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(−)-Hinokinin causes antigenotoxicity but not genotoxicity in peripheral blood of Wistar rats

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Abstract

(−)-Hinokinin, a dibenzylbutyrolactone lignan, exhibits significant trypanocidal activity both *in vitro* and *in vivo*, and was obtained by partial synthesis from (−)-cubebin isolated from the dry seeds of *Piper cubeba*. Considering the good trypanocidal activity of (−)-hinokinin, as well as its potential for the development of new drugs, it is extremely important to evaluate its possible mutagenic activity to allow its safe use in humans. In the present study, we evaluated the antimutagenic effect of (−)-hinokinin on the chromosome damage induced by the chemotherapeutic agent doxorubicin (DXR). The test system employed was the analysis of micronucleated polychromatic erythrocytes in peripheral blood of Wistar rats. Additionally, the antioxidant activity of (−)-hinokinin was evaluated in *in vitro* experiments by measuring the production of hydrogen peroxide and other peroxides. Our results showed that animals treated with different doses of (−)-hinokinin (10, 20, and 40 mg/kg b.w.) exhibited micronucleated cell frequencies similar to that of the negative control. In addition, treatment with combinations of (−)-hinokinin and DXR resulted in lower micronucleated cell frequencies than those observed for animals treated with DXR alone. The present study shows that (−)-hinokinin not only has no genotoxic effect, but is also effective in reducing the chromosome damage induced by DXR. (−)-Hinokinin exerted a significant antioxidant effect on parasite mitochondria in the protocol used, which might be one possible mechanism by which this compound may exert a protective effect on the chromosome damage induced by the free radicals generated by DXR.

Keywords: Micronuclei; Peripheral blood polychromatic erythrocytes; (−)-Hinokinin

1. Introduction

Chagas' disease is transmitted by the protozoan *Trypanosoma cruzi* and affects more than 18 million people in Latin America, causing approximately 400,000 deaths per year (Pozas et al., 2005). In Brazil, five to six million people are estimated to be infected, 300,000 of them living in the state of São Paulo (Da Silva Filho et al., 2004).

At present, treatment of Chagas' disease is based on two nitroheterocyclic drugs, nitrofuran nifurtimox (Lampit®), whose production has now been discontinued, and 2-nitroimidazole benznidazole (Rochagan®) (Croft, 1997; Docampo and Schmuñis, 1997; Paulino et al., 2005). However, these drugs have marked side effects and, because of their low effectiveness, are only used for the treatment of the acute phase of the disease. Blood transfusion is the most important mechanism of transmission, and gentian violet is the only effective compound able to eliminate the parasite from blood prior to transfusion. Despite its good activity, gentian violet alters blood color, is mutagenic,

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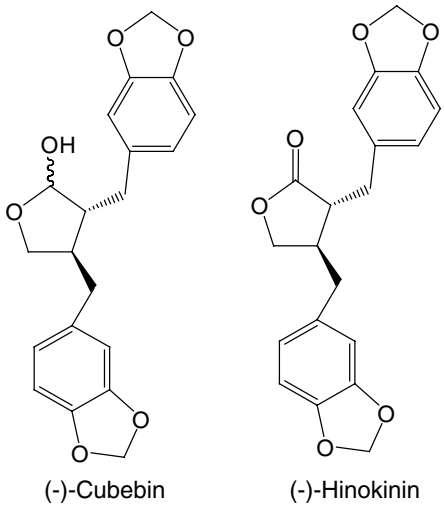

Fig. 1. Chemical structures of (−)-cubebin, isolated from *Piper cubeba*, and (−)-hinokinin, obtained by partial synthesis.

and is rejected by the patients (Rodrigues Coura and de Castro, 2002). Thus, the search for new chemoprophylactic trypanocidal agents has been the main goal in the prevention of Chagas' disease.

On the basis of these data, a comprehensive review of natural active principles with trypanocidal activity was published in 1996 (Sepulveda-Boza and Cassels, 1996). Many classes of secondary metabolites were described in this review, but there was no mention of a metabolite belonging to the lignan group. Many dibenzylbutyrolactone lignans exhibit a broad range of biological activities such as inhibition of platelet-aggregating factor, as well as antitumor, antiviral, and anti-inflammatory activities. These compounds also show significant trypanocidal activity, which makes the trypanocidal evaluation of derivatives of (−)-cubebin, isolated from the dry seeds of *Piper cubeba*, vitally important (Bastos et al., 1999). On the basis of this finding, (−)-hinokinin, an oxidized derivative of (−)-cubebin, was obtained by partial synthesis (Fig. 1) (Souza et al., 2005).

Because of the promising trypanocidal activity of (−)-hinokinin, as well as its potential use as an antichagasic drug, it is necessary to evaluate its possible mutagenic activity for safe use in humans. Therefore, the present study investigated *in vivo* the potential mutagenic and/or antimutagenic effects of (−)-hinokinin using the rat peripheral blood micronucleus test. Additional *in vitro* experiments were performed to understand the *in vivo* presumed antioxidant mechanism of action by measuring the production of hydrogen peroxide (H_2O_2) and other peroxides.

2. Materials and methods

2.1. Animals

For this experiment, four-week-old Wistar rats with a body weight (b.w.) of approximately 100 g obtained from the animal house of the Faculty of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, SP, Brazil, were used. The animals were kept in plastic boxes in an experimental room under controlled conditions of temperature (22 ± 2 °C) and humidity (50 ± 10%) on a 12 h light–dark cycle, with chow and water being available *ad libitum*. The study protocol was approved by the Ethics Committee for Animal Care of the University of Franca, Brazil (Process no. 001/05).

2.2. Drugs, reagents, solvents and compound identification procedures

Doxorubicin (DXR) was purchased from Pharmacia Brasil Ltd., São Paulo, Brazil. $CDCl_3$ and KCl were supplied by Merck Co., Darmstadt, Germany. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA), *t*-butyl hydroperoxide, dimethyl sulfoxide (DMSO), and Hepes-KOH were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. Methylene chloride and pyridinium chlorochromate were purchased from Acros Co., Pittsburgh, PA, USA. Acetone, chloroform, ethyl acetate, hexane, and methanol (MeOH) were supplied by Mallinckrodt Co., Xalostoc, Mexico. Ethanol was bought from a local distillery and purified by distillation. Silica gel was bought from Merck Co. Optical rotations were measured at $\lambda = 589$ nm on a Schmidt–Haensch polartronic HH8 polarimeter (Berlin, Germany) using a 1.0 cell. Infra Red (IR) spectra were recorded with a Nicolet FT-IR Protegé 520 instrument. Nuclear magnetic resonance (NMR) spectra were recorded with a Brucker DRX 400 spectrometer. Samples were dissolved in $CDCl_3$ and the spectra were calibrated at solvent signals of $\delta = 7.26$ (^{1}H) or $\delta = 77.0$ (^{13}C). A high-performance liquid chromatography (HPLC) apparatus equipped with an LC-10ADVP pump, an SPD-M10AVP diode array detector and a DGU-14A degasser (Shimadzu, Kyoto, Japan) was used for purity determination. Analysis was performed on a Shimadzu C-18 CLC-ODS column using an increasing gradient, starting with 50% MeOH:H_2O and ending with 100% MeOH, for 30 min. The solvents used in the reactions were generally distilled and dried before use.

2.3. Chromosome damage-inducing agent

DXR dissolved in distilled water was used as the inducer of micronuclei in rat peripheral blood cells (positive control).

2.4. Isolation of (−)-cubebin

Powdered seeds of commercially available *Piper cubeba* L. were exhaustively extracted by maceration with 96% ethanol. The concentrated crude extract was partitioned between the hexane and methanol/water (9:1) phases, providing 430 g of the dried methanol/water fraction. This mass was submitted to repeated column chromatography on 1.0 kg silica gel (12 × 120 cm). Elution with increasing ratios of hexane, dichloromethane, and ethyl acetate yielded six fractions. The cubebin-rich fractions (hexane/dichloromethane 1:1 and 100% dichloromethane) were submitted to repeated crystallization in hexane/acetone to provide crystalline (−)-cubebin (37 g), mp 130–131 °C, $[\alpha]_D^{26}$ −8.12° (*c* 0.46, $CHCl_3$). The chemical structure was confirmed by ^{1}H NMR and IR by comparison with data published in the literature (Koul et al., 1983). Purity was estimated to be 99% by both HPLC and spectral data analysis.

2.5. Preparation of (−)-hinokinin by partial synthesis

(−)-Cubebin (0.5004 g, in 10 ml dichloromethane) was treated with two equivalents (2.32 mM) of pyridinium chlorochromate at room temperature and the reaction mixture was stirred for 12 h. The solvent was removed under vacuum and the residue was chromatographed on silica gel in hexane–ethyl acetate (4:1), yielding 0.4926 g (98%) of an oily product ($[\alpha]_D^{26}$ −30 (26 °C) (*c* 0.99, $CHCl_3$)): ^{1}H NMR δ($CDCl_3$) 6.8–6.4 (m, 1 H), 5.9 (sl, 2H), 4.15 (dd, 1H, $J = 7.1$ Hz and $J = 9.3$ Hz), 3.85 (dd, 1H, $J = 7.1$ Hz and $J = 9.1$ Hz), 3.0 (dd, 1H, $J = 5.1$ Hz and $J = 14.2$ Hz), 2.85 (dd, 1H, $J = 7.3$ Hz and $J = 14.2$ Hz), 2.6 (d, 1H, $J = 7.1$ Hz), 2.55 (m, 1H), 2.45(d, 1H, $J = 8{,}6$ Hz), 2.4 (m, 1H); ^{13}C NMR δ($CDCl_3$) 178.4,

147.9, 147.8, 146.5, 146.4, 131.6, 131.3, 122.2, 121.55, 109.4, 108.8, 108.4, 108.3, 101.0, 71.2, 46.4, 41.3, 38.4, 34.8 (Souza et al., 2005).

2.6. Animal treatment

The doses of (−)-hinokinin were selected on the basis of its effectiveness in trypanocidal assays (Saraiva et al., 2006). The animals were divided into 10 treatment groups of six animals each (three males and three females) as follows: animals treated with three different doses of (−)-hinokinin (HK I = 10 mg/kg b.w.; HK II = 20 mg/kg b.w.; HK III = 40 mg/kg b.w.); DXR (90 mg/kg b.w.); DMSO (0.6 g/kg b.w.) and DXR; HK I and DXR; HK II and DXR; HK III and DXR, and negative (water) and solvent (DMSO) controls according to the protocol shown in Table 1. The doses of (−)-hinokinin were administered to the animals by gavage (1 ml/100 g b.w.), followed by the intraperitoneal (i.p.) injection of DXR (0.5 ml/100 g b.w.). The different doses of (−)-hinokinin were obtained from a stock solution of 4 mg/ml in 5.6% DMSO in water. The solvent control group was treated by gavage (1 ml/100 g b.w.) with the same DMSO dose as the animals that received 40 mg (−)-hinokinin/kg b.w. Peripheral blood samples were collected 24 h after the treatments.

2.7. Micronucleus test

The peripheral blood micronucleus assay was performed according to the protocol described by MacGregor et al. (1987). For determination of the frequency of micronucleated polychromatic erythrocytes (MNPCEs), 2000 polychromatic erythrocytes (PCEs) per animal were analyzed by light microscopy under oil immersion. The nuclear division index (NDI) was calculated by analysis of 400 erythrocytes (PCE/PCE + NCE [normochromatic erythrocytes]), in order to determine the cytotoxicity of (−)-hinokinin (Mersch-Sundermann et al., 2004).

The percent reduction in the frequency of MNPCEs (Waters et al., 1990) was calculated by the number of MNPCEs obtained after treatment with DXR minus the number found for the antigenotoxic treatments (HK I + DXR; HK II + DXR; HK III + DXR), divided by the number observed with DXR minus the number obtained for the negative control.

Table 1
Experimental groups and treatment protocol

Treatment	Group[a]	Dose
Control	1	Water
DMSO	2	0.6 g/kg b.w.
HK I	3	10 mg/kg b.w.
HK II	4	20 mg/kg b.w.
HK III	5	40 mg/kg b.w.
DXR	6	90 mg/kg b.w. (i.p.)
DMSO + DXR	7	As in (2) and (6)
HK I + DXR	8	As in (3) and (6)
HK II + DXR	9	As in (4) and (6)
HK III + DXR	10	As in (5) and (6)

DMSO = dimethyl sulfoxide; HK = (−)-hinokinin; DXR = doxorubicin.
[a] Number of animals in each group = 6.

2.8. Antioxidant activity of (−)-hinokinin

This activity was evaluated by measuring the production of H_2O_2 and other reactive oxygen species through the quantification of the oxidation product of H_2DCFDA, a dichlorofluorescein compound (Carthcart et al., 1983). For this, trypomastigote forms of *Trypanosoma cruzi* cultured in the LLC-MK_2 cell line (Hull et al., 1962) were suspended (1×10^6 parasites/ml) in a medium containing 125 mM sucrose, 65 mM KCl, and 10 mM Hepes-KOH, pH 7.2. Next, (−)-hinokinin was added to the medium at a concentration of 8, 32 and 64 μM, followed by the addition of 5 μM H_2DCFDA, and the system was incubated at room temperature for 35 min. The parasites were then removed by centrifugation (8000*g*, 4 °C, 10 min), and the fluorescence of the supernatant was determined at 485 nm as described by Fang and Beattie (2003).

t-Butyl hydroperoxide and a parasite-containing solution of 10% DMSO were used as positive and negative controls, respectively. The assays were performed in triplicate.

2.9. Statistical analysis

The data were analyzed statistically by analysis of variance for completely randomized experiments, with calculation of the *F* statistics and respective *P* values. When $P < 0.05$, treatment means were compared by the Tukey test and the minimum significant difference was calculated for $\alpha = 0.05$.

3. Results

The frequencies of MNPCEs in peripheral blood cells of Wistar rats treated with (−)-hinokinin and/or DXR are presented in Table 2. Analysis of animals treated with different doses of (−)-hinokinin showed no significant difference in the frequencies of micronucleated cells

Table 2
Frequency of micronucleated polychromatic erythrocytes (MNPCEs) and nuclear division indexes (NDI) of peripheral blood cells of animals submitted to treatment with different doses of (−)-hinokinin and/or DXR, and their respective controls

Treatment	NDI[a] (mean ± SD)	MNPCEs[b]		Reduction (%)
		Number	%	
Control	0.13 ± 0.03	19	0.16	–
DMSO	0.13 ± 0.02	12	0.10	–
HK I	0.13 ± 0.02	14	0.12	–
HK II	0.17 ± 0.03	10	0.08	–
HK III	0.19 ± 0.02	4	0.03	–
DXR	0.10 ± 0.02	158[c]	1.32	–
DMSO + DXR	0.11 ± 0.02	95[c]	0.80	–
HK I + DXR	0.12 ± 0.03	51[d]	0.42	76.9
HK II + DXR	0.10 ± 0.01	50[d]	0.42	77.7
HK III + DXR	0.09 ± 0.01	38[d]	0.32	86.3

[a] Four hundred erythrocytes were analyzed per animal, for a total of 2400 per treatment (PCE/PCE + NCE).
[b] Two thousand polychromatic erythrocytes (PCEs) were analyzed per animal, for a total of 12,000 per treatment.
[c] Significantly different from the control ($P < 0.05$).
[d] Significantly different from the DXR group ($P < 0.05$).

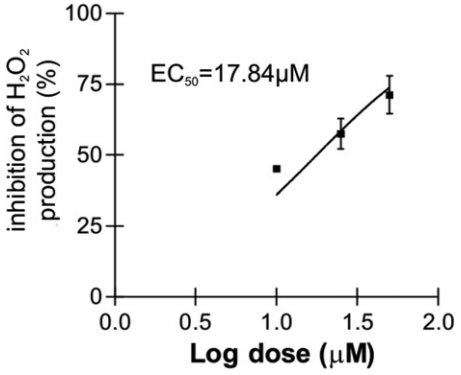

Fig. 2. EC_{50} value of (−)-hinokinin in the inhibition of H_2O_2 produced by *Trypanosoma cruzi* mitochondria.

(HK I = 0.12 ± 1.63%; HK II = 0.08 ± 1.86%; HK III = 0.03 ± 0.82%) compared to the control group (0.16 ± 1.60%), thus demonstrating the absence of a genotoxic effect. In contrast, animals treated with (−)-hinokinin and DXR presented a significant reduction in the frequencies of MNPCEs (HK I = 0.42 ± 4.32%; HK II = 0.42 ± 1.63%; HK III = 0.32 ± 3.78%) when compared to those treated with DXR only (1.32 ± 11.82%). However, this protective effect of (−)-hinokinin was not dose dependent.

The frequency of MNPCEs was lower in animals treated with DMSO and DXR (0.80 ± 5.23%) than in those treated with DXR alone (1.32 ± 11.82%), but this difference was not statistically significant.

Comparative analysis by the Tukey test showed no significant differences between male and female rats.

Mean NDI values and the respective standard deviations are presented in Table 2. No significant differences in NDI were observed between any treatment and the negative control group, demonstrating the absence of cytotoxicity in the different treatments under the conditions employed in this study.

Regarding the antioxidant assay, (−)-hinokinin inhibited the accumulation of H_2O_2 produced by mitochondria in the presence of the pro-oxidant compound, *t*-butyl hydroperoxide, at concentrations of 10, 25 and 50 μM by 46%, 57% and 72%, respectively. Fig. 2 shows the EC_{50} value for (−)-hinokinin obtained in the antioxidant assay. The positive and negative controls displayed 100% and 0% of oxidative effects, respectively.

4. Discussion

The aim of the present study was to assess the mutagenicity and/or antimutagenicity of (−)-hinokinin in Wistar rat peripheral blood. The results obtained here show that (−)-hinokinin does not increase the frequency of micronucleated cells; therefore, this compound has no genotoxic effect.

Regarding the test protocol employed here, Abramsson-Zetterberg et al. (1999) showed that because rats have been used as a model in conventional toxicological tests, it might be advantageous to use the micronucleus test in parallel as an indication of the genotoxic effect in rats. In the case of prolonged exposure of rats to drugs, which is common in toxicological tests, it should be possible to collect several peripheral blood samples from the same animal for the micronucleus test. The advantages of the possibility to use rat peripheral blood erythrocytes have encouraged several studies. The peripheral blood micronucleus test used in this study has been employed in many studies on the assessment of mutagenicity (Henderson et al., 1993; Sewerynek et al., 1996; Vijayalaxmi et al., 2001; Trosic et al., 2002; Hamanda et al., 2003). In addition, the increased frequency of MNPCEs observed in animals treated with the well-known clastogenic agent DXR shows that this test system may reveal an increase in the frequency of MNPCEs in animals treated with (−)-hinokinin.

In the *in vivo* experiments, a DXR dose of 90 mg/kg b.w. was chosen because this dose induces the increase in the frequency of MNPCEs necessary for the investigation of the antimutagenic potential of (−)-hinokinin. According to Chatterjee and Raman (1993), the full protective efficiency of an agent is elicited when the frequency of aberrations induced is high enough and the concentration of the protective agent administered is sufficient. On this basis, in the present study we decided that the minimum frequency of MNPCEs induced by DXR should be 1% or higher.

The present results also show that (−)-hinokinin causes a significant reduction in the frequency of MNPCEs induced by DXR. The antimutagenic effect of (−)-hinokinin might be due to its the ability to scavenge free radicals, acting as an antioxidant agent, since the genotoxic activity of the chemotherapeutic agent DXR has been attributed to its ability to produce free radicals (Keizer et al., 1990), which cause different types of damage to the cell including DNA cleavage. The chemical structure of DXR favors the generation of free radicals which can bind to iron and form complexes with DNA, inducing double-strand breaks (Eliot et al., 1984). Some studies have demonstrated that this oxidative damage is probably related to the formation of free radicals, with a concomitant reduction in antioxidant capacity (Myers et al., 1977).

In an attempt to better understand the mechanisms underlying the observed antimutagenic effect of (−)-hinokinin, we evaluated the antioxidant activity of (−)-hinokinin by measuring the production of H_2O_2 and other peroxides. (−)-Hinokinin displayed a significant antioxidant activity on the parasite mitochondria in the protocol used, suggesting that one possible mechanism by which this compound may exert a protective effect is by acting as a chemoprotective agent, preventing the DNA damage produced by the reactive oxygen species generated by DXR.

The observation that (−)-hinokinin does not exert a significant dose-dependent protection might be due to its erratic absorption by the cell membrane, leading to an inconstant bioavailability of the compound in the cell.

The lower frequency of MNPCEs observed for animals treated with DMSO and DXR compared to the group

treated only with DXR might be due to the ability of DMSO to scavenge reactive oxygen species (Waldren et al., 2004). However, this result was not statistically significant.

The present study shows that (−)-hinokinin not only had no genotoxic effect, but was also effective in reducing the chromosome damage induced by DXR. The exact mechanism of action of (−)-hinokinin is still unclear, but its anticlastogenic role observed here might be due to its antioxidant effect on the chromosome damage induced by the free radicals generated by DXR. Finally, our results contribute to a better understanding of the mechanism of action of this drug in the human body, which permits its more effective and safer use in clinical administrations.

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