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► **To cite this version:**

Ilaria de Stefano, Giuseppina Raspaglio, Gian Franco Zannoni, Daniele Travaglia, Maria Grazia Prisco, et al.. Antiproliferative And Antiangiogenic Effects Of The Benzophenanthridine Alkaloid Sanguinarine In Melanoma. *Biochemical Pharmacology*, 2009, 78 (11), pp.1374. 10.1016/j.bcp.2009.07.011 . hal-00524466

HAL Id: hal-00524466

<https://hal.science/hal-00524466>

Submitted on 8 Oct 2010

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Accepted Manuscript

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PII: S0006-2952(09)00643-1
DOI: doi:10.1016/j.bcp.2009.07.011
Reference: BCP 10268

To appear in: *BCP*

Received date: 8-6-2009
Revised date: 16-7-2009
Accepted date: 17-7-2009

Please cite this article as: De Stefano I, Raspaglio G, Zannoni GF, Travaglia D, Prisco MG, Mosca M, Ferlini C, Scambia G, Gallo D, Antiproliferative And Antiangiogenic Effects Of The Benzophenanthridine Alkaloid Sanguinarine In Melanoma, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.07.011

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ANTIPROLIFERATIVE AND ANTIANGIOGENIC EFFECTS OF THE
BENZOPHENANTHRIDINE ALKALOID SANGUINARINE IN MELANOMA

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Running title: Sanguinarine in melanoma

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ABSTRACT

1
2 This study was aimed at evaluating the potential application of benzophenanthridine
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4 alkaloids, sanguinarine and chelitrine, in the therapy of melanoma cancer. *In vitro*
5
6 antiproliferative activity of sanguinarine was higher than that of chelitrine against the B16
7
8 melanoma 4A5 cells. Both agents were able to produce DNA breaks, and the DNA unwinding
9
10 assay showed that they act as DNA intercalating agents. Sanguinarine was selected for
11
12 determination of its *in vivo* preclinical efficacy. Oral treatment with sanguinarine reduced the
13
14 tumor burden in a transplantable murine tumor grown in a syngenic host (B16 melanoma 4A5
15
16 in C57BL/6 mice), and in a human tumor xenograft grown in immunodeficient mice (A375
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18 human melanoma in athymic nude mice). In A375 tumors a significant decrease in the
19
20 proliferation marker Ki67, and a reduction in the activated mitogen-activated protein kinases
21
22 (p-p44/42 MAPK), and in protein kinase B (pAKT) were also observed. Three out of eleven
23
24 A375-bearing treated mice were tumor-free at the end of treatment, and did not develop any
25
26 tumor after a further, treatment-free, observation period of 60 days. Sanguinarine also showed
27
28 a striking anti-angiogenic activity in mice. Data from the present study support the concept
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30 that sanguinarine can be effective in melanoma skin cancer.
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44 Key words: sanguinarine, melanoma, angiogenesis, MAPK, mice
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1. INTRODUCTION

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2 Malignant melanoma is an aggressive, therapy-resistant malignancy of melanocytes. The
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4 incidence of melanoma has been steadily increasing worldwide, resulting in an increasing
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6 public health problem. In Europe, 26 368 males and 35 909 females are diagnosed each year
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8 with melanoma, and around 8 500 males and 8 000 females die because of it [1]. In the United
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10 States, about 62 480 cases of melanoma were expected to be diagnosed in 2008, with about 8
11
12 420 Americans dead from this cancer [2]. As with most malignant disorders, prognosis is
13
14 highly dependent on the clinical stage (extent of tumor burden) at the time of diagnosis, and
15
16 the patient's clinical stage at diagnosis dictates selection of therapy. If melanoma is diagnosed
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18 early it can be cured by surgical resection, and about 80% of cases undergo this treatment.
19
20 However, metastatic malignant melanoma is largely refractory to existing therapies, and has a
21
22 poor prognosis, with a median survival rate of 6 months, and a 5-year survival rate of less
23
24 than 5% [3]. There are several approved postoperative adjuvant therapies for malignant
25
26 melanoma [3, and references therein]. Interferon- α (IFN- α) is the most commonly used
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28 adjuvant immunotherapy for advanced melanoma, although its efficacy is still a matter of
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30 debate. High-dose interleukin-2 (IL-2) has also been approved, but response rates are low, and
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32 toxicity is a problem. Dacarbazine (DTIC) is the reference approved chemotherapeutic agent
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34 for the treatment of advanced melanoma, and drugs such as carmustine (BiCNU), paclitaxel
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36 (Taxol), temozolomide and cisplatin have shown single-agent activity in metastatic disease
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38 [4]. In addition, many different immunotherapies have been tested, but so far none of these
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40 approaches has reached regulatory approval [5]. Overall, despite decades of investigation, no
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42 systemic treatment that improves overall survival in patients with advanced metastatic
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44 melanoma has been developed, and new treatment options are urgently needed.
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Natural products have long been a fertile source of cure for cancer, with plant-derived drugs becoming increasingly explored and integrated into chemotherapy strategies; there are at least

1 250,000 species of plants, out of which more than one thousand have been found to possess
2 significant anticancer properties [6]. Recently, the benzophenanthridine alkaloids, which are
3
4 mainly distributed in Papaveraceae (*Chelidonium majus*, *Macleaya cordata*, and *Sanguinaria*
5
6 *canadensis* L), have been the focus of increasing attention for their anticancer activity [7-9].
7
8 Specifically, several studies have indicated that sanguinarine [13-methyl-
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10 (1,3)benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i] phenanthridinium], a compound endowed with
11
12 antimicrobial, antioxidant, and anti-inflammatory properties [10, and the references therein],
13
14 is able to inhibit the growth of various human cancer cell lines, at micromolar concentration,
15
16 inducing a selective apoptotic response in cancer cells *versus* normal cells [7, 9, 11]. Notably,
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18 a recent study has also shown that sanguinarine reduces *in vitro* cell proliferation of K1375-
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20 M2, a very invasive melanoma cell line; Authors reported that sanguinarine acts as a DNA
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22 damaging agent, showing also collateral damage to mitochondrial bioenergetics, in a fashion
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24 similar to doxorubicin [12].
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31 In the present study, we have explored the potential application of benzophenanthridine
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33 alkaloids sanguinarine and cheliritrine in the therapy of melanoma cancer. To this end, *in*
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35 *vitro* antiproliferative activity of the compounds was investigated, along with their mechanism
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37 of action; sanguinarine was selected for preclinical *in vivo* evaluations. Results obtained
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39 demonstrated that the antitumoral effect of sanguinarine relies on its activity on several
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41 critical steps in primary tumor progression, including cell proliferation and angiogenesis.
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2. MATERIALS AND METHODS

2.1. Drugs and reagents

For *in vitro* studies, sanguinarine chloride and chelerritrine chloride (donated by INDENA Milan, Italy) were diluted in DMSO. Solutions were further diluted at each experimental day in order to achieve a 0.1% final DMSO concentration. All reagents were purchased from Sigma (Sigma- Chemical Co. St. Louis, Mo.), unless indicated.

2.2. Cell Lines

B16 melanoma 4A5 cells, a cell line derived from subcutaneously inoculated B16F0 tumors in C57BL/6 strain mouse, and A375 cells, derived from a 54 year old female with malignant melanoma, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were grown in DMEM medium with 4500 mg/L glucose, supplemented with 10% fetal bovine serum, 1% non-essential amino acids mixture, 1% Kanamycin. Cells, propagated as a monolayer culture, were trypsinized twice weekly, and plated at a density of 10×10^4 cells per mL. All cultures were incubated at 37 °C under 5% CO₂ in a high humidity atmosphere.

2.3. Growth inhibition assay

B16 melanoma 4A5 cells were seeded (20 000 cells/well) in 96-well flat bottom plates (Viewplates, Perkin Elmer Life Science, Waltham, MA). After 24 hours, media were replaced and, after one washing, media containing the tested compounds were added. Three independent experiments were performed in quadruplicates. After 72 h of culture in the presence of the compounds, plates were harvested and the number of viable cells was estimated by dosing ATP, using the ATPlite kit (Perkin-Elmer Life Science), and the automated luminometer Topcount (Perkin-Elmer Life Science). For each compound a dose–response curve was plotted, and the IC₅₀ values were then calculated by fitting the concentration–effect curve data obtained in the three experiments with the sigmoid–Emax

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model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect [13].

2.4. *Single cell gel electrophoresis (Comet assay)*

Nuclei were isolated from B16 melanoma 4A5 cells by incubating whole cells in a buffer containing 5 mM MgCl₂, 1 mM EGTA, 1 mM KH₂PO₄, 150 mM NaCl, for 20 min on ice with gentle rocking. Plasma membrane disruption and nuclei integrity were checked under the microscope. Isolated nuclei were exposed to the drugs for 60 min. Drug-untreated or H₂O₂-treated (50 μM for 5 min on ice) samples were run as negative and positive controls for DNA damage, respectively. DNA breaks were detected as described [14]. The calculations of the %Tail DNA were carried out using the Kinetic Komet 5.0 software from Kinetic Imaging Ltd. (Liverpool, UK).

2.5. *Relaxation activity of Topoisomerase-I*

Topoisomerase-I (Topo-I) functional activity was assayed by relaxation of supercoiled plasmid DNA in a cell free system as described [14]. Images were acquired and quantified through the Phoretix 1D software package (Phoretix International Ltd, Newcastle, Tyne and Wear, UK).

2.6. *Measurement of DNA intercalation*

Intercalation was determined by the unwinding assay [15]. Supercoiled pBR322 DNA (Roche Diagnostics Corporation, Indianapolis, IN, USA) was relaxed with 300 U of Topo-I (Topogen, Port Orange, FL) (37 °C, 15 min) in Topo-I relaxation buffer (Topogen). To confirm full relaxation of DNA, one sample was terminated with SDS after 75 min. Inhibitors were added and the incubations were continued for another 60 min. The reaction was terminated by addition of 1% (w/v) SDS. After digestion with proteinase K, open circular and linear DNA were separated from intact supercoiled and relaxed form, by agarose gel electrophoresis in the presence of 1 μg/ml ethidium bromide, under the same conditions as for

1 the relaxation assay. The compounds were removed by extraction with chloroform/isoamyl
2 alcohol (24:1), and topoisomers were separated on 1% agarose in Tris-Borate buffer. The gel
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4 was stained with ethidium bromide and acquired as described above.
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7 *2.7. Animals*

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9 Female C57BL/6, and female athymic mice [Athymic Nude-nu], 6 weeks old, were obtained
10 from Charles River S.r.l. (Lecco, Italy), and housed under controlled conditions. The
11 UKCCCR guidelines for the welfare of animals in experimental neoplasia were followed [16].
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Studies were approved by the Animal Care and Use Committee of the Catholic University of
the Sacred Heart (Rome, Italy), and by the Italian Ministry of Health.

2.8. *Tumor Growth*

On the day of inoculation, a suspension of B16 melanoma 4A5 (2×10^5) or A375 (5×10^6)
cells was injected subcutaneously in C57BL/6 or athymic mice, respectively. Sanguinarine
was dissolved in PEG 300: sterile water (1:2). Tumor-bearing mice received via gavage either
5 mg sanguinarine/kg/day or vehicle (n=10-12 mice/group), daily, week-end off. Dosage used
in this study was selected on the basis of previous unpublished data showing that the
Maximum Tolerated Dose following oral chronic administration is 5 mg/kg/day. Body weight
and tumor dimensions were measured two times per week. Due to excessive tumor ulceration,
all mice bearing B16 melanoma 4A5 tumors were sacrificed on day 18 of the study; athymic
mice bearing A375 xenografts were sacrificed when tumor weight was in the range of 1300-
1500 mg. At sacrifice, blood was collected and serum frozen at -20°C for analysis; all tumors
were removed, and subsequently cut into two fragments: one-half of the tumor was snap-
frozen in liquid nitrogen, before storage at -80°C for protein analysis, the other half was fixed
in 10% formalin and subsequently dehydrated and blocked in paraffin.

2.9. Evaluation of anti-tumor activity

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2 Tumor weight was calculated from two dimensional measurements (mm) [17]: Tumor weight
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4 = length x width²/ 2. The ratio between the mean tumor weight of treated mice and that of
5
6 control mice x 100 (T/C%) was assessed on each day of measurement. Differences in efficacy
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8 between treatment groups were expressed as the percentage of maximum tumor weight
9
10 inhibition (TWI %), calculated as follows: TWI % = 100 –T/C%. The optimum value for
11
12 tumor weight inhibition obtained during the study was considered. In the A375 model,
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14 significant differences in tumor progression were evaluated by the Kaplan-Meier survival
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16 analysis followed by logrank test, using as end-point a tumor weight of 1000 mg.
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2.10. Immunohistochemical analysis

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23 Immunohistochemical analysis of monoclonal mouse anti-human Ki67 (M7240, DAKO
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25 Italia, Milano, IT) (1:50 dilution, 60 min, room temperature) was carried out on 3- μ m thick
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27 paraffin sections as described [18]. Immunohistochemical scoring was determined without
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29 any knowledge of which group the mice belonged to. The number of positive (brown stained)
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31 cells was determined as a percentage of the total number of cells counted in 5 separate fields
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33 of 100 cells, in non necrotic areas of 8 tumor section, in each group.
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2.11. Immunoblotting

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40 Samples (80 μ g aliquots as determined by the Bradford assay, Bio-Rad Laboratories, Inc.,
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42 Hercules, CA) were separated on SDS-PAGE and immunoblot analyses were carried out as
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44 described [19]. Antibodies used included: rabbit anti-phospho-p44/42 mitogen-activated
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46 protein kinase (p-p44/42 MAPK) (Thr202/Tyr204), and anti-total-p44/42 MAP kinase
47
48 (1:1000); rabbit anti-phospho-AKT (p-AKT) (Ser473), and anti-total-AKT (1:1000) (all from
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50 Cell Signaling Technology, Boston, MA); mouse anti- β -actina (1:4000). Protein level were
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52 quantified by densitometric analysis using the Scion Image Beta 4.02 software package
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54 (Scion Corporation, Frederick, MD, USA).
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2.12. Angiogenesis Assay

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2 The method described by Passaniti et al. [20] was used, with some modifications. Briefly, b-
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4 FGF (300 ng/ml) was incorporated into liquid Matrigel (12.5 mg/ml) and a plug of 0.45 ml
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6 Matrigel was injected subcutaneously into the ventral abdominal wall of each female athymic
7
8 mice. Mice (10/group) received via gavage sanguinarine (5 mg/kg/day) or vehicle, daily, for 6
9
10 days from the day of Matrigel injection. At day 7, the pellet was removed, and the
11
12 hemoglobin content was measured by the Drabkin's procedure (Drabkin reagent kit). Matrigel
13
14 and basic Fibroblast Growth Factor (bFGF) were obtained from Becton Dickinson (Bedford,
15
16 MA).
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2.13. Statistical methods

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22 Tumor weight data were analyzed by repeated-measures ANOVA, followed by the
23
24 Bonferroni method as post-test. To determine significant differences in tumor progression
25
26 between groups, the Kaplan-Meier survival analysis was used, followed by logrank test (A375
27
28 only). The remaining data were analyzed by the unpaired t test. Statistical analysis was carried
29
30 out with GraphPad Prism5 Software (San Diego, CA, USA). $P < 0.05$ was used as the critical
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32 level of significance.
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3. RESULTS

3.1. *In vitro* studies

The benzophenanthridine alkaloids sanguinarine and cheliritrine, tested in B16 melanoma 4A5 cell line, yielded IC₅₀ values of 1.96 ± 0.22 , and 2.9 ± 0.22 μM respectively, following 72 hours of drug exposure; these results clearly pointed out that sanguinarine was more active than cheliritrine against melanoma cancer cells, and, for this reason, it was selected for subsequent *in vivo* investigations. A potential effect of drugs on the nuclear structure was assessed by Comet assay and results are shown in **Fig. 1A and B**: both compounds were able to induce DNA breaks, but the effect was greater upon sanguinarine treatment. Topo-I was then investigated as a possible target. A representative experiment is shown in **Fig. 2A**.

Notably, both sanguinarine and cheliritrine were able to markedly inhibit the process of DNA relaxation. This result prompted us to investigate whether the substances under investigation, rather than a specific activity on Topo-I, were DNA intercalating agents. This hypothesis was tested through the DNA unwinding assay. Several topoisomers of a DNA plasmid are noticeable in normal isotonic buffers, but when an intercalating agent is added to DNA, the structure of DNA changes and the number of possible topoisomers decreases. Using again a p-Bluescript plasmid, and Ethidium Bromide as positive control, this assay clearly demonstrated that both the agents were able to intercalate into the DNA structure (**Fig. 2B**).

3.2. *In vivo* studies

3.2.1. *Antitumor efficacy*

In the first set of experiments, C57BL/6 female mice were injected s.c. with B16 melanoma 4A5 cells, a very aggressive syngenic tumor with a rapid growth rate, and treated orally with sanguinarine 5 mg/kg/day or vehicle, until the end of the study. Results showed a relevant therapeutic activity with suppression of tumor growth in comparison with control mice

1 (p<0.01, by repeated-measures ANOVA, followed by Bonferroni test, **Fig. 3A**). A TWI of
2 52% was recorded on day 18 of the study. Similar results were also obtained using A375, a
3 tumor with a slower rate of *in vivo* growth; also in this case, sanguinarine-treated mice had a
4 significant therapeutic effect, showing a significant tumor growth inhibition in comparison to
5 control mice (p<0.05, **Fig. 3C**). A TWI of 58% was recorded on day 60 of the study. In
6 addition, results from the Kaplan- Meier analysis (**Fig. 3E**) showed that treatment
7 significantly improved time to tumor progression versus control mice (p<0.05), the end point
8 criterion being tumors grown to 1000 mg. By day 77 of the study, there were three tumor-free
9 mice; these mice were no longer dosed, showing no evidence of tumor after a further
10 treatment-free observation period of 60 days (i.e. day 137 of the study). Body weight (**Fig. 3B**
11 and **3D**) and food consumption (data not shown) did not significantly differ among treatment
12 groups in both studies, thus confirming the absence of drug toxicity at the selected dose level.
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29 3.2.2. Effect on tumor proliferation

30 Proliferation of A375 cells significantly differed among groups (**Fig. 4A and B**). An average
31 value of $66.0 \pm 5.1\%$ (mean \pm SEM) Ki67 positive cells was recorded in control tumors. A
32 reduction in proliferation was observed in animal receiving 5 mg/kg/day sanguinarine, the
33 mean value of positive stained cells being $50.0 \pm 3.2\%$; this change reached statistical
34 significance, with respect to controls (p=0.03).
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44 3.2.3. Effect on pMAPK/MAPK and pAKT/AKT levels in tumors

45 Representative immunoblots of the total and the phosphorylated forms of the proteins are
46 shown in **Fig. 4C** and **4E**. Results obtained showed that the ratio p-p44/42 MAPK/p44/42
47 MAPK was decreased in A375 tumors following treatment with 5 mg/kg/day sanguinarine,
48 this change reaching statistical significance (**Fig. 4D**, p=0.04 vs controls). Tumors from
49 treated mice also tended to have a lower pAKT/AKT ratio compared to controls (**Fig. 4F**, p =
50 0.07).
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3.2.4. *Angiogenesis in vivo*

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2 Daily oral doses of 5 mg/kg sanguinarine for 6 days significantly reduced the angiogenic
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4 response induced by bFGF (**Fig. 5A and B**). The mean hemoglobin content of bFGF-
5
6 containing pellets was significantly lower ($p = 0.02$) in sanguinarine-treated mice ($0.05 \pm$
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8 0.009 g/dl, mean \pm SEM) than in vehicle-treated mice (0.11 ± 0.012 g/dl, mean \pm SEM).
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4. DISCUSSION

1
2 Experimental data from the present study support the concept that sanguinarine can be
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4 effective in melanoma skin cancer. During a cellular screening of benzophenanthridine
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6 alkaloids we found that the drug resulted more active than the analogous compound
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8 cheliritrine against the murine cell line B16 melanoma 4A5, and that it mainly acts as an
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10 intercalating agent producing DNA breaks. This finding is in keeping with earlier data from
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12 Maiti and colleagues [21], showing that sanguinarine strongly interacts with DNA, binding,
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14 like ethidium bromide, by monofunctional mode of intercalation. Importantly, previous
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16 Authors have suggested that the cytotoxic and the DNA damaging effects of sanguinarine are
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18 selective against cancer cells as compared to normal cells [7, 22], a finding, however, not
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20 confirmed by other studies [23]. We did not evaluate the effects of sanguinarine on normal
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22 melanocytes in the present work; while not addressing safety issues at this time, we believe,
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24 however, that properly designed *in vitro* and *in vivo* experiments are needed to exploit the
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26 undesired toxic effect of this alkaloid to normal tissue. Indeed, toxicity is a crucial matter in
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28 the development of anticancer drugs, often derailing clinical trials.

29
30 The activity of sanguinarine in melanoma was then confirmed *in vivo*, against a transplantable
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32 murine tumor grown in a syngeneic host (B16 melanoma 4A5 in C57BL/6 mice), and against
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34 a human tumor xenograft grown in immunodeficient mice (A375 in athymic nude mice).

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36 Because of an intact tumor host environment, the syngeneic transplantable mouse tumor
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38 model represents a valuable experimental model for the evaluation of therapies that require
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40 immune response, or that target specific components of blood vessels or the extracellular
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42 matrix. Conversely, the human xenograft tumor grown in nude mice exhibits a proliferative
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44 pattern with molecular and pathophysiological features similar to patient-tumor
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46 characteristics. In both experimental models, oral sanguinarine administration reduced by
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48 more than 50% tumor development, compared to controls. In A375 tumors, this result was
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1 associated with a significant decrease in the proliferation marker Ki67, and with a reduction
2 in the activated MAPK and AKT. These latter findings are particularly important when
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4 considering that the RAS/RAF/MEK/ERK (MAPK) and PI3K/AKT (AKT) signaling
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6 pathways are constitutively activated through multiple mechanisms, and appear to play a
7
8 major role in melanoma development and progression, this implying that combined targeting
9
10 of MAPK and AKT signaling pathways is a promising strategy for melanoma treatment [24].
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12 Notably, 3 out of 11 A375-bearing treated mice were tumor-free at the end of treatment (i.e.
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14 day 77 of the study), showing no evidence of tumor after a further, treatment-free, observation
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16 period of 60 days. It is worthy to note that preliminary pharmacokinetics data obtained in our
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18 laboratory indicated that within two hours following oral treatment with 5 mg/kg
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20 sanguinarine, the compound achieved relevant levels in plasma and in B164A5 tumors
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22 (unpublished data).
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29 The antiproliferative activity of sanguinarine has been mainly attributed to its involvement on
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31 critical molecular events regulating the cell cycle and the apoptotic machinery [7, 9, 25, 26],
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33 yet its observed therapeutic effect cannot be ascribed solely to a direct antiproliferative
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35 activity, as indicated by data from the present study, showing that the role of the alkaloid in
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37 tumor suppression is more complex than that of merely antiproliferative cytotoxic agent.
38
39 Rather, an effect on tumor angiogenesis seems to play a decisive role. Other Authors have
40
41 previously reported that sanguinarine is a potent antiangiogenic natural product, able to
42
43 suppress *in vitro* VEGF-induced endothelial cell migration, sprouting, and survival [27]. *In*
44
45 *vivo* studies confirmed its activity as a potent inhibitor of blood vessel formation in the mouse
46
47 Matrigel plug assay (the drug was incorporated into the Matrigel), and in the chicken
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49 chorioallantoic membrane assay (the drug was air-dried on plastic discs then applied to the
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51 CAM surface) [27]. To our knowledge, the present study is the first to report an effect of
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53 sanguinarine as angiogenesis inhibitor, following systemic administration to experimental
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1 animals. Moreover, Western analysis on A375 tumors showed a relevant reduction in AKT
2 activation in treated mice when compared to controls. Notably, this finding is in keeping with
3
4 previous findings reported by other Authors demonstrating, in *in vitro* models, that the
5
6 antiangiogenic effect of sanguinarine could occur partly through blocking the VEGF-
7
8 induced PI 3'-kinase/AKT activation, a pivotal event in angiogenesis signaling [27,28].
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11 One mechanism by which VEGF-targeted therapy may be of benefit in cancer treatment is by
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13 counteracting the up-regulation of VEGF expression following genotoxic stress induced by
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15 chemotherapy or radiation therapy [29]. Notably, treatment of human melanoma cells with
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17 dacarbazine caused an increase in secreted VEGF-A, and interleukin 8 (IL8) [30], and
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19 dacarbazine-resistant melanoma cell lines demonstrated increased growth *in vivo* with
20
21 increased microvessel density [31]. Altogether these findings support an exciting hypothesis
22
23 of opportunity for testing combination regimens between sanguinarine and cytotoxic drugs
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25 currently approved for the treatment of advanced melanoma.
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31 In conclusion, our results suggest that sanguinarine exerts a profound anti-cancerous effect
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33 through a combined action on several critical determinants of tumor progression, such as
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35 angiogenesis, and cell proliferation. To our knowledge the present study is the first reporting
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37 an antiproliferative and antiangiogenic effect of sanguinarine following oral administration to
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39 mice. Notably, its inhibitory effects on angiogenesis and tumor progression were observed in
40
41 mice at a dosage devoid of apparent toxicity, although additional studies need to be carried
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43 out to fully characterize the safety profile of the drug. Overall, findings from the present study
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45 make sanguinarine a likely candidate for further evaluation in melanoma treatment.
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Acknowledgements

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2 This study was supported in part by the Ministero Istruzione Università e Ricerca (Decreto
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4
5 No. 1587). Sanguinarine and Chelirtrine were donated by INDENA s.p.a.
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FIGURE LEGENDS

1
2 **Figure 1** –Comet assay performed in isolated nuclei in the presence of the vehicle (Control,
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4 DMSO 0.1%), sanguinarine (0.1, 0.25, 0.5, 1, 2.5 and 5 μM), chelerritrine (0.1, 0.25, 0.5, 1,
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6 2.5 and 5 μM), or H_2O_2 (50 μM) as positive control. **(A)** Representative image of the
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8 experiment reported in the bar chart. **(B)** Bar chart showing the results of the % Tail DNA.
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10 This experiment was repeated three times with similar results. * = $p < 0.05$, ** = $p < 0.01$, ***
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12 = $p < 0.001$ vs Control
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20 **Figure 2 (A)** Agarose gel electrophoresis for a cell free DNA relaxation assay of a
21
22 supercoiled pBluescript plasmid DNA, treated with the tested compounds from 2 to 30 min.
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24 Relaxed DNA topoisomers (rel) slowly migrate in the gel as compared to supercoiled DNA
25
26 (sc). After 30 min the full DNA relaxation is obtained in control lanes (DMSO 0.5%).
27
28 Sanguinarine and chelerritrine strongly inhibit the activity of Topoisomerase-I at 50 μM ; CPT
29
30 was used as a positive control. **(B)** Representative image of the agarose gel electrophoresis
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32 for DNA unwinding assay. Several topoisomers of a DNA plasmid are noticeable in normal
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34 isotonic buffer (rel, Ctrl 15'), but when an intercalating agent is added to DNA the structure
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36 of DNA changes, and the number of topoisomers decreases, as occurred in the presence of
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38 sanguinarine and chelerritrine (50 μM), with presence of supercoiled (sc) DNA. Ethidium
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40 Bromide was used as positive control. To confirm full relaxation of DNA, one sample was
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42 terminated with SDS after 75 min (Ctrl 75'). This experiment was repeated three times with
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44 similar results.
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54 **Figure 3** – **(A)** B16 melanoma 4A5 tumor growth, and **(B)** body weight, of female C57BL6
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56 mice receiving 0 (control) or 5 mg/kg/day sanguinarine (means \pm SEM, n=10/group);
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58 *different from control at that time, $p < 0.01$. **(C)** A375 xenograft growth, and **(D)** body weight
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1 of female athymic mice receiving 0 (control) or 5 mg /kg/day sanguinarine (mean \pm SEM,
2 n=11-12/group at the start of the study. Data are reported until n=5/group); *different from
3 control at that time, $p < 0.05$. (E) Kaplan-Meier curves showing the tumor progression of
4 female athymic mice with A375 xenografts (n=11-12/group). The end-point was tumor
5 weight = 1000 mg. * = $p < 0.05$ vs Control (Logrank test). By day 77 of the study, there were
6 three tumor-free mice; these mice were no longer dosed, showing no evidence of tumor after a
7 further treatment-free observation period of 60 days (i.e. day 137 of the study).
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19 **Figure 4 – (A)** Representative immunohistochemical localization of Ki67, and **(B)** tumor
20 proliferation, in A375 xenografts from female athymic mice receiving 0 (control), or 5
21 mg/kg/day sanguinarine (mean \pm SEM, n=8 tumors/group). Magnification 20x. **(C, E)**
22 Representative western blots, and **(D, F)** protein expression for p-p44/42MAPK/total-p44/42
23 MAPK and p-AKT/AKT, respectively, in A375 tumors from female athymic mice receiving 0
24 (control), or 5 mg/kg/day sanguinarine (mean \pm SEM, n=8 tumors/group).
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36 **Figure 5 - Effects of sanguinarine on *in vivo* angiogenesis in the Matrigel plug assay. (A)**
37 Macroscopic evaluation of the Matrigel plugs. Angiogenic plugs appear red-orange with
38 small curly vessels, non-angiogenic plugs appear translucent. **(B)** Hemoglobin content
39 (g/dl) of the pellets in control and sanguinarine (5 mg/kg/day os)-treated mice. The box
40 extend to the 25th and 75th percentiles; the horizontal bars indicate median values, and
41 whiskers minimum and maximum values (n=10/group).
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Figure 1

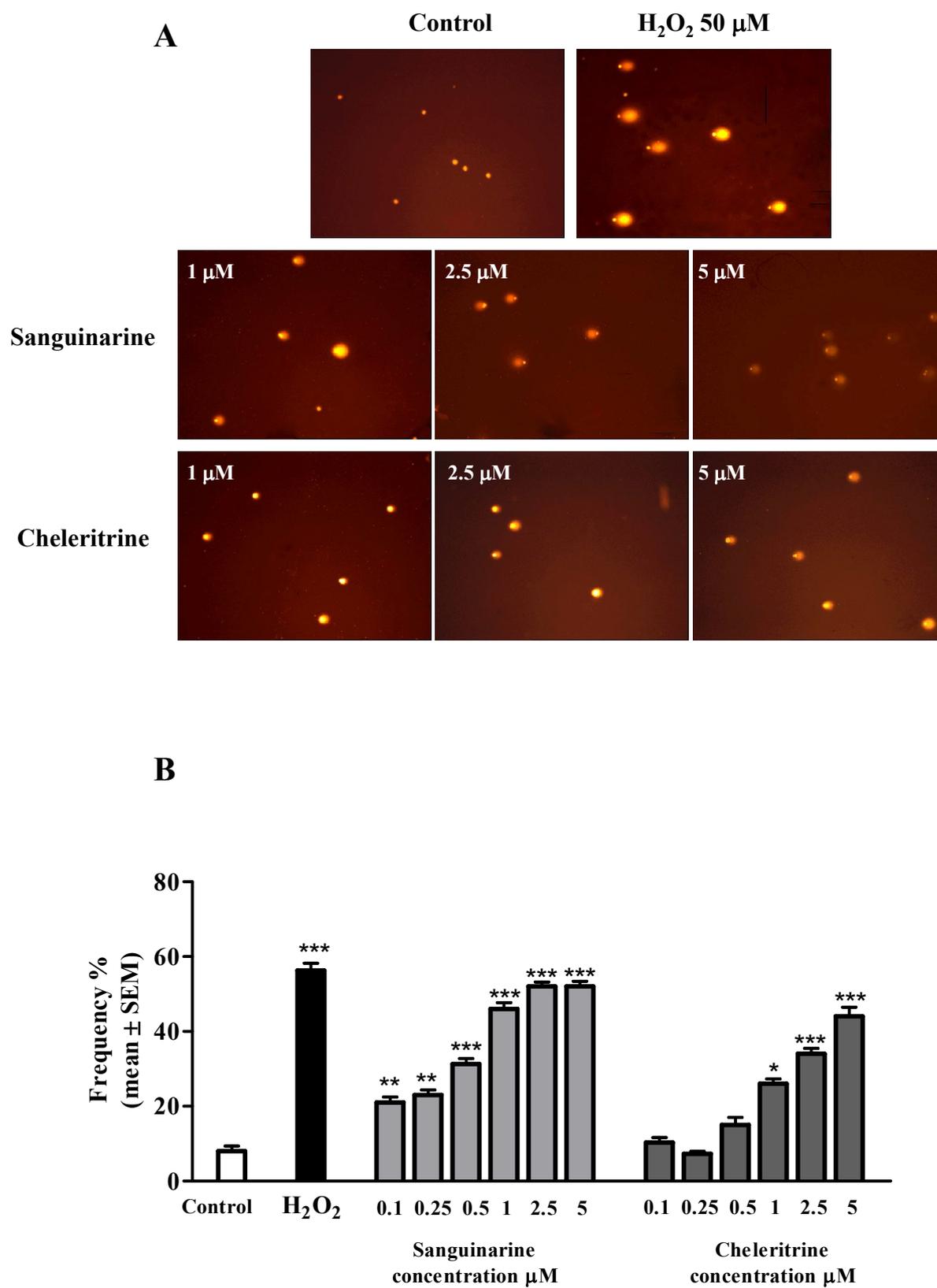
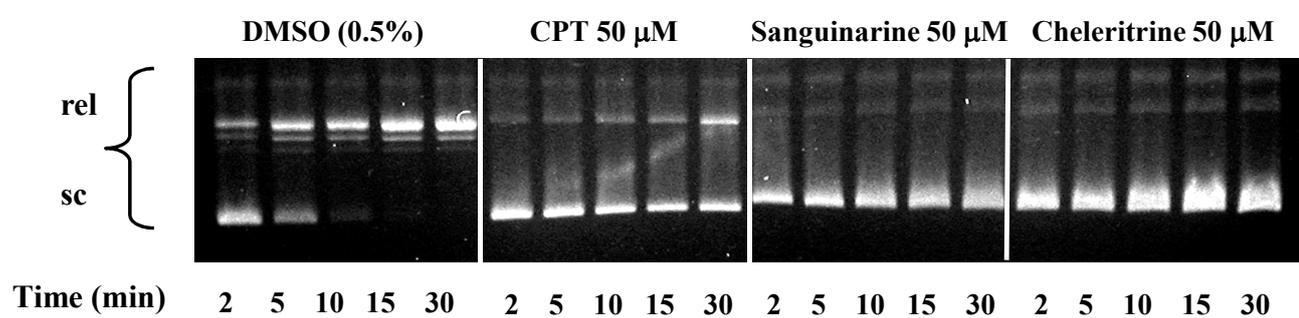


Figure 2

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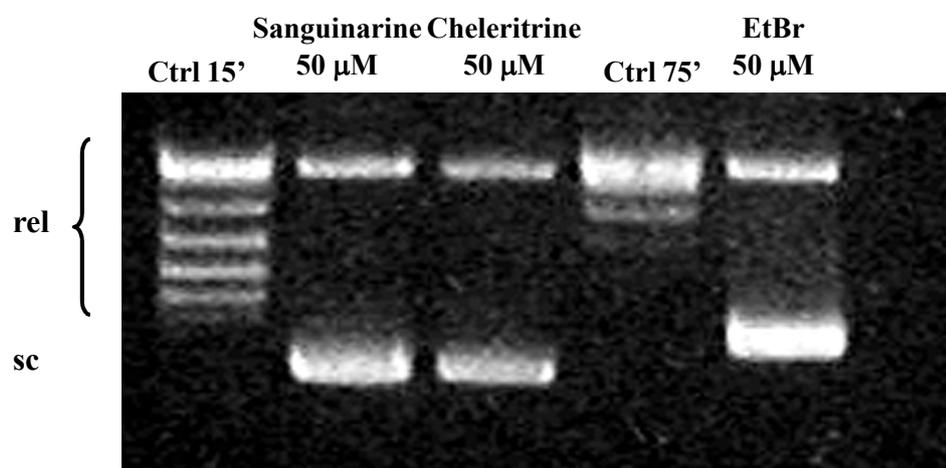


Figure 3

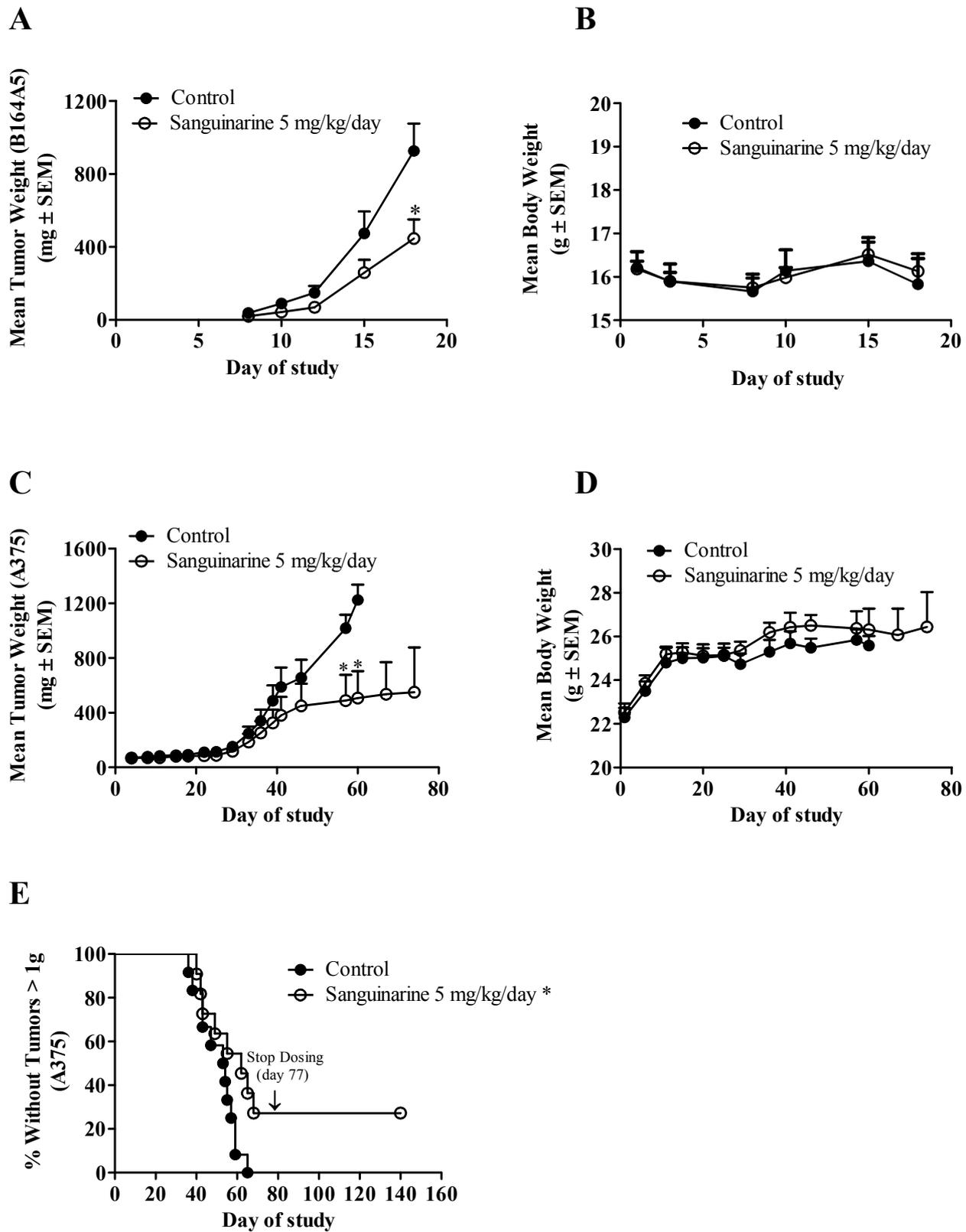


Figure 4

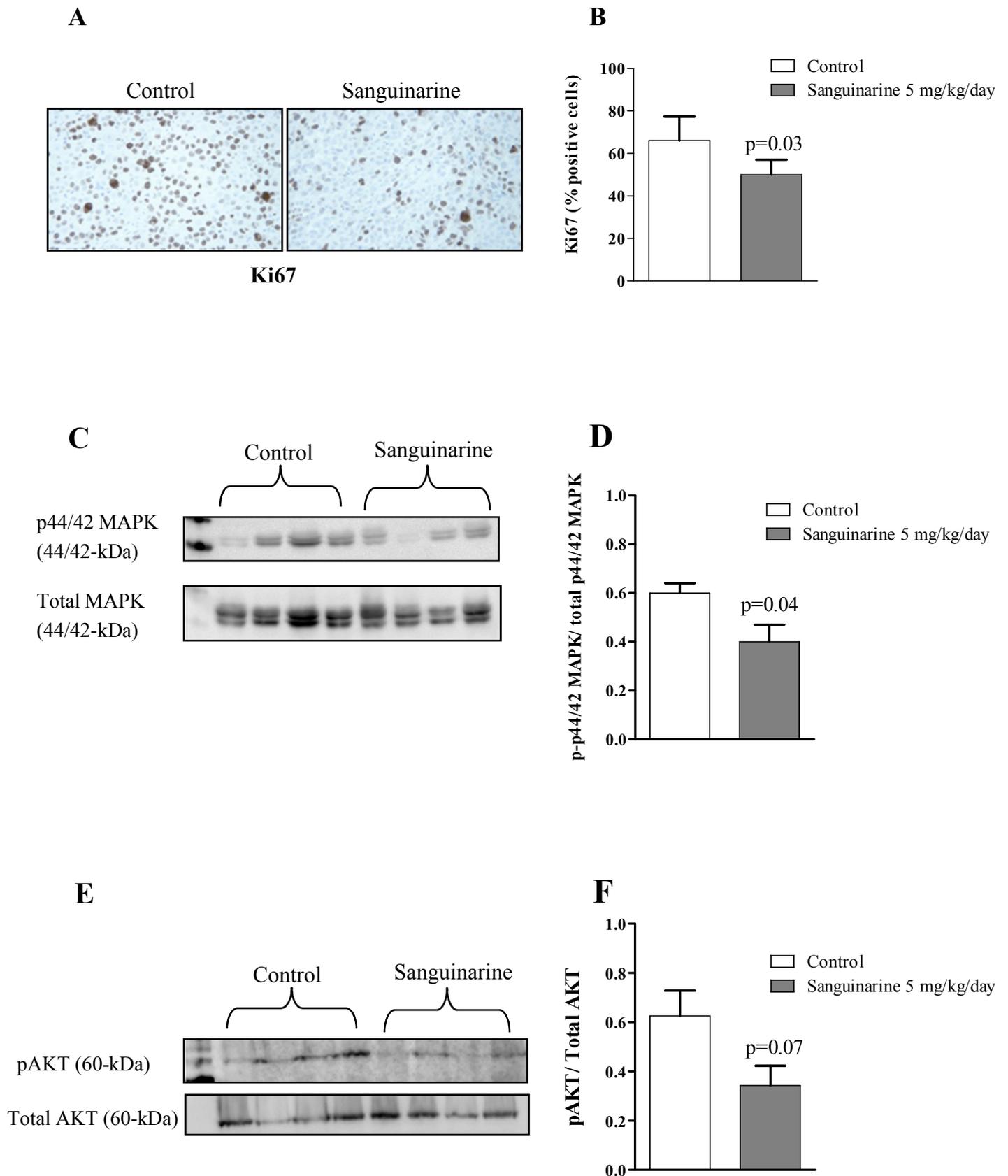
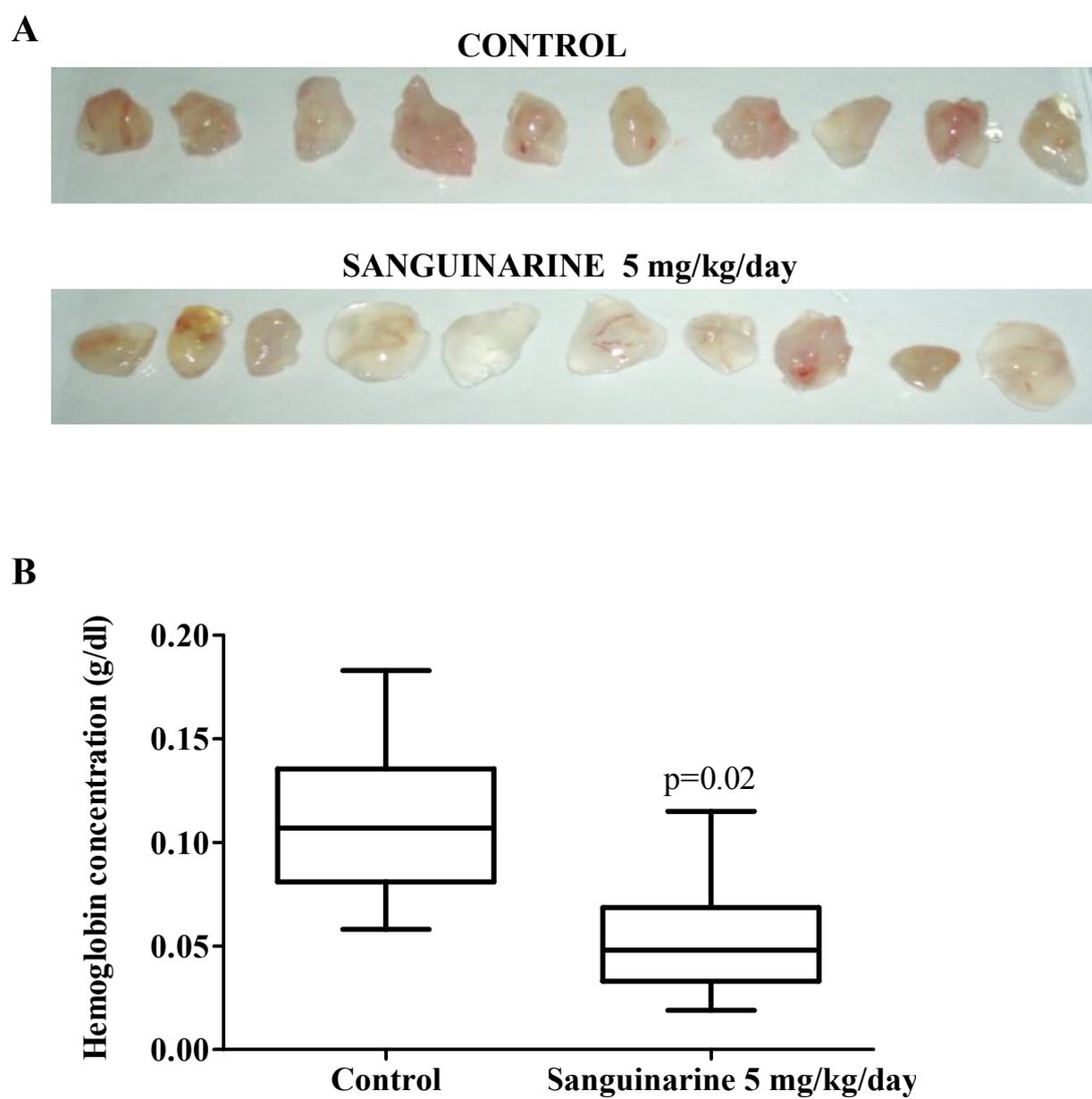


Figure 5



Preclinical *in vivo* antiproliferative and antiangiogenic effects of Sanguinarine in melanoma