The cytotoxic activity of sanguinarine in C32 human amelanotic melanoma cells

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Abstract: Sanguinarine, an alkaloid present in various medicinal plants, has been associated with detrimental effects on different types of cancer cells. The aim of our study was to analyze the cytotoxic effects of sanguinarine on C32 human amelanotic melanoma cells. The cells were exposed to various concentrations of sanguinarine (0.5, 1, and 2 µM) for 24 h and displayed a dose-dependent decrease in cell viability; the sanguinarine-induced oxidative stress in C32 cells affected the activity of antioxidant enzymes and generated lipid peroxidation. Moreover, we observed an enhanced expression of heat shock proteins 60, 70, and 90 in response to the stress exerted by this alkaloid. The increased p53 protein expression and Bax/Bcl-2 ratio revealed the susceptibility of C32 cells to apoptosis. Our study demonstrated that sanguinarine induced cytotoxicity in melanoma cells that ultimately resulted in cell death. These results emphasize the antigrowth efficiency of sanguinarine against amelanotic melanoma cells, but further research is needed in order to obtain more potent antitumor effects of this alkaloid.

Key words: Sanguinarine, melanoma, oxidative stress, lipid peroxidation, heat shock proteins

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1. Introduction

Amelanotic melanoma is a rare type of cutaneous melanoma characterized by minimal or no pigmentation that causes delayed diagnosis and poor prognosis (Joshi et al., 2012). Treatment usually consists of chemo- and radiotherapy, and the range of side effects is well documented. This makes the discovery of new and safer therapeutic agents a priority. In the past few decades, scientists have begun to study various natural compounds with anticancer effects.

Sanguinarine, a natural benzophenanthridine alkaloid, has been associated with a pronounced antiproliferative effect and oxidative-stress-induced cell death in different types of melanoma cells (Kemény-Beke et al., 2006; Serafim et al., 2008; Hammerová et al., 2011; Burgeiro et al., 2013). In addition, chelidone and sanguinarine extracted from Chelidonium majus L. induced apoptosis in human acute T-lymphoblastic leukemia MT-4 cells (Philchenkov et al., 2008) and leukemia cells (HL-60, L1210) (Nadova et al., 2008), as well as in human pancreatic carcinoma AsPC-1 and BxPC-3 cells (Ahsan et al., 2007). Sanguinarine has been shown to inhibit cell proliferation and metastasis in human breast cancer (Choi et al., 2009).

Further, it was reported that this alkaloid generated increased production of reactive oxygen species (ROS) and depletion of cellular antioxidants, such as reduced glutathione (GSH) (Matkar et al., 2008). The mechanism suggested for sanguinarine-induced apoptosis is based on the activation of caspases regulated by different proteins, including members of the Bcl-2 family (Lee et al., 2012). However, the mechanism of antiproliferative action of various anticancer compounds may or may not involve apoptosis (Ökten et al., 2015; Vejselova and Kutlu, 2015).

The aim of this study was analysis of the effects produced by three different concentrations of pure sanguinarine on amelanotic melanoma C32 cells. The antiproliferative activity of sanguinarine was previously investigated on melanin-producing melanoma C32 cells. The antiproliferative activity of sanguinarine was previously investigated on melanin-producing melanoma C32 cells. The antiproliferative activity of sanguinarine was previously investigated on melanin-producing melanoma C32 cells. The antiproliferative activity of sanguinarine was previously investigated on melanin-producing melanoma C32 cells. The antiproliferative activity of sanguinarine was previously investigated on melanin-producing melanoma C32 cells. The antiproliferative activity of sanguinarine was previously investigated on melanin-producing melanoma C32 cells.

In order to assess the cytotoxic effects of this alkaloid, we evaluated cellular antioxidant status and lipid peroxidation, as well as its cell-death–inducing effect.

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2. Materials and methods

2.1. Cell culture and treatment
Human amelanotic melanoma cells (C32 cell line) were purchased from the American Type Culture Collection (ATCC CRL-1585). They were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin and maintained in a humidified atmosphere with 5% CO₂ at 37 °C. The culture medium was changed every 2 days until cells reached 80% confluence, and subsequently, the cells were detached with 0.25% (w/v) Trypsin 0.53 mM EDTA solution (Sigma-Aldrich, USA). For treatment, 6 × 10⁴ cells/cm² were seeded into 25-cm² culture flasks and allowed to adhere until they reached 80% confluence. Afterwards, the cells were treated with 0.5, 1, and 2 µM sanguinarine chloride (>98% pure; Sigma-Aldrich) for 24 h. Untreated C32 cells were used as controls.

2.2. Cell viability assay
Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). After 24 h of treatment, the cells were incubated with 1 mg/mL MTT for 3 h at 37 °C. Then the medium was removed and 100 µL of isopropyl alcohol was added to each well for formazan crystal solubilization. The optical density was read at 595 nm (TECAN GENios plate reader). The cell viability was expressed as a percentage of the control.

2.3. Cell lysate preparation
C32 cells were harvested after 24 h of treatment and lysed by sonication on ice (30 s × 3 times). The homogenate was centrifuged at 3000 × g for 10 min at 4 °C, and the supernatant was used for biochemical and Western blot analyses.

2.4. Assessment of antioxidant enzymes
Catalase (CAT) (EC 1.11.1.6) activity was assayed by monitoring the decrease in absorbance of H₂O₂ at 240 nm, according to the method of Aebi (1974). One unit represented the amount of enzyme that catalyzed the conversion of 1 µmol of H₂O₂ in 1 min. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by the method of Paoletti and Mocali (1990), following the oxidation of NADH at 340 nm. One unit of SOD activity was defined as the amount of enzyme that inhibited the oxidation of NADH by 50%. Measurement of glutathione reductase (GRed) (EC 1.6.4.2) activity was performed according to the method of Goldberg and Spooner (1983). One unit of GRed activity was calculated as 1 µmol of NADPH consumed per minute. Glutathione peroxidase (GPX) (EC 1.11.1.9) activity was measured using Beutler's method (1984) by monitoring the oxidation of NADPH by t-butyl-hydroperoxide at 340 nm. One unit of activity was defined as the amount of enzyme that catalyzes the conversion of 1 µmol of NADPH per minute under standard conditions. Glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) activity was assayed using the method of Lohr and Waller (1974). The rate of NADPH formation during the reaction catalyzed by G6PDH was followed at 340 nm. Glutathione S-transferase (GST) (EC 2.5.1.18) activity was determined spectrophotometrically at 340 nm according to the method of Habig et al. (1974) by measuring the rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with GSH. One unit of GST activity was defined as the amount of enzyme that generated 1 µmol of conjugated product per minute. The recording of all enzymatic activities was performed in a physiological pH of 7.4, and the temperature was set at 37 °C for SOD activity or at room temperature (25 °C) for the rest of the enzymes. All enzymatic activities were calculated as specific activities (units/mg of protein) and expressed as percentage of the control values.

2.5. Lipid peroxidation assay
The malondialdehyde (MDA) content was assayed using the method described by Dinischiotu et al. (2013). Each sample (200 µL) was incubated with 0.1 M HCl (700 µL) for 20 min at room temperature. Then 900 µL of 0.025 M thiobarbituric acid was added, and the mixture was incubated for 1 h at 37 °C. Subsequently, samples were subjected to fluorescence analysis (λex/em = 520/549 nm) (Spectrofluorometer FP-6300, JASCO). Relative fluorescence units (RFU) recorded were converted to MDA nanomoles using 1,1,3,3-tetramethoxypropane as standard.

2.6 Western blot analysis
Equal amounts of total protein from the cell lysates collected for each sample were boiled (95 °C) for 5 min and subjected to 10% SDS-PAGE. Proteins were transferred to PVDF membranes in Tris-glycine buffer [48 mM Tris-HCl (pH 8.3), 39 mM glycine, 20% methanol] using a wet transfer unit (BIO-RAD, USA). Then the membranes were blocked overnight at 4 °C and incubated with specific mouse monoclonal IgG anti-Bax; Bcl-2; p53; heat shock proteins (Hsp) 60, 70, and 90; and β-actin (all from Santa Cruz Biotechnologies, USA). The Western blot chromogenic immunodetection kit containing secondary antibody anti-mouse IgG conjugated with alkaline phosphatase and BCIP/NBT substrate for alkaline phosphatase was used according to the manufacturer’s instructions. Densitometry data were obtained using GelQuantNET software.

2.7. Statistical analysis
The data were calculated as mean ± standard deviation (SD) of three independent experiments (n = 3) and expressed as
a percentage of the control. The results were analyzed for statistical significance using the GraphPad Prism software (version 5; GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between groups were evaluated by one-way ANOVA followed by Bonferroni’s post-hoc test, and P < 0.05 was considered statistically significant.

3. Results
3.1. Sanguinarine decreased the viability of C32 amelanotic melanoma cells
As shown in Figure 1A, the alkaloid was able to induce a reduction in C32 cell numbers after 24 h of incubation. The melanoma cells treated with 2 µM sanguinarine displayed distinct morphological changes (Figure 1A, arrows), including cell shrinkage, rounding up, and membrane blebbing. Furthermore, the results of the MTT assay (Figure 1B) revealed a dose-dependent decrease in cell viability after exposure to sanguinarine (8%, 28.5%, and 45% for 0.5, 1, and 2 µM, respectively).

3.2. Sanguinarine induced oxidative stress in C32 amelanotic melanoma cells
Figure 2 shows a dose-dependent increase in the CAT- and SOD-specific activities after sanguinarine exposure. SOD activity was elevated by 155.5%, 230%, and 348% and CAT by 133%, 161%, and 176% after exposure to 0.5, 1, and 2 µM sanguinarine, respectively, compared to the controls.

Figure 1. Sanguinarine induced morphological changes and decreased cell viability in C32 amelanotic melanoma cells. (A) Representative phase contrast images showing cellular morphology of C32 cells treated for 24 h with 0.5, 1, and 2 µM sanguinarine. Morphological changes of cells treated with 2 µM sanguinarine as indicated by arrows: cell shrinkage (a), rounding up (b), and membrane blebbing (c). Scale bar = 50 µm. (B) Cell viability was detected by MTT assay, as described in Materials and methods. The results were calculated as means ± SD (n = 3) and expressed as percentage of control (* P < 0.05 and *** P < 0.001).
Furthermore, insignificant decreases in GRed and GPX activities, down to 80% of control values corresponding to 2 µM sanguinarine exposure, were observed (Figures 2C and 2D). A decrease in G6PDH activity by 45%, 50%, and 64% was also noted for 0.5, 1, and 2 µM sanguinarine, respectively, compared to the control (Figure 2E). While treatment with 0.5 µM sanguinarine did not modify GST activity, a dose-dependent decrease in GST activity by 40% and 73% was observed for 1 and 2 µM sanguinarine, respectively (Figure 2F). Finally, as shown in Figure 3, sanguinarine induced a significant increase in MDA content in a dose-dependent manner compared to the control.

Figure 2. Sanguinarine induced a dose-dependent increase in CAT- (A) and SOD- (B) specific activity and decreased the activity of GRed (C), GPX (D), G6PDH (E), and GST (F) in C32 cells treated for 24 h with 0.5, 1, and 2 µM sanguinarine. Activities were calculated as means ± SD (n = 3) and expressed as percentage of control (** P < 0.01 and *** P < 0.001).
3.3. Sanguinarine promoted susceptibility to apoptosis and upregulated HSP expression in C32 amelanotic melanoma cells

As shown in Figures 4A and 4B, p53 expression in cells treated with 2 µM sanguinarine increased by 18% compared to the control. As far as Bax and Bcl-2 expressions are concerned (Figures 4C and 4D), we observed a significantly higher Bax protein level (by 50% for both concentrations; P < 0.001) and 25% and 70% decreases after treatment with 1 and 2 µM sanguinarine, respectively, compared to the control. Densitometric analysis provided Bax/Bcl-2 ratio values (Figure 4E), and these increased compared to the control (1.43 and 3.47 for 1 and 2 µM sanguinarine, respectively). In order to check the cellular responses activated to limit damage induced by the stress caused by sanguinarine, HSPs expression was examined by Western blotting (Figures 5A–5D). This exposure raised Hsp60 expression by similar levels (15%) for both concentrations tested, whereas 2 µM sanguinarine treatment upregulated Hsp90 and Hsp70 expression by 57% and 104%, respectively, compared to the control.

4. Discussion

Recent data suggested that sanguinarine is a very potent inducer of human melanoma (Burgeiro et al., 2013) and human colon cancer (Han et al., 2013) cell death by oxidative stress.

The C32 cell line selected as an in vitro model to assess the cytotoxic activity of pure sanguinarine on human skin melanoma in the present study has been considered a suitable cell culture model to study the therapeutic effects of alkaloids (Photiou et al., 1992; Siwak et al., 2005) and medicinal plants (Loizzo et al., 2007; Conforti et al., 2009). In our study, after 24 h of incubation with 2 µM sanguinarine, cell viability was suppressed by almost half compared to untreated cells, proving that the alkaloid had antiproliferative action. These results were similar to the IC50 value (i.e. the concentration that induces 50% inhibition of cell viability) obtained in a study on B16 melanoma 4A5 cells after 72 h of incubation (De Stefano et al., 2009), suggesting that there could be differences between the time at which antiproliferative efficiency was achieved against amelanotic and melanotic melanoma cells. In order to evaluate the mechanism involved in cell death induction, we analyzed the status of the antioxidant defense system after melanoma cell treatment. The significant increase in CAT and SOD activities of treated cells compared to control suggested that 2 µM sanguinarine-induced ROS were partially counteracted. Previous studies have shown that the induction of superoxide radicals by this alkaloid is indispensable for its ability to trigger apoptosis (Huh et al., 2006). The mitochondrion has been proposed as the most probable source of superoxide radicals due to the discovery of sanguinarine accumulation near the external side of inner mitochondrial membranes. Apparently, positively charged sanguinarine neutralized negative membrane charges, thus cancelling the membrane gradient, inhibiting ATP synthesis, and uncoupling oxidative phosphorylation (Faddeeva and Beliaeva, 1997).

It seems likely that ROS, escaped from SOD and CAT action, attacked polyunsaturated fatty acids from phospholipids, and the lipid peroxidation cascade occurred. Generated MDA could react with G6PDH through a nonenzymatic process causing its inhibition (Ganea and Harding, 2000). Consequently, the decreased G6PDH activity determined a low level of NADPH, which is used as a cofactor in the reaction for GSH regeneration from glutathione disulfide in the reaction catalyzed by GRed. The insignificant decrease in GRed activity could be due to the generation of NADPH in the reactions catalyzed by NADP+-isocitrate dehydrogenase 1, which converts isocitrate to α-ketoglutarate or malic enzyme (Zhang and Du, 2012). Furthermore, the insignificant decrease in GPX activity suggested that the quantity of hydrogen peroxide formed was high, and CAT was the most important factor in its removal. In addition, an important interaction between manganese SOD and GPX-1 was described in human cancer cells (Ekoue and Diamond, 2014). The elevated MDA level compared with the significant decrease in GST-specific activity indicated that this enzymatic activity was involved in MDA detoxification in an inefficient way. Some types of GST play an important role in regulating mitogen-activated protein kinase pathways; the latter are implicated in the cellular responses to apoptosis and proliferation and influence decisions concerning cell fate (Townsend and Tew, 2003).
It is likely that in order to counteract this tendency, cells activate multiple survival pathways in order to establish the cellular homeostasis. In this context, the heat shock proteins are stress induced and interact with different members of the apoptotic cascade to inhibit or to promote cell death (Arya et al., 2007; Lanneau et al., 2008). Elevated levels of Hsp60, 70, and 90 expression after sanguinarine exposure demonstrate that various signals are integrated by these proteins in order to modulate apoptosis in C32 cells. This increase could explain the 50% cell survival after exposure. The upregulated heat shock proteins could be responsible for a block in the apoptotic pathway that enables cells to escape death throughout proteasome-mediated degradation of apoptosis regulatory proteins (Gao et al., 2010).

**Figure 4.** Sanguinarine promoted susceptibility to apoptosis in C32 cells. (A) One representative Western blot analysis was figured for 24 h of treatment with 1 and 2 µM sanguinarine. Densitometric analysis of the immunoblot data for p53 (B), Bax (C), and Bcl-2 (D) proteins and for Bax/Bcl-2 ratio (E). The densities of protein bands were quantified, and the content was normalized against β-actin. The protein expression was calculated as mean ± SD (n = 3) and expressed as percentage of control (** P < 0.01 and *** P < 0.001).
While it is known that Hsp60 can induce cell survival by inhibiting Bax translocation into mitochondria, it is also involved in procaspase-3 maturation, which is essential for cell death (Chandra et al., 2007). Sanguinarine might have induced the dissociation of the Hsp60-Bax complex, resulting in the translocation of cytosolic Hsp60 to the plasma membrane and Bax to the mitochondria, as previously highlighted in the case of hypoxia (Gupta and Knowlton, 2002). Moreover, an Hsp60-based survival program that involves the stabilization of mitochondrial survival and restraint of p53 function (Ghosh et al., 2008) could also be activated during cell exposure to sanguinarine.

In our case, the elevated pro-apoptotic Bax protein expression and the diminished level of Bcl-2 protein expression provided a high Bax/Bcl-2 ratio, which could be relevant for increasing sensitivity of C32 cells to apoptosis after exposure to sanguinarine, as described in the literature (Raisova et al., 2001). It appears that sanguinarine is responsible for inducing cell death by
modulating Bax and Bcl-2 expression, which could further compromise mitochondrial integrity and might, in turn, trigger the release of apoptogenic proteins into the cytosol. Our data revealed that sanguinarine induced oxidative stress and cell death in C32 human amelanotic melanoma cells. The decrease in cellular viability and the increase in Bax/Bcl-2 ratio were evident for 2 µM sanguinarine. Nevertheless, the upregulation of Hsp60, Hsp70, and Hsp90 expression did not effectively counteract the death pathway. Taken together, these findings proved the antigrowth efficiency of sanguinarine against the amelanotic melanoma cells, but further research is needed to obtain more potent antitumor effects of this alkaloid.

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References


