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The effect of a Titanocene Dichloride derivative, Ti IV $(C_5H_5)_2$ NCS_2, on the haematopoietic response of Ehrlich tumour-bearing mice

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Abstract

The effects of the [Ti IV $(C_5H_5)_2$ NCS_2] metallocene (BCDT), a Titanocene Dichloride derivative, on the growth and differentiation of granulocyte-macrophage progenitor cells [colony-forming unit-granulocyte-macrophage (CFU-GM)] and bone marrow cellularity in normal and Ehrlich ascites tumour-bearing mice were studied. As expected for the Ehrlich ascites tumour-model, concomitant myelosuppression, increased number of spleen CFU-GM and changes in bone marrow cellularity were observed. The treatment of Ehrlich ascites tumour-bearing mice with BCDT (10–30 mg/kg/day) produced a dose-dependent increase in myelopoiesis, a reduction in splenic colonies and a restoration in the total and differential marrow cell counts. We also observed an increase in CFU-GM number when bone marrow cells obtained from normal mice were incubated in vitro with serum from normal mice treated with BCDT. In addition, BCDT prolonged, in a dose-dependent manner, the survival of mice inoculated with Ehrlich ascites tumour. Although it has been previously reported that substitutions in the two halides of the titanocene do not interfere with antitumoural effect, our results with BCDT demonstrated a reduction in antitumour efficacy when compared to previous results with the original titanocene produced in our laboratory. © 2002 Published by Elsevier Science B.V.

Keywords: Ehrlich ascites tumour; Metallocene; Titanocene Dichloride; Myelopoiesis; Bis-cyclopentadienylditiocianatetitanium (IV)

1. Introduction

After the discovery of its antitumour activity, *cis*-diaminedichloroplatinum (II) (cisplatin) has become one of the widely used drugs for the treatment of cancer. Its importance was derived largely from its ability to confer complete remission in patients with advanced testicular cancer, and it was subsequently found to exhibit significant therapeutic efficacy in ovarian, uterus, bladder, and head/neck cancer (Rosenberg et al., 1969; Calvert et al., 1995; Boyles et al., 2001). However, the remarkable antitumour effects of cisplatin coincided with marked toxic effects, including neurotoxicity, nephrotoxicity and severe emesis. In addition, there is a propensity for tumours to develop resistance to platinum, which constitutes a major problem in its clinical use. The effectiveness of this drug against a wide range of human tumours, as well as its limitations, has generated a broad interest in developing new organometallic complexes with lower toxicity and improved activity and spectrum. Despite decades of efforts, there has been little progress to date in developing more selective new platinum complex with the above characteristics. Thus far, the most effective second-generation platinum compound developed is the carboplatinum. The advantage of lower nephrotoxicity in association with a similar antitumour activity brings promise to this new compound (Christodoulou et al., 1988).

In addition to platinum other metal-containing complexes have been synthesised and subjected to tumour screening. The Bis (cyclopentadyenil) metal (IV) (metallocene) dihalides complexes [M IV $(C_5H_5)_2$ X_2] with M = titanium, vanadium or molybdenum as central metal atom were the first early transition metal compounds detected with antitumour activity (Kopf and Kopf-Maier, 1979; Kopf-Maier et al., 1980, 1986; Modski and Harding, 2001). These substances exhibit marked antineoplastic activity against fluid and solid Ehrlich tumour, fluid and solid Sarcoma 180, B16 melanoma, Lewis Lung carcinoma, mouse mammary tumour TA3Ha and colon 38 adenocarcinoma (Kopf-Maier

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et al., 1986; Kopf-Maier, 1988), and markedly inhibited the growth of xenografted human carcinomas of lung, breast, gastrointestinal tract and renal cell (Kopf-Maier and Kopf, 1986; Kopf-Maier, 1989, 1999).

There have been a large number of reports in which the systemic effects of Titanocene Dichloride have been investigated. Titanocene Dichloride, Ti IV $(C_5H_5)_2$ Cl_2, the most active neutral metallocene, is now undergoing clinical trials with promising results in patients with progressive advanced cancer (Christodoulou et al., 1988; Mross et al., 2000).

Although the in vitro properties and mechanism of action of the titanocenes are not totally clear, it is already known that the main drug's target of action is the DNA (Kopf-Maier, 1980; Harding and Mokdsi, 2000; Mross et al., 2000). Several efforts have aimed at elucidating the extension of the effects of these compounds. Studies in our laboratory have shown an important protective effect of Titanocene Dichloride against the myelosuppression induced by Ehrlich ascites tumour in mice. In this regard, we have observed a significant recovery in the reduced number of granulocyte-macrophage colony-forming cells [colony-forming unit-granulocyte-macrophage (CFU-GM)] found in tumour-bearing treated mice (Valadares et al., 1998).

Several observations performed by Kopf-Maier et al. (1981) on the structure–activity relationship of metallocene dihalides, using the Ehrlich ascites tumour cells, pointed out the following characteristics related to the antitumoural efficacy: the antitumour activity of the metallocenes is strongly dependent on the metal central atoms, the potency is not affect by substitution in the halides, $(C_5H_5)_2$ Ti X_2, and chemical modification of the cyclopentadyenil rings reduce the antitumoural activity. On the other hand, Boyles et al. (2001) observed increased effectiveness in vitro through the introduction of the electron withdrawing carbometoxy group into the cyclopentadyenil rings.

To further investigate the influence of structural changes in the antitumour activity of the titanocene dihalides, we have designed this study using a derivative with substitution of the halogens by pseudo halogens. Our findings are in contrast with the observations published by Kopf-Maier et al. (1981), since a reduced antitumoural efficacy in parallel with a higher toxicity was found in tumour-bearing mice treated with different doses of the Titanocene Dichloride derivative, when compared to the results obtained previously in our laboratory with the parental compound (Valadares et al., 1998).

2. Material and methods

2.1. Mice

The mice used in this study were bred at Unicamp Central Animal Facilities and raised under specific pathogen-free condition. Male BALB/c mice, 8–10 weeks old, were matched for body weight before use. The animals were housed 10/cage and were allowed free access to laboratory chaw and water. Animal experiments were done in accordance with institutional protocols and the guidelines of the Institutional Animal Care and Use Committee.

2.2. Tumour

Ehrlich Ascites Tumour was maintained in BALB/c mice in the ascites form by serial transplantation. Tumour cell suspensions were prepared in balanced salt solution at pH 7.4 to final concentration 6×10^6 viable cells/ml. In all experimental protocols described, 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.

2.3. In vitro treatment of bone marrow cells

Bis-cyclopentadienylditiocianatetitanium IV (BCDT) was dissolved in a dimethyl sulfoxide/saline [DMSO/saline (1:10 = v/v)] mixture. Concentrations of 0.3, 0.2, 0.1, 0.05 or 0.025 mg/Petri/ml dishes were added in a volume of 0.1 ml at the beginning of the bone marrow progenitor cell assay. Control cultures received only the same volume of the solvent.

2.4. Drug and mice treatment

The BCDT compound was kindly donated by Dr. Stanley I. Klein and Dr. Brasílio C.A. Pereira (Instituto de Química, Universidade Estadual Paulista (UNESP), Araraquara, SP, Brazil). The compound was prepared and purified according to Birmingham and Wilkinson (1954) and Pereira and Klein (2000). The compound was diluted in DMSO/saline (1:10) immediately before use in appropriate concentrations. Groups of normal and Ehrlich ascites tumour-bearing mice received (0.1 ml/mouse) 10, 15 or 30 mg/kg of BCDT injected i.p. in 1, 2 or 3 doses, one dose per day, in consecutive days. BCDT treatment started 24 h after tumour inoculation and progenitor cell assays were performed on the first day after the last injection. The femoral marrow and spleen were collected on day 4 following Ehrlich ascites tumour-bearing mice for the CFU-GM study. Each experiment included parallel control groups of normal and Ehrlich ascites tumour-bearing mice treated with an equivalent volume of the diluents (Fig. 1).

2.5. Haematopoietic stimulator

Recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was supplied by Sigma, St. Louis, MO. The rmGM-CSF is an acid glycoprotein with a molecular weight of 22 kDa expressed in *Escherichia coli*. Colony formation was stimulated by an inclusion in the cultures of 1 ng/ml rmGM-CSF when 1×10^5 bone marrow

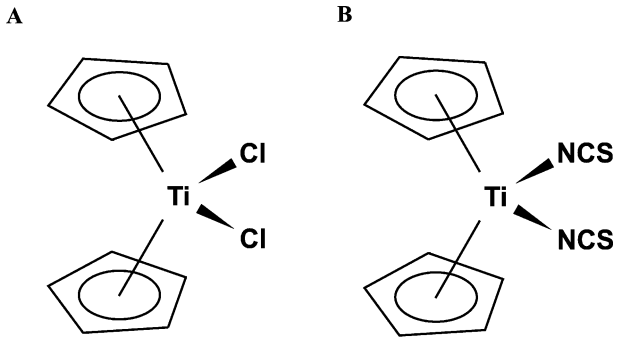


Fig. 1. Chemical structure of Titanocene Dichloride (A) and Bis-cyclopentadienylditiocianatetitanium IV (BCDT) (B).

cells were cultured in 1 ml of soft agar. This concentration of rmGM-CSF was determined from the linear portion of the dose–response curve performed in our laboratory before starting the experiments.

2.6. Haematopoietic tissues for progenitor cell assay

After the animals were killed by cervical dislocation, marrow cells and spleens were aseptically collected. The plug of the marrow was gently extruded into a sterile plastic tube by 1 ml of RPMI-1640 (Sigma) injected through the femur. The wormlike marrow plug was then converted into a dispersed cell suspension in 5 ml of RPMI medium by gently aspirating the suspension up and down 20 times using a sterile 5 or 10 pipette. Spleen cell suspensions were prepared in 5 ml of RPMI medium by gently pressing aseptically removed spleen through a stainless steel mesh net. As mentioned previously, bone marrow cells and spleens were collected on day 1 after the respective treatment.

2.7. Progenitor cell assay

Assays with cell suspensions from femoral marrow and spleen were performed in 1-ml agar cultures in 35-mm Petri dishes using 1×10^5 marrow cells or 2×10^5 spleen cells per culture. The medium used was Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 20% fetal calf and 0.6% of agar. Colony formation was stimulated by the addition of the rmGM-CSF described above. The cultures were incubated for 7 days in a fully humidified atmosphere of 10% CO_2, and colony formation (clones >50 cells) was scored at 35 × magnification using a dissection microscope (Metcalf, 1984).

2.8. Bone marrow cellularity

Femoral marrow was aseptically collected from mice treated with 3 × 10 mg/kg of BCDT after tumour inoculation. Cytocentrifuge preparations were made of marrow cells

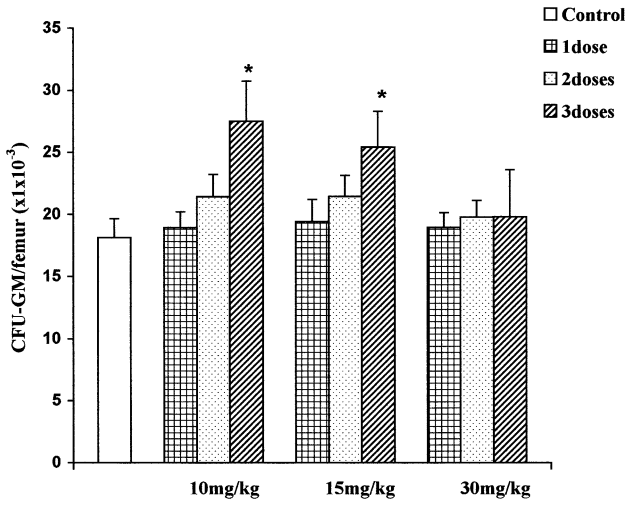


Fig. 2. Bone marrow granulocyte-macrophage colony-forming units (CFU-GM) in mice receiving 10, 15 or 30 mg/kg of BCDT injected i.p. in 1, 2 or 3 doses. Control mice received diluent only. Results represent the means ± S.D. for eight mice. ANOVA, Tukey test. * $P < 0.001$ compared with control group.

suspensions and stained with May–Grunwald–Giemsa. Total and differential cell counts were then performed. Measurements were taken 24 h after the last injection of BCDT.

2.9. Serum colony-stimulating factors (CSF)

Mice were bled from the heart, under ether anaesthesia, 24 h after treatment with 3 × 10 mg/kg of BCDT drug. Pooled blood was left at 37 °C for 30 min; clots were

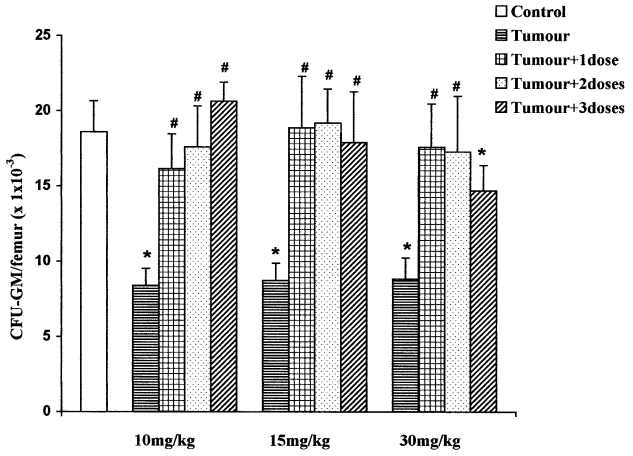


Fig. 3. Bone marrow granulocyte-macrophage colony-forming units (CFU-GM) in mice receiving 10, 15 or 30 mg/kg of BCDT injected i.p. in 1, 2 or 3 doses. Drug treatments started 24 h after the intraperitoneal inoculation of 6×10^6 Ehrlich ascites tumour cells and CFU-GM number was determined 24 h after the last BCDT injection. Control mice received diluent only. Results represent the means ± S.D. for seven mice. ANOVA, Tukey test. * $P < 0.001$ compared with control group; # $P < 0.05$ compared with tumour group.

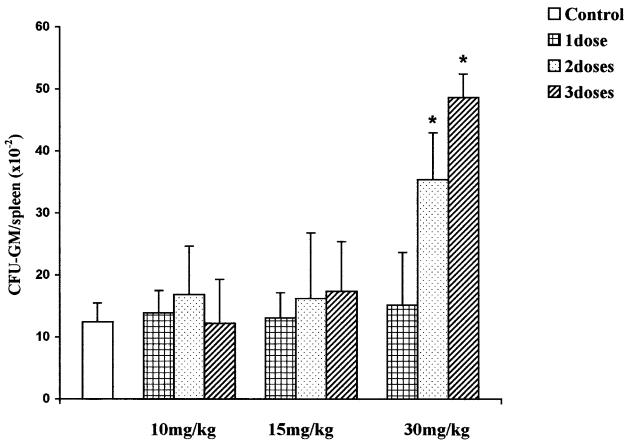


Fig. 4. Spleen granulocyte-macrophage colony-forming units (CFU-GM) in mice receiving 10, 15 or 30 mg/kg of BCDT injected i.p. in 1, 2 or 3 doses. Control mice received diluent only. Results represent the means ± S.D. for seven mice. ANOVA, Tukey test. * $P<0.001$ compared with control group.

allowed to retract overnight at 4 °C. Following centrifugation, the serum was removed and stored at − 20 °C. CSF was determinate by the ability of serum obtained from control and treated group to induce bone marrow cell proliferation and differentiation.

2.10. Statistical analysis

Comparisons of data among all groups were done by one-way analysis of variance (ANOVA). In cases of significant differences, Tukey test was used. Survivals of Ehrlich ascites tumour-bearing mice treated with BCDT were tested by Kaplan–Maier curve. Statistical significance was assigned when $P<0.05$.

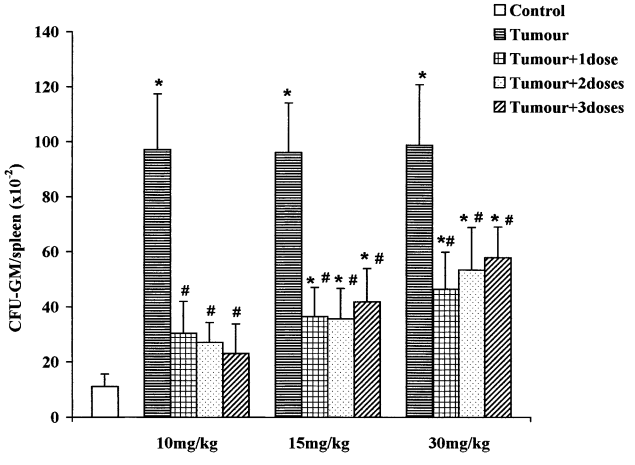


Fig. 5. Spleen granulocyte-macrophage colony-forming units (CFU-GM) in mice receiving 10, 15 or 30 mg/kg of BCDT injected i.p. in 1, 2 or 3 doses. Drug treatments started 24 h after the intraperitoneal inoculation of 6×10^6 Ehrlich ascites tumour cells and CFU-GM number was determined 24 h after the last BCDT injection. Control mice received diluent only. Results represent the means ± S.D. for seven mice. ANOVA, Tukey test. * $P<0.001$ compared with control groups; # $P<0.001$ compared with tumor groups.

Table 1
Effects of the BCDT on the spleen weight in Ehrlich ascites tumour-bearing mice

Groups	Spleen weight
Control	184.2 ± 8.9
BCDT 10 mg/kg	195.4 ± 10.9
BCDT 15 mg/kg	189.8 ± 15.8
BCDT 30 mg/kg	208.2 ± 19.3
Tumour	354.2 ± 23.4[a]
Tumour + 10 mg/kg	237.2 ± 10.2
Tumour + 15 mg/kg	243.4 ± 212.0
Tumour + 30 mg/kg	252.5 ± 27.2

Control and Ehrlich ascites tumour-bearing mice were treated with BCDT or diluents for 3 days, starting 24 h after tumour inoculation and, after the last injection, the measurements were done.

[a] $P<0.001$ when compared to control.

3. Results

3.1. Bone marrow and spleen progenitor cell assay

The effects of the treatment with 1, 2 or 3 injections of 10, 15 or 30 mg/kg of BCDT on bone marrow progenitor cells are shown in Figs. 2 and 3. As we can see from Fig. 2, the 3-dose schedules of the two lower-dosage regimens, 10 and 15 mg/kg, produced, in the normal animal, an increase of 50% and 38%, respectively, in the myelopoietic (CFU-GM) activity, as compared to nontreated animals and to all the other drug-treated groups ($P<0.001$). Conversely, no changes were observed in the number of CFU-GM with the administration of the 3-dose schedule of the 30 mg/kg regimen. In contrast, the evaluation of the extramedullar

Table 2
Effects of the serum from BCDT-treated mice with 3 doses of 10 mg/kg and direct BCDT on the bone marrow progenitor cells

Stimulus	Number of Colonies
rmGM-CSF	135 ± 10
Serum/treated 1:1[a,b]	17 ± 5
Serum/treated 1:2[a]	8 ± 2
Not treated[c]	5 ± 2
BCDT 0.3 mg	3 ± 1
BCDT 0.2 mg	4 ± 2
BCDT 0.1 mg	2 ± 1
BCDT 0.05 mg	5 ± 2
BCDT 0.025 mg	4 ± 1
Control[d]	3 ± 1

rmGM-CSF-recombinant murine granulocyte-macrophage colony-stimulating factor.

[a] Serum from drug-treated animals diluted at 1:1 and 1:2.

[b] $P<0.001$ when compared with not treated group.

[c] Serum from control animals.

[d] Control received the same volume of respective solvent. Results are expressed as mean ± S.D. of four mice per group.

Table 3
Changes in cell population in the bone marrow of mice bearing the Ehrlich ascites tumour after treatment with 3 doses of 10 mg/kg of BCDT[a]

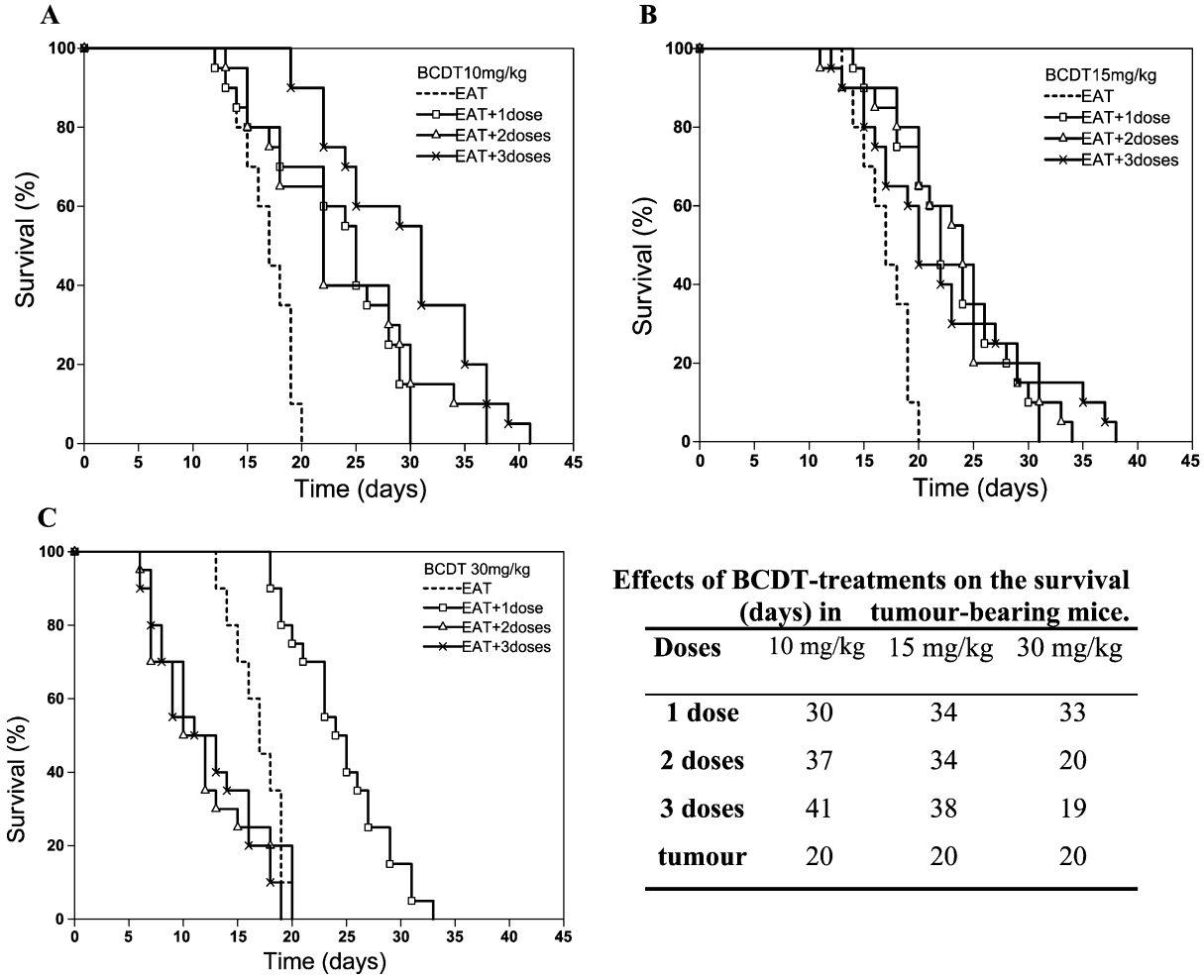
Parameters ($\times 10^6$)	Control	BCDT	Tumour	Tumour + BCDT
Femoral marrow cell counts	15.4 ± 1.5	15.5 ± 1.3	10.4 ± 1.2[b]	14.6 ± 1.4
Lymphoblasts	5.6 ± 0.5	4.9 ± 0.8	3.1 ± 0.3[b]	4.2 ± 0.8
Neutrophils and metamyelocytes	1.6 ± 0.3	1.2 ± 0.1	1.0 ± 0.5	0.9 ± 0.2
Promyelocytes and myelocytes	1.1 ± 0.2	0.8 ± 0.1	0.6 ± 0.1[b]	1.0 ± 0.1
Myeloblasts	6.9 ± 1.1	8.3 ± 1.2	5.3 ± 0.5[b]	7.7 ± 1.2

[a] Mice inoculated with Ehrlich ascites tumour cells were treated with BCDT or diluent for 3 days, starting 24 h after tumour inoculation and, after the last injection, measurements were taken.
[b] $P < 0.001$.

haematopoiesis demonstrated an increase in the number of CFU-GM in the spleen with the 2- and 3-dose schedule of 30 mg/kg regimen, when compared to nontreated mice ($P < 0.001$). No changes were observed in the spleen of mice treated with 1, 2 or 3 doses of 10 and 15 mg/kg and 1 dose of 30 mg/kg (Fig. 4).

In Ehrlich ascites tumour-bearing mice, tumour-induced myelosuppression was restored to normal values with all the treatment regimens. However, the increase in the CFU-GM number produced by the 3 doses of 30 mg/kg of BCDT was significantly lower in relation to the controls and all the other treated groups. In addition, toxicity with this higher drug-regimen was clearly demonstrated with the death of 33% of the treated animals. In this respect, toxicity-related deaths, using 3 doses of 30 mg/kg of titanocene, were also observed by Villena-Heinsen et al. (1998) (Fig. 5).

Contrasting with the myelosuppression produced by Ehrlich ascites tumour, we observed in this tumour-model the presence of splenomegaly associated with a dramatic increased in the number of CFU-GM in the spleen ($P < 0.001$) (Table 1). The treatment of these animals with BCDT decreased significantly ($P < 0.001$) the number of



Effects of BCDT-treatments on the survival (days) in tumour-bearing mice.

Doses	10 mg/kg	15 mg/kg	30 mg/kg
1 dose	30	34	33
2 doses	37	34	20
3 doses	41	38	19
tumour	20	20	20

Fig. 6. Effects of 10 (A), 15 (B) or 30 mg/kg (C) of BCDT on the survival of Ehrlich ascites tumour(EAT)-bearing mice. In all treatment schedules, mice were treated i.p., with 1, 2 or 3 injections starting 24 h after tumour inoculation (1×10^6 cell/ml). Control mice received the diluent only.

splenic colonies in all treated groups. However, only in the groups treated with 10 mg/kg did this effect reach values near to normal control ($P < 0.001$).

Considering that the treatment with 3 doses of 10 mg/kg of BCDT produced the higher CFU-GM stimulation, serum from mice treated with this drug-regimen was selected for the investigation of serum colony-stimulating activity. The results demonstrated a stimulating effect on the growth and differentiation of bone marrow cells from normal mice ($P < 0.001$) (Table 2), which suggest that some indirect effects are also underway in the response to BCDT. The colonies produced by serum were predominantly of the granulocyte-macrophage morphology. On the other hand, no changes were observed after the in vitro addition of BCDT to cultures of haematopoietic cells from untreated mice.

3.2. Bone marrow cellularity

As before, the 3 doses of 10 mg/kg of BCDT regimen was chosen to study the changes in total femur cell counts and in the composition of bone marrow cell population due to Ehrlich ascites tumour inoculation. The results, presented in Table 3, measured on the 4th day of Ehrlich ascites tumour inoculation, demonstrated a significant ($P < 0.001$) reduction in the total bone marrow cell number and in the relative distribution of lymphoblasts, promyelocytes/myelocytes and myeloblasts. BCDT treatment restored these changes back to control values.

In normal mice no changes in the composition of femur cells were observed.

3.3. Survival

As demonstrated in Fig. 6, treatment with the 10 and 15 mg/kg regimens produced an increase in the rate of survival in all the six groups studied. Non-treated-tumour-bearing mice died within 20 days. With the treated animals, better results were observed with the 3 doses of 10 mg/kg (41 days), followed by the 3 doses of 15 mg/kg (38 days) ($P < 0.001$). Lower protection was shown with the 1 dose of 10 mg/kg (30 days). On the other hand, no protection was afforded by the 2 or 3 doses of 30 mg/kg and all the animals died in the same time interval as the tumour-bearing controls.

4. Discussion

During the past 40 years, chemotherapy has achieved major progress towards the management of some types of cancer so that now it is possible to achieve by the use of anticancer agents complete tumour regression followed by long disease-free periods of survival. Nevertheless, major problems such as the toxic effects on the bone marrow cells produced by the majority of the antitumoural compounds still need to be overcome. The bone marrow is more sensitive than other organs because of its high metabolic and mitotic activity rate. As a result, leucopoenia develops as a complication and predisposes the patients to infectious diseases (Abdel-Dayem et al., 1999). Major efforts have currently been placed on the development of antitumour curative drugs that destroy the cancer cell without producing lasting and irreversible damage to the haematopoietic system of the host. In this respect, we have previously demonstrated that the antitumoural action of the compound Titanocene Dichloride in Ehrlich ascites tumour-bearing mice does not adversely affect haematopoietic tissue while it acts against tumour cells. On the contrary, it has a modulatory effect on haematopoiesis at the level of granulocyte macrophage progenitor cells with concomitant prolonged host survival (Valadares et al., 1998). The present study was designed to evaluate the effects on haematopoietic progenitor cells in tumour-bearing mice of a Titanocene Dichloride derivative, BCDT, which presents a substitution of the chloride ligands by pseudo halogens in the halides radical.

BCDT was administrated to normal and Ehrlich ascites tumour-bearing mice as 1, 2 or 3 injections of 10, 15 or 30 mg/kg doses. Our results demonstrated, in parallel to an antitumoural effect related to myelopoietic recovery in tumour-bearing mice, a significant increase in the number of bone marrow CFU-GM in the normal mice treated with the 10 and 15 mg/kg dose schedules. Although, the in vitro incubation of bone marrow cells from normal mice with BDCT did not induce any direct simulation on haematopoietic progenitor cells, the serum obtained from normal mice treated with BCDT induced a threefold increase in CFU-GM formation in vitro, when compared to control values. In this sense, we can suggest that adjuvant CSF produced by indirect effect of this titanocene could be significant to produce synergism with other factors in the inhibition of Ehrlich ascites tumour evolution. It is well established that tumour cells may constitutively produce CSF and CSF receptors (Subiza et al., 1989) as a result of genetic aberrations in growth factor signalling pathways and/or greater cytokine mRNA stability linked to the neoplastic transformation. The physiologic function of CSF of tumour origin is not well understood, although both local and systemic effects have been implicated (Ruiz de Morales et al., 1999). Systemic effects include a development of suppressor cells, a decrease in lymphoid response and in extramedullar haematopoiesis along with splenomegaly. At the local level, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) may promote both tumour cell growth or inhibition by acting in an autocrine fashion and/or inducing a host response leading to decreased tumourigenicity (Dedhar et al., 1988). In this regard, Ruiz de Morales et al. (1999), suggested, using two variants of Ehrlich ascites tumour cells, that the amount of GM-CSF and G-CSF released by Ehrlich ascites tumour cells might not be large enough to decrease their tumourigenicity. Moreover, the possibility of these cells to produce immunosuppressive factors subverting the host immune response and therefore counteracting the effect(s) of CSFs was also considered.

According to previous results from our laboratory (Valadares et al.,1998; Queiroz et al., 2001; Justo et al., 2001), there is in the 4th day of Ehrlich ascites tumour development a drastic reduction in bone marrow CFU-GM number and cellularity, which was restored close to normal values with the lower doses of BCDT. In addition, a dose-dependent increase in survival rate was observed with the BCDT-treatments. The better efficacy in prolonging life span was produced by the 3 doses of 10 mg/kg and this treatment was followed by a greater increase in bone marrow CFU-GM number. This dose schedule also produced the more favourable response for the reduction of splenic haematopoiesis and splenomegaly induced by the tumour. In line with other studies, we suggested that this phenomenon produced by the tumour evolution is attributed to an accelerated traffic of progenitors from the host bone marrow to the spleen, which is a highly reactive organ able not only to increase cellularity but also to become the site of active haematopoiesis (Segura et al., 1997; Valadares et al., 1998; Ruiz de Morales et al., 1999; Segura et al., 2000; Justo et al., 2000; Justo et al., 2001; Queiroz et al., 2001).

On the other hand, the 3 doses of 30 mg/kg produced a lower recovery in the number of bone marrow CFU-GM, when compared to other treated groups. This effect was followed by a reduced survival rate. Moreover, treatment with this higher dose also produced an increased extramedullar haematopoiesis in normal mice. Abdel-Dayem et al. (1999), in accordance with others, showed that the presence of extramedullar haematopoiesis followed by spleen hyperplasia are severe side effects that contribute to a less favourable prognosis. Additional evidence to the toxicity of this higher-dose regimen was provided by the 33% mortality induced in tumour-bearing animals.

These findings corroborate previous studies performed in our laboratory and by others (Smarajit et al., 1993; Justo et al., 2000; Justo et al., 2001; Queiroz et al., 2001), which demonstrated that the recovery of tumour-induced myelosuppression is often related to better antitumoural response.

In comparison with previous results from our laboratory addressing the effects of the Titanocene Dichloride on haematopoiesis, we observed a similar but less effective profile for BCDT. The myelomodulatory action found for both compounds was two times greater for Titanocene Dichloride. In addition, the sera from Titanocene Dichloride-treated mice increased more than twofold the CFU-GM formation, when compared to BCDT (Valadares et al., 1998).

The mechanism of all the antitumour titanocenes is most likely a complex pathway, probably involving a number of different biological molecules related to the transport and delivery of the Ti species into cancer cells, and, after hydrolysis, subsequent interaction with nucleic acids and/or proteins and/or other potential coordinating constituents present in the intracellular environment (Modski and Harding, 2001). The tendency to hydrolyse seems to be one of the hypotheses for the tumour-inhibiting potency of the titanocene dihalides (Kopf-Maier, 1980). In this regard, new structure activity in vitro studies including the design of hydrolytically stable metallocene and the preparation of highly water-soluble amino acid analogues have not led to improvement in the anticancer activity of Titanocene Dichloride (Bakhtiar and Ochiai, 1999; Harding and Mokdsi, 2000). Our in vivo results with BCDT reported here confirm and extend these findings with the observation of a lower efficacy and higher toxicity for a derivative that undergoes a less extensive hydrolysis as BCDT. The vastly different chemical and hydrolytic stability of each of the metallocene points to a unique mechanism of action of each metallocene in vivo. In this regard, the presence of chloride in the original compound allows for a more extensive hydrolysis and a greater availability of Ti species in comparison to the pseudo halogens of BCDT compound.

In spite of the differences found with these two titanocenes, BCDT and Titanocene Dichloride, our results showed that both have a significant ability to keep the balance between the positive and negative stimuli controlling the haematopoiesis and thereby to harass Ehrlich ascites tumour-evolution.

The precise mechanism by which the titanocenes modulate myelopoiesis is unknown at present. However, it is evident that the positive effects of these compounds might occur by the release of some biologically active material acting synergically with stimulatory cytokines present in the organism, enhancing proliferation in critical pools of haematopoietic stem and progenitor cells, leading finally to higher regeneration of the functional peripheral blood cell population.

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