Cancer Chemother Pharmacol (2011) 68:45–52
DOI 10.1007/s00280-010-1446-2

ORIGINAL ARTICLE

In vitro and in vivo antitumor effects of (4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone

Hemerson Iury F. Magalhães · Daniel P. Bezerra · Bruno C. Cavalcanti · Diego V. Wilke · Rodrigo Rotta · Dênis P. de Lima · Adilson Beatriz · Ana Paula N. N. Alves · Flávio da S. Bitencourt · Ingrid S. T. de Figueiredo · Nylane M. N. Alencar · Letícia V. Costa-Lotufo · Manoel Odorico Moraes · Claudia Pessoa

Received: 4 May 2010 / Accepted: 24 August 2010 / Published online: 7 September 2010

Abstract
Purpose (4-Methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT) is a phenstatin analog compound. PHT is a known tubulin inhibitor that has potent cytotoxic activity. In the present study, PHT was synthesized and its antitumor activity was determined using in vitro and in vivo experimental models.
Methods The in vitro cytotoxic activity of the PHT was determined by the MTT assay. The antimitotic and hemolytic effects were determined based on the inhibition of sea urchin embryo development and lysis of mouse erythrocytes, respectively. In vivo antitumor activity was assessed in mice inoculated with sarcoma 180 cells.
Results In vitro, PHT displayed cytotoxicity in tumor cell lines, showing IC_{50} values in the nanomolar range. In addition, it inhibited sea urchin embryo development during all phases examined, first and third cleavage and blastula stage. However, PHT did not induce hemolysis using mouse erythrocytes, suggesting that the cytotoxicity of PHT does not involve membrane damage. The in vivo study demonstrated tumor inhibition rates of 30.9 and 48.2% for PHT at doses of 20 and 40 mg/kg, respectively. In addition, PHT was also able to increase the response elicited by 5-fluorouracil (5-FU) from 33.3 to 55.7%. The histopathological analysis of liver, kidney, and spleen showed that they were just moderately affected by PHT treatment. Neither enzymatic activity of transaminases nor urea levels were significantly affected. Hematological analysis showed leukopenia after 5-FU treatment, but this effect was prevented when 5-FU was combined with PHT.
Conclusions In conclusion, PHT exhibited in vitro and in vivo antitumor effects without substantial toxicity.

Keywords Phenstatins · Tubulin inhibitor · Antitumor activity · Sarcoma 180 · Toxicity

H. I. F. Magalhães · B. C. Cavalcanti · D. V. Wilke · F. S. Bitencourt · I. S. T. de Figueiredo · N. M. N. Alencar · L. V. Costa-Lotufo · M. O. Moraes · C. Pessoa (✉)
Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil
e-mail: c_pessoa@yahoo.com

D. P. Bezerra (✉)
Departamento de Fisiologia, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brazil
e-mail: danielpbezerra@gmail.com

R. Rotta · D. P. de Lima · A. Beatriz
Departamento de Química (Laboratório LP4), Universidade Federal do Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil

A. P. N. N. Alves
Departamento de Clínica Odontológica, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil

Introduction

A new family of tubulin polymerization inhibitors called phenstatins has attracted much attention due to their interesting cytotoxic effect. Therefore, a large number of studies on phenstatins have been published, and the development of these compounds is protected by patents [1–5]. The first compound, phenstatin, was first synthesized by Pettit et al. [6] during research directed at the study of the structure–activity relationship of combretastatins, as an unexpected product of the oxidation of the silyl derivative of combretastatin A-4 (CA-4) with Jacobsen's complex. Phenstatin was found to be as effective as CA-4 in acting as a tubulin

polymerization inhibitor, preventing cancer cells from dividing [7–9].

(4-Methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT) (Fig. 1) is a known bisarylketone belonging to the phenstatin family. This compound has been studied because of its potent cytotoxicity and ability to inhibit tubulin assembly [4, 8, 10, 11]. Although it has been documented that the growth inhibitory effect of PHT in cancer cells is associated with antitubulin activity, the exact mechanism underlying the cytotoxic effects of PHT is incompletely understood. In particular, its in vivo antitumor activity remains unexplored.

In the present work, PHT was synthesized and tested for antitumor activity using in vitro and in vivo experimental models. In order to evaluate the toxicological aspects related to PHT treatment, hematological, biochemical, histopathological, and morphological analyses of the treated animals were performed.

Materials and methods

(4-Methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT) synthesis

The melting point was determined using a Uniscience of Brazil Mod. 498 apparatus. NMR spectra were recorded on a Bruker DPX-300 spectrometer; the chemical shifts were expressed in ppm (δ) relative to TMS ($\delta = 0.0$), and $CDCl_3$ was employed as the solvent. High-resolution electrospray ionization–mass spectrometry (ESI–MS) analyses were performed using a Q-TOF Micromass spectrometer in both positive- and negative-ion modes with capillary voltage set at ±3,000 V, cone voltage at ±40 V, and desolvation temperature at 100°C.

Thin-layer chromatography (TLC) was carried out on silica gel plates with fluorescence indicator F_{254} (0.2 mm, E. Merck); the spots were visualized under UV light and by spraying with 1% vanillin solution in ethanol or charring reagent. Purification of compounds was performed using column chromatography; the stationary phase was silica gel 60 (80–230 mesh) from ACROS (Brazil), silica gel 60 (230–400 mesh) from Merck and Celite.

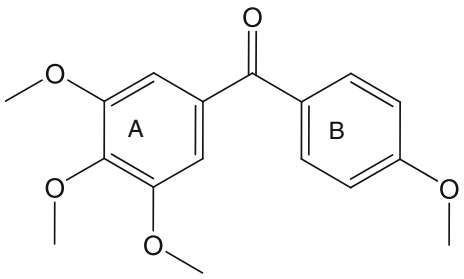

Fig. 1 Chemical structure of (4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT)

The reaction was carried out in a one-neck, 250-ml, round-bottomed flask fitted with a condenser with drying tube. Anhydrous dichloromethane (20 ml), 3,4,5-trimethoxybenzoic acid (1.4 g, 6.6 mmol), and thionyl chloride (1.57 g, 13.2 mmol) were added to the flask. The mixture was refluxed for 4 h, and after cooling to room temperature, the solvent was removed with a rotary evaporator. Dichloromethane (25 ml) was added to the flask and cooled to 0°C. With good stirring, anhydrous aluminum chloride (0.44 g, 3.3 mmol) and anisole (0.72 g, 6.6 mmol) were slowly tapped into the reaction vessel, which required 10 min. After the addition, the reaction mixture was stirred at room temperature for 30 min and then allowed to decompose by pouring ice-cold hydrochloric acid (20 ml) into the flask. After extraction with dichloromethane and washing with cold sodium bicarbonate solution and water, the organic layer was removed using a rotary evaporator. The residue was purified by flash chromatography using an eluent of 5:1 hexane:ethyl acetate. A colorless crystalline solid was obtained. Yield = 80%, m.p. = 67–68°C. NMR-^{1}H (300 MHz, $CDCl_3$): δ 3.82 (bs, 6H); 3.85 (bs, 3H); 3.87 (bs, 3H); 6.91 (d, 2H, J = 8.5 Hz); 6.96 (s, 2H); 7.76 (d, 2H, J = 8.5 Hz). NMR-^{13}C (75 MHz, $CDCl_3$): δ 55.3 (CH_3); 56.1 (2 × CH_3); 60.8 (CH_3); 107.3 (2 × CH); 113.4 (2 × CH); 130.1 (C); 132.2 (2 × CH); 133.9 (C); 141.4 (C); 152.7 (2 × C); 163.0 (C); 194.5 (C). ES/MS: 135.0832.

Animals

A total of 80 Swiss mice (male, 25–30 g), obtained from the central animal house of Universidade Federal do Ceará, Brazil, were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12:12 h light–dark cycle (lights on at 6:00 a.m.). Animals were treated according to the ethical principles for animal experimentation of COBEA (Colégio Brasileiro de Experimentação Animal), Brazil. The Animal Studies Committee of Universidade Federal do Ceará approved the experimental protocol (number 52/08).

Cells

The cytotoxicity of PHT was tested against HL-60 (leukemia), MDA-MB-435 (melanoma), SF-295 (brain), and HCT-8 (colon) human cancer cell lines, all obtained from the National Cancer Institute, Bethesda, MD, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin and incubated at 37°C in a 5% CO_2 atmosphere.

Sarcoma 180 tumor cells have been maintained in the peritoneal cavities of the Swiss mice in the Laboratory of Experimental Oncology of the Universidade Federal do Ceará since the mid-1980s.

In vitro biological evaluation of PHT

Determination of the effect of PHT on tumor cells in culture

Tumor cell growth was determined by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product [12]. For all experiments, cells were seeded in 96-well plates (10^5 cells/well for adherent cells or 0.5×10^5 cells/well for suspended cells in 100 µl of medium). After 24 h, PHT (9–5,000 ng/ml) was added to each well (using the HTS—high-throughput screening—Biomek 3000; Beckman Coulter Inc., Fullerton, CA, USA) and the cells incubated for 72 h. 5-Fluorouracil (5-FU–Sigma Chemical Co. St Louis, MO, USA) was used as the positive control. At the end of incubation, the plates were centrifuged and the medium was replaced by fresh medium (150 µl) containing 0.5 mg/ml MTT. Three hours later, the formazan product was dissolved in 150 µl DMSO, and the absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter Inc., Fullerton, CA, USA). The drug effect was expressed as the percentage of control absorbance of reduced dye at 595 nm.

Determination of the effect of PHT on sea urchin embryo development

The assay was performed following the method described by Jimenez et al. [13]. Adult sea urchins (*Lytechinus variegatus*) were collected at Lagoinha Beach, on the northeastern coast of Brazil. Gamete elimination was induced by injecting 3.0 ml of 0.5 M KCl into the urchin's coelomic cavity. For fertilization, 1 ml of a sperm suspension (0.05 ml of concentrated sperm in 2.45 ml of filtered sea-water) was added to every 50 ml of egg suspension. The assay was carried out in 24-multiwell plates. PHT was added immediately after fertilization (within 2 min) to obtain concentrations ranging from 100 to 100,000 ng/ml in a final volume of 2 ml. At appropriate intervals, 200-µl aliquots were fixed in the same volume of 10% formaldehyde to obtain embryos in the first and third cleavage and blastula stage. A total of 100 eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal cells.

Determination of the effect of PHT on mouse erythrocytes

The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method described by Jimenez et al. [14]. PHT was tested at concentrations ranging from 800 to 200,000 ng/ml. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the hemoglobin released was measured spectrophotometrically as the absorbance at 540 nm.

In vivo biological evaluation of PHT

Determination of the effect of PHT on tumor growth in mice

Ten-day-old sarcoma 180 ascites tumor cells (2×10^6 cells/500 µl) were implanted subcutaneously into the left hind groin of the mice (as described by Bezerra et al. [14]). One day after inoculation, PHT (20 or 40 mg/kg) alone or combined with 5-FU (PHT, 20 mg/kg + 5-FU, 10 mg/kg) was dissolved in 10% DMSO and administered intraperitoneally for 7 days in mice inoculated with sarcoma 180 tumor. The doses were based on previous studies obtained with the CA-4 [15]. 5-Fluorouracil (10 mg/kg) was used as the positive control. Negative control was treated with the vehicle used for diluting the tested substance (10% DMSO). On day 8, peripheral blood samples from control and treated mice were collected from the retro-orbital plexus under light ether anesthesia, and the animals were then sacrificed by cervical dislocation. The tumor, liver, spleen, and kidneys were excised, weighed, and fixed in 10% formaldehyde. The tumor inhibition rate (%) was calculated by the following formula: inhibition rate (%) = $[(A - B)/A] \times 100$, where A is the average tumor weight of the negative control, and B is that of the treated group. Body weights were measured at the start and at the last day of treatment. The blood samples were used for hematological and biochemical analyses.

Toxicological analyses

Determination of the effect of PHT on biochemical parameters

Blood samples of treated animals were collected from the retro-orbital plexus under light ether anesthesia. Biochemical analyses were performed on serum samples obtained after centrifugation of total blood without anticoagulants, at 2,500 rpm for 15 min. Spectrophotometric determination of alanine aminotransferase (ALT or TGP) and aspartate aminotransferase (AST or TGO) enzymatic activities and urea

levels was carried out with standardized diagnostic kits using a LABTEST® spectrophotometer (Lagoa Santa, MG, Brazil).

Determination of the effect of PHT on hematological parameters

For the hematological analysis, an aliquot of blood from each animal was mixed with ethylenediaminetetraacetic acid (EDTA), and hematological parameters (platelet count and total and differential leukocyte counts) were determined by standard manual procedures using light microscopy.

Histopathology and morphological analyses

After fixation with formaldehyde, tumors, livers, spleens, and kidneys were submitted to gross examination for size or color changes and hemorrhage. Portions of the tumor, liver, spleen, and kidney were then cut into small pieces, followed by staining of the histological sections with hematoxylin and eosin. Histological analysis was performed by light microscopy. The presence and extent of liver, kidney, or spleen lesions attributed to the drugs were considered.

Statistical analysis

Data are presented as mean ± SEM or IC_{50} values and their 95% confidence intervals (CI 95%) obtained by nonlinear regression. The differences between experimental groups were compared by ANOVA (analysis of variance) followed by the Student–Newman–Keuls or Bonferroni test ($P < 0.05$). All statistical analyses were performed using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA, USA).

Results

In vitro biological effects of PHT

The in vitro effects of PHT against tumor cell lines were determined, and the results are summarized in Table 1. MTT analysis showed that PHT exhibited cytotoxic activity against all tumor cell lines tested, with IC_{50} values in the nanomolar range after 72 h of incubation. In the preclinical anticancer drug-screening program used in this study, the lead compounds that show IC_{50} values below 1 μM are considered promising [16–18]. Therefore, PHT can be considered a very potent cytotoxic compound.

The antimitotic activity was determined as the ability to inhibit sea urchin embryo development. PHT induced a dose-dependent inhibition of embryo development during all stages examined, first and third cleavage and blastula stage. The IC_{50} values are presented in Table 1. According to Jacobs et al. [19], if a substance causes 100% inhibition in this assay at a concentration of 16,000 ng/ml or less, it may be considered to be very active. Thus, PHT could be considered very active, completely inhibiting sea urchin mitosis at concentrations less than 100 ng/ml. The compound was also tested for its ability to induce lysis of mouse erythrocytes (Table 1). However, PHT was not hemolytic even at the highest concentration tested (200,000 ng/ml).

Table 1 In vitro biological activity of (4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT)

Biological assay	PHT	5-FU
Cytotoxicity against cell lines		
Tumor cell lines		
HL-60	40 (130) 30–50	>5,000 (38,439)
MDA-MB-435	60 (210) 50–60	700 (5,381) 500–950
HCT-8	120 (400) 70–190	200 (1,538) 150–280
SF-295	<9 (30)	280 (2,153) 220–370
Sarcoma 180	90 (290) 60–140	40 (308) 30–60
Antimitotic activity in sea urchin embryos		
Stages examined		
1st cleavage	124 (413) 120–130	Nd
3rd cleavage	149 (596) 140–160	Nd
Blastula	125 (416) 120–130	Nd
Hemolytic assay		
Mouse erythrocytes	>200,000 (661,500)	Nd

Data are presented as IC_{50} values in ng/ml (nM) and 95% confidence interval obtained by nonlinear regression from three independent experiments performed in duplicate. 5-Fluorouracil (5-FU) was used as the positive control

Nd not determined

In vivo biological effects of PHT

The effects of PHT on mice transplanted with sarcoma 180 tumor are shown in Fig. 2. A significant reduction in tumor weight was observed in PHT-treated animals as well as for the PHT plus 5-FU-treated animals ($P < 0.05$). On day 8, the average tumor weight of control mice was 2.23 ± 0.14 g. In the presence of PHT (20 or 40 mg/kg),

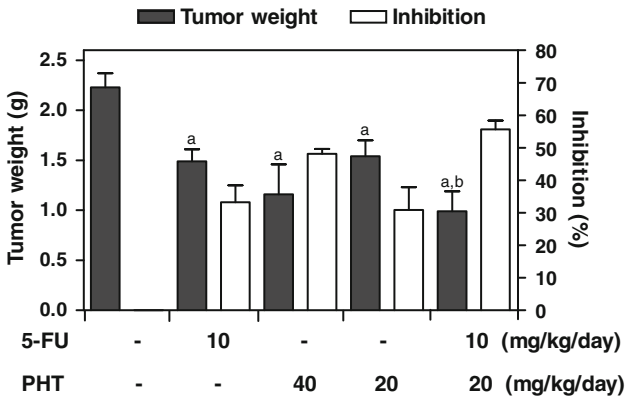

Fig. 2 Effect of (4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT) on mice inoculated with sarcoma 180 tumor. The graph shows tumor weight (g) and tumor growth inhibition levels. Negative control was treated with the vehicle used for diluting the test substance (10% DMSO). 5-Fluorouracil (5-FU) was used as a positive control. Data are presented as mean ± SEM of ten animals. [a] $P < 0.05$ compared with negative control group by ANOVA followed by the Student–Newman–Keuls test. [b] $P < 0.05$ compared with 5-FU only group and 20 mg/kg PHT only group by ANOVA followed by the Student–Newman–Keuls test

the average tumor weights were 1.54 ± 0.16 and 1.16 ± 0.03 g, respectively. Tumor growth inhibition rates were 30.9 and 48.2% for PHT treatment, 20 or 40 mg/kg, respectively. Additionally, PHT was also able to increase the response elicited by 5-FU from 33.3 to 55.7%.

In histopathological analyses, the tumors excised from control mice showed groups of large, round, and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei, and binucleation. Various degrees of cellular and nuclear pleomorphism were seen. Mitosis, muscle invasion, and coagulative necrosis were also noticed. In the tumors excised from treated animals, extensive areas of coagulative necrosis were observed.

Toxicological analyses

After killing the animals, the organs were weighed. No significant changes in the organ weights were seen in PHT or 5-FU-treated animals (Table 2). No significant gains in body weight were seen among the groups ($P > 0.05$) (Table 2).

No significant changes in the renal (urea levels) or liver (enzymatic activity of transaminases: aspartate aminotranspherase—AST and alanine aminotranspherase—ALT) parameters were seen in sarcoma 180-transplanted mice treated with PHT or 5-FU ($P > 0.05$) (data not shown). In the peripheral blood from mice transplanted with sarcoma 180 tumor, 5-FU induced a decrease in total leukocytes ($P < 0.05$). Additionally, the leukopenia observed after 5-FU treatment was prevented when the treatment was combined with PHT (Table 3).

Histopathological analyses of livers removed from PHT-treated animals showed Kupffer cell hyperplasia, hemosiderin-laden macrophages, and ballooning degeneration of hepatocytes. Microvesicular steatosis was also seen in livers removed from animals treated with 5-FU only or 5-FU plus PHT (Fig. 3). Besides the toxic effects observed in livers, histopathological analyses of kidneys removed from PHT-treated animals showed hydropic change, hyaline casts, and glomerular and tubular hemorrhage. On the other hand, glomerular structure was essentially preserved (data not shown). There was no alteration in the spleens removed from mice treated with PHT or PHT plus 5-FU (data not shown).

Table 2 Effect of (4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT) on organ and body weights

Drug	Dose (mg/kg/day)	Increase in body weight (g)	Liver (g/100 g body weight)	Spleen (g/100 g body weight)	Kidney (g/100 g body weight)
Healthy mice					
0.9% NaCl	–	3.24 ± 1.19	4.61 ± 0.19	0.26 ± 0.02	1.07 ± 0.03
10% DMSO	–	3.15 ± 1.02	4.49 ± 0.20	0.38 ± 0.09	1.21 ± 0.05
Mice transplanted with S180					
0.9% NaCl	–	4.99 ± 1.05	5.42 ± 0.07	0.79 ± 0.03	1.33 ± 0.03
10% DMSO	–	6.63 ± 0.10	5.63 ± 0.18	0.93 ± 0.10	1.28 ± 0.05
5-FU	10	5.20 ± 1.02	4.77 ± 0.24	0.81 ± 0.12	1.03 ± 0.08
PHT	40	7.29 ± 0.52	5.56 ± 0.54	0.81 ± 0.08	1.14 ± 0.12
PHT	20	9.20 ± 1.39	5.87 ± 0.39	0.79 ± 0.09	1.24 ± 0.08
PHT + 5-FU	20 + 10	5.56 ± 0.80	5.39 ± 0.38	0.69 ± 0.07	1.27 ± 0.06

Mice were injected with sarcoma 180 (2.0×10^6 cells/animal, s.c.). The animals were treated, starting one day after tumor implantation, for seven consecutive days

Data are presented as mean ± SEM of ten animals. Negative control was treated with the vehicle used for diluting the test substance (10% DMSO). 5-Fluorouracil (5-FU) was used as the positive control

Table 3 Effect of (4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT) on the hematological parameters in peripheral blood

Drug	Dose (mg/kg/day)	Platelets (10^5 cells/ml)	Total leukocytes (10^3 cells/ml)	Differential leukocyte count (%)			
				Eosinophils	Lymphocytes	Neutrophils	Monocytes
Healthy mice							
0.9% NaCl	–	7.5 ± 0.39	6.9 ± 0.27	2.0	66	28.0	4.0
10% DMSO	–	8.2 ± 0.58	5.5 ± 0.85	3.0	71	24.0	2.0
Mice transplanted with S180							
0.9% NaCl	–	7.1 ± 0.65	5.7 ± 0.52	0.7	67.8	26.2	5.3
10% DMSO	–	7.4 ± 0.81	5.8 ± 0.48	5.0	49.0	45.0	1.0
5-FU	10	6.4 ± 0.75	2.3 ± 0.29[a]	4.5	79.5[a]	12.5[a]	3.5
PHT	40	8.8 ± 0.64	8.9 ± 0.2[a]	1.5	69[a]	27[a]	2.5
PHT	20	6.9 ± 0.89	7.9 ± 0.1[a]	4.5	65[a]	25[a]	5.5
PHT + 5-FU	20 + 10	7.5 ± 0.49	4.9 ± 0.1[b]	2.0	75[a,b]	20[a,b]	3.0

Mice were injected with sarcoma 180 (2.0×10^6 cells/animal, s.c.). The animals were treated, starting one day after tumor implantation, for seven consecutive days

Data are presented as mean ± SEM of ten animals. Negative control was treated with the vehicle used for diluting the test substance (10% DMSO). 5-Fluorouracil (5-FU) was used as the positive control

[a] $P < 0.05$ compared to mice transplanted with tumor S180-treated negative control group by ANOVA followed by the Bonferroni test

[b] $P < 0.05$ for 5-FU-treated versus PHT + 5-FU-treated by ANOVA followed by the Bonferroni test

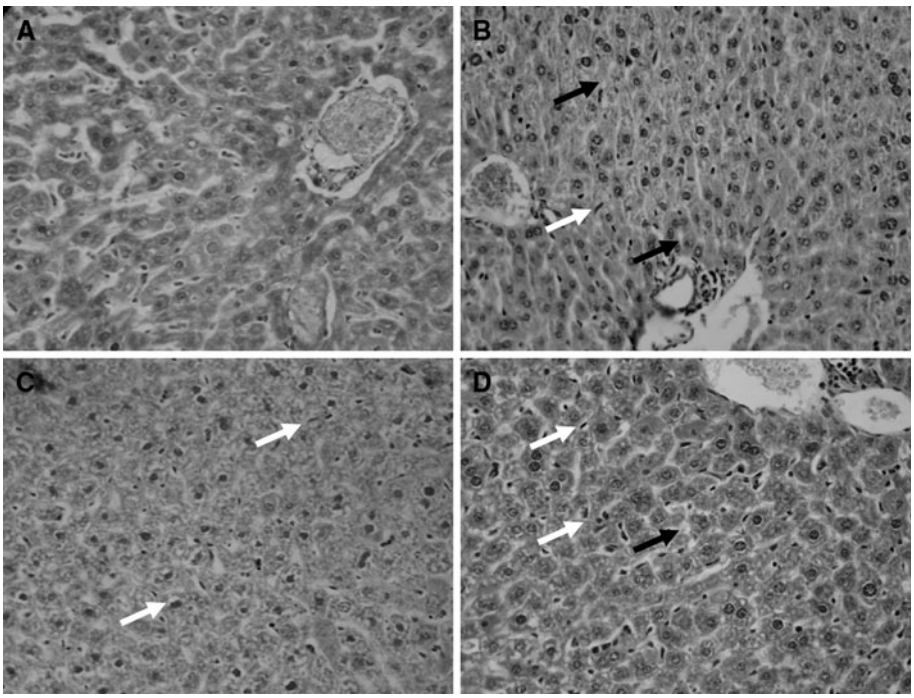

Fig. 3 Effect of (4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (PHT) on the liver of mice transplanted with the sarcoma 180 tumor. Photomicrographs show the histopathology of the livers from DMSO-treated (**a**), 10 mg/kg 5-FU-treated (**b**), 20 mg/kg PHT-treated (**c**), and PHT plus 5-FU-treated mice (PHT, 20 mg/Kg + 5-FU, 10 mg/Kg) (**d**) analyzed by light microscopy (×400). *White arrows* show Kupffer cell hyperplasia or degeneration of hepatocytes. *Black arrows* point to areas with microvesicular steatosis

Discussion

The antitumor activity of PHT was evaluated using different bioassays. In vitro, antimitotic activity was determined as the ability to inhibit sea urchin embryo development and the growth of tumor cell lines. Its lytic activity in mouse erythrocytes was also determined. The in vivo study was performed using sarcoma 180 as an experimental model. PHT showed both in vitro and in vivo antitumor effects. Its ability to inhibit the growth of tumor cell lines has been previously reported [4, 7, 8, 11], but PHT had never been investigated in in vivo experimental models.

As mentioned above, PHT cytotoxicity is already known. In the structure-activity relationship of combretastatins and/or phenstatins, the ring A (3,4,5-trimethoxyphenyl) has been reported to be essential for its potent cytotoxicity [6, 7, 15]. In ring B, the substitution of the hydroxyl in position 3 promotes the formation of compounds

with more potent cytotoxic activity [3, 6, 20]. PHT is a phenstatin compound with a modification of the hydroxyl in position 3 of the ring B, which is replaced by a hydrogen.

PHT was able to inhibit the sea urchin embryo development during all stages examined, first and third cleavage and blastula stage. These data corroborate the potent cytotoxicity found for PHT. This is the first report on PHT's effects on sea urchin eggs. This model has been used for decades to determine the cytotoxic, teratogenic, and antineoplastic activities of new compounds [21–23]. Additionally, PHT has been reported to be a tubulin polymerization inhibitor [4, 7, 8, 11]. In fact, when PHT was tested for its ability to induce lysis of mouse erythrocytes, it proved to be a non hemolytic compound, suggesting that its cytotoxicity is related to a more specific mechanism of the action.

PHT inhibited sarcoma 180 tumor growth in mice, showing in vitro and in vivo antitumor effects. Besides, when the tumor-bearing animals were treated with PHT plus 5-FU, the tumor inhibition rate increased additively. Although ideal drug combinations would be those that are synergistically active against tumor cells without increased systemic toxicity, additive antitumor activity with a favorable toxicity profile can also be clinically beneficial [24]. PHT exhibited additive in vivo antitumor effects without substantial toxicity. Although not reported in in vivo data with PHT or other phenstatin compounds, CA-4 analogs have been studied in in vivo experimental models. In vivo antitumor effect of CA-4 and its analogs also exhibited promising results for further studies because of their excellent effect and relatively low toxicity [15].

The liver plays a major role in metabolism and the kidney in the urinary system, and both have a number of functions in the body, including mostly detoxification and excretion of wastes, respectively. On the other hand, the spleen is an organ with important roles in regard to red blood cells and the immune system. Ordinarily, they are susceptible to various drugs, mostly the antineoplastic agents. Hepatic dysfunction induced by irinotecan, renal toxicity induced by docetaxel, and hematopoietic suppression induced by 5-FU are such examples [25–27]. Herein, hepatotoxic and nephrotoxic effects as well as changes in hematological parameters were checked.

Histopathological analyses of livers removed from PHT-treated animals showed Kupffer cell hyperplasia, hemosiderin-laden macrophages, and ballooning degeneration of hepatocytes. Microvesicular steatosis was seen only in livers removed from PHT plus 5-FU-treated animals, suggesting that this effect was not related to the PHT treatment alone. Nevertheless, except in the most deleterious cases, the hepatic tissue could regenerate. On the other hand, none of the treated groups showed a change in the serum level of transaminase enzymatic activity. Thus, the hepatic alterations observed after treatment with PHT alone or combined with 5-FU could be considered reversible [28–30].

In the histopathological analyses of kidneys, PHT-treated animals showed hydropic change, hyaline casts, and glomerular and tubular hemorrhage. However, these alterations could be considered reversible, since the interstitial tissues were preserved [31, 32]. Additionally, none of the treated groups showed any change in the serum levels of urea.

Immunotoxicity and/or hematopoietic suppression is one of the risks of radiotherapy/chemotherapy, which substantially increases the risk of infections. This is one of the most incapacitating side effects of cancer treatment [33, 34]. Unlike several chemotherapeutics, PHT did not reduce the numbers of hematopoietic cells. Additionally, treatment with PHT combined with 5-FU positively influenced the increase in total leukocytes. On the other hand, none of the treated groups showed a change in the histopathological analyses of spleens.

In conclusion, PHT displayed in vitro and in vivo antitumor effects without substantial toxicity. Moreover, this phenstatin compound enhanced the efficacy of 5-FU. The data presented here reinforce the anticancer potential of the phenstatin family.

Acknowledgments We wish to thank CNPq, CAPES, Instituto Claude Bernard, FUNCAP and FINEP for their financial support in the form of grants and fellowship awards. The authors also thank the National Cancer Institute (Bethesda, MD, USA) for the donation of the tumor cell lines used in this study. The authors thank Silvana França dos Santos, Luciana França and Maria de Fátima Teixeira for technical assistance. Dr. A. Leyva helped with English editing of the manuscript.

References

1. Vernon B, Powell S (2004) Localized delivery system for phenstatin using N-isopropylacrylamide, WO 2004009127
2. Pettit GR, Grealish MP (2001) Synthesis of hydroxyphenstatin and the prodrugs thereof as anticancer and antimicrobial agents, WO 2001081288
3. Alvarez C, Alvarez R, Corchete P, Pérez-Melero C, Peláez R, Medarde M (2008) Naphthylphenstatins as tubulin ligands: synthesis and biological evaluation. Bioorg Med Chem 16:8999–9008
4. Alvarez R, Alvarez C, Mollinedo F, Sierra BG, Medarde M, Peláez R (2009) Isocombretastatins A: 1,1-diarylethenes as potent inhibitors of tubulin polymerization and cytotoxic compounds. Bioorg Med Chem 17:6422–6431
5. Alvarez C, Alvarez R, Corchete P, Pérez-Melero C, Peláez R, Medarde M (2010) Exploring the effect of 2,3,4-trimethoxy-phenyl moiety as a component of indolephenstatins. Eur J Med Chem 45:588–597
6. Pettit GR, Toki B, Herald DL, Verdier-Pinard P, Boyd MR, Hamel E, Pettit RK (1998) Antineoplastic agents. 379. Synthesis of phenstatin phosphate. J Med Chem 41:1688–1695
7. Cushman M, Nagarathnam D, Gopal D, He HM, Lin CM, Hamel E (1992) Synthesis and evaluation of analogs of (Z)-1-(4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)ethene as potential cytotoxic and antimitotic agents. J Med Chem 35:2293–2306

8. Liou JP, Chang CW, Song JS, Yang YN, Yeh CF, Tseng HY, Lo YK, Chang YL, Chang CM, Hsieh HP (2002) Synthesis and structure-activity relationship of 2-aminobenzophenone derivatives as antimitotic agents. J Med Chem 45:2556–2562
9. Liou JP, Chang JY, Chang CW, Chang CY, Mahindroo N, Kuo FM, Hsieh HP (2004) Synthesis and structure-activity relationships of 3-aminobenzophenones as antimitotic agents. J Med Chem 47:2897–2905
10. Frank HR, Tarbell DS (1948) Friedel-Crafts reaction on highly methoxylated compounds. J Am Chem Soc 70:1276–1278
11. Barbosa EG, Bega LAS, Beatriz A, Sarkar T, Hamel E, Amaral MS, Lima DP (2009) A diaryl sulfide, sulfoxide, and sulfone bearing structural similarities to combretastatin A-4. Eur J Med Chem 44:2685–2688
12. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 16:55–63
13. Jimenez PC, Fortier SC, Lotufo TMC, Pessoa C, Moraes MEA, Moraes MO, Costa-Lotufo LV (2003) Biological activity in extracts of ascidians (Tunicata, Ascidiacea) from the northeastern Brazilian coast. J Exp Mar Biol Ecol 287:93–101
14. Bezerra DP, Castro FO, Alves APNN, Pessoa C, Moraes MO, Silveira ER, Lima MAS, Elmiro FJM, Costa-Lotufo LV (2006) In vivo growth-inhibition of sarcoma 180 by piplartine and piperine, two alkaloid amides from *Piper*. Braz J Med Biol Res 39:801–807
15. Wu M, Sun Q, Yang C, Chen D, Ding J, Chen Y, Lin L, Xie Y (2007) Synthesis and activity of Combretastatin A-4 analogues: 1, 2, 3-thiadiazoles as potent antitumor agents. Bioorg Med Chem Lett 17:869–873
16. Bezerra DP, Pessoa C, Moraes MO, Alencar NM, Mesquita RO, Lima MW, Alves AP, Pessoa OD, Chaves JH, Silveira ER, Costa-Lotufo LV (2008) In vivo growth inhibition of sarcoma 180 by piperlonguminine, an alkaloid amide from the *Piper* species. J Appl Toxicol 28:599–607
17. Pessoa C, Silveira ER, Lemos TL, Wetmore LA, Moraes MO, Leyva A (2000) Antiproliferative effects of compounds derived from plants of Northeast Brazil. Phytother Res 14:187–191
18. Costa-Lotufo LV, Silveira ER, Barros MC, Lima MA, De Moraes ME, De Moraes MO, Pessoa C (2004) Antiproliferative effects of abietane diterpenes from *Aegiphila lhotzkyana*. Planta Med 70:180–182
19. Jacobs RS, White S, Wilson L (1981) Selective compounds derived from marine organisms: effects on cell division in fertilized sea urchin eggs. Fed Proc 40:26–29
20. Alvarez C, Alvarez R, Corchete P, Pérez-Melero C, Peláez R, Medarde M (2007) Synthesis and biological activity of naphthalene analogues of phenstatins: naphthylphenstatins. Bioorg Med Chem Lett 17:3417–3420
21. Fusetani N (1987) Marine metabolites which inhibit development of echinoderm embryos. In: Scheur PJ (ed) Biorganic marine chemistry. Springer, Berlin, pp 61–92
22. Costa-Lotufo LV, Khan MT, Ather A, Wilke DV, Jimenez PC, Pessoa C, de Moraes ME, de Moraes MO (2005) Studies of the anticancer potential of plants used in Bangladeshi folk medicine. J Ethnopharmacol 99:21–30
23. Bezerra DP, Pessoa C, de Moraes MO, Silveira ER, Lima MA, Elmiro FJ, Costa-Lotufo LV (2005) Antiproliferative effects of two amides, piperine and piplartine, from *Piper* species. Z Naturforsch C 60:539–543
24. Kaufman DC, Chabner BA (2001) Clinical strategies for cancer treatment: the role of drugs. In: Chabner BA, Longo DL (eds) Cancer chemotherapy & biotherapy. Lippincott Williams & Wilkins, Philadelphia, pp 1–16
25. Zamagni C, Martoni A, Cacciari N, Gentile A, Pannuti F (1998) The combination of paclitaxel and carboplatin as first-line chemotherapy in patients with stage III and stage IV ovarian cancer: a phase I-II study. Am J Clin Oncol 21:491–497
26. Cao X, Cai R, Ju DW, Tao Q, Yu Y, Wang J (1998) Augmentation of hematopoiesis by fibroblast-mediated interleukin-6 gene therapy in mice with chemotherapy. J Interferon Cytokine Res 18:227–233
27. Saif MW (2009) Secondary hepatic resection as a therapeutic goal in advanced colorectal cancer. World J Gastroenterol 15:3855–3864
28. McGee JOD, Isaacson PA, Wright NA (1992) Oxford textbook of pathology: pathology of systems. Oxford University Press, New York
29. Scheuer PJ, Lefkowitch JH (2000) Drugs and toxins. In: Scheuer PJ, Lefkowitch JH (eds) Liver biopsy interpretation, 6th edn. W. B. Saunders, London, pp 134–150
30. Kummar V, Abbas A, Fausto N (2004) Robbins and cotran pathologic basis of disease, 7th edn. W.B., Saunders
31. Curran RC (1990) Color atlas of histopathology. Oxford University Press, New York
32. Olsen S, Solez K (1994) Acute tubular necrosis and toxic renal injury. In: Tisher CC, Brenner BM (eds) Renal pathology: with clinical and functional correlations. JB Lippincott, Philadelphia, pp 769–809
33. Mitchell JA, Gillam EM, Stanley LA, Sim E (1990) Immunotoxic side-effects of drug therapy. Drug Saf 5:168–178
34. Takiguchi N, Saito N, Nunomura M, Kouda K, Oda K, Furuyama N, Nakajima N (2001) Use of 5-FU plus hyperbaric oxygen for treating malignant tumors: evaluation of antitumor effect and measurement of 5-FU in individual organs. Cancer Chemother Pharmacol 47:11–14