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# GENOTOXIC ACTION OF THE SESQUITERPENE LACTONE GLAUCOLIDE B ON MAMMALIAN CELLS *in vitro* AND *in vivo*

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## ABSTRACT

Glaucolide B is a sesquiterpene lactone isolated from *Vernonia eremophila* Mart. (Vernonieae, Asteraceae) and has schistosomicidal, antimicrobial and analgesic activities. This study examined the cytotoxic and clastogenic activities of glaucolide B in human cultured lymphocytes and in bone marrow cells from BALB/c mice. The mitotic index (MI) and chromosomal aberrations were analyzed in both of the above systems, whereas sister chromatid exchanges (SCE) and the proliferation index (PI) were determined only *in vitro*. In human cultured lymphocytes, glaucolide B concentrations greater than 15 μg/ml of culture medium completely inhibited cell growth. At 4 μg/ml and 8 μg/ml of culture medium, glaucolide B significantly increased the frequency of chromosomal aberrations in lymphocytes and was also cytotoxic at concentrations ≥8 μg/ml; there was no increase in the frequency of SCE. Glaucolide B (160-640 mg/kg) did not significantly increase the frequency of chromosomal aberrations in mouse bone marrow cells nor did it affect cell division. Since glaucolide B showed no clastogenic action on mammalian cells *in vivo* but was cytotoxic and clastogenic *in vitro*, caution is needed in its medicinal use.

## INTRODUCTION

Plants synthesize toxic chemicals in large amounts, primarily as a defense against bacterial, fungal, insect and other animal predators. Various toxicological studies have assessed the mutagenic, teratogenic and carcinogenic properties of some of these chemicals (Ames, 1983; Konstantopoulou *et al.*, 1992; Varanda *et al.*, 1997). In many cases, the active compounds have been shown to be sesqui- and diterpenes (Gilbert *et al.*, 1970).

The sesquiterpene lactones (SL) from plants comprise a group of substances with a variety of biological effects. These compounds are terpenoids and are characteristic of the Asteraceae but may also occur in other angiosperm plant families as well as in plants of the genus *Hepatica*; their principal structural characteristic is the presence of α,β-unsaturated γ-lactone (Rodriguez *et al.*, 1976). SL possess antibacterial, antifungal, antitumor, antiprotozoal, antihelminthic, schistosomicidal, cytotoxic and analgesic activities (Picman, 1986). Glaucolide B (Figure 1) is an SL that is active against the embryos and adults of *Biomphalaria glabrata* snails, causing approximately 70 and 90% mortality, respectively, within 24 h; this compound has an analgesic action in mice and shows strong antimicrobial activity against *Bacillus cereus* (Alarcon *et al.*, 1990).

Based on these data, we have examined the clastogenic and cytotoxic activities of glaucolide B in temporary cultures of human lymphocytes and in bone marrow cells of BALB/c mice.

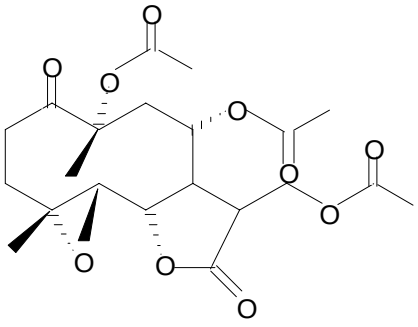

**Figure 1** - Structure of glaucolide B.

## MATERIAL AND METHODS

### Glaucolide B preparation

Glaucolide B extracted from *Vernonia eremophila* Mart. (family Asteraceae) (Alarcon *et al.*, 1990) was dissolved in dimethylsulfoxide (DMSO, Merck) and diluted in RPMI-1640 medium (Sigma) to the desired concentrations for the *in vitro* assays. For tests *in vivo*, the lactone was suspended in a solution of powdered milk at a concentration of 320 mg/kg body weight (b.w.).

### Assays with human peripheral blood lymphocytes

Human peripheral blood lymphocytes were obtained from six volunteers (three women and three men) and analyzed for chromosomal aberrations. For the analysis of sister chromatid exchanges (SCE) and proliferation index (PI), lymphocytes were obtained from three non-smoking, healthy volunteers (two women and one man) aged 18-21 years. Metaphase preparations were obtained as described by Moorhead *et al.* (1960). The lymphocytes

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were grown in 80% RPMI-1640 medium (Sigma) - 20% fetal calf serum (Cultilab) supplemented with penicillin (5 µg/ml) and streptomycin (10 µg/ml). The cells were stimulated with 2% phytohemagglutinin (Gibco). One milliliter of plasma was added to each 10 ml of culture medium, and the cultures incubated at 37°C for 48 h for chromosomal aberrations and 72 h for SCE studies.

Since preliminary tests showed that a glaucolide B concentration of 15 µg/ml totally inhibited lymphocyte division, concentrations of 2, 4 and 8 µg/ml of culture medium were used in subsequent experiments. Negative control cultures received no treatment. DMSO (final concentration, 0.104%), and 1-β-D-arabinofuranosylcytosine (Ara-C, concentration 0.0061 µg/ml) were used as the solvent and positive controls, respectively.

The cultures were treated with glaucolide B between $G_o$ and $G_1$ (6 h after incubation). The cells were harvested after 48 h to check for chromosomal aberrations and after 72 h to check for SCE. 5-Bromo-2'-deoxyuridine (BrdU; Sigma) was added to the SCE cultures to a final concentration of 10 µg/ml of culture medium. Mitotic arrest was achieved by adding colchicine (0.016%, 25 µl/10 ml; Sigma) 90 min before fixation. After hypotonic treatment (0.075 M KCl for 10 min) and fixation (methanol:glacial acetic acid, 3:1 v/v), the cells were stained with Giemsa diluted in 0.06 M phosphate buffer, pH 6.8, for the analysis of chromosomal aberrations and by the fluorescence plus Giemsa technique of Perry and Wolff (1974), in combination with the technique of Korenberg and Freedlender (1974) for SCE. One hundred metaphases from each culture were analyzed for chromosomal aberrations, and 2000 cells were scored to determine the mitotic index (MI) (48-h cultures). For SCE analysis, 50 second-division metaphases per culture were examined, and 200 metaphases from each culture were scored for first, second and third cell division (72-h cultures). The PI was obtained using the following equation (Degrassi *et al.*, 1989):

$$PI = [(M3 - M1) + 1],$$

where M1 and M3 are the percentages of the first- and third-division metaphases, respectively.

### Assay with BALB/c mice bone marrow cells

BALB/c mice (*Mus musculus*) weighing approximately 30 g were obtained from the Animal House of the Faculty of Medicine of Ribeirão Preto (USP). The mice were divided into groups of six animals (three females and three males) and were treated by gavage with glaucolide B diluted in a solution of powdered milk to give doses of 160, 320 and 640 mg/kg b.w. in a fixed volume of 0.5 ml. The highest dose was determined by the maximum solubility of glaucolide B. The mice were killed by ether inhalation 24 h after treatment and 90 min after intraperitoneal injection of 0.3 ml of a 1% colchicine solution (Sigma). The negative control group received distilled water, the solvent control group received a powdered milk solution (320 mg milk/kg b.w.) and the positive control group received 8 mg of cyclophosphamide/kg b.w.

Metaphase cell preparations were obtained from bone marrow cells by the technique of Ford and Hamerton (1956) adapted for mice by Rabello-Gay (1991). One hundred metaphases per animal were analyzed to determine the frequency of chromosomal aberrations. The mitotic index represented the number of metaphase cells detected in 2000 cells analyzed per animal and was expressed as a percentage.

### Statistical analysis

The Friedman and Kruskal-Wallis tests were used to analyze the results of the *in vitro* and *in vivo* assays, respectively, by comparing them with the corresponding negative control (Hollander and Wolf, 1973). P values = 0.05 were considered to indicate significance level. The positive control data were not included in the statistical analyses.

## RESULTS

### Human cultured lymphocytes exposed to glaucolide B

The chromosomal aberrations in human lymphocytes treated with glaucolide B are summarized in Table I. Gaps were the most consistent structural aberrations. The total frequency of chromosomal aberrations at glaucolide B concentrations of 4 and 8 µg/ml was significantly higher than the controls (P = 0.0387). There were no significant differences among individuals. Compared with the negative control, glaucolide B significantly increased number of cells with chromosome aberrations only at the highest concentration tested (P = 0.0407) (Table I). This concentration also significantly decreased the mitotic index compared to the controls (P = 0.0206). Glaucolide B did not increase the SCE frequency nor did it alter the cell PI (Table II).

### BALB/c mice bone marrow cells exposed to glaucolide B

The chromosomal aberrations seen in bone marrow cells from BALB/c mice treated with glaucolide B are presented in Table III. Most of the alterations were chromatid gaps. Glaucolide B did not significantly alter the total number of chromosomal aberrations or the number of cells with chromosomal aberrations. At the concentrations tested (160, 320 and 640 mg/kg), glaucolide B had no cytotoxic effect and did not significantly alter the mitotic index. The powdered milk solution used as the vehicle had no cytotoxic or clastogenic effects, although the number of chromosomal aberrations and the number of cells with aberrations were lower than in the treated and negative control groups (Table III).

**Table I** - The mitotic index (MI) and the number of chromosomal aberrations and abnormal metaphases in human cultured lymphocytes treated with glaucolide B.

| Treatment | | Chromosomal aberrations | | | | | | Aberrations per 100 cells | Abnormal metaphases | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MI (%) | G' | G" | B' | B" | OA | Total | | Total | (%) |
| **Control** | 6.9 | 12 | 0 | 7 | 2 | 0 | 21 | 3.5 | 21 | 3.5 |
| **DMSO#** | 7.2 | 9 | 0 | 5 | 2 | 0 | 16 | 2.7 | 15 | 2.5 |
| **Glaucolide B (µg/ml)** | | | | | | | | | | |
| **2** | 6.3 | 11 | 3 | 2 | 2 | 0 | 18 | 3.0 | 18 | 3.0 |
| **4** | 6.1 | 20 | 3 | 7 | 4 | 0 | 34* | 5.7* | 31 | 5.2 |
| **8** | 4.4* | 20 | 4 | 8 | 5 | 1 dic; 1 su | 39* | 6.5* | 35* | 5.8* |
| **Ara-C (0.0061 µg/ml)** | 5.7 | 33 | 12 | 39 | 50 | 2 dic | 136 | 22.7 | 129 | 21.5 |

A total of 600 cells were analyzed per treatment. G' = Chromatid gap; G" = chromosome gap; B' = chromatid break; B" = chromosome break; OA = other aberrations; dic = dicentric; su = sister union; DMSO = dimethylsulfoxide; Ara-C = Arabinofuranosylcytosine. #Final concentration of DMSO, 0.104%. *Significantly different from the controls (P<0.05).

**Table II** - Frequencies of sister chromatid exchange (SCE) and the proliferation index (PI) in human cultured lymphocytes treated with glaucolide B.

| Treatment | SCE | | Nº of metaphases | | | PI |
|---|---|---|---|---|---|---|
| | Mean ± SEM | Total | 1st cycle | 2nd cycle | 3rd cycle | |
| **Control** | 9.9 ± 0.3 | 1482 | 89 | 106 | 105 | 1.05 |
| **DMSO#** | 9.8 ± 0.4 | 1475 | 115 | 120 | 65 | 0.83 |
| **Glaucolide B (µg/ml)** | | | | | | |
| **2** | 8.9 ± 0.3 | 1338 | 123 | 97 | 80 | 0.86 |
| **4** | 10.7 ± 0.3 | 1611 | 104 | 154 | 42 | 0.79 |
| **8** | 10.0 ± 0.4 | 1496 | 138 | 110 | 52 | 0.71 |

A total of 150 and 200 cells were analyzed per treatment for SCE and PI, respectively. SEM = Standard error of the mean. #Final concentration, 0.104%.

**Table III** - The mitotic index (MI) and the number and frequency of chromosomal aberrations and abnormal metaphases in bone marrow cells from BALB/c mice treated by gavage with glaucolide B.

| Treatment | | Chromosomal aberrations | | | | | Aberrations per 100 cells | Abnormal metaphases | |
|---|---|---|---|---|---|---|---|---|---|
| | MI (%) | G' | G" | B' | B" | Total | | Total | (%) |
| **Control (water)** | 1.50 | 16 | 0 | 6 | 0 | 22 | 3.6 | 20 | 3.33 |
| **Milk (320 mg/kg b.w.)** | 1.45 | 9 | 1 | 3 | 0 | 13 | 2.1 | 13 | 2.17 |
| **Glaucolide B (mg/kg)** | | | | | | | | | |
| **160** | 1.25 | 10 | 0 | 10 | 0 | 20 | 3.3 | 20 | 3.33 |
| **320** | 1.47 | 14 | 0 | 7 | 1 | 22 | 3.7 | 22 | 3.67 |
| **640** | 1.40 | 12 | 0 | 9 | 0 | 21 | 3.5 | 21 | 3.50 |
| **CP (8.0 mg/kg)** | 2.45 | 13 | 0 | 70 | 0 | 83 | 13.8 | 57 | 9.5 |

The mice were sacrificed 24 h after treatment with glaucolide B. One hundred cells were analyzed per animal for a total of 600 cells per treatment. G' = Chromatid gap; G" = chromosome gap; B' = chromatid break; B" = chromosome break; CP = cyclophosphamide.

## DISCUSSION

Many plants and compounds derived from them have been tested for their toxicity and potential therapeutic action. Such studies have led to the discovery of new drugs which may be used in medicine. The SL are a group of plant substances with a variety of biological effects that are currently being tested for their possible therapeutic use. Evaluation of the cytotoxic and mutagenic potential of glaucolide B in human lymphocytes and in BALB/c mouse bone marrow cells is important because of the biological properties of this compound.

For many years, human lymphocyte cultures have been used to assess the clastogenic potential of test substances *in vitro* (Henderson *et al.*, 1997). However, since many drugs are (in)activated after their metabolization, tests *in vivo* of mutagenicity are recommended for assessing the clastogenic activity of certain compounds whose activity requires metabolic activation; such an approach also provides conditions closer to those found in humans (Legator and Ward Jr., 1991). Huggett *et al.* (1996) recommended that any assessment of genotoxicity should be based on studies *in vitro* and *in vivo*, and not simply on one or the other of these.

At concentrations above 15 µl/ml of culture medium glaucolide B was cytotoxic to lymphocytes, but did not change the frequency of chromosomal aberrations. Concentrations of 4 and 8 µl/ml of culture medium dose-dependently increased the frequency of chromosomal aberrations.

The frequency of chromosomal aberrations in the negative controls was 3.5/100 cells, which was within the range of 0-6.7% for spontaneous aberrations proposed by Kasuba *et al.* (1995).

Some authors do not recognize gaps as true chromosomal damage and attribute them to problems with the staining procedure (Preston *et al.*, 1987a). However, several studies on the clastogenic action of different compounds have suggested that gaps may indeed be associated with a mutagenic action (Goetz and Dohnalva, 1975; Tavares and Takahashi, 1996; Varanda *et al.*, 1997). Anderson and Richardson (1981) reported that the frequency of gaps was dependent on the dose of the mutagenic agent and that such gaps were as sensitive as other types of aberrations for indicating chromosomal damage. This conclusion agrees with our findings described above, particularly when the frequency of chromosomal gaps in cultured cells treated with glaucolide B was compared with that of the negative control.

SL may act directly on DNA and cause lethal cell damage (Jones *et al.*, 1981), inducing chromosomal breaks in human lymphocytes (Vaidya *et al.*, 1978). Parthenolide, a cytotoxic SL, inhibited DNA replication in HeLa cells, most likely by interfering with the DNA template. This lactone also caused single-strand breaks in HeLa cell DNA (Woynarowski and Konopa, 1981). This mechanism may explain the increased frequency of total chromosomal aberrations in lymphocytes exposed to glaucolide B. A similar clastogenic effect on human lymphocytes was found with the SL goyazensolide which significantly increased the number of chromosomal aberrations at a concentration of 0.6 µg/ml of culture medium (Mantovani *et al.*, 1993).

Concentrations of glaucolide B $\geq$ 15 µg/ml of culture medium were cytotoxic and inhibited the growth of cultured cells. A concentration of 8 µg/ml significantly reduced the MI when compared with the negative control. This effect may reflect the fact that lactones react strongly with the sulfhydryl (Hanson *et al.*, 1970) and thiol (Kupchan *et al.*, 1970; Smith *et al.*, 1972) groups of enzymes, thereby inhibiting activities which have biologically important functions. The inhibitory action of lactones may also be attributable to the presence of the O=C-C-$CH_2$ group which, according to Picman (1986), is responsible for the cytotoxicity. The inhibition of metabolic and enzymatic activities by SL may involve Michael-type reactions (Lee *et al.*, 1977).

SCE are frequently considered a parameter for assessing genotoxicity, since their frequency is clearly increased by exposure to many mutagenic and carcinogenic chemicals. However, SCE can occur spontaneously in an above normal quantity, even when there is no exposure to a known genotoxin (Bender *et al.*, 1992).

Glaucolide B did not increase the number of SCE compared to the controls. The frequency of SCE for the negative controls and DMSO were 9.9 and 9.8 SCE/cell, respectively, which is within the 5-10 SCE/cell basal frequency suggested by Natarajan and Obe (1986). There is considerable evidence that the mechanisms involved in the production of chromatid breaks are different from those causing SCE (Benedict and Jones, 1979).

In experiments with other lactones such as erematholide-C and 15 deoxygoyazensolide under the same conditions as in the present study, the frequency of SCE in human lymphocytes *in vitro* did not increase significantly (Vicentini-Dias, 1992). Similar results were observed for the pseudoguayanolide goyazensolide (Mantovani *et al.*, 1993) and eremanthine (Dias *et al.*, 1995).

The PI is a parameter which analyzes the kinetics of cellular division. The cytotoxicity of a compound can be assessed using PI because interference with the cell cycle delays or accelerates this index. The PI values obtained above show that there was little variation among the glaucolide B treated and control cells. Thus, glaucolide B does not influence the kinetics of cell growth.

Glaucolide B had no clastogenic effect in the assay in mice. Similar results were reported for eremanthine in BALB/c mouse bone marrow cells (Dias *et al.*, 1995), and for goyazensolide in Wistar rat bone marrow cells (Mantovani *et al.*, 1993).

The negative results with glaucolide B in the assays *in vivo* may indicate that the compound was bio-transformed in the liver, leading to its inactivation, or that there was rapid excretion of the metabolite with no effect on the bone marrow cells. In this regard, little is known of the

pharmacokinetics of glaucolide B in mice. It is also possible that bone marrow cells may not be the target organ for this compound.

Although various studies demonstrate the possibility of SLs having mutagenic activity, Woynarowski *et al.* (1981) suggested that SLs undergo some type of cellular metabolic transformation to be able to acquire the ability to damage DNA. However, no evidence of direct interaction between SL and DNA has been observed. DNA may not be a target molecule for the direct action of SLs, since most studies have demonstrated that SLs mainly act by inhibiting enzymes which play an important role in the maintenance of the integrity of cells and, consequently, of the organism.

In addition, the lactones can react with the nucleophilic centers of intracellular macromolecules (Jones *et al.*, 1981). Such a reaction with the thiol group of glutathione (Picman *et al.*, 1979; Schmidt, 1997), an important intracellular compound which participates in the inactivation of chemical substances (Hayes and Pulford, 1995), may be sufficient to protect the cell, and macromolecules such as DNA, from the effects of glaucolide B at the concentrations tested here. Woerdenbag *et al.* (1989) observed that glutathione depletion increased the extent of DNA damage caused by the SL eupatoriopicrin in tumor cells.

The MI of BALB/c mice showed that glaucolide B did not interfere with the growth and division of bone marrow cells. At the highest dose tested (640 mg/kg b.w.), no deathes were recorded.

Because it is only soluble in DMSO, intraperitoneal administration of glaucolide B requires a much greater volume of DMSO than is recommended by guidelines (0.1 ml) (Preston *et al.*, 1987b), so we opted for gavage, using powdered milk solution, which, besides not being toxic, was better accepted by the animals, assuring total drug ingestion. Milk was not cytotoxic or clastogenic, as observed by Tavares and Takahashi (1994) and Dias *et al.* (1995), who used milk to dilute the alkaloid boldine and the SL eremanthine, respectively.

In conclusion, glaucolide B had no significant clastogenic effect *in vivo*. However, since the compound did show cytotoxic and clastogenic effects in human lymphocytes *in vitro*, caution should be exercised in the use of this substance as a medicine.

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## RESUMO

O glaucolido B é uma lactona sesquiterpênica, γ-lactona α,β-insaturada, isolada da *Vernonia eremophila* Mart. (Vernonieae, Asteraceae); apresenta atividade esquistossomicida e antimicrobiana, além de atividade analgésica. A aceitação de uma substância para uso medicinal também depende de dados sobre sua toxicidade, além de sua eficiência medicinal. Assim, o objetivo deste trabalho foi testar a atividade clastogênica e citotóxica do composto glaucolido B *in vitro* e *in vivo*, utilizando linfócitos em cultura temporária e células da medula óssea de camundongos BALB/c, respectivamente. Analisaram-se o índice mitótico (MI) e as aberrações cromossômicas nos sistemas *in vitro* e *in vivo*, e trocas entre cromátides irmãs (SCE) e índice proliferativo (PI) somente no ensaio *in vitro*. Nas culturas de linfócitos humanos as concentrações superiores a 15 µg/ml de meio de cultura inibiram totalmente o crescimento celular. Os testes realizados com as concentrações 2, 4 e 8 µg/ml de meio de cultura demonstraram que o glaucolido B induziu aumento significativo na freqüência de aberrações cromossômicas nas culturas tratadas com as duas maiores concentrações, e mostrou-se citotóxico em concentrações iguais ou superiores a 8 µg/ml de meio de cultura, mas não aumentou a freqüência basal de SCE. A análise das células de medula óssea de camundongos não revelou aumento significativo na freqüência de aberrações cromossômicas com a administração de diferentes concentrações de glaucolido B (160, 320 e 640 mg/kg de peso corpóreo), e também não interferiu na divisão celular. Assim, este composto não apresentou ação clastogênica sobre células de mamíferos *in vivo*, no entanto teve efeito citotóxico e clastogênico *in vitro*, sendo necessário cautela no seu possível uso como medicamento.

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