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ORIGINAL ARTICLE

Alpha-tomatine activates cell cycle checkpoints in the absence of DNA damage in human leukemic MOLT-4 cells

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Summary
Alpha-tomatine is a major glycoalkaloid found in the roots, leaves, stems and fruit of tomatoes *Lycopersicon esculentum*. Recently, alpha-tomatine has been recognized as a potential anticancer drug. In the present study, we identified the signaling cascades involved in the antitumor effect of alpha-tomatine on MOLT-4 leukemic cells. Alpha-tomatine inhibited the proliferation and decreased the viability of MOLT-4 cells in a dose-dependent manner. An increase in the activity of caspases 9 and 3/7 was not observed. However, an increase in the amount of p53 and its phosphorylation on serine 15, as well as an increased amount of mitochondrial protein PUMA was detected 4 and 24 h after exposure to alpha-tomatine at a concentration of 1–3 μmol/l. Inhibition of the proliferation of MOLT-4 cells by alpha-tomatine is also associated with an increase in $p21^{WAF1/CIP1}$ and the activation of Chk2. The comet assay did not detect significant amounts of single or double DNA strand breaks in cells treated with alpha-tomatine at concentrations of 0.1–9 μmol/l. Our results thus contribute to the understanding of the anticancer action of alpha-tomatine.

Key words: alpha-tomatine; DNA damage; p53; PUMA; leukemia

Abbreviations:
Ann, Annexin V; DSB, double strand break of DNA; EC_{50}, concentration causing the effect in 50% of the cells; PI, propidium iodide

INTRODUCTION

Alpha-tomatine is a major glycoalkaloid found in the roots, leaves, stems and fruit of tomatoes *Lycopersicon esculentum*. During the maturation of tomatoes, the content of alpha-tomatine decreases – an immature green tomato contains up to 500 mg of alpha-tomatine per kg of fresh fruit weight, but as the tomato ripens, alpha-tomatine is degraded and its content in red tomatoes is around 5 mg/kg of fresh fruit weight (Friedman 2002). The molecule of alpha-tomatine consists of tomatidine (aglycon) attached by an O-glycosidic bond to the tetrasaccharide moiety of β-lycotetraose (consisting of D-galactose, D-xylose and 2 molecules of D-glucose) (Shih et al. 2009).

Simultaneously with the first isolation and purification of tomatine (the mixture of two distinct alkaloids, alpha-tomatine and dehydrotomatine) from tomatoes by Fontaine et al. in 1948, the antifungal effect of tomatine against *Candida albicans* was demonstrated (Ma and Fontaine, 1948). Even before this, the antimicrobial and antifungal effect of alpha-tomatine was reported by the same group (Irving et al.

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1946). Experiments with rats and mice demonstrated the anti-inflammatory activity of tomatine connected with several unusual properties which had never been described for any anti-inflammatory substance in general (Filderman and Kovacs 1969). Currently it is known that the anti-inflammatory action of tomatine is mediated by the inhibition of NF-κB and JNK signaling (Chiu and Lin 2008, Shih et al. 2009, Shieh et al. 2011).

Alpha-tomatine has low oral and local toxicity. Whereas chronic oral application does not cause any systemic changes or abnormalities, the parenteral administration of a few dozen milligrams per kg is fatal in rodents. After intravenous injection, hypotension, an increase in respiratory rate and depth, and hemolysis occur (Wilson et al. 1961). In addition, a toxic effect on rat heart cells was described; the mechanism of action is similar to that of heart glycosides (Bergers and Alink 1980).

Herbal compounds such as alkaloids, phenolics, polyphenols, quinones and terpenoids have been shown to be very effective alternatives to synthetic therapeutics (Berger 2011). Recently, it has been discovered that alpha-tomatine exhibits an antiproliferative effect on tumor cell lines derived from human prostate carcinoma (Lee et al. 2011), human colon (HT29) and liver (HepG2) carcinoma (Lee et al. 2004), and lung carcinoma (Sheih et al. 2011). The exact underlying mechanisms of the anticancer action, however, are far from clear. Programmed cell death, which serves as a protective process by eliminating cells that are abnormal and potentially dangerous (Fuchs and Steller 2011), could be potentiated by alpha-tomatine.

For our study, we chose the MOLT-4 cell line derived from human T-lymphoblastic leukemia. These cells express specific surface markers: $CD1^+$ (49%), $CD4^+$ (55%), $CD5^+$ (72%) and $CD7^+$ (77%). MOLT-4 cells also contain wild-type protein p53. Their high sensitivity to ionizing radiation and cytostatic drugs make them a useful model for studying the molecular response to DNA damage (Tichý et al. 2007).

In the present study, we identified the signaling cascades involved in the antitumor effect of alpha-tomatine on MOLT-4 leukemic cells and demonstrated that, despite the activation of p53 and Chk2, direct DNA fragmentation is not involved in the cytostatic action of alpha-tomatine.

MATERIAL AND METHODS

Cell cultures and culture conditions

Human leukemic T-lymphocytes (MOLT-4 cell line) were obtained from the American Type Culture Collection ATCC (Manassas, USA) and maintained in Iscove's modified Dulbecco's medium (Sigma, USA). The medium was supplemented with 20% fetal calf serum and all cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. Cell lines in the maximal range of up to 20 passages were used for the experiment.

Cytostatic treatment

Alpha-tomatine: A stock solution of alpha-tomatine (Santa Cruz Biotechnology, California, USA) was prepared by dissolving 2 mg of the substance in 1 ml of DMSO and 4 ml of MOLT-4 culture medium (final concentration c = 387 μmol/l). A new stock solution was prepared every day and stored at a temperature of 8 °C. For the experiments, the stock solution was diluted with the culture medium to final concentrations of 0.1–9 μmol/l.

Irradiation: The cells were irradiated in culture flasks at room temperature using a ^{60}Co γ-ray source (Chisotron, Chirana, Czech Rep.) at a distance of 1 m from the source and at a photon dose rate of 1 Gy/min.

Mitoxantrone: A stock solution of mitoxantrone (Sigma, USA) was prepared by dissolving 1.3 mg of the substance in 2.5 ml of distilled water (final concentration c = 1 mmol/l). For experiments the stock solution was diluted to a final concentration of 5 nmol/l.

Proliferation and viability

Cell proliferation and viability were determined 4, 24, and 72 hours after treatment with 1, 2, 3 and 4 μmol/l alpha-tomatine. The cultures were divided every second day by dilution to a concentration of 2×10^5 cells/ml. Cell membrane integrity was determined using the Trypan blue exclusion technique – mixing of 50 μl Trypan blue and 50 μl of cell suspension. The cell counts were carried out using a Bürker chamber.

Western Blotting

Whole-cell lysates (Cell Lysis Buffer, Cell Signaling Technology, Inc, USA) were prepared 4, 24 and 72 hours after the application of 1, 2 and 3 μmol/l of alpha-tomatine, and quantification of the protein content was performed using BCA assay (Sigma-Aldrich, USA). The lysates (20 μg purified protein) were loaded into each lane of a polyacrylamide gel. After electrophoretic separation, the proteins were transferred to a PVDF membrane (Bio-Rad, USA). Non-specific binding of the membranes was blocked for 1 hour in Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk. The membranes were washed twice with TBST, each time for 5 minutes, and once with TBS, again for 5 minutes.

Incubation with primary antibody (p53 – Exbio, Czech Republic; β-actin, $p21^{WAF1/Cip1}$ – Sigma-Aldrich, USA; p53_serine15 – Calbiochem-Merck, USA; Chk2, Chk2_threonin68, PUMA – Cell Signaling, Boston, MA, USA) was performed at 4 °C overnight. The following day the membranes were washed five-times with TBST, each time for 5 minutes, and once with TBS, again for 10 minutes, and then incubated with appropriate secondary antibody (DakoCytomation, Denmark) for one hour at room temperature. Band detection was performed using a chemiluminiscence detection kit (Roche, Switzerland). To ensure equal protein loading, each membrane was reprobed and β-actin was detected. Bands were quantified using an ImagePro 5.1 computer image analysis system (Media Cybernetics, Bethesda, MD, USA).

Flow-cytometry

Apoptosis was determined by flow cytometry using APOPTEST™-FITC kit (DakoCytomation, Denmark) according to the manufacturer's instructions. The APOPTEST™-FITC kit employs the property of fluorescein isothiocyanate (FITC) conjugated to Annexin V (Ann-FITC) to bind to phosphatidylserine in the presence of Ca^{2+}, and the property of propidium iodide (PI) to enter cells with damaged cell membranes and to bind to DNA. Measurement was performed immediately using a CyAn™ ADP (Beckman Coulter, Miami, FL, USA) flow cytometer. Listmode data were analyzed using Summit v4.3 software (Beckman Coulter, USA).

For the analysis of cell cycle distribution, the cells were washed with ice cold PBS and fixed with 70% ethanol. They were then incubated for 5 min at room temperature in buffer (192 ml 0.2 mol/l Na_2HPO_4 + 8 ml 0.1 mol/l citric acid, pH 7.8) and stained with propidium iodide (PI) in Vindelov's solution for 60 minutes at 37 °C. The DNA content was determined by means of a BD FACS Aria III flow cytometer (Becton, Dickinson and Company, New Jersey, USA) using a 13 mW Coherent® Sapphire™ solid-state laser with excitation at 488 nm; total emission above 560 nm was recorded. List mode data were analyzed using BD FACS Diva 6.1.3. software (Becton, Dickinson and Company, New Jersey, USA).

Activity of caspases

The induction of programmed cell death was determined by monitoring the activities of caspase 3/7 and caspase 9 by Caspase-Glo Assays (Promega, Madison, WI, USA) 4 and 24 hours after treatment with 1, 2 and 3 μmol/l of alpha-tomatine. The assays provide proluminogenic substrates in an optimized buffer system. The addition of Caspase-Glo Reagents results in cell lysis, followed by caspase cleavage of the substrate and the generation of a luminescent signal. A total of 1×10^4 cells were seeded per well using a 96-well-plate format (Sigma, St. Louis, MO, USA). After treatment, Caspase-Glo Assays reagents were added to each well (50 μl/well) and incubated for 30 minutes before luminescence was measured using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland).

Comet assay

DNA damage was measured using alkaline and neutral versions of comet assay as described previously (Olive at Banáth 1993, Collins et al. 1996, Calini et al. 2002). Briefly, cells embedded in 1% agarose (Sigma) on microscope slides were lysed in 10 mmol/l Tris-buffered 2.5 mol/l NaCl (pH 10.0; Penta, Prague, Czech Republic) containing 1% Triton X 100 (Merck) and 100 mmol/l EDTA (Penta) for 1 hour at 4 °C. In alkaline conditions (NaOH, EDTA), the electrophoresis was carried out at 40 V, 300 mA, for 30 minutes at 4 °C after 40 minutes of unwinding. The electrophoresis in the neutral conditions (90 mmol/l Tris, 90 mmol/l boric acid, 2 mmol/l EDTA, pH 8.0) was performed at 29 V, 6 mA, for 40 minutes at 4 °C after washing in borate buffer. DNA damage was analyzed by the comet module of Lucia 6.20 image analysis (Laboratory Imaging, Prague, Czech Republic) after the cells were stained with ethidium bromide (Sigma). The percentage of DNA in the comet tail was measured. At least fifty cells per slide were analyzed.

Statistics

The statistical data for comet assay results were calculated with STATISTICA 10.0 software (StatSoft CR, Czech Republic). Differences within the groups were evaluated by the Kruskal-Wallis test. All experimental data were expressed as median and 25^{th} and 75^{th} percentiles. Other results were evaluated with descriptive statistics, and the charts were made using Microsoft Office Excel (Microsoft Inc., Redmond, WA, USA). The values were expressed as arithmetic means with standard deviation (SD) unless otherwise indicated. Significant differences between groups were analyzed using the Student t-test. Experimental data were evaluated at the significance level $2\alpha=0.05$.

RESULTS

Proliferation and viability

The effect of alpha-tomatine was assessed by comparing cell proliferation and viability in the MOLT-4 leukemic cell line exposed to alpha-tomatine

in concentrations of 1, 2, 3 and 4 μmol/l for 4, 24 and 72 h with the untreated control cell line (Fig. 1). The application of 1 and 2 μmol/l alpha tomatine had no pronounced effect on cell viability and slightly inhibited proliferation within 24 h of treatment. After incubation with alpha-tomatine at the concentration of 3 μmol/l, the number of cells decreased initially (to 360 × 10^3 after 4 hours and remained at about 400 × 10^3 for 24 hours). Proliferation recovered after 72 hours and the number of cells increased to 1355 × 10^3 (control – 1700 × 10^3). The changes in viability after exposure to the concentration of 3 μmol/l were only slight – 93% of the cells were viable after 4 hours, 82% after 24 hours, and 87% after 72 hours. The treatment with 4 μmol/l alpha-tomatine led to a significant decrease in viability (after 4 hours only 29% of cells remained viable; after 24 hours, 12%; and after 72 hours, 14%) and a rapid decrease in the number of cells (after 4 hours 20 × 10^3, compared to 500 × 10^3 in control).

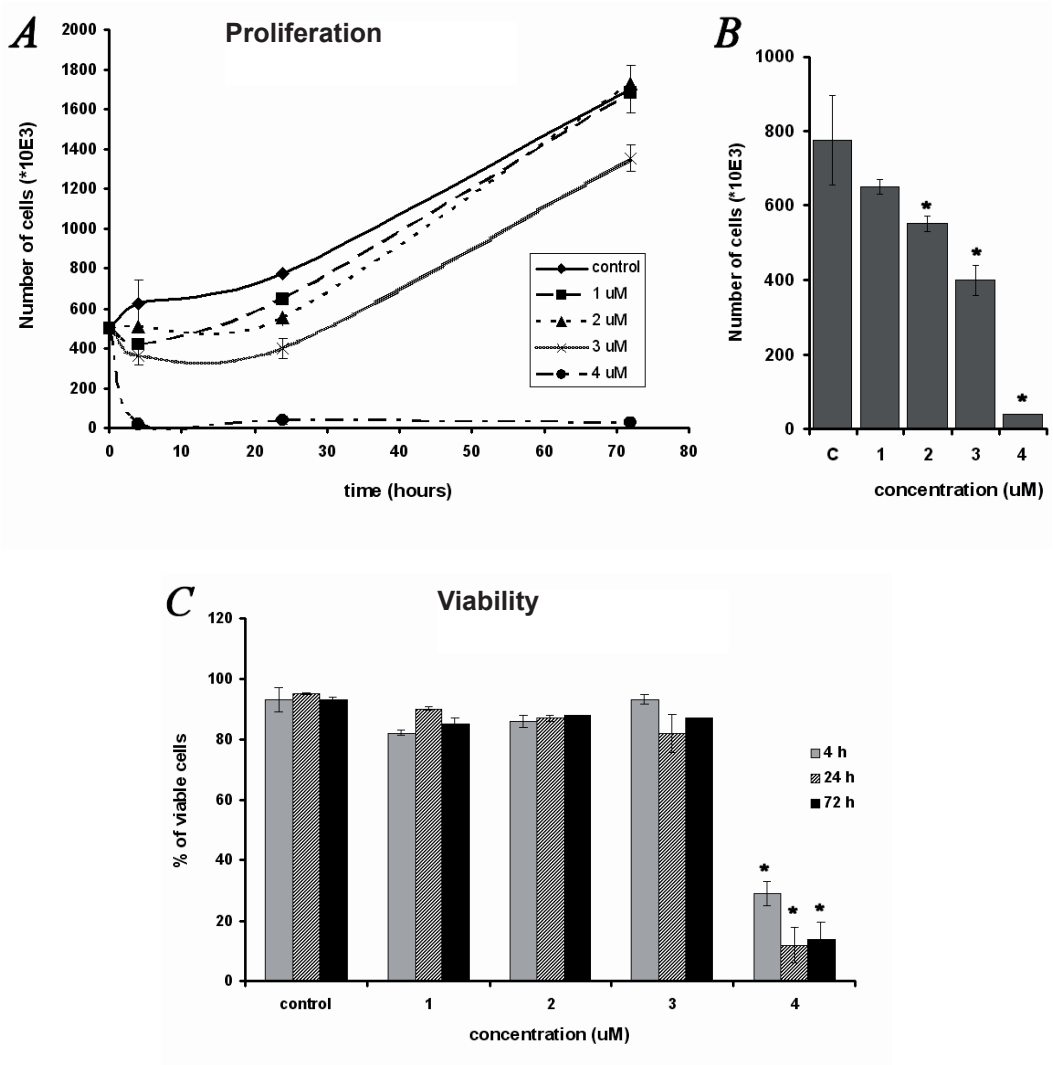


Fig. 1. **Effect of alpha-tomatine (1–4 μmol/l) on the proliferation and viability of human MOLT-4 leukemic cells.** Results are shown as mean ± SD from three experiments. * Statistically significant as compared with controls. (*A*) Changes in the number of cells during the 3 days after treatment. (*B*) Details of changes in the number of cells after 24 hours of treatment, during the period of maximal effect of tomatine at concentrations below 4 μmol/l. (*C*) Changes in the viability of cells during the 3 days after treatment. The percentage of viable cells was determined using Trypan blue exclusion staining.

Changes in p53, p21 and Chk2, and cell cycle distribution

First, we detected changes in the levels of the proteins p53 and $p21^{WAF1/CIP1}$. The amount of tumor suppressor protein p53 increased after 4 and 24 hours. The exposure of MOLT-4 cells to alpha-tomatine also induced phosphorylation of p53 on serine 15 after the same periods of incubation. An inhibitor of cyclin-dependent kinases, $p21^{WAF1/CIP1}$ increased after 4 h (Fig. 2). These changes in $p21^{WAF1/CIP1}$ disappeared after 24 h. After 72 hours, we did not detect any changes in the p53-p21 pathway, and the amount of p53 and $p21^{WAF1/CIP1}$ was the same as in the control group (data not shown).

To further elucidate the mechanisms inhibiting proliferation, we determined the changes in Chk2, particularly the phosphorylation of threonine 68. After 4 and 24 hours, there was a concentration-dependent increase in the level of Chk2 phosphorylated on threonine 68, while the overall amount of Chk2 did not change significantly (Fig. 2). No changes in Chk2 phosphorylation after 72 hours were observed.

To determine cell cycle distribution and possible cell cycle arrest after alpha-tomatine treatment, we employed cell cycle analysis of DNA content using flow cytometry. The treatment of MOLT-4 cells with 3 μmol/l alpha-tomatine resulted in cell cycle arrest and in an accumulation of cells in the G1 phase, accompanied by a decrease in cells in the G2 phase. 24 hours after the application of 3 μmol/l alpha-tomatine, 60% of cells were in the G1 phase, while in untreated control samples, the proportion was 52% (Fig. 3).

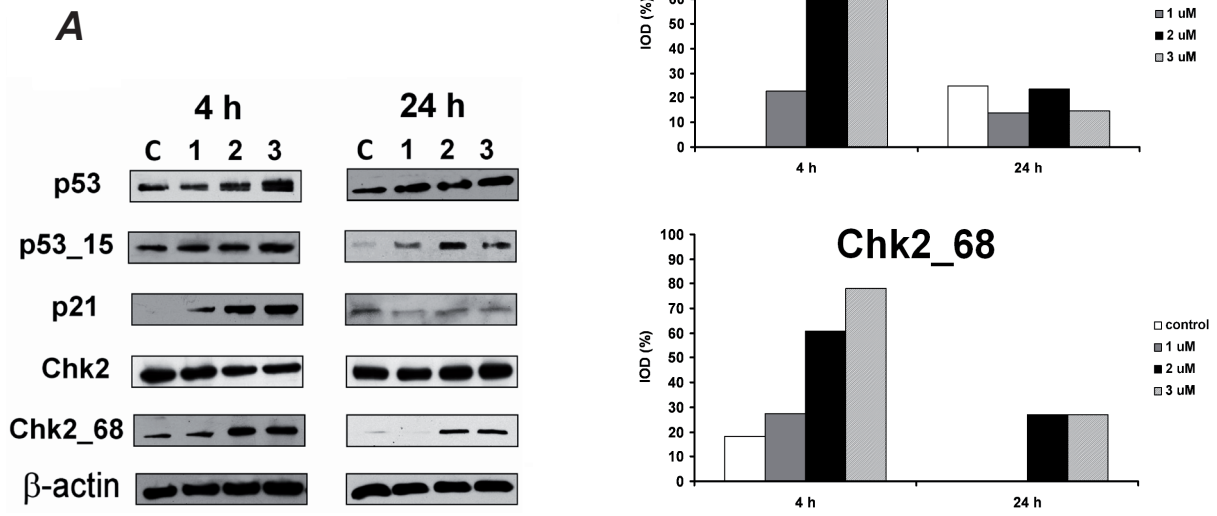


Fig. 2. **Induction and activation of p53, $p21^{WAF1/Cip1}$ and Chk2 in MOLT-4 cells exposed to 1, 2 and 3 μmol/l alpha-tomatine 4 and 24 hours** after the application of the drug detected by western blot (*A*). To ensure equal protein loading, membranes were reincubated with β-actin. Data were quantified densitometrically and are expressed as the mean ± SD from two independent experiments. The values of the integrated optical density were expressed as a percentage of β-actin. Untreated control; p53_15 – p53 phosphorylated on serine 15; Chk2_68 – Chk2 phosphorylated on threonine 68 (*B*).

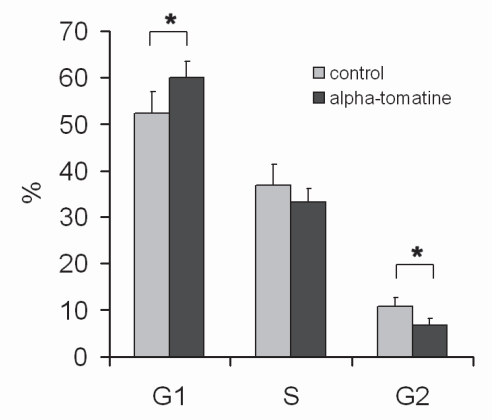


Fig. 3. **Analysis of the cell cycle of MOLT-4 cells 24 h after the application of 3 µmol/l alpha-tomatine** – percentage of cells in G1, S, and G2/M phase. Results are shown as mean ± SD from five measurements from three independent experiments. * Statistically significant as compared with controls.

DNA fragmentation – comet assay

The most common reason for an increase in the amount of p53 is cellular stress related to DNA damage. Therefore, using comet assay, we examined whether alpha-tomatine induces breaks in the DNA. Our results show that in untreated control groups MOLT-4 cells were intact. Using the alkaline version of comet assay, we evaluated the amount of single strand breaks in cells. No significant DNA damage was found in cells treated with 0.1–9 µmol/l of alpha-tomatine for 4 h (Fig. 4A–C, G). The neutral version of comet assay was used to evaluate the number of double strand breaks (DSB) in cells. Again, no significant amount of DSB was found in cells treated with 0.1–9 µmol/l of alpha-tomatine for 4 h (Fig. 4D–F, H). Furthermore, neither significant phosphorylation of H2AX nor the formation of DNA damage-associated foci were observed after exposure to 3 µmol/l alpha-tomatine for 4 or 24 h (data not shown).

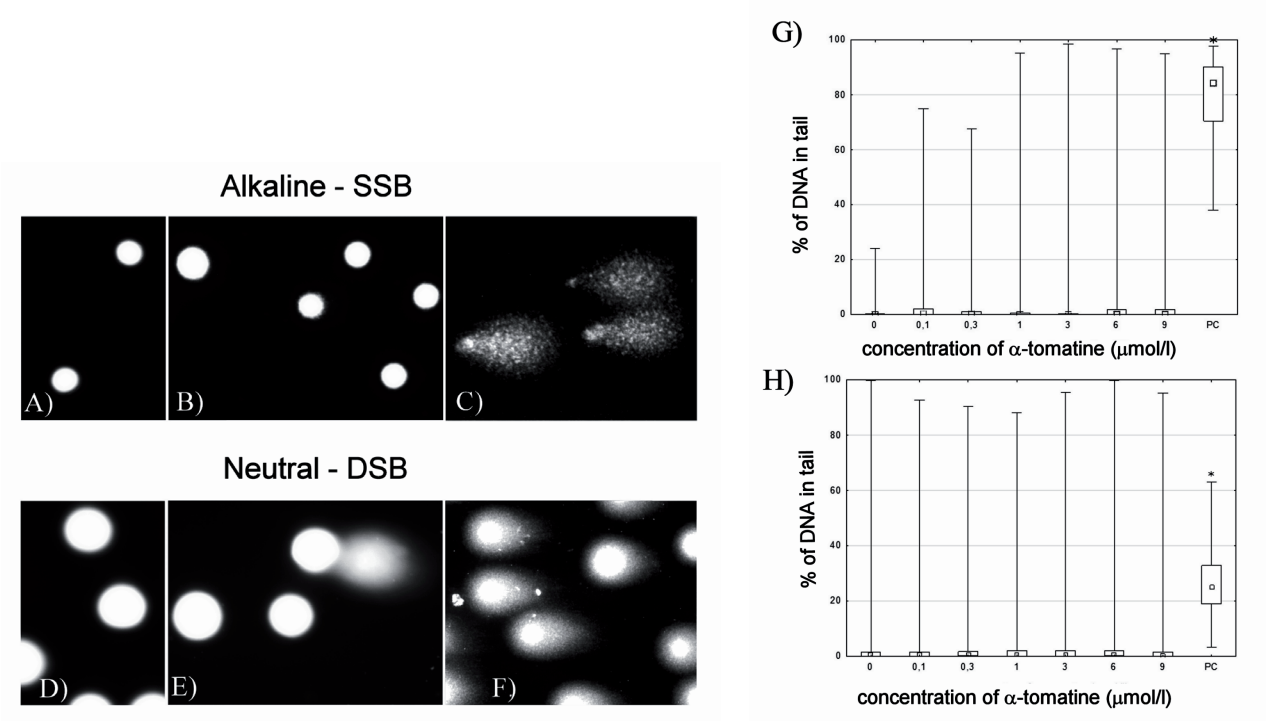
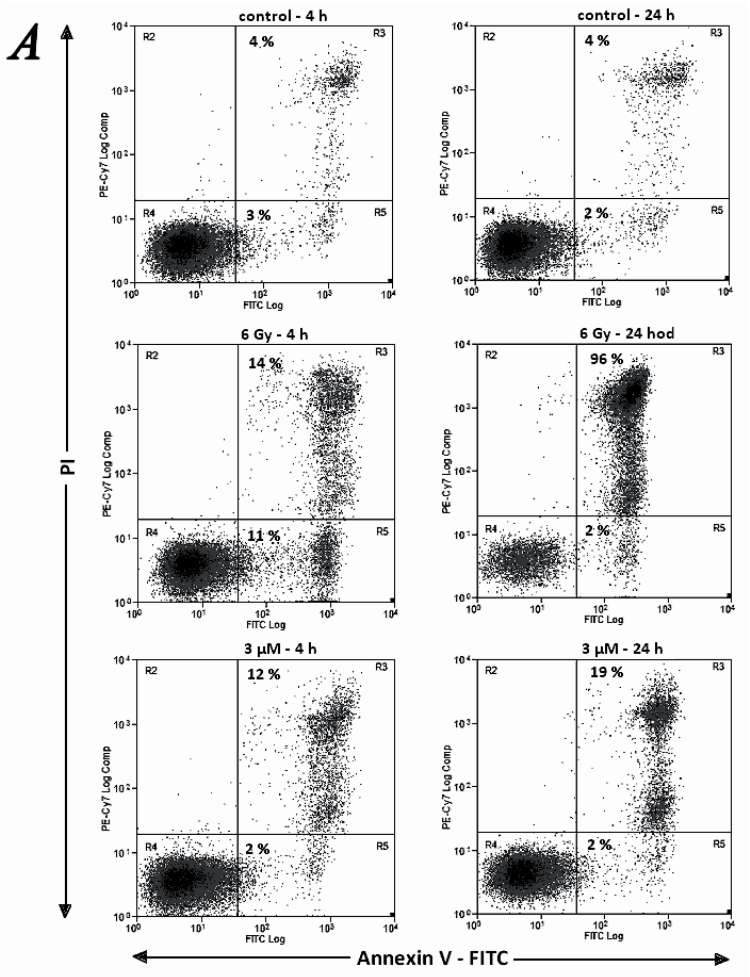
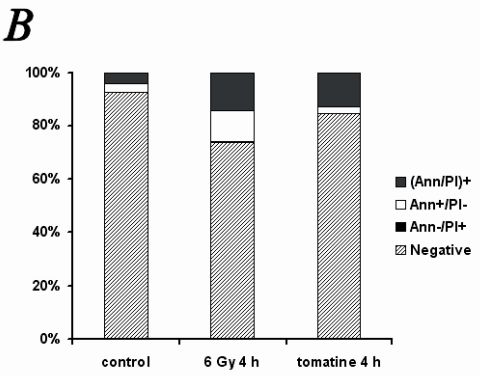
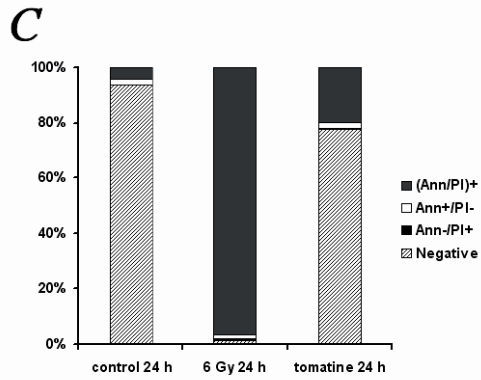
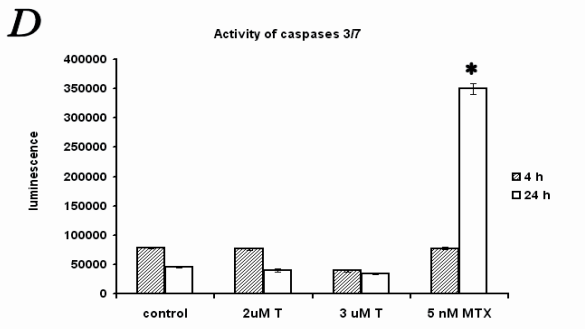


Fig. 4. **Detection of DNA damage by comet assay in MOLT-4 cells exposed to 0.1–9 µmol/l alpha-tomatine.** (*A–C*) Digital camera photograph of the alkaline version of comet assay. Control MOLT-4 cells without exposure to alpha-tomatine (*A*), cells treated with 3 µmol/l of alpha-tomatine (*B*), and 1.5% H_2O_2-treated cells (positive control) (*C*). (*D–E*) Digital camera photograph of the neutral version of comet assay. Control MOLT-4 cells without exposure to alpha-tomatine (*D*), cells treated with 3 µmol/l of alpha-tomatine (*E*), and cells after exposure to 20 Gy of irradiation (positive control) (*F*). (*G*) The dependence of DNA single-strand breaks on the concentration of alpha-tomatine after 4 hours of exposure. PC – positive control (1.5% H_2O_2). (*H*) The dependence of DNA double-strand breaks on the concentration of alpha-tomatine after 4 hours of exposure. PC – positive control (20 Gy gamma radiation). * Statistically significant as compared with controls.









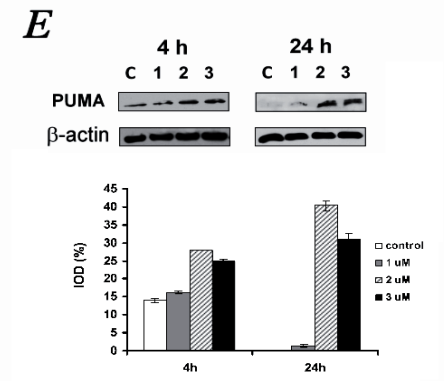


Fig. 5. **Effect of alpha-tomatine on the induction of programmed cell death in human MOLT-4 leukemic cells.** (*A–C*) Programmed cell death induction was determined by Annexin V and propidium iodide staining. Representative dotplots of one of three experiments (*A*). Quantification of programmed cell death 4 h (*B*) and 24 h (*C*) after the application of 3 µmol/l of alpha-tomatine. Irradiation by 6 Gy of gamma radiation was used as a positive control. Ann – Annexine V; PI – propidium iodide. (*D*) Activity of caspases 3/7 was determined 4 and 24 h after exposure to 2 and 3 µmol/l of alpha-tomatine. 5 nmol/l of mitoxantrone was used as a positive control. T – alpha-tomatine; MTX – mitoxantrone. (*E*) Changes in pro-apoptotic PUMA protein detected by western blot followed by densitometry 4 and 24 h after exposure to 1, 2 and 3 µmol/l of alpha-tomatine. To ensure equal protein loading, membranes were reincubated with β-actin. The densities of protein bands were quantified and expressed as a percentage of the integrated optical density related to β-actin. Results are shown as mean ± SD from three independent experiments.

Mechanisms of cell death

The analysis of apoptosis induction in the MOLT-4 cells was performed after a 4h- and 24h-long incubation with 3 μmol/l alpha-tomatine, using Annexin V (Ann) binding to phosphatidylserine on the cell surface of apoptotic cells. Propidium iodide (PI) was used as a marker for cell membrane permeability. To investigate the effect of alpha-tomatine, a positive control of γ-irradiated cells (6 Gy) was used. The treatment with a single dose of 3 μmol/l alpha tomatine did not lead to an increase in the number of early apoptotic cells (Ann+/PI-), but to a quantitative increase in the number of late apoptotic/necrotic cells (Ann+/PI+) after 4 h (12%) and 24 h (19%) – in comparison with the control group (4%). The cells affected by 6 Gy of γ-radiation were found to be in the stage of early apoptosis (11% of Ann+/PI- cells), transformed to late apoptosis/necrosis (96%) 24 h later (Fig. 5A–C). Next, we evaluated the activation of caspases 9 and 3/7. A significant increase in the activity of these caspases after exposure to 3 μmol/l alpha-tomatine was not observed up to 24 h post-treatment (Fig. 5D). However, an increase in the amount of PUMA protein from the bcl-2 family was detected 4 and 24 h after exposure to 1–3 μmol/l alpha-tomatine (Fig. 5E).

DISCUSSION

Recently, alpha-tomatine has been recognized as a potential anticancer drug. However, several recent studies report different antiproliferative mechanisms; thus, the underlying mechanisms are far from clear. In human prostatic adenocarcinoma PC-3 cells, alpha-tomatine induced programmed cell death, which is not associated with cell cycle arrest and inhibits NF-κB nuclear translocation (Lee et al. 2011). In NCI-H460 human non-small cell lung cancer cells, alpha-tomatine inactivated the FAK/PI3K/Akt signaling pathway and reduced the DNA-binding activity of NF-κB (Shieh et al. 2011). In MCF-7 (breast) cancer cells, it suppressed the protein kinase α/ERK/NF-κB pathway, which led to the downregulation of the 12-*O*-tetradecanoylphorbol-13-acetate-induced activation of metalloprotease 2/9 and thereby inhibited the migration and invasion of the cells (Shi et al. 2012). The study by Lee at al. reporting antiproliferative effects on human colon (HT29) and liver (HepG2) cancer cells did not identify a specific mechanism. They show that the effect of alpha-tomatine was more pronounced on liver cells than on colon cells (Lee et al. 2004). PC-3 cells appear to be particularly susceptible to inhibition, when compared with breast (MDA-MB-321) or gastric (KATO-III) cancer cells, or normal cells (Choi et al. 2012). Interestingly, the removal of one, two or three sugar molecules from alpha-tomatine results in a decrease in antitumor activity. The aglycon tomatidine had the lowest activity (Lee et al. 2004, Friedman et al. 2009, Choi et al. 2012).

After treatment with 4 μmol/l alpha-tomatine, we detected an abrupt decrease in cell number already after 4 hours, which was accompanied by a significant decrease in cell viability. After treatments with lower alpha-tomatine concentrations (1–3 μmol/l), we observed only a transient inhibition of proliferation. The proliferation and viability of cells treated with 1–3 μmol/l of alpha-tomatine recovered after 72 hours. The study by Lee et al. demonstrates that concentrations of alpha-tomatine effective on PC-3 cells are in a similar range: PC-3 cells treated with 2.5 and 5.0 μmol/l alpha-tomatine showed a decrease in viability and the EC_{50} value at 24 h post treatment was estimated at 1.67±0.3 μmol/l. Similarly, the cell viability of NCI-H460 cells significantly decreased after 24 h treatment with alpha-tomatine at concentrations between 2–4 μmol/l. Non-cytotoxic concentrations reduced cell invasion and migration (Shieh et al. 2011). In a recent study by Chao et al., the EC_{50} values at 24 h post treatment for the HL-60 and K526 leukemic cell lines were 1.92 and 1.51 μmol/l respectively (Chao et al. 2012).

How exactly alpha-tomatine induces cell death remains an unanswered question. The treatment of PC-3 prostate cancer cells with 2 μmol/l alpha-tomatine for 24 hours resulted in an increase in the number of early apoptotic cells as well as late apoptotic cells, and the activation of caspase-3, caspase-8 and caspase-9 was confirmed (Lee et al. 2011). In contrast, in HL-60 and K562 leukemic cells, alpha-tomatine induced caspase-independent programmed cell death – caspase-3, -8, -9 were not activated (Chao et al. 2012). Our data support the findings of Chao et al. In our study on MOLT-4 leukemic cells, the 3 μmol/l concentration of alpha-tomatine induced a slight decrease in cell viability 24 h after application of the drug. Also, after 24 hours, 20 % of the cells bound Annexin V and propidium iodide, indicating late apoptosis/necrosis. We did not detect a significant increase in the activity of caspases 9 and 3/7. However, an increase in the amount of mitochondrial protein PUMA was detected 4 and 24 h after exposure to 1–3 μmol/l alpha-tomatine.

PUMA protein belongs to the group of pro-apoptotic factors from the Bcl-2 family, characterized by the presence of only the BH3 domain. The role of these BH3-onlies is to mediate apoptotic signalization dependent on cell integrity. BH3-onlies bind to anti-apoptotic Bcl-2-like factors and enable

oligomerization of Bax-like pro-apoptotic factors and permeabilization of the mitochondrial membrane. In undamaged cells, these factors are absent or exist in an inactive form (Bogner et al. 2010). The treatment with alpha-tomatine led to an increase in PUMA protein level after 4 and 24 hours. This fact correlates with the observed increase in p53, because one of the p53 roles is to increase the transcription of PUMA. Our study is the first to report changes in BH3-onlies induced by alpha-tomatine; however, our findings are consistent with recent findings that alpha-tomatine causes a decrease in membrane potential, the upregulation of pro-apoptotic Bax-like protein Bak, and the release of programmed cell death-inducing factor (AIF) from mitochondria (Chao et al. 2012).

Transcription of the PUMA gene (*BBC3*) can be activated by protein p53 (Lakin and Jackons 1999, Erlacher et al. 2005). Human p53 is a tumor suppressor phosphoprotein; its most investigated role is the ability to act as a specific activator of gene transcription. In this way, p53 is involved in regulation of the cell cycle, programmed cell death, senescence, DNA repair, cell differentiation, and angiogenesis (Sionov and Haupt 1999). The p53 in normal cells is degraded by the proteasome 26S. DNA damage and other stressors induce post-translational modifications of p53, mainly phosphorylations, participating in its stabilization and activation (Bai and Zhu 2006, Lavin and Gueven 2006). Our experimental data showed a dose-dependent increase in p53 and in its phosphorylation on serine 15 after 4 and 24 hours of exposure to alpha-tomatine. Phosphorylation of serine 15 on the N-terminal domain of p53 can be mediated directly by ATM kinase or through Chk1/Chk2 kinase, and it inhibits the interaction of p53 with its negative regulator oncoprotein Mdm2 (Shieh et al. 1997, Yap et al. 2004).

P53 readily reacts to genotoxic stressors, mainly to the induction of single and double strand breaks. However, the examination by comet assay did not detect significant amounts of single and double DNA strand breaks in cells treated with 0.1–9 mmol/l of alpha-tomatine. The increase in p53 must therefore be due to another type of stress. Different forms of stress, such as DNA damage (ultraviolet or ionizing radaition, chemical agents), oxidative stress, hypoxia, ribonucleotide depletion, and deregulated oncogene expression induce an increase in the amount of p53 (Prives and Hall 1999). Alpha-tomatine interacts with cholesterol and can cause damage to the integrity of biomembranes (Keukens et al. 1995, 1996), which may be responsible for the observed p53 increase.

Our study also showed a dose-dependent increase in p21 after 4 hours as well as the activation of Chk2 (phosphorylated at threonine 68) after 4 and 24 hours. Both of these proteins are involved in cell cycle regulation. Chk2 is a protein responsible for regulation of the cell cycle and controls the checkpoints in G1/S and G2/M transition by the inhibition of Cdc25A and Cdc25C (Bartek and Lukas 2003). The gene *CDNK1A* encoding protein $p21^{WAF1/CIP1}$ (wild type activated fragment 1, cyclin-dependent kinase inhibitor protein 1) is another target of p53. $P21^{WAF1/CIP1}$ triggers mainly cell cycle arrest in the G1/S phase, but also participates in cell cycle arrest at the G2/M checkpoint (Taylor and Stark 2001, Cazzalini et al. 2010). A previous study revealed that alpha-tomatine did not affect cell cycle distribution (Chao et al. 2012); however, the increased amounts of p21 and phosphorylated Chk2 (threonine 68) correlate well with the transient decrease in cell proliferation, indicating an overall slow-down of the cycle.

We may conclude that alpha-tomatine does not cause direct DNA damage. It induces caspase-independent cell death associated with an increase in p53 and the BH3-only protein PUMA. The inhibition of proliferation by alpha-tomatine is associated with an increase in $p21^{WAF1/CIP1}$ level and the activation of Chk2. Alpha-tomatine is a potential antitumor agent; however, the mechanism of its anticancer effect requires additional study.

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