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Natural killer cell activity and anti-tumour effects of dehydrocrotonin and its synthetic derivatives

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Abstract

In this work, the anti-tumour properties of dehydrocrotonin and its derivatives were investigated in vitro and in vivo using the Ehrlich ascites tumour model. Treatment of Ehrlich ascites tumour-bearing mice with 20 mg/kg dehydrocrotonin for 4 days significantly increased survival, whereas administration of dehydrocrotonin derivatives was ineffective in affording protection. Compound IV exhibited little activity against Ehrlich tumour cells in vitro. Investigation of the effects of dehydrocrotonin treatment on total natural killer (NK) cell activity of tumour-bearing mice as a possible mechanism of dehydrocrotonin action in vivo revealed that this sesquiterpene lactone significantly improved NK cytotoxicity against YAC-1, a Moloney virus-induced mouse T-cell lymphoma of A/SN origin. As expected, tumour growth in non-treated mice markedly suppressed NK cell cytolysis. No effects on NK functional activity were observed in normal mice receiving dehydrocrotonin. In summary, only the natural compound exhibits anti-tumour efficacy and immunomodulatory actions in vivo, which may be related to its chemical structure.

Keywords: Dehydrocrotonin; Tumour; Cytotoxicity; Natural killer cell; Anti-tumour

1. Introduction

The bark and leaves of *Croton cajucara* Benth. (Euphorbiaceae), an Amazonian medicinal plant called ‘‘sacaca’’, are commonly used as an infusion in the powdered or dried pill form to treat a large number of gastrointestinal, kidney and liver disorders (Maciel et al., 2000). Recently, leaves have been by over-weight people slimming purposes, but the prolonged use required has been correlated with frequent toxic hepatitis in the Amazonian region (Maciel et al., 1998). A clerodane nor-diterpene, *trans*-dehydrocrotonin, which is the major secondary metabolite present in the bark of sacaca, has been studied for its anti-ulcerogenic (Souza-Brito et al., 1998; Hiruma-Lima et al., 1999) and anti-tumour activities (Grynberg et al., 1999). However, hepatotoxicity has been reported to be a possible limitation to its clinical application (Rodriguez and Haun, 1999; Melo et al., 2002). Thus, efforts have been made to develop new semi-synthetic derivatives with fewer side effects. In vitro experiments performed with the dehydrocrotonin derivative IV indicated that it has a greater selectivity than the original diterpene (Anazetti et al., 2003). To aid in the design of new derivatives with improved in vivo activities, a clear understanding of the effects of chemical modifications introduced in the natural compound on its in vivo efficacy is necessary. The best-known naturally occurring diterpene, taxol, extracted from the bark of *Taxus brevifolia*, has significant efficacy against human breast and ovarian tumours (Kosmas et al., 2000).

The Ehrlich ascites tumour is a rapidly growing carcinoma, with a very aggressive behaviour, that has been widely used in the literature to investigate the anti-tumour properties of several new agents. Progressive Ehrlich tumour growth is characterized by profound alterations in the immune response and by a high rate of glutamine consumption (Márquez et al., 1989; Lobo et al., 2000), which has been associated with phenotypic and metabolic alterations leading to decreased immunocompetence (Subiza et al.,

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1989; Segura et al., 2001; Latha et al., 2000; Justo et al., 2000, 2001, 2003). In this respect, a dramatic inhibition of T and natural killer (NK) cell responses has been reported to occur in parallel with an increase in suppressive macrophages and in down-regulatory humoral factors (Parhar and Lala, 1988; Subiza et al., 1989; Fecchio et al., 1990a,b; Segura et al., 1997, 2000; Ruiz de Morales et al., 1999; Justo et al., 2000, 2003; Valadares et al., 2003).

To further expand our knowledge of the chemotherapeutic potential of diterpenes, in the present study, the in vivo and in vitro anti-tumour activities of dehydrocrotonin and its semi-synthetic derivatives were evaluated using the Ehrlich ascites tumour. The effects of dehydrocrotonin on total NK cell activity in normal and tumour-bearing mice were also examined in an attempt to determine a possible role of the immune system in its in vivo anti-tumour activity.

2. Materials and methods

2.1. Dehydrocrotonin and derivatives

Dehydrocrotonin was obtained from *C. cajucara* bark as described by Souza-Brito et al. (1998). The lactone of the dehydrocrotonin molecule was opened to yield Compound IV by a modification of the dimethylamine reaction described by Cromwel and Cook (1958). The ketone group of dehydrocrotonin was reduced according to Itokawa et al. (1989), using a methanol solution of dehydrocrotonin treated with an excess of sodium borohydride. Compound III was obtained by reducing dehydrocrotonin through reaction with $LiAlH_4$ in tetrahydrofurane (Shimoma et al., 1998). The purity and chemical structures of compounds I–IV (Fig. 1) were confirmed by nuclear magnetic resonance (NMR), ultraviolet spectroscopy (UV), infrared spectroscopy (IR) and mass spectroscopy (MS) techniques (Melo et al., 2001). These compounds were chosen based on previous structure–activity studies of the toxicity and biological activities of dehydrocrotonin and its semi-synthetic derivatives II, III and IV (Rodriguez and Haun, 1999; Melo et al., 2001, 2002).

2.2. Mice

The mice used in this study were bred at UNICAMP Central Animal Facilities (CEMIB-Universidade Estadual de Campinas, Campinas, SP) and maintained under specific pathogen-free conditions. Male BALB/c mice, 6–8 weeks old, were matched for body weight before use. Animal experiments were done in accordance with institutional

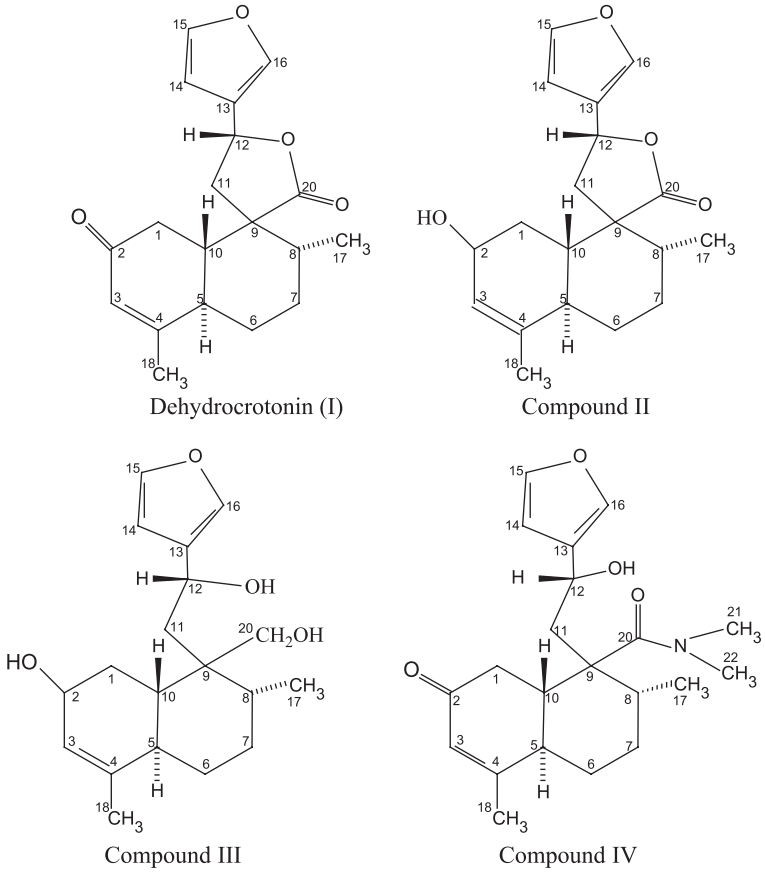

Fig. 1. Chemical structures of dehydrocrotonin (DHC) and its derivatives.

protocols and the guidelines of the UNICAMP Institutional Animal Care and Use Committee that follows the recommendations of the Canadian Council on Animal Care.

2.3. Mouse tumour model

Ehrlich ascites tumour was maintained in male BALB/c mice by serial transplantation. Tumour cell suspensions were prepared in balanced salt solution at pH 7.4 to final concentrations of 6×10^7 viable cells/ml. Mice were inoculated intraperitoneally (i.p.) on day 0 with 6×10^6 viable tumour cells per mouse in a volume of 0.1 ml. Viability, assessed by the Trypan blue dye exclusion method, was always found to be 95% or more (Justo et al., 2000).

2.4. Treatment regimen

Dehydrocrotonin and its derivatives were supplied in balanced salt solution containing 12% Tween 80 (pH 7.4) and diluted immediately before use in appropriate concentrations. Doses of 2.5, 5.0, 10 or 20 mg/kg were administered for 4 consecutive days to groups of normal and tumour-bearing mice by i.p. injection of 0.1 ml per mouse. Treatment started 24 h after tumour inoculation. Each experiment included parallel control groups of normal and tumour-bearing mice treated with an equivalent volume of the vehicle. The in vivo anti-tumour activity of the compounds was determined by the increase in the survival time of treated mice compared to that of the non-treated control mice. The experiments were repeated at least once.

2.5. Cytotoxicity assay

Tumour cell cultures were derived from ascitic tumour cells harvested by peritoneal lavage from mice 8 to 10 days after tumour transplantation. The cells were washed twice, resuspended in RPMI 1640 medium (Sigma, USA) containing 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin (enriched medium) and 10% foetal calf serum (Gibco, USA) and placed in tissue culture flasks. Ehrlich tumour cells were used in cytotoxicity experiments after two serial passages in tissue culture flasks. Aliquots of 3×10^5 tumour cells were seeded in quadruplicate into 96-well flat microtit plates (Corning, USA) in enriched RPMI 1640 medium supplemented with 10% foetal calf serum. The compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium. The final DMSO concentration was 0.2% (v/v). The cultures were maintained at 37 °C in a 5% CO_2 atmosphere for 72 h. After incubation, cell viability was determined in the presence or absence of dehydrocrotonin or its derivatives, using the standard reduction of the

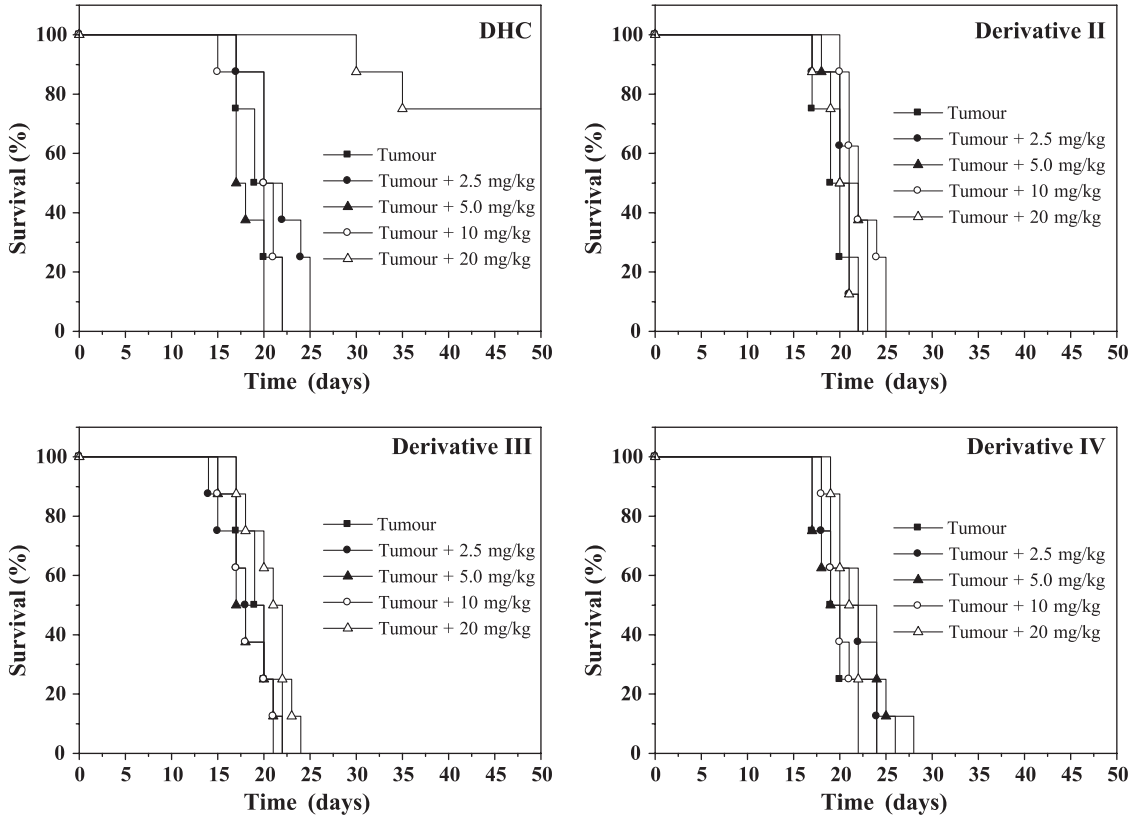

Fig. 2. Effects of different doses of dehydrocrotonin (DHC) and its derivatives on the survival of Ehrlich ascites tumour (EAT)-bearing mice. Mice received i.p. doses of 2.5–20 mg/kg of DHC or its derivatives for 4 days after the inoculation of 6×10^6 tumour cells. Control tumour-bearing mice received vehicle only. Groups of eight mice were checked daily survival.

tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the phosphatase activity assays according to Anazetti et al. (2003).

2.6. Preparation of effector cells for the NK cell assay

Spleens from mice were collected and passed through a stainless-steel mesh net to obtain single-cell suspensions. The resultant mononuclear cells were isolated from the cell suspension by Ficoll-Hypaque (Sigma) gradient separation, washed three times and resuspended in enriched RPMI 1640 culture medium supplemented with 10% foetal calf serum. Cell suspensions were placed in 150-mm tissue culture dishes and incubated at 37 °C under 5% CO_2 for 90 min to remove adherent cells. Non-adherent cells were then harvested by gentle pipetting. The cells were washed three times and the cell concentration was adjusted to 5×10^6 cells/ml as described by Justo et al. (2003).

2.7. Preparation of target cells for the NK cell assay

YAC-1, a Moloney virus-induced mouse T-cell lymphoma of A/SN origin, was used as target cell in the 4-h ^{51}Cr-release assay. Briefly, 5×10^6 pelleted YAC-1 cells were resuspended in 0.2 ml of foetal calf serum and labelled with 10 μCi of sodium chromate (^{51}Cr) (IPEN, Brazil) for 90 min at 37 °C in a shaking water bath. After labelling, the cells were washed twice with RPMI 1640 culture medium and

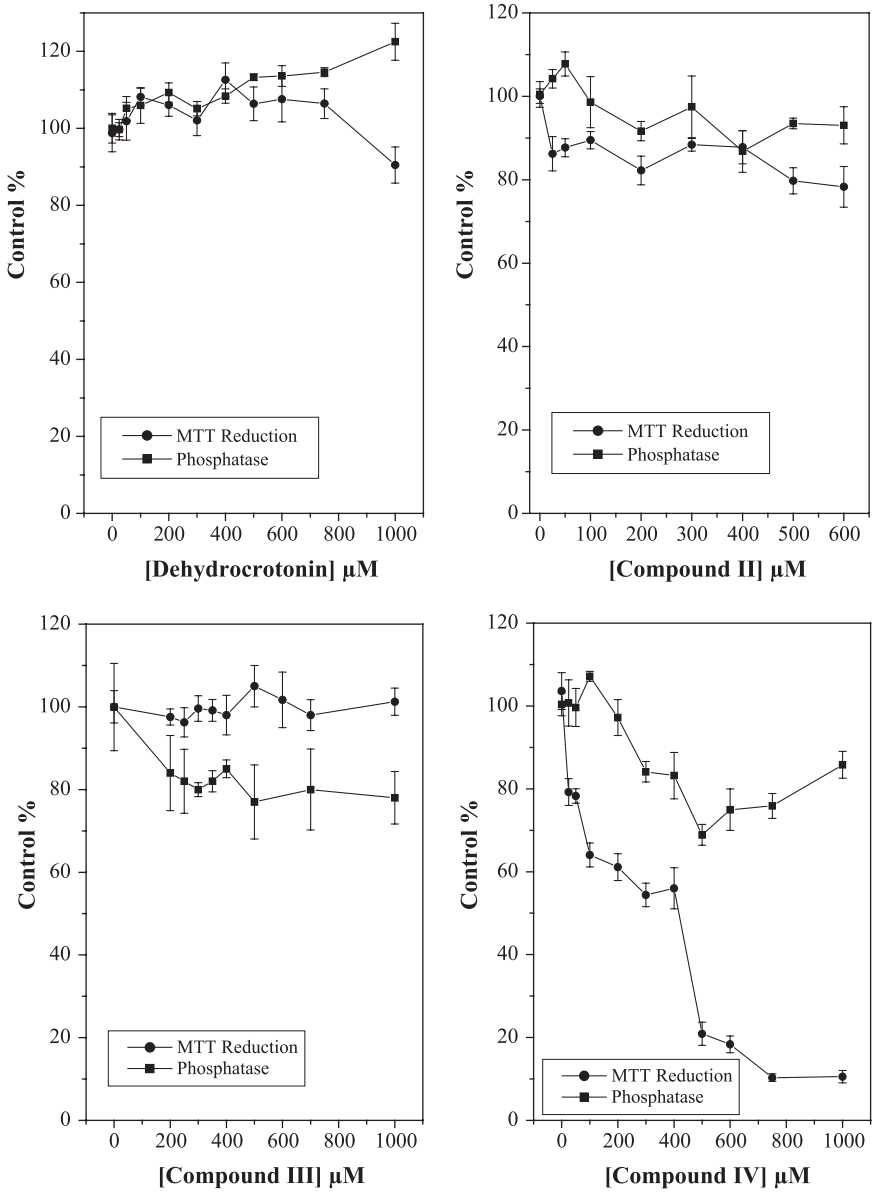

Fig. 3. Viability of Ehrlich tumour cells after treatment with different concentrations of dehydrocrotonin (DHC) or its derivatives for 72 h. Endpoints evaluated: MTT reduction and phosphatase activity. Each point represents the mean ± S.D. of three experiments with four replicates.

resuspended at a concentration of 1×10^5 cells/ml in enriched RPMI 1640 culture medium supplemented with 10% foetal calf serum.

2.8. NK cell cytolytic assay

NK activity of effector cells was measured in a 4-h ^{51}Cr-release assay using YAC-1 target cells. Effector cells and targets were dispensed in triplicate into 96-well round-bottom microtit plates (Corning), producing effector to target ratios of 50:1, 25:1, 12.5:1 and 6.25:1. Plates were centrifuged at 800 rpm for 5 min and incubated for 4 h at 37 °C in a humidified CO_2 incubator. After the incubation period, the plates were centrifuged again at 1200 rpm for 10 min and 0.1 ml of the supernatant was collected to count radioactivity in a Beckman Biogama Counting System (Beckman 5500 B, Irvine, USA). Spontaneous release was determined by adding 100 labelled target cells to 0.1 ml of medium in the absence of effector cells and was always less than 10% of the maximum release, which was determined by exposure of labelled target cells to 0.05% Tween-20. Percentage of cytotoxicity, as measured by specific ^{51}Cr release, was calculated by using the formula: (cpm experimental − cpm spontaneous)/(cpm maximal − cpm spontaneous) × 100.

2.9. Statistical analysis

Comparisons of data among all groups were done by analysis of variance (ANOVA). In case of significant differences, the Tukey test was used. Survival of Ehrlich ascites tumour-bearing mice treated with the compounds was analysed by the Log-rank test. Statistical significance was assigned when $P < 0.05$.

3. Results

The effects of treatment of tumour-bearing mice with doses of 2.5–20 mg/kg of dehydrocrotonin and its derivatives on survival are presented in Fig. 2. The diterpene dehydrocrotonin had significant activity against the Ehrlich ascites tumour at a dose of 20 mg/kg. All the untreated tumour-bearing mice died within 20 days, whereas treatment with 20 mg/kg of dehydrocrotonin significantly increased survival to 80% ($P < 0.005$). The dehydrocrotonin derivatives were ineffective to protect mice against tumour growth, using the same dose-schedule as that for dehydrocrotonin.

To verify the in vitro toxicity of these compounds, the cytotoxicity of the diterpenes on ascitic Ehrlich cells was measured by MTT reduction and phosphatase activity. Fig. 3 indicates that dehydrocrotonin and compounds II and III were not cytotoxic to Ehrlich tumour cells even at high concentrations. Conversely, an IC_{50} value of 400 μM was determined for compound IV in the MTT reduction assay, whereas the protein phosphatase activity was inhibited by only 20% at the same concentration.

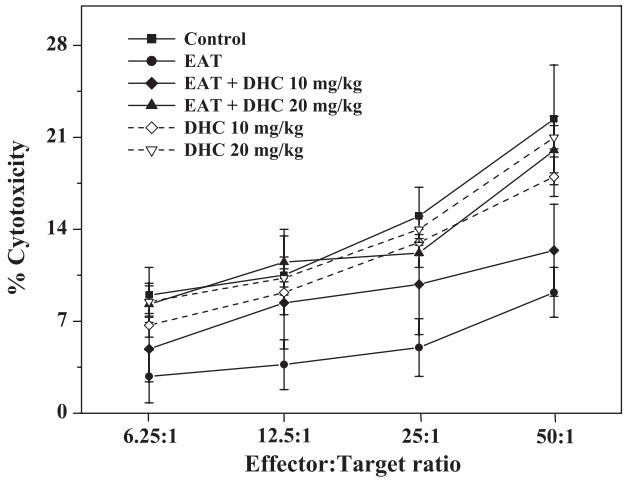

Fig. 4. Splenic NK cell activity in Ehrlich ascites tumour (EAT)-bearing mice receiving 10 or 20 mg/kg dehydrocrotonin (DHC) for 4 days. Treatment started 24 h after the inoculation of 6×10^6 tumour cells and NK cell activity was evaluated 24 h after the last dose. Control mice received vehicle only. Results are given as the means ± S.D. for eight mice per group.

In order to better understand the in vivo anti-tumour effect of dehydrocrotonin, we next evaluated a possible action of this compound on total NK cell-mediated cytolysis. The influence of dehydrocrotonin, administered to mice at doses of 10 and 20 mg/kg for 4 days, on NK cell cytotoxic function is presented in Fig. 4. Treatment of normal mice with dehydrocrotonin produced no changes in NK cell activity when compared with that of the controls. In the tumour-bearing group, this activity was markedly reduced in relation to the control levels ($P < 0.01$). Treatment of these animals with 20 mg/kg of dehydrocrotonin restored to normal values the total NK cell cytotoxicity at all effector/target cell ratios ($P < 0.05$), whereas the dose of 10 mg/kg did not significantly stimulate NK activity in Ehrlich ascites tumour mice. As previously reported (Ruiz de Morales et al., 1999; Justo et al., 2000, 2003), Ehrlich ascites tumour-bearing mice also presented with splenomegaly and had a significant tumour burden. However, after dehydrocrotonin administration, surviving mice were free of ascitic fluid in the peritoneal cavity and no signs of tumour-induced splenomegaly were found.

4. Discussion

The clerodane-type diterpene dehydrocrotonin from *C. cajucara* has been shown to exhibit a broad spectrum of biological activities in vivo, including anti-tumour properties in different tumour models (Grynberg et al., 1999; Maciel et al., 2000; Anazetti et al., 2003). The mechanism(s) for this anti-tumour effect has not been well defined. Since the therapeutic efficacy of dehydrocrotonin may be restrict-

ed by a dose-limiting hepatotoxicity (Rodriguez and Haun, 1999; Melo et al., 2002), much attention has focused on chemical modification of the natural compound to enhance its selectivity and therapeutic potential. Anazetti et al. (2003) have recently reported that the cytotoxicity of dehydrocrotonin and compound IV was similar, while an earlier study showed that this derivative was less cytotoxic to hepatocytes (Melo et al., 2002). Additionally, changes in the chemical structure of the parent molecule may also provide further understanding of the structure–activity relationship of this class of compounds. In the present study, in vitro and in vivo experiments were performed to determine the anti-tumour potential of dehydrocrotonin and three different semi-synthetic derivatives of dehydrocrotonin against the Ehrlich ascites tumour.

Cytotoxicity against ascitic Ehrlich tumour cells in vitro was evaluated by using the MTT and phosphatase activity assays. Dehydrocrotonin and compounds II and III did not affect the viability of Ehrlich cells up to a concentration of 1 mM, whereas compound IV showed little direct effect on tumour cells, measured by the MTT reduction, when compared with other cytotoxic compounds described in the literature such as crotonin (Grynberg et al., 1999) and artemisinin (Woerdenbag et al., 1993) with IC_{50} values of 16 and 29.8 μM, respectively.

In addition, none of the dehydrocrotonin derivatives inhibited Ehrlich tumour growth in vivo, as demonstrated by survival analysis of Ehrlich ascites tumour-bearing mice receiving different doses of these compounds. However, dehydrocrotonin was effective in treating Ehrlich ascites tumour inasmuch as six of eight (75%) mice were rendered disease-free by the administration of four consecutive daily doses of 20 mg/kg ($P<0.001$). These observations are consistent with some evidence in the literature suggesting that dehydrocrotonin-induced anti-tumour effects are at least partially indirect, by modulation of immune function (Grynberg et al., 1999).

During Ehrlich ascites tumour growth, the level of NK cell activity in spleen cells is decreased (Parhar and Lala, 1985a,b, 1988; Justo et al., 2003; Valadares et al., 2003). This effect on NK cell function was recently studied in SCID Ehrlich ascites tumour-bearing mice, suggesting that NK cells together with cytokines contribute significantly to the natural resistance to growth of this murine tumour model (Valadares et al., 2003). Additionally, accumulated evidence supports a significant role for cytokines such as interleukin-2 and interferon-γ in the control of NK cell-mediated cytotoxicity in this tumour model (Parhar and Lala, 1988; Lala et al., 1990; Segura et al., 1997; Justo et al., 2003). NK cells have also been shown to produce and secrete potent immunoregulatory cytokines, particularly interferon-γ (Kim et al., 2000), which increases cell reactivity and activates macrophages (Misawa et al., 2000; Miller, 2001; Justo et al., 2003). Based on the critical role of NK cells in immune surveillance, we thus examined the effect of dehydrocrotonin on the anti-tumour cytotoxicity mediated by NK cells.

The results presented here demonstrate that only the dose of 20 mg/kg of dehydrocrotonin induced significantly higher levels of splenic NK activity in tumour-bearing mice. This same dose also reduced the spleen enlargement observed in Ehrlich ascites tumour mice. These results suggest that at least part of the dehydrocrotonin-induced anti-tumour effect is mediated through augmentation of NK cell activity. Previous data indicate that there can be several mechanism(s) by which indirect effects of dehydrocrotonin are mediated. Interestingly, increased amounts of tumour necrosis factor-α have been shown in Ehrlich ascites tumour-bearing mice treated with this diterpene, suggesting that dehydrocrotonin-induced cytokines could also serve as anti-tumour mediators (Grynberg et al., 1999). Considerable evidence exists on the role of tumour necrosis factor-α either alone or in combination with interferon-γ in macrophage-mediated regulation of host anti-tumour responses (Yamamoto et al., 1995). Therefore, it is also possible that dehydrocrotonin alters the subset composition in various organs, such that the percentage of NK-active lymphocytes is increased, which would lead to an increase in NK activity. Studies to investigate the ability of dehydrocrotonin to alter subset composition are in progress.

The present studies extend our previous findings of a greater effectiveness of dehydrocrotonin and compound IV in comparison with other dehydrocrotonin derivatives (Anazetti et al., 2003). Structure–activity studies have shown that three common functional moieties are present in lactones with cytotoxic activity (Giordano et al., 1992): an α-methylene-γ-lactone, an α-β-unsaturated cyclopentenone system, and an $O{=}C{-}C{=}CH_2$ system; the last of these moieties functions as an essential alkylating centre. Kupchan et al. (1971) have shown that the α-methylene-γ-lactone system participates in a Michael-type reaction with biological nucleophiles and inactivates key enzymes. The presence of the lactone in the chemical structure of dehydrocrotonin appears essential for its anti-tumour activity in vivo, because compound IV, which possesses the Michael acceptor group ($C(O)N(CH_3)_2$), was ineffective in affording protection against Ehrlich ascites tumour growth. In addition, the reactive electrophilic chemical species ($O{=}C{-}C{=}CH_2$) of dehydrocrotonin could bind to biomolecules, thus contributing to the significant anti-tumour activity of this compound in comparison with that of derivative II, which lacks this moiety. Finally, the absence of the lactone and the $O{=}C{-}C{=}CH_2$ system in compound III may be responsible for its lack of anti-tumour activity. These findings provide insight into the structural requirements for the anti-tumour activity of sesquiterpene lactones and may help in the design of new compounds with this pharmacological activity.

Taken together, these studies showed that compounds II, III and IV differed in their toxicity from dehydrocrotonin and provided some evidence on the mechanism of toxicity of sesquiterpene lactones. Only the natural compound significantly enhanced the survival of tumour-bearing mice,

without affecting tumour cell growth in vitro. It is clear that the effect produced by dehydrocrotonin on total NK cell activity contributes to the anti-tumour efficacy of this compound, suggesting a role for effector leukocytes in the immunomodulatory activity of this natural compound.

Acknowledgements

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