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ORIGINAL ARTICLE

Antitumor and angiostatic peptides from frog skin secretions

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Abstract The discovery of new molecules with potential antitumor activity continues to be of great importance in cancer research. In this respect, natural antimicrobial peptides isolated from various animal species including humans and amphibians have been found to be of particular interest. Here, we report the presence of two anti-proliferative peptides active against cancer cells in the skin secretions of the South American tree frog, *Phyllomedusa bicolor*. The crude skin exudate was fractioned by size exclusion gel followed by reverse-phase HPLC chromatography. After these two purification steps, we identified two fractions that exhibited anti-proliferative activity. Sequence analysis indicated that this activity was due to two antimicrobial α-helical cationic peptides of the dermaseptin family (dermaseptins B2 and B3). This result was confirmed using synthetic dermaseptins. When tested in vitro, synthetic B2 and B3 dermaseptins inhibited the proliferation of the human prostatic adenocarcinoma PC-3 cell line by more than 90%, with an EC_{50} of around 2–3 μM. No effect was observed on the growth of the NIH-3T3 non-tumor mouse cell line with Drs B2, whereas a slight inhibiting effect was observed with Drs B3 at high dose. In addition, the two fractions obtained after size exclusion chromatography also inhibited PC-3 cell colony formation in soft agar. Interestingly, inhibition of the proliferation and differentiation of activated adult bovine aortic endothelial cells was observed in cells treated with these two fractions. Dermaseptins B2 and B3 could, therefore, represent interesting new pharmacological molecules with antitumor and angiostatic properties for the development of a new class of anticancer drugs.

Keywords Antimicrobial peptide · Dermaseptin · Antitumor activity · Angiogenesis

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Introduction

Over the past two decades, amphibian skin secretions have become a pivotal model for the discovery of new bioactive molecules with potential therapeutic activities, such as antimicrobial, antidiabetic, antineoplastic, analgesic, and sleep-inducing properties. Frog dermatous glands synthesize and excrete an extraordinarily rich variety of mammalian-like hormones and neuropeptides, including thyrotropin-releasing hormone, angiotensin, bombesin, skin peptide tyrosine-tyrosine, calcitonin gene-related peptide, and bradykinin, as well as opioid peptides that have potential pharmacological interest for human health (Lazarus and Attila 1993). In addition, these glands also produce huge quantities of cytolytic and cationic antimicrobial peptides (CAPs), thought to be involved in the defense of naked frog skin against microbial invasion and in wound repair (Mangoni 2009). Most CAPs rapidly kill a broad range of bacteria, fungi, yeasts, and protozoa by compromising the structural and functional integrity of the plasma membrane of the target cells and/or inactivating intracellular targets (Matsuzaki 1999; Nicolas 2009; Shai 1999; Yang et al. 2000).

Interestingly, recent studies have reported that some CAPs produced by amphibians and mammals [e.g., aurein 1.2, citropin 1.1, bovine lactoferricin, cathelicidin-derived antimicrobial peptides (AMPs), defensins, and magainin 2] exhibit direct cytotoxic activity against human cancer cells (Hoskin and Ramamoorthy 2008; Mader and Hoskin 2006). Although cancer treatment by conventional chemotherapy is hindered by toxic side effects and frequent development of multi-drug resistance by cancer cells, innate immunity polypeptides seem to overcome these limitations via a unique mechanism of cancer cell killing that involves membrane lysis. Unfortunately, the development of most of these peptides as therapeutic anticancer agents is limited by the fact that they are either not at all or barely tumor selective, causing hemolysis and/or lysis of normal human leukocytes, epithelial cells, and fibroblasts.

We have exploited the properties of skin secretions of the South American tree frog, *Phyllomedusa bicolor,* to exploit a huge library of biologically active peptides, including hormones, neuropeptides, and CAPs in the aim of characterizing strong anti-proliferative activities against human tumor and normal endothelial cells. We used bioassay-driven identification of antitumor and angiostatic activities to isolate dermaseptins B2 (Drs B2) and B3 (Drs B3), two components of *P. bicolor* skin secretions that demonstrated both anti-angiogenic and direct anticancer activities. Drs B2 and Drs B3, which respectively consist of 33 and 28 residues, belong to the dermaseptin gene-based superfamily of AMPs found in hylid frog skin secretions (Amiche et al. 2008; Charpentier et al. 1998; Nicolas and El Amri 2009). These peptides share low micromolar antitumor and angiostatic activities and broad spectrum sterilizing antimicrobial activity, with no hemolytic effects. They could, therefore, represent promising model compounds for the use in the rational development of a new class of antitumor drugs.

Experimental procedures

Materials

Dulbecco's modified Eagle's medium (DMEM) GlutaMAX containing 1,000 mg/L D-glucose and pyruvate, DMEM GlutaMAX containing 4,500 mg/L glucose, RPMI-1640 GlutaMAX, fetal bovine serum (FBS), phosphate buffered saline (PBS), and gentamycin (50 mg/mL) were obtained from Invitrogen Life Technologies Corporation (Cergy Pontoise, France). Bovine serum albumin (BSA) and agar were from Sigma-Aldrich (St Quentin en Fallavier, France), and trypsin (0.05% trypsin and 0.02% EDTA in Dulbecco's PBS (D-PBS)) was from PAA Laboratories (Les Mureaux, France). SDS 20% was obtained from MP Biomedicals Inc. (Vannes, France) and the BCA Protein Assay Kit from Pierce (Brebières, France). Crystal violet was obtained from Gurr–Searle Diagnostic (High Wycombe, Bucks, UK). Collagen type I was isolated in our laboratory (Laaroubi et al. 1994). Recombinant human basic fibroblast growth factor-2 (FGF-2) was purified in the laboratory by sequential heparin-Sepharose and Mono-S chromatography from bacteria (Dauchel et al. 1989; Hamma-Kourbali et al. 2008). Fmoc-protected amino acids and Fmoc-Rink amide PEG MBHA resin were purchased from IRIS Biotech GmbH (Marktredwitz, Germany), Fmoc-Ala Novasyn TGT resin preloaded came from Merck Novabiochem (Darmstadt, Germany), and solvents from SDS (France).

Methods

Breeding of the Amazon tree frog P. bicolor

Phyllomedusa bicolor frogs were housed in large wooden cages (120 × 90 × 90 cm) covered on three sides by plastic mosquito net, as described previously (Seon et al. 2000). Phyllodendron, Potos and Dracena plants were used as perches, and water bowls were provided for nocturnal baths. The frogs were fed crickets. Relative humidity was maintained at 65% by a constantly operating humidifier. The temperature was maintained at 25 ± 1°C. All experiments involving frogs adhered to the ARVO resolution on the use of animals in research and the guidelines of the INSERM (French National Institute of Health and Medical Research) ethics committee on animal research, and were carried out by authorized investigators.

Extraction and purification of peptides from frog skin secretions

Peptide exudates were harvested from four living frog specimens by gentle squeezing of the latero-dorsal portion of the skin. The secretions obtained were dissolved in 10 mL of water, sonicated for 1 min, centrifuged (13,000*g* for 10 min at 4°C), and the resulting supernatant recovered and lyophilized. The dry material (approximately 8 mg) was dissolved in 0.5 mL of buffer (PBS 1×, EDTA 5 mM, pH 6), centrifuged (13,000*g* for 10 min at 4°C), and passed through a 0.22-μm pore-size filter. Finally, the filtered extract was fractionated by size exclusion chromatography (Superdex 75 10/300 GL, GE Healthcare, Orsay, France). Elution was carried out using PBS buffer (pH 6) containing 5 mM EDTA, at a flow rate of 0.5 mL/min. Fractions of 4 mL were collected in polypropylene tubes. The fractions were lyophilized, reconstituted in 500 μL H_2O, desalted (PD-10 desalting column, GE Healthcare; Orsay, France), and tested for anti-proliferative activity. Active peak

fractions were analyzed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC), using a Lichrospher C-18 analytical HPLC column (5 µm, 250 × 4.6 mm; Interchim; Montlucon, France) with a gradient of 20–60% acetonitrile (ACN; 0.5%/min) containing 0.07% trifluoroacetic acid (TFA) in 0.1% TFA/H_2O at a flow rate of 0.75 mL/min. Peaks with absorbance at 220 nm were collected and used for further study of antitumor activity.

Protein determination

The quantity of total protein was determined using the microtiter plate procedure according to the BCA Protein Assay Kit instructions. BSA was used as the standard (Bradford 1976).

Cell lines and culture

The human epithelial prostate adenocarcinoma PC-3 cell line and the mouse fibroblast NIH-3T3 cell line were obtained from the American Type Culture Collection ATCC (Manassas, USA). Adult bovine aortic endothelial (ABAE) cells were isolated as previously described (Gospodarowicz et al. 1983). The NIH-3T3 cells were cultured in DMEM supplemented with 10% (v/v) FBS, the ABAE cells were cultured in DMEM supplemented with 10% FBS and 5 ng/mL FGF-2, and the PC-3 cells were cultured in RPMI supplemented with 5% FBS at 37°C in a controlled humidified environment with 7% CO_2.

In vitro proliferation assay

The cells were seeded at a density of 10^4 cells/well in 24 well plates in complete medium and incubated at 37°C in a controlled humidified 7% CO_2 environment. On the first, third, and fifth days after plating, the cells were treated with chromatography fractions or synthetic peptide. The cells were fixed with absolute ethanol 24 h after the last treatment, and cell counting was carried out with crystal violet staining, as previously described (Gillies et al. 1986).

In vitro angiogenesis assay

Differentiation of the ABAE cell line induced by FGF-2 was tested on three-dimensional collagen gels prepared according to the Montesano procedure, with minor modifications (Laaroubi et al. 1994). In brief, 10^5 ABAE cells per well of a 4-well culture plate were seeded onto the collagen layer in complete medium supplemented with 5% FBS and FGF-2 (20 ng/mL). Immediately and 48 h after plating, the cells were treated with the fast protein liquid chromatography (FPLC) fractions at a final concentration of 5 µg/mL. After 48 h of the last treatment, tubular network structures were observed and quantified, using an Axiovert 10 photozoom inverted microscope connected to a digital camera (Axiocam MRm Zeiss, Germany). Quantification of capillaries was determined by analyzing five randomly chosen fields per well in triplicate. The results are expressed as the number of pseudo capillaries per field.

Soft agar assay

PC-3 cells (10^4 cells/well) were added to 1 mL of 0.35% top agar in complete medium containing the chromatography fractions and plated onto a 0.8% agar bottom in 12-well plates. After polymerization, the agar/cell layer was covered with 0.5 mL of complete medium. The cells were first treated with the fractions (5 µg/mL) 24 h after plating, then every 48 h for 10 days. A photozoom inverted Axiovert 10 microscope connected to a digital camera (Axiocam MRm Zeiss; Germany) equipped with a 10× magnification measuring grid was used to count the number of colonies per mm^2 of diameter greater than 50 µm, which were scored as positive. The number of colonies was determined by analyzing five fields per well in triplicate.

Mass spectrometric analysis and N-terminal sequencing

MALDI-TOF–MS

HPLC fractions with anti-proliferative activity were analyzed with a Voyager DE-PRO matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and 4700 Proteomic Analyzer MALDI-TOF-TOF (Applied Biosystems; Courtabœuf, France), using the α-cyano-4-hydroxycinnamic acid matrix. Samples (1 µL) were mixed with a saturated matrix solution (1 µL in acetonitrile: H_2O 0.1% TFA 1:1) and spotted onto a sample plate. The MS positive ion spectra were carried out in the reflector mode with external calibration using the 4700 standard kit.

Automated Edman's degradation

Primary structures of the isolated peptides were determined by automated Edman's degradation, using an Applied Biosystems model 492cLC Procise sequanator (Courtabœuf, France). The amino acid sequences obtained were compared with those of known proteins in the Swiss-Prot, PIR, Genpept, and PDB databases, using the FASTA Web-accessible search program.

Solid-phase peptide synthesis

Drs B2 and B3 were synthesized using solid-phase Fast-Moc chemistry procedures on an automated peptide

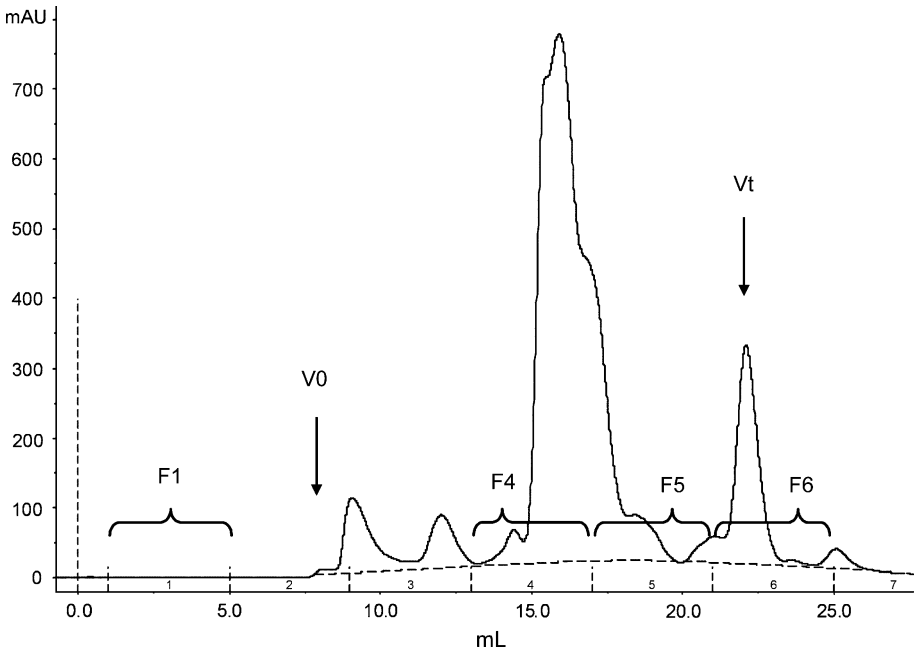

Fig. 1 Size exclusion chromatogram of *P. bicolor* skin secretions: skin secretions from *Phyllomedusa bicolor* were fractionated by FPLC using Superdex 75 10/300 GL column. *Vertical arrows* indicate the volume of elution of the V_o (7.82 mL) and V_t (21.45 mL) of the column, respectively. *F1*, *F4*, *F5* and *F6* correspond to the fractions (4 mL) collected

synthesizer 433A (Applied Biosystems; Courtabœuf, France). For each peptide, the peptidyl-resin (Fmoc-Rink amide PEG MBHA resin for Drs B2 and Novasyn TGT resin preloaded with Fmoc-Ala for Drs B3) and side chain protection bonds were cleaved by incubating in a mixture of 95% TFA, 2.5% triisopropylsilane, and 2.5% H_2O for 2 h at room temperature (Auvynet et al. 2006). The resulting mixture was filtered to remove the resin and the crude peptide precipitated with cold ether. It was recovered by centrifugation at 5,000*g* for 15 min at 4°C, washed three times with cold ether, dried, dissolved in 10% acetic acid, and lyophilized. The crude peptides were purified by RP-HPLC on a Waters RCM compact preparative cartridge module (300 Å; 25 × 100 mm) and eluted at a flow rate of 8 mL/min by a 0–60% linear gradient of ACN (0.07% TFA) in 0.1% TFA/H_2O (1% ACN/min). The homogeneity and identity of the synthetic peptides were assessed by MALDI-TOF mass spectrometry (Voyager DE-PRO, Applied Biosystems, Courtabœuf, France) and analytical RP-HPLC with a Lichrospher 5 mm C-18 column (Interchim; Montluçon, France).

Statistical analysis

The results were expressed as mean ± SEM of at least three determinations for each test from three independent experiments. Statistical analyses were performed using the program GraphPad Prism 5 (GraphPad Software) and using the unpaired *t* test. The statistical significance of the differences is given as $*p < 0.05$; $**p < 0.01$, and $***p < 0.001$ (the * is used to indicate comparisons versus control conditions).

Results

To investigate whether skin secretions of *P. bicolor* contained molecules with anti-proliferative activity toward tumor cells, we fractionated the skin extract into six separated fractions (F1–F6) by size exclusion chromatography in a Superdex 75 column. As shown in Fig. 1, more than 80% of the total material was collected in fractions F4, F5, and F6, with retention volumes of 7.82 and 21.45 mL, corresponding to dextran blue and potassium bichromate markers used to respectively determine the V_o and the V_t of the column. We first tested these three fractions on the proliferation of normal (mouse fibroblast NIH-3T3) and tumor (human prostate adenocarcinoma PC-3) cell lines. Fraction F1, corresponding to elution of the buffer with no detectable material, was used as the negative control.

Effects of various chromatography fractions on the proliferation of normal and tumor cells in vitro

We evaluated in vitro proliferation of the adherent NIH-3T3 and PC-3 cell lines following treatment with various concentrations of the F1, F4, F5, and F6 fractions. As shown in Fig. 2a, no significant effect on cell proliferation in the NIH-3T3 non-tumor cell line treated with the F1, F4, and F5 fractions was found, whereas a slight stimulation of was observed with the F6 fraction. When a similar experiment was carried out on the PC-3 tumor cell line, significant dose-dependent inhibition was observed with the F4 and F5 fractions, when compared with untreated cells or to the control group treated with fraction F1 (Fig. 2b). It is worth noting that when cells were treated with 5 or 10 μg/mL of

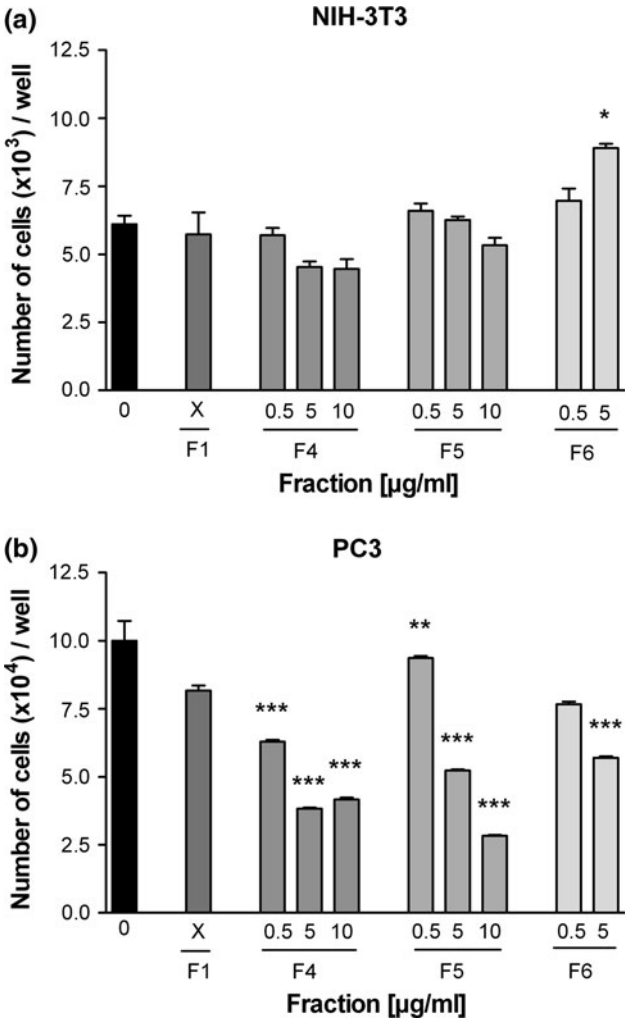

Fig. 2 Effect of different fractions extracted from frog skin secretion on proliferation of the non-tumor cell line NIH-3T3 and the tumor cell line PC-3: proliferation of **a** the non-tumor cell line NIH-3T3 (immortalized mouse fibroblasts) and of **b** the tumor cell line PC-3 (human prostate) was performed on plastic 24 wells plates (1.91 cm^2; cell density of 1×10^4 cells/well/0.5 mL). 24 and 72 h after plating, the cells were treated with the fractions (*F1*, *F4*, *F5* and *F6*) at different concentrations. *X* volume used for P1 is equal to the highest volume which was used in this test. 24 h after the last treatment, cell counting was performed with crystal violet staining. The results are expressed in the number of cells per well. The results represent the mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus F1 as negative control

fraction F4 and F5 proteins, the inhibition obtained was more than 50% in comparison with the control.

Effects of the various chromatography fractions on PC-3 colony formation in vitro

Because anti-proliferative activity was found when the PC-3 tumor cell line was treated with fractions F4, F5, and F6, we wondered whether this effect would be observed when PC-3 cells were cultured in soft agar, a model used to evaluate anchorage-independent growth, a hallmark of malignant transformation. As shown in Fig. 3a and b, treatment with 5 µg/mL of F4 and F5 protein fractions significantly inhibited PC-3 cell colony formation. The inhibition observed with the F4 fraction was as effective as that obtained with 5 µM of 5-fluoro-uracil (5-FU), used as a positive control. Surprisingly, the same concentration of fraction F6 had no effect on colony formation, as did fraction F1, used as the control. These experiments confirmed the anti-proliferative activity of the F4 and F5 fractions in the PC-3 human tumor cell line.

Effects of various chromatography fractions on endothelial cell proliferation and differentiation in vitro

Because angiogenesis is essential for the development and growth of tumors, we investigated whether the various fractions had an inhibitory effect on the differentiation and proliferation of endothelial cells in vitro. As seen in Fig. 4, a significant inhibition of ABAE cell proliferation was alone observed in the presence of 10 µg/mL of the F4 and F5 fractions. No inhibition was observed with the F6 fraction, but a slight stimulation of cell proliferation.

We then evaluated the effects of various fractions on endothelial cell differentiation in the Montessano model. As represented in Fig. 5a and b, when a monolayer of ABAE cells was cultured on a three-dimensional collagen I layer in the presence of the FGF-2 angiogenic factor, tubular network formation was observed within 72 h. As observed, when the ABAE cells were cultured in the presence of 5 µg/mL of fraction F4 or F5, the capillary network formation was strongly inhibited by more than 88%. It is noteworthy that these fractions had no effect on the endothelial monolayer itself. Taken together, these data demonstrate the anti-angiogenic activities of fractions F4 and F5. As the previous results showed and the F4 fraction seemed to present the most antitumor and anti-angiogenic activities. The material present in this fraction was therefore further purified by reverse-phase HPLC chromatography.

Purification and identification of the active component of fraction F4

As seen in Fig. 6a, five different peaks were obtained from chromatography of fraction F4, which were then harvested and tested for activity on PC-3 cell proliferation. Figure 6b shows that treatment of these cells with 2.5 and 5 µg/mL of the F4-peak 1 (F4-p1) and the F4-peak 4 (F4-p4) resulted in significant decreases in cell growth, respectively, 15 and 55% for F4-p1 and 60 and 95% for F4-p4, when compared with the untreated control group. Cell proliferation was

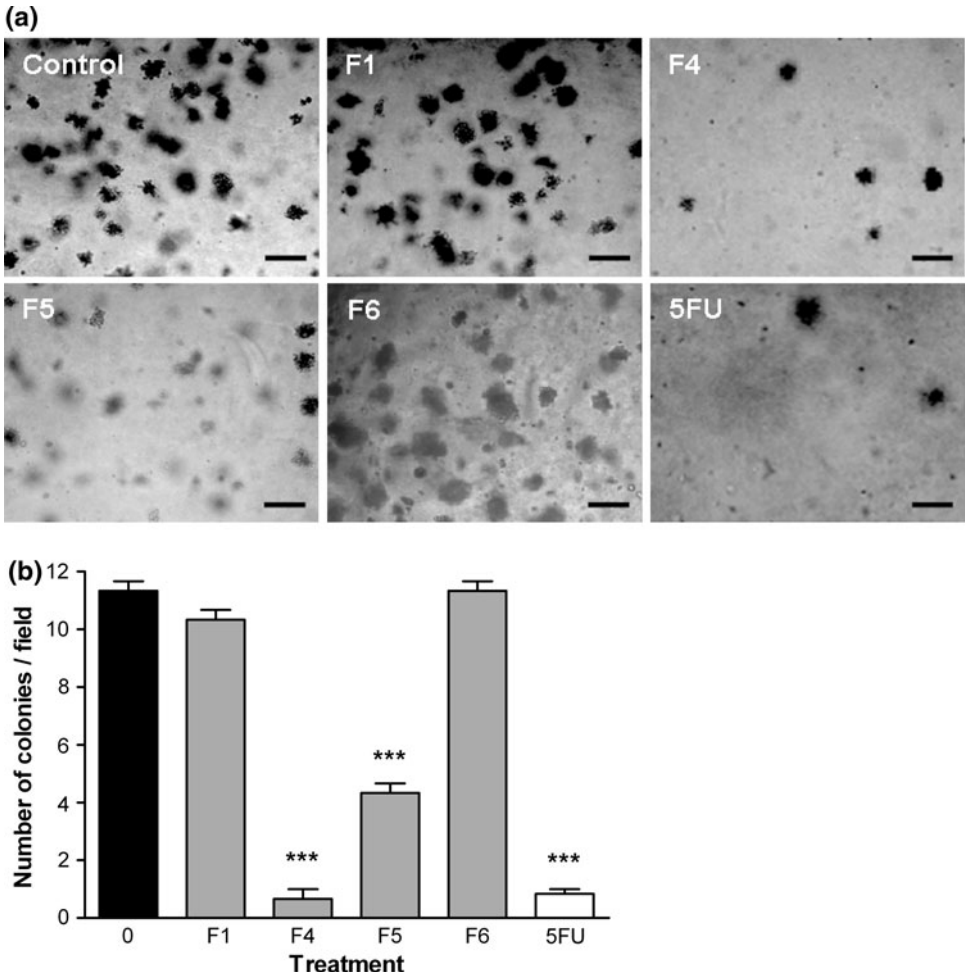

Fig. 3 Effect of different fractions extracted from frog skin secretion on colony formation of the tumor cell line PC-3: the effect of fraction *F1*, *F4*, *F5* and *F6* on colony formation of the tumor cell line PC-3 was tested into soft agar (12 wells plates, 3.8 cm^2; cell density of 1×10^4 cells/well/1 mL). Directly after plating and every 48 h during 10 days, cells were treated with the different fractions (5 µg/mL). Volume used for F1 is equal to the highest volume which was used in this test. After 10 days, the colonies per field, larger than 50 µm were counted. **a** Photos of colony formation (10× magnification, *bar* 100 µm). **b** Quantification of these results is expressed in the number of colonies per field. The results represent the mean of five fields ± SEM of three determinations. ***$p < 0.001$ versus F1 as negative control

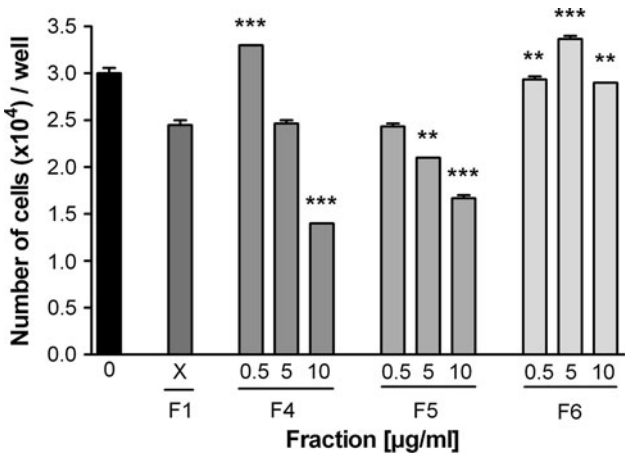

Fig. 4 Effect of fraction *F1*, *F4*, *F5* and *F6* extracted from frog skin secretions on proliferation of the aortic bovine endothelial cell (ABAE): the experiment was performed as described in Fig. 2. The results are expressed in the number of cells per well. The results represent the mean ± SEM of three determinations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus F1 as negative control

also diminished after treatment with F4-p2 and F4-p3 at a concentration of 5 µg/mL, but in smaller quantities, respectively, 20% for F4-p2 and 40% for F4-p3. In contrast, the treatment of these cells with F4-p5 at concentrations between 0.1 and 2.5 µg/mL showed a slight (bell shaped) increase in cell proliferation, which reached maximal effect at 20%, when compared with untreated cells treated at a concentration of 1 µg/mL. Because F4-p1 and F4-p4 exhibited strong anti-proliferative activity on PC-3 cells, we decided to characterize the primary structures of the bioactive molecule(s) present in these peaks by automated Edman degradation. Surprisingly, we found that the sequences determined in the F4-p1 and F4-p4 peaks, respectively, corresponded to the AMPs dermaseptin B2 (Drs B2) (*GLWSKIKEVGKEAAKAAAKAAGKAALGAVSE AV-CONH2*) and dermaseptin B3 (Drs B3) (*ALWKNML KGIGKLAGQAALGAVK TLVGA-COOH*) (Mor et al. 1994). To confirm this observation, the materials in F4-p1 and F4-p4 were subjected to an electrospray mass spectrometric analyzer. The molecular mass of the F4-p1 ion product

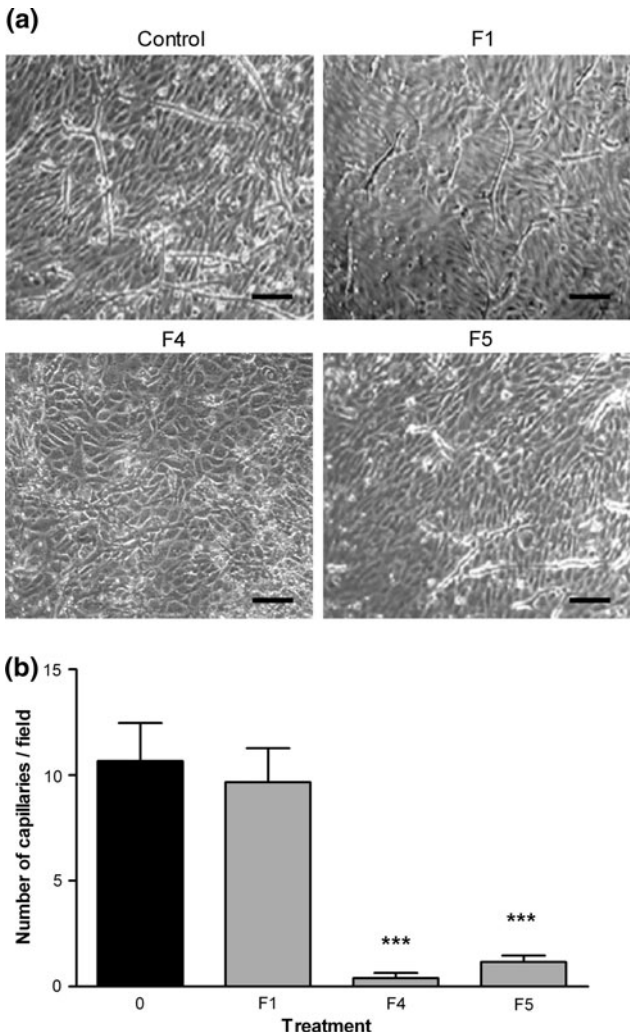

Fig. 5 Effect of different fractions extracted from frog skin secretions on differentiation of the endothelial cell line ABAE: the effect of fraction *F1*, *F4* and *F5* on differentiation of ABAE cells on collagen induced by FGF-2 (20 ng/mL) was tested with the Montesano test (12 wells plates, 3.8 cm^2; cell density of 1×10^5 cells/well/1 mL). Directly and 48 h after plating, cells were treated with the fractions (5 μg/mL). Volume used for F1 is equal to the highest volume which was used in this test. After 48 h of the last treatment, pseudo capillaries were counted. **a** Photos of capillary formation (expansion ×10, *bar* 100 μm). **b** Quantification of these results is expressed in the number of pseudo capillaries per field. The results represent the mean of five fields ± SEM of three determinations. ***$p < 0.001$ versus F1 as negative control

$[M+H]^+$ was 3,179.9, which corresponds to Drs B2 (calculated monoisotopic mass 3,178.8) and that of the F4-p4 product was $[M+H]^+$ 2,780.3, corresponding to Drs B3 (calculated monoisotopic mass 2,778.6).

Effect of dermaseptin B2 and dermaseptin B3 on in vitro NIH-3T3 and PC-3 proliferation

Since Drs B2 and Drs B3 were identified as the components present in F4-p1 and F4-p4, respectively, we decided to confirm their anti-proliferative activity on PC-3 cells using synthetic peptides. Drs B2 and B3 were also tested on the proliferation of NIH-3T3 cells as a control. As represented in Fig. 7a, dose-dependant inhibition of PC-3 cell proliferation was observed with synthetic Drs B2 with a median effective concentration (EC_{50}) of about 2 μM and more than 90% inhibition when treated at a concentration of 5 or 7.5 μM. In contrast, synthetic Drs B2 had no inhibiting effect when tested on NIH-3T3 cells (Fig. 7b); in fact, a slight stimulation was observed. The treatment of these cell lines with synthetic Drs B3 showed the same pattern as for Drs B2 (Fig. 7c, d) with EC_{50} on PC-3 cells at around 3 μM. The treatment with 7.5 μM of Drs B3 resulted in more than 90% inhibition of PC3-cell proliferation. In contrast, low concentrations of Drs B3 (0.25–1 μM) had a slight stimulating effect on NIH-3T3 cells; however, when treated with 7.5 μM, a slight inhibition (23%) was observed (Fig. 7d). These data confirmed those previously obtained with the natural peptide contained in the skin secretion of *P. bicolor*.

Discussion

Despite the recent advances in treatment, cancer remains a major cause of mortality worldwide. Surgery and irradiation are often used to treat local tumors, but more advanced or metastatic cancers are usually treated with chemotherapy. Unfortunately, cancer cells frequently become resistant to chemotherapeutic agents. Therefore, one of the future goals of cancer research is the development of new classes of anticancer drugs with new modes of action that could selectively target cancer cells, but that lack the toxicity of conventional chemotherapeutic agents and that are unaffected by common mechanisms of chemoresistance.

Here, we report the presence of two peptides that are present in skin secretions of the *P. bicolor* frog to have anti-proliferative activity against cancer cells. After two purification steps, we identified Drs B2 and Drs B3 as candidates for this activity. Drs B2 has previously been described as a 33-residue-long cationic-, α-helical AMP that possesses membrane-damaging activities against a broad spectrum of microorganisms, including bacteria, yeasts, fungi, and protozoa (Amiche et al. 2008; Galanth et al. 2009; Mor and Nicolas 1994). Drs B3 is a 28 amino acid-containing peptide that is highly active against Gram-positive and Gram-negative bacteria with a minimal inhibitory concentration in the 1–10 μM range (Charpentier et al. 1998). The dermaseptin family comprises a large class of membrane-damaging polycationic (Lys-rich) peptides of various lengths (28–34 residues) and amino acid sequences that undergo coil-to-helix transition upon binding to the lipid bilayer of many microorganisms. More than

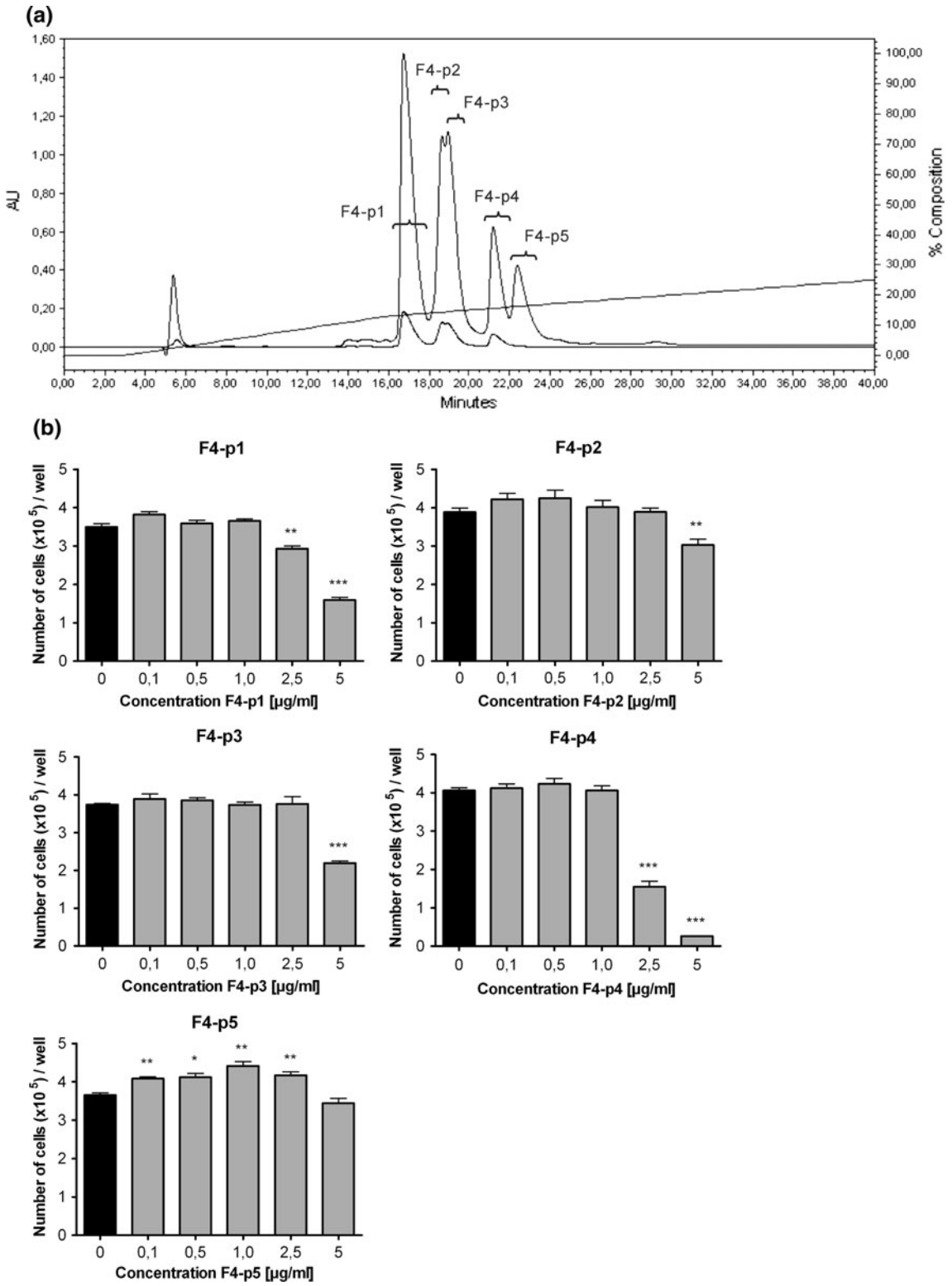

Fig. 6 Elution profile of fraction F4 on reverse-phase chromatography and evaluation of the eluted materials on the proliferation of the tumor cell line PC-3: **a** HPLC chromatogram of the fraction F4, which results from previous fractionation of skin secretion by FPLC (see Fig. 1) and showing five separated peaks (*F4-p1* to *F4-p5*). **b** Proliferation of the tumor cell line PC-3 was performed on plastic 24 wells plates (1.91 cm^2; cell density of 1×10^4 cells/well/0.5 mL). On the first, third and fifth day after plating, the cells were treated with peaks *F4-p1* to *F4-p5* fraction at different concentrations. After 24 h of the last treatment, cell counting was performed with crystal violet staining. The results are expressed in the number of cells per well. The results represent the mean ± SEM of three determinations. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus control (untreated cells)

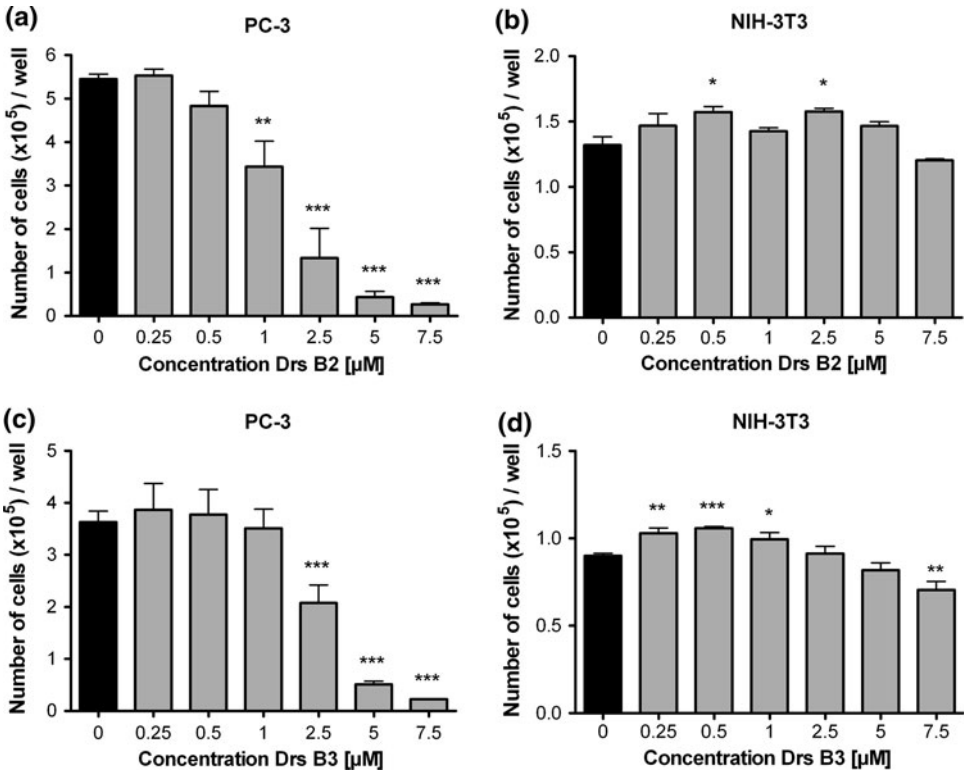

Fig. 7 Effect of dermaseptin B2 and dermaseptin B3 on proliferation of the non-tumor cell line *NIH-3T3* and the tumor cell line *PC-3*: proliferation of **a**, **c** the tumor cell line *PC-3* and of **b**, **d** the non-tumor cell line *NIH-3T3* was performed on plastic 24-well plates (1.91 cm^2; cell density of 1×10^4 cells/well/0.5 mL). On the first, third and fifth day after plating, the cells were treated with **a**, **b** Drs B2 or **c**, **d** Drs B3 at different concentrations. After 24 h of the last treatment, cell counting was performed with crystal violet staining. The results are expressed in the number of cells per well. The results represent the mean ± SEM of three determinations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control (untreated cells)

50 dermaseptins (in the narrow sense) with cationical α-helical amphipathic peptides have been isolated from only 12 species of the phyllomedusinae subfamily (Amiche et al. 2008). They all have a conserved Trp residue at position 3 and an AA[A/G]KAAL[G/N]A sequence consensus motif in the mid-region. Among all the dermaseptins which have been isolated from the skin of *P. bicolor*, Drs B3 is the smallest peptide and the only one that is not amidated at its carboxy terminal extremity (Amiche et al. 2008; Charpentier et al. 1998).

The presence of Drs B2 and Drs B3 in the higher molecular weight fractions (range 25–13 kDa) obtained from the Superdex size exclusion chromatography elution was surprising for peptides of molecular weights of 3.18 and 2.78 kDa, respectively. It should be noted that this chromatography was carried out in PBS buffer. Indeed, Drs B2 and Drs B3 had been previously purified from *P. bicolor* skin secretions in a two-step procedure that included exclusion chromatography under acidic conditions, followed by reverse-phase chromatography in an HPLC system (Charpentier et al. 1998; Mor and Nicolas 1994). Under these conditions, Drs B2 and Drs B3, like the other members of the dermaseptin B family, were eluted in the Sephadex chromatography column from the low molecular weight fraction. It is therefore tempting to speculate that peptides from the skin secretions of frogs could be aggregated in such a way as to be protected from proteases. Further investigations are needed to verify this assumption.

Drs B2 and Drs B3 belong to the class of α-helical CAPs that are isolated from the skin of amphibians. This class of peptides include magainins from the African clawed frog *Xenopus Laevis* (Cruciani et al. 1991; Cruz-Chamorro et al. 2006), gaegurins from the Korean frog *Rana rugosa* (Kim et al. 2003; Won et al. 2006) and aurein 1.2 from the Australian bell frog *Litoria raniformis* (Rozek et al. 2000) that already have been described to have anti-proliferative activities against tumor cells. The molecular mechanism(s) by which these peptides kill selectively the tumor cells is still unclear, but the negatively charged components of the cancer cells membranes were supposed to play an important role in attracting the CAPs to the plasma membrane of the tumor cells like for the lytic activity of CAPs on bacteria (Hoskin and Ramamoorthy 2008). Further studies are needed to determine if such a difference in negatively charged components of the tumor membranes is necessary for the anti-proliferative activity of Drs B2 and Drs B3.

Although magainins or gaegurins have been reported to be cytotoxic for human tumor cells, the reported EC_{50}s were in the 10–100 µM ranges. Similarly, a recent report has described dermaseptin L1, a member of the dermaseptin family isolated from Lemur leaf frog *Hylomantis lemur*, with a selective cytolytic activity against the human hepatoma cell line HepG2 (EC_{50} 45 µM) when compared with erythrocytes (EC_{50} 200 µM) (Conlon et al. 2007). In contrast with these peptides, the observed EC_{50} of Drs B2 and Drs B3 on PC-3 cells were in the low micromolar

range. Furthermore, Drs B2 is not cytotoxic against erythrocytes (median lethal concentration $LC_{50} > 250$ μM) (Galanth et al. 2009) and is devoid of anti-proliferative activity in normal cells even when tested at the highest concentration (7.5 μM).

The difference in sensitivity and toxicity of Drs B2 and Drs B3 towards normal and tumor cells represents a real advantage for their use as pharmacological tools in cancer therapy, and further experiments are in progress to test Drs B2 and Drs B3 on other human cancer cell lines to confirm their potent anticancer activity. Another finding of interest is the ability of Drs B2 and Drs B3 to inhibit normal endothelial cell proliferation and differentiation, which is an essential step in tumor growth (Folkman 1971).

In conclusion, our study describes that Drs B2 and Drs B3 as associated with antitumor and angiostatic activities and could represent interesting basis for the design of analogs that act against the proliferation of human tumor cells with a high selectivity. They now must be evaluated in animal models in vivo. The mechanism of action of Drs B2 and Drs B3 against tumor cells remains to be clarified before further pharmacological development.

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