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Comparative Effectiveness of Two Metronomic Chemotherapy Schedules—Our Experience in the Preclinical Field

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Metronomic chemotherapy refers to the chronic, equally spaced, delivery of low doses of chemotherapeutic drugs, without extended interruptions. Previously, we developed two combined metronomic schemes for the treatment of murine mammary tumors. The aim of this study was to compare their effects on tumor and metastasis growth, survival, and toxicity. Metronomic chemotherapy with Cyclophosphamide + Celecoxib (Cy + Cel) showed higher antimetastatic power than Cyclophosphamide + Doxorubicin (Cy + Dox), while being similar in other aspects. That difference, plus the advantage that represents its oral administration, suggests that the Cy + Cel combination is more suitable than Cy + Dox for metronomic chemotherapy of mammary tumors and could be proposed to the translation to the clinic.

Keywords: Metronomic chemotherapy, Combined treatment, Cyclophosphamide, Doxorubicin, Celecoxib, Mammary adenocarcinomas

INTRODUCTION

Metastatic breast cancer, as most of advanced tumors, remains incurable, and its treatment is limited to palliative management, with the objective to prolong progression-free survival, overall survival, and provide an acceptable quality of life. The problem of metastatic breast cancer management persists, in spite of having a good response, at least in local stages and despite the inclusion of targeted agents like Trastuzumab (1), Everolimus (2), Lapatinib (3), Pertuzumab (4, 5), Trastuzumab Emtansine (T-DM1) (6, 7).

The concept of metronomic chemotherapy (MCT) is well known in the oncology research area. Briefly, it refers to the chronic administration of low doses of chemotherapeutic drugs, at frequent and regular intervals, without extended rest periods, allowing a continuous and chronic treatment for different kinds of tumors, without side effects or severe toxicity (8). Prolonged rest periods, needed after a standard chemotherapy for the recovery of patients from common toxicities, represent an opportunity for specific and resistant cancer cells to re-grow. Those facts underline the importance of avoiding or reducing rest periods.

Several mechanisms of action like inhibition of angiogenesis, restoration of antitumor immune response, and induction of tumor dormancy, has been proposed to explain MCT therapeutic effect (9–11).

Cox-2 plays an important role in carcinogenesis and tumor growth and progression (12–15). This enzyme is frequently expressed in invasive and *in situ* breast cancers (16, 17). The use of Cox-2 inhibitors in cancer therapy has proved to be effective, inhibiting cell proliferation and angiogenesis.

In the same way, Cyclophosphamide, an alkylating agent that has been used for decades and it is presently used in standard chemotherapy, is one of the first and most studied drugs in metronomic or low-dose administration settings. Its antiangiogenic and immunomodulating effects were probed in different experimental tumor-models and also in the clinic (8).

Doxorubicin is an anthracycline widely used in cancer chemotherapy, commonly utilized for treating several types of cancers. Different authors found that the metronomic administration of this drug, alone or in combination with Cy, brings about an antitumor and antimetastatic effect (11, 18, 19).

Considering the high incidence of mammary tumors in humans, we had studied the therapeutic efficacy and the mechanism/s of action of MCT with cyclophosphamide (Cy) as a single drug and combined with Celecoxib (Cel) (20), or with doxorubicin (Dox) (11), in two mouse mammary adenocarcinomas (MA) tumor-models.

The aim of the present work was to compare the results previously obtained on efficacy and toxicity in animals bearing two different MA, treated with two different MCT regimens: Cy + Cel or Cy + Dox.

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MATERIALS AND METHODS

Animals

Inbred BALB/c and CBi female mice were obtained from our breeding facilities. Animals were fed with commercial chow and water *ad libitum* and maintained in a 12-hr light/dark cycle. All the experiments were developed during the first half of the light cycle. Tumor-bearing mice were euthanized by CO_2 exposure. The animals were treated in accordance to the Canadian Council on Animal Care guidelines (21).

Drugs

Cyclophosphamide (Laboratorio Filaxis, SA, Argentina) was dissolved in sterile distilled water at a concentration of 20 mg/mL and diluted in the drinking water to reach .12 mg/mL. Drinking water was replaced every other day and the mice's daily Cy intake/kg body weight (BW) was calculated.

Doxorubicin (Laboratorio Filaxis, SA, Argentina) was dissolved in sterile saline immediately before its intraperitoneal injection.

Celecoxib (Pfizer Corp, Chicago, USA) was dissolved in dimethylsulfoxide at a concentration of 200 mg/mL. Immediately before its administration by gavage, it was further diluted with phosphate buffer saline to a concentration of 2 mg/mL (14).

Tumors

The mouse mammary tumors M-234p and M-406, established in our laboratory, were used. Both tumors are negative for estrogen receptor, progesterone receptor, and Her2/neu (ER^- PR^- $HER2^-$).

M-234p: It is a type B (22) moderately differentiated mammary adenocarcinoma that shows a mixed pattern and develops lung metastasis. 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 semidifferentiated mammary adenocarcinoma 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.

Treatments

MCT Cy + Cel: Adult BALB/c or CBi female mice were implanted subcutaneously in their right flanks with ≅1 mm^3 M-234p (I) or M-406 (II) tumor fragments, respectively. Five (for M-234p) or 8 (for M-406) days later, when the tumors reached ≅150 mm^3, the animals were distributed in four groups. ($N = 6$–7 and $N = 5$–6/group for M-234p and M-406, respectively) and treated as follows: *Control*: regular drinking water without drug administration; *Cy*: In drinking water (≅30 mg/kg BW/day); *Cel*: Oral Cel (≅30 mg/kg p.o.), five times/week; *Cy + Cel*: treatments combined.

MCT Cy + Dox: Adult BALB/c or CBi mice were implanted s.c. with ≅1 mm^3 M-234p (I) or M-406 (II) tumor fragments, respectively. Five (M-234p) or 8 (M-406) days later, when tumors reached ≅150 mm^3, animals ($N =$ 5–8/group) were distributed and treated as follows: *Control*: regular drinking water without drug administration; *Cy*: in drinking water (≅30 mg/kg BW/day); *Dox*: 0.5 mg/kg/BW, i.p. three times/week; *Cy + Dox*: treatments combined.

Antitumor and antimetastatic effects

Antitumor effect

Tumor sizes were measured with Vernier calipers, and tumor volumes were calculated as follows: $v = 0.4$ (ab^2), where $v =$ volume (mm^3), $a =$ largest diameter (mm), and $b =$ smallest diameter. Animals were weighed twice/week, and blood samples were obtained on day 0 and day 24 (M-234p) or 25 (M-406) for white blood cell count. When the first animal reached the largest ethically permitted tumor volume (LPV), animals belonging to the four groups were euthanized. 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 and 2×10^5 M-406 cells in 0.1 mL saline, respectively. On day 3, animals were distributed in four groups and treated as indicated above (MCT Cy + Cel and MCT Cy + Dox). 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 then fixed in Bouin's solution to determine the number and size of metastatic foci. With both data, the total metastatic burden/mouse was calculated.

Treatment comparison

As we had previously demonstrated that the therapeutic efficacy of the combined treatment groups was significantly higher than that achieved with each individual drug, the data herein analyzed were those belonging to the groups of animals that received MCT with both drugs. For the efficacy comparison, we calculated the percentages of reduction with respect to each control group of both, tumor and lung metastatic volumes of each group of combined treatment. In the same way, the percentages of survival increase with respect to controls were also determined and statistically compared.

Statistical analysis

Kruskal–Wallis and Dunn's Multiple Comparison 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 $p < .05$.

RESULTS

The results previously obtained with respect to tumor growth and survival in the s.c. studies for both tumor models and both drug combinations are shown in Figures 1 and 2.

As previously informed, both treatments significantly inhibited tumor growth (11, 20). The% of reduction of tumor volume of animals in the combined treated group with respect to control group without treatment [median

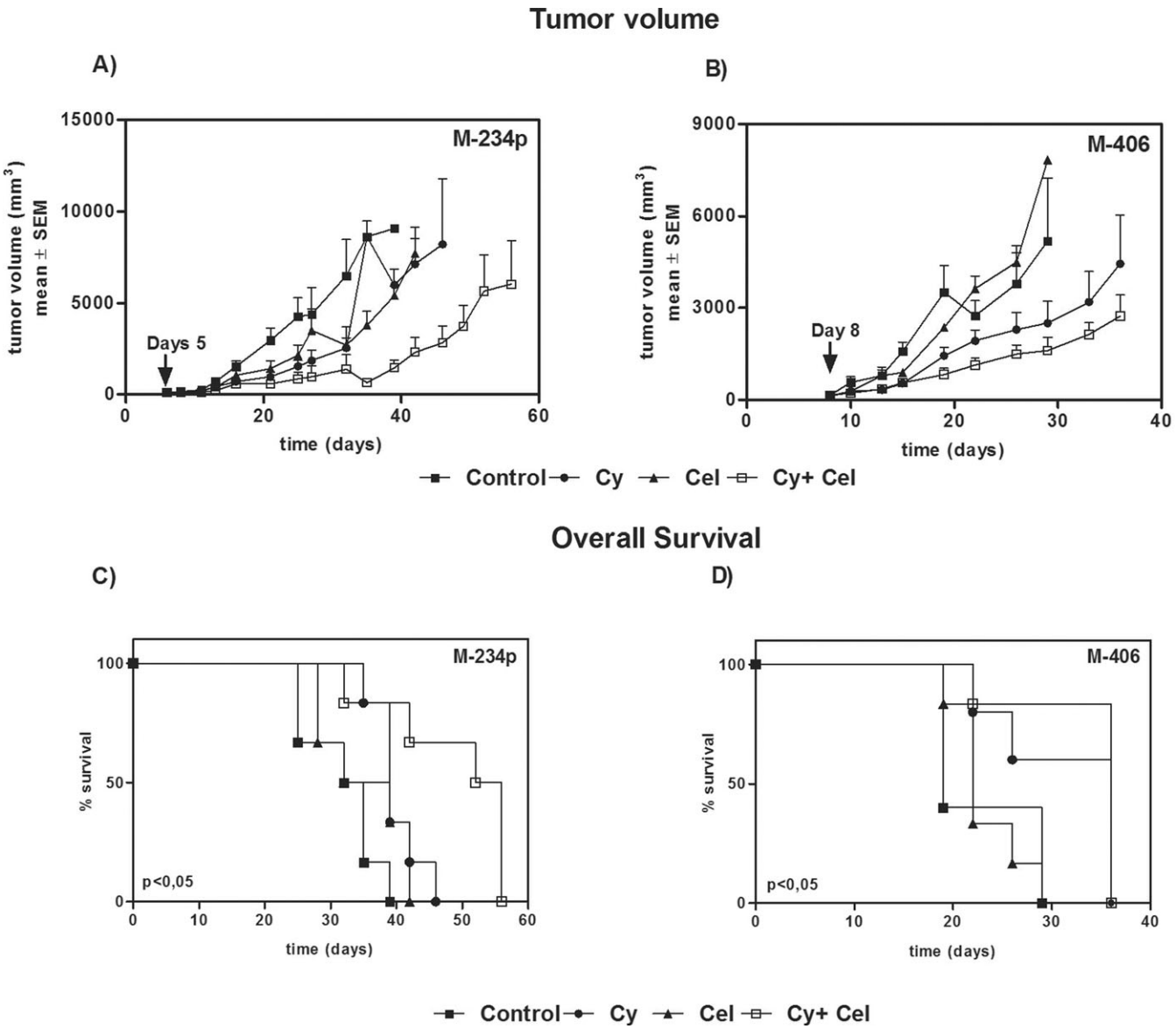

Figure 1. Tumor growth and overall survival: On day 5 (M-234p) and day 8 (M-406) when tumor volume reached 100 to 140 mm^3, animals were distributed in four groups and treated as indicated in the Material and Methods Section. *Tumor growth assessment*: (M-234p: $N = 7$/group; M-406: $N = 5$ or 6/group), Data for each time-point are Mean ± SEM. (**A**) Day 35: Control *vs.* Cy + Cel ($p < .01$); Day 39: Cy + Cel *vs.* Cy ($p < .01$), *vs.* Cel ($p < .05$). (**B**) Day 19: Control *vs.* Cy ($p < .05$) and *vs.* Cy + Cel ($p < .01$). (ANOVA and Tukey's Multiple Comparison Test). *Overall survival:* $N = 6$ and $N = 5$ or 6/group for M-234p and M-406, respectively. (**C**) Control *vs.* Cy + Cel: $p < .001$ and *vs.* Cy: $p < .05$; Cy and Cel *vs.* Cy + Cel $p < .05$. (**D**) Control *vs* Cy + Cel: $p < .01$ and *vs.* Cy $p < .05$; Cel *vs.* Cy + Cel $p < .01$ (Logrank/Mantel-Cox Test) (Reproduced with permission of the publisher from: Mainetti LE et al. *J Cancer Res Clin Oncol 137*: 151, 2011).

(range): AI: 84.4% (30–99.4), AII: 77.9% (50.9–89.9), BI: 75.5% (62.5–96.8), BII: 95.6 (57–99.6)] did not differ between treatments or between tumor models (Kruskal–Wallis non-parametric ANOVA) (Table 1).

Table 1. Percentage of Reduction of Tumor Volume with Respect to Control Group. ANOVA (Kruskal–Wallis): N.S.

	Percentage of tumor volume reduction (median-range)	
Tumor	Cy + Cel	Cy + Dox
I. M-234p	84.4% (30–99.4)	75.5% (62.5–96.8)
II. M-406	77.9% (50.9–89.9)	95.6% (57–99.6)

Mice that received MCT with Cy + Cel or Cy + Dox, showed a significantly higher survival than the corresponding control mice in both tumor models in the s.c. studies. When comparing the treatments between each other, while in the M-234p tumor model the Cy + Cel treatment increased twice as much the survival with respect to the Cy + Dox treatment [AI: 77.6% (5.3–84.2); BI: 36.1% (12.4–113), respectively], in the M-406 tumor model, on the contrary, survival with the Cy + Dox treatment [BII: 110.9% (60–308.2)] doubled that obtained with Cy + Cel treatment [AII: 56.5% (−4.3–56.5)]. Also, the differences observed between tumors for each treatment were not significant, regardless of the high disparity in the median percentages showed in Cy + Dox

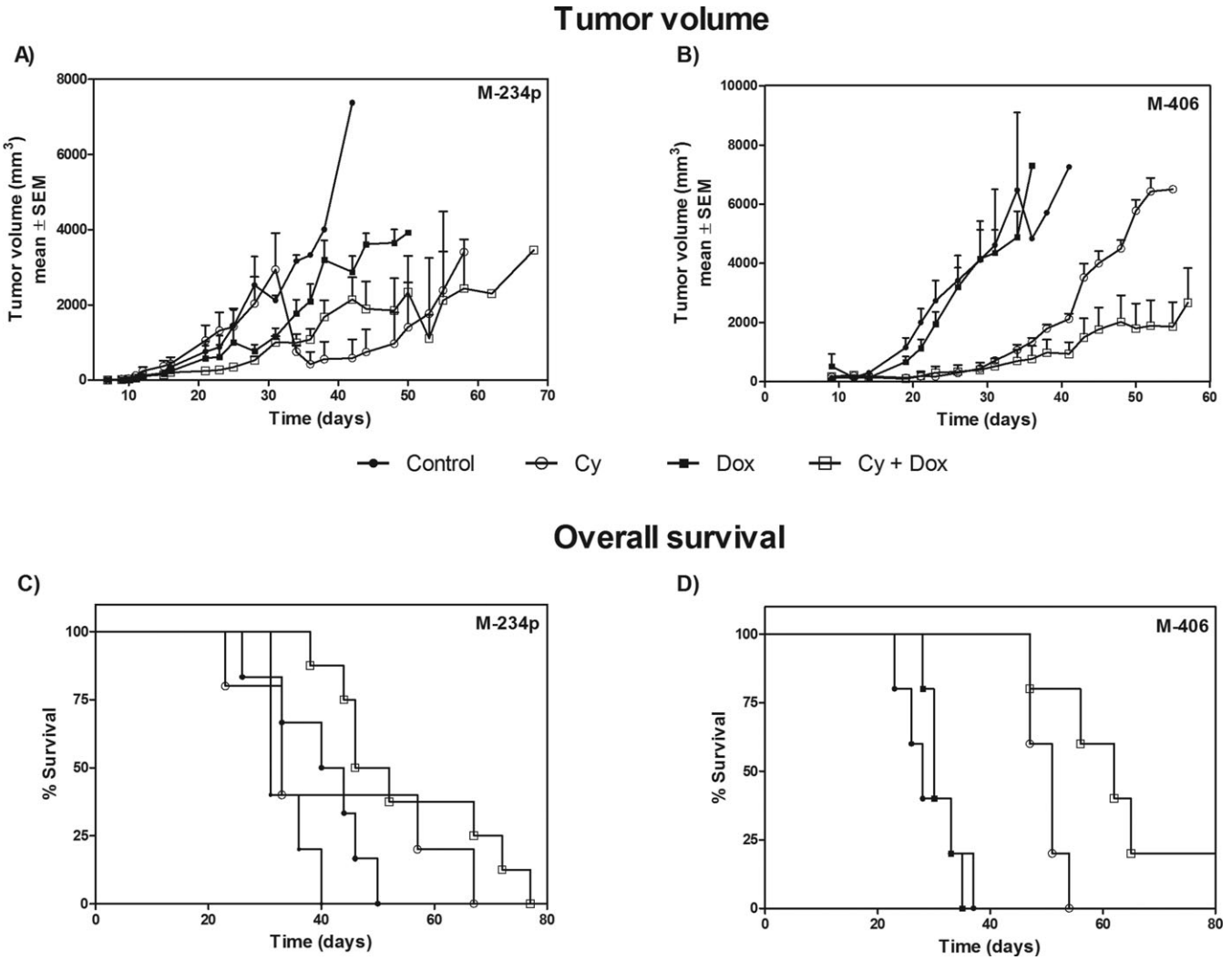

Figure 2. Tumor growth and overall survival: *Tumor growth assessment*: Data for each time-point in mm^3 are mean ± SEM. (**A**) Day 28: *Control* vs. *Cy* and *vs*. *Cy* + *Dox* ($p < .05$); Day 31: *Cy* vs. *Dox* and vs. *Cy* + *Dox* ($p < .05$); (**B**) Day 31: *Control* vs. *Cy*; *Dox* vs. *Cy* + *Dox* ($p < .05$) One way ANOVA/Tukey. Day 5: *Cy* vs. *Cy* + *Dox* ($p < .05$) *t*-test. *Overall survival:* (**C**) ($p < .05$); (**D**) ($p < .01$), Log-rank Test. (Reproduced with permission of the publisher from: Mainetti LE et al. *Ann Oncol* 24:2310, 2013).

treatment (BI: 36.1% (12.4–113); BII: 110.9% (60–308.2). The statistical comparison with Kruskal–Wallis test did not reach significance, in spite of being close to it ($p = .054$). Also the post tests (Dunn's Multiple Comparison Test) comparing all the groups between each other were not significant (Table 2).

The lung metastatic burden was found to be diminished in both therapeutic schemes. The% of reduction of lung metastatic volume [AI: 99.7% (98.7–100), AII: 99.8% (97.1–99.2), BI: 90% (48.4–99.3), BII: 90.6% (35.9–96.6)] was significantly different among all the groups ($p < .01$); Dunn's post-test showed differences in AI *vs*. BI ($p < .05$) (Table 3).

The surrogate markers of morbidity/toxicity monitored, namely the motor activity, fur quality, food intake, response to stimuli and breathing, plus the evolution of body weight and total leucocytes count showed no differences with respect to their respective controls, along the experiments, independently of the tumor-models or treatments (Data not shown).

Table 2. Percentage of Survival Increase with Respect to Control Group. ANOVA (Kruskal-Wallis): $P = 0.054$

	Percentage of survival increase (median-range)	
Tumor	Cy + Cel	Cy + Dox
I. M-234p	77.6% (5.3–84, 2)	36.1% (12.4–113)
II. M-406	56.5% (−4.3–56, 5)	110.9% (60–308.2)

Table 3. Percentage of Reduction of Lung Metastatic Burden with Respect to Control Group. ANOVA (Kruskal-Wallis): $P < 0.01$; I. M-234p: Cy+Cel *vs* Cy+ Dox, $P < 0.05$ (Dunn's Multiple Comparison Test)

	Percentage of lung metastatic burden reduction (median-range)	
Tumor	Cy + Cel	Cy + Dox
I. M-234p	99.7% (98.7–100)	90% (48.4–99.3)
II. M-406	99.8% (97.1–99.2)	90.6% (35.9–96.6)

DISCUSSION

The combination of two or more existing chemotherapy agents in order to achieve therapeutic synergism is an interesting goal in most of the metronomic schedules assayed.

A number of authors have studied the therapeutic effect of metronomic chemotherapy in either the pre-clinical or the clinical field, using different drug combinations. Just to mention a few: Vinblastine plus anti-VEGFR antibody (23), TNP-470 (24), imatinib (25), peptide ABT-510 (26), tirapazamine (27), Metronomic paclitaxel plus Cetixumab (28), Metronomic Cy plus bevacizumab, Cetuximab or trastuzumab (29), Cy plus Bevacizumab and Sorafenib (30), 5-fluorouracil pro-drug UFT (31), axitinib (32), low dose of gemcitabine (33), and celecoxib (34–36). Some of them were developed in tumor models of mammary adenocarcinomas (24, 27, 29, 31, 36)

The therapeutic results achieved with the different drugs combinations were variable. Also, those therapeutic schedules were accompanied by the presence or absence of toxic effects. Hence, it is somewhat difficult to identify which is, for a determined type of tumor, the best metronomic drug combination in terms of efficacy and derived toxicity, two properties that in turn, will determine the extension and the quality of life of the tumor bearers.

Following this line of thought, we decided to compare the antitumor and the antimetastatic efficacy of the two drug combinations tested in our lab to treat two murine mammary adenocarcinomas.

In spite of the limits of the nonorthotopic s.c. models compared to orthotopic ones, in order to study the effect of the treatments on tumor growth, we utilized s.c. tumor injections, because of its simplicity. We prefer to challenge the animals with tumor fragments instead of cell suspensions obtained by disrupting cell–cell or cell–extracellular matrix interactions. The inoculation of tumor fragments in the mammary fat pad would have to be done in anaesthetized mice, a procedure that we want, if possible, to avoid, due to the influence of anesthesia on the immune response and its importance in metronomic chemotherapy (10).

The antitumor efficacy did not show statistical differences, either among treatments or tumor-models. The same happened with the increase in survival. Nevertheless, in spite that both tumors have the same histological features and receptor status, each one showed differences, although not significant, in the effect that either treatment caused in survival, On the other hand, the combination of Cy + Cel was superior than Cy + Dox related to antimetastatic power, suggesting its potential use at the adjuvant setting.

In matter of toxicity, both treatments showed low to null toxic effects. No weight losses or differences in the total leucocytes count 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 (11, 20). Therefore, the quality of life in both combinations would be similar. Nevertheless, if we take into consideration that the administration of Cy + Cel is exclusively oral, while the Cy + Dox schedule has the drawback of the Dox intraperitoneal injection, the scale tilts into the Cy + Cel direction.

The statistical comparison we made allows us to choose the Cy + Cel treatment as the best of our MCT treatments. But, what about the different schedules and combinations tested by other researchers in other models? Are they better, similar or worse, in efficacy and toxicity, than that achieved with our treatments? Which one would be the better choice to translate to the clinic? Speaking particularly about mammary adenocarcinoma treatment, it would be of interest that other authors calculate their own percentages of decrease in tumor and metastasis volume and the percentages of survival increase with respect to controls. The availability of such data would enable to compare different schedules and combinations of MCT for mammary tumors for their translation to the clinic.

In the meantime, in the clinical field, metronomic Cy + Cel schedule for treating advanced breast cancer patients is being tested, showing a good response and low to null toxicity (36). Also, other metronomic schedules in which a chemotherapeutic drug is administered orally, combined with new targeted agents like Lapatinib (37) for breast cancer, or Everolimus (38) for renal cell cancer, have been tested.

In conclusion, although both combined metronomic treatments were fairly similar in respect to the absence of toxicity and to the inhibition of tumor growth, leading to an increased survival, the election of the Cy + Cel combination as the better one, was based in its antimetastatic power and also because of the advantage that represents its oral administration. Although idarubicin may represent a step ahead the use of anthracycline treatments because of the convenience of its oral administration (39), more information in needed about both, the antitumor effect achieved and its cardiotoxicity. The possibility of chronic oral treatment is not a minor advantage, since the development of these oral chemotherapies allows an effective treatment with an easy drug administration, with less significant adverse effects, providing better outpatient management without the emotional burden that intravenous chemotherapy represents.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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