ORIGINAL ARTICLE

THERAPEUTIC EFFICACY OF METRONOMIC CHEMOTHERAPY WITH CYCLOPHOSPHAMIDE AND DOXORUBICIN ON MURINE MAMMARY ADENOCARCINOMAS

[1,2,§]L. E. Mainetti, [1,§]M. J. Rico, [1]M. V. Fernández-Zenobi, [1]H. A. Perroud, [1]E. A. Roggero, [1,3]V. R. Rozados, [1,3]O. G. Scharovsky

[1]Institute of Experimental Genetics, School of Medical Sciences, National University of Rosario, Rosario, Argentina

[2] Present address: Departments of Urology and Pathology, Wayne State University School of Medicine, Detroit, MI, USA

[§]Contributed equally to this work

[3]V. R. Rozados and O. G. Scharovsky contributed equally to this work and should both be considered as senior authors.

Corresponding author:

Dr. O. G. Scharovsky

Institute of Experimental Genetics, School of Medical Sciences, National University of Rosario.

Santa Fe 3100

(S2002KTR) Rosario, Argentina

TE: + 54-341-4804558/63 Ext: 244

Fax: + 54-341-4804569

e-mail: graciela.scharovsky@gmail.com

SUMMARY

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 approximately 150 mm^3 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 toxicity. Combined MCT was more effective than each monotherapy causing decrease in VEGF serum concentration and tumor proliferation rate plus increase in tumor apoptosis. *Conclusions:* The therapeutic benefits of combined MCT with Cy and Dox on mammary adenocarcinomas together with its low toxicity profile suggest the possibility of future translation into the clinic.

KEY WORDS

Angiogenesis, Cyclophosphamide, doxorubicin, mammary adenocarcinomas, metronomic chemotherapy, toxicity

INTRODUCTION

Metronomic chemotherapy (MCT) involves the chronic administration of doses of chemotherapy drugs that are below the maximum tolerated dose (MTD), at frequent and regular intervals, without extended rest periods [1]. It aims to achieve a balance between efficacy in tumor killing and lack of toxicity. 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, toxicity 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 CBi [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 CO_2 exposure.

Drugs

Cyclophosphamide: It was dissolved in sterile distilled water at a concentration of 20mg/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.

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 ≅1 mm^3 M-234p or M-406 tumor fragments, respectively. Five (M-234p) or eight (M-406) days later, when tumors reached ≅150 mm^3, animals (*N*=5-8/group) were distributed and treated as follows: *Control*: no treatment; *Cy*: in drinking water (≅30 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, Tregs 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 $5x10^5$ M-234p cells or $2x10^5$ M-406 cells in 0.1mL saline. On day 3, animals (M-234p: *N*=7-8/group; M-406: 5-6/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 sacrificed when it showed signs of metastatic illness.

VEGF serum concentration

Serum VEGF was quantified with Quantikine© ELISA kit (R & D Systems Inc, Minneapolis, U.S.A) and performed 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. Five μm thickness sections were used for immunohistochemical studies or stained with hematoxylin-eosin.

Tumor microvascular density (MVD) and area (MVA): They were determined using immunostaining for CD31 endothelial marker (eBioscience). The number of $CD31^+$ blood vessels/field was calculated in three hot spots areas at 400X. For MVA (vessel wall+lumen area) quantification ImageJ program (U. S. NIH, Bethesda, Maryland, USA) was utilized.

Ki67 proliferation marker: Tumor sections were incubated with anti-Ki-67 antibody (Abcam, Cambridge, MA USA). The percentage of $Ki67^+$ cells was determined in 30 fields (1000X) 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 (1000X).

Treg cells quantification

Circulating natural Treg cells ($CD4^+CD25^+Foxp3^+$) were determined by flow cytometry using the Mouse regulatory T cell staining kit (eBioscience, San Diego CA, USA). Cells were analyzed in a Coulter Epics XL (Coulter Corp. Miami, FL, USA) cytometer. Acquired data were analyzed with WinMD1 2.8 data analysis software (Scripps Research Institute, La Jolla, Ca, USA).

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 sacrificed (Fig.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 to *Control* and *Dox* groups (Fig. 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, 1/6 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

M234-p and M-406 bearing mice that received MCT with *Cy+Dox* showed a longest survival rate (P<0.05, P<0.01, respectively) (Fig.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 (Fig. 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 (Fig. 2A). M-406 developed less metastasis in *Cy+Dox* than in *Control* group (P<0.05) (Fig. 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 (Fig. 2C). In the M-406 tumor-model all the treated groups showed lower values, although non-statistically different, than in *Control* (Fig. 2D). The total metastatic burden for the three treated groups was significantly lower than in *Control* group in both tumor-models (Fig. 2E and 2F).

Survival

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

Evaluation of toxicity

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, S.1A and S.1B).

No decrease in white blood cells counts was found in either experimental group (S.1C and S.1D). The experimental metastasis assays showed a similar lack of toxicity.

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) (Fig. 4A). 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) (Fig. 4B).

Histological and Immunohistochemical Studies

Tumor microvascular density and area

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 (S.2).

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) (Fig. 4A). *Cy+Dox* group showed the lowest values in M-406 tumors, though they did not differ from the other groups (Fig. 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 (Fig. 4C and 4D). 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.

Treg cells quantification

The percentage of circulating $CD4^+CD25^+Foxp3^+$ Treg cells in treated groups did not show differences with respect to *Control* group in M-234p (day 24) (S.3A) and M-406 (day 25) (S.3B) 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 toxicities. The possibility of obtaining similar therapeutic results, but avoiding toxicity through the administration of metronomic combination of Cy+Dox prompted us to develop pre-clinical studies in two 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 found an antimetastatic effect of MCT of Cy plus low-dose liposomal Dox [10] for a lung tumor. Also, the activity and toxicity 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*. This 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 lab, administering MCT with Cy+Celecoxib [5] or by other, utilizing different tumor-models and/or other drug combinations. Cruz-Munoz obtained a reduction of human melanoma metastasis with metronomic topotecan [13], 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 toxicity. 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/toxicity monitored. The possibility that a chronic administration of Dox could induce cardiac toxicity cannot be ruled out when translating to the clinic, though such event has not yet been reported.

Although MCT modulate the serum concentration of pro-angiogenic molecules, such as VEGF [3, 17, 18] and anti-angiogenic 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 Treg 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 Tregs measured in the treated groups of both tumor-models. Some authors found that MCT induced a decrease of Tregs [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 toxicity profile was shown. The decrease of VEGF concentration and tumor cell proliferation together with the increase of tumor cell apoptosis would be responsible, at least in part, for the therapeutic effect achieved.

ACKNOWLEDGEMENTS

Work in O.G.S. laboratory is supported by grants from National University of Rosario (Argentina). O.G.S. is a member of the scientific career of the Research Council of the National University of Rosario (CIUNR, Argentina).

FUNDING

This work was supported by National University of Rosario [1MED210 to O.G.S.; 1MED283 to O.G.S.].

DISCLOSURE

The authors have declared no conflicts of interest

REFERENCES

1. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 2000; 105: 1045-1047.
2. Orlando L, Cardillo A, Ghisini R et al. Trastuzumab in combination with metronomic cyclophosphamide and methotrexate in patients with HER-2 positive metastatic breast cancer. BMC Cancer 2006; 6: 225.
3. Colleoni M, Rocca A, Sandri MT et al. Low-dose oral methotrexate and cyclophosphamide in metastatic breast cancer: antitumor activity and correlation with vascular endothelial growth factor levels. Ann Oncol 2002; 13: 73-80.
4. Rozados VR, Sanchez AM, Gervasoni SI et al. Metronomic therapy with cyclophosphamide induces rat lymphoma and sarcoma regression, and is devoid of toxicity. Ann Oncol 2004; 15: 1543-1550.
5. Mainetti LE, Rozados VR, Rossa A et al. Antitumoral and antimetastatic effects of metronomic chemotherapy with cyclophosphamide combined with celecoxib on murine mammary adenocarcinomas. J Cancer Res Clin Oncol 2011; 137: 151-163.
6. DiMasso R, Abdala S, Sanchez S, Font M. Respuesta a la selección divergente por conformación corporal en el ratón. Mendeliana 1991; 9: 79-92.
7. Squartini F, Pingitore R. Tumours of the mammary gland. IARC Sci Publ 1994; 47-100.
8. Pasquier E, Kavallaris M, Andre N. Metronomic chemotherapy: new rationale for new directions. Nat Rev Clin Oncol 2010; 7: 455-465.
9. Bonadonna G. Evolving concepts in the systemic adjuvant treatment of breast cancer. Cancer Res 1992; 52: 2127-2137.
10. Shiraga E, Barichello JM, Ishida T, Kiwada H. A metronomic schedule of cyclophosphamide combined with PEGylated liposomal doxorubicin has a highly antitumor effect in an experimental pulmonary metastatic mouse model. Int J Pharm 2008; 353: 65-73.
11. Todorova VK, Kaufmann Y, Klimberg VS. Increased efficacy and reduced cardiotoxicity of metronomic treatment with cyclophosphamide in rat breast cancer. Anticancer Res 2011; 31: 215-220.
12. Dellapasqua S, Mazza M, Rosa D et al. Pegylated liposomal doxorubicin in combination with low-dose metronomic cyclophosphamide as preoperative treatment for patients with locally advanced breast cancer. Breast 2011; 20: 319-323.
13. Cruz-Munoz W, Man S, Kerbel RS. Effective treatment of advanced human melanoma metastasis in immunodeficient mice using combination metronomic chemotherapy regimens. Clin Cancer Res 2009; 15: 4867-4874.
14. Tran Cao HS, Bouvet M, Kaushal S et al. Metronomic gemcitabine in combination with sunitinib inhibits multisite metastasis and increases survival in an orthotopic model of pancreatic cancer. Mol Cancer Ther 2010; 9: 2068-2078.
15. Fedele P, Marino A, Orlando L et al. Efficacy and safety of low-dose metronomic chemotherapy with capecitabine in heavily pretreated patients with metastatic breast cancer. Eur J Cancer 2012; 48: 24-29.
16. Perroud H, Rico M, Alasino C et al. Safety and therapeutic effect of metronomic chemotherapy with cyclophosphamide and celecoxib in advanced breast cancer patients. Future Oncology 2013 -in press.

17. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 2004; 4: 423-436.
18. Colleoni M, Orlando L, Sanna G et al. Metronomic low-dose oral cyclophosphamide and methotrexate plus or minus thalidomide in metastatic breast cancer: antitumor activity and biological effects. Ann Oncol 2006; 17: 232-238.
19. Bocci G, Francia G, Man S et al. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. Proc Natl Acad Sci U S A 2003; 100: 12917-12922.
20. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005; 307: 58-62.
21. Rico MJ, Rozados VR, Mainetti LE et al. Regulatory T cells but not NKT I cells are modulated by a single low-dose cyclophosphamide in a B cell lymphoma tumor-model. Exp Oncol 2012; 34: 38-42.
22. Ghiringhelli F, Menard C, Puig PE et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007; 56: 641-648.
23. Schwartzentruber DJ, Lawson DH, Richards JM et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med 2011; 364: 2119-2127.
24. Rozados VR, Mainetti LE, Rico MJ et al. The immune response and the therapeutic effect of metronomic chemotherapy with cyclophosphamide. Oncol Res 2010; 18: 601-605.

LEGENDS TO FIGURES

Figure 1: ***Tumor growth and overall survival***: *Tumor growth assessment*: Data for each time-point in mm^3 are mean ± SEM. **A**) Day 28: *Control* vs *Cy* and vs *Cy+Dox* (P<0.05); Day 31: *Cy* vs *Dox* and vs *Cy+Dox* (P<0.05); **B**) Day 31: *Control* vs *Cy*; *Dox* vs *Cy+Dox* (P<0.05) One way ANOVA/Tukey. Day 5: *Cy* vs *Cy+Dox* (P<0.05) t Test. *Overall survival:* **C**) (P<0.05); **D**) (P<0.01), Log-rank Test.

Figure 2: ***Antimetastatic effect****: Number of lung* metastatic *foci* (median, range): **A**) *Control* vs *Cy+Dox* (P<0.05), vs *Cy* (P<0.001); **B**) *Control* vs *Cy+Dox* (P<0.05), Kruskal-Wallis/Dunn´s. *Metastases diameter* (mm; mean ± SEM): **C)** *Cy+Dox* vs *Control* (P<0.001) vs *Cy* and *Dox* (P<0.01); **D**) NS, One way Anova/Tukey. *Total metastatic burden* (mm^3; mean ± SEM): **E**) *Control* vs *Cy* and *Cy+Dox* (P<0.001) and vs *Dox* (P<0.05), *Dox* vs *Cy+Dox* (P<0.01); **F**) *Control* vs *Cy* and *Dox* (P<0.01), vs *Cy+Dox* (P<0.001), One way Anova/Tukey. *Overall survival:* **G)** P<0.0001; **H**: P<0.0001; Log-rank Test.

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 ± SEM serum concentration. **A)** *Control* vs Day 0, vs *Dox*, vs *Cy* and vs *Cy+Dox* (P<0.001); **B)** *Control* vs Day 0 (P<0.01) vs *Dox* and vs *Cy+Dox* (P<0.05), ANOVA/Tukey.

Figure 4: *Ki-67 expression.* The score (median, range) of $Ki67^+$ cells and sections of M-234p **(A)** and M-406 **(B)** tumors (X100 magnification), obtained on days 31 and 26, respectively, is shown. **A)** *Control* vs *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 (X100 magnification), obtained on days 31 and 26, respectively. is shown. **C)** NS**, D)** *Control* vs *Cy+Dox* (P<0.05), Kruskal–Wallis/Dunn's.

Supplementary Material 1. *Evolution of body weight*: data for each time-point are the percentage of initial body weight (mean ± SEM): **A)** M-234-p; **B)** M-406. No significant body weight losses were detected in either tumor-model, during treatment. ***Total leukocytes count,*** **C)** Day 0 vs Control and vs Dox (P< 0.01), **D)** Day 0 vs Control (P<0.01), vs Dox (P<0.05), Kruskal–Wallis/Dunn's.

Supplementary Material 2. *Tumor microvascular density (MVD) and area (MVA).* NS, Kruskal–Wallis /Dunn's.

Supplementary Material 3. *T regulatory cells.* Percentage of circulating $CD4^+CD25^+Foxp3^+$ Treg cells (median, range). **A)** NS, **B**) Kruskal Wallis Test (P<0.01), Dunn's Multiple Comparison Test, NS.

Figure 1

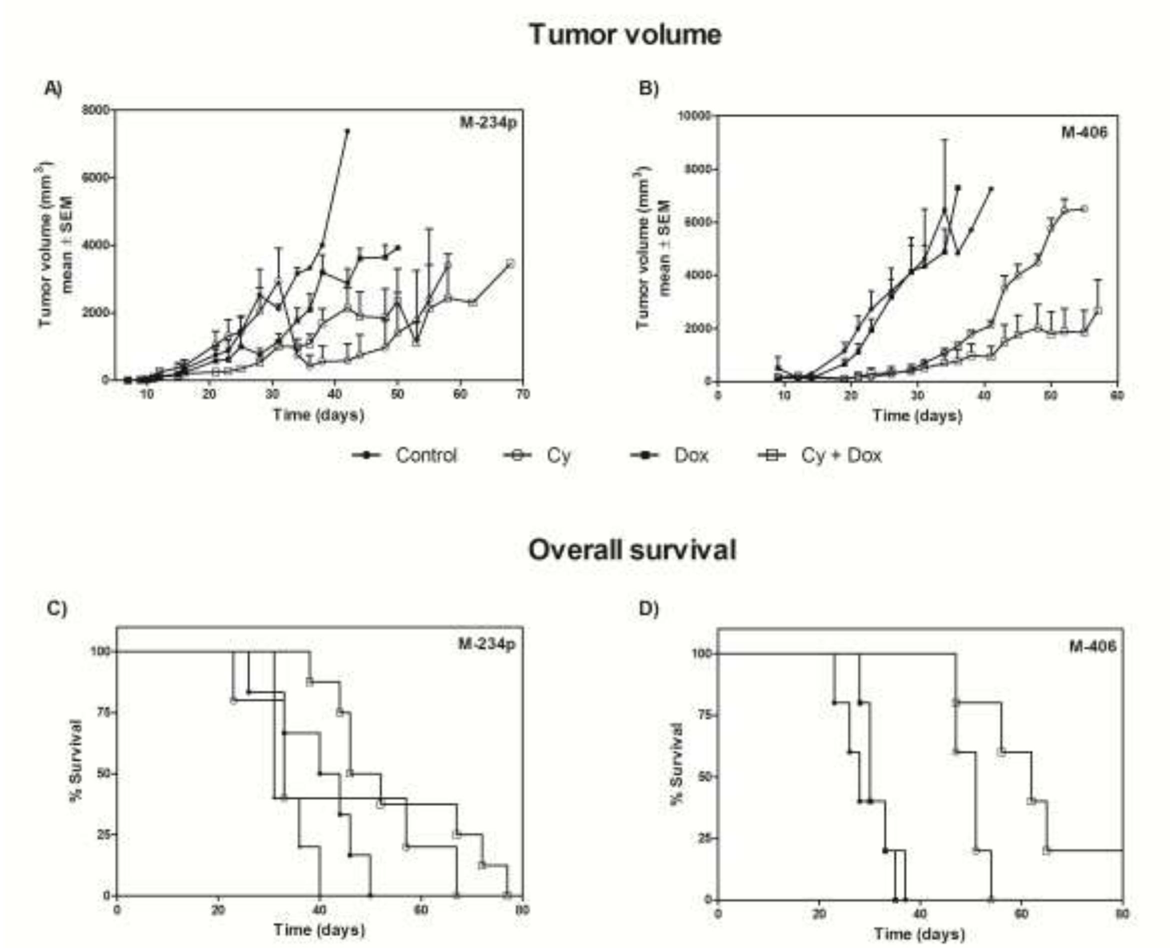


Figure 2 21

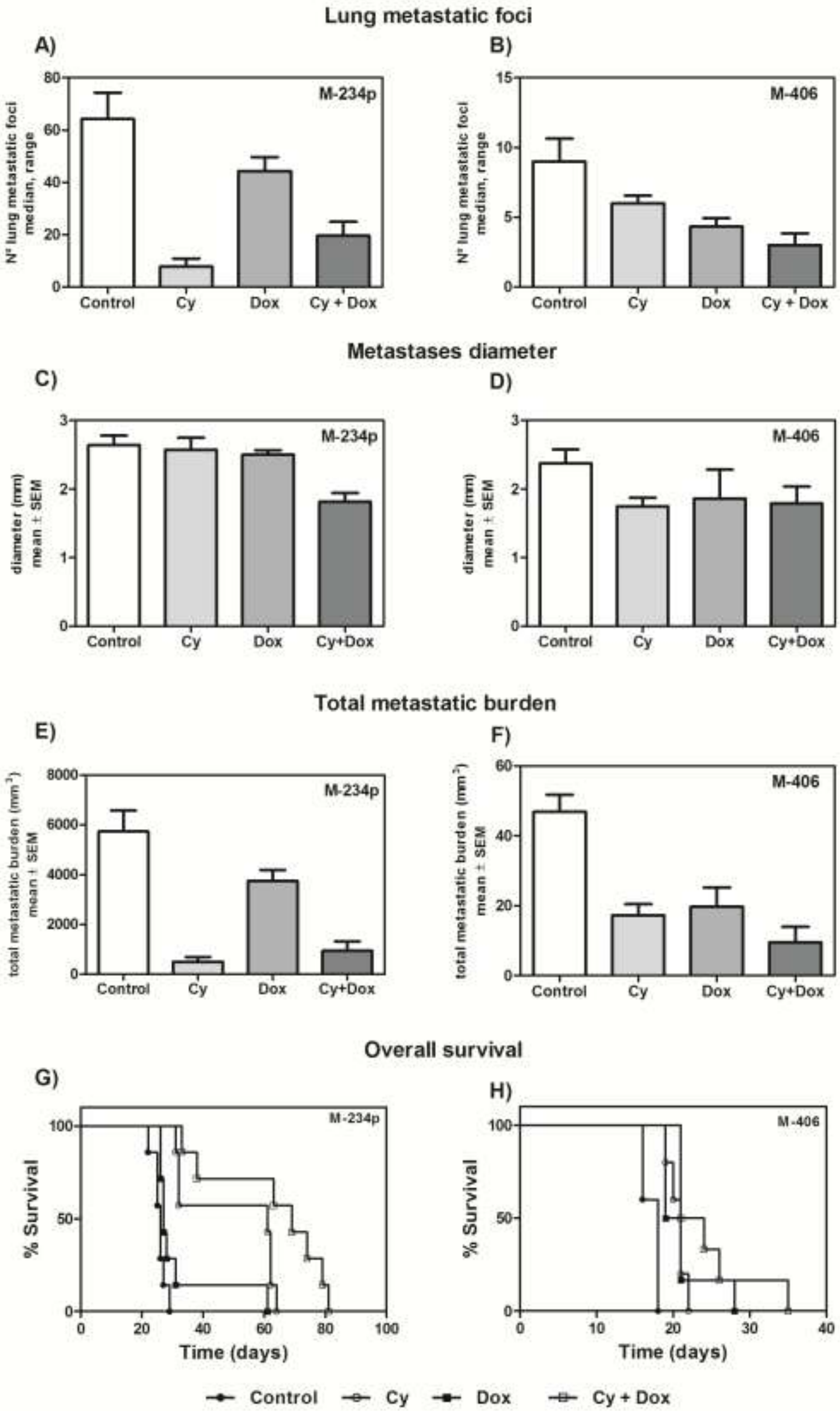

Lung metastatic foci
A)
B)
M-234p
M-406
N° lung metastatic foci
median, range
Control
Cy
Dox
Cy + Dox
Metastases diameter
C)
D)
diameter (mm)
mean ± SEM
Cy+Dox
Total metastatic burden
E)
F)
total metastatic burden (mm³)
mean ± SEM
Overall survival
G)
H)
% Survival
Time (days)
Control
Cy
Dox
Cy + Dox

Figure 3

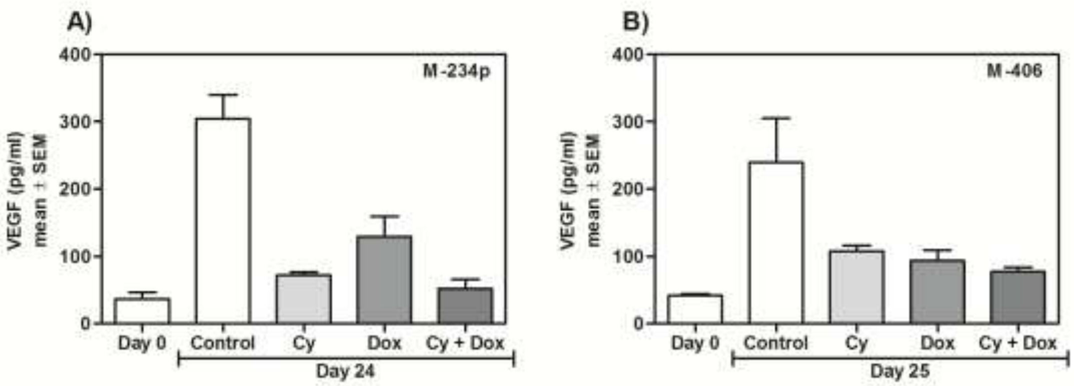
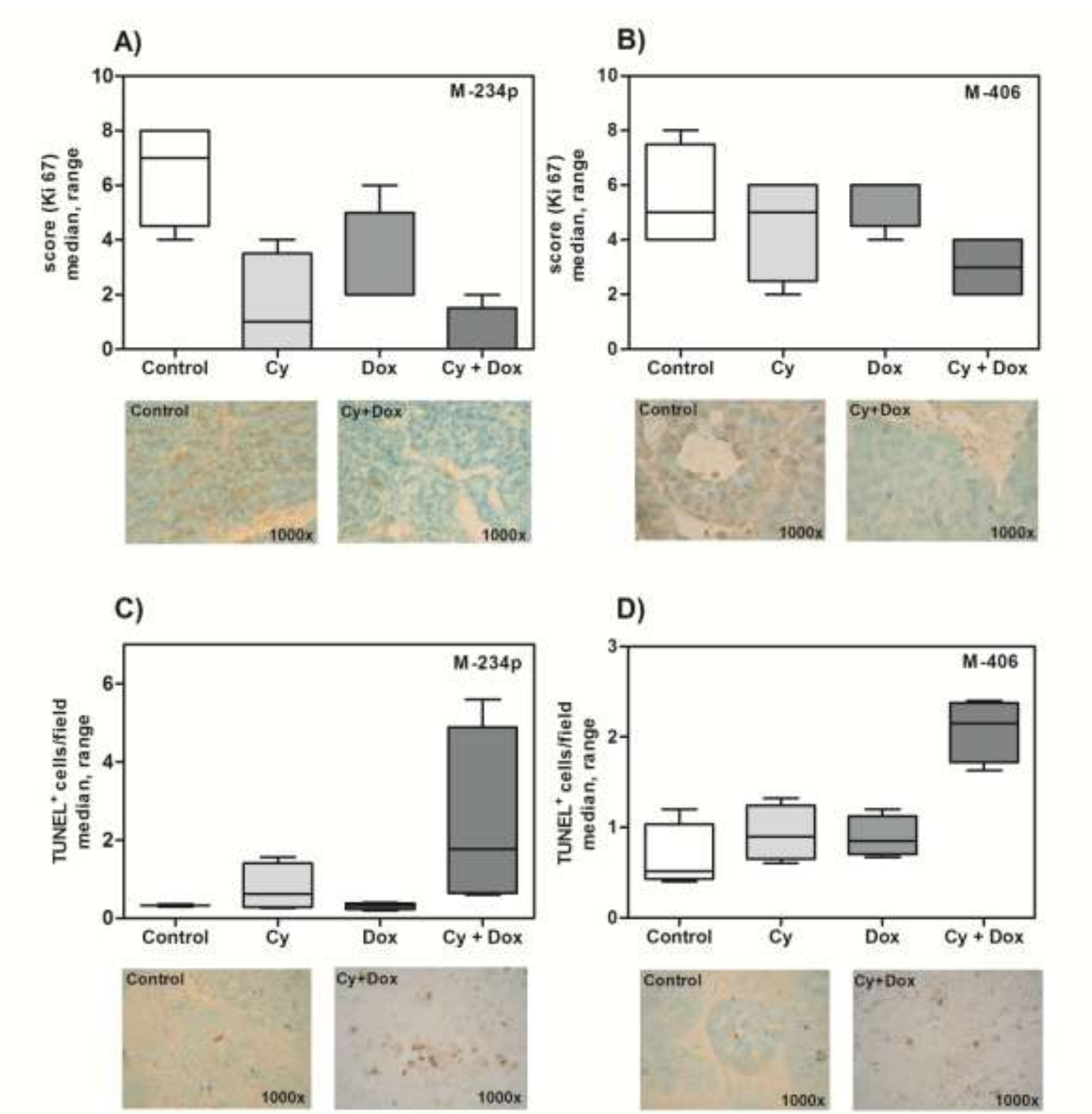
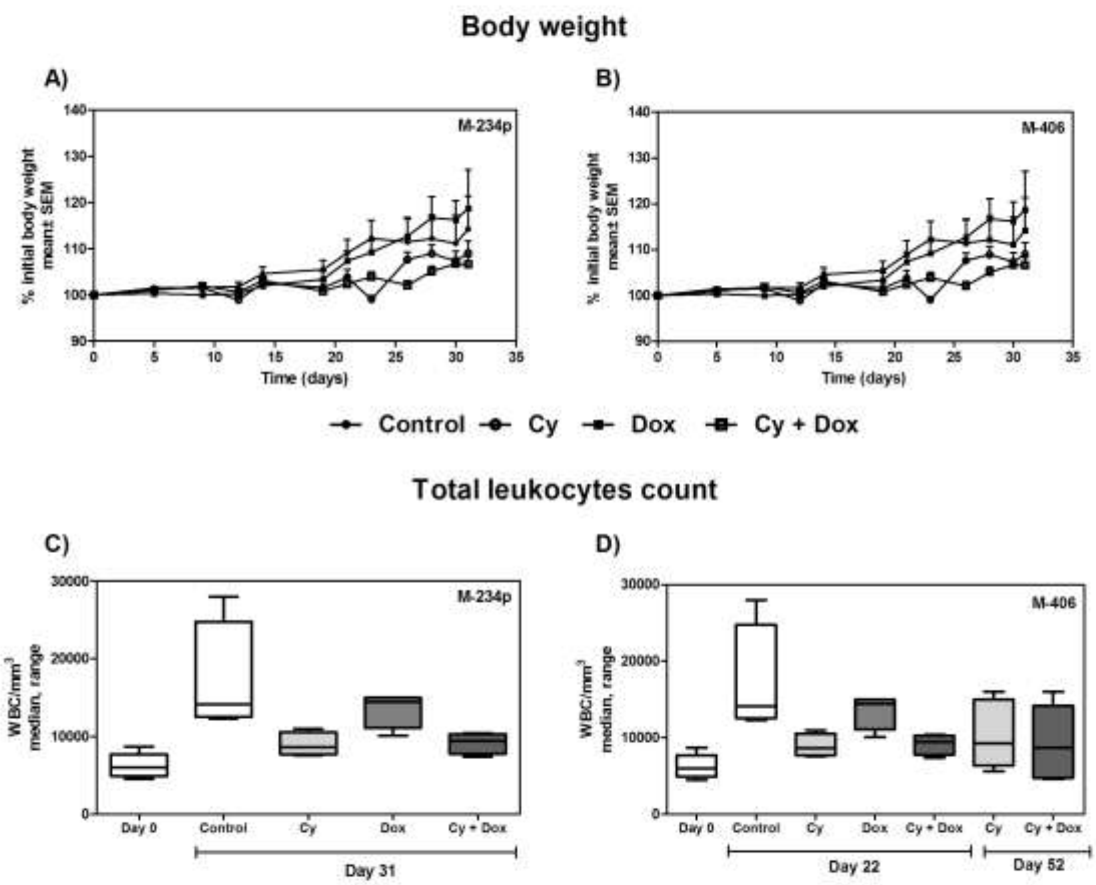

A)
M-234p
VEGF (pg/ml) mean ± SEM
400
300
200
100
0
Day 0
Control
Cy
Dox
Cy + Dox
Day 24
B)
M-406
VEGF (pg/ml) mean ± SEM
400
300
200
100
0
Day 0
Control
Cy
Dox
Cy + Dox
Day 25

Figure 4



A)
M-234p
score (Ki 67) median, range
Control
Cy
Dox
Cy + Dox
Control
Cy+Dox
1000x
B)
M-406
score (Ki 67) median, range
C)
M-234p
TUNEL⁺ cells/field median, range
D)
M-406

Supplementary material 1



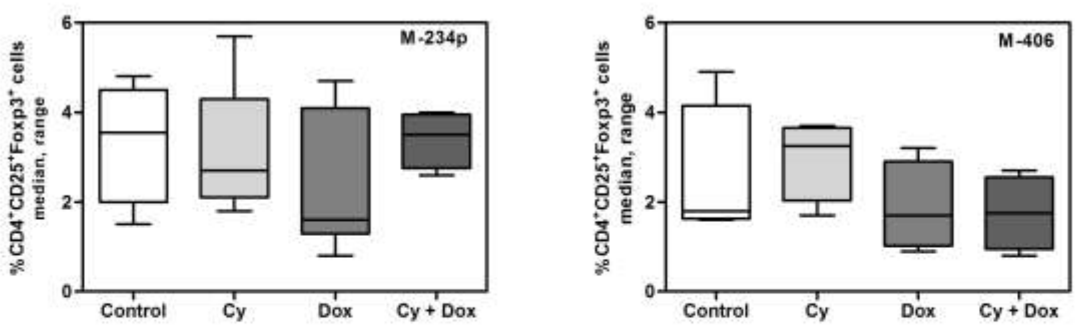
Supplementary material 2

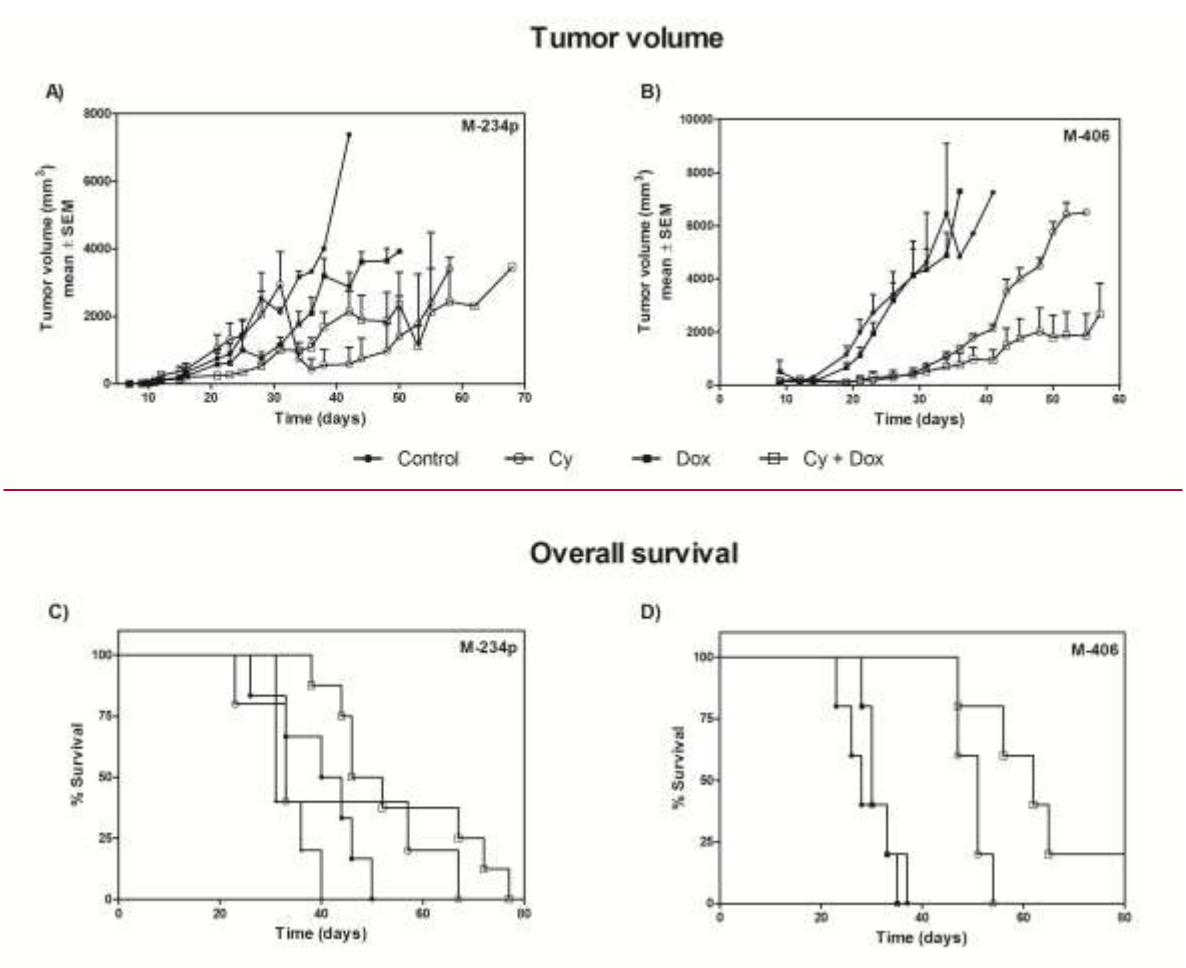
Experimental Groups	**M-234p**		**M-406**	
	MD[a]	**MA[b]**	**MD[a]**	**MA[b]**
Control	19 (17-35)	19 (8.3-35.2)	21 (16-26)	31 (19.2-47.5)
Cy + Dox	17 (13-20)	11 (9.4-14.4)	19 (18-20)	18 (10.9-29.5)

[a] N° of $CD31^+$ vessels/field (400X); median (range)

[b] % of tissue occupied by vessels/field (400X); median (range)

Supplementary material 3




Tumor volume
A)
M-234p
Tumor volume (mm3) mean ± SEM
Time (days)
B)
M-406
Tumor volume (mm3) mean ± SEM
Time (days)
Control
Cy
Dox
Cy + Dox
Overall survival
C)
M-234p
% Survival
Time (days)
D)
M-406
% Survival
Time (days)