Rapid human melanoma cell death induced by sanguinarine through oxidative stress

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Abstract

Sanguinarine is a natural isoquinoline alkaloid derived from the root of *Sanguinaria canadensis* and from other poppy *fumaria* species, and is known to have a broad spectrum of pharmacological properties. Here we have found that sanguinarine, at low micromolar concentrations, showed a remarkably rapid killing activity against human melanoma cells. Time-lapse videomicroscopy showed rapid morphological changes compatible with an apoptotic cell death, which was further supported by biochemical markers, including caspase activation, poly(ADP-ribose) polymerase (PARP) cleavage and DNA breakdown. Pan-caspase inhibition blocked sanguinarine-induced cell death. Sanguinarine treatment also induced an increase in intracellular calcium concentration, which was inhibited by dantrolene, and cleavage of BAP-31, thus suggesting a putative role for Ca^{2+} release from endoplasmic reticulum and a cross-talk between endoplasmic reticulum and mitochondria in the anti-melanoma action of sanguinarine. Sanguinarine disrupted the mitochondrial transmembrane potential ($\Delta\Psi_m$), released cytochrome *c* and Smac/DIABLO from mitochondria to cytosol, and increased the generation of reactive oxygen species (ROS). Overexpression of Bcl-X_L by gene transfer did not prevent sanguinarine-mediated cell death, ROS generation or release of mitochondrial apoptogenic proteins. However, preincubation with *N*-acetyl-*L*-cysteine (NAC) prevented sanguinarine-induced oxidative stress, PARP cleavage, release of apoptogenic mitochondrial proteins, and cell death. Pretreatment with glutathione (GSH) also inhibited the anti-melanoma activity of sanguinarine. Thus, pretreatment with the thiol antioxidants NAC and GSH abrogated the killing activity of sanguinarine. Taking together, our data indicate that sanguinarine is a very rapid inducer of human melanoma caspase-dependent cell death that is mediated by ROS generation.

1. Introduction

Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium) is a natural isoquinoline alkaloid (Malikova et al., 2006) that can be found in plants of the *Papaveraceae* family, such as *Sanguinaria canadensis*. Sanguinarine has a broad spectrum of pharmacological activities, including antitumor (Faddeeva and Beliaeva, 1997), anti-bacterial (Mitscher et al., 1978), and anti-inflammatory (Lenfeld et al., 1981) actions. Sanguinarine has been reported to induce cell cycle arrest and apoptosis in distinct cancer cells (Adhami et al., 2004; Ahmad et al., 2000; Ahsan et al., 2007; Ding et al., 2002; Holy et al., 2006; Kemeny-Beke et al., 2006; Serafim et al., 2008; Weerasinghe et al., 2001).

Melanoma remains one of the most deadly skin cancers and its incidence has been steadily rising throughout the past decades (Cho and Chiang, 2010). Chemotherapy has had little impact on the survival of patients with metastatic melanoma (Hersey et al., 2009). So far, there is no efficient therapy against melanoma, hence the need of searching for new effective agents. Some previous reports have shown that sanguinarine was effective in treating melanoma in xenograft assays (De Stefano et al., 2009), and acted as a DNA damaging agent and partially affected mitochondrial-mediated processes in mouse melanoma K1735-M2 cell line (Serafim et al., 2008).

Mitochondria play a role in the chemotherapeutic action of a number of molecules that leads to cancer cell death. Mitochondrial damage, which involves permeabilization of the outer mitochondrial membrane, activates a series of events leading to cell death (Caroppi et al., 2009). The activation of the intrinsic apoptotic pathway involves release of cytochrome *c* and other proapoptotic factors from the mitochondrial intermembrane space. In the cytosol, cytochrome *c* binds to the apoptosis protease-activation factor (APAF-1) and forms a complex known as apoptosome, which then recruits and activates procaspase-9, thus triggering a cascade of events leading to apoptosis (Acehan et al., 2002). One of the consequences of mitochondrial dysfunction is excessive reactive oxygen species (ROS) production. Oxygen is initially converted to superoxide anion by electrons that can leak from both complexes I and III in the electron transport system present in mitochondria (Ishii, 2007). Oxidative stress results in damage to the cellular components, including mitochondrial structures, and eventually leads to apoptosis (Ishii, 2007). However, despite mitochondria constitute a major origin of ROS, additional sources have been reported (Vaquero et al., 2004).

Here, we have analyzed the action of sanguinarine on human melanoma cells as well as the underlying mechanisms, with special relevance to the putative role of mitochondria.

The results reported here provide novel insights into the sanguinarine-mediated anti-melanoma activity.

2. Materials and methods

2.1. Reagents

Sanguinarine chloride was from Alexis Biochemicals (Lausen, Switzerland) and prepared in dimethyl sulfoxide (DMSO). Total volume of vehicle (DMSO) in all experiments was lower than 0.2%, and it rendered negligible effects (*data not shown*). Pan-caspase inhibitor z-VAD-fmk was from Alexis Biochemicals (Lausen). Trypan blue, propidium iodide, RNase A, *N*-acetyl-*L*-cysteine (NAC), dantrolene, glutathione (GSH), dihydroethidine (DHE), and BSA were from Sigma (St. Louis, MO). 30% Acrylamide/Bisacrylamide, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine were from Bio-Rad (Hercules, CA). Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes, and ECL Western blotting detection reagents were from GE Healthcare (Princeton, NJ). Fluo-4 AM, Hoechst 33342, 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)], and tetramethyl rhodamine methyl ester (TMRM) were from Molecular Probes (Leiden, The Netherlands). Lipofectamine 2000 reagent was from Invitrogen (Eugene, OR). RPMI-1640, FBS, antibiotics, L-glutamine, NaP, NEAA, 0.25% Trypsin-EDTA were purchased from Gibco (Carlsbad, CA).

2.2. Cell culture

The human melanoma cell lines SK-MEL-2, SK-MEL-5, SK-MEL-28 and MALME-3M were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All these cells were derived from malignant melanoma. However, SK-MEL-2, SK-MEL-5 and MALME-3M cells were also metastatic cell lines, their metastatic sites being the skin of thigh, axillary node and lung, respectively. Cell lines were grown in RPMI-1640 supplemented with 15% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin at 37°C in 5% CO₂ humidified air. For SK-MEL-2, SK-MEL-5 and SK-MEL-28 cell lines, the medium was also supplemented with 1 mM of sodium pyruvate (NaP) and 1% non-essential amino acids (NEAA). Cells were periodically tested for *Mycoplasma* infection and found to be negative.

2.3. Cell viability and cell death analysis

Sanguinarine was incubated at various concentrations with human melanoma cell lines for the indicated times, and then cell viability was determined by using Trypan Blue dye reagent

(Sigma). Non-viable cells stained blue due to Trypan Blue uptake, while viable cells excluded Trypan Blue and showed normal refringent cytoplasm. Cell samples were counted under the light microscope, and the percentage of non-viable cells was determined. Apoptotic cell death was analyzed *in situ* by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique using the Fluorescein Apoptosis Detection System Kit (Promega, Fitchburg, WI), according to the manufacturer's instructions, labeling the 3'-OH ends generated by DNA fragmentation through incorporation of fluorescein-12-dUTP (Cabaner et al., 1999; Gavrieli et al., 1992). Fluorescent cells were visualized and scored with a Zeiss LSM 310 laser scan confocal microscope.

2.4. Western blotting in total cell extracts

Cells at approximately 70% of confluence (about 1.0×10^6) were lysed with 60 μ l of 25 mM Hepes (pH 7.7), 0.3 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β -glycerophosphate and 0.1 mM sodium orthovanadate supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml aprotinin, 20 μ g/ml leupeptin). Fifty micrograms of protein extract were run on SDS-polyacrylamide gels, transferred to hybond ECL nitrocellulose membranes, blocked with 5% (w/v) defatted milk powder in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 60 min at room temperature, and incubated for 1 h at room temperature, or overnight at 4°C, with the following specific antibodies: anti-17 and 19 kDa active caspase-3 rabbit polyclonal antibody (1:750 dilution, Cell Signaling Technology, Beverly, MA); N-15 anti-50 kDa caspase-4 goat polyclonal antibody, that also detects the active cleaved p20 subunit (1:250 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); anti-35 kDa caspase-7 mouse monoclonal antibody, that also recognizes the cleaved p11, p20 and p32 subunits (1:750 dilution, BD Biosciences, San Jose, CA); 1C12 anti-57 kDa caspase-8 mouse monoclonal antibody, that also detects the cleaved p43 and p18 subunits (1:500 dilution, Cell Signaling Technology); anti-47 kDa caspase-9 rabbit polyclonal antibody, that also recognizes the cleaved p10, p17 and p38 subunits (1:250 dilution, Calbiochem, Gibbstown, NJ); C2.10 anti-116 kDa poly(ADP-ribose) polymerase (PARP) mouse monoclonal antibody, that also detects the 85 kDa cleavage product (1:750 dilution, BD Biosciences); anti-58 kDa caspase-10 rabbit polyclonal antibody, that also recognizes the p17 and p23 subunits (1:50 dilution, Calbiochem); C-15 anti-31 kDa BAP-31 goat polyclonal antibody, that also detects the 20 kDa cleavage product (1:500 dilution, Santa Cruz Biotechnology); AC-15 anti-42 kDa β -actin mouse monoclonal antibody (1:5,000 dilution, Sigma). Secondary antibodies were anti-mouse biotinylated (GE

Healthcare, Princeton, NJ); anti-rabbit biotinylated (GE Healthcare) and anti-goat horseradish peroxidase (Santa Cruz Biotechnology). When secondary biotinylated antibodies were used, membranes were subsequently incubated with streptavidin-horseradish peroxidase conjugate (GE Healthcare). Signals were detected using an ECL kit (GE Healthcare).

2.5. Analysis of mitochondrial protein release

Release of apoptogenic proteins from mitochondria to cytosol was analyzed from 3×10^6 cells by Western blot as previously described (Gajate et al., 2003), using the following specific antibodies: anti-15 kDa cytochrome *c* mouse monoclonal antibody (1:500 dilution, BD Biosciences); anti-22 kDa Smac/DIABLO mouse monoclonal antibody (1:500 dilution, BD Biosciences); AC-15 anti-42 kDa β -actin monoclonal antibody (1:5,000, Sigma). Blots were then incubated with biotinylated anti-mouse immunoglobulin antibodies (GE Healthcare), followed by incubation with streptavidin-horseradish peroxidase conjugate (GE Healthcare). Signals were developed using an ECL kit (GE Healthcare).

2.6. Cytofluorimetric analysis of intracellular calcium and mitochondrial transmembrane potential

To evaluate changes in intracellular calcium concentration, cells were incubated with or without 3 μ M Sanguinarine for 2 h, and 30 min before the end of the treatment, 2 μ M Fluo-4 AM (*green* fluorescence) was used to measure intracellular calcium. Cells were then washed with PBS, harvested and analyzed on a flow cytometer FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). To evaluate changes in mitochondrial transmembrane potential ($\Delta\Psi_m$), cells were incubated with or without 3 μ M Sanguinarine for 2 h, and 30 min before the end of the treatment 100 nM tetramethyl rhodamine methyl ester (TMRM) (*red* fluorescence) (Molecular Probes) was added to measure mitochondrial membrane potential ($\Delta\Psi_m$). Cells were then washed with PBS, harvested and analyzed on a flow cytometer FACSCalibur (Becton Dickinson). In addition, mitochondrial transmembrane potential ($\Delta\Psi_m$) was also examined by incubating cells (10^6 /ml) in PBS with 20 nM 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] (*green* fluorescence) (Molecular Probes) for 20 min at 37°C, followed by analysis on a flow cytometer FACSCalibur (Becton Dickinson).

2.7. Measurement of reactive oxygen species (ROS) generation by flow cytometry

To evaluate the generation of ROS, cells were incubated in PBS with 2 μ M dihydroethidine (DHE) (*red* fluorescence after oxidation) for 40 min at 37°C, followed by analysis on a FACScalibur flow cytometer as previously described (Gajate et al., 2000).

2.8. Confocal microscopy

SK-MEL-2 cells in glass-bottom dishes were incubated with 3 μ M sanguinarine for 2 h. Then, cells were double labeled with Fluo-4 AM (2 μ M) and Hoechst 33342 (2 μ g/ml), added to the cell culture 1 h and 30 min, respectively, or with DHE (2 μ M) and Hoechst 33342 (2 μ g/ml), added to the cell culture 40 and 30 min, respectively, before the end of drug treatment. Then, cells were analyzed by confocal microscopy using a Leica laser scanning confocal microscope (Bannockburn, IL). Fluo-4 AM signal was acquired using an air-cooled argon laser. DHE signal was acquired using a green He-Ne laser. Hoechst 33342 signal was obtained by using a violet diode laser. The antifading reagent SlowFade[®] Gold (Invitrogen, Eugene, OR) was used to preserve fluorescence signal intensity.

2.9. Overexpression of *bcl-x_L* by stable transfection

SK-MEL-2 cells were transfected with 8 μ g of pSFFV-Neo expression vector containing the human *bcl-x_L* open reading frame, driven by the long terminal repeat of the splenic focus-forming virus (pSFFV-Bcl-X_L) (Mollinedo et al., 1997), by using Lipofectamine reagent (Invitrogen, Eurogene, OR) and following the manufacturer's instructions. As a control, transfection was performed with empty pSFFV-Neo plasmid. Transfectants were selected by growth in complete culture medium supplemented with 800 μ g/ml G418 (Sigma), and then grown in the presence of 250 μ g/ml G418. Stable transfectants were achieved after about 30 days. The different clones were isolated and monitored for Bcl-X_L expression by Western blot, using the anti-17 and -26 kDa Bcl-X rabbit polyclonal antibody (1:250 dilution, BD Biosciences).

2.10. Statistical analysis

Data are expressed as means $\pm$ SD of the number of experiments indicated in the figure legends. Multiple comparisons were performed using one-way analysis of variance followed by a Bonferroni post hoc test. Single comparisons between two experimental groups were made by using Student's *t*-test. Significance was accepted with *p* value < 0.05.

3. Results

3.1. Sanguinarine induces a rapid killing of human melanoma cells

We found that sanguinarine induced a rapid killing of SK-MEL-2 melanoma cancer cells when incubated at low micromolar concentration for only 2 h incubation, as assessed by Trypan blue staining (Fig. 1A). A potent melanoma cell-killing activity was evident at 3 μ M sanguinarine when incubated for either 2 or 24 h (Fig. 1A). The tumor killing activity of sanguinarine was increased with drug concentration and incubation time, but sanguinarine induced a remarkable and strong antitumor activity at early incubation times (Fig. 1A). Furthermore, 3 μ M sanguinarine induced a decrease in cell viability following 2 h incubation in a number of human melanoma cell lines, including SK-MEL-2 (47.4 ± 8.0 % cell death), SK-MEL-5 (21.2 ± 1.5 % cell death), SK-MEL-28 (15.6 ± 1.5 % cell death) and MALME-3M (28.1 ± 1.7 % cell death), as assessed by Trypan blue staining (Fig. 1B). Treatment of SK-MEL-2 cells with 3 μ M sanguinarine induced an increase in non-viable cells at very early incubation times in a time-dependent manner (Fig. 1C), which was also confirmed by morphological changes (Fig. 1D). Time-lapse videomicroscopy of untreated (Supplementary Video 1) and 3 μ M sanguinarine-treated (Supplementary Video 2) SK-MEL-2 melanoma cells showed a remarkably rapid killing of melanoma cells by sanguinarine, which displayed the typical morphological changes of apoptosis (cell shrinkage, rounding up, blebbing). The onset of this sanguinarine-induced killing of melanoma cells was achieved after only 25 min of drug incubation (Supplementary Video 2). The induction of sanguinarine-induced cell death by an apoptosis-like process in melanoma cells was further supported by TUNEL assay (data not shown).

3.2. Involvement of caspase activation in sanguinarine-induced melanoma cell killing

Because SK-MEL-2 cells were the most sensitive melanoma cells to sanguinarine treatment (Fig. 1B), this cell line was used in the following experiments to analyze the underlying mechanism involved in sanguinarine-mediated melanoma cell killing. Sanguinarine treatment of SK-MEL-2 cells induced a rapid activation of several caspases, including initiator caspases 8, 10, 9 and 4, as well as executioner caspases 3 and 7 (Fig. 2A). Cleavage of the caspase-3/7 substrate PARP, a nuclear enzyme involved in DNA repair and programmed cell death, was also rapidly detected (Fig. 2A). Pre-incubation of melanoma cells with the pan-caspase inhibitor z-VAD-fmk prevented cell killing induced by 3 μ M sanguinarine after treatment for either 2 or 24 h incubation (Fig. 2B). These results suggest that caspase activation is required for sanguinarine-induced melanoma cell killing, and that several caspases are triggered by sanguinarine in a similar time frame.

3.3. Sanguinarine increases intracellular calcium and induces BAP-31 cleavage in melanoma cells

We found that sanguinarine treatment caused an increase in intracellular calcium concentration in SK-MEL-2 cells, as detected by Fluo-4 AM and analyzed by both confocal microscopy (Fig. 3A) and flow cytometry (Fig. 3B). In addition, sanguinarine induced cleavage of BAP-31 (Fig. 3C), an endoplasmic reticulum membrane protein that is a caspase-8 substrate (Ng et al., 1997). The p20-BAP31 cleavage product has been shown to direct proapoptotic signals between endoplasmic reticulum and mitochondria (Breckenridge et al., 2003). Interestingly, incubation of SK-MEL-2 melanoma cells with dantrolene significantly inhibited the killing activity of sanguinarine (Fig. 3D). Dantrolene prevents excessive Ca^{2+} release from the endoplasmic reticulum by inhibiting ryanodine receptors (Inan and Wei, 2010; Zhao et al., 2001). These data suggest that Ca^{2+} release endoplasmic reticulum might play a role in the action of sanguinarine on melanoma cells. Our data suggest that sanguinarine might induce some endoplasmic reticulum-mediated responses that could affect mitochondria.

3.4. Sanguinarine induces ROS generation, $\Delta\Psi_m$ loss and release of mitochondrial apoptogenic proteins

Next, we analyzed the effect of sanguinarine on mitochondrial-mediated cell death. Sanguinarine (3 μM , 2 h incubation) induced a potent and rapid generation of ROS in SK-MEL-2 cells, as assessed by dihydroethidine (DHE) fluorescence using flow cytometry (Fig. 4A), and confocal microscopy (Fig. 4B). Sanguinarine also promoted $\Delta\Psi_m$ loss in SK-MEL-2 melanoma cells in a concentration-dependent manner, as assessed by measurements of DiOC₆(3) fluorescence (green fluorescence), a cationic probe that accumulates into mitochondria as a function of its potential (Fig. 4C). The increase in the percentage of melanoma cells with low $\Delta\Psi_m$ following sanguinarine treatment paralleled its cell-killing activity (cf. Figs. 1A and 4C). Sanguinarine also promoted a dramatic $\Delta\Psi_m$ loss in melanoma cells, as assessed by TMRM fluorescence (*red* fluorescence) to measure $\Delta\Psi_m$ (Fig. 4D), and the release of mitochondrial cytochrome *c* and Smac/DIABLO to the cytosol after only 30-min treatment (Fig. 4E). However, cytochrome oxidase subunit II, used as a marker of mitochondrial membrane, remained in the mitochondrial extract and was not released to the cytosol (*data not shown*). No alterations in ATP/ADP ratio or energy charge were detected after 30 min-, 1 h- and 2 h-drug treatment (*data not shown*). These results suggest that

sanguinarine exerts a rapid effect on mitochondrial integrity, leading to depolarization and release of proapoptotic proteins.

3.5. Sanguinarine-mediated cell death is not prevented by Bcl-X_L overexpression

Because Bcl-X_L preserves mitochondrial integrity, abrogating cytochrome *c* release, $\Delta\Psi_m$ loss and apoptosis (Gajate and Mollinedo, 2007; Gajate et al., 2000), we overexpressed Bcl-X_L in SK-MEL-2 cells to examine the role of mitochondria in sanguinarine-induced cell death. SK-MEL-2 cells were stably transfected with pSFFV-*bcl-x_L* (SK-MEL-2-Bcl-X_L), containing the human *bcl-x_L* open reading frame, or with control pSFFV-Neo plasmid (SK-MEL-2-Neo). SK-MEL-2-Neo cells behaved as non-transfected SK-MEL-2 cells in all parameters studied. Western blot analysis showed a high expression of Bcl-X_L in SK-MEL-2-Bcl-X_L cells as compared to the low Bcl-X_L endogenous level of SK-MEL-2-Neo cells (Fig. 5A, *inset*). Interestingly, while Bcl-X_L overexpression inhibited cell death induced by a number of proapoptotic agents, including edelfosine and berberine (*data not shown*), as previously reported (Burgeiro et al., 2011; Gajate and Mollinedo, 2007), it did not protect against cell death, PARP degradation, ROS generation or the release of mitochondrial cytochrome *c* and Smac/DIABLO to cytosol induced by sanguinarine (Fig. 5, A-D). These data show that the action of sanguinarine on mitochondria is not inhibited by Bcl-X_L overexpression.

3.6. N-Acetyl-L-cysteine and glutathione (GSH) protect melanoma cells against sanguinarine-induced cell death

Pretreatment of SK-MEL-2 cells with the antioxidant N-acetyl-L-cysteine (NAC) prevented sanguinarine-induced cell death (Fig. 6A) and PARP cleavage (Fig. 6B). NAC pretreatment also blocked oxidative stress (Fig. 6C), and release of mitochondrial cytochrome *c* and Smac/DIABLO to the cytosol (Fig. 6D). Because NAC is known to induce intracellular glutathione (GSH) levels by acting as a precursor for the synthesis of GSH (Duyndam et al., 2001; Menon et al., 2005), we also examined the action of GSH on the cell killing activity of sanguinarine. As shown in Fig. 7, we found that GSH inhibited significantly the anti-melanoma activity of sanguinarine when incubated for either 2 or 24 h. Thus, pretreatment with the thiol antioxidants NAC and GSH abrogated the cell-killing activity of sanguinarine. These results suggest the involvement of ROS generation in the rapid action of sanguinarine on mitochondria and cell killing.

4. Discussion

Here, we report that sanguinarine induces an extremely rapid caspase- and ROS-dependent cell killing in human melanoma cells. Time-lapse videomicroscopy showed the rapid shrinkage, rounding up and blebbing of melanoma cells following sanguinarine treatment. These morphological changes, together with a positive TUNEL reaction, suggest the induction of sanguinarine-induced cell death by an apoptosis-like process in melanoma cells. The activation of different caspases upon sanguinarine addition suggests the triggering of distinct signaling pathways. The rapid activation of caspases-4 and -9 suggests the involvement of endoplasmic reticulum and mitochondria in the antitumor action of sanguinarine.

Adenoviral expression of the p20 cleavage fragment from the endoplasmic reticulum membrane protein BAP-31 has been reported to cause an early release of Ca^{2+} from the endoplasmic reticulum, concomitant Ca^{2+} uptake by mitochondria, and mitochondrial recruitment of Drp1, a dynamin-related protein that mediates scission of the outer mitochondrial membrane, resulting in fission of the mitochondrial network (Breckenridge et al., 2003). Generation of the p20-BAP-31 fragment was detected after 2 h sanguinarine incubation, coincident with caspase-8 activation. Sanguinarine also caused an increase in cytosolic $[\text{Ca}^{2+}]$. Elevation of cytosolic $[\text{Ca}^{2+}]$ can result from Ca^{2+} -influx from the extracellular space or from Ca^{2+} -release from intracellular stores, especially from endoplasmic reticulum (Bergner and Huber, 2008). The inhibition of sanguinarine-induced cell killing by dantrolene, a ryanodine receptor inhibitor that prevents Ca^{2+} release from the endoplasmic reticulum (Inan and Wei, 2010; Zhao et al., 2001), further supports a role of endoplasmic reticulum in the anti-melanoma action of sanguinarine. The increase in cytoplasmic $[\text{Ca}^{2+}]$ following sanguinarine treatment might originate, at least in part, from extracellular Ca^{2+} , as sanguinarine-induced cardiac muscle contracture has been shown to be inhibited by chelation of extracellular Ca^{2+} or addition of the Ca^{2+} antagonist La^{3+} (Hu et al., 2005). In this regard, it is worthy to note that the process known as calcium-induced calcium release (CICR), by which calcium is able to activate calcium release from intracellular Ca^{2+} stores (e.g.: endoplasmic reticulum), is primarily mediated by the ryanodine receptor and Ca^{2+} release from the endoplasmic reticulum (Islam et al., 1992; Verkhratsky and Shmigol, 1996). In addition, we have found that pretreatment of SK-MEL-2 cells with the extracellular calcium chelator EGTA partially reduces the killing activity of sanguinarine (*data not shown*).

Previous studies showed that sanguinarine treatment increased cytosolic levels of cytochrome *c*, and active forms of caspases-3, -7, -8 and -9, leading to PARP cleavage and apoptotic cell death in immortalized human HaCaT keratinocytes (Adhami et al., 2003). Our

data show that sanguinarine compromised mitochondrial integrity, resulting in mitochondrial depolarization and release of apoptogenic proteins. However, overexpression of Bcl-X_L, an antiapoptotic protein that mediates its effects through heterodimerization with the BH3 region of proapoptotic molecules, did not prevent sanguinarine-induced cell death, PARP cleavage, release of mitochondrial apoptogenic proteins, or oxidative stress response. It has been reported that sanguinarine, in addition to be effective in displacing bound BH3 peptides, binds at the BH1 region of Bcl-X_L, thus sanguinarine might antagonize other pro-survival mechanisms in addition to the one that involves BH3 region binding (Zhang et al., 2006). In this regard, a number of reports indicate that sanguinarine downregulates Bcl-2, thus altering the Bcl-2/Bax ratio in different tumor cells (Han et al., 2008; Kim et al., 2008; Lee et al., 2012; Tsukamoto et al., 2011). Furthermore, recent evidence suggests that the anti-proliferative activity of sanguinarine in malignant melanoma cells is independent of their p53 status (Hammerova et al., 2011).

Sanguinarine treatment has been reported to induce apoptosis in PC3 human prostatic adenocarcinoma cells through an early and severe glutathione (GSH)-depleting effect (Debiton et al., 2003). GSH is an endogenous antioxidant (Dodd et al., 2008) that acts as a redox buffer to preserve the reduced intracellular environment (Fraternale et al., 2006). Our present results show that sanguinarine induces a significant increase in oxidative stress in SK-MEL-2 cells, and the thiol antioxidant NAC, which increases GSH level (Duyndam et al., 2001; Menon et al., 2005), prevents an increase in ROS level, the release of mitochondrial apoptogenic proteins to the cytosol, as well as cell death. In addition, we report here that GSH inhibits significantly the anti-melanoma activity of sanguinarine. Taken together, our data indicate that the oxidative stress triggered in melanoma cells by sanguinarine underlies the subsequent mitochondrial depolarization and release of mitochondrial apoptogenic proteins.

By using sanguinarine self-fluorescence, nuclear accumulation of this alkaloid in mouse melanoma K1735-M2 cells has been observed (Serafim et al., 2008). Mitochondrial dysfunction was associated with nuclear DNA damage, and p53 was translocated to mitochondria upon sanguinarine treatment of mouse melanoma cells (Serafim et al., 2008). However, sanguinarine has also been reported to cause oxidative DNA damage and rapid apoptosis, that is not mediated by p53-dependent DNA damage signaling (Matkar et al., 2008a; b). Here, we have found that human melanoma cells undergo a very rapid cell death response, which involves oxidative stress, following sanguinarine treatment. Thus, the

induction of a potent oxidative response seems to be critical for the anti-melanoma action of sanguinarine.

The results reported here indicate that ROS generation, which in turn leads to mitochondrial dysfunction, is critical in sanguinarine-induced cell death in human melanoma cells. The rapid antitumor action of sanguinarine suggests that this drug could have a potential pharmaceutical application for the treatment of hyperproliferative skin disorders, including melanoma.

5. Conclusions

The data reported here show that sanguinarine induces a rapid caspase-dependent cell death in human melanoma cells, partially involving endoplasmic reticulum- and mitochondria-mediated responses. This cell death is dependent on the generation of reactive oxygen species, and is not prevented by Bcl-X_L overexpression. The fact that sanguinarine induces a very rapid cell death with apoptotic features in melanoma cells, together with the lack of inhibition by overexpressing Bcl-X_L, highlight the potent anti-melanoma activity of this isoquinoline alkaloid and suggest its potential in the treatment of skin cancer.

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FIGURE LEGENDS

Fig. 1. Decrease of cell viability by sanguinarine in human melanoma cells. (A) SK-MEL-2 cells were treated with 1, 2, 3, 4, 5 or 10 μ M sanguinarine (*Sang*) for 2 and 24 h, and non-viable cells were counted using the Trypan Blue Dye Exclusion assay. Untreated control cells (C) were run in parallel. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control 2 h; # $p < 0.05$ vs. untreated control 24 h, one-way ANOVA test, Bonferroni post-test. (B) Different melanoma cell lines were treated with 3 μ M sanguinarine (*Sang*) for 2 h, and non-viable cells were counted using the Trypan Blue Dye Exclusion assay. Untreated control cells (C) were run in parallel. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control, Student's t test. (C) Time-course of the decrease in cell viability induced by 3 μ M sanguinarine in SK-MEL-2 cells. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control, one-way ANOVA test, Bonferroni post-test. (D) Representative images of sanguinarine-treated SK-MEL-2 cells for the indicated times. These images were extracted from time-lapse videomicroscopy recordings, in which untreated control SK-MEL-2 cells or SK-MEL-2 cells treated with 3 μ M sanguinarine were recorded for 2 h. The images were captured by using differential interference contrast (DIC). Bar, 50 μ m.

Fig. 2. Caspase involvement in the cell-killing activity of sanguinarine on SK-MEL-2 cells. (A) Cells untreated (C) or treated with 3 μ M sanguinarine for the indicated times were analyzed by Western blot using specific antibodies. The migration positions of full-length PARP (116 kDa), and its cleavage product p85, as well as the cleavage products of several caspases are indicated. β -actin was used as a loading control in Western blot analyses. Blots are representative of two separate experiments performed. (B) SK-MEL-2 cells were preincubated with or without 50 μ M of z-VAD-fmk for 1 h, and then incubated in the presence or absence of 3 μ M sanguinarine (*Sang*) for 2 and 24 h. Then dead cells were counted using Trypan Blue Dye Exclusion method. Untreated control cells were run in parallel. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control 2 h; Φ $p < 0.05$ vs. *Sang* 2 h; # $p < 0.05$ vs. untreated control 24 h; θ $p < 0.05$ vs. *Sang* 24 h, one-way ANOVA test, Bonferroni post-test.

Fig. 3. Sanguinarine raises intracellular free calcium concentration in SK-MEL-2 cells. (A) Cells were incubated with 3 μ M sanguinarine (*Sang*) for 2 h and then Fluo-4 AM (green

fluorescence) was used to visualize intracellular calcium. Hoechst 33342 stained nuclear DNA (*blue* fluorescence). Control image bar, 20 μm ; sanguinarine image bar, 10 μm . (B) Cells were incubated with 3 μM sanguinarine (*Sang*) for 2 h, and then Fluo-4 AM (*green* fluorescence) was used to measure intracellular calcium by flow cytometry. Untreated control cells were run in parallel. Data shown are mean $\pm$ SD of five independent experiments. * $p < 0.05$ vs. untreated control, student's t test. (C) SK-MEL-2 cells were untreated (C) or treated with 3 μM sanguinarine for the indicated times and analyzed by immunoblotting with anti-BAP-31 antibody (SDS-15% polyacrylamide gels). The migration position of its cleavage product p20 is indicated. Immunoblotting for β -actin was used as an internal control for equal protein loading in each lane. Blots are representative of two independent experiments performed. (D) SK-MEL-2 cells were preincubated with or without 10 μM dantrolene for 1 h, and then incubated in the presence or absence of 3 μM sanguinarine (*Sang*) for 2 h and 24 h. Then dead cells were counted using Trypan Blue Dye Exclusion method. Untreated control cells were run in parallel. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control 2 h; Φ $p < 0.05$ vs. *Sang* 2 h; # $p < 0.05$ vs. untreated control 24 h; θ $p < 0.05$ vs. *Sang* 24 h, one-way ANOVA test, Bonferroni post-test.

Fig. 4. Sanguinarine induces mitochondrial dysfunction and ROS generation in SK-MEL-2 cells. (A) Cells were incubated with 3 μM sanguinarine (*Sang*) for 2 h and then DHE was used to detect ROS production. Untreated control cells were run in parallel. Data shown are mean $\pm$ SD of four independent experiments. * $p < 0.05$ vs. untreated control, Student's t test. (B) Cells were incubated in the absence (*Control*) and in the presence of 3 μM sanguinarine (*Sang*), and then DHE (*red* fluorescence) was used to visualize oxygen radicals by confocal microscopy. DHE is oxidized into fluorescent ethidium by the superoxide ion. Hoechst 33342 stained nuclear DNA (*blue* fluorescence). Bar, 25 μm . (C) SK-MEL-2 cells were treated with 1, 2, 3, 4, 5 or 10 μM sanguinarine (*Sang*) for 2 and 24 h, and then DiOC₆(3) (*green* fluorescence) was used to measure the percentage of cells with high and low mitochondrial membrane potential ($\Delta\Psi_m$) by flow cytometry. Untreated control cells were run in parallel. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control 2 h; # $p < 0.05$ vs. untreated control 24 h, one-way ANOVA test, Bonferroni post-test. (D) Cells were incubated without or with 3 μM of sanguinarine for 2 h and then TMRM (*red* fluorescence) was used to measure mitochondrial membrane potential ($\Delta\Psi_m$) by flow cytometry. Untreated control cells were run in parallel. Data shown are mean $\pm$ SD of four independent experiments. * $p < 0.05$ vs. untreated control, Student's t test. (E) SK-MEL-

2 cells treated with 3 μ M sanguinarine for the indicated times, and cytosolic proteins were analyzed by immunoblotting with anti-cytochrome *c* (Cyt *c*) and Smac/DIABLO antibodies. β -actin serves as an internal control for equal protein loading in each lane. Data shown are representative of two experiments performed.

Fig. 5. Bcl-X_L overexpression does not prevent sanguinarine-induced decrease in cell viability, ROS generation and release of mitochondrial apoptogenic proteins in SK-MEL-2 cells. (A) *Neo* and *Bcl-X_L* transfected cells were untreated (C) or treated with 3 μ M sanguinarine (*Sang*) for 2 h and assayed for cell viability by the Trypan Blue Dye Exclusion assay. Data shown are mean $\pm$ SD of five independent experiments. * $p < 0.05$ vs. untreated control *Neo*; # $p < 0.05$ vs. untreated control *Bcl-X_L*, one-way ANOVA test, Bonferroni post-test. *Inset*, Bcl-X_L protein expression in SK-MEL-2-*Neo* and SK-MEL-2-*Bcl-X_L* cells was analyzed by SDS-PAGE of 40 μ g cell extract protein and immunoblotting. β -actin was used as a control for protein loading. (B) *Neo* and *Bcl-X_L* transfected cells were untreated (C) or treated with 3 μ M sanguinarine (*Sang*) for 2 h and assayed for PARP cleavage. The migration positions of full-length PARP (116 kDa) and its cleavage product p85 are indicated. Immunoblotting for β -actin was used as an internal control for equal protein loading in each lane. Blots are representative of two separated experiments. (C) Bcl-X_L overexpression did not prevent sanguinarine-induced ROS production. SK-MEL-2 cells (*Neo*) and Bcl-X_L-transfected SK-MEL-2 cells (*Bcl-X_L*) were treated with 3 μ M sanguinarine (*Sang*) for 2 h, and then analyzed for ROS generation (using DHE). Untreated control cells (control) were run in parallel. Data shown are mean $\pm$ SD of four independent experiments. * $p < 0.05$ vs. untreated control *Neo*; # $p < 0.05$ vs. untreated control *Bcl-X_L*; + $p < 0.05$ vs. *Sang Neo*, one-way ANOVA test, Bonferroni post-test. (D) Bcl-X_L overexpression did not prevent sanguinarine-induced release of apoptogenic proteins from mitochondria. *Neo* and *Bcl-X_L* transfected cells were untreated (C) or treated with 3 μ M sanguinarine (*Sang*) for 2 h and cytosolic fractions were analyzed by immunoblotting with anti-cytochrome *c* (Cyt *c*) and Smac/DIABLO antibodies. β -actin serves as an internal control for equal protein loading in each lane. Data shown are representative of two separated experiments.

Fig. 6. Involvement of ROS generation in the antitumor action of sanguinarine in SK-MEL-2 cells. (A) SK-MEL-2 cells were preincubated without or with 10 mM NAC for 30 min, and then incubated in the presence or absence of 3 μ M sanguinarine (*Sang*) for 1 h, and dead cells were determined using Trypan Blue. Untreated control cells (C) were run in

parallel. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control; # $p < 0.05$ vs. *Sang*, one-way ANOVA test, Bonferroni post-test. (B) SK-MEL-2 cells were preincubated with or without 10 mM NAC for 30 min, and then incubated in the presence or absence of 3 μ M sanguinarine (*Sang*) for 2 h and analyzed by immunoblotting with anti-PARP antibody (SDS-8% polyacrylamide gels). The migration positions of full-length PARP (116 kDa) and its cleavage product p85 are indicated. Immunoblotting for β -actin was used as an internal control for equal protein loading in each lane. Blots are representative of two experiments performed. (C) Cells were preincubated with or without 10 mM NAC for 30 min, and then incubated in the presence or absence of 3 μ M sanguinarine (*Sang*) for 2 h. Then ROS production was measured by flow cytometry. Data shown are mean $\pm$ SD of four independent experiments. * $p < 0.05$ vs. untreated control; # $p < 0.05$ vs. *Sang*, one-way ANOVA test, Bonferroni post-test. (D) Cells were preincubated with or without 10 mM NAC for 30 min, and then incubated in the presence or absence of 3 μ M sanguinarine (*Sang*) for 2 h. Cytosolic proteins were analyzed by immunoblotting anti-cytochrome *c* (Cyt *c*) and Smac/DIABLO antibodies. β -actin serves as an internal control for equal protein loading in each lane. Untreated control cells (C) were run in parallel in A-D. Data shown are representative of two experiments performed.

Fig. 7. Glutathione preincubation inhibits the sanguinarine-induced decrease in cell viability of SK-MEL-2 melanoma cells. SK-MEL-2 cells were preincubated with or without 10 mM GSH for 1 h, and then incubated in the presence or absence of 3 μ M sanguinarine (*Sang*) for 2 and 24 h. Then non-viable cells were counted using Trypan Blue Dye Exclusion method. Untreated control cells were run in parallel. Data shown are mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. untreated control 2 h; Φ $p < 0.05$ vs. *Sang* 2 h; # $p < 0.05$ vs. untreated control 24 h; θ $p < 0.05$ vs. *Sang* 24 h, one-way ANOVA test, Bonferroni post-test.

SUPPLEMENTARY MATERIAL (VIDEOS)

Supplementary Video 1. Time-lapse videomicroscopy of untreated control SK-MEL-2 cells, grown in complete RPMI-1640 culture medium supplemented with 15% heat-inactivated FBS. Recording time: 3 h. Magnification: 20x.

Supplementary Video 2. Time-lapse videomicroscopy of the effect of sanguinarine on SK-MEL-2 cells. Film recording started immediately after addition of 3 μ M sanguinarine on SK-MEL-2 cells grown in complete RPMI-1640 culture medium supplemented with 15% heat-inactivated FBS. Recording time: 3 h. Magnification: 20x.

Figure 1.
Burgeiro et al.

Figure 2.
Burgeiro et al.

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Figure 3.
Burgeiro et al.

Figure 4.
Burgeiro et al.

Figure 5.
Burgeiro et al.

Figure 6.
Burgeiro et al.

Figure 7.
Burgeiro et al.