

## EVALUATION OF THE GENOTOXIC ACTIVITY OF THE SESQUITERPENE LACTONE GOYAZENSOLIDE IN MAMMALIAN SYSTEMS *in vitro* AND *in vivo*

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### ABSTRACT

The sesquiterpene lactone goyazensolide has schistosomicidal and cytotoxic activities. This chemical substance was tested to determine clastogenic effects on mammalian systems *in vitro* (human peripheral blood lymphocyte cultures) and *in vivo* (Wistar rat bone marrow cells). The concentrations tested *in vitro* were 0.1, 0.3, 0.6 and 1.0 µg/ml culture medium, and *in vivo* were 0.4, 0.8, 1.6 and 3.2 mg/100 g body weight. All *in vitro* treatments with goyazensolide and only the 0.8 mg *in vivo* treatment had significantly more chromosome gaps than the controls. However, only *in vitro* analysis of the number of cells with chromosome breaks demonstrated that treatment with 0.6 µg goyazensolide/ml culture medium has a clastogenic action. Goyazensolide was found to have no effect on SCE induction *in vitro*. The toxic dose was determined both *in vitro* (1.0 µg/ml) and *in vivo* (3.2 mg/100 g).

### INTRODUCTION

Many plant extracts and their active principles have been described and utilized as therapeutic agents (Farnsworth *et al.*, 1985). Among them, we find many of the sesquiterpene lactones (SLs) which are components of compositae species. In plants, the SLs or their metabolites are believed to participate in protective mechanisms against

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pathogenic organisms, herbivorous insects and mammals, and they also appear to function as allelopathic agents in competition with other plants. SLs have antileukemic, hypotensive, analgesic, cytostatic, antimicrobial and antihelminthic activities and they produce allergic contact dermatitis (Picman, 1986).

Goyazensolide, an SL with schistosomicidal and cytotoxic properties (Vichnewski *et al.*, 1976; Herz and Goedken, 1982), has potential for use in the treatment and prophylaxis of schistosomiasis. We investigated the clastogenic effects of goyazensolide on mammalian systems *in vivo* and *in vitro*.

## MATERIAL AND METHODS

### *Chemical agent*

The sesquiterpene lactone goyazensolide (C<sub>19</sub>H<sub>20</sub>O<sub>7</sub>) is a secondary metabolite of *Eremanthus* Less. Our sample was extracted from *E. mattogrossensis* kuntze (family Asteraceae) by Prof. Walter Vichnewski.

Goyazensolide was dissolved in the organic solvent dimethyl sulfoxide (DMSO) and diluted with distilled water to the desired concentrations.

### *In vitro* assay in human peripheral blood lymphocyte cultures for the determination of chromosome aberrations and sister chromatid exchanges

Blood samples were obtained from five healthy non-smoking individuals (three women and two men) aged 20 to 30 years. Twelve cultures were made with the samples from each individual, six of them for the analysis of chromosome aberrations (CA) and six for the analysis of sister chromatid exchanges (SCE). Lymphocytes were incubated in 10 ml of culture medium consisting of 80% RPMI 1640 medium (4% phytohemagglutinin, 0.01 mg streptomycin/ml and 0.005 mg penicillin/ml), and 20% normal human serum. Cultures were incubated at 37°C and harvested after 48 hours for CA determination and after 72 hours for SCE determination. An aliquot of 0.05 ml colchicine solution (0.016 mg/ml) was added to the cultures one hour and 45 minutes before fixation. At time zero, 0.1 ml of the respective treatment was added to each culture. The six cultures for CA determination were divided into the following treatments: 1. H<sub>2</sub>O (negative control); 2. 0.025% DMSO (solvent control); 3. 0.1 µg goyazensolide/ml culture medium; 4. 0.3 µg goyazensolide/ml culture medium; 5. 0.6 µg goyazensolide/ml culture medium; 6. 1.0 µg goyazensolide/ml culture medium. The six cultures for SCE determination were divided into the same treatments performed for CA. Metaphase preparations for chromosome analysis were obtained by the macroculture technique of Moorhead *et al.* (1960). The slides obtained from 72-hour cultures for SCE determination

were stained by the fluorescence plus Giemsa technique of Perry and Wolff (1974), in combination with the technique of Korenberg and Freedlender (1974).

#### *In vivo assay with Wistar rat bone marrow cells for the determination of chromosome aberrations*

Forty-two Wistar rats (*Rattus norvegicus*) weighing approximately 100 g were supplied by the main Animal Rearing Facility of the Faculty of Medicine of Ribeirão Preto. The animals were divided into seven groups of three males and three females each, submitted to the following respective treatments: 1. H<sub>2</sub>O (negative control); 2. 0.025% DMSO (solvent control); 3. 0.4 mg goyazensolide/100 g body weight; 4. 0.8 mg goyazensolide/100 g body weight; 5. 1.6 mg goyazensolide/100 g body weight; 6. 3.2 mg goyazensolide/100 g body weight; 7. 1.5 mg cyclophosphamide (CP)/100 g body weight (positive control). All animals were treated with i.p injections (1.0 ml) and sacrificed 24 hours later; one hour and 30 minutes before sacrifice, the animals received i.p. 0.5 ml of 0.16% colchicine solution. Metaphase preparations were obtained by the technique of Ford and Hamerton (1956).

#### *Slide analysis*

One-hundred metaphases per culture (*in vitro* assay) or per animal (*in vivo* assay) were analyzed for the presence of chromosome aberrations (gaps and breaks). For SCE analysis (*in vitro* assay), 50 metaphases per culture were analyzed. The mitotic indices (MI) reported for the *in vitro* and *in vivo* assays correspond to the number of metaphase cells detected in 1000 cells analyzed per culture or per animal and are expressed as percentages.

Data concerning chromosome aberrations for the *in vitro* and *in vivo* assays are reported as the number of cells with breaks or with gaps, detected in each treatment. Data concerning SCE number (*in vitro* assay) are reported as mean SCE number per cell detected in each treatment.

#### *Statistical analysis*

The data were analyzed statistically by the conditional test for the detection of rare events (Pereira, 1991), with the level of significance set at  $\alpha = 0.05$ .

## RESULTS

#### *In vitro assay*

The number of cells with chromosome gaps presented was significantly greater ( $p < 0.007$ ) in cultures treated with goyazensolide when compared to the controls (negative control and DMSO) (Table I).

Table I - Mitotic index (MI), frequency of cells with chromosome aberrations and mean SCE numbers detected in lymphocyte cultures treated with goyazensolide. A total of 500 and 250 cells were analyzed per treatment for chromosome aberrations and SCE, respectively.

Treatment	Number of cells with chromosome aberrations		MI %	SCE: $\bar{X} \pm SE$
	With gaps	With breaks		
Control	2	5	4.2	11.10 $\pm$ 0.27
DMSO	6	4	4.1	10.53 $\pm$ 0.25
0.1 $\mu\text{g}^*$	18+	9	3.7	11.40 $\pm$ 0.30
0.3 $\mu\text{g}^*$	16+	9	3.3	10.53 $\pm$ 0.28
0.6 $\mu\text{g}^*$	13+	20+	3.3	10.68 $\pm$ 0.29
1.0 $\mu\text{g}^*$	----- toxic dose -----			

\* -  $\mu\text{g}$  goyazensolide/ml culture medium; SCE - sister chromatid exchange; MI - mitotic index; DMSO - dimethyl sulfoxide (0.025%); + - statistically significant difference ( $P < 0.007$ ); SE - standard error.

Only the 0.6  $\mu\text{g}$  treatment induced a significantly greater number of cells with chromosome breaks than the controls (Table I), for 0.1  $\mu\text{g}$  and 0.3  $\mu\text{g}$  the increase was not significant ( $P > 0.10$ ).

Mitotic indices were not significantly affected by goyazensolide-treatment cultures ( $P > 0.15$ ), though 1.0  $\mu\text{g}/\text{ml}$  culture medium was toxic, inhibiting cell growth (Table I).

Mean SCE number/cell was about 11.0 for all treatments (Table I). There was no significant difference between goyazensolide-treated cultures and controls ( $P > 0.50$ ).

### *In vivo assay*

The number of cells with gaps was significantly greater than in controls (negative control and DMSO) only for the 0.8 mg goyazensolide/100 g body weight treatment ( $P < 0.005$ ), for 0.4 mg and 1.6 mg, there was no significant difference ( $P > 0.77$  and 0.20, respectively) (Table II).

Goyazensolide treatment did not significantly affect the number of cells with chromosome breaks (0.4 mg,  $P > 0.10$ ; 0.8 mg,  $P > 0.20$ ; 1.6 mg,  $P > 0.50$ ) (Table II). With cyclophosphamide (CP) treatment the number of altered cells was higher than for the controls and similar to data reported in the literature (Table II).

Table II - Mitotic index (MI) and frequency of cells with chromosome aberrations detected in bone marrow cells of wistar rats treated with goyazensolide. A total of 600 cells were analyzed per treatment.

Treatment	Number of cells with chromosome aberrations		MI %
	With gaps	With breaks	
Control	12	9	2.4
DMSO	13	4	2.5
0.4 mg*	10	11	2.3
0.8 mg*	27+	10	2.1
1.6 mg*	17	6	2.0
3.2 mg*	----- toxic dose -----		
CP	26	79	1.6

\* - mg goyazensolide/100 g body weight; DMSO - dimethyl sulfoxide (0.025%); CP - 1.5 mg cyclophosphamide/100 g body weight (positive control); MI - mitotic index; + - significantly different from the controls.

Goyazensolide treatment did not significantly affect mitotic index values (0.4 mg,  $P > 0.45$ ; 0.8 mg,  $P > 0.30$ ; 1.6 mg,  $P > 0.25$ ) (Table II). Treatment with 3.2 mg goyazensolide/100 g body weight was fully toxic, inhibiting cell division.

## DISCUSSION

Some SLs are genotoxic (MacGregor, 1977; Norman *et al.*, 1976; Manners *et al.*, 1978; Vaidya *et al.*, 1978; Woyanorowski *et al.*, 1981). Our *in vitro* tests demonstrated that goyazensolide causes a significant increase in gaps, whereas only the 0.6  $\mu$ g treatment induced chromosome breaks. In the *in vivo* assay, none of the goyazensolide concentrations tested had a clastogenic effect and only the 0.8 mg concentration induced a significant increase in the number of gaps. Goyazensolide was found to have no effect on SCE induction *in vitro*.

The evaluation of goyazensolide concentrations close to the toxic dose and below such a dose both *in vivo* and *in vitro* permitted us to assess the possible effects over a broad spectrum of activity.

In our evaluation of the effect of goyazensolide we did not consider the increase in chromosome gaps detected as clastogenic activity since many investigators recommend this type of procedure (Natarajan and Obe, 1986; Brusick, 1987; Preston *et*

*al.*, 1987a,b). We detected a significant induction of gaps in treatments with goyazensolide, both *in vitro* and *in vivo*. The induction of gaps by goyazensolide may be related to inhibition of DNA repair enzymes (Picman, 1986).

In comparing the results obtained for gaps (which increased both *in vitro* and *in vivo*) with those obtained for breaks (which increased only *in vitro*), we observed no correlation between them, but rather a relation between the induction of these changes and the toxic doses and test system used.

The clastogenic concentration (0.6  $\mu\text{g}$ ) observed *in vitro* was very close to the toxic dose (1.0  $\mu\text{g}$ ), whereas the lower concentrations tested (0.3 and 0.1  $\mu\text{g}$ ) had no clastogenic effect. This leads us to conclude that, *in vitro*, the limit between the toxic effect and clastogenic effect of goyazensolide, in terms of concentration, is very narrow.

Klimek *et al.* (1981) suggested that the  $\alpha$ -methylene- $\gamma$ -lactone portion, when present in the SL molecule, may be responsible for the inhibition of nucleic acid synthesis in human lymphocyte cultures. This has been determined on the basis of the marked inhibition of [ $^3\text{H}$ ]-uridine incorporation into RNA and of [ $^3\text{H}$ ]-thymidine into DNA by the SLs alatalide, eupatoriopicrin and hydroxysonobilin. This may be the mechanism of action of goyazensolide in our lymphocyte culture assay, where the compound was clastogenic at the concentration of 0.6  $\mu\text{g}$ , since its molecule also has the  $\alpha$ -methylene- $\gamma$ -lactone portion. The inhibition of DNA and RNA synthesis by SLs has also been reported for other cell types both *in vivo* and *in vitro* (Lee *et al.*, 1977; Hall *et al.*, 1977, 1980; Hladon and Twardowski, 1979; Spring *et al.*, 1982; Woynarowski and Konopa, 1980).

Although the goyazensolide concentrations tested *in vivo* were close to the toxic dose (3.2 mg), no clastogenic effect was observed. This difference in chromosome breaks between the two systems (no significant activity *in vivo* and more breaks *in vitro*) indicates that goyazensolide is less effective *in vivo* than *in vitro*, at the DNA level, probably due to the *in vivo* interaction of goyazensolide with the organism as a whole, which permits metabolism, inactivation and excretion of the compound.

SLs metabolism seems not to affect the ability of these substances to produce clastogenic effects (MacGregor, 1977), as demonstrated by the data obtained here for both assays. However, Woynarowski *et al.* (1981) suggested that SLs undergo some type of cellular metabolic transformation to be able to acquire the ability to damage DNA. Nevertheless, at present there is no evidence that might permit a generalization of these results in terms of the need for metabolic transformation for SL to produce DNA damage.

SLs are highly interactive with cellular enzymes presenting sulphhydryl groups, which they inhibit (Picman, 1986). This may be the mechanism by which toxic doses of goyazensolide (1.0  $\mu\text{g}$  *in vitro* and 3.2 mg *in vivo*) cause growth inhibition or cell death. It has been suggested that the inhibition of the metabolism and activity of cellular enzymes by SLs occurs by Michael-type reactions (Lee *et al.*, 1977).

The presence of glutathione (GSH) in cells, a substance capable of reacting with SLs (Picman *et al.*, 1979), may be sufficient to protect the cells when they are exposed to goyazensolide concentrations below the toxic dose *in vivo* and to concentrations below the clastogenic dose (0.6 µg) *in vitro*, since GSH is an important cell compound which participates in the mechanism of inactivation of chemical substances (Greim *et al.*, 1981). Woerdenbag *et al.* (1989) observed that GSH depletion (by buthionine sulfoximine) in tumoral cells increases the extent of DNA damage caused by the SL eupatopicrin. Also SLs may damage DNA and this damage may be repaired (Jones *et al.*, 1981).

Although various studies demonstrate the possibility of SLs having mutagenic activity, 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.

### ACKNOWLEDGMENTS

We are grateful to Miss Sueli Aparecida Neves, Mr. Luiz Augusto da Costa Junior and Mr. Silvio Avelino dos Santos for technical assistance.

We are indebted to Dr. Marco Aurelio Sicchiroli Lavrador, FCFRP-USP, for statistical analysis of the data.

Research supported by FINEP, CAPES and CNPq. Publication supported by FAPESP.

### RESUMO

A lactona sesquiterpênica goyazensolide apresenta atividades esquistossomocida e citotóxica. Essa substância química foi testada em sistemas de mamíferos *in vitro* (cultura de linfócitos do sangue periférico humano) e *in vivo* (células de medula óssea de ratos Wistar) para determinar seu efeito clastogênico. *In vitro* foram testadas as concentrações de 0,1; 0,3; 0,6 e 1,0 µg/ml de meio de cultura, e *in vivo* as concentrações de 0,4; 0,8; 1,6 e 3,2 mg/100 g de peso corpóreo. A análise do número de células com *gaps* cromossômicos demonstrou que todos tratamentos com goyazensolide *in vitro* e somente o tratamento com 0,8 mg *in vivo* foram significativamente maiores do que nos controles. Contudo, na análise estatística do número de células com quebras cromossômicas demonstramos, somente *in vitro*, que o tratamento com 0,6 mg goyazensolide/ml de meio de cultura tem uma ação clastogênica. O goyazensolide não causou efeito sobre a indução de SCE *in vitro*. A dose tóxica foi determinada tanto *in vitro* (1,0 µg/ml) como *in vivo* (3,2 mg/100 g).

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(Received March 5, 1993)