Cytotoxic Effect of the Diterpene Lactone Dehydrocrotonin from *Croton cajucara* on Human Promyelocytic Leukemia Cells

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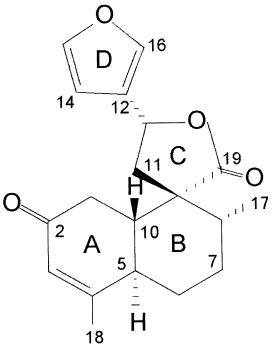

Fig. **1** Chemical structure of *trans*-dehydrocrotonin.

Abstract

Diterpenes exhibit potent antineoplastic properties against human and murine carcinoma cell lines. *trans*-Dehydrocrotonin from *Croton cajucara*, a Brazilian medicinal plant, is a nor-diterpene with antiulcerogenic activity. In this work, we examined the effect of *trans*-dehydrocrotonin (*t*-DCTN) on the vitality of HL60 cells by assessing the MTT reduction, protein content and phosphatase activity of these cells. Protein quantification indicated that *t*-DCTN reduced the number of cells with an IC_{50} of 500 μM; mitochondrial function (MTT reduction), was also inhibited (IC_{50} = 300 μM), when the cells were treated for 24 h. In contrast, when the cells were treated with this lactone in the initial plating and cultured for 96 h, *t*-DCTN was more toxic for all parameters analyzed: MTT and phosphatase activity (IC_{50} = 180 μM) and protein content (IC_{50} = 150 μM). The flavonoid utilized as positive control myricetin and the following IC_{50} values were obtained after 24 h of treatment: 300 and 192 μM for protein content and MTT reduction, respectively. According to the chemical characteristics of both compounds, the cytotoxic effect of *t*-DCTN could be explained through two mechanisms: adduct formation with DNA and proteins and/or oxidative stress induction.

Croton cajucara is a plant found in the Amazonian region, where it is used by local populations as a medicinal plant. A 19-nor-clerodane diterpene, trans-dehydrocrotonin, is an important bioactive compound isolated from the bark of *Croton cajucara* Benth. (Euphorbiaceae), commonly known as sacaca [1]. Previous studies have shown that this lactone (*t*-DCTN; Fig. **1**) has a similar diterpenolactone structure to that found in other *Croton* species, and that it has antiulcerogenic [2] and antineoplastic properties against Ehrlich tumor [1]. Cytotoxic drug therapy remains the principal method for treating myeloid leukemia. One limitation with this therapy is its serious toxicity, including myelosuppression and immunodeficiency. Cytotoxic drug therapy should ideally achieve a balance between its efficacy and toxicity to leukemic and normal myelocytes [3]. Phosphatases are hydrolases that catalyze the hydrolysis of monoester phosphates and are divided into three groups: acid phosphatase, alkaline phosphatase and protein phosphatase. Protein phosphorylation can be reversed by protein phosphatases, which are divided into serine/threonine phosphatases and tyrosine phosphatases, based on their substrate specificity [4]. The aim of this work was to examine the cytotoxicity of *t*-DCTN *in vitro* by assessing its effect on MTT reduction [(3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide], protein content and protein tyrosine phosphatase activity. Previous studies in our laboratory have shown that the major protein phosphatase from HL60 cell is a protein tyrosine phosphatase.

t-DCTN was used at concentrations up to 500 μM prepared in 0.8% of DMSO. At this concentration DMSO has no effect on HL60 vitality. As shown in Fig. **2A**, the IC_{50} values for the effect on protein content and MTT reduction were 500 μM and 300 μM, respectively. The decrease in protein content shown in Fig. **2A** and **B** induces cell death, probably through apoptosis. Mitochondrial function was also affected by *t*-DCTN, perhaps through an action on succinate dehydrogenase and/or a decrease in the cell number. In contrast to the other parameters, it was not possible to calculate an IC_{50} value for phosphatase activity. The influence of the length of exposure to *t*-DCTN on the above parameters was also examined. *t*-DCTN was more toxic when the cells were treated with the lactone during plating and for the next 96 h. Under these conditions we obtained the following IC_{50} values: MTT and phosphatase activity (180 μM) and protein content (150 μM) as shown in Fig. **2B** and for myricetin the IC_{50} values were 300 and 192 μM for protein content and MTT reduction, respectively (Fig. **3**).

Sesquiterpene lactones have functional groups that are responsible for their biological effects. The structure of *t*-DCTN contains three highly reactive functional groups: O = C-C = C (ring A), a lactone (ring C) and cyclopentenone (ring D). Since the structure of *t*-DCTN is not planar, the most available group is O = C-C = C, which can function as a Michael acceptor to interact with the SH groups of proteins, GSH and nitrogen bases, mainly guanine, through nucleophilic attack [5], [6]. Other diterpenes isolated from *C. incanus* and *Laetia corymbulosa* also exhibited cytotoxic effect on several tumor cell lines, and the authors suggested that their effect was due to interaction with macromolecules [7], [8]. Furthermore, recently, some authors have shown that several sesquiterpene lactones may generate a reactive intermediate capable of damaging cellular constituents [8]. The oxi-

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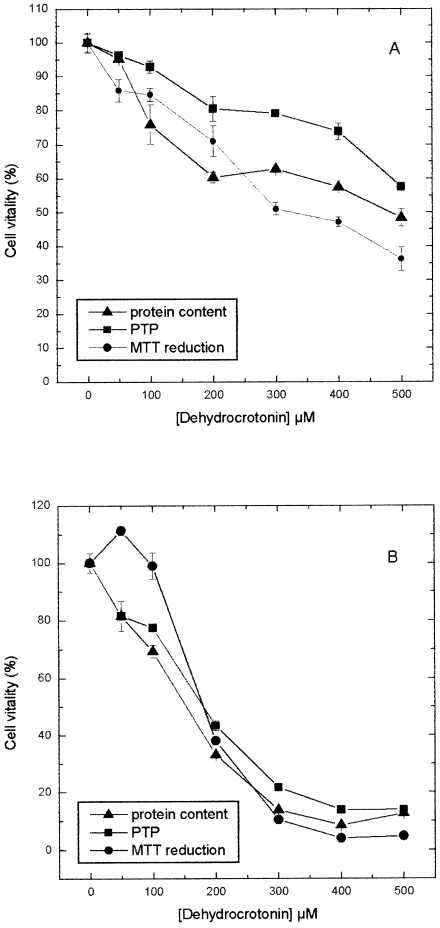

Fig. **2** Effect of *t*-DCTN on cell vitality. The curves show the effects of *t*-DCTN on MTT reduction, protein content and protein tyrosine phosphatase activity in HL60 cells. The inhibition was expressed relative to normal cell vitality (100%) and the points represent the mean ± SD of at least three experiments run in quadruplicate. Panels **A** and **B** show the cell vitality following treatment with *t*-DCTN for 24 h and 96 h, respectively.

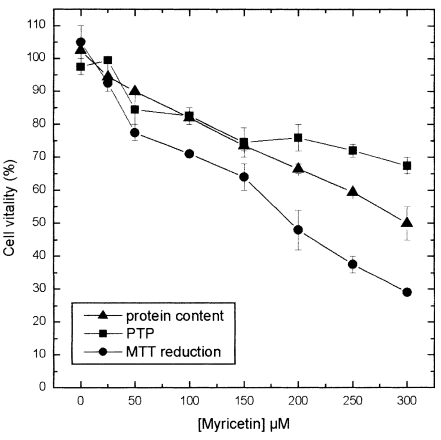

Fig. **3** Effect of myricetin on cell vitality. The curves show the effects of myricetin on MTT reduction, protein content and protein tyrosine phosphatase activity in HL60 cells. The inhibition was expressed relative to normal cell vitality (100%) and the points represent the mean ± SD of at least three experiments run in quadruplicate. The figure shows the cell vitality following treatment with myricetin for 24 h.

dative stress provoked by reaction of these compounds with molecular oxygen results in the formation of hydrogen peroxide, altering the intracellular redox equilibrium. These data suggest that the oxygen reactive species generated also play an important role in *t*-DCTN's cytotoxicity.

In conclusion, analyzing these results together, it is possible that the cytotoxic effect of *t*-DCTN could be due to the formation of adducts with macromolecules and/or induction of oxidative stress.

Materials and Methods

HL60 cells were kindly supplied by Dr. Rui Curi/Valdemir Vieira Colleone of the University of São Paulo (São Paulo, Brazil). The extraction of the powdered bark of *Croton cajucara* was carried out with hexane by a standard method and *t*-DCTN was characterized by spectroscopic methods such as IR, UV, MS and ^{1}H- and ^{13}C-NMR and $[\alpha]_D^{25}$ + 10.6° ($CHCl_3$, *c* 0.6) as recently described [1], [9]. The purity was over 99% as assessed by NMR [10], [11]. Myricetin was obtained from Sigma Co. All other chemicals were of the highest purity available.

HL60 cells were routinely grown in suspension in RPMI (Roswell Park Memorial Institute) medium containing glutamine (0.200 g/L) and antibiotics (100 IU penicillin/mL, 100 µg streptomycin/mL) and supplemented with 10% heat-inactivated fetal calf serum, in a 5% CO_2 humidified atmosphere at 37 °C. The cells were treated with *t*-DCTN at different times, 96 h and 24 h. HL60 cells were inoculated with 3×10^5 cells/mL (24-well tissue culture plates) and incubated with this drug for 96 h. When the cells were treated only for 24 h, *t*-DCTN was added 72 h after plating of the cells; in this case the cell density was 9×10^5 cells/mL. Under both conditions, after 96 h the cytotoxicity assays (MTT reduction, protein quantification and phosphatase activity) were performed. The lactone was used in the concentration range of 50 – 500 µM. At all concentrations utilized the t-DCTN was prepared in 0.8% DMSO.

MTT assay: The medium containing *t*-DCTN was removed and 1 mL of MTT solution (0.5 mg MTT/mL of culture medium) was added to each well. After incubation for 4 h at 37 °C, the medium was removed and the formazan solubilized in 1 mL of ethanol. The plate was shaken for 5 min on a plate shaker and the absorbance then measured at 570 nm [12], [13].

Protein phosphatase assay: The enzyme was obtained by lysing the cells with 1 mM acetate buffer, pH 5.5. The reaction mixture (final volume, 1 mL) contained 100 mM acetate buffer, pH 5.5, 5 mM *p*-nitrophenyl phosphate and enzyme (cell extract). After a 30 min incubation at 37 °C, the reaction was stopped by adding 1 mL of 1 M NaOH. The amount of *p*-nitrophenol produced was measured at 405 nm [14].

Protein quantification: Protein concentrations were determined by a modification of Lowry's method [15].

Acknowledgements

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