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# Anticancer Effect of a Spiro-acridine Compound Involves Immunomodulatory and Anti-angiogenic Actions

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**Abstract.** *Background/Aim: Studies with acridine compounds have reported anticancer effects. Herein, we evaluated the toxicity and antitumor effect of the (E)-1'-((4-chlorobenzylidene)amino)-5'-oxo-1',5'-dihydro-10H-spiro[acridine-9,2'-pyrrole]-4'-carbonitrile (AMTAC-06), a promising anticancer spiro-acridine compound. Materials and Methods: The toxicity of AMTAC-06 was evaluated on zebrafish and mice. Antitumor activity was assessed in Ehrlich ascites carcinoma model. Effects on angiogenesis, cytokine levels and cell cycle were also investigated. Results: AMTAC-06 did not induce toxicity on zebrafish and mice ($LD_{50}$ approximately 5000 mg/kg, intraperitoneally). No genotoxicity was observed on micronucleus assay. AMTAC-06 significantly reduced the total viable Ehrlich tumor cells and increased sub-$G_1$ peak, suggesting apoptosis was triggered. Moreover, the compound significantly decreased the density of peritumoral microvessels, indicating an anti-angiogenic action, possibly dependent on the cytokine modulation (TNF-$\alpha$, IL-1$\beta$ and IFN-$\gamma$). No significant toxicological effects were recorded for AMTAC-06 on tumor transplanted animals. Conclusion: AMTAC-06 has low toxicity and a significant antitumor activity.*

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*Key Words:* Spiro-acridine, antitumor activity, anti-angiogenic activity, immunomodulation, zebrafish, Ehrlich ascites carcinoma model.

The mechanisms underlying the transformation of normal cells into malignant cells have been thoroughly studied, aiming to better understand the biology of cancer and develop new treatments. Researchers have described common traits acquired during tumorigenesis, including sustaining proliferative signaling, induction of angiogenesis, deregulation of cell cycle, resistance to cell death and tumor-promoting inflammation (1-3). These hallmarks are important therapeutic targets for new antitumor drug development (3, 4).

Acridines are heterocyclic molecules containing a planar ring, which have shown anti-inflammatory, anticancer, antiparasitic, antiviral, and antimicrobial activities, among others (5). Their antitumor action is based on DNA binding and topoisomerase inhibition (6), which may cause apoptosis and cell cycle arrest (7). Nevertheless, acridine compounds

have high toxicity and drug resistance (5). Recently, a new series, called spiro-acridines, have been obtained by condensation reactions followed by spontaneous cyclization, resulting in rings of five or six members linked directly to acridine C-9 carbon (6, 8). Previous studies showed that the (E)-1'-((4-chlorobenzylidene)amino)-5'-oxo-1',5'-dihydro-10H-spiro[acridine-9,2'-pyrrole]-4'-carbonitrile (AMTAC-06) inhibited topoisomerase IIα, however, no antitumor activity data has been reported yet (8). Herein, we evaluated the toxicity and antitumor effect of AMTAC-06 on *in vivo* models.

## Materials and Methods

*Chemicals*. The spiro-acridine compound (E)-1'-((4-chlorobenzylidene)amino)-5'-oxo-1',5'-dihydro-10H-spiro[acridine-9,2'-pyrrole]-4'-carbonitrile (AMTAC-06) (Figure 1) was synthesized in the Laboratory of Synthesis and Vectorization of Molecules (LSVM) from the State University of Paraíba (UEPB), João Pessoa, Paraíba, Brazil, according to the methodology previously described (8).

*Animals*. Female Swiss albino mice (*Mus musculus*) (28-32 g), were kept under controlled conditions (21±1˚C, 12/12 h light-dark cycle) at the Dr. Thomas George animal facilities (Research Institute in Drugs and Medicines/Federal University of Paraíba, Brazil). Animal handling procedures were approved by the Ethical Committee on the Use of Animals (CEUA)/UFPB, code n˚ 9129090919 (ID 000786).

*Zebrafish embryos*. The zebrafish (*Danio rerio*) embryos were obtained from the Unconventional Organisms Production Unit (UniPOM), from the Federal University of Paraíba (João Pessoa, Brazil). The embryos were obtained using egg traps, which were placed overnight in a tank containing male and female specimens (1:1 ratio), one day before the experimental tests. Eggs were collected 1 h after the beginning of the light cycle and rinsed with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$). Viable fertilized eggs were selected in a stereomicroscope, for use in the acute embryo toxicity test. All the procedures used were previously approved by the CEUA/UFPB, code n˚ 5900310718/2018.

*Acute toxicity test in zebrafish embryos*. Acute toxicity of AMTAC-06 was assessed in the zebrafish model, according to the fish embryo acute toxicity (FET) test, guideline n˚ 236 of the Organisation for Economic Co-operation and Development (OECD), with some modifications (9). Zebrafish embryos with up to 3 h post-fertilization were incubated with AMTAC-06 (7.88-126.2 μM), for 96 h. For each AMTAC-06 concentration tested, 20 fertilized eggs were used on 96-well plate (1 embryo per well), in addition to E3 medium (negative control) and solvent (DMSO 0.5%) groups (n=20 embryos/group). The exposures were performed under static conditions (without renewing of E3 medium or test compound). Embryos were evaluated daily regarding the following endpoints that represent embryos or larvae death: lack of detachment of the tail-bud from the yolk sac, egg coagulation, lack of heartbeat, and absence of somite formation (10). The $LC_{50}$ (median lethal concentration) was estimated from the number of deaths obtained.

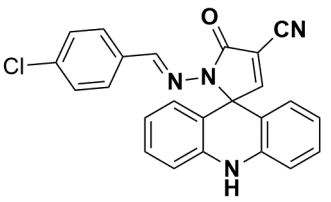


Figure 1. *Spiro-acridine compound AMTAC-06 chemical structure*.

*Acute non-clinical toxicity in mice*. Acute toxicity analysis of mice was performed based on the Guidelines for Testing of Chemicals n˚ 423, OECD (11). Mice (n=3/group) were treated with AMTAC-06 [single dose of 2,000 mg/kg, intraperitoneally (*i.p.*)] or vehicle alone [control group, which received Tween 80 at 12% (v/v) in saline]. The $LD_{50}$ (dose responsible for the death of 50% of the animals) was estimated (12).

*Evaluation of genotoxicity*. Micronucleus assay was performed according to the "Mammalian Erythrocyte Micronucleus Test" guideline n˚ 474, OECD (13). Three groups of mice were tested (n=6 animals/group); in the first group, animals were treated with AMTAC-06 (a single dose of 2,000 mg/kg, *i.p.*), the second group received the standard drug, cyclophosphamide (50 mg/kg, *i.p.*), and in the third group, animals were injected with the vehicle alone [Tween 80 at 12% (v/v) in saline]. After 48 h, peripheral blood was collected from retro-orbital plexus and used to prepare blood smears (three per animal). The number of micronucleated erythrocytes was estimated by counting at least 2,000 cells (14).

*Antitumor activity on Ehrlich ascitic carcinoma model*. To evaluate the *in vivo* antitumor activity of AMTAC-06, mice were injected (*i.p.*) with $4\times10^6$ cells/ml of Ehrlich cells (0.5 ml/animal) to induce cancer. Ehrlich cells were kindly donated by Pharmacology and Toxicology Division, CPQBA, UNICAMP, São Paulo, Brazil. After 24 h, 6 groups of animals (n=8/group) were created, each one treated with different doses of AMTAC-06 (3.12, 6.25, 12.5 or 25 mg/kg), vehicle alone [Tween 80 at 12% (v/v) in saline], or the standard drug 5-fluorouracil (5-FU; 25 mg/kg). After 7 days of treatment, blood samples were obtained, and the animals were euthanized to collect ascitic fluid from the peritoneal cavity. Then, tumor volume (ml) was measured, while the total number of viable cancer cells ($\times10^7$) was estimated in a hemocytometer using trypan blue assay (10).

*Analysis of cell cycle*. Ascitic fluid cells ($1\times10^6$) of each animal from the following groups: tumor control; 12.5 mg/kg AMTAC-06; 25 mg/kg 5-FU were harvested, fixed by dripping the sample into 70% cold alcohol and kept at –20˚C until analysis. Cells were pelleted (400 × *g*, 10 min, 4˚C), washed twice in PBS, and then incubated with RNase (0.1 mg/ml) to eliminate RNA traces and propidium iodide (PI) to stain the DNA (0.05 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) (37˚C, 30 min, in the dark) (15). The samples were analysed using a FACSCanto™ II flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). At least 10,000 events per sample were recorded. The DNA content was estimated based on the amount of PI fluorescence detected on the orange-red light 564 to 606 nm detector. The proportion of cells in each cell

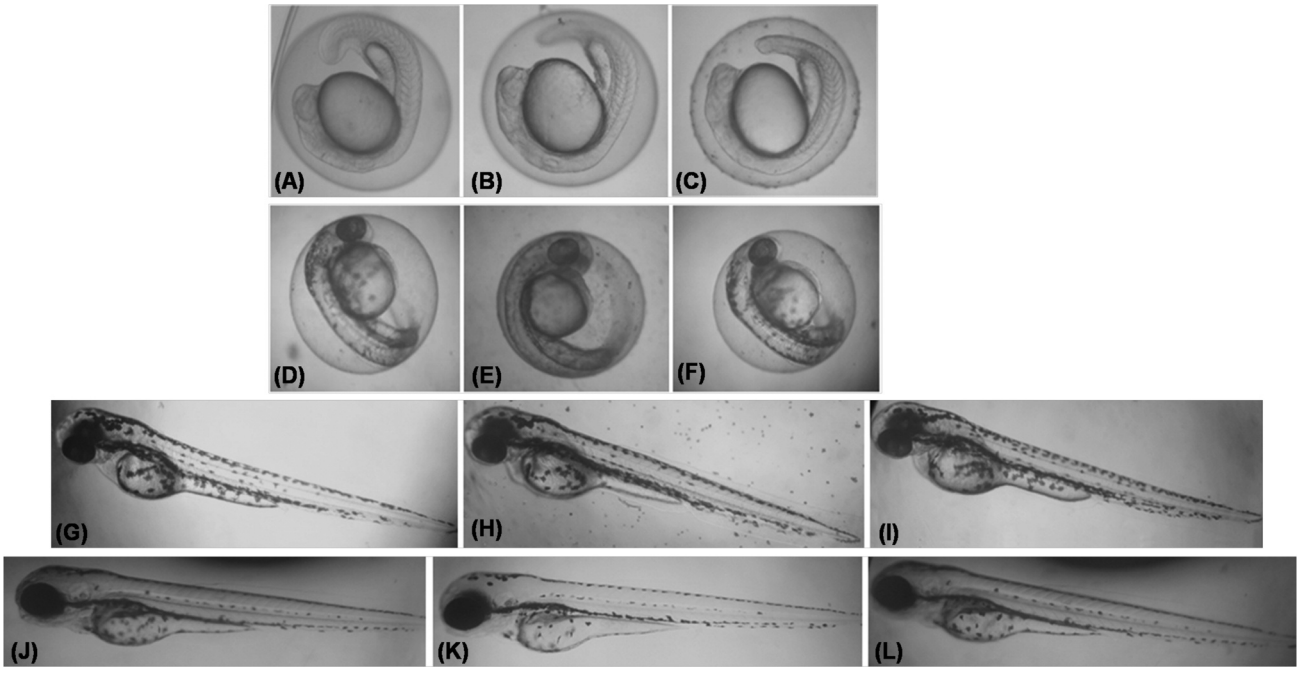


Figure 2. *Representative images of zebrafish embryos and larvae after exposure to AMTAC-06 (126.2 μM) or DMSO (0.5%) and E3 medium controls for 24 (A-C), 48 (D-F), 72 (G-I) and 96 h (J-L). E3 medium: (A, D, G, J); 126.2 μM AMTAC-06: (B, E, H, K); DMSO 0.5%: (C, F, I, L).*

cycle phase was estimated. Flowing software version 2.5.1 (Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland) was used to analyze the data.

*Evaluation of angiogenesis*. To evaluate the anti-angiogenic effect, animal's peritoneum from the control, 12.5 mg/kg AMTAC-06 and 25 mg/kg 5-FU groups was incised and images of peritoneum cavities lining were captured. The microvessel density was calculated as the blood vessel area per field divided by total area, using AvSoft Bioview Spectra 4.0.1® software (AVSoft, São Paulo, Brazil) (12).

*Quantification of cytokine levels*. The ascitic fluid collected from the peritoneal cavity of the control [Tween 80 at 12% (v/v) in saline] and treated animals (12.5 mg/kg AMTAC-06 and 25 mg/kg 5-FU) was used to quantify the levels of the cytokines IFN-γ, IL-1β, IL-4, IL-12 and TNF-α using ELISA according to the manufacturer's protocol (Bioscience, Inc. Science Center Drive, San Diego, CA, USA). Absorbance was measured at 450 nm on an ELISA plate reader (Sinergy HT microplate reader, BioTek Instruments, Winooski, VT, USA) (15).

*Toxicity evaluation on Ehrlich tumor transplanted mice*. General parameters, including water/food consumption and body weight (at the beginning and end of the treatment), were recorded daily, for the 7 days of treatment on Ehrlich transplanted mice. Biochemical analyzes (levels of urea and creatinine and activities of the enzymes alanine aminotransferase - ALT and aspartate aminotransferase - AST) were performed from serum samples, while the erythrogram and leukogram was determined using heparinized whole blood (14).

*Statistical analysis*. One-way ANOVA was used to verify differences among groups, followed by Tukey test. Differences were considered significant at $p<0.05$. Results are expressed in mean±standard error (SEM).

## Results

*Acute toxicity test in zebrafish embryos*. AMTAC-06 induced no lethal effects or morphological changes during embryonic and larval development (Figure 2). Thus, the $LC_{50}$ (median lethal concentration) value was higher than 126.2 μM after 96 h of exposure.

*Acute non-clinical toxicity in mice*. AMTAC-06 (2,000 mg/kg) did not cause mice death. The $LD_{50}$ (lethal dose 50%) value estimated was approximately 5,000 mg/kg.

*Evaluation of genotoxicity*. AMTAC-06 (2,000 mg/kg) induced no changes in the number of micronucleated erythrocytes (9.14±0.91), in relation to control (9.50±0.76). However, cyclophosphamide increased the number of micronucleated erythrocytes (18.40±0.52; $p<0.05$, compared to the control).

*Antitumor activity on Ehrlich ascitic carcinoma model*. There was a significant decrease in tumor volume after AMTAC-06 treatment (0.32±0.03 ml; $p<0.0001$, for 25 mg/kg), in relation to control (8.04±0.60 ml) (Figure 3A). Additionally, a

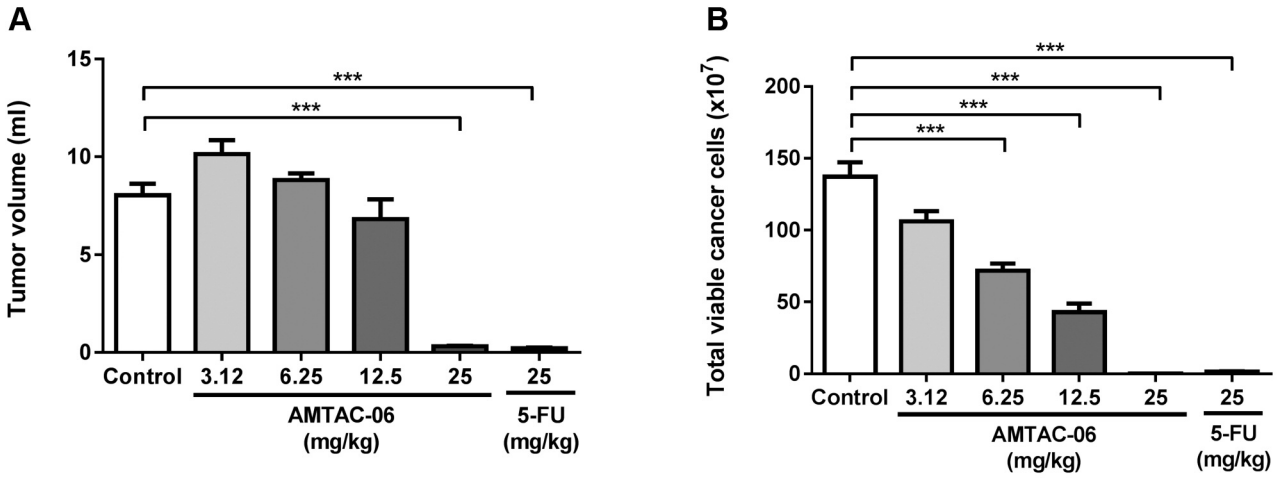


Figure 3. *Effects of 7-day treatment with AMTAC-06 (3.12, 6.25, 12.5 or 25 mg/kg) or 5-fluorouracil (5-FU; 25 mg/kg) on tumor volume (A) and total viable cancer cells (B) in mice transplanted with Ehrlich ascites carcinoma. Data are presented as mean±SEM of eight animals. ***p<0.0001 compared to the control group.*

significant reduction in total viable cancer cells was also observed after treatment with AMTAC-06 (6.25 mg/kg: 71.8±4.92×$10^7$ cells, 12.5 mg/kg: 43.0±5.96×$10^7$ cells, 25 mg/kg: 0.20±0.02×$10^7$ cells; $p$<0.0001 for all), compared to the control (137.2±9.98×$10^7$ cells) (Figure 3B). Treatment with 5-FU also significantly reduced both evaluated parameters ($p$<0.0001), compared to the control cells (Figure 3).

*Analysis of cell cycle*. AMTAC-06 (12.5 mg/kg) induced a significant increase in the sub-$G_1$ peak (51.36±3.69%; $p$<0.0001), while reduced cells in the $G_0/G_1$ phase (25.27±1.49%; $p$<0.0001), compared to the control (24.78±2.54% and 43.89±2.07%, respectively). The 5-FU treatment caused an significant increase in the sub-$G_1$ peak ($p$<0.0001) and a reduction on percentage of cells in the G0/G1 ($p$<0.0001), S ($p$<0.0001) and $G_2$/M ($p$<0.001), compare to the controls (Figure 4).

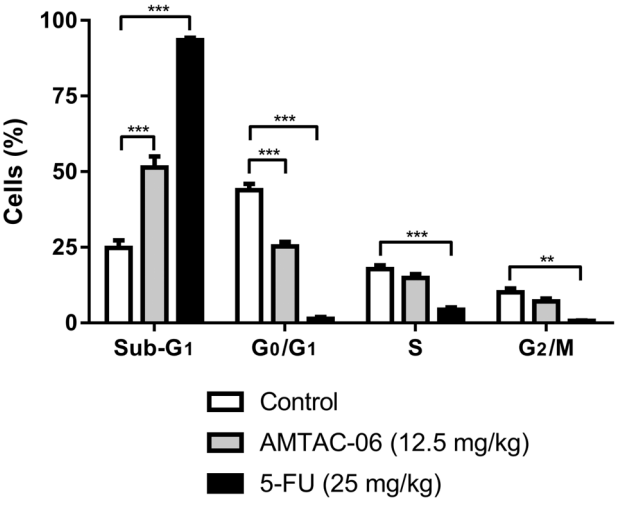


Figure 4. *Cell cycle analysis of Ehrlich ascites carcinoma cells after 7-day treatment with AMTAC-06 (12.5 mg/kg) or 5-FU (25 mg/kg). The proportion of cells (%) in each cell cycle phase was estimated. Data are presented as mean±SEM of eight animals. **p<0.001 and ***p<0.0001 compared to the control group.*

*Evaluation of angiogenesis*. There was a significant reduction in tumor microvessel density (48.67±6.39%; $p$<0.001) for AMTAC-06 (12.5 mg/kg) treatment, in relation to control (100±8.27%), which was also observed for 5-FU treatment ($p$<0.0001) (Figure 5).

*Quantification of cytokine levels*. AMTAC-06 (12.5 mg/kg) induced a significant increase in TNF-α (162.20±29.36 pg/ml; $p$<0.05) and IL-1β (15.74±4.12 pg/ml; $p$<0.05) levels, in relation to control (46.59±9.82 pg/ml and 4.74±1.0 pg/ml, respectively). In contrast, there was a decrease in IFN-γ levels (511.50±16.12 pg/ml; $p$<0.0001), compared to the control (3,206±254.1 pg/ml). No significant effects were observed on IL-4 and IL-12 levels. Regarding 5-FU treatment, only a significant decrease in IL-12 levels ($p$<0.05, compared to the contol) were recorded (Figure 6).

*Toxicity on Ehrlich tumor transplanted mice*. No significant changes in body weights, consumption of water and feed (Table I), as well as biochemical and hematological parameters (Table II) were recorded in animals.

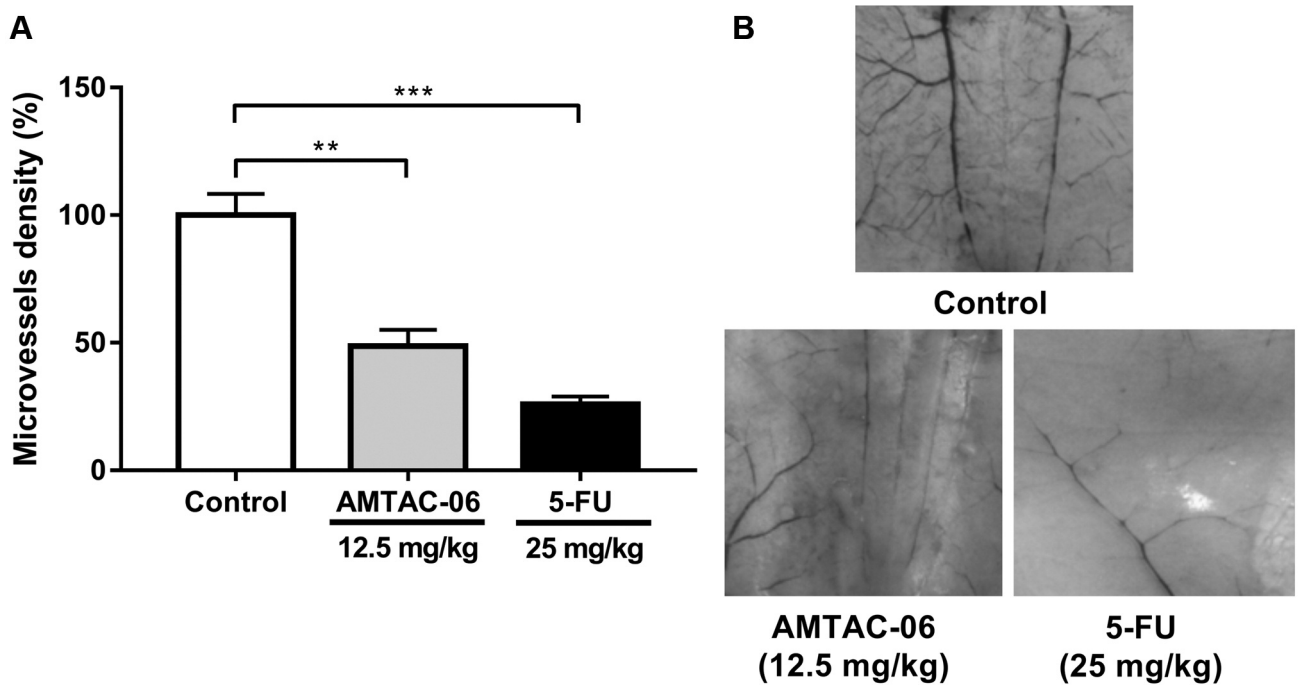


Figure 5. *Microvessel density (%) of mice transplanted with Ehrlich ascites carcinoma and treated for seven days with AMTAC-06 (12.5 mg/kg) or 5-FU (25 mg/kg). Microvessel density (A) is expressed as the blood vessel area peer field divided by total area. Representative images of the peritoneal cavity lining from animals treated with AMTAC-06, 5-FU, or control are shown (B); a decrease in the neovascularized areas was observed after treatments. Data are presented as mean±SEM of eight animals. **p<0.001 and ***p<0.0001 compared to the control group.*

## Discussion

Spiro-acridines are a new class of acridine compounds. Considering that AMTAC-06 inhibits topoisomerases (8), we investigated the toxicity and antitumor potential of this spiro-acridine compound.

The zebrafish (*Danio rerio*) is an emerging and alternative experimental model for assessing the toxicity of new drugs (16) since their toxicological responses are very similar to humans (17, 18). Herein, the results for AMTAC-06 in this model showed low toxicity. Until now, there are no literature reports about toxicity of acridine compounds on zebrafish model.

Acute non-clinical toxicity in mice has been evaluated to establish safe doses for *in vivo* pharmacological assays (12). In our study, the high $LD_{50}$ value obtained for AMTAC-06 (5,000 mg/kg) suggests low toxicity in mice. In general, a substance is considered a good candidate for other studies if its $LD_{50}$ is three times higher than the minimum effective dose (19). Regarding acridine compounds, both low (14, 15, 20) and high toxicity (21, 22) have been reported in literature.

Genotoxicity is one of the side effects of many antineoplastic drugs on non-tumor cells (23). In this study, AMTAC-06 did not increase the number of micronucleated erythrocytes, suggesting no genotoxicity, which corroborates the literature for other acridine analogs (14). In contrast, the best known acridine compound used in the clinic, called Amsacrine, has been shown to cause genotoxicity in mouse bone marrow cells (24).

Ehrlich ascites carcinoma is a murine mammary adenocarcinoma widely used for test promising antitumor drugs (10, 12, 14, 15). AMTAC-06 showed antitumor effect, although reduction on tumor volume was observed only at 25 mg/kg, reversing the tumor-induced peritoneal ascites. Considering that at 25 mg/kg no tumor cells were obtained in the ascitic fluid for the performance of subsequent assays, 12.5 mg/kg dose was selected for the study of AMTAC-06 mechanisms of action.

The cell cycle regulates cell proliferation, constituting an important target of antitumor drugs, including acridine compounds (14, 25, 26). In our study, AMTAC-06 increased the sub-$G_1$ peak indicating apoptosis, according to literature data (27). Increase in the sub-$G_1$ peak followed by apoptosis for acridine compounds has been previously shown (15, 28, 29). Our results suggest that the antitumor effect of AMTAC-06 may involve DNA damage and apoptosis induction.

Angiogenesis plays an essential role in sustaining tumor proliferation and metastasis (30, 31). Therefore, anti-angiogenic therapy represents a promising strategy for cancer treatment (32). The reduction of the peritumoral microvessels

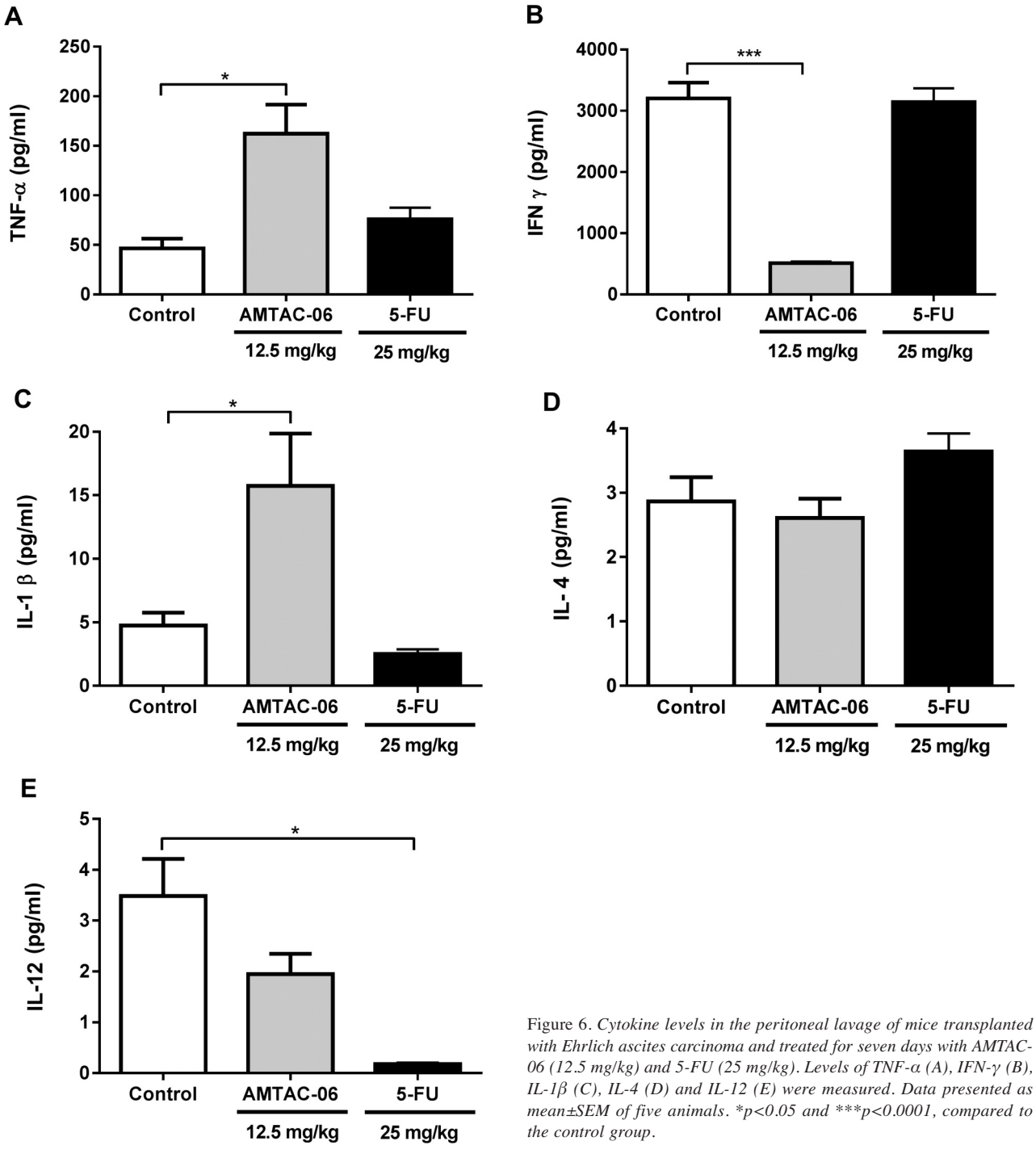


Figure 6. *Cytokine levels in the peritoneal lavage of mice transplanted with Ehrlich ascites carcinoma and treated for seven days with AMTAC-06 (12.5 mg/kg) and 5-FU (25 mg/kg). Levels of TNF-α (A), IFN-γ (B), IL-1β (C), IL-4 (D) and IL-12 (E) were measured. Data presented as mean±SEM of five animals. *p<0.05 and ***p<0.0001, compared to the control group.*

density by AMTAC-06 suggests anti-angiogenic action, corroborating literature for acridine (14, 33) and spiro-acridine compounds (15).

Cytokines are components of the tumor microenvironment involved in different stages of tumor progression, including angiogenesis modulation (31). We therefore evaluated the modulation of cytokines by AMTAC-06. The dual and opposing role of several cytokines in tumor response are currently discussed in the literature (34-36). TNF-α and IL-1β are pleiotropic cytokines involved in inflammation and

Table I. *Effects of 7-day treatment with AMTAC-06 or 5-fluorouracil (5-FU) on body weight and water and feed consumption of mice transplanted with Ehrlich ascites carcinoma.*

| Groups | Dose (mg/kg) | Water intake (ml) | Food intake (g) | Starting weight (g) | Final weight (g) |
|---|---|---|---|---|---|
| Control | - | 40.0±3.65 | 33.83±5.87 | 29.38±0.62 | 26.50±1.48 |
| AMTAC-06 | 12.5 | 45.0±5.16* | 42.66±5.49* | 28.60±0.40* | 24.80±0.80* |
| 5-FU | 25 | 29.0±2.96 | 21.80±1.51 | 30.67±1.0 | 28.67±0.33 |

Data presented as mean±SEM of eight animals analyzed by analysis of variance (ANOVA) followed by Tukey test. *$p<0.05$ compared to the 5-FU group.

Table II. *Biochemical and hematological parameters of peripheral blood of mice transplanted with Ehrlich ascites carcinoma and treated for seven days with AMTAC-06 and 5-fluorouracil (5-FU).*

| Parameters | Healthy group* | Control** | AMTAC-06 (12.5 mg/kg) | 5-FU (25 mg/kg) |
|---|---|---|---|---|
| Erythrocytes ($10^6$/mm$^3$) | 6.57±0.21 | 6.91±0.75 | 6.60±0.37 | 5.17±0.29 |
| Hemoglobin (g/dl) | 12.96±0.39 | 12.54±1.03 | 12.18±0.55 | 11.43±0.50 |
| Hematocrit (%) | 35.81±1.24 | 34.29±3.85 | 30.00±2.26 | 32.75±1.25 |
| MCV (fm$^3$) | 54.26±1.24$^b$ | 49.89±2.52 | 44.99±1.82$^b$ | 63.68±2.52$^a$ |
| MCH (pg) | 19.65±0.33$^b$ | 18.46±0.57 | 18.54±0.34$^b$ | 21.98±0.35$^a$ |
| MCHC (g/dl) | 19.60±1.26$^{a,b}$ | 37.63±2.30 | 41.55±1.36 | 37.12±2.60 |
| Total leukocytes ($10^3$/mm$^3$) | 11.58±0.82$^b$ | 10.13±1.60 | 8.01±1.04$^b$ | 3.02±0.06$^a$ |
| Lymphocytes (%) | 81.38±3.43 | 78.43±3.31 | 78.57±3.60 | 88.00±1.09 |
| Neutrophils (%) | 2.00±0.29$^{a,b}$ | 13.80±1.77 | 16.33±1.64$^b$ | 9.40±0.87 |
| Monocytes (%) | 5.42±0.64$^{a,b}$ | 10.33±0.66 | 8.66±1.08$^b$ | 1.00±0.31$^a$ |
| Eosinophils (%) | 2.00±0.30 | 2.60±0.24 | 2.12±0.29 | 1.60±0.24 |
| AST (U/l) | 101.0±7.27$^b$ | 120.5±17.03 | 108.8±5.54$^b$ | 162.60±2.27$^a$ |
| ALT (U/l) | 113.6±7.97$^b$ | 106.3±14.20 | 80.50±3.40$^b$ | 176.6±3.33$^a$ |
| Urea (mg/dl) | 41.60±4.11 | 45.56±6.63 | 37.25±6.79 | 39.75±2.45 |
| Creatinine (mg/dl) | 0.92±0.04 | 1.03±0.15 | 0.84±0.10 | 0.82±0.06 |

MCV: Mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; AST: aspartate aminotransferase; ALT: alanine aminotransferase. Data presented as mean±SEM of eight animals analyzed by analysis of variance (ANOVA) followed by Tukey test. $^a p<0.05$ compared to the control group; $^b p<0.05$ compared to the 5-FU group. *Animals without Ehrlich tumor (healthy), submitted to the same experimental conditions. **Animals transplanted with the Ehrlich tumor and treated with the vehicle only.

Th1 response profile, activating macrophages and neutrophils that induce cytotoxic mechanisms against cancer cells (34, 37). Furthermore, there is evidence of the anti-angiogenic effect of these cytokines (38, 39). Regarding IFN-γ, recent data show its pro-tumoral activity (36, 40). Therefore, we suggest that the antitumor and anti-angiogenic effect induced by AMTAC-06 may be dependent on the modulation of these cytokine levels. Limited data regarding immunomodulation by acridine compounds have been described (15, 41).

Toxicity data for AMTAC-06 on Ehrlich tumor transplanted mice showed no general toxicity, considering water and feed consumption as well as weight evolution. Urea and creatinine serum levels were used to investigate renal toxicity, and AST and ALT were quantified for hepatic evaluation. No changes were observed on these biochemical parameters, suggesting no renal or liver toxicity. The absence of renal toxicity of AMTAC-06 corroborates data in the literature for other acridine derivatives (14, 21), nevertheless, several studies have reported that some analogs induce liver toxicity (14, 21, 22). Unlike most anticancer agents, AMTAC-06 did not change hematological parameters. However, another acridinic compound has been shown to induce few changes in the hematological parameters of Ehrlich tumor transplanted animals (14).

## Conclusion

AMTAC-06 has low toxicity and exerts antitumor activity by inducing cell cycle alterations, immunomodulatory effects on cytokine profiles, and anti-angiogenic actions. This study provides additional evidence for the anticancer potential of acridine compounds, especially spiro-acridines, encouraging the discovery of new candidates for antitumor drugs.

## Conflicts of Interest

The Authors declare no conflicts of interest.

## Authors' Contributions

Ricardo Olímpio de Moura, Rawny Galdino Gouveia and Jamire Muriel da Silva synthesized and provided the AMTAC-06. Sâmia Sousa Duarte and Marianna Vieira Sobral wrote the paper. All other Authors were responsible for substantial contributions to the conception, design, and execution of the experiments as well as data analysis.

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