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Research Paper

Cytotoxicity and antiangiogenic activity of grandisin

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Abstract

Objectives The antitumoural properties of grandisin, a tetrahydrofuran neolignan from *Piper solmsianum*, were investigated by in-vitro and in-vivo assays using the Ehrlich ascites tumoural (EAT) model.

Methods Viability of the tumour cells was evaluated by Trypan blue exclusion and MTT methods, after incubation with grandisin (0.017–2.3 μM). The effects of grandisin on the activity of caspase-3, -6, -8, and -9 were also investigated using colorimetric protease kits. In-vivo studies were performed in EAT-bearing mice treated intraperitoneally with 2.5, 5 or 10 mg/kg grandisin for 10 days.

Key findings Grandisin inhibited the growth of EAT cells, by both methods, with IC50 values less than 0.25 μM. The results showed that the activity of all the caspases studied increased in grandisin-treated cells, when compared with control, non-treated cells. Administering grandisin to EAT-bearing mice increased survival of the animals, in a dose-dependent manner. Simultaneously, we detected a 66.35% reduction of intraperitoneal tumour cell burden in the animals treated with 10 mg/kg grandisin. Additionally, in these animals, the marked increase of vascular endothelial growth factor (VEGF) levels, induced by EAT development, was decreased with treatment with grandisin, resulting in a reduction of 32.1% of VEGF levels in the peritoneal washing supernatant, when compared with the control.

Conclusions The results demonstrated that grandisin induced in-vitro cytotoxicity and antiangiogenic effects in mice while it acted against tumour evolution, prolonging host survival.

Keywords antitumour; cytotoxicity; grandisin; vascular endothelial growth factor

Introduction

Natural products from plants have been a major source of biologically active compounds. The search for plant-derived cytotoxic agents continues to be an important line in the discovery of modern anticancer drugs.[1] Lignans are a family of natural products that originated as secondary metabolites through the shikimic acid pathway with different characteristic chemical and several biological properties, such as reverse transcriptase inhibition and anti-HIV activity, immunomodulatory activity, antileishmaniosis, antifungal and, particularly, as antitumoural compounds.[2–7] It is worth mentioning that lignans, such as podophyllotoxin, have been investigated extensively due to their antitumoural properties. Two of its semisynthetic derivatives, etoposide and teniposide, have been included in a variety of cancer chemotherapy protocols, such as in the treatment of Wilms tumours, genital tumours, non-Hodgkin and other lymphomas, and lung cancer.[6–8]

In-vitro studies with grandisin, a tetrahydrofuran neolignan from *Piper solmsianum*, have reported potent antimalarial activity against *Plasmodium falciparum* and trypanocidal activity against the trypomastigote form of *Trypanosoma cruzi*.[9–11]. De Oliveira *et al*.[12] demonstrated that grandisin was an effective *T. cruzi* trypanothione reductase inhibitor against both forms of this parasite: amastigote and trypomastigote. Despite the high in-vitro

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effectiveness of this compound as an antiparasitic compound, the in-vivo studies have been precluded by its high lipophilicity, which was considered a limiting factor to address further investigations.[11]

Angiogenesis is the formation of new blood vessels in a given vascular bed and has been described as one of the hallmarks of cancer, playing an essential role in tumoural growth, invasion and the process of metastasis.[13,14] The angiogenesis process has been associated with vascular endothelial growth factor (VEGF), and so any VEGF inhibiting compound is capable of acting on angiogenesis development and thus suppressing tumour growth *in vivo*.[15] Consequently, antiangiogenic tumour therapy has gained much interest in preclinical and clinical pharmacological assessment. In addition, it was found that well-known and widely applied chemotherapeutic agents, e.g. cyclophosphamide and etoposide, also show antiangiogenic activity.

The Ehrlich ascites tumour (EAT) is an experimental model extensively used in preclinical studies to investigate the antitumour properties of compounds.[14,16–18] EAT development induces a marked angiogenesis associated with cytokines, mainly VEGF.

In this study, the in-vitro cytotoxic effects of grandisin on EAT cells and, for the first time, the in-vivo antitumour activity in EAT bearing-mice have been evaluated. The ability of grandisin to suppress VEGF was investigated also, and because grandisin is not a water-soluble compound, it was evaluated in a micellar dispersion to improve its hydrodispersion in biological systems.

Material and Methods

Chemicals

Phosphatidyl choline, RPMI 1640 medium, chloroform and Trypan blue dye were purchased from Sigma-Aldrich (St Louis, MO, USA). MTT-tetrazolium was from Boehringer Mannheim (Indianapolis, IN, USA). Dimethylsulfoxide (DMSO) was from Merck, Germany. Colorimetric protease kits and VEGF Kit were from R&D Systems (Minneapolis, MN, USA). Bio-Rad Protein assay was from Bio-Rad Laboratories Inc., USA. Ketamine was from König (São Paulo, SP, Brazil) and xylazine was from Syntec (Cotia, SP, Brazil).

Grandisin

Grandisin (molecular weight: 432.214) was isolated from *P. solmsianum* leaf extracts according to the literature by Dr Massuo Jorge Kato (Laboratório de Química de Produtos Naturais, Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo).[12]

Grandisin loaded micelles

This compound was prepared as a micellar dispersion to allow its dissolution in water. The micelles were prepared as follows: first, 50 μg soy phosphatidyl choline (PC), sunflower oil 4 : 1 (mol/mol PC), and grandisin (10 mg) were dissolved in 1.0 ml chloroform. The mixture was dried under a nitrogen atmosphere and freeze-dried overnight to ensure the complete removal of chloroform. Water (1 ml) was added to the dried lipid film to promote the hydration of the lipid–drug mixture. After 1 h, the mixture was sonicated for 10 min in a Ti-probe sonicator to obtain a homogeneous dispersion of small micelles. For the control group, the micellar dispersion was prepared without grandisin.

Mice

The experiments were carried out on adult male Swiss mice (28–30 g; aged 6–8 weeks) obtained from Indústria Química do Estado de Goiás (IQUEGO). All mice were kept under constant environmental conditions with a 12 : 12 light–dark cycle. The mice were fed with standard granulated chow and water was freely given. Animal experiments were carried out in accordance with institutional protocols and the guidelines of the Canadian Council on Animal Care. The experimental protocol was approved by the Institutional Ethics Committee of this University.

Mouse tumour model

EAT was maintained in male Swiss mice by serial transplantation. Tumour cell suspensions were prepared in phosphate-buffered saline (PBS (in mM): 22.2 Na_2HPO_4; 5.6 KH_2PO_4; 123.3 NaCl) at pH 7.4 to a final concentration of 2×10^6 viable cells/ml. Mice were inoculated intraperitoneally (i.p.) on day 0 with 6×10^6 viable tumour cells/ml in a volume of 0.1 ml. Viability, assessed by the Trypan blue dye exclusion method, was always found to be 95% or higher. Tumour cell cultures were derived from ascitic tumour cells harvested by peritoneal lavage from mice eight to ten days after tumour transplantation.

In-vitro assays

Cytotoxicity assay

The growth and viability of EAT cells were first evaluated by the Trypan blue exclusion method. Briefly 2×10^6 viable Ehrlich ascites tumour cells were seeded in quadruplicate into 96-well flat microtitre plates (Corning, USA) in enriched RPMI 1640 medium supplemented with 10% fetal calf serum. Cultures were incubated with grandisin (0.017–2.3 μM) for 24 and 48 h. Trypan blue solution (in phosphate buffer) cell suspensions were then mixed in equal volumes (0.1 ml) and the number of cells counted using a haemocytometer. Cells stained were scored as dead.

The MTT-tetrazolium reduction assay was performed using 2×10^6 viable cells, which were seeded in quadruplicate into 96-well flat microtitre plates (Corning, USA) in enriched RPMI 1640 medium supplemented with 10% fetal calf serum and incubated with grandisin for 24 h. The cells were washed twice with PBS before adding MTT 10 μg/well (5 mg/ml). After incubation for 4 h, the culture medium was removed and 100 μl DMSO was added to each well to solubilize the formazan formed. The plates were shaken gently for 10 min and the absorbance was measured at 560 nm.

Caspase activity

Direct measurements of caspase activity were performed using colorimetric protease kits (R&D Systems, USA) according to the manufacturer's recommendations, after the incubation of cells with grandisin for 24 h (0.75 μM). The caspase activity assay is based on the spectrophotometer detection of the *p*-nitroanilide (pNA) chromophore after cleavage from the substrates X-*p*NA, where X stands for amino acid sequences

recognized by the specific caspase-3, caspase-6, caspase-8 and caspase-9. According to the procedure, 2×10^6 EAT cells were pelleted by centrifugation and lysed on ice. The protein concentration in the lysate was measured using a Bio-Rad Protein assay (Bio-Rad Laboratories Inc., USA). This assay was based on the method of Bradford[19] in which 200 μg total protein was incubated with each X-*p*NA substrate (200 μM final concentration) at 37°C in a microtitre plate. The optical density of samples was measured at 405 nm. After subtraction of the background, the increase in the caspase activity was determined by comparing these results with the levels of the control.

In-vivo assays

Antitumoural evaluation

The groups of tumour-bearing mice received (0.1 ml/mouse) 2.5, 5 or 10 mg/kg per day grandisin (i.p.) for 10 consecutive days. Treatment started 24 h after tumour inoculation. Each experiment included parallel control groups of normal mice treated with an equivalent volume of the micellar dispersion without grandisin. The in-vivo antitumour activity of grandisin was determined by the increase in the survival time of treated mice (n = 10 per group) compared with non-treated control mice. The ascitic fluid from the peritoneal cavity of tumour-bearing mice (n = 6 per group) treated with 2.5, 5 or 10 mg/kg per day grandisin, for 10 days, was quantitatively isolated by peritoneal washings 24 h after the administration of the last injection. The total number of tumour cells was counted by the Trypan blue exclusion method. The micelle-loaded grandisin was prepared immediately before use. The mice were killed with a mixture of ketamine/xylazine and the abdominal wall of each animal was removed and photomicrographs (Canon PowerShot S80) were taken for visual analysis.

VEGF production

The levels of the cytokine VEGF, released by tumour cells, in the peritoneal washing supernatant of EAT-bearing mice (n = 6 per group) treated with 2.5, 5 or 10 mg/kg per day grandisin, for 10 days, were determined, 24 h after the last injection, by use of a VEGF ELISA Kit according to the manufacturer's instructions (R & D System).

Statistical analysis

The evaluation of cytotoxicity was carried out by three separate experiments. The results were transformed to percentage of the control and the IC50 (concentration that produces a 50% inhibitory effect on the evaluated parameter) was graphically obtained from the dose–response curve. Results were expressed as the mean ± SD of four replicates. The VEGF and EAT inhibition statistical analysis were performed using non-parametric Mann-Whitney U test comparing the treated groups with controls and Kruskal–Wallis test between treated groups. The probability of survival of the EAT-bearing mice treated with grandisin was calculated by the Kaplan–Meier curve and the difference between the groups was compared using the log-rank test. Statistical significance was considered when $P < 0.05$.

Results

Grandisin cytotoxicity to EAT cells was investigated after incubation periods of 24 and 48 h, using the Trypan blue dye exclusion method. As shown in Figure 1a, by using this method tumour cell proliferation was inhibited, in a concentration–dependent manner, in response to increasing concentrations of grandisin micellar dispersion (0.017–2.3 μM). The 50% of inhibition (IC50 value) was obtained with concentrations of approximately 0.22 and 0.25 μM grandisin, for 24 and 48 h, respectively. The IC50 values were then used for the investigation of the cytotoxicity by the MTT method, and produced inhibition of approximately 55% on the growth and viability of Ehrlich ascites tumour at 24 h exposure (data not shown). Analysis of the morphological changes of grandisin exposed and non-exposed EAT cells were studied using Giemsa staining. Figure 1b shows the representative morphological changes of EAT cells. Non-exposed cells appeared normal and the nuclei were round and homogeneous. After being exposed to 0.22 μM grandisin, the cells exhibited the characteristic features of apoptosis.

These results prompted us to investigate whether the grandisin antiproliferative activity could be a consequence of

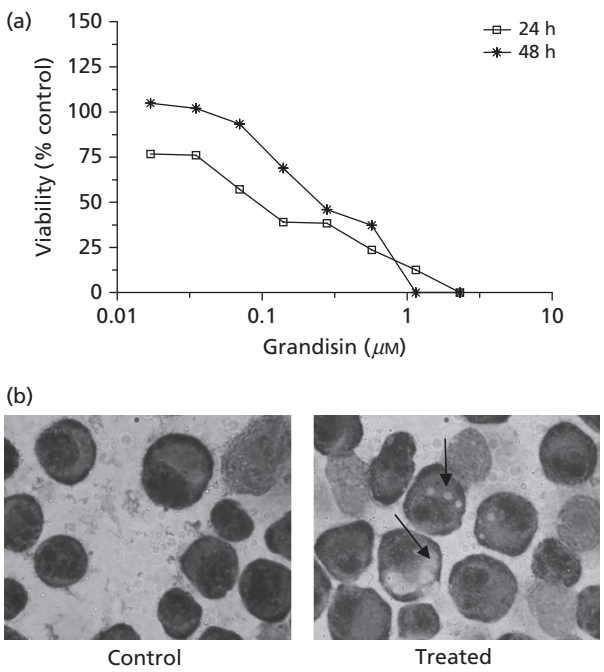

Figure 1 Grandisin cytotoxicity to Ehrlich ascites tumour cells. (a) Ehrlich ascites tumour (EAT) cells (2×10^6/ml), were treated with different concentrations of grandisin (0.017–2.3 μM) for 24 or 48 h in 96-well microtitre plates. Cell viability was determined by the Trypan blue exclusion method. In the absence of compound, the viability was considered as 100%. Results represent the mean ± SD of three experiments run in three replicates. (b) Morphological features of grandisin-induced apoptosis in EAT cells (2×10^4/ml) treated with 0.22 μM grandisin for 24 h. The arrows show the apoptotic nucleui. The control cells were stained with Giemsa and showed a normal nucleus. Representative photomicrographs of two independent experiments.

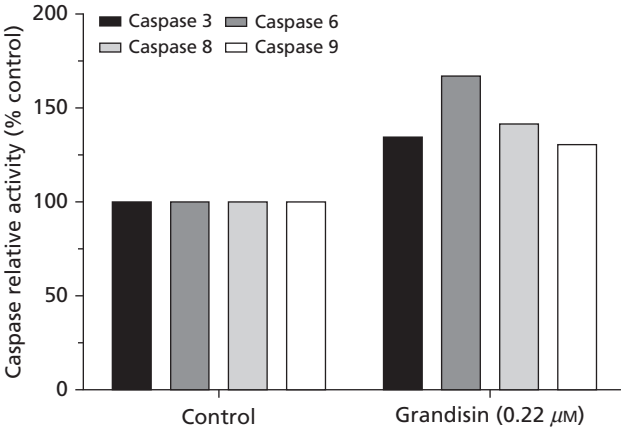

Figure 2 Effects of grandisin on the activity of caspase-3, -6, -8 and -9 using Ehrlich ascites tumour cells. Grandisin-induced Ehrlich ascites tumour (EAT) cell death was caspase dependent, as shown by caspase relative activity in EAT (2×10^6/ml) cells after treatment with 0.22 μM grandisin for 24 h. Colorimetric assay was performed to determine caspase-3, -6, -8 and -9 activity. The percentage of enzyme activity was relative to control. In the absence of grandisin the activity was considered to be 100%.

apoptosis induced by proteases, such as caspases. Thus, we evaluated the effects of grandisin (0.22 μM) on the activity of caspase-3, -6, -8, and -9 using EAT cells after incubation with this compound for 24 h. The results revealed that the activity of all caspases studied significantly increased in grandisin-treated cells compared with the control, non-treated cells. The assessment of caspase-3, -6, -8, and -9 activity showed enhancement of 34, 65, 40, and 35%, respectively, in relation to control, after incubation of tumour cells with 0.22 μM grandisin for 24 h (Figure 2).

In relation to in-vivo studies, the 10-day treatment using the highest dose of grandisin (10 mg/kg per day) significantly enhanced survival rate (71.0%), when compared with the control group, as shown in Table 1. In addition, we observed increases in the survival rate of mice treated with 2.5 and 5 mg/kg per day of 35.7 and 50.0%, respectively, compared with the control group. Moreover, the administration of grandisin after EAT inoculation resulted in the reduction of tumour cells, in a dose-dependent manner, compared with the untreated group (Table 1). The treatment using 10 mg/kg per day resulted in 66.35% reduction in intraperitoneal tumour cell burden on day 11. We observed tumour growth inhibition of 33.65 and 35.26% respectively using 2.5 and 5 mg/kg grandisin. In parallel, the treatment with grandisin also reduced, in a dose–dependent manner, the VEGF 'levels in the peritoneal washing supernatant induced by EAT development. In the group of mice treated with 10 mg/kg, the marked increase in VEGF levels was significantly reduced by 32.1% in the peritoneal washing supernatant. Furthermore, non-treated mice showed tortuous dilated and congested vessels when compared with the control. After being treated with 2.5, 5 or 10 mg/kg grandisin, the capillaries were markedly reduced, showing features similar to the control group (Figure 3).

Table 1 Effects of grandisin on the survival of Ehrlich ascites tumour-bearing mice, tumour inhibition and on the concentration of the cytokine vascular endothelial growth factor released by tumour

	Survival (days – %)	Inhibition (cells – %)	VEGF (pg/ml – %)
EAT	14 – 0%	10.40 – 0%	127.83 – 0%
EAT + 2.5 mg/kg	19 – 35.70%	6.90 – 33.65%	121.08 – 5.28%
EAT + 5 mg/kg	21 – 50.00%	6.70 – 35.26%	99.65 – 22.04%*
EAT + 10 mg/kg	24 – 71.00%*	3.50 – 66.35%*	86.67 – 32.10%*

Grandisin was administered intraperitoneally at 2.5, 5 or 10 mg/kg per day. In all treatment schedules, mice were treated intraperitoneally for 10 days starting 24 h after tumour inoculation (2×10^6 cell/ml). For survival analyses groups of 10 animals were checked daily. The levels of the cytokine vascular endothelial growth factor (VEGF), released by tumour cells, in the peritoneal washing supernatant of Ehrlich ascites tumour (EAT)-bearing mice (n = 6 per group) were determined, 24 h after the last injection, by VEGF ELISA Kit. Control mice received only the diluents. In the absence of grandisin the concentration was considered to be 100%. *$P < 0.05$ compared with control.

Discussion

Several relevant chemotherapeutics in clinical practice do not lead to a cure or long-term survival for most cancers.[20] Cancer is a huge public health problem, and it is clear that new agents with different mechanisms of action are required for the

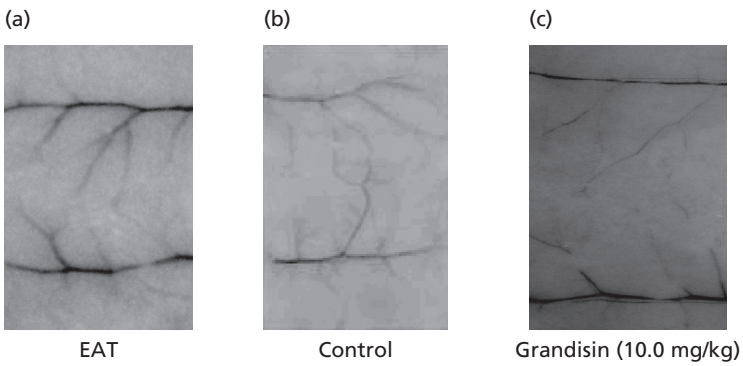

Figure 3 Photomicrograph of the vascular pattern in Ehrlich ascites tumour transplanted mice. (a) Ehrlich ascites tumour (EAT) transplanted mice. (b) Control mice. (c) Mice inoculated with EAT and treated with grandisin (10.0 mg/kg per day; i.p.) for 10 days, starting 24 h after tumour inoculation (2×10^6 cell/ml). Morphological analysis was performed after the last injection.

treatment of cancer and as adjuvants to improve combination chemotherapy, as well as to overcome antitumoural multidrug resistance mechanisms. Extensive research makes the lignans an important family for the development of new chemotherapeutic agents.[1] In this study we have reported our investigation of the in-vitro cytotoxic effects of grandisin, a neolignan from *P. solmsianum*, on EAT cells and its antitumour activity in EAT-bearing-mice.

Most antitumour agents currently used in clinical practice present cytotoxic activity.[21] In our research, the in-vitro cytotoxic assay showed that the use of less than 0.25 μM grandisin micellar dispersion decreased viability of EAT cells by 50%. On the other hand, this molecule also showed slight inhibitory effects on normal human peripheral blood lymphocytes (data not shown). In this context, since caspases are very well established as the main players in apoptotic mechanisms, we evaluated the effects of grandisin on the activity of caspase-3, -6, -8, and -9. The results showed that the activity of all caspases studied increased in grandisin-treated cells, when compared with control, non-treated cells. The highest enhancement in this activity was observed with caspase-6. Hence, we suggest that grandisin killed cells via caspase-dependent apoptotic mechanisms, especially by interfering with caspase-6 activation.

Derivatives from the lignan podophyllotoxin, such as etoposide and teniposide, are widely used as anticancer drugs and show acceptable clinic efficacy against several types of neoplasms. Nonetheless, the detailed antitumoural mechanism of podophyllotoxin derivatives remains to be elucidated, although it has already been well established that these drugs are potent irreversible inhibitors of DNA topoisomerase II. Additionally, their action is based on the formation of a nucleic acid–drug–enzyme complex, which induces breaks in single- and double-stranded DNA as the initial step in a series of biochemical transformations that eventually lead to cell death.[1,6,22] As a consequence, these drugs have marked cytotoxicity on tumour and normal cells, in general, associated to induction of apoptosis by caspase-induced mechanisms. Several studies have revealed the importance of caspases-3, -6, and -8 in etoposide-induced apoptosis, whereas others have demonstrated the importance of caspase-8 in apoptotic mechanisms, regardless of any death receptor pathway.[23,24] It has been proven that pro caspase-8 can be triggered by other proteases, such as granzyme B.[24] Sohn *et al.*[25] reported that caspase-3 and -6 were required for the efficient activation of caspase-8.

Corroborating these in-vitro findings, in-vivo results showed that the micellar dispersion of grandisin presented a significant dose-dependent efficacy against EAT growth, increasing survival time. The best results in prolonging the life span were produced with the 10 mg/kg per day dose. Simultaneously, we detected a 66.35% reduction of intraperitoneal tumour cell burden in the animals under study.

During the development of EAT, a high peritoneal wall vascularization was observed as a direct/indirect consequence of growth factors, mainly VEGF at high concentrations.[14,26] As mentioned before, VEGF plays a crucial role in the growth, invasion and metastasis of tumours. In this study, the marked increase of VEGF levels induced by EAT development was significantly decreased in the treatment using the highest dose of grandisin, resulting in a reduction of 32.1% of VEGF levels in the peritoneal washing supernatant, when compared with the control. Economou *et al.*[27] showed that mice treated with the cyclolignan picropodophyllin, administered intraperitoneally or orally, presented respectively 22 and 32% decrease in experimental choroidal neovascularization associated to a reduction in VEGF levels. Moreover, Lennernäs *et al.*[28] reported dose–response effects of fluorouracil, paclitaxel, doxorubicin, cisplatin, methotrexate, cyclophosphamide and etoposide on decrease of VEGF165/164-mediated angiogenesis, using the rat mesenteric-window angiogenesis assay.

Conclusions

Although the precise antitumoural mechanism of grandisin remains unknown, it was evident that the treatment using this neolignan induced in-vitro cytotoxic, by apoptotic, and antiangiogenic effects in mice while it acted against tumour evolution, significantly prolonging host survival. Further studies are required to characterize the toxicological and pharmacological properties of grandisin, so as to consider it as a candidate anticancer agent.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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