Biol Res 43: 317-322, 2010

Betamethasone inhibits tumor development, microvessel density and prolongs survival in mice with a multiresistant adenocarcinoma TA3

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ABSTRACT

Tumor resistance to traditional cancer treatments poses an important challenge to modern science. Thus, angiogenesis inhibition is an important emerging cancer treatment. Many drugs are tested and corticosteroids have shown interesting results. Herein we investigate the effect on microvessel density, survival time and tumoral volume of mice with TA3-MTX-R tumors. Twenty six mice were inoculated with $1x10^6$ tumor cells, 4-5 days after injection, six mice were injected with PBS (group A) and twenty mice were treated with β-met (group B). All animals from Group A died on day 22**.** Group B was divided into B1 (treated discontinued) and B2 (treated daily) and observed until day 88. All mice were processed for histo-immunohistochemical analysis and the blood vessels were counted. A decrease in microvessel density and tumoral volume and longer survival times were observed in the treated group. We propose that the antiangiogenic β-met effect explains, at least partially, its tumor inhibitory properties. As an important perspective, we will experimentally combine these strategies with those recently described by us with regard to the important antiangiogenic-antitumor effects of *Trypanosoma cruzi* calreticulin. Since the molecular targets of these strategies are most likely different, additive or synergic effects are envisaged.

Key terms: angiogenesis, betamethasone treatment, metastasis.

INTRODUCTION

Angiogenesis, the development of new blood vessels, is a fundamental physiological process that promotes embryonic development, tissue repair and fertility, although it also promotes chronic inflammation, tumor growth and tumor metastasis (Carmeliet, 2005; Aranda & Owen, 2009). It is now widely accepted that when the effect of proangiogenic and antiangiogenic molecules is tipped in favor of the former, the process is activated (Malonne *et al.*, 1999; Hanahan and Weinberg, 2000; Bouck *et al.*, 1996, Pike Se *et al.*, 1999, Ferreira *et al.*, 2005) New blood vessels in tumors can grow by sprouting from pre-existing vessels or by recruitment of rare, circulating bone marrow-derived endothelial progenitor cells (Avraamides *et al.*, 2008). Tumor cells, macrophages and fibroblasts within tumors secrete factors, such as vascular endothelial growth factor (VEGF), that induce blood vessel growth in tumors (Adams and Alitalo, 2007; Schmid and Varner, 2007). Basic and clinical studies indicate that suppression of angiogenesis can inhibit tumor progression and metastasis (Folkman, 1971). Angiogenic protein array monitored both pro-and anti-angiogenic proteins show that 17 out of 24 proteins are pro-angiogenic factors. The majority of them (G-CSF, GM-CSF, IGF-11, IL-1α, IL-β, IL-G, IL-9, TNF-α, MCP1, eotaxin, bFGF, VEGF, leptin, and thrombopoietin) are involved in all tumor angiogenesis steps and most of these factors have pro-inflammatory effects that strengthen their pro-angiogenic effects and support tumor growth. Moreover, IL-6 has an anti-apoptotic effect on cancer cells by inhibition of p53 induced-apoptosis. On the other hand, FasL helps tumor cells to escape immune surveillance by inducing apoptosis of T cells (Banciu *et al.*, 2006). New findings indicate that select integrins can modulate lymphangiogenesis and may thereby affect tumor metastasis (Avraamides *et al.*, 2008). Angiogenesis is stimulated by tumor-associated macrophages. Circulating bone marrow-derived cells (monocytes) migrate into tumors in response to tumor secreted chemokines and differentiate into macrophages. Pro-angiogenic tumor macrophages release a number of potent proangiogenic cytokines, such as VEGFA, VEGFC, tumor necrosis factor alpha (TNF-α), interleukin 8 (IL8) and basic fibroblast growth factor (bFGF, also known as FGF2) (Molina *et al.*, 2005; Schmid and Varner 2007; Lin and Pollard, 2007) and express a broad array of extracellular matrix (ECM) degrading proteases, including urokinase type plasminogen activator (uPA, also known as PLAU), and the matrix metalloproteinases MMP2, MMP7, MMP9 and MMP12, and elastase (Schmid and Varner, 2007).

Glucorticoids (GC) exert a broad variety of activities on mammalian cells, including immunosupressive, anti-inflammatory, apoptotic, necrotic and anti-angiogenic effects. In principle, all these effects, both at the genomic and non-genomic level, could play a role in the antitumor activity exerted by GC. The genomic mechanisms are induced by very low concentrations of GC. These mechanisms are determined by the interaction of GC with their cytosolic receptors (cGCR) followed by cGCR activation and translocation into the nucleus. Once in the nucleus, the GC/cGCR complexes modulate the activity of transcription factors, such as activator protein-1 (AP-1), nuclear factor kB (NF-kB) and nuclear factor of activated T cells (NF-AT). This leads to regulation of the expression of genes for many immunoregulatory and inflammatory cytokines (TNF-α, GM-CDF, IL-1a, 2, 6, 8, 11), for apoptotic

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Received: January 15, 2010. In revised form: June 22, 2010. Accepted: June 26, 2010.

proteins (members of the Bcl-2 family such as Bcl-x_s, Bad, Bax, Bid, Fasl), as well as pro-angiogenic proteins (like bFGF and VEGF) (Amsterdam *et al.*, 2002; Schmidt *et al.*, 2004; Smoak and Cidlowski, 2004). Higher dosages increase cGCR occupation, which intensifies the GC effects at the genomic level. If cGCR are saturated, GC can additionally induce non-genomic effects. Non-genomic actions involve three mechanisms: 1) cGCR-mediated inhibition of arachidonic acid release, 2) intercalation of GC molecules in cellular membranes, thus altering cationic transport through the plasma membrane and increasing proton leak out of the mitochondria, and 3) binding of GC to specific membrane-bound receptors (Buttgereit *et al.*, 2004). The responses induced by GC non-genomic mechanisms include immunosuppressive and anti-inflammatory effects and necrosis induction.

Previously, we have shown that 10^{-9}M of Betamethasone (β-met) (minimal antiangiogenic concentration) mediated a considerable inhibition of neovascularization promoted by murine A/J TA_3 tumor supernatant on the chorioallantoic membrane (CAM) of 12-day chick embryos (Lemus *et al.*, 2001). On the other hand, the association of β-met with ketoprofen produced a synergistic effect, significantly decreasing tumor angiogenesis on CAM (Zúñiga *et al.*, 2003). Moreover, inflammatory angiogenesis promoted by polyurethane sponge on mice, was strongly inhibited by low concentrations of β-met or sulindac, alone or combined (Illanes *et al.*, 2002).

The purpose of this study was to investigate the *in vivo* β-met effects on a murine TA3-MTX-R tumor model, with a focus on tumor growth, angiogenesis and survival time.

MATERIALS AND METHODS

Eight-week-old, 25-g females A, J mice, clinically healthy, were obtained from our Central Animal Facility and kept under standard conditions, with 12-hour light, dark cycles and food and water provided *ad libitum*. Experiments were performed according to the national regulations and were approved by the local animal experimentation ethics committee. Twenty-six animals were used in this work. For tumor induction, all mice were inoculated intramuscularly (day 1) in the gluteal region with 0.2 ml of ascites containing $1x10^6$ multiresistant A, J mice TA_3 –MTX-R tumor cells (Zipper *et al.*, 1995; Gajardo *et al.*, 2001; Plaza *et al.*, 2008; Plaza *et al.*; 2009). 95% viability of these tumor cells was determined by trypan blue exclusion counted in a Neubauer's chamber. Palpable and measurable tumors appeared 4-5 days after injection. These were measured daily and the volume estimated by the formula $V = 0.52 \times a^2 \times b$ where a = longest diameter, b = shortest diameter (O´Reilly *et al.*, 1994). Two groups were considered: (A) control n = 6, injected intraperitoneally (ip) with PBS and (B) experimental n = 20, inoculated ip with 10 µg/animal of β-met, a dose known to inhibit angiogenesis (Illanes *et al.*, 2002). On day 22, the first death occurred in the control group. On that day, the rest of the control mice (n = 5) and 2 mice from (B), were killed by continuous ether inhalation. Thus, tissue samples from lymphatic regional ganglia, lung, heart and spleen, as well as tumor and intestinal samples (as a control for Ki-67 effects), were isolated and fixed in 10% formaldehyde. A search for metastasis sites was performed, using a meso-microscopic study with standard Hematoxylin-eosin staining. To examine the effect of β-met on survival, the remaining 18 mice (B) were equally divided into B1 and B2 subgroups. In B1 (n = 8), the treatment with β-met was discontinued while in B2 (n = 10) was continued. In double blind experiments, three independent observers evaluated the total number of capillaries present in 0.081 mm^2 tissue sections, at 400X. The tumor periphery and metastasis sites were used to avoid the central heterogeneity reported (central necrosis, fibrosis, etc.) (Folkman, 1971). Five histological slides per organ for each mouse were prepared, and counts in 4 fields in the periphery of the tumor and metastasis of every Arteta stained tissue section were performed. In order to assess blood vessel formation, 15 fields per group for every organ studied were counted. Arteta staining improves the visualization of blood vessels in a tissue.

Cell proliferation was studied by immunohistochemical detection of Ki-67 antigen. The polyclonal antibody (Novocastra, Cat#NCL–Ki67-P) labels Ki-67 antigen in the granular components of the nucleolus during late G1, S, G2 and M phases. Negative and Positive Controls were performed. (Scholzen and Gerdes, 2000). Immunohistochemical evaluation was made with a semi-quantitative estimation of a field and observed by three observers in double blind technique.

Comparisons of blood vessel density and tumor volume between controls and the different experimental conditions were performed using Student's t test for independent samples. Survival was calculated using Kaplan-Meier (STATA program).

RESULTS

β-met inhibits the growth of a murine TA3-MTX-R tumor

As indicated in Fig. 1, palpable and measurable tumors appeared 4 days after injection. Significant differences in volume could be detected starting day 8 after treatment when tumor volume in controls (n = 6) was 1594 ± 76.55 mm^3, versus 854 ± 29 mm^3 in the experimental group (n = 18) ($P< 0.001$). On day 22, tumor volume was 5425 ± 282 mm^3 in the control group versus 3856 ± 122 mm^3 in the experimental ($P< 0.001$). When treatment for B1 group was discontinued, the tumor volume increased significantly when compared to the B2 group ($p<0,0001$), and all animals of the B1 group died 48 days after injection while the B2 group the tumor volume decreased gradually during the experiment, showing stabilization between days 50 and 58 after treatment with an average tumor volume of 379 ± 67 mm^3. Later, tumor growth progressively reached 8125 ± 87.6 mm^3 on day 88, and the remaining mice died.

Associated with the tumor growth rate control group (A), we observed an important and rapid progress of their clinical deterioration, characterized by detriment of the general state, loss of hair, increase in leg volume, inflammation, lameness with necrosis, distal hemorrhage of the posterior right extremity, and progressive decrease in food ingestion. All these signs were not found in the treated group (B), where we observed a minor general detriment, more reactivity and attentiveness to environmental stimuli, and improved mobility and appetite.

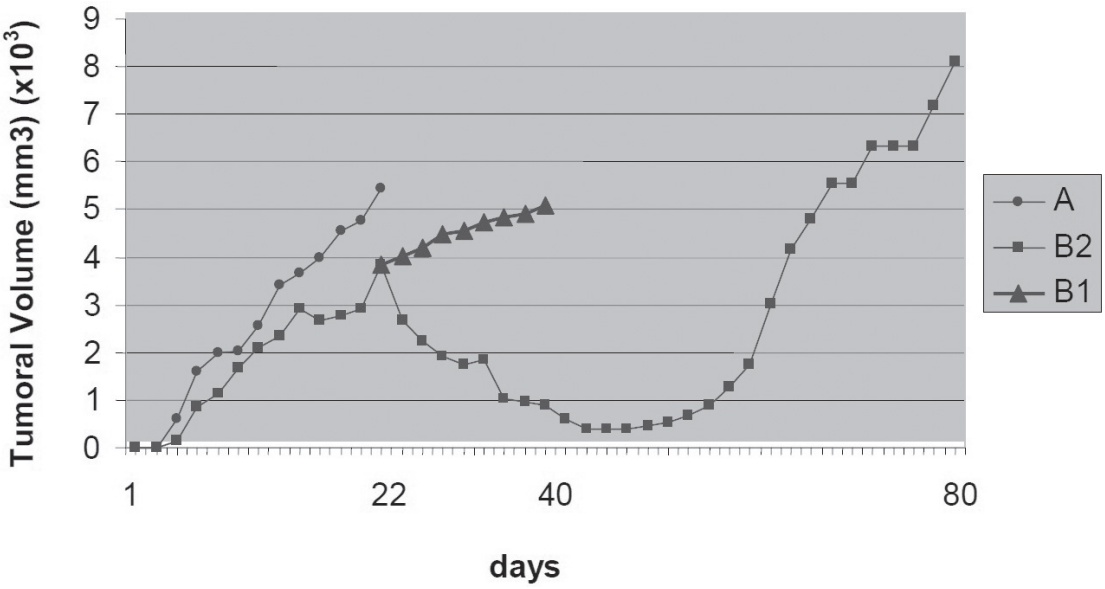

Figure 1: β-met inhibits the growth of a murine TA3-MTX-R tumor. Treatment started from day 4. Significant differences were seen among the control group (A) (●), treated discontinued (B1) (▲) and treated daily group (B2) (■).

β-met decrease microvessel density in a murine organ with a TA3-MTX-R tumor.

As summarized in Table I, a significant decrease in the number of vessels per area was detected in the β-met group versus the placebo group in every studied tissue. In group A (control), we detected an average of 8.6 ± 1.0 (mean ± sem) vessels/area (v/ac) versus 4.0 ± 0.6 v/ac in group B (β-met) when we observed tumor slides.(Figure 3A) In regional lymph nodes, 4.4 ± 0.6 vessels in A versus 2.9 ± 0.3 vessels in B, in lungs, 55.7 ± 3.25 in A versus 39.9 ± 2.5 in B; in heart, 14.8 ± 1.9 in A, *versus* 13 ± 2.0.

β-met decreases cell proliferation in TA3-MTX-R tumors

The immunohistochemical detection of Ki-67 showed a significant decrease in Group B when compared to Group A ($P< 0.001$). Group B showed many zones with negative Ki-67 cells, while Group A showed major cell proliferation in the entire tumor. (Figure 3 B) The marker is brown and the staining interpretation was made in cells with a nuclear staining pattern.

Table I

β-met inhibits angiogenesis in a murine TA3-MTX-R tumor

Organ	n	PBS (mean±sem)	β-met (mean±sem)	P
Tumor	15	8.6±1.0	4.0±0.6	0.0005
Lymph nodes	15	4.4±0.6	2.9±0.3	<0.05
Lung	15	55.7±3.25	39.9±2.5	0.0006
Heart	15	14.8±1.9	13.0±2.0	0.5194*

*: Not statistically significant
Blood vessels were examined in the Arteta stained organ sections of mice that died on day 22 and in the organs of mice remaining alive. Dates are expressed as number of vessels in 0.0081 mm^2.

β-met increases the survival of A/J mice bearing TA3-MTX-R tumors.

To examine the effect of β-met on mice survival, the animals were divided in two groups: (B1) β-met-treated discontinued and (B2) β-met-treated continued.

Figure 2 shows that in B1, the median survival was 32 days and in B2 the median was 63 days. Treatments (PBS-control and β-met) were started 4-5 days after tumor injection and continued for 88 days. The day of death represents the number of days after the injection. Some 90% of PBS-treated died by day 22, while β-met-treated mice survived until day 88.

DISCUSSION

The GCs exert a broad variety of effects on mammalian cells, including anti-inflammatory, immunosuppressive, anti-angiogenic, apoptotic, and necrotic effects. These actions are mediated by genomic and non-genomic mechanisms. The present study provides insight into the mechanism of antitumor action of β-met. In fact, in the clinical setting, GCs have been used for their anti-inflammatory and anti-emetic effects. In addition, GC has been applied clinically in the treatment of hematological malignancies because it has efficient cytolytic activity on cells of lymphoid origin (Schiffelers *et al.*, 2005). On the other hand, there are indications that GCs have inhibitory actions on solid tumor growth due to suppressive effects on tumor angiogenesis and inflammation (Folkman *et al.*, 1983; Banciu *et al.*, 2006) Preclinical studies on solid tumor growth inhibition indicate that GC-induced antitumor effects are obtained by using substantially higher doses than the minimal ones needed to achieve inhibition of inflammation. For example, to obtain antitumor effects in tumor-bearing mice, frequent and high doses of cortisone in the range of 500-700 mg/kg are needed (Folkman *et al.*, 1983; Penhaligon and Camplejohn, 1985; Banciu *et al.*, 2008). In these studies, some animals died due to opportunistic infections, indicating that severe systemic

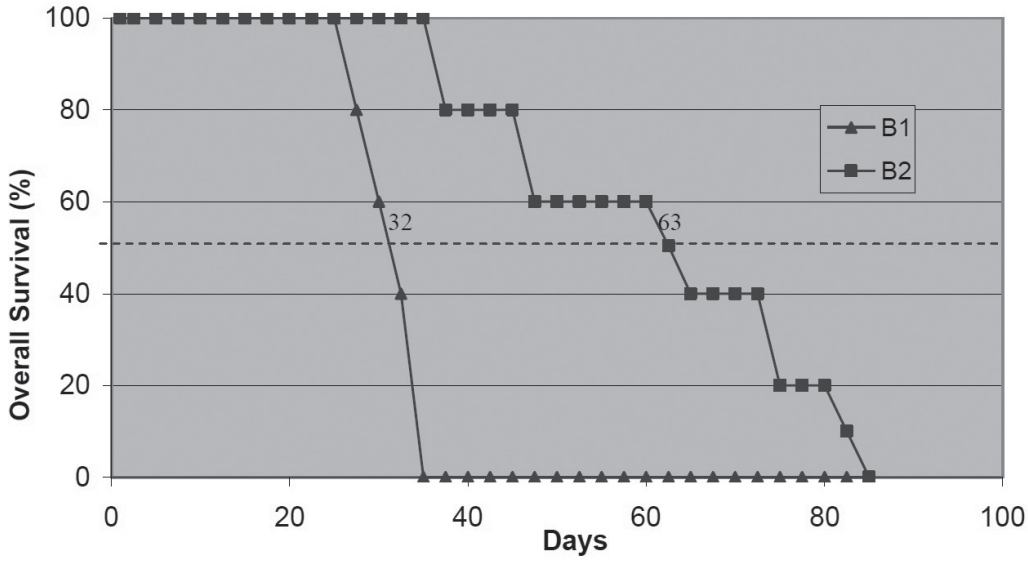

Figure 2: β-met increases the survival of A/J mice bearing TA3-MTX-R tumors. The median duration of survival (indicated by the dotted lines) was 32 days in the group where β-met treatment was discontinued, as compared to 63 days in the group receiving continuous treatment ($p<0.05$, Kaplan-Meier Estimate of Survival).

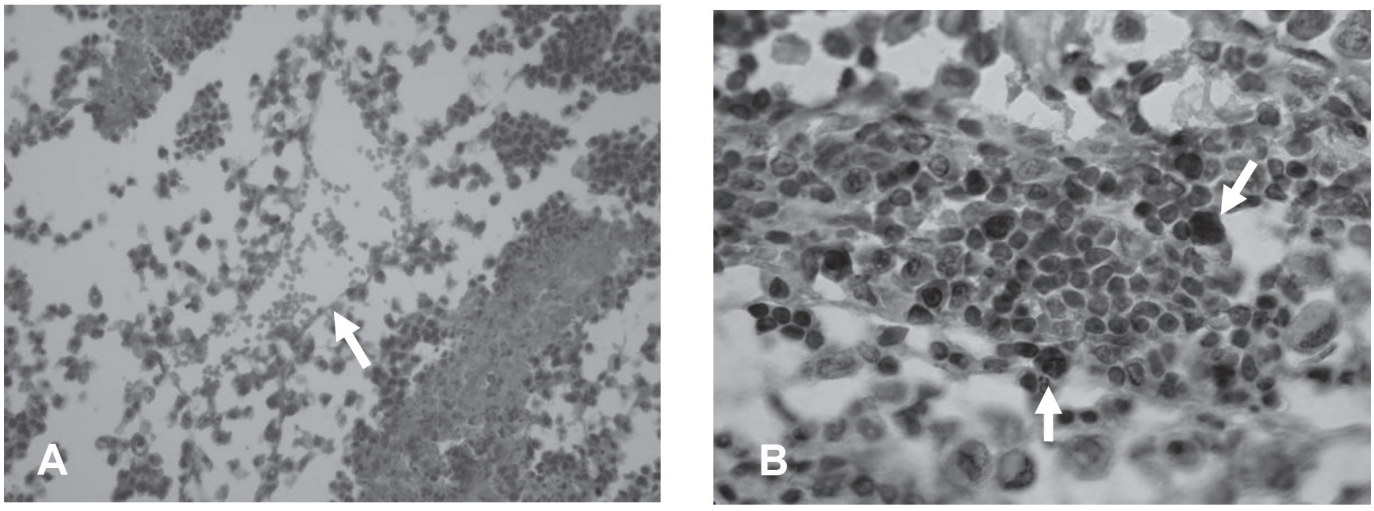

Figure 3: A: Arteta Staining in TA3-MTX-R tumor. The white arrow indicates a blood vessel. This staining was used for blood vessel counting (Arteta, 400x). B: Ki-67 immunodetection in TA3-MTX-R tumor. Ki-67 positive cells are marked in brown and indicated with a white arrow (IHC anti-Ki67, 1000x).

immune suppression can occur (Penhaligon and Camplejohn, 1985). On the other hand, GC regulate the production and activity of a broad variety of inflammatory and angiogenic proteins. These include enzymes responsible for the synthesis of key mediators of inflammation, for the degradation of basal membranes and for the reorganization of extracellular matrix of blood vessels; peptide growth factors; mediators of inflammatory reactions; and cell adhesion molecules (Yang *et al.*, 2002; Lubet *et al.*, 2004; Banciu *et al.*, 2008). These actions are exerted by GC at genomic and nongenomic levels. Additional contributing factors could be the inhibition of phospholipase A_2 transcription produced by stimulus in the production of proteins, such as Lipocortin 1 (Buttgereit, 1998). This decrease in phospholipase A_2 levels produces an inhibition in the cascade of arachidonic acid, thus diminishing the production of some well-known proinflammatory and proangiogenic mediators, such as prostaglandins, tromboxans and leucotriens. Prostaglandins stimulate directly and indirectly the transcription of numerous important proangiogenic factors (Höper, 1997; Cheng, 1998). In fact, one of the main inhibitory actions of GC on tumor inflammation is the downregulation of expression of genes encoding for enzymes involved in synthesis of prostaglandin E_2 (PGE_2), such as cytosolic phospholipase A_2 ($cPLA_2$) and cyclooxygenase 2 (COX-2) (Croxtall *et al.*, 2002; Farooqui *et al.*, 2007). Inhibition of the production of PGE_2, a key mediator of inflammation, leads to a supplementary reduction of growth factors, cell adhesion molecules, and metalloproteinases involved in different steps of tumor angiogenesis (Vassiliou *et al.*, 2004). Remarkably, GC appears to have no reducing effects on the levels of most of the anti-angiogenic and anti-inflammatory proteins, allowing these

proteins to mediate downregulation of genes encoding for pro-angiogenic factors (VEGF, bFGF, MMP_s) (Reichenstein *et al.*, 2004; Banciu *et al.*, 2006, 2008). These effects of GC on the production of angiogenic/inflammatory factors in tumors can cause a shift in the balance between pro- and anti-angiogenic factors in favor of inhibition of inflammation-associated angiogenesis, thus diminishing tumor growth. Our *in vivo* studies indicate that β-met inhibits tumor growth (Figure 1) by virtue of its capacity to interfere with angiogenesis (Table I). This inhibition of tumor angiogenesis may be due to a reduction of intratumor levels of pro-angiogenic factors, as well as to a direct inhibition of endothelial and tumoral cell proliferation.

Our results show that, the smaller tumoral volume in group B1 (Figure 1), could be related with the β-met antiangiogenic and proapoptotic properties.

Interestingly, starting on day 57 post-inoculation, tumor size in group B2 began a rapid progression until the animals died (Figure 1). Perhaps this difference between the two B groups can be explained by tumor drug resistance given by the emergence of more proangiogenic and aggressive clones within the tumoral masses. Hypoxia**,** proangiogenic through different mechanisms (Forsythe *et al.*, 1996; Brown *et al.*, 1997; Carmeliet, 2000), is produced by rapid tumor growth associated with a poor vascularized environment (Griffioen, 2000). This information refutes several studies proposing that an acceptable antiangiogenic therapy for cancer should not elicit medium term resistance (O'Reilly *et al.*, 1994; 1997). It has also been demonstrated that hypoxia in tumors tends to select for a more malignant phenotype, increases mutation rates, increases expression of genes associated with angiogenesis and tumor invasion, and is associated with a more metastatic phenotype of human cancers. Therefore, hypoxia has a key negative role in tumor prognosis, both because it causes resistance to standard therapies and because it promotes a more malignant phenotype (Brown and Wilson, 2004)

Capillary counting in Arteta stained sections, as an indicator of angiogenesis, shows a significant decrease in the number of vessels, thus corroborating the effect of β-met in other models, and correlates with the tumor size evolution (A vs. B1, Figure 1) in our model. Finally, the secondary increase of the tumor aggressiveness in our model resulted in the increase of the tumor size and the number of vessels towards the end of the experience in group B2 (Figure 1). For this reason, this treatment could complement other experimental chemotherapies against multiresistant tumors. As well, it could be used to decrease the tumor size before surgery (in tumors like the one used in this experiment, TA3-MTX-R), or associated with other drugs. As an important perspective, we will experimentally combine these strategies with those recently described by us with regard to Trypanosoma *cruzi* calreticulin important antiangiogenic - antitumor effects. Since the molecular targets of these strategies are most likely different, additive or synergic effects are envisaged. As an important perspective, we will soon experimentally combine these strategies with those recently described by us with regard to *Trypanosoma cruzi* calreticulin important antiangiogenic - antitumor effects (López *et al.*, submitted). Since the molecular targets of these strategies are most likely different, additive or synergic effects are envisaged.

ACKNOWLEDGMENTS

The authors thank the reviewers for their valuable comments on the manuscript. This paper is part of the thesis for the Master Degree in Medical Sciences (Morphology) by Osvaldo Garrido.

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