

Therapeutic efficacy of metronomic chemotherapy with cyclophosphamide and doxorubicin on murine mammary adenocarcinomas

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Background: Metronomic chemotherapy (MCT) refers to the chronic and equally spaced administration of low doses of different chemotherapy drugs, without extended rest periods. Herein, we investigated the therapeutic efficacy of metronomic cyclophosphamide (Cy) combined with doxorubicin (Dox) in two mouse mammary adenocarcinoma models.

Materials and methods: Mice were s.c. challenged with M-234p or M-406 mammary tumors, and when the tumors reached ~150 mm³, they were treated with: (I) no treatment (controls); (II) Cy in the drinking water (30 mg/kg body weight/day); (III) Dox (0.5 mg/kg body weight i.p. three times/week); (IV) treated as (II) + (III). Mice challenged i.v. with M-234p or M-406 tumor cells received, on day 3, the same treatments.

Results: We found that MCT with Cy plus Dox inhibited tumor growth, decreased lung metastases, and increased the median survival time, while having low toxic effect. Combined MCT was more effective than each monotherapy causing decrease in VEGF serum concentration and tumor proliferation rate plus increase in tumor apoptosis.

Conclusion(s): The therapeutic benefits of combined MCT with Cy and Dox on mammary adenocarcinomas together with its low toxic effect profile suggest the possibility of future translation into the clinic.

Key words: angiogenesis, cyclophosphamide, doxorubicin, mammary adenocarcinomas, metronomic chemotherapy, toxic effect

Introduction

Metronomic chemotherapy (MCT) involves the chronic administration of doses of chemotherapy drugs that are below the maximum tolerated dose, at frequent and regular intervals, without extended rest periods [1]. It aims to achieve a balance between efficacy in tumor killing and lack of toxic effect. The inhibition of angiogenesis would explain its therapeutic effect [2, 3].

We have demonstrated the antitumor efficacy of MCT with cyclophosphamide (Cy) as a single drug [4] and in combination with celecoxib on mice mammary adenocarcinomas (MA) [5]. Considering the high incidence of

mammary tumors in humans, in this study, we analyze the therapeutic efficacy, toxic effect, and mechanism/s of action of MCT combining Cy and doxorubicin (Dox), in mouse MA tumor-models.

Materials and methods

Animals

Inbred BALB/c and C57BL/6 female mice were obtained from our breeding facilities. Animals were fed with commercial chow and water *ad libitum* and maintained in a 12-h light/dark cycle and were treated in accordance to the Canadian Council on Animal Care guidelines. Tumor-bearing mice were euthanized by CO₂ exposure.

Drugs

cyclophosphamide. It was dissolved in sterile distilled water at a concentration of 20 mg/ml and diluted in the drinking water to reach 0.12 mg/ml. Drinking water was replaced every other day and the mice's daily Cy intake/kg body weight (BW) was calculated.

doxorubicin. It was dissolved in sterile saline immediately before its intraperitoneal injection.

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tumors

M-234p. It is a moderately differentiated type 'B' MA [7]. It spontaneously arose in a BALB/c female mouse, and it is maintained *in vivo* by serial subcutaneous passages in syngeneic mice, with 100% of incidence.

M-406. It is a type 'B' MA, which appeared spontaneously in an inbred CBI female mouse. It is maintained *in vivo* by serial intraperitoneal passages in syngeneic mice, with 100% of incidence.

experimental models

antitumor effect. Adult BALB/c or CBI mice were implanted s.c. with $\cong 1 \text{ mm}^3$ M-234p or M-406 tumor fragments, respectively. Five (M-234p) or eight (M-406) days later, when tumors reached $\cong 150 \text{ mm}^3$, animals ($n = 5\text{--}8/\text{group}$) were distributed and treated as follows: *Control*: no treatment; *Cy*: in drinking water ($\cong 30 \text{ mg/kg BW/day}$); *Dox*: 0.5 mg/kg BW, i.p. three times/week; *Cy + Dox*: *Cy* and *Dox* treatments combined. Tumors were measured and tumor volumes calculated. Animals were weighed twice/week, and blood samples were obtained on day 0 and days 24 (M-234p) or 25 (M-406) for white blood cell count, T_{reg} s, and VEGF determinations. When the first animal reached the largest ethically permitted tumor volume (LPV), animals belonging to the four groups were euthanized, and tumors were excised and processed for histology and immunohistochemistry, as described below. For survival studies, in a duplicate experiment, animals were euthanized when each one reached LPV.

antimetastatic effect. Adult BALB/c and CBI mice were injected intravenously with 5×10^5 M-234p cells or 2×10^5 M-406 cells in 0.1 ml saline. On day 3, animals (M-234p: $n = 7\text{--}8/\text{group}$; M-406: $n = 5\text{--}6/\text{group}$) were treated as indicated above. The animals were controlled daily and weighed twice/week. All the mice were euthanized by the time the first mouse showed signs of metastatic illness. Lungs were excised, weighed, and fixed and the number and size of metastatic foci determined. The effect of the different treatments on survival was assessed in a duplicate experiment in which each animal was killed when it showed signs of metastatic illness.

VEGF serum concentration

Serum VEGF was quantified with Quantikine® ELISA kit (R & D Systems Inc, Minneapolis, MN) and carried out in duplicates.

histological and immunohistochemical studies

Tumors were excised on days 31 (M-234p) and 26 (M-406), fixed in 10% buffered formalin and paraffin-embedded. About 5- μm -thickness sections were used for immunohistochemical studies or stained with hematoxylin-eosin.

tumor microvascular density and area. They were determined using immunostaining for CD31 endothelial marker (eBioscience). The number of CD31⁺ blood vessels/field was calculated in three hot-spot areas at 400 \times . For microvascular area (MVA) (vessel wall + lumen area) quantification ImageJ program (U. S. NIH, Bethesda, MD) was utilized.

Ki67 proliferation marker. Tumor sections were incubated with anti-Ki-67 antibody (Abcam, Cambridge, MA). The percentage of Ki67⁺ cells was determined in 30 fields (1000 \times) utilizing the following score: 0 = 0%, 2 < 20%, 4 = 20–40%, 6 = 40–60%, 8 > 60%.

Apoptosis. Tumor sections were immunostained by the TUNEL method (ApopTag® In Situ Apoptosis Detection Kit, Millipore, MA). Apoptotic cells were counted in 30 fields (1000 \times).

T_{reg} -cell quantification

Circulating natural T_{reg} cells (CD4⁺CD25⁺Foxp3⁺) were determined by flow cytometry using the Mouse regulatory T cell staining kit (eBioscience, San Diego CA). Cells were analyzed in a Coulter Epics XL (Coulter Corp., Miami, FL) cytometer. Acquired data were analyzed with WinMDI 2.8 data analysis software (Scripps Research Institute, La Jolla, CA).

statistical analysis

ANOVA and Tukey–Kramer multiple comparison tests, Kruskal–Wallis and Dunn's post test, and log-rank test were used to examine the differences between groups with GraphPad Prism® version 3.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

results

antitumor effect

tumor growth inhibition. M-234p in *Cy* and *Cy + Dox* groups showed smaller volumes than that in *Control* group ($P < 0.05$) on day 28, just before almost all the animals in this group were killed (Figure 1A). Tumor volume in *Cy + Dox* group varied from lower to higher values than those of the *Cy* group, ending (day 68) with a volume significantly smaller than that in *Cy* group.

M-406 showed that tumor volume in *Cy* or *Cy + Dox* groups were, on day 23, lower ($P < 0.01$) compared with *Control* and *Dox* groups (Figure 1B). On day 50 the tumor volume in *Cy + Dox* group was significantly lower than that observed in the *Cy* group ($P < 0.05$). Interestingly, one of six animals in *Cy + Dox* group showed a complete tumor regression which, despite of the withdrawal of treatment, lasted until the end of the experiment (day 120).

survival. M-234p- and M-406-bearing mice that received MCT with *Cy + Dox* showed a longest survival rate ($P < 0.05$, $P < 0.01$, respectively) (Figure 1C).

In the M-406 tumor-model (day 54), all the animals in *Cy + Dox* group were alive, while 100% of those belonging to *Dox*, *Control*, and *Cy* groups were dead on days 35, 37, and 54, respectively (Figure 1D).

antimetastatic effect

metastasis inhibition. M-234p showed fewer lung metastatic foci in *Cy* ($P < 0.001$) or *Cy + Dox* ($P < 0.05$) than in *Control* group (Figure 2A). M-406 developed less metastasis in *Cy + Dox* than in *Control* group ($P < 0.05$) (Figure 2B).

The metastasis diameter in M-234p-challenged animals in *Cy + Dox* group was lower than *Control* ($P < 0.001$) and *Cy* and *Dox* ($P < 0.01$) groups (Figure 2C). In the M-406 tumor model, all the treated groups showed lower values, although nonstatistically different, than in *Control* (Figure 2D). The total metastatic burden for the three treated groups was significantly lower than in *Control* group in both tumor models (Figure 2E and F).

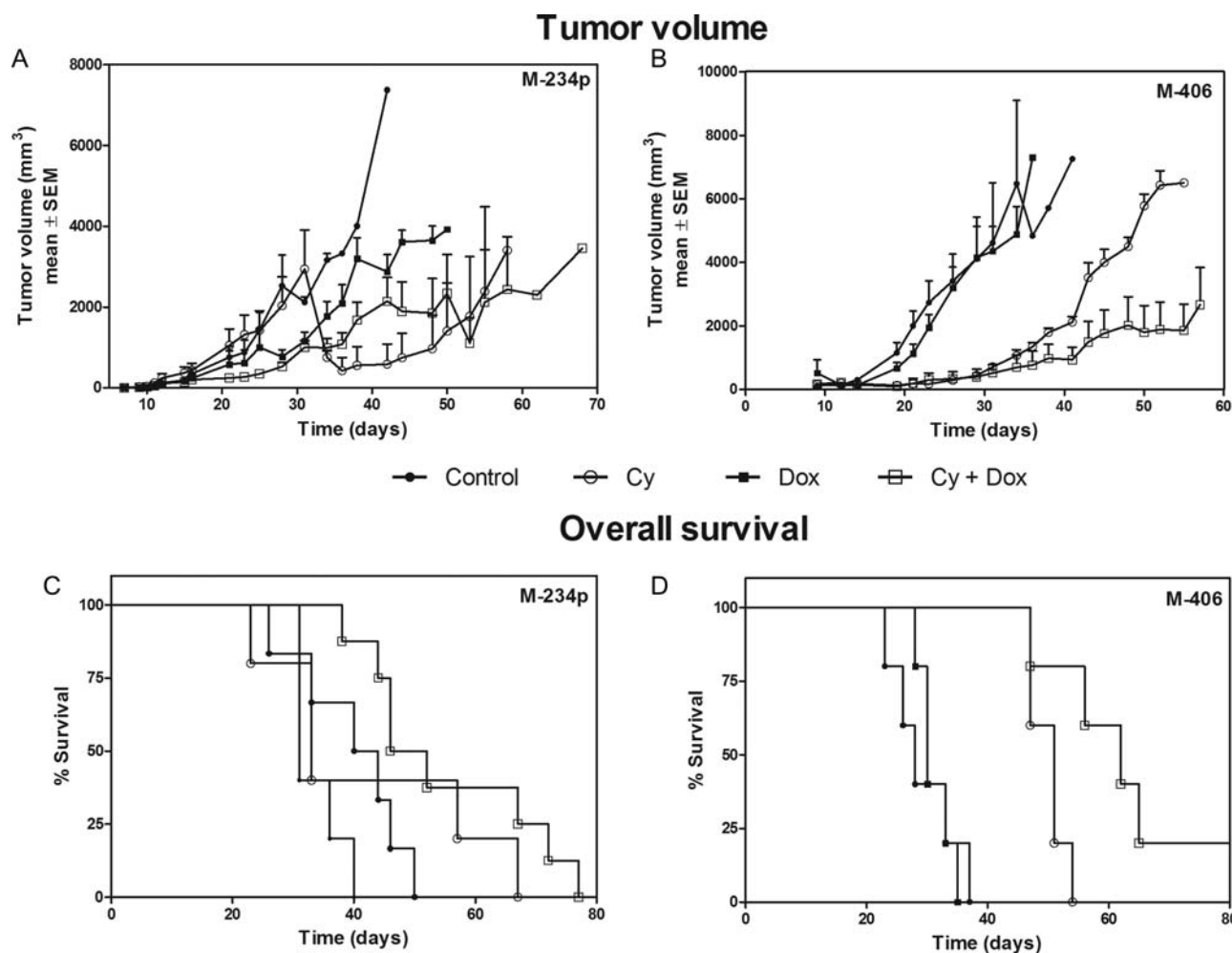


Figure 1. Tumor growth and overall survival: tumor growth assessment: data for each time-point in mm^3 are mean \pm SEM. (A) Day 28: Control versus Cy and versus Cy + Dox ($P < 0.05$); day 31: Cy versus Dox and versus Cy + Dox ($P < 0.05$); (B) day 31: Control versus Cy; Dox versus Cy + Dox ($P < 0.05$), one-way ANOVA/Tukey. Day 50: Cy versus Cy + Dox ($P < 0.05$) *t*-test. Overall survival: (C) ($P < 0.05$); (D) ($P < 0.01$), Log-rank test.

survival. Animals in Cy + Dox groups lived longer than those in the other groups for both tumors ($P < 0.0001$) (Figure 2G and H).

evaluation of toxic effect

Treated animals, monitored for their motor activity, fur quality, food intake, response to stimuli and breathing, showed normal characteristics throughout the experiment and did not experience weight loss in any of the tumor-models (Supplementary Material, S1A and S1B, available at *Annals of Oncology* online).

No decrease in white blood cells counts was found in either experimental group (Supplementary Material, S1C and S1D, available at *Annals of Oncology* online). The experimental metastasis assays showed a similar lack of toxic effect.

VEGF serum concentration

VEGF serum concentration in animals bearing. M-234p tumors, which had received either monotherapy or the combined treatment, were lower than those in Control group on day 24 ($P < 0.001$) (Figure 3A). In M-406-bearing animals,

those belonging to Dox and Cy + Dox groups showed, on day 25, lower values than in Controls ($P < 0.05$) (Figure 3B).

histological and immunohistochemical studies

tumor microvascular density and area. Microvascular density (MVD) and MVA were determined in tumors of animals belonging to groups that showed the highest (Control) and lowest (Cy + Dox) VEGF levels without showing statistical differences for either parameter (Supplementary Material, S.2, available at *Annals of Oncology* online).

Ki67 expression. In M-234p tumor model, all the treatments diminished Ki67 tumor expression, although only Cy + Dox differed from Control group ($P < 0.05$) (Figure 4A). Cy + Dox group showed the lowest values in M-406 tumors, although they did not differ from the other groups (Figure 4B). Photomicrographs of M-234p and M-406 tumors in Control and Cy + Dox groups are shown.

apoptosis. In both tumor models, the Cy + Dox group showed higher number of TUNEL⁺ cells than the Control group

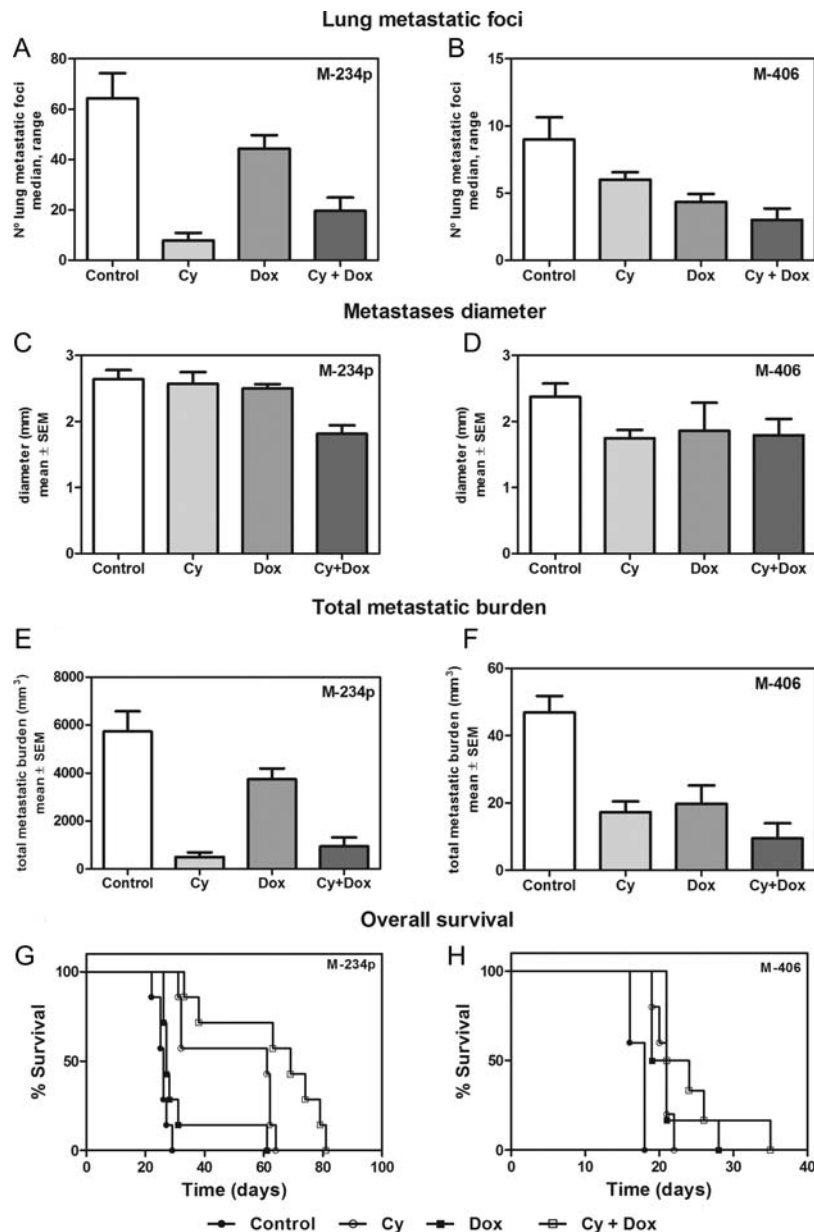


Figure 2. Antimetastatic effect: Number of lung metastatic foci (median, range): (A) *Control* versus *Cy + Dox* ($P < 0.05$), versus *Cy* ($P < 0.001$); (B) *Control* versus *Cy + Dox* ($P < 0.05$), Kruskal–Wallis/Dunn’s. Metastases diameter (mm; mean \pm SEM): (C) *Cy + Dox* versus *Control* ($P < 0.001$) versus *Cy* and *Dox* ($P < 0.01$); (D) NS, one-way ANOVA/Tukey. Total metastatic burden (mm³; mean \pm SEM): (E) *Control* versus *Cy* and *Cy + Dox* ($P < 0.001$) and versus *Dox* ($P < 0.05$), *Dox* versus *Cy + Dox* ($P < 0.01$); (F) *Control* versus *Cy* and *Dox* ($P < 0.01$), versus *Cy + Dox* ($P < 0.001$), One-way ANOVA/Tukey. Overall survival: (G) $P < 0.0001$; (H): $P < 0.0001$; Log-rank test.

(Figure 4C and D). For M-406 tumors, that difference reached statistical significance ($P < 0.05$). Photomicrographs of M-234p and M-406 tumors in *Control* and *Cy + Dox* groups are shown.

T_{reg}-cell quantification

The percentage of circulating CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in treated groups did not show differences with respect to *Control* group in M-234p (day 24) (Supplementary Material, S3A, available at *Annals of Oncology* online) and M-406 (day 25) (Supplementary Material, S3B, available at *Annals of Oncology* online) tumor models.

discussion

The importance of metronomic scheduling in cancer therapeutics accounts for the increasing number of clinical protocols utilizing MCT in the past few years [8]. *Cy* and *Dox* are frequently used for the treatment of breast cancer, as monotherapies or in combination with other drugs [9]. Generally, the good results obtained are transitory and they usually have mild to severe toxic effects. The possibility of obtaining similar therapeutic results, but avoiding toxic effect through the administration of metronomic combination of *Cy + Dox* prompted us to develop pre-clinical studies in two

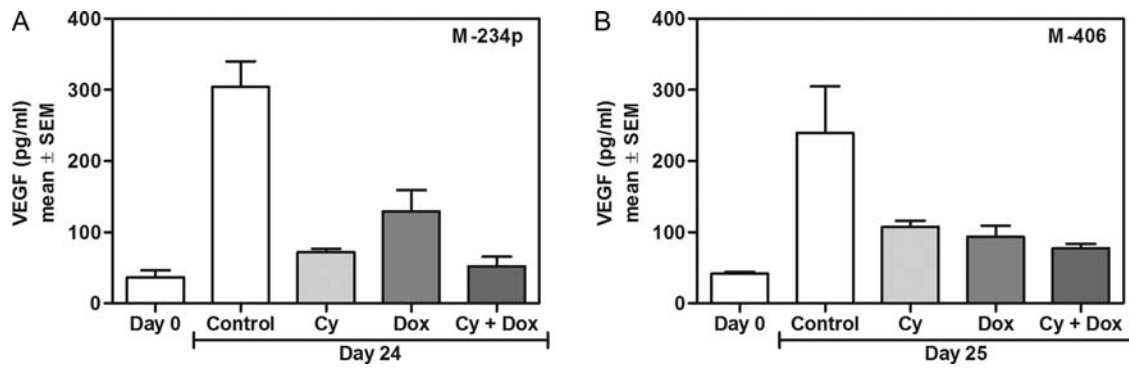


Figure 3. VEGF serum concentration: blood samples were taken on day 0 and on days 24 and 25 from mice bearing M-234p (A) and M-406 (B) tumors, respectively. Data for each time-point are mean \pm SEM serum concentration. (A) Control versus Day 0, versus Dox, versus Cy and versus Cy + Dox ($P < 0.001$); (B) Control versus Day 0 ($P < 0.01$) versus Dox and versus Cy + Dox ($P < 0.05$), ANOVA/Tukey.

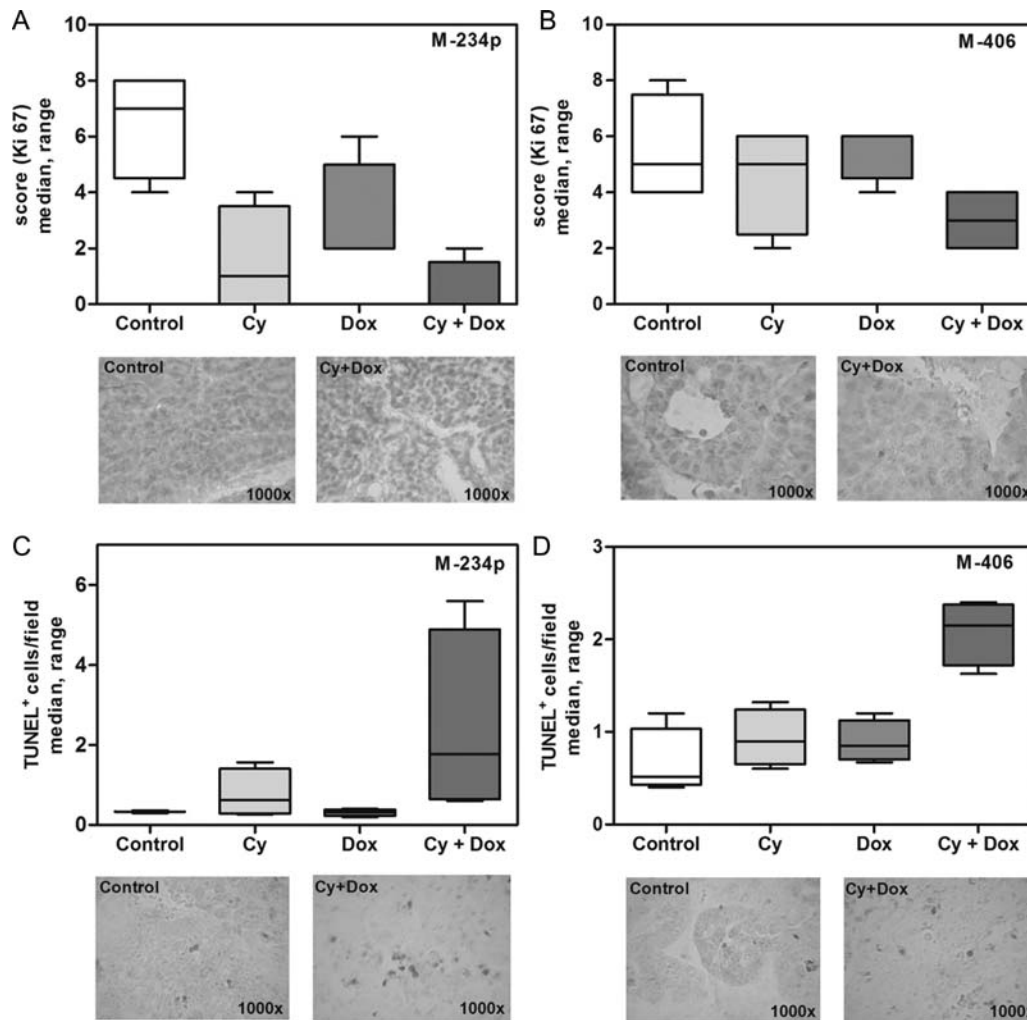


Figure 4. Ki67 expression. The score (median, range) of Ki67⁺ cells and sections of M-234p (A) and M-406 (B) tumors (100 \times magnification), obtained on days 31 and 26, respectively, is shown. (A) Control versus Cy + Dox ($P < 0.05$), (B) NS, Kruskal–Wallis /Dunn's. Apoptosis. The number of TUNEL⁺ cells/field (median, range) and sections of M-234p (C) and M-406 (D) tumors (100 \times magnification), obtained on days 31 and 26, respectively, is shown. (C) NS, (D) Control versus Cy + Dox ($P < 0.05$), Kruskal–Wallis/Dunn's.

MA tumor models. The experimental models were designed in order to mimic the clinical situation of a cancer patient who starts adjuvant chemotherapy. Our results using MCT showed

that the combined treatment, in both tumor models, was most efficient in tumor growth inhibition, resulting in a significant increase in the overall survival.

As far as we know, this is the first time that MCT with combined Cy and Dox is utilized as an intervention therapeutic strategy for MA, both at the experimental and clinical level. Shiraga [10] found an antimetastatic effect of MCT of Cy plus low-dose liposomal Dox for a lung tumor. Also, the activity and toxic effect of MCT with Cy or Dox was studied in a rat breast cancer model, but the drugs were administered as monotherapies [11]. In the clinical field, patients with locally advanced breast cancer were treated before surgery with pegylated liposomal Dox combined with metronomic Cy, achieving limited therapeutic activity [12].

The development of metastasis is an important hurdle for a successful cancer treatment. Interestingly, the antimetastatic activity of the treatment was evidenced by a reduction in the number and diameter of lung metastatic nodules, although this was not significant for M-406, hence leading to a significant decrease in the lung metastatic burden. The groups *Cy + Dox* and *Cy* did not differ in some of the evaluated parameters; nevertheless, when the overall survival was calculated the group with combined treatment was the one that lived longer, with a survival rate significantly higher than that of all the other groups, including *Cy*. These data would indicate that the combined treatment may interfere with the seeding capacity of both tumor cell types and with metastatic growth, at least for M-234p cells.

These results agree with those obtained by our laboratory, administering MCT with *Cy + Celecoxib* [5] or by other, utilizing different tumor models and/or other drug combinations. Cruz-Munoz [13] obtained a reduction of human melanoma metastasis with metronomic topotecan, while MCT with gemcitabine and sunitinib inhibited metastasis in pancreatic cancer [14]. In the clinical setting, advanced breast cancer patients received diverse metronomic treatments, showing transitory inhibition of progression [15, 16].

The treatment showed low/null toxic effect. No weight losses were detected throughout the experiment in any of the groups of both tumor models. Also, no alterations were found in the markers of morbidity/toxic effect monitored. The possibility that a chronic administration of Dox could induce cardiac toxic effect cannot be ruled out when translating to the clinic, although such event has not yet been reported.

Although MCT modulate the serum concentration of proangiogenic molecules, such as VEGF [3, 17, 18] and antiangiogenic factors, such as TSP-1 [19], these effects do not always correlate with changes in the intratumoral MVD [20]. In both tumor models, VEGF concentration was significantly lower in tumor-bearing mice treated with *Cy + Dox* than in *Control* mice. It still remains uncertain whether the reduction in VEGF levels observed in the *Cy + Dox* group is a sign of an antiangiogenic effect, or whether it is partially a consequence of the reduced tumor volumes. Moreover, those reductions in VEGF do not correlate with a concomitant diminution in MVD and MVA. Additional experimental research should be done to address the possibility that normalization, instead of reduction, of the MVD could be occurring [20].

The evaluation of tumor proliferation and apoptosis showed a decrease in proliferating cells, mainly in M-234p cells and an increase in apoptotic cells, mainly in M-406 cells.

We demonstrated that a single-low dose of Cy downregulates the percentage of circulating natural and inducible T_{reg} cells of tumor bearers [21], while, in the metronomic setting that was not the case. No changes with respect to controls were observed in circulating T_{regs} measured in the treated groups of both tumor models. Some authors found that MCT induced a decrease of T_{regs} [22], while others reported the opposite [23]. So, the controversy is still ongoing. Nevertheless, concerning the involvement of the immune response in the therapeutic effect of MCT with Cy, as demonstrated in a rat lymphoma model [24], the possibility exists that the immune system would be involved in the antitumor effect obtained through mechanisms to be determined.

In brief, the antitumor and antimetastatic benefits of combined MCT with Cy and Dox on MA together with its low toxic effect profile was shown. The decrease in VEGF concentration and tumor cell proliferation together with the increase in tumor cell apoptosis would be responsible, at least in part, for the therapeutic effect achieved.

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disclosure

OGS is a member of the scientific career of the Research Council of the National University of Rosario (CIUNR, Argentina). The authors have declared no conflicts of interest.

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