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Short Communication

Chemoprotective effect of the tetrahydrofuran lignan grandisin in the in-vivo rodent micronucleus assay

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Abstract

Objectives The chemoprotective effect of the tetrahydrofuran lignan grandisin against DNA damage induced by cyclophosphamide (200 mg/kg) has been evaluated using the *in vitro* rodent micronucleus assay.

Methods The effects of a daily oral administration of grandisin (2, 4, or 8 mg/kg) for five days before exposure to cyclophosphamide on the frequency of micronucleus in the bone marrow of normal mice exposed and unexposed to cyclophosphamide were investigated ($n = 5$ per group). Electrochemical measurements were applied to investigate whether the antimutagenic effects of grandisin could be, at least in part, a consequence of its or its metabolite's antioxidant properties.

Key findings Grandisin did not show mutagenic effects on the bone marrow cells of exposed mice. On the other hand, the oral administration of grandisin (2, 4, or 8 mg/kg) per day reduced dose-dependently the frequency of micronucleus, induced by cyclophosphamide, in all groups studied. Cyclic voltammograms showed two peaks for a grandisin metabolite, which were absent for grandisin.

Conclusions Under the conditions tested herein, this study has shown that mice treated with grandisin presented, in a dose-dependent manner, a protective effect against cyclophosphamide-induced mutagenicity. This effect could be, at least in part, associated to grandisin bioactivation. These data open new perspectives for further investigation into the toxicology and applied pharmacology of grandisin.

Keywords antimutagenic; grandisin; lignan; micronucleus

Introduction

Genomic damage induced by different factors, such as radiation, lifestyle, and genetics, has been associated to degenerative diseases, including cancer.[1] Recently, interest in compounds that can protect or minimize genotoxic effects has been growing. Several researches have pointed out that natural products have chemoprotective effects, such as antimutagenic or anticarcinogenic activity, considered effective in preventing mutations and cancer. According to Duthie,[2] there is strong and convincing evidence that extracts or phytochemicals modulate both biomarkers of DNA damage and the indicators of malignant transformation *in vitro* and *in vivo*.

Natural polyphenols include numerous classes of compounds, for example cardanols, flavonoids, lignans, and neolignans, which present a wide range of biological activity including antitumoral, antimitotic, antiviral, cardiovascular, immunosuppressive, and antioxidant properties.[3–7]

Lignans have a number of skeletal types and among a variety of substituents the phenolic groups are frequently involved in antioxidant activity. This was the case for lignans from *Euterpe oleracea*, in which chemoprotective and antioxidant effects were demonstrated.[3] The antioxidant properties of aryltetralin lignans from *Virola* species have been recorded, but its potency was increased when methylenedioxy groups were deprotected to catechol

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function.[8] Grandisin is a tetrahydrofuran lignan described from *Virola* and *Piper* species and several important biological properties have been described, including antimalarial and trypanocidal activity against *Plasmodium falciparum* and *Trypanosoma cruzi*, respectively.[9–13] Further investigation showed that grandisin was active against both amastigote and trypomastigote by inhibition of trypanothione reductase.[12]

Recently, in-vitro effectiveness of grandisin as a larvicidal agent against *Aedes aegypti* and *Chrysomya megacephala* F. has been demonstrated.[14,15]

In relation to in-vivo studies, Carvalho *et al*.,[16] demonstrated that grandisin had antinociceptive and anti-inflammatory properties. More recently, we demonstrated that this lignan presented a marked antitumoral activity against the Ehrlich ascites tumour experimental model. On the other hand, administration of grandisin was shown to be toxic to bone marrow with a significant reduction of production and distribution of leucocytes.[16–18]

We have evaluated the mutagenic and antimutagenic effects of grandisin against DNA damage induced by cyclophosphamide, using the micronucleus test in rodent bone marrow, to expand our knowledge on the balance of its toxicological/therapeutic potential.

Materials and Methods

Grandisin and its metabolite

Grandisin was isolated from *Piper solmsianum* leaf extracts according to the literature by Dr Massuo Jorge Kato, in Laboratório de Química de Produtos Naturais, Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo. Grandisin was isolated as the major compound in the leaf extracts as colourless crystals (mp 118–120°C, MeOH; $[\alpha]$ D = –57.1° (c 0.35 in $CHCl_3$)) and was readily identified by analysis of its ^1H and ^{13}C NMR spectra.[11] For the in-vivo studies, grandisin was diluted in saline containing 5% dimethyl sulfoxide (DMSO) immediately before use.

To investigate the structure of the grandisin metabolite, microbial models of mammalian metabolism were used to prepare the major metabolite of grandisin in a scale required to carry out the in-vitro antioxidant assay.[19–21] *Cunninghamella echinulata* ATCC 9244 was grown for 70 h in 10 Erlenmeyer flasks, each containing 100 ml liquid medium.[22] Grandisin (50 mg) dissolved in ethanol (1 ml) was added and the incubation process continued at 27°C with orbital shaking (200 rev/min) for 72 h. Filtration of the incubation mixture and extraction of biomass by acetone yielded 300 mg of a crude brown oil. Pure metabolite 4-*O*-demethylgrandisin was recovered as a white powder after crystallisation from EtOAc : cyclohexane. Melting point 134–138°C, MH^+ 418, ^1H, and ^{13}C NMR were similar to those previously determined by Barbosa-Filho *et al*.[23]

Mutagenic and antimutagenic studies

The experiments were carried out on adult male Swiss mice obtained from Indústria Química do Estado de Goiás (IQUEGO). All mice (28–30 g) were kept under constant environmental conditions with a 12 : 12 light–dark cycle. Animals were fed with standard granulated chow and drinking water was freely available. Animal experiments were carried out in accordance with institutional protocols and the Canadian Council on Animal Care Guidelines.[24] The experimental protocol was approved by the Institutional Ethic Committee of this University. For the mutagenicity test groups of mice ($n = 5$) received grandisin at doses of 2, 4, or 8 mg/kg per day (0.2 ml per mouse) for five consecutive days, by gavage. The micronuclei frequency assessment was performed 24 h after the treatment. For the antimutagenicity test the mice were treated as described above but were exposed to cyclophosphamide (200 mg/kg) 24 h after the end of the treatment, administrated as a single dose diluted in physiological saline solution. Each experiment included parallel control groups of normal and exposed mice to an equivalent volume (0.2 ml per animal) of the vehicle (saline containing 5% of DMSO). The micronuclei frequency was performed 24 h after the exposition to cyclophosphamide.

Micronucleus test

The mutagenicity and antimutagenicity of grandisin were evaluated using the micronucleus test by counting a total of at least 1000 erythrocytes for bone marrow per animal per treatment group.[25,26] The cells were stained with Leishmann, the slides were coded, and the cells blindly scored by light microscope (1000×). The frequency of micronucleated erythrocytes in individual mice was used as the experimental unit, with variability (standard deviation) based on differences among animals within the same group.

In-vitro antioxidant assay by electrochemical measurements

All electrochemical experiments with grandisin and its major metabolite were carried out on a potentiostat/galvanostat Autolab (model PGSTAT 30-Eco Chemie, Utrecht, Holland) connected with an electrochemical cell based on a tree electrode system. The work electrode was a carbon paste electrode modified with grandisin or its metabolite. The composition of the paste was 40 mg carbon powder, 4 mg grandisin or the metabolite and 20 mg mineral oil; the counter electrode was platinum wire and the reference electrode was Ag/AgCl/KCl (sat).

The solid state voltammetry was carried out in phosphate buffer 0.1 mol/l solution, pH 6.5, as support electrolyte; a scan rate of 100 mV/s in the range of –0.50–1.25 V was used.

Statistical analysis

Data were analysed for statistically significant experimental differences using analysis of variance and the Tukey test. Probability values greater than 0.05 were considered nonsignificant. In all cases at least three independent experiments were conducted. The percentage of reduction in the frequency of cyclophosphamide-induced DNA damage was calculated according to Manoharan and Banerjee[27] by the following formula:

Table 1 Effects of the prophylactic oral treatment with grandisin for five days on the frequency of micronucleated erythrocytes in the bone marrow of mice not exposed or exposed to cyclophosphamide

Treatment	Mean ± SD	Micronucleated erythrocytes %
Control	5.3 ± 1.37	0.5 ± 0.13
Grandisin 2 mg/kg	6.5 ± 1.66	0.6 ± 0.16
Grandisin 4 mg/kg	4.3 ± 1.31	0.4 ± 0.13
Grandisin 8 mg/kg	4.9 ± 1.25	0.4 ± 0.12
Grandisin 2 mg/kg + cyclophosphamide	5.4 ± 1.14*	0.5 ± 0.11
Grandisin 4 mg/kg + cyclophosphamide	4.0 ± 0.70*	0.4 ± 0.07
Grandisin 8 mg/kg + cyclophosphamide	3.2 ± 1.2*	0.3 ± 0.08
Cyclophosphamide	9.2 ± 2.62	0.9 ± 0.26

1000 Micronuclei were scored per animal. Data presented as the mean and standard deviation (SD) among mice. *$P < 0.05$ in relation to cyclophosphamide.

$$\%\ \text{reduction} = \frac{(\text{average frequency of the damage in group A}) - (\text{average frequency of the damage in group B})}{(\text{average frequency of the damage in group A}) - (\text{average frequency of the damage in group C})} \times 100$$

Where A is the positive control treated with cyclophosphamide, B is the group treated with cyclophosphamide plus grandisin, and C is the control group.

Results

The effects of a daily oral administration of grandisin (2, 4, or 8 mg/kg) for five days to normal mice before exposure (or not) to cyclophosphamide on the frequency of micronucleated erythrocytes in the bone marrow are shown in Table 1. These results showed that no statistically significant difference in the frequency of micronuclei between the negative control and the groups treated with grandisin could be detected. Therefore, grandisin did not present a mutagenic effect on the bone marrow cells of treated mice.

As expected, the results clearly showed that cyclophosphamide was able to induce a significant ($P < 0.05$) enhancement of the micronuclei, over the basal level in bone marrow cells, corroborating previous results obtained by our research group.[28] In contrast, when the antimutagenicity profile for grandisin was evaluated, significant decreases, in a dose-dependent manner ($P < 0.05$), in the frequency of cyclophosphamide-induced micronuclei were observed in all groups studied. Figure 1 shows the percentage of reduction on the frequency of micronuclei in mice orally pretreated with grandisin. Daily grandisin doses of 2, 4, or 8 mg/kg reduced the frequency of micronuclei to 64.7, 85.2, and 97.0%, respectively.

These results prompted us to investigate whether the chemoprotective effects of grandisin could be a consequence of an antioxidant property of the compound itself or of its major metabolite, obtained from biotransformation by the fungus *C. echinulata*. Usually, fungus is used as a microbial model of mammalian metabolism.[29] This process resulted in

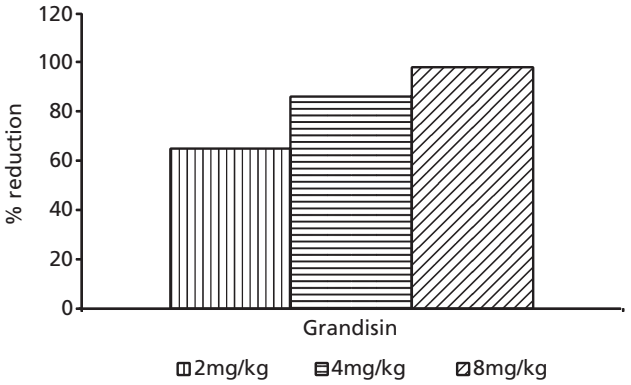

Figure 1 Reduction (%) of micronucleated erythrocytes in mice orally pretreated with grandisin exposed to cyclophosphamide. Grandisin was administered at 2, 4 or 8 mg/kg per day for five days. Dose of cyclophosphamide was 200 mg/kg. $n = 5$.

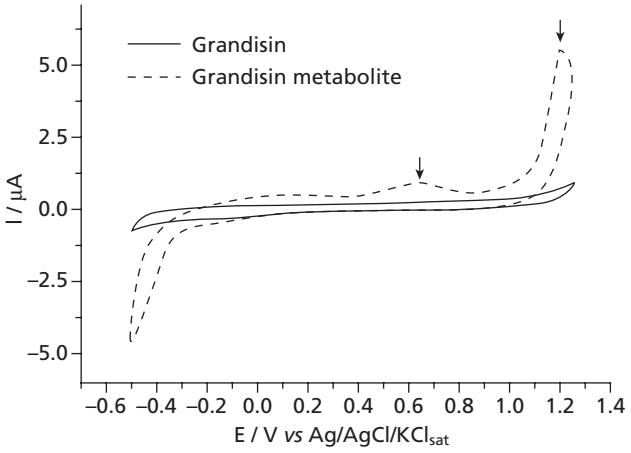

Figure 2 Cyclic voltammograms obtained with carbon paste electrodes modified with grandisin and its metabolite 4-O-demethylgrandisin. Phosphate buffer 0.1 mol/l, pH = 6.5; –0.5 to 1.25 V; 100 mV/s.

a demethylated form, 4-O-demethylgrandisin. According to the cyclic voltammograms shown in Figure 2, two peaks could be observed for grandisin metabolites, at 0.63 and 1.22 V, which were not detected with the parent compound. These peaks were associated to the oxidation process of phenolic groups to the quinone form, responsible for the antioxidant activity of polyphenols.

Discussion

Lignans, such as grandisin, are considered an important class of compounds when the development of new therapeutic agents is concerned. However, its high lipophilicity has been a limiting factor for in-vivo studies and, consequently, a constraint on the advance of our knowledge about the therapeutic/toxicological potential of grandisin.[13,30]

We have demonstrated that grandisin presented an antimutagenic effect against the genotoxicity induced by cyclophosphamide. The effects of the daily oral administration of 2, 4, or 8 mg/kg grandisin on the frequency of micronucleated

erythrocytes in the bone marrow of mice exposed or not to cyclophosphamide were investigated. In these assays, the results showed that grandisin did not show mutagenic effects on the bone marrow cells of exposed mice. In this regard, Figueiredo *et al*.,[18] demonstrated that mice exposed to 100 μg/g grandisin showed reduced leucocyte production. On the other hand, the daily oral administration of 2, 4, or 8 mg/kg grandisin for five days before exposure to cyclophosphamide reduced, dose-dependently, the frequency of micronucleated erythrocytes induced by cyclophosphamide in all groups studied.

The mechanism by which grandisin exhibits a protective effect against chromosomal fragmentation has not been elucidated. Many epidemiological studies have demonstrated that a reduced cancer risk is associated with frequent consumption of polyphenols, which act as chemopreventive agents.[31] Plant polyphenols are well recognized for their antioxidative activity since they are capable of scavenging free radicals, thus breaking the free radical chain reaction in the lipid peroxidation process.[31] In this context, although grandisin is not a phenolic compound, we investigated its ability to scavenge free radicals. Previous studies performed by our group on the antiradical action of grandisin used the free radical 1,2diphenylpicrylhydrazil (DPPH), regarded as standard (unpublished data). In spite of the lack of antioxidant activity of grandisin using the DPPH test, the electrochemical measurements of the major metabolite of grandisin showed two peaks in the cyclic voltammograms, which were not observed in the parent compound. These peaks could be associated to the oxidation process of phenolic groups to the quinone form, responsible for the antioxidant activity of polyphenols. These results suggested that the antioxidant activity of grandisin required biological activation (pro-antioxidant), since the activity could be observed for its metabolite only. Indeed, Ramos *et al*.[32] showed that insects fed grandisin were capable of biotransforming the tetrahydrofuran grandisin to mono- and di-O-demethylated phenolic derivatives. According to those authors, the antioxidant properties of the phenolic compounds could eventually represent a benefit for the herbivorous insects against their predators or to their overall health. Thus, the metabolite of grandisin, 4-O-demethylgrandisin, a demethylated phenolic form, could justify its antioxidant/chemoprotective action found in our studies in mice.

Data from the literature indicated that cytoprotectant compounds were potent anticarcinogenic agents due to various mechanisms including modulation of carcinogen activation and detoxification, decreased DNA binding of the carcinogen, inhibition of oxidative DNA damage, alteration in cell signalling and malignant transformation and inhibition of cell invasiveness and metastasis.[2]

Conclusion

Taken together and under the conditions herein tested, the mice treated with grandisin presented, in a dose-dependent manner, a chemoprotective effect against cyclophosphamide-induced mutagenicity. This effect could be, at least in part, associated to grandisin bioactivation following the inhibition of oxidative DNA damage. The mechanisms involved in grandisin antimutagenic activity are not completely understood and so studies are necessary to investigate its toxicological and pharmacological potential.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

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