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ORIGINAL CONTRIBUTION

Potential antitumor properties of a protein isolate obtained from the seeds of *Amaranthus mantegazzianus*

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Abstract
Background Amaranth is a crop that can be grown in different soils and climates, being resistant to high temperatures, drought, and some pests. The amaranth plant has nutritional qualities and desirable biological properties.
Aim of the study The aim of the study is to investigate the potential antitumor properties of *Amaranthus-mantegazzianus*-protein isolate (MPI) and to elucidate the possible mechanism of action.
Methods We use four different tumor-derived and in vitro-transformed cell lines with different morphology and tumorigenicity (MC3T3E1, UMR106, Caco-2, and TC7).
Results The MPI showed an antiproliferative effect on four cell lines with different potencies. The tumor-cell line UMR106 was the most sensitive (IC_{50}: 1 mg/ml). This antiproliferative effect of the MPI was enhanced by protease treatment (IC_{50}: 0.5 after 30% hydrolysis). In addition, the MPI produced morphological changes and caused a rearrangement of the cytoskeleton in UMR106 cell line. In an attempt to elucidate the mechanism of action, we observed that the MPI inhibited cell adhesion and induced apoptosis and necrosis in the UMR106 cell line. In reversibility studies, we were able to observe both temporary and permanent cytostatic and cytotoxic effects on the part of the MPI, depending on its concentration.
Conclusions we report a protein isolate from the seeds of *Amaranthus mantegazzianus* that exhibit potential antitumor properties and propose a putative mechanism of action.

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Introduction

Most of the prevalent chronic diseases in the world have a significant nutritional dimension that either contributes directly to the cause, enhances the risk through the phenomenon of promotion, exerts a beneficial effect by decreasing the risk, or even prevents the disease altogether. Healthy eating habits and the incorporation of functional food should, therefore, help minimize or even eliminate certain chronic human illnesses. Epidemiological evidence suggests that dietary factors play an important role in the etiology of different kinds of cancer [1]. For instance, diets rich in soybean products are associated with lower cancer-mortality rates; particularly for cancers of the colon, breast, and prostate [19]. Amaranth has drawn the attention of researchers because of its nutritional qualities and desirable agricultural properties, which characteristics include drought resistance and amenability to cultivation in climatic changes [5]. The nutritional composition of the amaranth grain has been extensively studied, especially with regard to its high quality and quantity of protein [6, 7]. The amino acid composition of the grain proteins are similar to the optimum balance required in the human diet [10]. In addition, these proteins have a variety of physical and chemical properties that would point to the amaranth as providing a potential new raw material for incorporation into a novel functional food [4, 28].

In susceptible populations, the amaranth plant may play an important role in the fight against hunger and malnutrition as well as prevent certain diseases of the population. A reappraisal of the beneficial effects from the dietary

intake of the seed, which results are the basis for various health claims, is currently taking place. Proteins and peptides are responsible for some of the observed biological and functional properties of the several species of amaranth seed: in these seeds, several bioactive proteins with different properties have been found, such as alpha-amylase inhibitors in *Amaranthus hypochondriacus* [8, 29] and antimicrobial inducing peptides in *Amaranthus caudatus* and *Amaranthus retroflexus* [13, 23, 24]. In recent years, antitumor activity has been reported in the leaves of the plant *Amaranthus tricolor* [17]. Several bioactive peptides have been detected in *Amaranthus hypochondriacus* through the use of bioinformatics tools [37]. The seed proteins of this plant were found to have, among others, antithrombotic, immunomodulatory, opioid, antioxidant, protease-inhibitory, and antihypertensive activities with these kinds of tools. At the moment, the only lunasin-like peptide with potential antitumor properties has been found in *A. hypochondriacus* storage proteins [37]. This article constitutes the first report of an antiproliferative activity in *Amaranthus mantegazzianus*-protein isolate (MPI) and presents its putative mechanism of action on different tumor and nontumor cell lines in vitro.

Materials and methods

Materials

Tissue culture materials were purchased from Trading New Technologies (Buenos Aires, Argentina) and Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) and fetal-bovine serum (FBS) obtained from GBO Argentina S.A (Buenos Aires, Argentina). Trypsin–EDTA was provided by Gibco (Gaithersburg, MD). Phalloidin came from Invitrogen Corporation (Buenos Aires, Argentina). Annexin V conjugated with FITC and propidium Iodide (PI) was from Molecular Probes (Eugene, OR). Triton X-100 was bought from Sigma (St Louis, MO, USA). All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

Biological material

Mature seed of *A. mantegazzianus* from commercial variety (*Pass cv Don Juan* Inta Anguil, La Pampa) were cultivated in Santa Rosa, La Pampa, Argentina from November to March, 2006/07/08. Flour was obtained by grinding whole seeds in an Udy mill, 1-mm mesh and screened by 10-mm mesh. The flour so obtained was defatted for 24 h with hexane in a 10% (w/v) suspension under continuous stirring, air-dried at room temperature, and finally stored at 4 °C until used. Protein content of the flour, as determined by the Kjeldhal method, was 17.0% (w/w). The preparation of MPIs and hydrolysates with alcalase were realized as described by Martinez and Añón [27]. The composition of MPIs was: carbohydrates: 10.4%, protein: 84.4%, ash: 3.4%, and lipid: 1.8%. The degree of hydrolysis was determined by quantification of amine groups with 2,4,6-trinitrobenzene sulfonic acid [2]. In order to evaluate the possible effect of soluble component of low molecular weight, we separate this trough ultra-filtration with a membrane of 3 kDa using Amicon equipment and then freeze-dried both fractions (*fraction 1*: molecular weigh >3 kDa and *fraction 2*: lower than 3 kDa). The MPI (*fraction 1*) without low molecular weight component was denominated: MPI-UF.

Structural characterization of *A. mantegazzianus*-protein isolate

Amaranthus mantegazzianus-protein isolates were analyzed by SDS-PAGE. Separating gels 12% (w/v) in polyacrylamide with a stacking gel of 4% (w/v) polyacrylamide. Running gels (12% w/v polyacrylamide) and stacking gel (4% w/v polyacrylamide) were arranged in minislabs (BioRad Mini Protean II Model). Runs were carried out according to the Laemmli method [22] as modified by Petruccelli and Añón [33]. Samples were run under reducing conditions; in this case, samples were boiled for 1 min in a sample buffer containing 5% (v/v) 2-mercaptoethanol (2-ME) before centrifugation. Molecular masses of polypeptides were calculated using the following protein standards (Pharmacia): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), R-lactalbumin (14.4 kDa). MPI samples were dissolved in sample buffer (0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 1% (w/v) SDS, and 0.05% (w/v) bromophenol blue). For reducing conditions, 5% (v/v) 2-mercaptoethanol (2-ME) was added and samples were heated (100 °C, 3 min). Gels were fixed and stained with Coomassie Brilliant Blue stain.

The electrophoretic pattern of MPI under denaturing conditions exhibited several polypeptides, including two bands of 61.5 and 57 kDa corresponding to the AB subunits and the P-54 polypeptide, a band of 45 kDa, probably a constituent of 7S globulin, some bands of MW between 35 and 38 kDa, corresponding to type A polypeptides naturally free in the amaranth seed and other bands of MW between 22 and 19 kDa that correspond to type B polypeptides. Also a band of 13.8 kDa, probably correspond to the albumin fraction was detected. These results are in agreement with others previously obtained in our laboratory. The isolates were composed primarily of the characteristic globulin-11S and globulin-P storage protein

fractions as previously shown by Martinez and Añón [27, 28].

The soluble fraction of MPI in culture medium shown the same polypeptide composition. The molecular mass of the protein bands detected were: 61.5, 56, 41, 36.9, 34.4, 20, 18.4, 14, and 12.5 kDa. In this fraction the polypeptide of the low MW, 12.5 kDa, presented a higher intensity than in MPI electrophoretic profile.

Preparation of protein isolates solutions

Dispersions of MPI, MPI-UF, or hydrolysates protein in DMEM (10–100 mg/ml) were gently stirred for 1 h at room temperature and centrifuged at 22,000*g* for 30 min at room temperature. The resulting supernatant was then sterilized by filtration through a 0.22-μm membrane and the soluble protein content measured by the Kjeldahl method ($N \times 5.85$) [6]. The 30% of the protein (MPI) was soluble in DMEM and the composition of this fraction was: protein: 79.6% and carbohydrates 17.1% (w/w) on dry weight bases (the content of lipids not was detectable). All protein determinations were carried out in triplicate.

Cell culture

MC3T3E1 osteoblastic mouse calvaria-derived cells, UMR106 rat osteosarcoma-derived cells, and the human colon-tumor lines (Caco-2 and TC7) were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) FBS in a humidified atmosphere of air plus 5% CO_2 at 37 °C. When 70–80% confluence was reached, the cells were subcultured by means of 0.1% (w/v) trypsin 1 mM EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS: 11 mM KH_2PO_4, 26 mM Na_2HPO_4, 115 mM NaCl, pH: 7.4). For experiments, cells were grown in multiwell plates. When cells reached the degree of confluence appropriate for each type of experiment, the monolayers were washed twice with DMEM and then incubated under the different experimental conditions.

Cell-proliferation assay

A mitogenic bioassay was carried out as described by Okajima et al. [32] with some modifications. Stated in brief, cells were grown in 48-well plates. When the cultures reached 60% confluence, the monolayers were washed twice with serum-free DMEM and incubated with DMEM with 1% (v/v) FBS (basal condition) or different dilutions of soluble protein plus FBS (1% final concentration by volume). After 24-h incubation, the monolayers were washed with PBS and fixed with 5% (v/v) glutaraldehyde in PBS at room temperature for 10 min, then stained with 0.5% (w/v) crystal violet in 25% aqueous methanol for 10 min. The dye solution was then discarded and the plate was washed with water and dried. The dye taken up by the cells was extracted with 0.5 ml/well of 0.1 M glycine/HCl buffer, pH 3.0 in 30% aqueous methanol and transferred to test tubes. The absorbance at 540 nm was read at a convenient sample dilution. Under these conditions, the colorimetric bioassay strongly correlated with cell proliferation as measured by cell counting in a Neubauer chamber.

Cell morphology

Cells grown on glass coverslips were incubated in DMEM with 1% FBS (basal condition) or with different dilutions of soluble protein plus FBS (1% final concentration) for 24 h. The cultures were then fixed and stained with Giemsa. The stained samples were finally viewed under light microscopy and photographs were taken for later evaluation.

Cytoskeletal rearrangement

To visualize actin filaments, a staining with fluorescence isothiocyanide-(FITC-phalloidin) was performed. Cells were grown on glass coverlips to 70% confluence and were then incubated for 24 h at 37 °C with DMEM plus 1% FBS (basal condition) or different dilutions of soluble protein plus FBS (1% final concentration). Next the cells were fixed, permeabilized with absolute ethanol (chilled to −20 °C) for 4 min at room temperature and washed with PBS. After an initial washing, the cells were incubated with FITC-phalloidin for 2 h, washed again, and mounted on slides. The cells labeled with green fluorescence were visualized by means of a fluorescence microscope and photographs were taken for later evaluation.

Cell adhesion

The cell-adhesion assay was performed as previously reported by McCarty et al. [30] with some modifications. Cells were plated in DMEM with 10% FBS in 24-well plates at a seeding density of 10E(5) cells/well in the presence of different doses of the MPI. The cells were left to adhere for 4 h at 37 °C. Each well was then washed with PBS and the crystal-violet assay was performed to determine the final cell number.

Lactate dehydrogenase (LDH) activity

Cultures were grown in 24-well plates. When the cells were confluent, the monolayers were washed twice with serum-free DMEM and incubated either with that basal medium

plus 1% FBS (basal condition) or with different dilutions of the MPI in DMEM plus 1% FBS. After 24 h of incubation, the culture media were removed and then centrifuged at 5,000*g* for 5 min (conditioned media). The monolayer was lysed with 0.1% (v/v) Triton X-100 (cellular extract). The LDH activity in the conditioned media and the cellular extract was measured with an optimized ultraviolet method (LDH-P kit from Wiener lab., Argentina). The results are expressed as follow-up: LDH (conditioned medium)/LDH (basal extract).

Determination of the apoptosis ratio

Apoptosis was evaluated by flow-cytometric analysis through the use of Annexin V conjugated with FITC and PI. After treatment of cells grown in 24-well plates with medium plus 1% FBS (v/v; basal condition), or with different dilutions of the MPI in DMEM plus 1% FBS, the cells were washed with PBS and then trypsinized. After centrifugation at 1,000*g*, the cells were resuspended in 300 µl of binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with Annexin V-FITC and PI by incubation at 4 °C for 30 min in the dark. The apoptotic cells (Annexin-V-positive/PI-negative cell) necrotic cells (Annexin-V-positive/PI-positive cell) and total cells (Annexin-V-negative/PI-negative cell) were analyzed quantitatively by means of the FACCalibur (BD) and the Cell Quest software.

Reversibility assay

Cells were grown in 48-well plates. At 60% confluence, the monolayers were washed twice with serum-free DMEM and incubated in that medium plus 1% FBS either without (basal condition) or with different dilutions of the MPI. After 24 h of incubation, one set of the MPI-treated samples was used to determine the cell number and the other set was washed thrice with DMEM; then fresh DMEM with 10% FBS was added to all wells (both the basal and the MPI-treated cultures), and the plates were incubated for another 24 h. Next, the cell number was determined by the crystal-violet assay. At all time points, one set each of basal and MPI-treated samples were included in the proliferation assay, and the experiments were performed in triplicate.

Statistical methods

At least three independent experiments were performed for each experimental condition. The PROBIT method was used to calculate the concentration that inhibits 50% of the cell proliferation (IC_{50}). The results are expressed as the mean ± SD, and statistical differences were analyzed by means of the Student *t*-test.

Results

Inhibition of cell proliferation by amaranth-protein isolates

The effect of the MPI, MPI-UF, and different protein hydrolysates on cell proliferation was determined by the crystal-violet bioassay, which method records the number of live cells. Figure 1 shows the effect of different MPI concentrations on the proliferation of the MC3T3-E1 and UMR106 cell lines. After 24 h of culture, MPI and MPI-UF showed an antiproliferative effect on both cell lines, and this growth inhibition was more pronounced with the UMR106 neoplastic (IC_{50} 1.0 mg/ml) line than with the MC3T3-E1 cells (IC_{50} 2.5 mg/ml, Table 1), thus suggesting a potential antitumor effect. We have furthermore examined the growth-inhibitory effect of the MPI on other cell lines derived from human-colon tumors (Caco-2 and TC7). Table 1 also shows the IC_{50}s for the effect of the

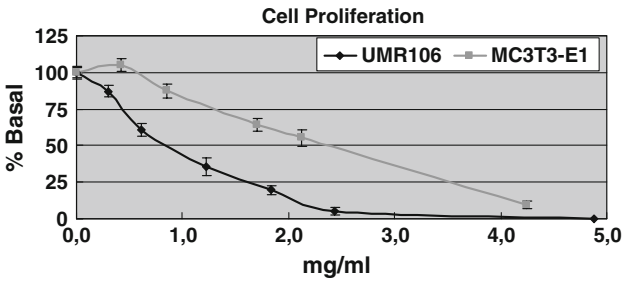

Fig. 1 Effect of MPI on UMR106 and MC3T3-E1 cell proliferation. Cells were incubated with DMEM with 1% FBS (Basal) or different dilutions of MPI plus FBS (1% final concentrations of FBS) at 37 °C for 24 h. To evaluate cell proliferation, crystal-violet assay was used as can be seen in "Material and methods" section. Results are expressed as % basal and represent the mean ± SD, $n = 18$

Table 1 The concentration of MPI, MPI-UF, BSA, and BBI that inhibits the 50% of cell proliferation (IC_{50}) on fourth cell lines studied

Samples	Cell lines			
	UMR106 (IC_{50}) [mg/ml]	MC3T3-E1 (IC_{50}) [mg/ml]	Caco-2 (IC_{50}) [mg/ml]	TC7 (IC_{50}) [mg/ml]
MPI	1.0 ± 0.05	2.5 ± 0.06	1.5 ± 0.1	2.5 ± 0.08
MPI-UF	1.1 ± 0.05	2.4 ± 0.06	ND	ND
BSA	No effect[a]			
BBI	No effect[a]			

To evaluate cell proliferation, crystal violet assay was used as can be seen in "Material and methods" section

ND not determinated

[a] No growth inhibition was observed within the range of concentrations tested (0.2–20 mg/ml)

MPI on these two lines (1.5 and 2.5 mg/ml, respectively). Different sensitivities to the MPI were thus observed for the four cell lines. In an attempt to evaluate nonspecific inhibitory effects on cell proliferation on the part of high quantities of protein in the culture medium or from the presence of the protease inhibitors like Bowman-and-Birk (BBI) present in soy, we examined the effect of bovine-serum albumin (BSA) and commercial soybean BBI on cell proliferation. After 24 h of incubation, we observed no growth-inhibitory effect on the fourth cell lines with either protein (Table 1).

Various studies have identified bioactive peptides within the amino acid sequences of native proteins [9]. Since hydrolytic reactions, such as those catalyzed by digestive proteases, can result in the release of such peptides, we were prompted to investigate the effect of protein hydrolysis on the MPI and on BSA. Table 2 shows the IC_{50} for the growth-inhibitory action of the hydrolyzed proteins from the amaranth and from BSA. Proteolytic hydrolysis improved the inhibitory effect of two protein sources. For the UMR106 cells, the most potent inhibitor was the MPI hydrolysate at a 30% degree of hydrolysis (MPIDH30; IC_{50}: 0.5 mg/ml). The MPI with a 2% degree of hydrolysis (MPIDH2) showed no improvement in the inhibitory action over that observed with the untreated isolate, while the effect on proliferation of the MPI with a high degree of hydrolysis (60%) was similar to that of the MPIDH30 (at an IC_{50} of 0.6 mg/ml). The hydrolysate from BSA at a 30% degree of hydrolysis also evinced a significant antiproliferative action on the tumor cells (IC_{50}: 6.0 mg/ml) as well as on the MC3T3-E1 line, though with these latter cells their inhibition was less pronounced and their action of a lower degree of potency (with IC_{50}:15.0 mg/ml).

Table 2 The concentration of MPI, BSA, and hydrolysates that inhibits the 50% of cell proliferation (IC_{50}) on UMR106 and MC3T3-E1 cell lines

Samples	Cell Lines	
	UMR106 (IC_{50}) [mg/ml]	MC3T3-E1 (IC_{50}) [mg/ml]
MPI	1.0 ± 0.05	2.5 ± 0.06
MPI (GH: 2)	1.1 ± 0.05	2.3 ± 0.04
MPI (GH: 30)	0.5 ± 0.06	1.7 ± 0.06
MPI (GH: 60)	0.6 ± 0.02	1.8 ± 0.05
BSA	No effect[a]	
BSAH (GH: 30)	6.0 ± 0.07	15.0 ± 0.2

To evaluate cell proliferation, crystal-violet assay was used as can be seen in "Material and methods" section

[a] No growth inhibition was observed within the range of concentrations tested (0.2–20 mg/ml)

As the tumor line UMR106 proved to be more sensitive to growth inhibition by the MPI, we focused the remainder of our investigation on these cells.

Changes in cell morphology induced by amaranth-protein isolates

Taking into account the deleterious effects of the MPI on cell proliferation, we investigated the action of this amaranth-protein isolate on the morphology of the UMR106 tumor-cell line. Figure 2A shows the morphological characteristics of the UMR106 osteosarcoma line after Giemsa staining. These cells exhibited a polygonal morphology with well-stained irregular-shaped nuclei and a well-defined vacuole-containing cytoplasm. Cells were interconnected by lamellar processes. Condensation was observed in the nucleus and cytoplasm after 24 h of incubation with the MPI at 1 mg/ml (Fig. 2A). At this time point, the number of processes between the cells had diminished along with the number of cells per field. In addition, at 2 mg/ml, cellular pyknosis and fragmented nuclei typical of apoptotic cells were observed, and the cell borders were also poorly defined. The cells exhibited a dense nucleus surrounded by a very small and highly condensed cytoplasm.

Changes in cytoskeletal proteins induced by amaranth-protein isolates

The staining of the actin-containing microfilaments with FITC-phalloidin has been used to visualize cytoskeletal architecture in the UMR106 cells [16]. Figure 2B shows the characteristics of the actin filaments in those cells under our experimental conditions. In the control field, the cells display an organized architectural distribution of the microfibrils: as is seen here, the actin microfilaments are normally oriented in the direction of the main axis of the cells. When the UMR106 cells were exposed to different concentrations of the MPI for 24 h, the regularity of this pattern was lost. At 1 mg/ml of MPI proteins, we observed a partial disorganization of the actin microfilaments in the cytoplasm as well as an alteration in the shape of the cells (Fig. 2B). This result is in accordance with the morphological alterations observed after Giemsa staining as well as with the proliferation results obtained in the crystal-violet assay.

Inhibition of cell adhesion by amaranth-protein isolates

Cell adhesion is an essential aspect of cell survival, in particular, for metastatic tumor cells [31]. Within this context, we therefore evaluated the action of the MPI on cell adhesion in the UMR106 cell-culture model. Here, the MPI inhibited cell adhesion in a dose-dependent manner

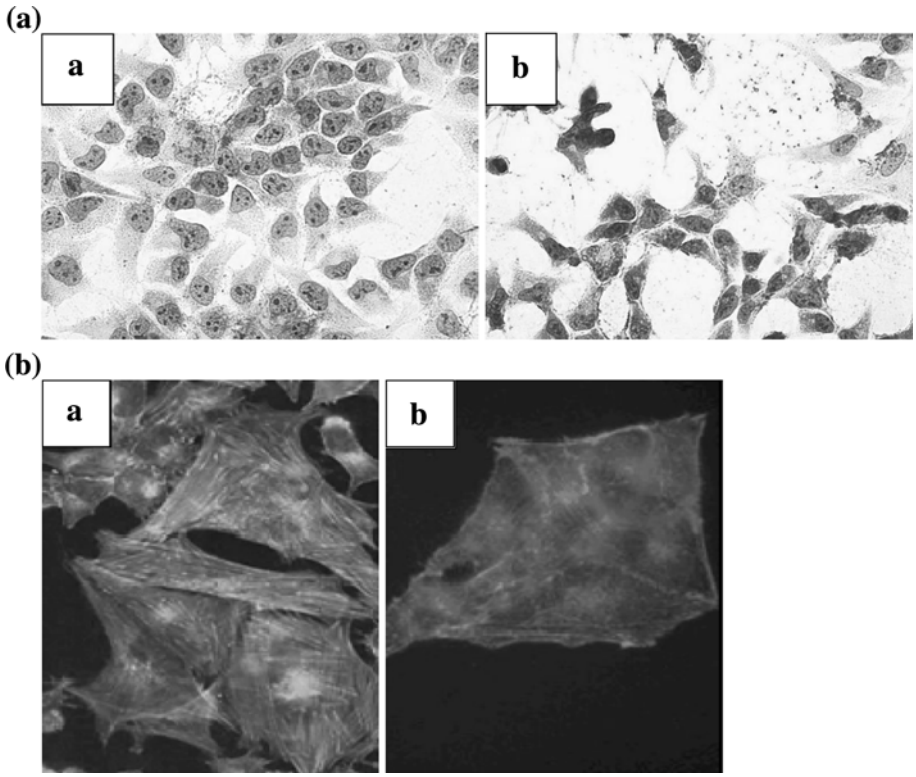

Fig. 2 **A**(*a*) UMR106 osteosarcoma cells were incubated with DMEM 1% FBS or (*b*) DMEM 1% FBS plus MPI (1 mg/ml). Then, the cells were fixed with methanol and stained with Giemsa. Samples were observed under light microscopy and pictures were taken for posterior evaluation (magnification 63×). **B**(*a*) UMR106 osteosarcoma cells were incubated with DMEM 1% FBS or (*b*) DMEM 1% FBS plus MPI (1 mg/ml). Then, the cells were fixed with cold ethanol and stained with FITC-phalloidin. Samples were observed under fluorescent microscopy and pictures were taken for posterior evaluation (magnification 63×)

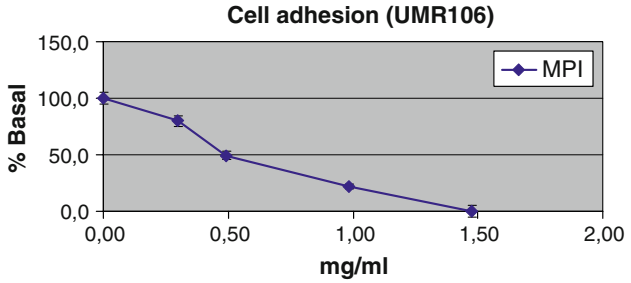

Fig. 3 UMR106 osteosarcoma cells were allowed to adhere for 4 h in the presence of different concentration of MPI as is indicated in the figure. Then, the number of adhering cells per well were evaluated by crystal-violet assay. Results are expressed as % basal and represent the media ± SD of three independent experiments

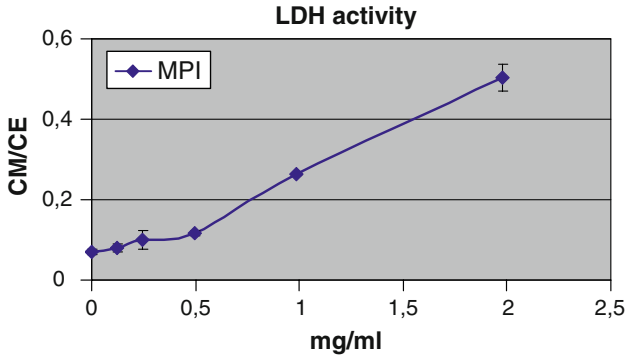

Fig. 4 UMR106 osteosarcoma cells were incubated with DMEM with 1% FBS (basal) or different dilutions of MPI plus FBS (1% final concentrations of FBS). After 24 h of incubations, the LDH activity in the conditioned media (CM) and cellular extract (CE) was measured using an optimized UV-method (LDH-P kit from Wiener lab., Argentina). The results are expressed as the ratio: LDH (conditioned medium)/LDH (cellular extract)

(Fig. 3), exhibiting a 50% inhibition at 0.5 mg/ml, as determined by the PROBIT method.

Induction of cell death through apoptosis and necrosis by amaranth-protein isolates

In an attempt to elucidate the mechanism of cell death induced by the MPI, we investigated the possible involvement of necrosis and apoptosis. Lactate dehydrogenase (LDH) release is commonly used as a marker for necrotic cell death or post-apoptotic cell death in vitro [21, 23]. When cells are lethally injured, the resulting loss of membrane integrity can be assessed by monitoring the activity of LDH in the culture medium. As Fig. 4 illustrates, the LDH activity

Table 3 UMR106 osteosarcoma cells were incubated with DMEM with 1% FBS (basal) or different dilutions of MPI plus FBS (1% final concentrations of FBS)

Concentrations MPI (mg/ml)	AnnexinV$^+$/PI$^-$ Apoptotic cell	AnnexinV$^+$/PI$^+$ Necrotic cell	AnnexinV$^-$/PI$^-$ Non-affected cell
0	7.0	3.1	90.9
0.1	6.2	4.3	89.5
0.5	8.4	6.5	75.1
1	12.6	8.1	79.3
2	25.1	11.9	63.0

After 24 h of incubations, the apoptotic cells were evaluated by flow cytometric analysis using Annexin-V/PI and were analyzed quantitatively by means of the FACCalibur (BD) and the Cell Quest software. In all determinations, 10,000 cells were counted. Results are expressed as % apoptotic cell (Annexin-V$^+$/PI$^-$), % necrotic cell (Annexin-V$^+$/PI$^+$), % non-affected cell (Annexin-V$^-$/PI$^-$) and represent the media of three independent experiments

in the culture media was not modified by exposure to the MPI until a level of 0.5 mg/ml of the isolate was present. Concentrations higher than 1 mg/ml evoked an increment in LDH values of four-fold or more over the basal condition, up to a value of seven-fold at 2 mg/ml. These result are accordingly the flow-cytometric analysis (Table 3).

Apoptosis was assessed by flow-cytometric analysis after labeling with Annexin-V/PI. After 24-h incubation, the MPI increased the proportion of apoptotic cells in a dose-dependent fashion from a level of 1 mg/ml of the isolate upwards, reaching a five-fold increase at 2 mg/ml (Table 3).

Induction of reversible and irreversible cytotoxicity by amaranth-protein isolates

We considered that the inhibitory effects of the MPI on cell proliferation after 24-h exposure, as measured by the crystal-violet assay, could possibly result from a growth inhibition and programmed death of the cells at concentrations lower than the IC_{50}. We, therefore, exposed the UMR106 cells to different MPI doses for 24 h and then washed the cells to allow their recovery in a MPI-free medium (the reversibility assay; Fig. 5). The results demonstrate that cells subjected to 0.1 mg/ml of MPI for 24 h failed to proliferate thereafter but nevertheless retained viability. By contrast, at a concentration of 0.3 mg/ml of the isolate or higher, the proliferation of the cells became blocked and they lost viability as well.

Discussion

Amaranth proteins could play an extremely important role in the fight against hunger and protein malnutrition as well

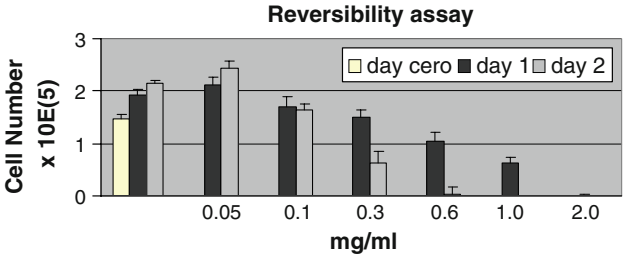

Fig. 5 UMR106 cells were exposed to different concentration of MPI for 24-h and then washed to allow their recovery in a MPI-free medium with 10% FBS. Cell number was determined at different time points using crystal-violet assay (0, 24, and 48 h). The experiments were carried out by triplicate and represent the media ± SD

as contribute to the prevention of certain diseases as a result of the nutritional and potential biological properties of these molecules. At the present time, several bioactive peptides in amaranth seeds of different biologic properties have been indicated. Silva-Sánchez et al. [37] reported the presence of lunasin-like peptides in the amaranth storage proteins: this peptide, present in diverse seeds, has been described as having antitumor properties [18]. To investigate further the potential antitumor characteristics of the amaranth-protein isolates, we examined the effects of the MPI on different tumor-derived and in vitro-transformed cell lines. Since that different cell lines might exhibit differing sensitivities to a given antitumor agent, we employed four lines here, with each one being from a separate source and possessing an individual morphology and tumorigenicity.

Tumor and transformed cell lines were incubated in the presence of different concentrations of the MPI to evaluate the effect of these isolate on cell proliferation. When the cells were incubated with doses higher than 0.75 mg/ml of the MPI, cell proliferation was inhibited. The antiproliferative effect was dose- and cell-type-dependent, with the UMR106 cell line being the most sensitive.

In an attempt to evaluate inhibitory effects on cell proliferation on the part of low molecular weight component like flavones, saponins, or salts that may be present in the MPI isolate, we eliminate these trough ultra-filtration with a membrane of 3 kDa using Amicon equipment. Nevertheless, as can be seen in Table 1, the MPI-UF showed similar result than MPI suggesting that the inhibitory action of the isolate is due to protein component and is not caused by other chemical component of low molecular weight.

As protease inhibitors had been largely reported to be present in MPIs, we examined the effect of commercial soybean BBI in this in vitro model system in order to evaluate if this kind of peptide might be responsible of the antiproliferative activity. No growth inhibition with BBI was observed, however, within the range of concentrations tested (0.2–20 mg/ml). These results would suggest that

bioactive peptides with antiproliferative actions different from those of protease inhibitors may be present in the MPI, one possibility being the lunasin-like peptides present in MPI [37]. At all events, we were able to discard the possibility of a nonspecific inhibition by high quantities of protein in the culture medium, as concentrations of BSA in excess of 20 mg/ml failed to inhibit cell proliferation.

Bioactive peptides have been identified within the amino acid sequences of native proteins [9, 20]. Hydrolytic reactions, such as those catalyzed by digestive enzymes, could release such peptides or expose bioactive domains on the external surface of minimally cleaved macromolecular proteins. To this end, we evaluated the effect on cell proliferation of hydrolysates of the MPI and BSA. All of these digests exhibited an antiproliferative action, with the MPI hydrolysate being the most potent. Furthermore, increasing the degree of hydrolysis augmented the inhibitory capacity of the MPI up to a maximum effect at 30% of hydrolyses. The BSA hydrolysate also showed a more potent antiproliferative effect than the undigested. We need to stress here that native BSA failed to manifest an inhibitory effect at the maximum doses examined (20 mg/ml); nevertheless, its hydrolysate exhibited an antiproliferative activity. These results would indicate the presence of bioactive amino acid sequences within certain protein domains in the MPI and BSA. Kim et al. [20] have reported an antitumor peptide from hydrolyzed soybean protein. These investigators hydrolyzed delipidated soybean protein with thermoase and extracted the hydrophobic peptides with ethanol. Thereafter, a peptide fraction from Sephadex-G-25 chromatography showed an IC_{50} of 0.16 mg/ml. Novel peptides of this type may be released by digestive enzymes within the gastrointestinal tract or by proteolysis in fermented amaranth-derived foods. Results such as these would point to the existence of bioactive peptides in the MPI in keeping with the indications already seen through the use of bioinformatics tools [37].

As the tumor line UMR106 proved to be more sensitive to the action of the MPI, we focused our attention on these cells line and attempted to investigate the possible mechanism of action.

Cytotoxicity is one of the chemotherapeutic targets of antitumor activity. Several mechanisms have been proposed to explain these correlations [35, 36]. A cytotoxic effect can be evaluated through morphological changes and alterations in the cytoskeleton. The organization of the cytoskeleton is essential for many cellular functions; disruption of the actin filaments affects multiple cellular functions including motility, signal transduction, and cell division and ultimately causes cell death [3, 39]. We, thus, investigated the effects of the MPI on cell morphology and cytoskeletal architecture. After a 24-h incubation, the MPI (at 1 mg/ml) induced morphological changes and a modification of the cytoskeleton in the UMR106 tumor line as determined by Giemsa and phalloidin staining. The morphologic alterations caused by the MPI involved marked changes in the characteristics of the nucleus and cytoplasm. These effects were intensified in a dose-dependent manner and resulted in a great loss of cells and cellular structure. At 1 mg/ml, we also found numerous dead cells detached from the monolayers, which observation correlated with the decrease in cell proliferation.

In accordance with this observation, we have investigated if the MPI interferes with cell adhesion and triggers programmed cell death. Adhesion is an important property for cell survival and particularly for the metastasis of invasive tumor cells [12, 31]. The MPI inhibited the adhesion of these cells in a dose-dependent fashion, with a 50% decrease occurring at 0.5 mg/ml. This interference with cellular adhesion by the isolate could be partially explained by two phenomena. First, the surface attachment of these cells is dependent on the interactions between various cell-associated adhesion proteins [14], a process that was seen to be inhibited by a synthetic polypeptide (the β-peptide) [25]. The MPI might contain different peptides or proteins whose domains exhibit sequences similar to those of the β-peptide and thus interfere with cell attachment. Second, the loss of cell viability per se that occurs at similar concentrations of the isolate (0.3 mg/ml) might be responsible for the detachment of cells as an indirect cause (*cf.* Fig. 3).

In an attempt to elucidate the mechanism by which the MPI induced cell death, we observed that the isolate (1 mg/ml) induced apoptosis and necrosis after 24 h of incubation. These results coincide with the antiproliferative action of the MPI. Moreover, all three effects—the interference with cell proliferation and the induction of apoptosis and necrosis—were dose-dependent for the UMR106 line. Similar results had been observed for lunasin, the antitumor peptide of soybean, which molecule induces apoptosis by binding preferentially to the deacetylated histone H4 in vitro [15]. Genotoxins and microtubule-binding toxins are used in chemotherapy cocktails for the treatment of several different cancers because of their differing mechanisms of action [11, 34, 38]. Both of these toxins generally bring about cell death by means of apoptosis [26]. In some in vitro experimental systems postapoptotic cells subsequently develop into necrotic cells [21], and such a process might represent a second possible mechanism of cell death after exposure to the MPI.

In order to examine the possibility of cellular recovery from the action of the MPI in blocking cell proliferation and causing cell damage, we carried out the following rescue experiment. The UMR106 cells were exposed to different doses of the isolate for 24 h and then washed to allow their recovery in MPI-free DMEM with 10% FBS

(the reversibility assay). Cells exposed to 0.1 mg/ml of the MPI for 24 h failed to proliferate but did not lose viability. Concentrations of the isolate higher than 0.3 mg/ml, however, produced an irreversible loss of cellular viability. These results suggest that 0.1 mg/ml of the MPI block cell proliferation exclusively, while doses higher than 0.3 mg/ml also promote a permanent loss of cell viability culminating in cell death.

In conclusion, we report a protein isolate from the seeds of *A. mantegazzianus* that exhibit potential antitumor properties. The antiproliferative effect of the MPI was enhanced by protease treatment. The MPI also produced morphological alterations in the cells and effected a rearrangement of the cytoskeleton. The mechanism of action of the antiproliferative activity appears to involve an inhibition of cell proliferation and cell adhesion along with the production of cell damage resulting in a permanent loss of cell viability. The processes of apoptosis and necrosis might be involved in the mechanism of cell death. These observations would suggest that the cytostatic and cytotoxic effects exerted by the MPI on tumor cells would point to its use as a potential ingredient in functional food in order to decrease the risk of human diseases such as cancer, or even prevent such pathology altogether.

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