Anti-Tumour Activity of Two 19-Nor-clerodane Diterpenes, *trans*-Dehydrocrotonin and *trans*-Crotonin, from *Croton cajucara*

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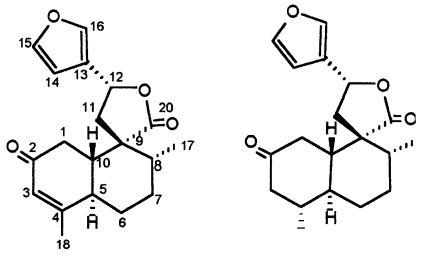
Abstract: The effects of two nor-diterpenes, *trans*-dehydrocrotonin (DCTN) and *trans*-crotonin (CTN) from *Croton cajucara* (Euphorbiaceae), on the survival of mice bearing Sarcoma 180 and Ehrlich carcinoma ascitic tumours, on the proliferation of cultured Ehrlich cells and TNFα activity were determined. When the mice were treated with 80 and 120 mg/kg of DCTN or 38 mg/kg of 5-FU a significant anti-tumour activity was obtained (%T/C of 128 – 140). The cytotoxicity against Ehrlich carcinoma was 16 μM for DCTN and CTN whereas the flavonoid quercetin was cytotoxic at 44 μM in 48 h cell culture. No apoptosis was seen on *in vitro* electrophoresis of DNA extracted from the tumour cells treated with DCTN and CTN. A significant TNFα activity was detected in Ehrlich tumour-bearing mice treated with DCTN suggesting an enhanced immune function.

Key words: *Croton cajucara*, Euphorbiaceae, clerodane diterpene, anti-tumour activity, TNFα activity.

Introduction

Croton cajucara is a plant found in the Amazonian region, where it is used by local populations as a medicinal plant. A clerodane nor-diterpene, *trans*-dehydrocrotonin (DCTN) is an important bioactive compound of *Croton cajucara* (1).



DCTN **CTN**

Since the structure determination of the first member (–)clerodine (2), of the clerodane diterpenes, this class of compounds has been shown to have a wide distribution among plants, marine species, and microorganisms. The differences exhibited among the members of this group are the levels of oxidation at the various positions of the decalin skeleton. In spite of their abundance in nature, the bioactivities of most clerodanes are unexplored. Although only a small number of compounds have been screened, a large number of physiological properties were found such as anti-microbial (3) and anti-inflammatory (4).

Diterpenes from *Euphorbia esula* L. (5) and *Premna schimperi* (6) showed a significant cytotoxic activity against human and murine carcinoma cell lines. The most notable naturally occurring diterpene, taxol, extracted from the bark of *Taxus brevifolia* has significant efficacy against human breast and ovarian tumours (7).

To obtain more information on diterpenes in cancer chemotherapy, in the present communication we report the evaluation of *in vivo* and *in vitro* anti-tumour activity of *trans*-dehydrocrotonin (DCTN) and of *trans*-crotonin (CTN), previously isolated from the bark of *Croton cajucara* (Euphorbiaceae), against two murine tumours Sarcoma 180 (S180) and Ehrlich carcinoma. The anti-tumour activities of these drugs were compared with the anti-metabolite 5-fluorouracil (5-FU). In addition, the diterpenes abilities to induce apoptosis *in vitro* and tumour necrosis factor-α (TNFα) activity *in vivo* were evaluated.

Materials and Methods

Materials

5-FU, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and quercetin were obtained from Sigma Co. (USA). Plant material, *Croton cajucara* Benth (local name Sacaca) was collected in Jacundá-PA (Amazon region, Brazil) and botanically authenticated by Dr. Nelson A. Rosa, Museum Parense Emilio Goeldi. A voucher specimen number 247 is deposited at the herbarium of the same Museum.

Extraction, isolation and identification of DCTN

The extraction of the powdered bark of *Croton cajucara* was carried out with hexane by a standard method and characterized by spectroscopic methods as IR, UV, MS and ^{1}H- and ^{13}C-NMR, as recently described (8).

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Hydrogenation of trans*-dehydrocrotonin*

DCTN in 95% ethanol was hydrogenated over prereduced 10% palladized charcoal. The reduction was complete after 2 h. CTN was then obtained by recrystallization twice from hexane-Me_2CO. The identity of *trans*-crotonin was confirmed by routine spectroscopic methods.

Animals and tumours

Female inbred DBA/2 mice weighing 20 – 25 g from Fiocruz Animal House (RJ) were used. Groups of 5 mice were housed in plastic cages under standard laboratory conditions. A total of 15 animals were used per group. S180 and Ehrlich carcinoma ascitic tumours were maintained by weekly *i.p.* passages in mice.

For *in vivo* assays, each mouse received 5×10^5 cells *i.p.* harvested from a mouse bearing a 7-day-old tumour.

Administration of drugs and anti-tumour evaluation

The administration of drugs was *i.p.*, in two or three doses (in 0.04 mL of DMSO/saline) 24 h after tumour inoculation. The control animals received the same vehicle. The anti-tumour effects of the drugs were determined by the increase in the survival time of treated mice (T) as compared to that of the control group (C), and expressed as %T/C as described earlier (9), (10). A reproducible %T/C ≥ 125 indicates significant anti-tumour activity (10). The experiments were repeated at least once.

TNFα assay

Animals were treated in the same conditions as described above. The animals were sacrificed 7 days after the last drug inoculation and ascitic fluid was collected from treated and control Ehrlich tumour-bearing mice. The exposure of cell line WEHI 164 to 0.01 – 30 ng/mL of recombinant mouse TNFα (Gibco) or ascitic supernatant for 20 h, was performed as previously described (11). The cytotoxicity was measured by MTT assay (12). The results were presented as % of growth inhibition ± SD for triplicate determination.

Cytotoxicity assay

Tumour cell cultures were started from mice Ehrlich ascites with at least one passage, prior to use. Aliquots of 1×10^5 cells were seeded in quadruplicate onto 96-wells flat microtiter plates in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 100 IU/mL penicillin and 100 μg/mL streptomycin. The drugs dissolved in DMSO at various concentrations were added to the culture and adjusted to a final DMSO concentration of 0.2% (v/v). The cultures were maintained in 5% CO_2 in air at 37 °C. Cellular viability was determined in the presence or absence of diterpenes and quercetin used as a positive control, using the standard MTT assay (12). Briefly, drug effects were observed after 24 – 48 h cell culture incubation, MTT was added to samples and the absorbance at 570 nm was measured. For each compound the concentration required to reduce absorbance by 50% (IC_{50}) in comparison to control cells was determined.

Analysis of DNA fragmentation by agarose gel electrophoresis

After 48 h of cell culture essentially as described above, following diterpenes and control treatment, cellular DNA was extracted by described methods (13).

Statistical analysis

The results of the TNFα and MTT assays are presented as means ± S.D. of 3 experiments. Statistical significance was assessed by the Student's t test. $P < 0.05$ was considered to show a significant difference.

Results and Discussion

In vivo *anti-tumour activity*

DCTN is the major constituent in the hexane extract of *Croton cajucara* bark and CTN was obtained by hydrogenation of DCTN. These two nor-diterpenes were used for our studies on *in vivo* and *in vitro* anti-tumour activity. The results of the *in vivo* anti-tumour evaluation of DCTN, CTN and 5-FU, used as a positive control, are shown in the Table **1**. We performed preliminary tests in order to detect signs of toxicity due to treatment with the drugs. Then we started the tests with the maximum tolerated doses in this treatment schedule. The diterpene DCTN presented a significant activity against ascitic murine tumours S180 and Ehrlich at doses of 84 and 120 mg/kg, %T/C = 137 and 128, respectively. A similar anti-tumour activity against S180 was obtained for 5-FU in the same range of concentration, 292 μmol/kg, compared with 257 μmol/kg for DCTN. A borderline anti-tumour activity was observed in the maximum soluble dose in the schedule of the treatment against S180 with the CTN derivative. For Ehrlich carcinoma a higher dose of DCTN given on days 1, 2 and 3, was necessary for significant activity, in contrast there was no activity after CTN treatment. The anti-tumour activity is dose response dependent for both tumours, since doses of DCTN lower than 84 mg/kg were not effective. Furthermore, there was no weight loss or evidence of toxicity by macroscopic examination of the organs in non-tumour bearing mice treated with the diterpenes. DCTN treatment of mice has the same anti-tu-

Table 1 *In vivo* effect of *trans*-dehydrocrotonin (DCTN), *trans*-crotonin (CTN) and 5-FU in ascitic S180 and Ehrlich tumour growth.

Drugs	Tumour	Treatment (days)	Total (mg/kg)	Dose mmol/kg)	% T/C[a]
5-FU	S180	1 and 2	38	0.292	140[b]
DCTN	S180	1 and 2	84	0.257	137[b]
		1 and 6	33.3	0.102	91
CTN	S180	1 and 2	80	0.244	121
DCTN	Ehrlich	1, 2 and 3	120	0.368	128[b]
CTN	Ehrlich	1, 2 and 3	120	0.365	110
5-FU	Ehrlich	1, 2 and 3	80	0.615	144[b]

[a] The efficiency of the ascitic tumour treatment was determined by the increase in the survival time of the treated mice (T) as compared to that of the control group (C) using the expression %T/C = 100 × median survival time of treated animals (days)/median survival time of control animals (days).

[b] %T/C ≥ 125 for significant antitumour activity.

mour effect as 5-FU a recognized anti-cancer drug on S180 and Ehrlich tumours.

In vitro *cytotoxic activity*

The cytotoxicity activity of the diterpenes on ascitic Ehrlich cells was measured by MTT assay and the IC_{50} determined in μg/ml or μM. The cell growth inhibitory effects show that the IC_{50} was 52.2 μg/ml (16 μM) and 51.8 μg/ml (16 μM) for DCTN and CTN, respectively and for flavonoid quercetin, 15 μg/ml (44 μM), used for positive control.

No induction of nucleosomal DNA fragmentation, was observed on analysis by agarose gel electrophoresis of cells treated with DCTN or CTN up to a dose of 50 μg/ml.

These results led us to assay for *in vivo* TNFα induction in Ehrlich tumour-bearing mice treated with diterpenes. A significant activity, $p < 0.05$, of TNFα was obtained in ascitic supernatant after treatment with DCTN at the dose of 120 mg/kg (Fig. **1**). The prolongation in survival time in mice treated with DCTN might be due to a direct tumour killing activity of the increased TNFα secreted at the area of tumour cell growth. It is known that macrophages are the primary source of TNF and that they are present in murine tumors in large numbers (14). Also, other diterpenes like taxol enhanced TNFα production (7).

Compounds containing an α,β-unsaturated carbonyl moiety have been shown to bind to receptors that induce increased activities of phase II enzymes responsible for metabolizing xenobiotic agents (15). DCTN containing this reactive electrophilic chemical species, may be binding to biomolecules and a significant anti-tumour activity was obtained in comparison with CTN which lacks this moiety.

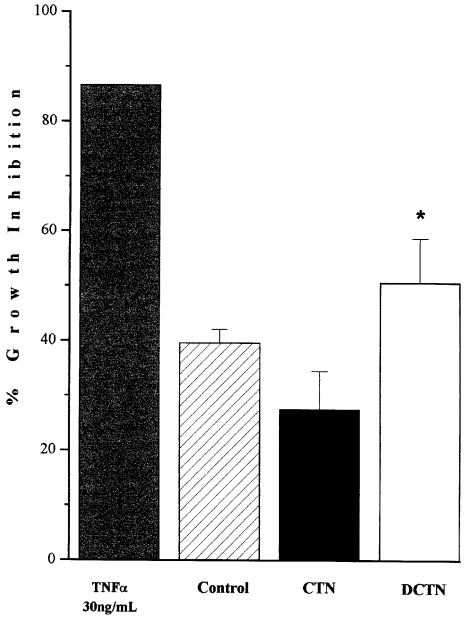

Fig. 1 Results of TNFα *in vivo* induction in tumor-bearing mice treated with diterpenes. In brief, after 24 h *i.p.* injection of 5×10^5 Ehrlich tumour cells, DCTN, CTN and solvent vehicle were *i.p.* injected on days 1, 2 and 3 in a total dose of 120 mg/kg. 7 days after the last injection ascitic supernatants were collected and TNFα content was evaluated using a cytotoxicity assay with the TNFα sensitive cell line WEHI-164. This assay was performed using a recombinant mouse TNFα (30 ng/mL) in medium RPMI. $^*p < 0.05$.

In our recent communication, the anti-estrogen effect of DCTN in rats was observed (16). Anti-estrogens have established a key place in the treatment of hormone-dependent cancer. Since DCTN has an anti-estrogen effect and in this work DCTN showed an anti-tumour activity against Ehrlich carcinoma, a spontaneous tumour derived from mammary adenocarcinoma, DCTN merits further studies in breast cancer cell lines. Thus, our findings with the diterpene DCTN indicate that other treatment schedules and other tumours may be realized in an attempt to obtain better anti-tumour activity.

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