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# Effect of goniothalamin on the development of Ehrlich solid tumor in mice

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ABSTRACT

In this work the antiproliferative activity of goniothalamin (**1**), both in racemic and in its enantiomeric pure forms, in a solid tumor experimental model using laboratory animals is described. The antiedematogenic activity displayed by racemic **1** in the carrageenan edema model in mice together with the reduction of Ehrlich solid tumor model suggest a relationship between anticancer and antiinflammatory activities with the antiinflammatory activity favoring the antiproliferative activity itself.



## 1. Introduction

The styryl lactone goniothalamin (**1**, Fig. 1) is a secondary metabolite widely distributed among plants of the genus *Goniothalamus* (Annonaceae). Recent studies have shown the cytotoxic and antiproliferative properties of this compound.[1–3] The natural enantiomer (*R*)-**1** has displayed antiproliferative activity against a variety of cancer cell lines, including cervical (Hela), gastric (HGC-27), kidney (786-0), breast carcinomas (MCF-7, T47D and MDA-MB-231), and leukemia (HL-60, Jurkat and CEM-SS).[1,4–9]

Studies in our laboratories have shown that the non-natural isomer (*S*)-**1** also displayed antiproliferative activity against several cancer cell lines with a promising selectivity against kidney cancer cell line 786-0 and adriamycin resistant ovarian cancer cells (NCI-ADR/RES).[1,10] Other reports point to the apoptotic activity of (*R*)-**1** in cancer cells via different mechanisms, including the increase in the expression of caspases 3,7, and 9, increase in the expression of pro-apoptotic protein Bax, inhibition of constitutive NOS and inhibition of nucleus–cytoplasm transport mediated by CRM1.[1,4,5,11]

The development of experimental models has contributed to the study of antineoplasic compounds and to the understanding of their mode of action. Solid tumors are structures resembling organs in their complexity and heterogeneity, with a microenvironment formed by tumor and stroma cells which are embedded in the extracellular matrix and in the presence of a vascular network. Inside these tumors there are differences in pH, oxygen pressure, and nutrient flux which often contribute to tumor resistance to chemotherapy due to irregular distribution of drugs inside the tumor matrix. Therefore, the development of experimental models to complement in vitro drug screening is necessary due to the limitations inherent to cell cultures to predict the behavior of solid tumors to chemotherapy.[12,13]

There are a number of in vivo experimental models based on laboratory animals including the Ehrlich solid tumor, derived from the mouse breast adenocarcinoma which is an aggressive and fast-growing carcinoma able to develop both in the ascitic or in the solid form depending whether inoculated intraperitoneously or subcutaneously, respectively.[14–17] The advantage of utilizing transplanted tumors is due to the previous knowledge of the amount and initial features of the tumor cells to be inoculated and to the fast development of neoplasia thus reducing the duration of the study.[18]

While several results based on in vitro studies have been already described for (*R*)- and (*S*)-**1**, including their proposed mode of action, much less is known about their role in animal models of cancer (in vivo). In this study, we report the in vitro activity of

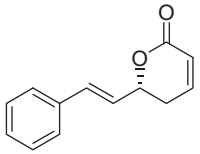

**Figure 1.** Naturally occurring (*R*)-goniothalamin.

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racemic **1** and its activity, as well as of its constituent enantiomers (*R*)-**1** and (*S*)-**1**, on the development of Ehrlich solid tumor in mice and on the carrageenan-induced paw edema model motivated by the evidence reported in the literature between cancer and inflammation.[19]

## 2. Results and discussion

### 2.1. In vitro antiproliferative activity of racemic, (*R*) and (*S*)-1 in human tumor cell lines

Considering that different cell lines display different sensitivities toward the same cytotoxic compound, in the present study we have selected cell lines of various histological origin: endodermal (NCI-460, HT-29), ectodermal (UACC-62, MCF-7, NCI-ADR/RES, U251), and mesodermal (OVCAR-03, PC-3, 786-0). Cell proliferation was determined by spectrophotometric assay using sulforhodamine B as protein-binding dye.[20] Racemic, (*R*) and (*S*)-goniothalamin (**1**) were used at concentrations between 0.25 and 250 μg/mL and doxorubicin (DOX 0.025–25 μg/mL) as positive control. Concentration that elicits total growth inhibition (TGI) was determined after 48 h of cell treatment.[21] The initial in vitro screening evaluations were planned to select the best candidates for anticancer drug development in animal models and, eventually, in clinical trials.

Both enantiomers and racemic **1** displayed antiproliferative activity against the cancer cell lines tested (Table 1). (*R*) and (*S*)-**1** showed lower TGI values against kidney cancer cell line 786-0 and adriamycin resistant ovarian cancer cells (NCI-ADR/RES), as described by de Fátima et al.[9,10] Racemic-**1** has displayed similar activity profile as (*R*)-**1** and (*S*)-**1** against NCI-ADR/RES, 786-0, NCI-460, PC-03, and U251 but, in analogy to (*R*)-**1**, it displayed higher potency against UACC-62, MCF-7, OVCAR-03, and HT-29 than (*S*)-**1**.

### 2.2. In vivo assays

#### 2.2.1. Acute toxicity

No evidence of toxicity was observed 4 h after administration of 50, 100, and 300 mg/kg dose of goniothalamin either in racemic, (*R*) and (*S*) forms as well as during the following 15 days when the animals were kept under observation. Therefore, the above dosages were considered safe for the following experiments and in vivo animal tests were carried out with 30, 100, and 300 mg/kg of racemic-**1** and 50 mg/kg of (*R*)-**1** and (*S*)-**1**.

#### 2.2.2. Ehrlich solid tumor

After the seventh day of the experiment, treatments with 30, 100, and 300 mg/kg of racemic-**1** and 10 mg/kg of 5-Fluorouracil (5-FU) inhibited 50.0 ± 19.3%, 41.7 ± 13.9%, 35.7 ± 2.5%, and 41.7 ± 17.8% tumor growth respectively, as compared to negative control, with statistically equal inhibition for all treatments investigated ($p$ <0.05). The activity above was maintained through the end of the experiment (four treatments) when tumor growth was

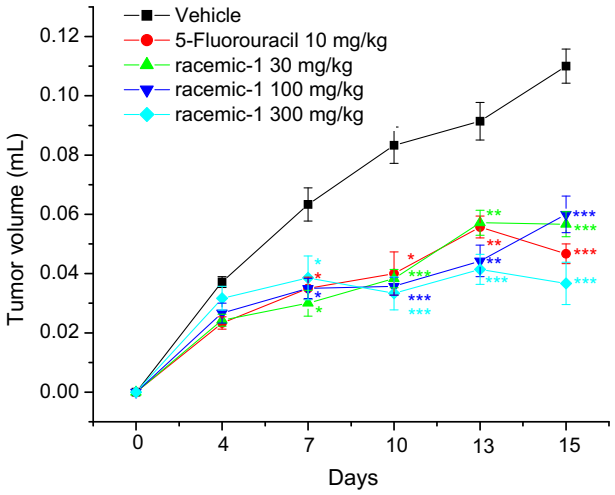


**Figure 2.** Effect of racemic-**1** on Ehrlich solid tumor expressed as tumor volume (mL) per day of treatment. Treatments with racemic-**1** at the doses 30, 100, and 300 mg/kg resulted in significant tumor growth inhibition. 5-Fluorouracil was the positive control and vehicle was saline 0.9%. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 statistically different from the group treated with vehicle (ANOVA).

inhibited by 57.6 ± 7.4% in the group treated with 5-FU (Fig. 2) and 48.5 ± 9.4%, 40.9 ± 9.5%, and 66.7 ± 15.9% upon treatment with racemic **1** at 30, 100, and 300 mg/kg, respectively ($p$ <0.001).

In accordance to these data, the relative tumor weight (tumor weight/animal weight ratio) was inhibited by 46.1 ± 7.8% in the group treated with 5-FU and 41.8 ± 26.0%, 31.6 ± 22.9%, and 36.7 ± 15.9% in the groups treated with racemic **1** at 300, 100, and 30 mg/kg dose, respectively ($p$ <0.05, Fig. 3).

Our initial in vitro studies have shown that (*S*)-**1** was significantly more potent against kidney cancer cells (786-0) and adriamycin resistant ovarian cancer cells (NCI-ADR/RES) when compared to the natural (*R*)-enantiomer.[10] As the importance of absolute stereochemistry on the pharmacological profile is well known since the marketing of racemic thalidomide and considering the promising results obtained with racemic **1** in Ehrlich solid tumor model, we decided to evaluate (*R*)-**1** and (*S*)-**1** in order to study the role of absolute configuration in experiments with Ehrlich solid tumor model in mice.[22] As the results of racemic **1** were similar for all treatments evaluated ($p$ <0.001), the 50 mg/kg dose of (*R*)-**1** and (*S*)-**1** was selected for further evaluation and doxorubicin (3 mg/kg) was employed as the positive control.

Goniothalamin enantiomers started to inhibit tumor growth after the 10th day, after administration of the third dose (Fig. 4). Enantiomer (*S*)-**1** inhibited the increase in tumor volume by 53.7 ± 20.9% at the 10th day a profile which was maintained up to the end of the experiment when inhibition of 48.7 ± 9.3% was observed. In animals treated with (*R*)-**1**, the inhibitory effect was observed after the 13th day of treatment (21.9 ± 15.6%), with similar results when compared with the other experimental groups at the end of experiment (37.4 ± 10.8%) ($p$ <0.001). Doxorubicin has

**Table 1**
TGI values, given in μM, for (*R*)-, (*S*)- and racemic goniothalamin [(*R*)-, (*S*)- and racemic-**1**] and doxorubicin (DOX) necessary for total inhibition of tumor cell proliferation[a]

| | UACC-62 | MCF-7 | NCI-ADR/RES | 786-0 | NCI-460 | PC-03 | OVCAR-03 | HT-29 | U251 |
|---|---|---|---|---|---|---|---|---|---|
| (*R*)-1 | 21.56 | 21.68 | 1.20 | 18.57 | 32.36 | 13.47 | 8.31 | 21.98 | 13.37 |
| (*S*)-1 | 76.35 | 77.03 | 11.46 | 28.65 | 44.06 | 24.03 | >100 | 57.55 | 24.05 |
| Racemic-1 | 23.25 | 22.68 | 6.38 | 22.38 | 36.35 | 18.24 | 18.81 | 29.21 | 18.16 |
| DOX[b] | 0.32 | 10.99 | 2.83 | 7.86 | 2.37 | 4.03 | 10.25 | 16.40 | 1.42 |

[a] Concentration that elicits total growth inhibition (TGI) was determined from non-linear regression analysis using the ORIGIN 7.5® (OriginLab Corporation).
[b] Doxorubicin (DOX) was the positive control.

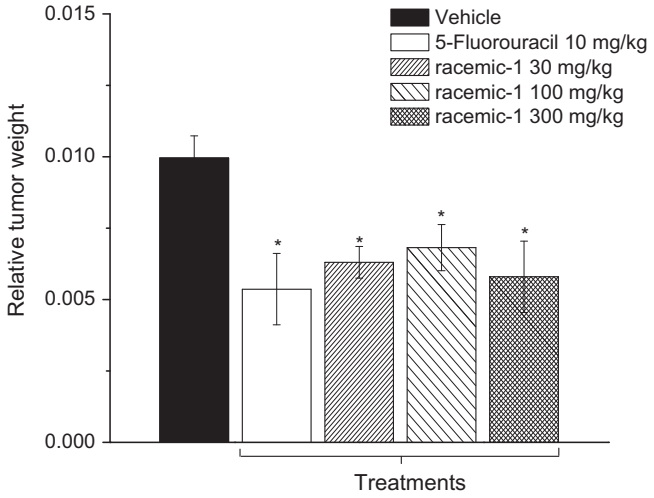


**Figure 3.** Relative tumor weight, expressed by the difference between the weights of the paw with tumor and of the healthy paw divided by the corporal weight. Treatments with racemic **1** at the doses 30, 100, and 300 mg/kg and with 5-Fluorouracil (10 mg/kg) resulted in significant reduction of relative tumor weight. Vehicle was saline 0.9%. $^{*}p$ <0.05, statistically different from the group treated with vehicle (ANOVA).

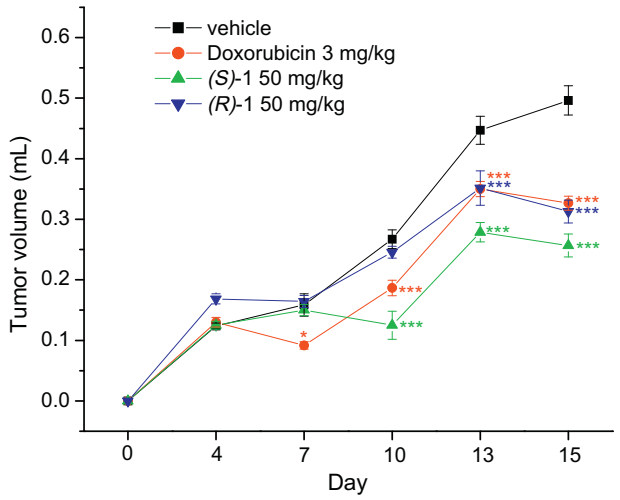


**Figure 4.** Effect of (*R*) and (*S*)-**1** on Ehrlich solid tumor expressed as tumor volume (mL) per day of treatment. Treatments with (*R*) and (*S*)-**1** at the dose 50 mg/kg resulted in significant tumor growth inhibition. Doxorubicin was the positive control and vehicle was saline 0.9%. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 statistically different from the group treated with vehicle (ANOVA).

inhibited tumor growth when compared to the negative control, after the seventh day (42.7 ± 9.2%) which was maintained up to the end of the experiment (34.7 ± 5.6%).

The data for tumor volume reduction were corroborated by the relative tumor weight (Fig. 5) which was kept at 28.0 ± 11.0% for doxorubicin while with (*R*)-**1** and (*S*)-**1** it was inhibited 25.3 ± 16.2% and 39.7 ± 20.3%, respectively ($p$ <0.001). During the experiments, animals were weighed and observed for toxicity of the evaluated compounds. After the experiments (four treatments), no sign of toxicity or weight loss was observed for the animals treated with racemic-**1** at 30, 100, and 300 mg/kg dosage, (*R*)-**1** and (*S*)-**1** (50 mg/kg), and 5-FU (10 mg/kg) while animals treated with doxorubicin (3 mg/kg) displayed loss of body weight (2.5 g loss at the end of the experiment). Doxorubicin is a highly potent antitumor agent that is one of the most active antineoplastic drugs developed to date. However, its application is limited by its dose-

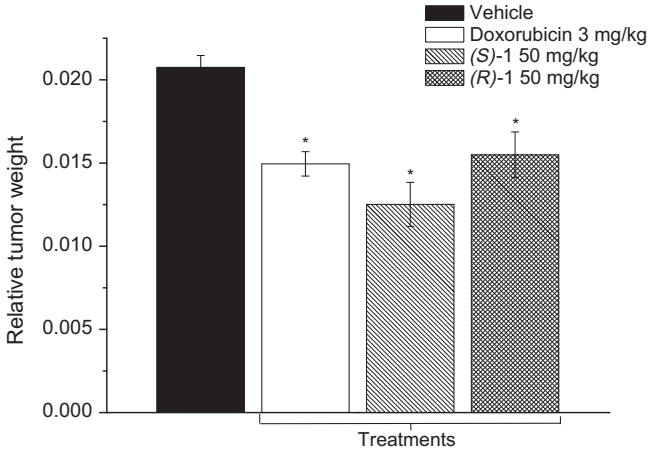


**Figure 5.** Relative tumor weight, expressed by the difference between the weights of the paw with tumor and of the healthy paw divided by the corporal weight. Treatments with (*R*) and (*S*)-**1** at the dose 50 mg/kg and with doxorubicin (3 mg/kg) resulted in significant reduction of relative tumor weight. Vehicle was saline 0.9%. $^{*}p$ <0.05, statistically different from the group treated with vehicle (ANOVA).

related side effects that include bone marrow toxicity, gastrointestinal disorders, stomatitis, alopecia, acute, and cumulative cardiotoxicity as well as edema.[23]

The results obtained in the Ehrlich solid tumor model at the end of the experiments with (*R*)- and (*S*)-**1** have shown that there is no statistically significant difference for both enantiomers as well as between them and the racemic form of goniothalamin. The toxicity displayed by most of the drugs available for cancer chemotherapy is one of the major challenges for the development of new drugs which ideally should display high therapeutic indexes being highly selective for tumor cells.[24] Therefore, the lack of toxicity displayed by goniothalamin is a promising result for the ensuing pre-clinical studies of this molecule.

The Ehrlich tumor is a fast-growing and aggressive adenocarcinoma which generates a local inflammatory response characterized by the increased vascular permeability which accounts for edema formation and promotes cell migration.[18]

Several studies have established the link between cancer and chronic inflammatory processes. Virchow reported the presence of inflammatory mediators in developing tumors with chronic edema. Epidemiological evidences support such relationship and suggest that more than 25% of cancer diseases are related to chronic infections or other types of inflammation.[19]

Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis.[25] Cancer-associated inflammation includes the infiltration of white blood cells, prominently phagocytic cells called macrophages (TAM), the presence of polypeptide messengers of inflammation (as tumor necrosis factor, interleukin-1, and chemokines) and the occurrence of tissue remodeling and angiogenesis.[26]

Based on these evidences and on its low toxicity and better yield, racemic **1** was evaluated in the carrageenan-induced paw edema model in mice.

### 2.2.3. Carrageenan-induced paw edema

The carrageenan edema model in mice has two distinct phases: the early phase starts immediately after carrageenan injection and it lasts for about 6 h while the late phase starts after 6 h and it ends about 72 h after carrageenan injection, with an inflammatory peak being observed between 48 and 72 h.[27,28] Serotonin, phospholipase $A_2$ ($PLA_2$), histamine, kinins, arachidonate metabolites (prostaglandins, leukotrienes), and nitric oxide (NO) have been strongly linked to the inflammatory process in the early phase, while the media-

tors in the late phase are suspected to be producing an edema dependent on mobilization of neutrophils, when COX-2 expression is increased and NO production is decreased.[28–30]

At the initial stage of the carrageenan edema model, the antiedematogenic activity of racemic **1** was noticed at its highest dose (300 mg/kg) with piroxicam (20 mg/kg) employed as the positive control. While goniothalamin has displayed antiinflammatory activity during the early phase of the experiment, piroxicam had lost it at the end of this phase (Fig. 6a). Compared to the negative control, no significant antiinflammatory activity was observed for the lower dose of racemic **1** in the early phase, while the 100 mg/kg dose inhibited edema only during part of this phase (between 3 and 4.5 h, Fig. 6b). In the late phase (after 6 h until the end of the experiment), the antiinflammatory activity of racemic **1** at the highest dose was observed with inhibition of the inflammatory peak which occurs between 48 and 72 h after induction of inflammation (Fig. 6a). Interestingly, the 100 mg/kg dose of goniothalamin, which did not display a pronounced antiinflammatory activity in the early phase, was effective after 24 h and kept its effect until the end of the experiment (Fig 6b). Similarly, the 30 mg/kg dose displayed antiinflammatory effect after 48 h as well as piroxicam employed as the positive control (Fig. 6a and b). In the event, all protocols evaluated have significantly inhibited the inflammatory edema when compared with the negative control ($p > 0.05$).

The antiinflammatory activity of doxorubicin (5 mg/kg) was also evaluated in the inflammatory edema assay induced by carrageenan. In this experiment, doxorubicin only started to reduce mice paw edema after 24 h of the inflammation onset (Fig. 6a), during the late phase when intense neutrophil mobilization occurs and leukotrienes are involved. In fact, the activity plot for doxorubicin nicely correlates with that for goniothalamin (30 mg/kg) which is consistent with goniothalamin displaying an activity profile which is dose dependent: high doses inhibit all phases while lower doses preferentially act in the late phase of the inflammation process.

Prostaglandins and leukotrienes promote tumor growth due to their role in the relation between tumor and stroma cells thus establishing a tumor microenvironment which facilitates angiogenesis and evades the immune system attack. Moreover, leukotrienes modulate proliferation, tumor differentiation, apoptosis and interfere with migration, and invasion of the tumor cells. In the tumor microenvironment, cells of the immune system and endothelium are recruited to produce more pro-inflammatory mediators including eicosanoids, growth, and angiogenic factors.[31]

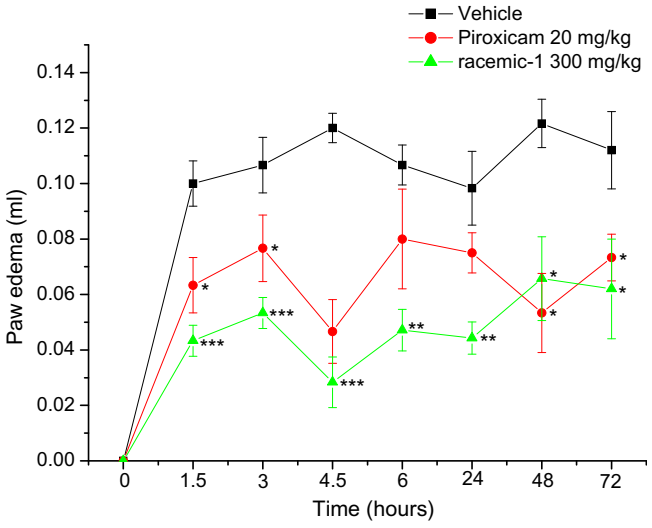


**Figure 6a.** Effect of racemic-**1** on carrageenan-induced paw edema expressed as paw edema (mL) per time after inflammation induction. Mice were treated with racemic-**1** (300 mg/kg), saline 0.9% or Piroxicam (20 mg/kg) 30 min before intraplantar carrageenan 2.5% injection. Paw edema was evaluated in plethysmometer 1.5, 3.0, 4.5, 6.0, 24, 48, and 72 h after inflammation induction $p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ statistically different from the group treated with vehicle (ANOVA).

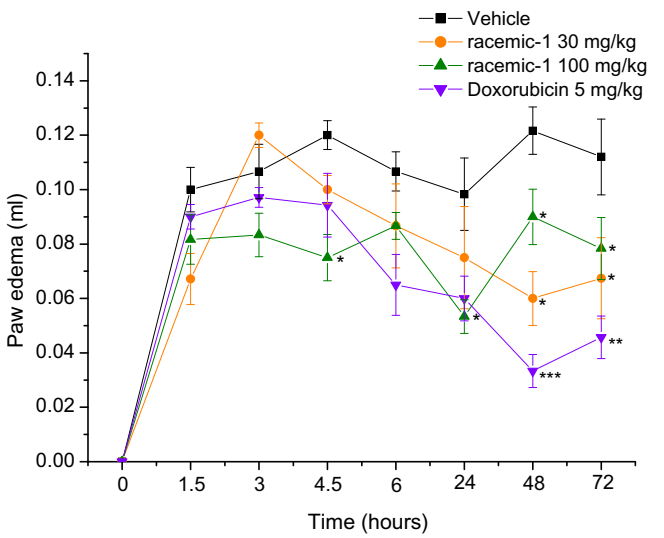


**Figure 6b.** Effect of racemic-**1** on carrageenan-induced paw edema expressed as paw edema (mL) per time after inflammation induction. Mice were treated with racemic-**1** (30 mg/kg and 100 mg/kg), saline 0.9% or doxorubicin (5 mg/kg) 30 min before intraplantar carrageenan 2.5% injection. Paw edema was evaluated in plethysmometer 1.5, 3.0, 4.5, 6.0, 24, 48, and 72 h after inflammation induction. $p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ statistically different from the group treated with vehicle (ANOVA).

The participation of the cellular response can be observed in the activity profile of doxorubicin (Fig. 6b), which induces severe leukopenia, reducing only the second phase of the inflammatory response.

Solid tumors are one of the leading causes of death in the western countries with an increasing number of cancer patients every year. Although the prognosis of these patients has improved during the last decade, there is still a need for novel treatments.[32] Therefore, the inhibition of the initial and late phases of the inflammation process by goniothalamin points to a dual strategy for the control of the tumor growth progression which may be promising for the development of novel chemotherapics for the treatment of solid tumors.

Additionally, the activity of goniothalamin in the Ehrlich solid tumor assay shows its systemic action as after intraperitoneous treatment the drug reached the foot paw. Generally, the complexity of the tumor biology related to its interaction with the associated stroma often leads to failure when drugs with a good in vitro profile enter in vivo experimentation.[24] This is however not the case for goniothalamin (**1**) which has displayed promising in vivo activity which supports its further evaluation in in vivo models in order to shed light into its mode of action.

## 3. Conclusions

In this work we were able to demonstrate the antiproliferative activity of goniothalamin (**1**), both in racemic or enantiomeric pure forms, in a solid tumor experimental model using laboratory animals. No evidence of toxic effects were found in the animals after single and repeated doses. The antiedematogenic activity displayed by racemic **1** in the carrageenan edema model in mice together with the reduction of Ehrlich solid tumor model, suggest a relationship between anticancer and antiinflammatory activities with the antiin-

flammatory activity favoring the antiproliferative activity itself. Further studies will be carried out in order to evaluate the antiinflammatory activity of each enantiomer as well as the mode of action of racemic **1** which leads to the observed antiedematogenic activity.

## 4. Materials and methods

### 4.1. Racemic and (*R*)- and (*S*)-goniothalamin

(*R*)- and (*S*)-goniothalamin enantiomers were prepared according to previously described methodology.[9,10] The racemic form was prepared accordingly except for the utilization of allylmagnesium bromide in substitution to the enantioselective allylation step.[9,10]

### 4.2. In vitro antiproliferative assay

#### 4.2.1. Cell lines

Human tumor cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-460 (lung, non-small cells), OVCAR-03 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), U251 (glioma) and NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance) were obtained from National Cancer Institute at Frederick, MA, USA.

#### 4.2.2. Cell culture

Stock cultures were grown in medium containing 5 mL RPMI 1640 (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS, GIBCO) at 37 °C with 5% $CO_2$. Penicillin:streptomycin (1000 μg/L: 1000 U/L, 1 mL/L) was added to the experimental cultures.

#### 4.2.3. Antiproliferative assay

Cells in 96-well plates (100 μL cells well$^{-1}$) were exposed to (*R*)-, (*S*)- and racemic-**1** concentrations in DMSO (Merck)/RPMI (0.25, 2.5, 25, and 250 μg mL$^{-1}$) at 37 °C, 5% of $CO_2$ in air for 48 h. Final DMSO concentration did not affect cell viability. Afterwards cells were fixed with 50% trichloroacetic acid (Merck) and cell proliferation determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay.[20]

Using the concentration–response curve for each cell line, the TGI (concentration that produces total growth inhibition or cytostatic effect) were determined through non-linear regression analysis (Table 1) using software ORIGIN 7.5® (OriginLab Corporation).[21]

### 4.3. In vivo assays

#### 4.3.1. Animals

Balb/C mice (20–35 g) obtained from CEMIB-Unicamp were maintained in a room with controlled temperature 25 ± 2 °C for 12 h light/dark cycle, with free access to food and water. Animal care, research and animal sacrifice protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Biology Institute/UNICAMP—Ethical Committee for Animal Research (2124-1 and 2126-1).

#### 4.3.2. Drugs

Doxorubicin (Europharma), 5-Fluorouracil (Europharma), Piroxicam (Pfizer), and Carrageenan (Sigma–Aldrich, USA).

#### 4.3.3. Acute toxicity

Balb/C mice were treated intraperitoneally with racemic **1**, (*R*)-**1**, and (*S*)-**1** (50, 100, and 300 mg/kg). Groups were observed during 4 h and then daily for 14 days. The following parameters were evaluated: general toxicity signals like body weight loss, locomotion, behavior (agitation, lethargy), respiration, salivation, tearing eyes, cyanosis, and mortality.[33,34]

#### 4.3.4. Ehrlich solid tumor

**4.3.4.1. Cells maintenance and preparation.** Ehrlich tumor cells were maintained in the ascites form by peritoneal passages in mice by weekly transplantation of $5 \times 10^5$ tumor cells. For testing, cells were prepared at a density of $2.5 \times 10^6$ cells/60 μL/animal.[35]

**4.3.4.2. Experimental procedure.** Experiments were designed according to Kleeb et al. (1999), with several modifications.[35] Balb/C mice ($n = 8$/group) right hind paw basal volume was measured using a plethysmometer (Panlab, Spain) and then were inoculated with 60 μL ($2.5 \times 10^6$ cells) into the right hind footpad. Tumor volume was determined by the difference between measured volume and basal volume, and evaluated every third day, until 15 days after cell inoculation, when animals were sacrificed. Then, both paws were removed and weighed, providing the relative tumor weight, calculated as the difference between the weights of the paw with tumor and of the healthy paw divided by the corporal weight. The animals were treated intraperitoneally every three day since third day after cell inoculation, as follows: negative control (vehicle-saline 0.9%), positive controls (Doxorubicin 3 mg/kg or 5-FU 10 mg/kg) and experimental groups (racemic **1** at 30, 100, and 300 mg/kg doses, (*R*)-**1** and (*S*)-**1** at 50 mg/kg dose.

#### 4.3.5. Carrageenan induced paw edema

**4.3.5.1. Experimental procedure.** Experiments were designed according to Posadas et al. (2004) and Nunes et al. (2007), with several modifications.[27,28] Right hind paw basal volume of Balb/C mice ($n = 8$/group) was measured using a plethysmometer (Panlab, Spain) and then were treated intraperitoneally as follows: negative control (vehicle-saline 0,9%), positive control (Piroxicam 20 mg/kg) and experimental groups (racemic **1** at doses 30, 100, and 300 mg/kg and doxorubicin 5 mg/kg). After 30 min, inflammation was induced by inoculation of 40 μl of carrageenan 2.5% into the right hind footpad and edema was determined by difference between measured volume and basal volume and evaluated 1.5, 3.0, 4.5, 6.0, 24, 48, and 72 h after carrageenan inoculation.

#### 4.3.6. Statistical analysis

Results were expressed as % inhibition ± standard deviation. All results were submitted to one way analysis of variance (ANOVA), considering as critical level $p \leqslant 0.05$ to evaluate significant difference between the control and treated groups, followed by Duncan's Test, using StatSoft® software. Graphs were designed using the Origin® software.

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