

Metronomic therapy with cyclophosphamide induces rat lymphoma and sarcoma regression, and is devoid of toxicity

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Background: Our aim was to investigate the clinical efficacy and toxicity of metronomic administration of low-dose cyclophosphamide (Cy) in lymphoma and sarcoma rat tumour models.

Methods: Adult inbred rats were challenged with lymphoma TACB and sarcoma E100 s.c. on day 0. Animals were divided into two groups: group I, control, injected with saline three times a week; and group II, treated with Cy 10 mg/kg three times a week, from day 10 until the tumour was non-palpable, or 5 mg/kg three times a week from day 7. Tumours were measured and animals were weighed twice weekly. Periodic blood samples were taken for determination of urea, creatinine, serum glutamic-oxaloacetic transaminase, lactate dehydrogenase and haematological parameters.

Results: The administration of low-dose Cy eradicated established rat lymphomas and sarcomas; there was neither metastatic growth nor recurrence at primary sites for 100% of the lymphomas and 83% of the sarcomas. In addition, the treatment did not cause weight loss, and was devoid of haematological, cardiac, hepatic and renal toxicity.

Conclusions: Metronomic administration of Cy at low doses on a thrice weekly schedule to already grown rat lymphomas and sarcomas demonstrated itself to be a successful antitumour therapy that did not cause weight loss and was devoid of haematological, cardiac, hepatic and renal toxicity.

Key words: cyclophosphamide, lymphoma, metronomic dosing, sarcoma, toxicity

Introduction

One of the great advances in cancer treatment during last half of the 20th century was the development and utilisation of chemotherapeutic drugs. These compounds have demonstrated antitumour efficacy through their damaging action against cellular DNA, which prevents proliferation and, frequently, drives the cell to its death. Since such an effect is only exerted on a fraction of tumour cells, the administration of higher drug doses are required to achieve better clinical results. However, the application of the highest drug doses tolerated by the patient brings about the problem of drug toxicity. Thus, it is mandatory to establish rest periods, which not only allow regrowth of tumour cells but also growth of selected therapy-resistant clones. After the successful first cycles of treatment, tumours acquire resistance to the chemotherapeutic

drugs. Thus, the growth of those resistant cells involve the development of more aggressive and malignant tumours.

In order to avoid the problems caused by traditional chemotherapeutic treatments, several researchers, including ourselves, recently began to search for new modalities of drug administration oriented towards a more efficient and non-toxic antitumoural and/or antimetastatic therapy. We have already demonstrated that a single low dose of cyclophosphamide (Cy), a treatment completely devoid of toxicity, inhibits the growth of spontaneous and experimental metastasis of a rat lymphoma, while it does not affect primary tumour growth [1]. Such an effect would mainly be due to modulation of the immune response [2–5]. These results proved that in our tumour model, the targets of low-dose chemotherapy were the immune cells and that metastatic cells were affected indirectly by the drug.

On the other hand, Kerbel, Folkman and colleagues have demonstrated the efficacy of some of the most widely used chemotherapeutic drugs as antiangiogenic agents [6–9]. The proposal implies that a change should be made with respect to the rationale behind chemotherapy, involving a different target. This new concept includes the possibility of treating tumours that no longer respond to traditional chemotherapy.

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Results obtained at the clinical level [10] provide a point of departure for future studies in this interesting area of cancer therapy.

Our objective in this study was to investigate the possibility of obtaining an antitumoral effect by the metronomic administration of low-dose Cy. Another main focus of our work was to determine the toxicity of the treatment. We report here the effect of administration of (i) Cy 10 mg/kg on a thrice weekly schedule, from day 10 until approximately day 60, on lymphoma TACB (L-TACB) growth; and (ii) Cy 5 mg/kg on the same schedule, from day 7 until approximately day 30, on sarcoma E100 (S-E100) growth, along with its toxicity.

Methods

Animals

Inbred adult IIM *e*/Fm and Fm-*m* rats (*e* and *m*, respectively) [11] from the Facultad de Ciencias Médicas, Universidad Nacional de Rosario breeding facilities, were used. Rats were fed with commercial chow and water *ad libitum*, and were maintained in a 12h light/dark cycle. All the experiments were carried out during the first half of the light cycle. The animals were treated in accordance with the guidelines issued by the Canadian Council on Animal Care.

Drugs

Cy (Endoxan-Asta, Labinca SA, Argentina) was dissolved in sterile, distilled water to a concentration of 20 mg/ml.

Tumours

L-TACB is a poorly differentiated B-cell lymphoma, which arose spontaneously in an inbred *e* rat [12]. When L-TACB is injected subcutaneously, lymph nodes are the exclusive site of metastatic growth.

S-E100 is a fibrosarcoma that appeared spontaneously in an outbred population of IIM rats in 1955 and is maintained by subcutaneous passage in rats of the allogeneic inbred line *m*, with 100% lethality [13].

Experimental models

Adult *e* or *m* rats were challenged with L-TACB or S-E100, respectively, subcutaneously by trocar, on day 0, and distributed as follows. L-TACB: group I control rats ($n=7$) were injected i.p. with saline three times a week, from day 10 until animals were killed; and group II rats ($n=6$) were injected i.p. with Cy (10 mg/kg body weight) three times a week, from day 10 until the day on which the tumour was no longer palpable. S-E100: group I control animals ($n=12$) were injected i.p. with saline three times a week, from day 7 until animals were killed; and group II rats ($n=12$) were injected with Cy 5 mg/kg i.p., three times a week, from day 7. In the case of the lymphoma tumour model, a third experimental group was added (group III), in which naïve animals ($n=4$) were injected with Cy, employing the same schedule and dose as in the respective group II.

Tumours were measured twice weekly using a calliper. Tumour volumes were calculated as follows: $v = 0.4 (ab^2)$, where v = volume (cm^3), a = largest diameter (cm) and b = smallest diameter (cm). Animals were weighed weekly. On days 0, 20, 40 and 60, blood samples were taken and each rat was subjected to an electrocardiogram (ECG). Group II animals were clinically controlled and the appearance of tumour recurrences and metastasis was checked until the end of the experiment (day 150 for L-TACB and day 120 for S-E100).

Haematological and serological parameters

Blood and serum samples were obtained from animals belonging to all experimental groups [L-TACB: group I ($n=7$), group II ($n=6$) and group III ($n=4$); S-E100: groups I and II ($n=4$)] for the determination of haematological and serological parameters, including haemoglobinemia, haematocrit, total leukocyte count and proportion of white blood cell (WBC) types (May Grünwald–Giemsa colouration), and serum concentration of urea (direct colorimetric method), creatinine (Jaféé reaction), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH, E.C.1.1.1.27) by the optimised ultraviolet method.

Electrocardiograms

The ECG DII derivation at a velocity of 50 mm/s was registered with awake animals, employing a conventional electrocardiograph. Cardiac rate, PR_i (PR interval), QT_i (QT interval) and $\hat{A}QRS$ (main ventricular depolarisation vector) were measured, and the presence of arrhythmia or other manifestations of myocardial lesions were analysed.

Statistical analyses

Analysis of variance and the Tukey–Kramer Multiple Comparison tests, the Mann–Whitney non-parametric test and Student's *t*-test (InStat, GraphPad Software, Inc.) were used. Results were considered significant if *P*-values were <0.05 .

Results

Tumour growth assessment

After subcutaneous implantation of L-TACB or S-E100 in *e* and *m* rats, respectively, tumours were allowed to grow to up to 0.35 cm^3 for L-TACB and 0.25 cm^3 for S-E100 when Cy treatment began. As can be seen in Figure 1A, the volume of L-TACB tumours in control animals was significantly higher than that in treated animals ($P < 0.01$) at day 20. At that time point, while control animals were euthanised, the inhibition of tumour growth began in treated rats. Complete regression of the tumours was reached between days 30 and 60, and the therapy was discontinued from the moment each tumour was no longer palpable. Neither recurrences at the primary site nor metastatic growth were observed until the end of the experiment on day 150. Figure 1B shows the evolution of S-E100 tumour size after Cy treatment. The treated tumours increased in size until day 14, when all the tumours began to regress. At day 21 regression was complete for all the treated tumours and therapy was discontinued. Nevertheless, by day 28 tumour recurrence was observed at the primary site in two out of 12 animals, and despite re-entry into the treatment schedule for 10 more days, tumours continued growing until the animals were killed. The rest of the treated animals were observed until day 120 without showing recurrences. S-E100 control animals were euthanised on day 20, their mean tumour volume being significantly higher than that of treated rats ($P < 0.0001$).

Evaluation of toxicity

Body weight

Modification of body weight, a good indicator of general health status, for both types of tumours showed no

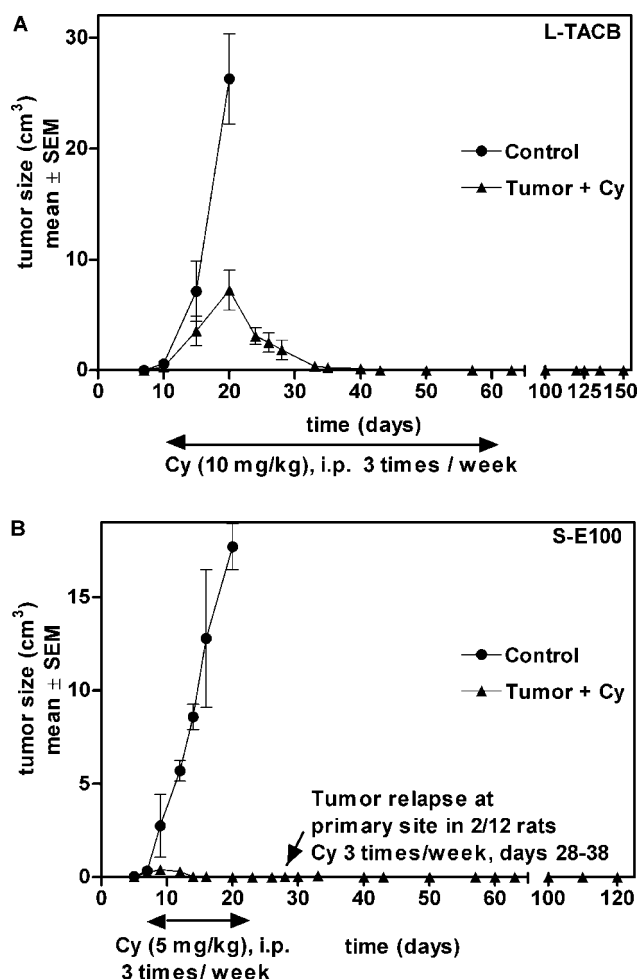


Figure 1. Regression of established tumours by chronic administration of low-dose cyclophosphamide (Cy). (A) Lymphoma TACB (L-TACB), control versus tumour + Cy ($P < 0.001$). (B) Sarcoma E100 (S-E100), control versus tumour + Cy ($P < 0.001$). Data shown represent mean values \pm SEM (standard error of the mean).

significant differences between control and treated animals (Figure 2A and B), except for an increase in body weight on day 20 in the L-TACB control group, which was probably due to a concomitant rapid increase in tumour mass that did not differ statistically from the weight of the L-TACB-bearing, Cy-treated rats. There were no weight losses in the treated groups of rats, including those without tumour and those treated with Cy. On the contrary, with the exception of isolated non-statistically significant decreases, weights increased steadily in both experiments.

Haematological parameters

The significant decrease in haemoglobin concentration in control L-TACB- and S-E100-bearing rats by day 20 compared with basal values ($P < 0.001$ and < 0.05 , respectively) was not observed in the corresponding treated groups (Figure 3A and B), which maintained steady haemoglobin levels over time. Also, in the group of rats without tumour but treated with Cy, there was no evidence of any significant modification in

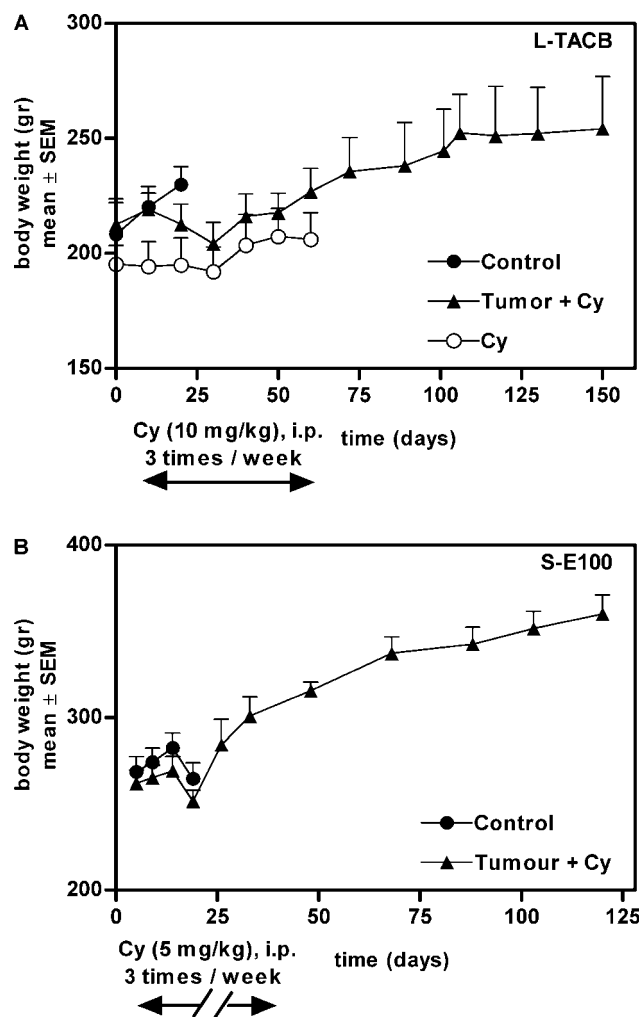


Figure 2. Evolution of body weight during treatment of established lymphoma TACB (L-TACB)-bearing rats (A) and sarcoma E100 (S-E100)-bearing rats (B) with low-dose cyclophosphamide. No body weight losses were detected in either tumour model. Data shown represent mean values \pm SEM of body weight (g).

haemoglobinemia (Figure 3A). Haematocrit determination yielded similar results for all the treated groups (Figure 3C and D). Conversely, L-TACB and S-E100 control groups had diminished haematocrit levels on day 20 compared with day 0 ($P < 0.001$ for L-TACB; $P =$ not significant for S-E100). Total leukocyte count did not show significant variation between the groups treated for both tumour types, while control animals presented an increase in total leukocytes for L-TACB ($P < 0.001$) and S-E100 ($P = 0.057$) (Figure 3E and F). On the other hand, animals belonging to the group without tumour but treated with Cy showed a significant decrease in WBC count to $\sim 35\%$ of basal values ($P < 0.05$). The proportion of each WBC type to total leukocytes also showed variation (mainly for neutrophils and lymphocytes) in the experiments. By day 20 and/or 40, L-TACB and S-E100 Cy-treated rats showed an increase in neutrophils and a decrease in lymphocytes, albeit to different degrees, with both tending to normalise towards day 60 (Table 1). The same tendency was observed for control rats at day 20. In the Cy-treated group

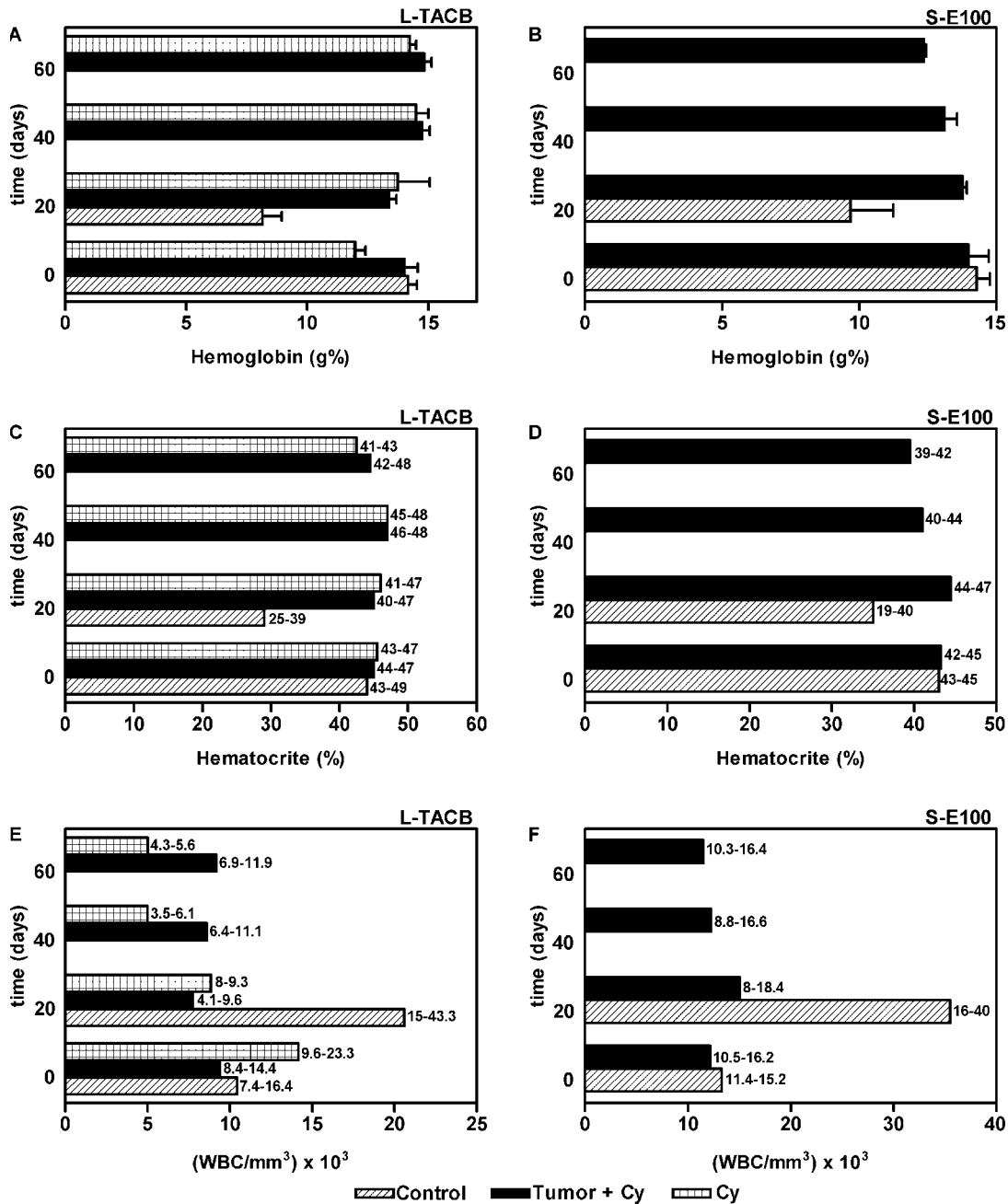


Figure 3. (A and B) Haemoglobin concentration (mean \pm SEM) in lymphoma TACB (L-TACB)- and sarcoma E100 (S-E100)-treated animals, respectively. Control L-TACB and S-E100 rats, day 0 versus day 20: $P < 0.001$ and $P < 0.05$, respectively. (C and D) Haematocrit values [median (range)] in L-TACB- and S-E100-treated animals, respectively. Control L-TACB rats, day 0 versus day 20: $P < 0.001$. (E and F) Total leukocyte count [median (range)] in L-TACB- and S-E100-treated animals, respectively. Control L-TACB and S-E100 rats, day 0 versus day 20: $P < 0.001$ and $P = 0.057$, respectively; naïve rats + Cy, day 0 versus days 40 and 60: $P < 0.05$. WBC, White blood cell count.

without tumour, no important modifications in the proportion of both WBC types were observed (Table 1).

Serologic parameters

Concentration of serum urea showed no significant variation, either in control or treated groups for L-TACB-bearing rats (Figure 4A). The same result was obtained for creatinine serum concentration, determined only in samples from L-TACB tumour models (Figure 4C). In the S-E100 tumour

model, urea concentration increased at day 20 in both experimental groups (Figure 4B), with the level in the control group being higher than that in the treated group ($P = 0.053$). Rejection of the tumours was accompanied on days 40 and 60 by a normalisation of serum urea concentration.

A striking elevation of AST concentration was observed at day 20 in L-TACB control animals (>15-fold increase) and S-E100 control animals (5-fold increase). While serum samples of S-E100 Cy-treated rats did not show any significant

Table 1. Evolution of the proportion of neutrophils and lymphocytes to total leukocytes after metronomic dosing with Cy in L-TACB and S-E100 tumour models

Group	Day	Percentage of cells [median (range)]			
		L-TACB		S-E100	
		Neutrophils	Lymphocytes	Neutrophils	Lymphocytes
Control	0	25 (18–26)	68 (63–74)	22.5 (20–25)	70 (65–71)
L-TACB (<i>n</i> = 7)	20	27 (21–41)	54 (37–69)	27 (14–37)	66.5 (55–78)
S-E100 (<i>n</i> = 4)					
Tumour + Cy	0	25.5 (19–34)	63 (55–73)	16.5 (14–21)	77.5 (76–80)
L-TACB (<i>n</i> = 6)	20	35 (20–45)	53.5 (42–73)	25 (17–41)	67.5 (58–77)
S-E100 (<i>n</i> = 4)	40	47 (39–48)	47.5 (40–57)	27 (15–43)	65 (53–76)
	60	34.5 (27–27)	67 (53–70)	21.5 (15–24)	70.5 (65–80)
Cy (<i>n</i> = 4)	0	33.5 (26–41)	58.5 (50–71)		
	20	27 (23–34)	64.5 (58–70)		
	40	31 (24–40)	58 (50–62)		
	60	28.5 (20–35)	59 (50–63)		

Cy, cyclophosphamide; L-TACB, lymphoma TACB; S-E100, sarcoma E100.

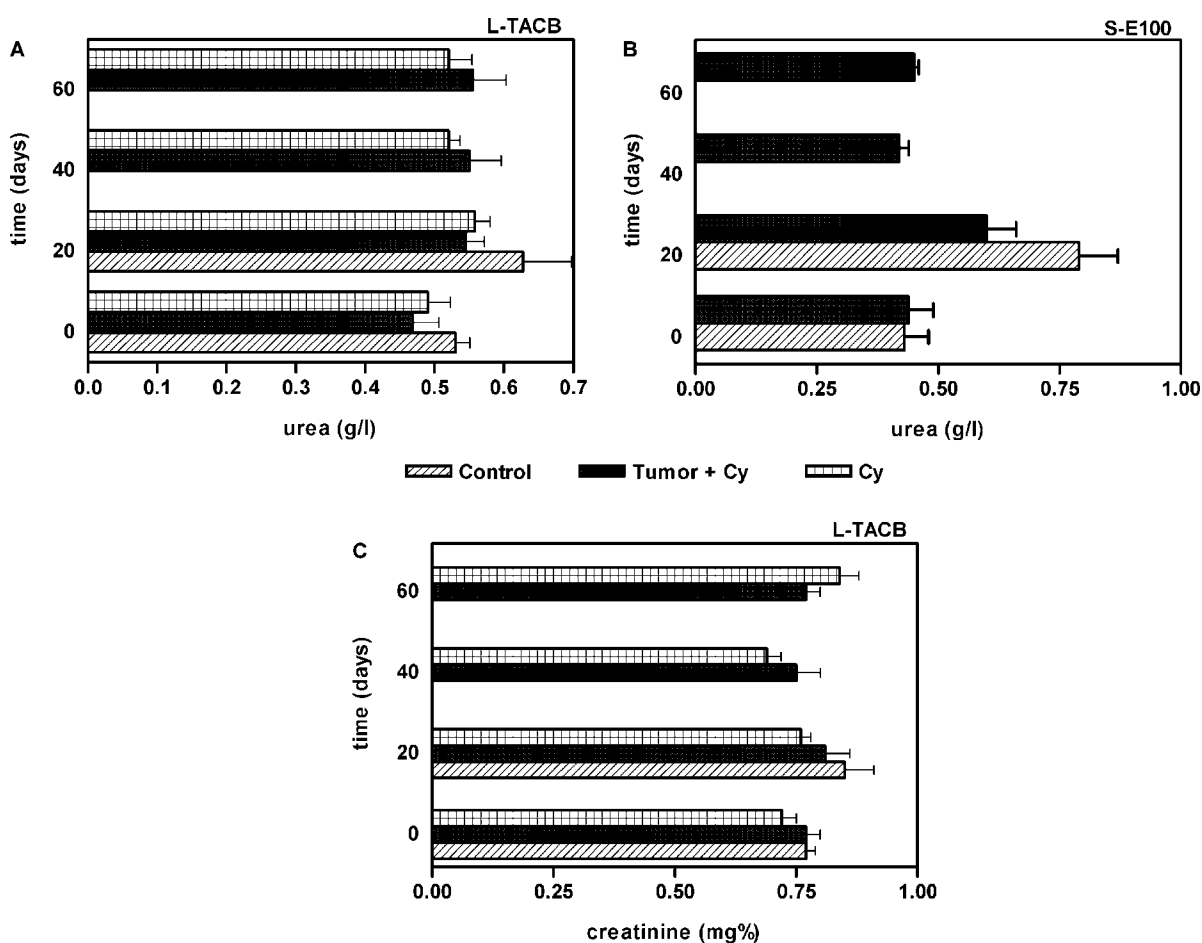


Figure 4. (A and C) Urea and creatinine serum concentration (mean \pm SEM), respectively, in the lymphoma TACB (L-TACB) tumour model. (B) Serum urea concentrations in the sarcoma E100 (S-E100) tumour model. Day 20, control versus tumour + Cy: $P = 0.053$.

variation in AST levels throughout the experiment, samples from L-TACB Cy-treated rats showed a transient, significant elevation of AST concentration ($P < 0.05$) on day 20 compared with basal values, which returned to normal on day 40 (Figure 5A and B). The modifications observed in serum LDH concentration were similar in both tumour models, albeit at different levels. Significant elevations were seen in serum LDH from both control groups at day 20 compared with basal values. The treatment of both tumour types diminished LDH levels by day 20 compared with those of control rats (Figure 5C and D), with values that were approaching statistical significance. Normality in LDH levels was finally reached on day 60. Interestingly, serum samples from the group without tumour but treated with Cy showed a significant decrease in LDH levels ($P < 0.05$) by days 40 and 60 (Figure 5C).

Electrocardiograms

Neither modifications of PR_i , QT_i and $\hat{A}QRS$ nor manifestations of myocardial lesions or arrhythmia were observed in control or treated rats. All the animals and experimental groups showed a similar ECG (data not shown).

Discussion

Historically, chemotherapy regimens have been controversial: which way should the scale be tipped between efficacy in tumour killing and lack of toxicity? On one hand, there is the ability of chemotherapeutic drugs to disrupt the DNA of tumour cells, rendering them unable to replicate and finally killing them, the befitting corollary being 'the higher the dose the better' [14]. On the other hand, toxicity is expressed at several organ sites, which not only diminishes quality of patient life but also conspires against a good resolution of the cancer treatment, adding illness to that which already exists [15–17]. The introduction of maximum tolerated doses in usual treatment protocols made it necessary to impose periods of rest between cycles of therapy. During such rest periods, recovery of 'good' cells is frequently accompanied by recovery of 'bad' cells, i.e. tumour cells resistant to the drug cell killing effect. The possibility of finding a treatment modality that avoids toxicity without diminishing effectiveness is still a matter for study and discussion. Several groups, including ours, have begun to address this issue. The experimental findings of Browder [7], Klement [6, 8] and Man [9] and colleagues successfully introduced a novel strategy for cancer

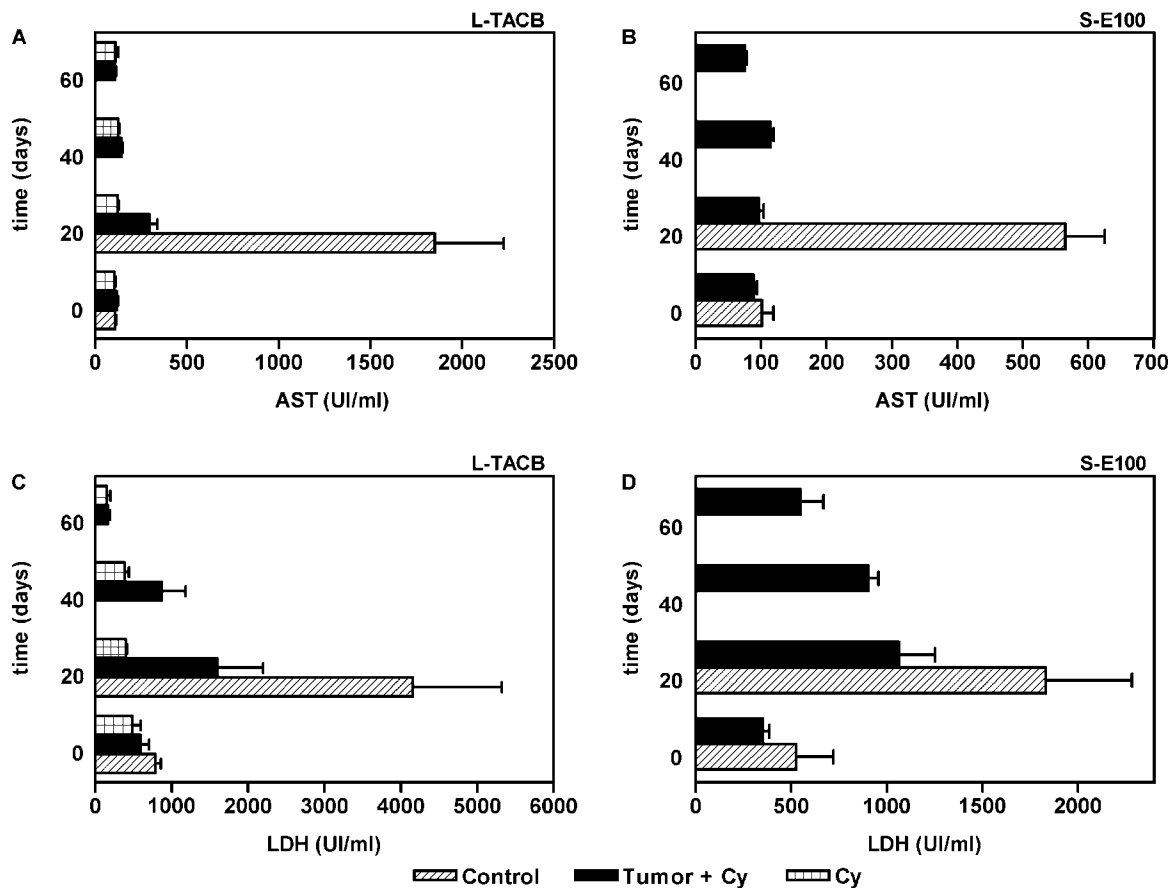


Figure 5. (A and B) AST serum concentration (mean \pm SEM) in lymphoma TACB (L-TACB)- and sarcoma E100 (S-E100)-treated animals, respectively. L-TACB+Cy rats, day 0 versus day 20: $P < 0.05$; day 20, control versus tumour+Cy: $P < 0.001$. (C and D) LDH serum concentration (mean \pm SEM) in L-TACB- and S-E100-treated animals, respectively. Cy-treated L-TACB rats and L-TACB and S-E100 control groups, day 0 versus day 20: $P < 0.05$. Naïve rats+Cy, day 0 versus days 40 and 60: $P < 0.05$.

treatment. The novel component comprised a cell target switch, which now aims towards the genetically stable tumour endothelial cells, along with a change in the schedule and dose of drug administration, thereby introducing metronomic chemotherapy [18]. We have previously demonstrated the effectiveness of a unique low dose of Cy, with no toxicity, in inhibiting lymphoma metastasis through modulation of the immune response [1–5]. In this study, we wished to determine whether the same low dose of Cy, administered chronically, could achieve an antitumour effect while maintaining a lack of toxicity. We studied not only the evolution of body weight, a suitable indicator of health status, but also serological and haematological parameters alongside ECG determinations.

The experimental models were designed to resemble the clinical situation of a patient with a recently detected tumour (not too large, but large enough to be easily detected) who begins therapy immediately after being diagnosed. Metronomic therapy began after 10 and 7 days of the tumour challenge, for L-TACB and S-E100, respectively. The response of L-TACB-bearing rats to treatment was complete in 100% of the animals, achieving total regression between days 30 and 60. Importantly, neither recurrences nor metastases were detected until the end of the experiment. The behaviour of S-E100 after Cy treatment was similar to that of L-TACB until day 28, when tumour growth at the primary site resumed in two out of 12 rats. The rest of the animals remained recurrence-free until the end of the experiment. These results, obtained with tumour types and species different from those assayed up to now, provide additional experimental evidence for the antitumour effect of metronomic therapy. The demonstration that different kinds of tumours can be eliminated or significantly diminished [6–10] by chronic administration of low doses of very different kinds of chemotherapeutic drugs [19] increases the likelihood of successful treatment of human cancer. It also encourages the development of phase I and II clinical trials for different cancer types using different drugs, which, ultimately, will determine the efficacy of metronomic therapy for the treatment of human cancers.

Other authors have demonstrated clearly the antiangiogenic nature of the mechanism of action of several drugs administered by metronomic dosing. Moreover, the combination of such treatment with specific antiangiogenic reagents increased significantly the observed antitumour effect of metronomic dosing [6–8, 20, 21]. Hence, the antitumour effect described herein is probably due to an antiangiogenic mechanism. Some recent findings by Mauceri et al. [22] support this hypothesis. She found that the addition of Cy to angiostatin, an internal fragment of plasminogen with antiangiogenic properties that is generally secreted by tumours [23], produced inhibition of tube formation. Moreover, recent preliminary results, from our laboratory, utilising the Matrigel plug angiogenesis assay in *e* rats showed an antiangiogenic effect exerted by four injections of Cy (10 mg/kg) administered over 6 days. Nevertheless, we do not dismiss the existence of other mechanisms contributing to the antitumour effect, mainly the modulation of the immune response, as we have demonstrated for the antimetastatic effect

of single low-dose Cy in the L-TACB tumour model [2–5]. The recurrence of the tumour in 17% of the Cy-treated, S-E100-bearing rats suggested that, in this case, the cell target of Cy would not be the genetically stable endothelial cells, which in all likelihood would not generate drug resistance. Future studies will address this important matter.

Determination of body weight showed no weight loss in either treatment group for each tumour model. The body weight of treated animals increased steadily, with the exception of transient, non-significant decreases. Concomitantly, animals were monitored for the appearance of any other clinical sign of drug toxicity, which never appeared.

From a haematological point of view, determinations of the haematological parameters throughout the study demonstrated the lack of toxicity of the treatment. Haemoglobin concentration and haematocrit did not suffer variation in any of the Cy-treated groups throughout the experiments, while in both control groups there were significant decreases in both values. Also, while the WBC count increased significantly with tumour growth, a significant decrease was observed in the group of naïve Cy-treated rats. These opposite effects caused by tumour growth and Cy treatment, respectively, might account for the lack of WBC changes observed in Cy-treated L-TACB- and S-E100-bearing rats. The altered WBC proportions observed in treated tumour-bearing rats, namely an increase in neutrophils and a decrease in lymphocytes, tended to return to basal values by day 60. Such changes are mainly due to tumour growth, because naïve rats treated only with Cy showed no important modifications to these proportions. Moreover, the Cy-treated tumour-bearing rats returned to normal values between days 40 and 60, despite continuation of chemotherapy.

Similarly, the evaluation of biochemical parameters showed a lack of renal, hepatic or cardiac toxicity with the treatment. Serum concentrations of urea and creatinine in L-TACB-bearing rats remained unaltered, while in the S-E100 tumour model, the significant increase in serum urea in control rats, caused by tumour growth, was moderated by the treatment. Cy-treated S-E100-bearing rats showed normalised uraemia by days 40–60; therefore, there was no renal toxicity when measured using these parameters.

The growth of L-TACB or S-E100 was accompanied by a striking elevation in AST concentration. AST is an enzyme present in high concentrations in tissues with high metabolic activity. Elevated levels of AST have been correlated with certain malignancies [24]. Severely damaged or dead cells release AST into the blood in quantities directly proportional to the amount of tissue damage [25]. Interestingly, the treatment administered herein did not modify the normal enzyme levels in S-E100-treated rats. Besides, in L-TACB Cy-treated rats there was a transient increase in AST concentration at day 20; an increase that, nevertheless, was significantly lower than that of control rats ($P < 0.01$), returning to normal levels by day 40.

Many tissues, including the liver, red blood cells and the brain, produce LDH, an enzyme normally found in the blood. The fact that tumour growth is frequently associated with an increase in LDH serum levels is already known,

and this protein can be considered as a tumour marker [26, 27]. Also, LDH, as well as AST, are protein markers associated with different types and degrees of cardiac damage [28]. The significant increases in LDH serum concentration found in control rats belonging to both tumour models were moderated by the treatment at day 20 and continued to decrease towards day 60. The augmentation of serum LDH during tumour growth would probably be compensated for by the decrease observed during Cy treatment in naïve rats. The AST and LDH results obtained herein indicate a lack of hepatic and cardiac toxicity with the treatment. Moreover, there were no changes in ECG results during the treatment, a fact that confirmed the absence of gross cardiac alterations.

In summary, metronomic administration of Cy at low doses on a thrice weekly schedule to already grown rat lymphomas and sarcomas was demonstrated to be a successful antitumour therapy that, interestingly, was devoid of toxicity. Further studies will help to clarify the mechanism of action and what type of cells are being targeted by this chronic chemotherapy.

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