Antitumor effects of the benzophenanthridine alkaloid sanguinarine in a rat syngeneic model of colorectal cancer

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To evaluate the in-vivo preclinical antitumor activity of sanguinarine in a rat syngeneic model of colorectal cancer. The effects of sanguinarine on DHD/K12/TRb colorectal adenocarcinoma cells were first evaluated *in vitro* by means of ^{3}H-thymidine incorporation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (MTS) assay, and terminal transferase dUTP nick end labeling (TUNEL) microscopy. For the in-vivo studies, DHD/K12/TRb cells (1.5×10^6 cells/0.3 ml of sterile saline/animal) were injected subcutaneously in syngeneic BDIX rats, which were chronically treated with sanguinarine (5 mg/kg/day *per os*) or control diluent. Tumor growth, body weight, hematologic, and clinical chemistry measurements were monitored in individual animals at defined time intervals. After killing, subcutaneous tumors were explanted from experimental animals for histopathological examination. *In vitro*, micromolar concentrations of sanguinarine inhibited dose-dependently DHD/K12/TRb cell proliferation and metabolism and induced cell death by apoptosis. *In vivo*, oral administration of sanguinarine induced a significant inhibition of tumor growth ($P < 0.01$ vs. untreated controls), in the absence of any toxic or side effects. Marked apoptosis and reduced peritumoral vascularization were observed in tumors from sanguinarine-treated rats as compared with the controls. Additional basic studies are needed to fully characterize the mechanism/s underlying the inhibitory effects of sanguinarine on angiogenesis and tumor growth as well as the pharmacological and safety profile of this drug in experimental tumor models. Overall, findings from this study suggest that sanguinarine is a likely candidate for further evaluation in cancer therapy. *Anti-Cancer Drugs* 23:32–42 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Colorectal cancer (CRC) is the second leading cause of death from tumors in the world with a worldwide incidence of more than one million cases each year [1]. Advances in screening, surgical techniques, adjuvant chemotherapy, and surveillance programs have improved the survival rate, which, however, remains low mainly due to liver metastases [2].

Postoperative or 'adjuvant' systemic therapy has become standard for stage III colon cancer but should also be strongly considered in stage II patients [2]. The optimal choice of adjuvant chemotherapy has recently changed from a 5-fluorouracil (5-FU)-based chemotherapy alone to a course of infusional 5-FU plus leucovorin and oxaliplatin (OXA) that seems to have a greater therapeutic efficacy [3].

Active agents, in addition to the original 5-FU, which have been approved recently by the Food and Drug Administration for metastatic CRC include capecitabine, OXA, bevacizumab, cetuximab, panitumumab, and irinotecan [2]. The last one is a derivative of camptothecin (CPT), found in *Camptotheca acuminate*, a plant native to China. It potently inhibits topoisomerase I, an enzyme that facilitates the uncoiling and recoiling of DNA during replication by cleaving one strand and subsequently reattaching this strand.

Natural products have long been a fertile source of cure for cancer, with plant-derived drugs becoming increasingly explored and integrated into chemotherapy strategies. So far, there are at least 250 000 species of plants, out of which more than 1000 have been found to possess significant anticancer properties [4].

Sanguinarine (13-methyl-[1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5-*i*] phenanthridinium) is a benzophenanthridine alkaloid that has significant structural homology to chelerythrine, which is derived from the root of *Sanguinaria*

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canadensis and other poppy *Fumaria* species [5–7]. Sanguinarine has been shown to possess antimicrobial, antioxidant, and anti-inflammatory properties [8–10]; when used at micromolar concentrations, it is able to inhibit the growth of various human cancer cell lines inducing a selective apoptotic response in cancer cells versus normal cells [11–13]. So far, the antiproliferative and/or proapoptotic activities of sanguinarine have been demonstrated in cells derived from several human cancers including epidermal [11], prostate [12,14], cervical [13,15], breast [16,17], lymphoma [18], melanoma [19,20], colon [21], gastric [22], pancreatic [23], lung [24], neuroendocrine [25], osteosarcoma [26], and in rat glioblastoma cells [27].

So far, there have been only a few studies on the in-vivo effects of oral sanguinarine administration [28–34] and even fewer in experimental tumor models [20,29].

The aim of this study was to investigate the antitumor potential of sanguinarine in a preclinical model of CRC, obtained by subcutaneous (s.c.) injection of DHD/K12/TRb colorectal adenocarcinoma cells in syngeneic immunocompetent BDIX rats. DHD/K12/TRb tumor cells [35] display many of the features of human epithelial CRC tissue, including the expression of human tumor-associated antigens [36,37]. We have used this preclinical model of CRC previously for evaluating the antitumor activity of different combination therapy approaches based on the use of adjuvants, cytokines, chemotherapeutic agents, and tumor peptide vaccination [36–39].

Methods

Tumor cells

The colonic adenocarcinoma cell line DHD/K12/TRb (European collection of cell culture ECACC Salisbury, UK), originally established from a 1,2-dimethylhydrazine-induced colon adenocarcinoma in syngeneic BDIX rats [35], was used. DHD/K12/TRb cells were maintained in monolayers using Dulbecco's minimal essential medium (DMEM, Flow, Irvin, UK) containing 10% heat-inactivated fetal bovine serum (Flow), L-glutamine (2 mmol/l), penicillin (100 IU/ml), and streptomycin (100 μg/ml), and incubated in a humidified atmosphere of 5% CO_2 at 37°C. Cells were serially passaged every 72 h after detachment from culture flasks with 0.05% trypsin and 0.02% EDTA solution (Flow). Cell growth and viability were evaluated by phase-contrast microscopy and the trypan blue dye exclusion test, as a routine procedure. Before each experiment, cell viability was determined and was found to be greater than 95% in all cases.

Drugs and reagents

For the in-vitro studies, sanguinarine chloride (donated by Indena, Milan, Italy) was diluted in dimethyl sulfoxide and stored at −20°C. Stock solutions were further diluted in DMEM at each experimental day to achieve a final dimethyl sulfoxide concentration less than or equal to 0.2%.

For the in-vivo studies, sanguinarine was provided by Indena already dissolved in sterile water:propylene glycol (2 : 1) and ready for use, together with a separate vial of diluent alone for control animals.

Etoposide (VP-16), 5-FU, CPT and OXA were purchased from Sigma (Sigma-Chemical Co., St Louis, Missouri, USA) and diluted in DMEM to obtain the concentrations to be used in the experiments. All the other reagents were from Sigma, unless indicated.

In-vitro studies

(1) Cell proliferation was evaluated on triplicate samples by [methyl-^{3}H]-thymidine (^{3}H-TdR) (Amersham Pharmacia Biotech, Buckingshamshire, UK) pulse incorporation into the cells. Briefly, DHD/K12/TRb cells (1×10^4/100μl/well) were seeded into 96-well microtiter plates (Falcon, Becton Dickinson and Company, Franklin Lakes, New Jersy, USA) in complete culture medium at the onset of cultures. After an overnight incubation, sanguinarine (0–10 μmol/l) was added to the cultures, which 6 h later were pulsed with ^{3}H-TdR (1 μCi/well) for an additional 18 h. Cells were harvested using a Microtiter Cell Harvester (Titertek 530 Flow Labs, Flow Laboratories Inc., Rockville, Maryland, USA) and the incorporated radioactivity was measured using a scintillation β-counter (LKB, Bromma, Sweden). Results were expressed as mean counts per minute of radioactivity ± SE of triplicate samples from three independent experiments.

(2) For the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay, cells were seeded at a density of 5×10^3 cells/100 μl/well into flat-bottomed 96-well plates, in complete culture medium, allowed to attach for an overnight, and then exposed or not to sanguinarine (0–10 μmol/l). After 24 h of treatment, 20 μl of CellTiter 96 A_{queous} One Solution Reagent (Promega, Madison, Wisconsin, USA) was added directly to the culture wells, according to the manufacturer's instructions. Viable cells were quantified by measuring absorbance at 490 nm using a LabSystem Multiskan Bichromatic plate reader (Thermofisher, New Hampshire, USA). At least 12 measurements (four wells from three independent experiments) were averaged for each data point. Results were expressed as mean absorbance ± SE of quadruplicate samples from three independent experiments.

(3) For apoptosis evaluation, the 'The DeadEnd Fluorometric TUNEL System' (Promega) was used. Briefly, DHD/K12/TRb cells (2.5×10^4/well) were grown in a Chamber Slide, Lab-Tek (Nunc, New York, USA). After a 24 h treatment with micromolar concentrations of sanguinarine, 5-FU, OXA, CPT, VP-16, or control diluent, medium was removed, the wells were rinsed twice with phosphate-buffered saline (PBS),

and the slides were fixed in 4% methanol-free formaldehyde in PBS at + 4°C for 10 min. Then, they were washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS, and stained with fluorescein-12-dUTP, according to the manufacturer's instructions. After two washes with PBS, the slides were stained at room temperature (RT) for 15 min with a solution of propidium iodide (100 μg/ml in PBS), washed three times with PBS, blotted dry, and mounted using Vectashield mounting medium for fluorescence (Vector Laboratories, California, USA). Samples were analyzed under a fluorescence microscope (Olympus BX50, Hamburg, Germany), and for each condition approximately 500 cells were counted. Results were expressed as the mean value of percentage of apoptotic cells ± SE obtained from three independent experiments.

The in-vitro cell sensitivity to sanguinarine was also expressed in terms of IC50 (drug concentration producing 50% inhibition of cell growth or cell metabolism) and in terms of EC25 (drug concentration causing 25% induction of cell apoptosis), which were calculated as reported previously [40].

In-vivo studies

Animals

Eight-week-old inbred male BDIX rats (Charles River Italia, Calco, Italy), with a mean weight of 287 ± 22.5 g, were used. Animals were housed in individual cages with wide wire bottoms to prevent coprophagia, maintained under constant conditions (relative temperature of 20 ± 2°C, relative humidity of 55 ± 5%, 12 h dark–light cycle), and acclimatized for at least a week before starting the experiments. Animals received basic rat chow and tap water *ad libitum*. Studies were performed in accordance with the European Community guidelines and were approved by the Animal Care and Use Committee of the University of Rome 'Tor Vergata'.

Experimental design

On day 0, DHD/K12/TRb cells (1.5×10^6 viable cells/0.3 ml of sterile saline/animal) were injected s.c. into the shaved left flank of BDIX rats. On day 1, the tumor-injected animals were randomized and divided into two experimental groups of 10 animals each: (a) tumor-bearing rats treated with sanguinarine; (b) tumor-bearing rats treated with control diluent.

The animals received through gavage either sanguinarine (5 mg/kg in 1 ml of volume) or control diluent daily, weekend off, throughout the experimental period. The dosage and treatment schedule were chosen according to recently published data [20] and on the basis of our preliminary experiments on oral chronic administration of sanguinarine in normal BDIX rats (data not shown).

Body weight and tumor size of the experimental animals were measured weekly throughout the experimental period. Tumor size was measured, using a Vernier calipers, in two perpendicular diameters (a the longest, and b the shortest), and tumor volume (mm^3) was calculated according to the formula $(a \times b^2)/2$, as described previously [39].

On days 0, 15, and 36, all the experimental animals were anesthetized by inhalation of halothane and blood was collected by intracardiac puncture for the assessment of blood cell counts and clinical chemistry parameters whose variations have been associated with hemathologic, liver, and/or pancreatic toxicity. Blood cell counts were performed on samples collected from individual animals and immediately placed in a tube containing EDTA (K2 EDTA, Becton Dickinson and Company), using the automatic analyzer Simply cell (BPC BioSed srl, Rome, Italy). Meanwhile, blood samples collected in an appropriately sized tube without anticoagulant to allow clotting and collection of sera were used for clinical chemistry analyses [blood urea nitrogen; total cholesterol; triglycerides (TRI); aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP); amylase (AMY); lactate dehydrogenase (LDH), which were performed using the automatic analyzer Keylab (BPC BioSed).

Owing to excessive tumor ulceration in the control animals, all the experimental rats were killed by an overdose of anesthetic on day 36 of the study. Tumors were excised from individual rats and immersed in water in a graduated glass cylinder for volumetric evaluation, and tumor volume was expressed as the difference in water displacement. Each experiment was repeated at least two times.

Tumor tissue specimens for ex-vivo analyses

After killing, the s.c. tumors were explanted from sanguinarine-treated rats and controls, measured, fixed in 10% buffered formalin and embedded in paraffin as a routine procedure. Sections from each paraffin block were cut and stained with hematoxylin–eosin for histopathological examination or collected on 3-aminopropyltriethoxy silane (Dako, Glostrup, Denmark)-coated slides for immunohistochemistry of the proliferation marker Ki67 and for TUNEL assay. Paraffin-embedded tissues were examined for tumor cell proliferation assessment and in-situ apoptosis detection as follows:

(1) Tumor cell proliferation in sanguinarine-treated rats and controls were assessed by evaluating nuclear positivity for the proliferation marker Ki67 after specific immunostaining with the anti-Ki67 rabbit monoclonal antibody (Abcam, Massachusetts, USA). Tissue sections were preincubated in 1% BSA for 15 min at RT to block nonspecific background. Slides

were then incubated overnight at 4°C with the specific antibody (working dilution 1 : 100). Primary antibody reaction was revealed using the kit Dako Cytomation LSAB 2 System HRP (Liquid DAB) that uses the streptavidin–biotin complex method. After peroxidase reaction, sections were counterstained with hematoxylin and observed under a Zeiss Axioplan microscope (Carl Zeiss Inc., Thornwood, New York, USA). For the analysis, a minimum of 600 nuclei/section were counted and results for each group of animals were given as the mean value of percentage of cells exhibiting nuclear Ki67 positivity. Quantitative assessment was performed in a blinded manner.

(2) The TUNEL assay for in-situ apoptosis detection was carried out using the 'In situ Cell Death Detection Kit' (Roche, Mannheim, Germany), according to the manufacturer's instructions. In detail, after unmasking with citrate buffer (0.1 mol/l) at pH 6.0 under microwave irradiation at 750 W for 1 min, de-waxed formalin-fixed sections were treated with Tris-HCl (50 mmol/l), pH 7.5 containing 3% BSA for 30 min at RT, blocked with TUNEL buffer (Roche) for 10 min at RT, and then incubated for 1 h at 37°C in a humidified atmosphere with the TUNEL mixture containing biotin (5 µmol/l) plus terminal deoxynucleotidyl transferase (TdT) (300 U/50 µl) in TUNEL buffer (all reagents from Roche). After washing three times in PBS, sections were incubated for 1 h at 37°C in a humidified atmosphere with AlexaFluor-647-conjugated streptavidin (Molecular Probes Inc., Eugene, Oregon, USA; working dilution 1 : 200). Positive control of TUNEL staining consisted of sections incubated with DNAse (1 U/µl) in Tris-HCl; negative controls consisted of sections incubated with the TUNEL mixture without TdT or without TdT and biotin. Nuclei were then counterstained with Hoechst (Sigma; working dilution 1 : 4000) and were observed by the Leica TCS SP5 confocal laser scanning microscopy (Leica Instruments, Wetzlar, Germany). For the analysis, a minimum of 400 nuclei/section were counted and results for each group of animals were given as the mean value of percentage of cells exhibiting nuclear TUNEL staining. Quantitative assessment was performed in a blinded manner.

Statistical analysis

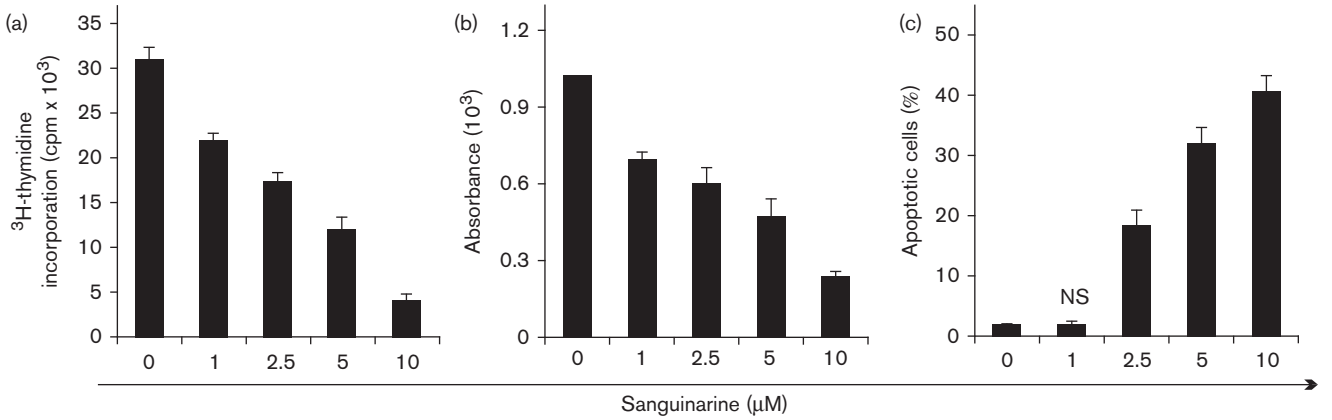
Data were analyzed for differences by Student's *t*-test or by the Wilcoxon's rank sum test where appropriate, and $P < 0.05$ was used as the critical level of significance.

Results

In-vitro studies

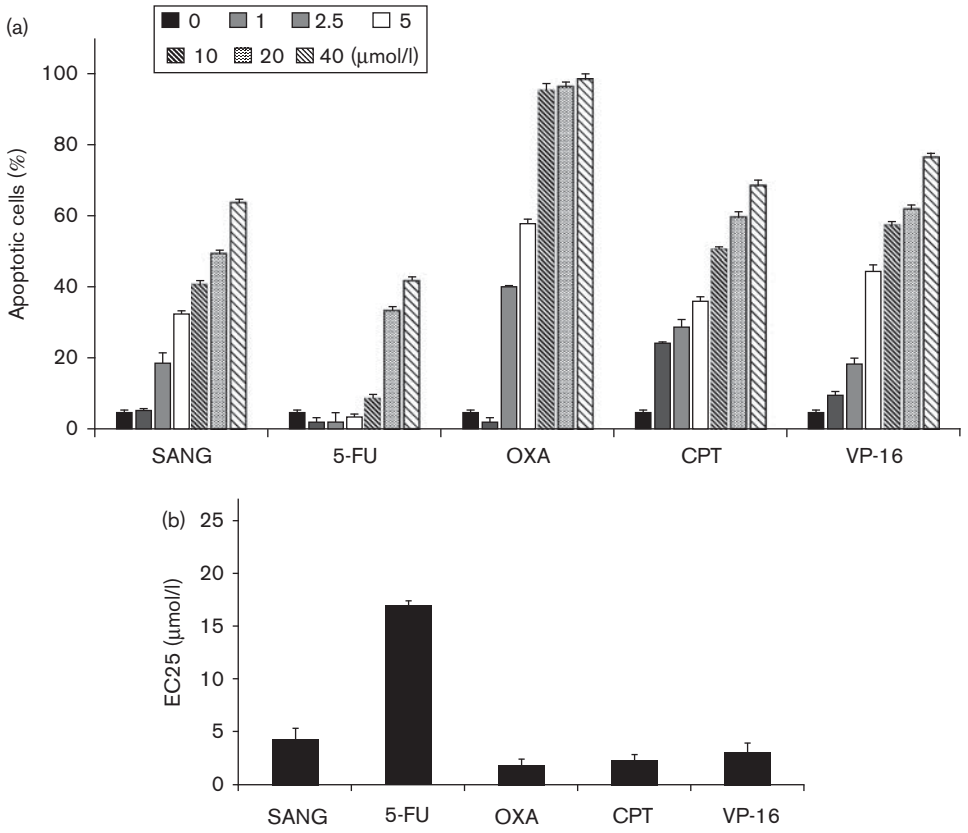
The in-vitro effects of different concentrations of the benzophenanthridine alkaloid sanguinarine on DHD/K12/TRb cells are shown in the Fig. 1. Briefly, we found that the addition of sanguinarine to the cultures inhibited dose-dependently tumor cell proliferation and metabolism (Fig. 1a and b; $P < 0.001$ for each point vs. control) and induced cell death by apoptosis (Fig. 1c; $P < 0.001$ vs. control for sanguinarine ≥ 2.5 µmol/l; $P = 0.78$ for 1 µmol/l sanguinarine). The IC50 of sanguinarine (mean ± SD) for ^{3}H-thymidine incorporation assay and for MTS assay was 4.99 ± 0.20 and 4.35 ± 0.78 µmol/l, respectively.

Fig. 1



In-vitro proliferation, metabolism, and apoptosis of DHD/K12/TRb cells after a 24 h incubation with sanguinarine (0–10 µmol/l). The experiments were performed as described in detail in Methods. (a) ^{3}H-thymidine incorporation assay. Results were expressed as mean counts per minute (cpm) of radioactivity ± SE of triplicate samples from three independent experiments; (b) MTS assay. Results were expressed as mean absorbance ± SE of quadruplicate samples from three independent experiments; (c) TUNEL assay. Results were expressed as the mean value of percentage of apoptotic cells ± SE obtained from three independent experiments. Statistical analysis of differences of sanguinarine-treated samples versus control: $P < 0.001$ by Student's *t*-test in each panel, except where indicated as NS (not significant, $P = 0.78$).

Fig. 2



In-vitro effects of different concentrations of sanguinarine (SANG), 5-fluorouracil (5-FU), oxaliplatin (OXA), camptothecin (CPT), or etoposide (VP-16) on apoptosis in DHD/K12/TRb cells. (a) Results were expressed as the mean value of percentage of apoptotic cells ± SE obtained from three independent experiments by TUNEL assay. (b) Results were expressed as mean values ± SD of the EC25 (drug concentration causing 25% induction of cell apoptosis) calculated in three independent experiments.

In an attempt to compare the proapoptotic activity of sanguinarine with that of selected cytotoxic drugs, which are effective in the control of human CRC cells growth, we exposed in-vitro DHD/K12/TRb cells to different concentrations of sanguinarine, 5-FU, OXA, CPT, and VP-16. The results of dose–response experiments are shown in Fig. 2a. The EC25 for induction of apoptosis was then calculated and used for comparison of sanguinarine versus all cytotoxic agents (Fig. 2b). Interestingly, the EC25 of sanguinarine was very close to that of OXA, CPT, and VP-16 but was considerably lower than that of 5-FU.

These results clearly pointed out that sanguinarine was active against the tested colorectal carcinoma cell line and, thus, was suitable for subsequent in-vivo investigations.

In-vivo studies

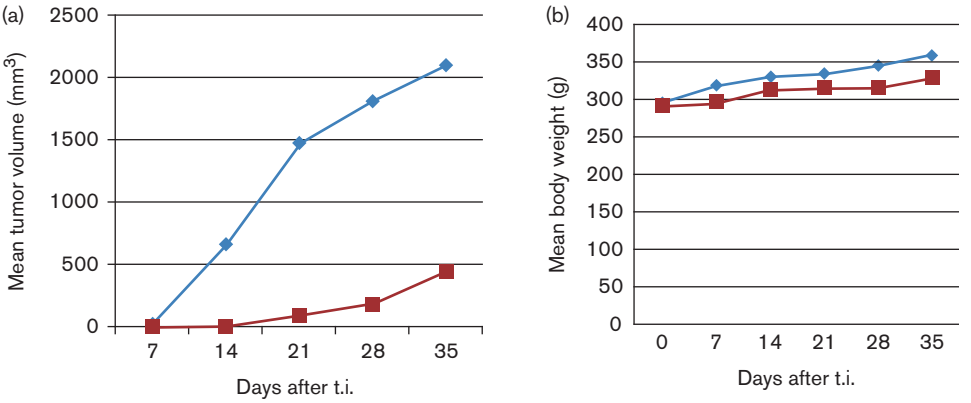
In our experiments, BDIX male rats were injected s.c. with DHD/K12/TRb cells and treated orally with sanguinarine (5 mg/kg/day) or control diluent, daily, week-end off, throughout the experimental period. Results showed a clear therapeutic activity of sanguinarine administration, which resulted in a more than 70% inhibition of s.c. tumor growth as compared with the tumor-bearing controls ($P < 0.01$ by the Wilcoxon rank sum test; Fig. 3a).

Body weights (Fig. 3b) did not significantly differ between the two treatment groups, thus suggesting the absence of drug toxicity at the selected dose level. The sanguinarine-treated rats did not show any other sign of toxicity related to the treatment, such as reduced food consumptions, loss of vigor, reduced activity in hind feet, or decreased body temperature (data not shown). Finally, the repeated evaluation of blood cell counts and of clinical chemistry parameters, which was performed in individual animals throughout the experimental period, showed neither hematologic (Table 1), nor liver and pancreatic (Table 2) toxicity in sanguinarine-treated rats as compared with the controls.

Ex-vivo analyses of cell proliferation and apoptosis

Histopathological analysis carried out on tumors explanted from sanguinarine-treated rats showed the

Fig. 3



Effects of chronic oral administration of sanguinarine on tumor growth and body weight in BDIX rats injected subcutaneously (s.c.) with DHD/K12/TRb cells. On day 0, 8-week-old BDIX rats were injected s.c. with DHD/K12/TRb cells (1.5×10^6/300 µl sterile saline/animals). On day 1, after tumor inoculation (t.i.), the animals were randomized and divided into two groups (10 rats/group): (◆) rats treated with control diluent; (■) rats chronically treated with sanguinarine (5 mg/kg/day *per os*). The experiment was repeated at least twice with similar results. (a) Kinetics of s.c. DHD/K12/TRb tumor growth. Points represent the mean value of tumor volume obtained from all rats in each group (10 animals/group); the SD for each point was less than 10% of the mean value reported; (b) kinetic of body weight of animals in the two experimental groups above indicated. Points represent the mean body weight obtained by repeated weighting of individual rats in each group (10 animals/group); the SD for each point was less than 10% of the mean value reported.

Table 1 Effects of chronic oral administration of sanguinarine on hematologic measurements in BDIX rats injected subcutaneously with DHD/K12/TRb tumor cells

		Day 15		Day 36	
	Day 0	T	T + Sang	T	T + Sang
RBC (10^6/mm^3)	10.1 ± 0.1	11 ± 1.3	11 ± 0.3	10.7 ± 0.8	9.3 ± 0.8
MCV (µm^3)	47.1 ± 0.1	47.5 ± 0.1	47.8 ± 0.8	47.5 ± 0.6	47.1 ± 0.8
HCT (%)	47.6 ± 0.1	52.3 ± 3	52.7 ± 2.2	51 ± 3.3	44 ± 3.1
PLT (10^3/mm^3)	605 ± 50	556 ± 55	629 ± 20	815 ± 8	545 ± 38
MPV (µm^3)	5.8 ± 0.1	5.9 ± 0.1	5.8 ± 0.1	5.9 ± 0.2	5.8 ± 0.1
WBC (10^3/mm^3)	10.7 ± 0.1	12.9 ± 2	12.4 ± 0.7	10.4 ± 1.1	8.5 ± 0.0
HGB (g/dl)	18.1 ± 0.1	19.9 ± 2.1	20.1 ± 0.9	18.9 ± 1.5	16.6 ± 1.0
MCH (pg)	17.9 ± 0.1	18 ± 0.2	18.3 ± 0.4	17.5 ± 0.1	17.7 ± 0.7
MCHC (g/dl)	38.1 ± 0.1	38 ± 0.4	38.2 ± 0.1	37 ± 0.6	37.7 ± 1.6
RDW (%)	16.4 ± 0.1	16.9 ± 0.7	16.9 ± 0.5	17.7 ± 0.4	17 ± 0.7
LYM (%)	6.8 ± 0.1	8.1 ± 0.8	8 ± 0.4	8 ± 0.3	6.1 ± 0.3
MID (%)	2.8 ± 0.0	3.7 ± 0.0	3.3 ± 0.0	1.6 ± 0.0	1.7 ± 0.0
GRAN (%)	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	0.8 ± 0.0	0.7 ± 0.0
LYM (10^3/mm^3)	64.2 ± 1.3	62.8 ± 1	65.5 ± 1.2	77.7 ± 2	72 ± 4.3
MID (10^3/mm^3)	26.9 ± 2.6	28.6 ± 2.3	25.8 ± 3.2	15 ± 0.9	20.6 ± 1.3
GRAN (10^3/mm^3)	8.9 ± 0.1	8.7 ± 0.8	8.7 ± 0.8	7.3 ± 0.1	7.4 ± 0.3
PCT (%)	0.4 ± 0.0	0.3 ± 0.0	0.36 ± 0.0	0.48 ± 0.0	0.31 ± 0.0
PDW (fl)	8.5 ± 0.3	8.7 ± 0.5	8.7 ± 0.2	8.6 ± 0.1	8.6 ± 0.3
(%)	5.4 ± 0.0	5.2 ± 0.0	5 ± 0.0	8.5 ± 0.0	5.2 ± 0.0

Sang, sanguinarine; T, tumor cells.

Table 2 Effects of chronic oral administration of sanguinarine on clinical chemistry measurements in BDIX rats injected subcutaneously with DHD/K12/TRb tumor cells

		Day 15		Day 36	
	Day 0	T	T + Sang	T	T + Sang
PCR (mg/dl)	3.27 ± 0.3	4.80 ± 4.3	5.3 ± 0.4	4.27 ± 0.4	2.72 ± 0.3
BUN (mg/dl)	36 ± 1.7	35 ± 1.8	41 ± 2.3	34.5 ± 1.3	30 ± 2.3
CHO (mg/dl)	48 ± 8.0	60 ± 7.0	64 ± 5.9	40.5 ± 5.4	48 ± 0.0
TRI (mg/dl)	248 ± 25	265 ± 26	259 ± 30	249 ± 22	252 ± 32.3
AST (U/l)	112 ± 8.3	139 ± 8.2	122 ± 9.0	193 ± 26	168 ± 13.8
ALT (U/l)	57.2 ± 5.3	59.2 ± 6.3	53.4 ± 8.3	44.8 ± 9.3	52 ± 9.7
ALP (U/l)	486 ± 3	476 ± 5	603 ± 9	620 ± 23	596 ± 9.2
BIL (mg/dl)	0.35 ± 0.2	0.4 ± 0.1	0.3 ± 0	0.35 ± 0.5	0.4 ± 0.0
AMY (U/l)	2996 ± 92	3096 ± 102	3089 ± 122	3190 ± 244	3522 ± 279
LDH (U/l)	944 ± 79	964 ± 99	682 ± 101	2717 ± 516	2274 ± 145

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; AST, aspartate aminotransferase; BIL, bilirubin; BUN, blood urea nitrogen; CHO, total cholesterol; LDH, lactate dehydrogenase; Sang, sanguinarine; T, tumor cells; TRI, triglycerides.

presence of a higher number of apoptotic/necrotic cells as compared with untreated controls (Fig. 4), suggesting that the inhibitory activity of sanguinarine on tumor growth was possibly due to cell death induction rather than cell differentiation, leading to tumor reversion.

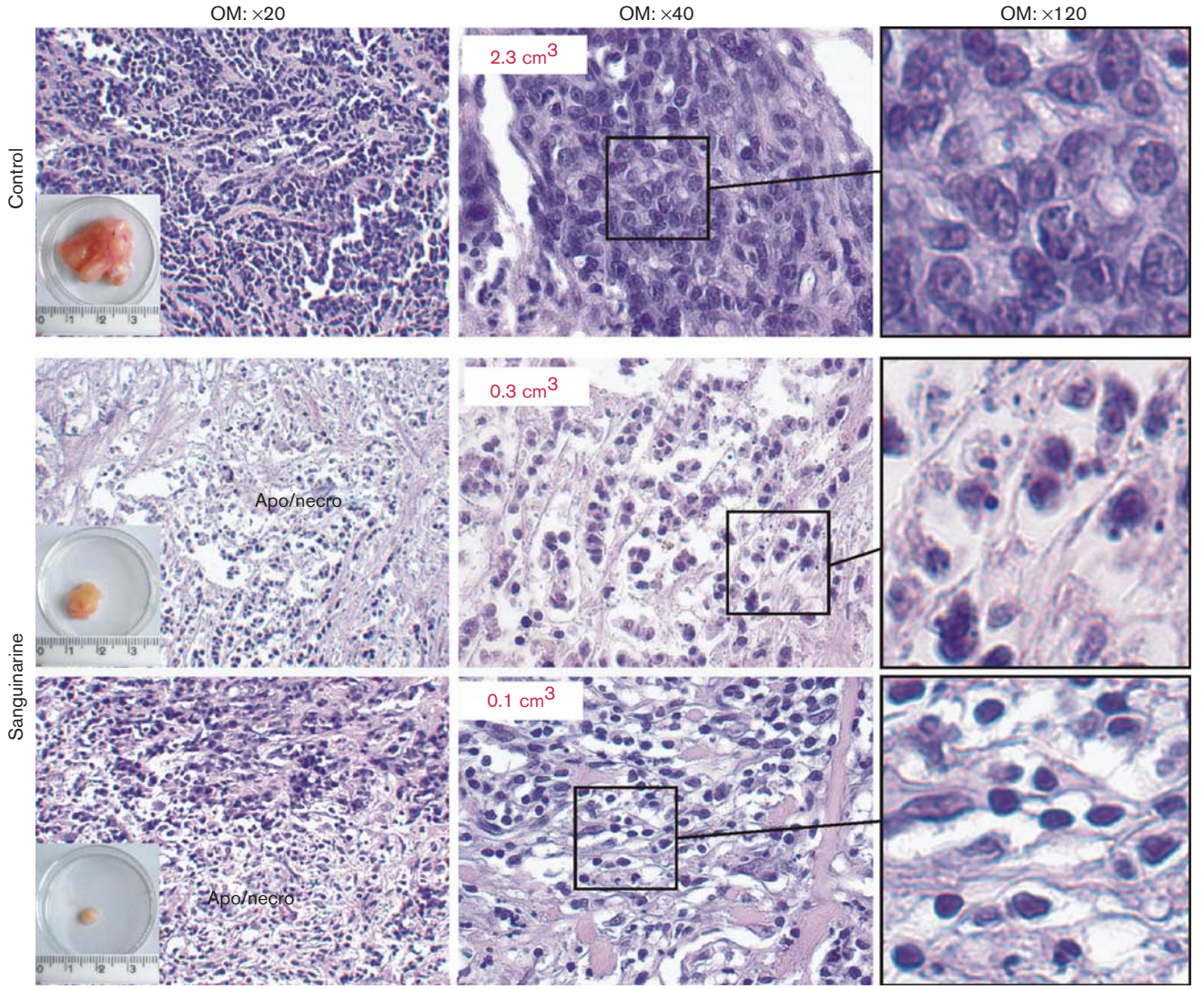
This was also confirmed by the TUNEL assay that tumors from sanguinarine-treated rats, recorded a mean increment in the percentage of apoptotic cells of approximately 13-fold as compared with controls (Fig. 5a; $P < 0.005$). Meanwhile, cancer cell proliferation, evaluated by specific immunostaining of Ki67-positive cells (Fig. 5b), was found to be inhibited, albeit at lower level (1.5-fold; $P < 0.01$).

Furthermore, a dramatic reduction of the peritumoral vascularization, mainly affecting the small endothelial vessels, was observed in tumor explanted from sanguinarine-treated rats (Fig. 6), suggesting that an antiangiogenic process might concur with apoptosis induction.

Discussion

Experimental data from this study support the concept that the in-vivo oral administration of sanguinarine can be effective in the control of DHD/K12/TRb colorectal adenocarcinoma growth in syngeneic rats in the absence of any toxic or side effects to the animals.

Fig. 4



Representative images of hematoxylin–eosin-stained sections of tumors explanted from control and sanguinarine-treated BDIX rats at the end of the study (day 36). Apoptotic/necrotic areas (Apo/Necro) are marked in tumors from treated animals. OM, original magnification. Images of the explanted tumors and their size in cm^3 are also reported.

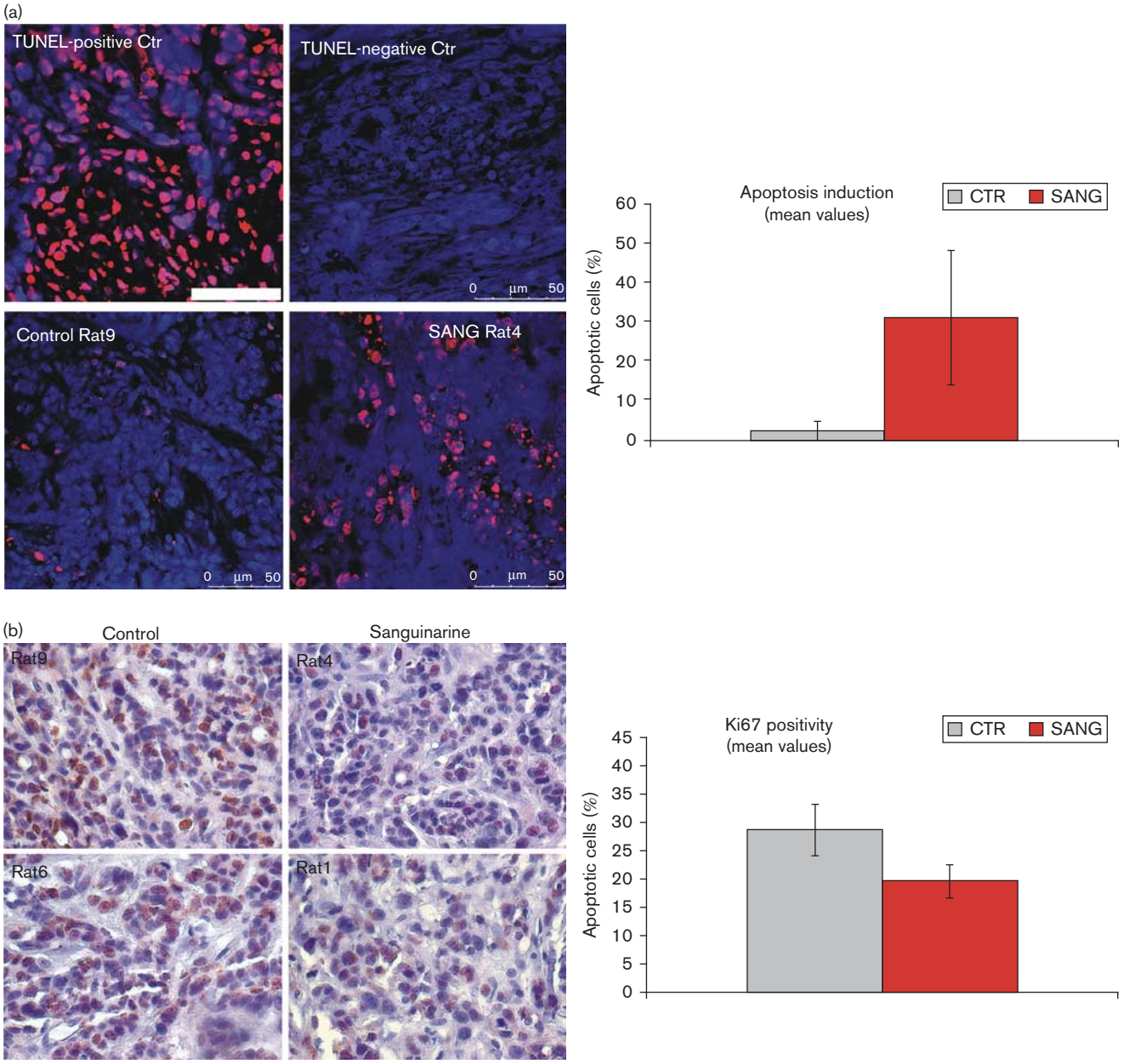
During a preliminary in-vitro screening, we found that micromolar concentrations of the drug were able to inhibit dose-dependently DHD/K12/TRb cell proliferation and metabolism. In the same cell line, sanguinarine was also capable of inducing cell death by apoptosis at levels comparable with those induced by cytotoxic drugs commonly used for the treatment of human CRC [2].

These findings are in keeping with previous observations showing that the cytotoxic and DNA-damaging effects of sanguinarine are selective against cancer cells as compared with normal cells [11,41,42], a finding that is not confirmed by other studies, however [16].

Sanguinarine-induced apoptosis has been shown occurring through multiple pathways, which involve inhibition of nuclear factor-kB activation [10], cell cycle arrest [12], and mitochondrial damage [43].

Reactive oxygen species generation, decrease in the protein levels of Bcl-2, c-IAP2, XIAP, and c-FLIPs, and activation of caspases have been found to be associated with sanguinarine-induced apoptosis in human breast cancer cells [44]. Consistently, pretreatment of these cells with *N*-acetylcysteine or glutathione antagonizes sanguinarine-induced apoptosis, outlining the critical role of reactive oxygen species generation in this cell death [44]. It has been also reported that sanguinarine sensitizes breast cancer cells to TRAIL-mediated apoptosis, which highlights the possibility that a strategy using sanguinarine plus TRAIL or conventional

Fig. 5

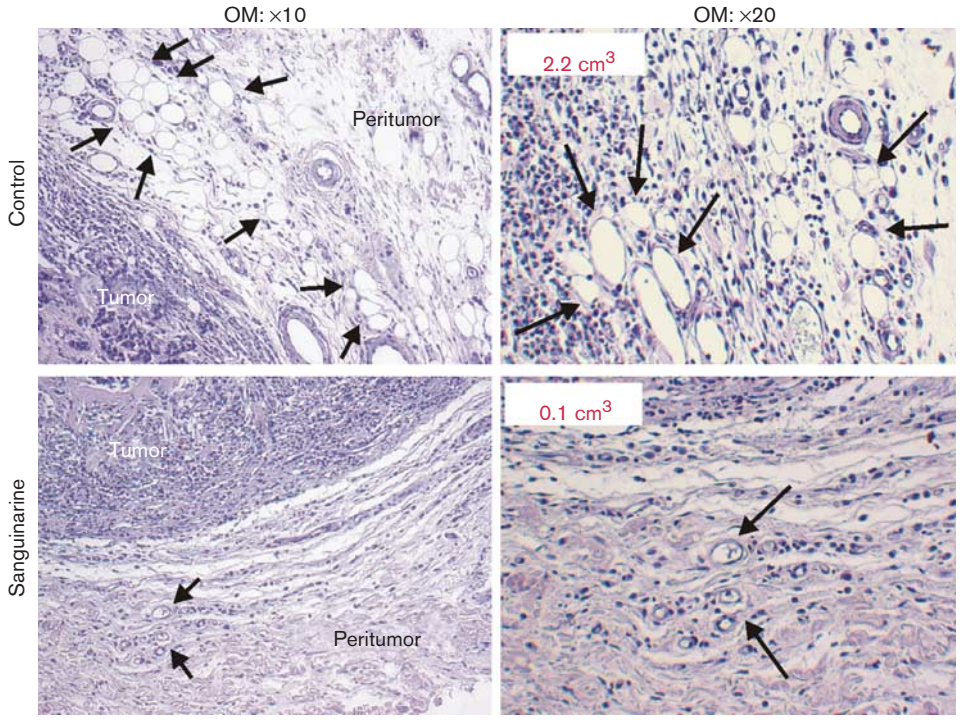


Ex-vivo analyses of tumor cell proliferation and apoptosis. (a) In-situ apoptosis detection by the TUNEL assay. Confocal microscopic images representative of TUNEL staining of tumors explanted from control and sanguinarine-treated BDIX rats on day 36 (left panels); positive and negative controls of TUNEL staining consisted of sections incubated with DNAse (1 U/µl) or with the TUNEL mixture without TdT, respectively; bar: 50 µm. For quantitative analysis of apoptosis induction (right panel), a minimum of 400 nuclei/section were counted and results are given as the mean value of percentage of cells exhibiting nuclear TUNEL staining ± SD. Statistical analysis of differences: $P < 0.005$ by Student's *t*-test; (b) immunohistochemical analysis of Ki67 expression (brown hue) in tumor explanted from untreated and sanguinarine-treated rats on day 36 (left panels); representative images of two controls and two treated animals are shown. Original magnification: ×40. For quantitative analysis (right panel), a minimum of 600 nuclei/section were counted and results are given as the mean percentage of cells exhibiting nuclear Ki67 positivity ± SD. Statistical analysis of differences: $P < 0.01$ by Student's *t*-test.

chemotherapeutic drugs may be effective against drug-resistant breast cancer cells overexpressing Bcl-2 or Akt [44].

Sanguinarine induces apoptosis in human lung cancer cells through a mechanism associated with the glutathione depletion-dependent caspase activation [24]. Caspase activation is also critical for sanguinarine-induced apoptosis in cells derived from human breast cancer [43], primary effusion lymphoma [18], gastric cancer [22], neuroendocrine tumors [25], and rat glioblastoma [27].

Fig. 6



Histopathological examination of tumors from BDIX rats injected with DHD/K12/TRb cells and treated or not with sanguinarine. Representative images of hematoxylin–eosin-stained sections of subcutaneous tumors explanted on day 36 from control and sanguinarine-treated BDIX rats, showing the dramatic reduction of the peritumoral vascularization, mainly at the small endothelial vessels (arrows), in tumor explanted from sanguinarine-treated rats as compared with controls. The tumor sizes in cm^3 are also reported. OM, original magnification.

It has been shown that cyclooxygenase 2 may rescue prostate cancer cells from sanguinarine-induced apoptosis by a mechanism involving inhibition of NO synthase activity, supporting the suggestion that combined administration of COX-2 inhibitors with sanguinarine may be developed as a strategy in the management of human prostate cancer [14].

It has been also shown that sanguinarine causes DNA damage and p53-independent cell death in human colon cancer cell lines [12].

Despite the above reported and the many other studies on sanguinarine, the complete signaling cascade of its proapoptotic activity and the mechanisms underlying its anticancer activity are only partially known and strictly depend on the cell type studied. Thus, there is a general agreement that the role of this alkaloid in tumor suppression is probably more complex than that presently known or previously hypothesized.

So far, there have been only a few studies about the effects of the in-vivo sanguinarine administration in experimental tumor models, [20,29] and the possible use of this alkaloid in the treatment of cancer remains an open question.

In an attempt to answer this question, we have studied the effects of oral sanguinarine administration in syngeneic BDIX rats injected s.c. with DHD/K12/TRb colorectal adenocarcinoma cells.

Herein, reported results indicate that oral sanguinarine administration is capable of inhibiting more than 70% DHD/K12/TRb tumor growth when compared with untreated tumor-bearing controls. We are not allowed to present survival data as, due to excessive tumor ulceration in the untreated controls, all tumor-bearing rats were killed on day 36 of the study because of bioethical considerations.

Histopathological examination performed on tumor sections from sanguinarine-treated rats showed the presence of a higher number of apoptotic/necrotic cells than in untreated controls. This datum was further confirmed by the TUNEL assay, which documented a 13-fold increase in the percentage of apoptotic cells in tumors from treated animals as compared with untreated controls. At the same time, the ex-vivo analysis of cancer cell proliferation performed by immunohistochemical staining with Ki67, evidenced a 1.5-fold reduction of positive cells in tumors from sanguinarine-treated animals when compared with untreated controls.

As above reported, the antiproliferative activity of sanguinarine has been mainly attributed to its involvement on critical molecular events regulating the cell cycle and apoptotic machinery; yet, its observed therapeutic effect cannot be ascribed solely to a direct antiproliferative activity, as also indicated by data from this study, but more likely it occurs through the induction of a dramatic proapoptotic and antiangiogenic effect. Consistently, we found that the sanguinarine-induced inhibition of tumor development was associated with a dramatic reduction of peritumoral vascularization mainly affecting the small endothelial vessels, which highlights the possibility that an antiangiogenic process might concur with apoptosis induction.

It has been reported that sanguinarine is able to suppress dose-dependently VEGF-induced endothelial cell migration, sprouting, and survival *in vitro*, and is able to inhibit blood vessel formation *in vivo* in mouse Matrigel plugs and the chorioallantoic membrane of chick embryos [20,45]. In keeping with these data, which were obtained in normal animals, our observation that in a rat syngeneic model of CRC the antitumor activity of sanguinarine is associated with marked reduction of peritumoral vascolarization represents the first in-vivo evidence of the antiangiogenic effect of this alkaloid in an experimental tumor model.

Finally, our study presents several limitations. Among these are the limited number of experimental animals studied, the lack of survival data, and the lack of the evaluation of the effects of sanguinarine *in vitro* on normal rat intestinal epithelial cells, the natural counterparts of DHD/K12/TRb tumor cells.

Although bioethical considerations have conditioned the first two limits of our study, the third one was largely overcome by the in-vivo evaluations of the toxicity profile of sanguinarine that we have presented herein. In fact, we provided to address safety issues in our experimental tumor model by exploring the possible toxic or side effects of this alkaloid to cell blood counts as well as to various biochemical and clinical parameters of individual animals belonging to the different experimental groups.

Indeed, sanguinarine had been indicated as one of the principle constituents responsible for the toxicity of *Argemone mexicana* seed oil [46]. In a recent report, serum AST, ALT, and LDH activities, hepatic vacuolization, lipid accumulation, and lipid peroxidation of the liver were found to be increased, and TRI decreased in mice treated with high-dose sanguinarine, indicating damage to the liver [47].

We monitored the serum AST, ALT, ALP, and LDH activities, bilirubin, AMY, blood urea nitrogen, total cholesterol, and TRI in our experimental animals throughout the experimental period and found them to be unmodified by the chronic oral administration of sanguinarine at the dose tested. In clinical medical application, it is known that increases of AST, ALT, and LDH are associated with hepatocyte cytotoxicity whereas ALP and bilirubin values are a better index of biliary diseases. Therefore, our results indicate that sanguinarine, at the dose tested (5 mg/kg/day), neither injured hepatocytes nor did it cause bile stasis and bile canaliculi injury. Similarly, the normality of serum AMY indicated no pancreatic toxicity in rats treated with this dose of sanguinarine. Other authors have recently obtained a significant inhibition of experimental and human melanoma cell growth *in vivo*, using the same dose of sanguinarine without apparent toxicity [18].

It is worth mentioning that tumor-bearing rats chronically treated with oral sanguinarine showed blood cell counts very similar to those of the untreated controls. The lack of hematologic toxicity of sanguinarine in our experimental animals deserves special attention as it is associated with significant tumor growth inhibition. It is known that several anticancer drugs with proven efficacy in the control of experimental and human cancers, unfortunately, induce high hematologic toxicity.

In conclusion, we report that oral sanguinarine administration is able to induce a significant inhibition of the s.c. DHD/K12/TRb tumor development in syngeneic BDIX rats. This effect seems to be due not only to the inhibition of tumor cell proliferation, but more importantly also to the marked cell apoptosis and reduced peritumoral vascularization induced by the sanguinarine administration. Interestingly, this effect occurs in the absence of any toxic or side effects to the animals at the dose tested in this study.

Further in-vivo studies are needed to fully characterize the mechanism/s underlying the inhibitory effects of sanguinarine on angiogenesis and tumor growth, as well as the pharmacological and safety profile of this drug in experimental tumor models. Overall, findings from this study suggest that sanguinarine is a likely candidate for further evaluation in cancer therapy.

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Conflicts of interest

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The authors declare that there are no conflicts of interest relevant to the article.

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