 1970;40:190–211.
[21] Van Loon LC, Van Strein EA. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 1999;55:85–97.
[22] García-Olmedo F, Rodríguez-Palenzuela P, Molina A, Alamillo JM, López-Solanilla E, Berrocal-Lobo M, et al. Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defence. FEBS Lett 2001;498:219–22.
[23] Lindholm P, Goransson U, Johansson S, Claeson P, Gullbo J, Larsson R, et al. Cyclotides: a novel type of cytotoxic agents. Mol Cancer Ther 2002;1:365–9.
[24] Luo MJ, Yang XY, Liu WX, Xu Y, Huang P, Yan F, et al. Expression, purification and anti-tumor activity of curcina. Acta Biochim Biophys Sin (Shanghai) 2006;38:663–8.
[25] Ngai PH, Ng TB. Phaseococcin, an antifungal protein with antiproliferative and anti-HIV-1 reverse transcriptase activities from small scarlet runner beans. Biochem Biochem Cell Biol 2005;83:212–20.
[26] Wang YX, Jacob J, Wingfield PT, Palmer I, Stahl SJ, Kaufman JD, et al. Anti-HIV and anti-tumor protein MAP30, a 30 kDa single-strand type-I RIP, share similar secondary structure and beta-sheet topology with the A chain of ricin, a type-II RIP. Protein Sci 2000;9:138–44.
[27] Arazi T, Huang PL, Huang PL, Zhang L, Shiboleth YM, Gal-On A, et al. Production of antiviral and antitumor proteins MAP30 and GAP31 in cucurbits using the plant virus vector ZYMV-AGII. Biochem Biophys Res Commun 2002;292:441–8.
[28] González De Mejía E, Prisecaru VI. Lectins as bioactive plant proteins: a potential in cancer treatment. Crit Rev Food Sci Nutr 2005;45:425–45.
[29] Chu KT, Ng TB. Smilaxin, a novel protein with immunostimulatory antiproliferative, and HIV-1-reverse transcriptase inhibitory activities from fresh *Smilax glabra* rhizomes. Biochem Biophys Res Commun 2006;340:118–24.
[30] Büssing A, Stein GM, Wagner M, Wagner B, Schaller G, Pfuller U, et al. Accidental cell death and generation of reactive oxygen intermediates in human lymphocytes induced by thionins from *Viscum album* L. Eur J Biochem 1999;262:79–87.
[31] Davies DR. The structure and function of the APs. Annu Rev Biophys Chem 1999;19:189–215.
[32] Rawling ND, Barret AJ. Families of aspartic peptidases, and those of unknown catalytic mechanism. Met Enzymol 1995;248:105–20.
[33] Mutlu A, Gal S. Plant aspartic proteinases: enzymes on the way to a function. Physiol Plant 1999;105:569–76.
[34] Guevara MG, Oliva CR, Machinandearena M, Daleo GR. Purification and properties of an aspartic protease from potato tuber that is inhibited by a basic chitinase. Physiol Plant 1999;106:164–9.
[35] Guevara MG, Daleo GR, Oliva CR. Purification and characterization of an aspartic protease from potato leaves. Physiol Plant 2001;112:321–6.
[36] Guevara MG, Oliva CR, Huarte M, Daleo GR. An aspartic protease with antimicrobial activity is induced after infection and wounding in intercelullar fluids of potato tubers. Eur J Plant Pathol 2002;108:131–7.
[37] Guevara MG, Veríssimo P, Pires E, Faro C, Daleo GR. Potato aspartic proteases: induction, antimicrobial activity and substrate specificity. J Plant Pathol 2004;86:233–8.
[38] Mendieta JR, Pagano MR, Muñoz FF, Daleo GR, Guevara MG. Antimicrobial activity of potato aspartic proteases (*St*APs) involves membrane permeabilization. Microbiology 2006;1152:2039–47.
[39] Cesari A, Falcinelli A, Mendieta JM, Pagano MR, Daleo GR, Guevara MG. Spermicidal activity of *St*APs (*Solanum tuberosum* aspatic proteases) involves membrane damage. Fertil Steril 2007;88:1248–55.
[40] O'Donovan MR, Johns S, Wilcox P. The effect of PHA stimulation on lymphocytes sub-population in whole-blood cultures. Mutagenesis 1995;10:371–4.
[41] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[42] Oakley BR, Kirsch DR, Morris NR. A simplified ultrasensitive silver stain for detecting proteins in polyacrilamide gels. Anal Biochem 1980;105:361–3.
[43] Hongo T, Mizuno Y, Haraguchi S, Yohida TO. A new anticancer drug sensitivity test using the microplate culture and surviving tumor cell staining method. Gan to Kagaku Ryoho 1986;13:247–54.
[44] Andersson M, Gunne H, Agerberth B, Boman A, Bergman T, Sillard R, et al. NK-lysin, a novel effector peptide of cytotoxic T and NK cell. Structure and cDNA cloning of the porcine dorm, induction by interleukin 2, antibacterial and antitumor activity. EMBO J 1995;14:1615–25.
[45] Andreu D, Carreño C, Linde C, Boman HG, Andersso M. Identification of an anti-mycobacterial domain in NK-lysin and granulysin. Biochem J 1999;344:845–9.

[46] Peña SV, Krensky AM. Granulysin, a new human cytolytic granule-asociated protein with possible involvement in cell-mediated cytotoxicity. Immunol 1997;9:117–25.
[47] Xia L, Ng TB. Isolation of allumin, a novel protein with antimicrobial and antiproliferative activities from multiple-cloved garlic bulbs. Peptides 2005;26:177–83.
[48] Evett GE, Donaldson DM, Vernon LP. Biological properties of *Pyrularia* thionin prepared from nuts of *Pyrularia pubera*. Toxicon 1986;24:622–5.
[49] Lee JC, Lee KY, Son YO, Choi KC, Kim J, Truong TT, et al. Plant-originated glycoprotein, G-120, inhibits the growth of MCF-7 cells and induces their apoptosis. Food Chem Toxicol 2005;43:961–8.
[50] Eliassen LT, Berge G, Sveinbjornsson B, Svendsen JS, Vorland LH, Rekdal O. Evidence for a direct antitumor mechanism of action of bovine lactoferrin. Anticancer Res 2002;22:2703–10.
[51] Schröder-Borm H, Bakalova R, Andrä J. The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increases surface levels of negatively charged phosphatidylserine. FEBS Lett 2005;579:6128–34.
[52] Risso A, Braidot E, Sordano MC, Vianello A, Macri F, Skerlavaj B, et al. BMAP-28, an antibiotic peptide of innate immunity, induces cell death through opening of the mitochondrial permeability transition pore. Mol Cell Biol 2002;22:1926–35.