

# **Metronomic chemotherapy following the maximum tolerated dose is an effective anti-tumour therapy affecting angiogenesis, tumour dissemination and cancer stem cells**

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## **Research article**

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### **Novelty and impact of the work**

In this work we report that standard chemotherapy at the maximum tolerated dose followed by metronomic maintenance administration, known as the chemo-switch schedule, is a potentially useful clinical strategy for treating various tumours with different drugs. We also demonstrate that this chemo-switch treatment is a multi-targeted schedule, inhibiting angiogenesis, cancer stem cells and tumour dissemination. Our study paves the way for a generalised use of the chemo-switch schedule in the treatment of tumours of diverse origin.

## **ABSTRACT**

In this article we report the effectiveness of a multi-targeted chemo-switch schedule (C-S) that combines metronomic chemotherapy (MET) after treatment with the maximum tolerated dose (MTD). We tested this schedule with gemcitabine in two distinct human pancreatic adenocarcinoma orthotopic models and with cyclophosphamide in an orthotopic ovarian cancer model. In both models the C-S schedule had the most favourable effect, achieving at least 80% tumour growth inhibition without increased toxicity. Moreover, in the pancreatic cancer model, while peritoneal metastases were observed in control and MTD groups, no dissemination was observed in the MET and C-S groups. C-S treatment caused a decrease in angiogenesis and its effect on tumour growth was similar to that produced by the MTD followed by antiangiogenic DC101 treatment. C-S treatment combined an increase in thrombospondin-1 expression with a decrease in the number of CD133+ cancer cells and triple-positive CD133+/CD44+/CD24+ cancer stem cells.

These findings confirm that the chemo-switch schedule is a challenging clinical strategy with demonstrable inhibitory effects on tumour dissemination, angiogenesis and cancer stem cells.

## INTRODUCTION

Metronomic chemotherapy (regular administration of conventional chemotherapeutic drugs at low, minimally toxic doses, with no prolonged drug-free breaks) appears to fulfil many of the requirements of an optimal multitarget therapy (minimal toxicities to allow for combinations, targeting not only the tumour but also the tumour microenvironment, etc.)<sup>1</sup>.

Although the effectiveness and rationale of MTD-based chemotherapy regimens and dose-escalation strategies have been questioned for many years, especially in patient populations with poor-prognosis tumours<sup>2</sup>, we have lacked sufficient convincing preclinical data to validate the potential of other alternative schedules of drug administration. Nevertheless, one such groundbreaking preclinical study was published ten years ago by Browder et al.<sup>3</sup> working in Judah Folkman's laboratory, the results being confirmed in Robert Kerbel's laboratory<sup>4</sup>. Using transplantable tumours<sup>3</sup> and xenograft models<sup>4</sup>, the two teams demonstrated that the frequent administration of low-dose chemotherapy could produce potent anticancer effects through the inhibition of angiogenesis.

In contrast to conventional MTD chemotherapy, the main primary target of metronomic chemotherapy is thought to be the tumour's neovasculature, although this is considered to be only an initial site of action, and additional mechanisms are probably involved<sup>5</sup>. Kerbel and Kamen<sup>5</sup> suggested that combining standard MTD chemotherapy with metronomic chemotherapy might have a synergistic effect. MTD chemotherapy would debulk the tumour before the initiation of metronomic chemotherapy, which would then have an antiangiogenic effect, while abolishing the endothelial cell rebound<sup>6</sup>. The concepts of combining metronomic chemotherapy with MTD chemotherapy or with agents targeting VEGF and PDGF receptors were brought together in a single preclinical study reported by Pietras and Hanahan<sup>7</sup>.

In this article, we generalise this chemo-switch concept to other tumour models and other drugs, evaluating its effects on angiogenesis and cancer stem cell (CSC) number.

## **MATERIAL AND METHODS**

### **Drugs**

Gemcitabine and cyclophosphamide (CTX) were provided by the Pharmacological Department of our institution. Both were dissolved in buffered saline solution at the corresponding concentrations according to the different doses and schedules.

Gemcitabine was administered by intraperitoneal injection in all treatment schedules, while cyclophosphamide was administered intraperitoneally (i.p.) when given at the maximum tolerated dose and orally by gavage when following the metronomic low dose.

DC101, an anti-VEGFR2 blocking antibody, was purified from a hybridoma culture (American Type Culture Collection). It was administered twice a week i.p. to mice at a dose of 4 mg/mouse, as previously described <sup>8</sup>.

### **Orthotopic models**

Three orthotopic tumour models were used in this study, all of which originated after implantation of fresh surgical tumour samples obtained from primary tumour patients. NP9 is a poorly differentiated tumour obtained from a human pancreatic peritoneal metastasis that had been perpetuated by orthotopic implantation in nude mice. It presents *TP53* and *KRAS* point mutations, which are common in pancreatic cancers <sup>9</sup>. TP11 was generated from a pancreatic human tumour. It is a moderately differentiated adenocarcinoma that, in addition to *TP53* and *KRAS* point mutations, presents a deletion of part of exon1 of *CDK2N* (*p16*) (Ginesta et al, manuscript in preparation). OVA17 is a serous ovarian carcinoma that was also directly generated by orthotopic implantation in mice of a human primary tumour (A. Villanueva et al, manuscript in preparation).

Four-week-old male athymic nude mice weighing 18–22 g (Harlan Laboratories, France) were used for NP9 or TP11 human pancreatic tumour implantation and expansion. Similarly, five-week-old female nu/nu Swiss mice (Harlan Laboratories) were used for the OVA17 ovarian tumour. Briefly, athymic mice were anaesthetised by isopropyl alcohol inhalation, and implantation was performed by sewing the tumour fragments to the pancreas or the ovaries with prolene 7.0 suture following an abdominal incision. Animals were housed in a sterile environment; cages, water, and bedding were autoclaved and food was  $\gamma$ -ray-sterilised. All protocols were approved by the local committee for animal care (IDIBELL).

### **Treatment schedules**

In pancreatic tumour models, gemcitabine treatment started when the tumour volume reached roughly 0.5 to 1.0 cm<sup>3</sup> (palpable tumour volume) by 15 days post-implantation for NP9 and 4 weeks post-implantation for TP11. Once the tumour volume was palpable, mice (n=30) were randomised into four cohorts and distributed to control (n=7) and three different treatment groups, receiving the MTDG schedule (n=8), the METG schedule (n=8) or the C-SG schedule (n=7). The control group received sham injections daily with vehicle alone (saline serum). Treatment was 28 days long, reproducing one cycle of treatment. Animals were weighed twice per week during treatment. At sacrifice, animals were bled from cardiac puncture and blood was collected for the study of haematological variables. Tumours were excised, measured and processed for the various analyses.

For the maximum tolerated dose gemcitabine (MTDG) schedule, we chose 100 mg/kg delivered four times at 3-day intervals by intraperitoneal injection (days 0, 3, 6, and 9 every 28 days, where day 0 is counted as the day of palpable volume). We had previously shown significant effectiveness in dose-finding experiments at this dose, and this has been established as the MTDG in the literature <sup>10, 11</sup>. Nevertheless, when we continuously

administered MTD during the entire cycle we found unacceptable toxicity in mice, so this option was discarded.

Animals treated with gemcitabine on the metronomic low-dose schedule (METG), received 1 mg/kg daily until sacrifice, as established by previous studies <sup>11</sup>.

Administration of the gemcitabine chemo-switch (C-SG) was performed combining both schedules: MTDG followed by METG until sacrifice (100 mg/kg on days 0, 3, 6 and 9, followed by 1 mg/kg daily from day 12 until day 28).

Finally, as a combination schedule of gemcitabine at the MTD followed by an antiangiogenic maintenance treatment, we performed an additional experiment: once NP9 tumours were palpable, 25 nu/nu mice were randomly distributed to a control (n=6) and three experimental treatment groups: METG (n=5), C-SG (n=7) and MTDG+DC101 (n=7). In the latter group, animals received the MTDG schedule followed by antiangiogenic treatment until sacrifice (antibody DC101 at 4 mg/mouse twice a week from day 12 to day 28) <sup>8</sup>. As in the previous *in vivo* study, the treatment lasted 28 days, after which the animals were sacrificed; tumours were excised and processed as described above.

In the *in vivo* ovarian cancer experiment, OVA17 ovarian tumour fragments were implanted in female nude mice, one in each ovary. Once tumours were palpable (by day 50 post-implantation) animals were randomised to the cyclophosphamide chemo-switch treatment (C-S CTX) (n=5) and control (CTL) (n=5) groups. The C-S CTX treatment was performed by combining the MTD and metronomic cyclophosphamide doses and schedules, as described in previous studies <sup>6, 12</sup>, and adapting these protocols to OVA17 tumour growth behaviour. During the 28-day treatment cycle, animals in the CS CTX group received first 150 mg/kg (MTD) three times at 2-day intervals by intraperitoneal injection (days 0, 2, 4), followed by 20 mg/kg daily and orally by gavage (metronomic dose) from day 13 until sacrifice. CTL mice received saline serum i.p. or orally by gavage as required.

### **Peritoneal dissemination analysis by quantification of mesenteric nodes**

After sacrifice, the mesenteric region of each mouse was excised and carefully exposed. Photographs were taken of all samples. We used the Volocity Demo program (PerkingElmer Inc.) to count the absolute number of nodes in the mesentery of each mouse and to calculate the ratio of the tumour area relative to the total area of mesentery.

### **Immunofluorescence staining**

Three-micrometer-thick OCT sections were cut and used for immunofluorescence staining analysis.

Proliferative activity was analysed by Ki67 (NeoMarker) staining. The tumour Ki67+ index was determined from counts of Ki67+ cellular nuclei relative to the total number of cells per field. We analysed four fields (40x) per tumour from five mice from each treatment and control group.

Blood vessel density was analysed by CD31 (BD PharMigen; endothelial marker) and laminin (Sigma Aldrich; basement membrane marker found surrounding blood vessels) double-staining. Tumour microvessels were analysed by measuring the relative area of CD31+ or laminin+, which was determined as the area of CD31- or laminin-stained vessels per measured field. We analysed four fields (20x) per tumour from four mice from each treatment and control group. Quantification was expressed in both cases as the positive relative area and was calculated using the Volocity Demo (PerkingElmer Inc) program.

### **Western blot**

NP9 tumours were lysed in Laemmly sample buffer (63 mM Tris HCl, 2% SDS, 10% glycerol, pH 6.8). Insoluble material was removed by centrifugation at 11,000 rpm for 10

min at 4°C. The protein concentration of the lysate was quantified by the Pearce BCA protein quantification method (Thermo Scientific). Equal amounts of protein were loaded and separated by SDS-polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk powder in TBS (150 mM NaCl, 50 mM Tris, pH 7.4) for 1 h and probed with primary antibodies against TSP-1 (NeoMarkers) or monoclonal anti-tubulin (Sigma Aldrich) overnight at 4°C. After washing with TBS-0.1% Tween, blots were incubated with anti-mouse IgG and revealed with the ECL system (GE Healthcare).

Quantitative densitometry was carried out using the Quantity One (BioRad Laboratories, INC) program.

### **Flow cytometry analyses**

Single-cell suspensions were prepared from NP9 tumours by mechanical and enzymatic dissociation. Enzymatic digestion was performed by incubation with collagenase IV (200 U/ml; Sigma, Aldrich) for 10 min at 37°C with stirring. The cell suspension was passed through a 70-µm filter and washed with PBS + 0.1% BSA + 1.5 mM EDTA. Dead cells were eliminated with the Hoechst 58 viability dye, the live cell population being between 100,000 and 300,000 cells. To identify pancreatic CSCs, dissolved cells were stained using anti-CD133-PE (Miltenyi Biotech), anti-CD44-Alexa 700 (BD Biosciences) and anti-CD24-APC (BioLegend), incubated for 15 min and then washed with PBS+0.1% BSA + 1.5 mM EDTA. Data were acquired and analysed with the Gallios™ flow cytometer (Beckman Coulter, Inc.) using the FCS Express 4 Image Cytometry program (De Novo Software, Los Angeles, CA).

### **Statistical analysis**

Statistical analyses were done with SPSS v.13.0 (SPSS, Inc., Chicago, IL). Differences in tumour volume, Ki67 proliferation index, mesenteric peritoneal dissemination, relative endothelial immunostained area levels and CD133+ or CD133+CD44+CD24+ cell subpopulations were compared by the two-tailed Mann-Whitney *U* test. Tumour volumes were summarised as medians (with ranges). Values of  $p \leq 0.05$  were considered statistically significant in all comparisons.

## **RESULTS**

### **Gemcitabine following the chemo-switch schedule blocked NP9 tumour growth**

Using the MTD schedule gemcitabine inhibited tumour growth to a similar extent as achieved by metronomic administration (median of 1.9 cm<sup>3</sup> vs. 1.76 cm<sup>3</sup> in the MDTG and METG groups). In both cases, inhibition was approximately 40%, a level that was not statistically significantly different from that of the control group (M=3.56 cm<sup>3</sup>). Administration of gemcitabine following C-S produced the best anti-tumour response, reducing tumour growth by up to 80% (M=0.64 cm<sup>3</sup>) (Fig. 1A). This effect on tumour volume was correlated with a significantly lower expression of the proliferation marker Ki67 (Fig. 1B).

### **Treatment following the chemo-switch schedule did not increase conventional treatment toxicity**

During gemcitabine administration no significant differences in the mean weight of mice in the control, MTD, MET and C-S groups (Sup. Fig. 1A). Moreover, to study the potential toxicity of the C-SG treatment, blood samples were collected on sacrifice day (day 28) to analyse haematological characteristics: segmented neutrophils, haematocrit and platelet count. There were no significant differences between the C-SG and the MTDG or METG

groups, suggesting that the C-SG schedule did not increase toxicity relative to other treatments (Sup. Fig. 1B).

### **Treatment with gemcitabine following the chemo-switch schedule blocked TP11 tumour growth**

To reproduce the gemcitabine tumour growth effects in a different pancreatic cancer model, we repeated the same *in vivo* experiments using TP11, a different orthotopic adenocarcinoma pancreatic model. Similarly to NP9, in the TP11 model the MTDG reduced tumour growth in the same proportion as did METG (M=1.19 cm<sup>3</sup> and 1.2 cm<sup>3</sup>, respectively). Inhibition was 50% compared with the controls (M=2.4 cm<sup>3</sup>), the difference being statistically significant (Fig 2A). Combining the two treatments in the C-SG schedule, tumour growth was significantly lower than in all other groups (M=0.71 cm<sup>3</sup>; P=0.014 compared with CTL; P=0.047 compared with MTDG and METG).

### **Cyclophosphamide following the chemo-switch schedule blocked ovarian tumour growth**

To determine whether the results obtained in pancreatic cancer models could be reproduced in a different cancer model, we studied the chemo-switch schedule in OVA17, an ovarian serous tumour model. Cyclophosphamide is the most widely explored agent for a metronomic approach in this type of tumour<sup>13</sup>. In OVA17, the cyclophosphamide C-S schedule had a dramatic effect (M=1.57 cm<sup>3</sup>), inhibiting tumour growth by almost 90% compared with the control group (M=0.15 cm<sup>3</sup>; P=0.014) (Fig 2B), while not having a significant effect on mouse weight (Sup. Fig. 1C).

### **NP9 tumour peritoneal dissemination was blocked by gemcitabine following the metronomic and chemo-switch schedules**

Gemcitabine treatment schedules had an effect on tumour peritoneal dissemination that was macroscopically observed in mice mesenteries (Fig 3A). CTL mice showed extensive macroscopic tumour peritoneal nodes, which were reduced to a few small, but visible, nodes in the MTDG group. In contrast, no dissemination was observed in the METG and C-SG groups.

To evaluate this effect, we counted the number of tumour nodes per mesentery (Fig 3B) and measured the relative tumour area per mesentery (Fig 3C). Peritoneal analysis showed that mesenteric nodes were larger and more abundant in the CTL group than in the MTDG group. Macroscopic peritoneal dissemination was completely inhibited in the METG and C-SG groups.

#### **Treatment with gemcitabine following the metronomic and chemo-switch schedules reduced blood vessel density in NP9 tumours and increased thrombospondin-1**

We evaluated the effect of the different gemcitabine treatments on the angiogenic pattern of NP9 pancreatic tumours (Fig. 4A). We found no significant differences in endothelial relative areas (CD31 and laminin staining) between the MTDG treatment and control groups. In contrast, in the METG and the C-SG groups, we found a similar and marked reduction of the endothelial area (Fig 4B and C). These results suggest that a potential antiangiogenic mechanism of action was at least partially responsible for the effect of C-SG on tumour growth.

To confirm this possibility, we performed a new *in vivo* experiment in NP9 tumours to compare the C-SG regimen with a new combination group: mice receiving the MTD-G schedule followed by an antiangiogenic treatment based on administration of the antibody DC101 until sacrifice (MTDG+DC101). This enabled us to confirm previous anti-tumour growth results in the METG and C-SG treatment groups compared with the control (M=0.6 cm<sup>3</sup>, 0.18 cm<sup>3</sup> and 1.24 cm<sup>3</sup> respectively) (Fig. 5A). The marked anti-tumour effect in the

C-SG group was significantly stronger than that produced by the metronomic regimen. When we analysed the effect of MTDG+DC101 treatment ( $M=0.13 \text{ cm}^3$ ), we found tumour growth to be reduced by the same proportion as in C-SG: 80% compared with the control group.

Analysing tumour microvessel density in this second *in vivo* experiment showed that anti-endothelial effect was quite similar in the METG and C-SG treatment groups and equivalent to the expected effect of the antiangiogenic MTDG+DC101 treatment group (Fig. 5B).

Induction of the antiangiogenic factor thrombospondin-1 is one of the most important effects of the metronomic treatment. To establish whether the C-S schedule also affects thrombospondin-1 we measured protein levels by western blot. Metronomic and chemo-switch treatments induced thrombospondin-1 (x4 and x2.5 respectively, Fig. 5C). In contrast, anti-VEGFR2 DC101 treatment did not induce this protein, confirming a completely different antiangiogenic mechanism mediated by VEGFR2 inhibition.

### **Gemcitabine following the metronomic and the chemo-switch schedules reduced cancer stem cells levels**

Finally, given that the presence of cancer stem cells (CSCs) may contribute to tumour resistance to classical chemotherapy<sup>14</sup>, and the previously described effects of metronomic chemotherapy on circulating endothelial cell progenitors<sup>5</sup>, we decided to investigate whether metronomic and/or chemo-switch schedules could affect CSC populations in our NP9 pancreatic tumour model. To this end, after treatment, tumours were disaggregated and human cells analysed using flow cytometry with specific antibodies. We measured the surface expression of three antigens: CD133, CD44 and CD24. The amount of human CD133+, a general marker of progenitor cells, was not significantly modified by MTDG treatment (2.12% CD133+ cells in control group, 1.88% in MTDG group) (Fig. 6A). In

contrast, the metronomic schedule caused a significant drop of 67% in CD133+ cells (0.75% CD133+ cells), an effect that was even stronger (a 77% drop) with the chemo-switch treatment (0.67% CD133+ cells). Finally, we analysed the effect of these treatments on CD133+/CD24+/CD44+ triple-positive cells, a tumour cell population with pancreatic cancer stem cell characteristics<sup>15</sup>. As shown in Fig. 6B, MTDG did not cause a significant change in number of these cells (0.95% compared with 0.69% in control tumours). The Metronomic schedule caused a drop in the proportion of these cells (0.44%), but only the chemo-switch schedule significantly reduced the proportion of CD133+/CD24+/CD44+ triple-positive cells in the tumour (0.38%). These results showed a clear effect of metronomic-based schedules on CSC populations. This could explain the anti-tumour and anti-dissemination efficacy of this therapy.

## DISCUSSION

In the present study we sought to explore the anti-tumour efficacy of a metronomic gemcitabine schedule in two different orthotopic pancreatic cancer models (NP9 and TP11), both of which are characterised by aggressive cancer growth and, in the case of the NP9 model, by metastatic development. We aimed not only to reproduce our previous results<sup>11</sup> in these more aggressive models, but also and more importantly, to test a combinatorial chemo-switch schedule in which metronomic chemotherapy was given after conventional MTD treatment. In our pancreatic tumour models, this C-S schedule had the most favourable effect, yielding at least 80% of tumour growth inhibition but with no increased toxicity. On the other hand, since 2007, when Samaritini and co-workers reported on cyclophosphamide metronomic chemotherapy for palliative treatment of a young patient with advanced epithelial ovarian cancer, several studies have been made of metronomic cyclophosphamide administration in this tumour type. Furthermore, in experimental models, the combined use of metronomic chemotherapy with antiangiogenic therapies produced a marked inhibition of tumour growth<sup>13</sup>. In our work in a serous ovarian cancer model, we reproduced an impressive anti-tumour response with cyclophosphamide following the C-S schedule that resulted in the complete inhibition of tumour growth. The chemo-switch schedule that we propose (which would first have a direct effect on tumour cells, followed by antiangiogenic effects) contrasts with other schedules combining antiangiogenic therapy with classical chemotherapy. For example, some years ago, Jain and co-workers proposed the normalisation theory, which proposes that an initial antiangiogenic treatment normalises aberrant tumour vasculature and increases the amount of the chemotherapeutic agent that reaches the tumour<sup>16</sup>. However, this theory is controversial and has been questioned by different groups. For example, Van der Veldt and co-workers<sup>17</sup> recently reported a rapid decrease in perfusion and uptake of chemotherapy when it is administered with bevacizumab (an anti-VEGF monoclonal antibody),

suggesting that other inhibitors of the VEGF pathway may produce similar effects. These authors proposed the administration of antiangiogenic agents after other anti-cancer drugs, since the immediate decrease in tumour perfusion should reduce the rate of clearance of drugs from tumours. Our most effective antitumour strategy, chemo-switch administration, emulates this treatment sequence. Moreover, we obtained similar results from the administration of gemcitabine at the maximum tolerated dose followed by a classical antiangiogenic strategy (antibody DC101), confirming the effectiveness of this combination in pre-clinical pancreatic cancer models.

To date, chemotherapy has sought to achieve complete tumour suppression, although this goal has only rarely been achieved. Tumour elimination has been elusive, with metastasis accounting for an important part of this failure. For example, Tran Cao and co-workers recently found that metronomic gemcitabine at a dose of 1 mg/kg/day suppressed metastasis at multiple sites in a pancreatic cancer mouse model, the effect being enhanced by sunitinib. They suggested a potential beneficial effect of this therapeutic schedule against pancreatic cancer in the adjuvant and maintenance settings<sup>18</sup>. Our results show that gemcitabine treatment schedules affects tumour peritoneal dissemination, with a reduction of tumour peritoneal nodes in the MTDG group and a striking disappearance of tumour peritoneal involvement in the MET and C-SG groups. The remarkable anti-tumour and anti-dissemination effects of C-SG could be due to the combined advantages of different therapeutic strategies: it maintains a constant pharmacological pressure throughout the entire tumour but with no apparent toxic effects, it directly attacks tumour cells through the MTD, inhibits angiogenesis (inducing thrombospondin-1) and reduces CSC number (metronomic administration). Concerning this last result, pancreatic carcinomas have a subpopulation of putative CSCs defined by their capacity for self-renewal and differentiation, their exclusive *in vivo* tumorigenicity and ability to drive metastasis<sup>15, 19</sup>. Most importantly, CSCs have also been proposed as the major source of

resistance to conventional chemotherapy and radiotherapy and the high recurrence rate after clinical remission in pancreatic cancer <sup>19, 20</sup> Therefore, novel therapies are urgently needed that are capable of eliminating CSCs while leaving normal stem cells unaffected. Lee et al first reported the identification of human pancreatic CSCs, which are defined by the expression of cell-surface marker CD44+CD24+epithelial-specific antigen (ESA+) and account for 0.5% to 1.0% of all pancreatic cancer cells <sup>15</sup>. CD 133 was also identified on the surface of fast proliferative primary pancreatic cancers <sup>19</sup>. Cells expressing these markers are highly tumorigenic and have the ability to self-renew and produce differentiated progeny. Signalling cascades that are integral to tumour metastasis are also upregulated in the pancreatic CSC <sup>19</sup>. Our results indicate that metronomy and C-SG both affect the pancreatic CSC population, in contrast with standard doses of chemotherapy. Similar results have been obtained in a glioma subcutaneous model <sup>21</sup> and a hepatocarcinoma orthotopic tumour model <sup>22</sup> with cyclophosphamide metronomic administration. Together, these and our results suggest metronomy-based schedules are a more effective treatment focused on target CSC.

As reviewed elsewhere <sup>23, 24</sup>, metronomic chemotherapy has attracted considerable attention in the clinic and showed promising results in phase II clinical studies for the treatment of adult patients with various types of advanced and/or refractory tumours, such as metastatic breast, ovarian and prostate cancers. Several phase III clinical trials are also currently underway ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). The preliminary evidence of disease stabilisation obtained from patients with varying and progressing tumours, and the low toxicity profile registered when metronomic regimens are administered, support metronomic implementation in the clinical setting with predicted better survival and life quality. Moreover, not only metronomic administration alone, but also therapeutic schedules combining MTD with metronomic chemotherapy and other targeted drugs are now being explored in the clinical environment <sup>25</sup>. Cyclophosphamide-based metronomic

chemotherapy has a clinical effect when given alone and enhances the activity of other agents<sup>13, 22, 26</sup>. In this context, randomised phase II trials are more appropriate for measuring the impact of cyclophosphamide-based metronomic and sequential C-S chemotherapy schedules. Furthermore, an oral pro-drug of gemcitabine is currently under clinical development<sup>27</sup>. This would facilitate the reproduction of these results in the clinical environment.

Taken together, these findings confirm that a schedule of standard chemotherapy followed by metronomic maintenance administration, known as chemo-switch, is a potentially effective clinical strategy that warrants further investigation in the clinical milieu.

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## **FIGURE LEGENDS**

### **Figure 1. Treatment with gemcitabine following the chemo-switch schedule inhibited NP9 tumour growth.**

A) Mice bearing the orthotopically implanted NP9 tumour were treated with vehicle, MTDG, METG or C-SG. After 28 days of treatment mice were sacrificed and tumour volumes measured. Results are expressed as median volumes.

B) Ki67 proliferation index quantified by immunofluorescence staining analyses. Four fields (40x) per tumour from five mice from each treatment and control group were analysed. Mean  $\pm$  SD of the percentage of Ki67-positive nuclei in a viable tumour area are shown. There was a decrease in the expression of the Ki67 proliferation marker in the MTDG group (36.9%) compared with the control group (47.8%;  $P=0.10$ ). This tendency was significantly different in the METG compared with the control group (31%;  $P=0.045$ ). The 21% decrease in the Ki67 index in the C-SG group was significant compared with the control ( $P=0.006$ ) and MTDG ( $P=0.018$ ) groups, and close to significance in comparison with the METG group ( $P=0.055$ ).

### **Figure 2. Treatment following the chemo-switch schedule inhibited pancreatic TP11 and ovarian OVA17 tumour growth.**

A) Mice bearing the orthotopically implanted TP11 tumour model were treated with vehicle, MTDG, METG or C-SG. After 28 days of treatment mice were sacrificed and tumour volumes were measured. Results are expressed as the median volume.

B) Mice bearing the orthotopically implanted OVA17 ovarian serous tumour model were treated with vehicle or C-S cyclophosphamide (CTX). After 28 days of treatment mice were sacrificed and tumour volumes measured. Results are expressed as median volumes.

**Figure 3. Treatment with gemcitabine following the chemo-switch schedule inhibited tumour peritoneal dissemination.**

A) Small bowel mesentery images from the control and three experimental treatment groups. Extensive macroscopic tumour peritoneal nodes were observed in the control group, while only a few small, but visible, nodes were present in the MTD-G group (arrows).

B) Mesenteric node quantification (mean  $\pm$  SD; n=7 in CTL and C-SG groups, n=8 in MTDG and METG groups). There were fewer mesenteric nodes in the MTDG group than in the control group (21.8 vs. 44.5; P=0.164).

C) Quantification of relative mesenteric tumour node area (mean %  $\pm$  SD; n=7 in CTL and C-SG groups, n=8 in MTDG and METG groups). Nodes in the control group were larger than in the MTDG group (5.77% vs. 1.05%).

**Figure 4. Treatment with gemcitabine following the metronomic and chemo-switch schedules reduced blood vessel density in tumours.**

A) Immunofluorescence staining for CD31 (red) and laminin (green) on endothelial cells in the control and experimental groups (20x); both markers co-localise.

B) Relative CD31 area quantification. Four fields (20x) per tumour from four mice from each treatment and control group were analysed. Results are expressed as the percentage of relative CD31+ area (mean  $\pm$  SD).

C) Relative laminin area quantification. Four fields (20x) per tumour from four mice from each treatment and control group were analysed. Results are expressed as the percentage of relative laminin+ area (mean  $\pm$  SD).

**Figure 5. Chemo-switch anti-tumour growth effect had an antiangiogenic effect based on TSP-1 protein induction.**

A) Mice bearing the orthotopically implanted NP9 tumour were treated with vehicle, METG, C-SG or MTDG+DC101. After 28 days of treatment mice were sacrificed and tumour volumes measured. Results are the median of n=6 samples in the CTL group, n=5 in the METG group and n=7 in the C-SG and MTDG+DC101 groups.

B) Relative CD31 area quantification by immunofluorescence staining analysis. Four fields (20x) per tumour from four mice from each treatment and control group were analysed.

c) Thrombospondin-1 protein levels relative to CTL group measured by western blot. Densitometric quantifications of western blots relative to actin are shown. Results are the mean  $\pm$  SD of n=8 individuals in the CTL group, n=9 in the METG group, n=9 in the C-SG group and n=3 in MTDG+DC101 group, and are represented as arbitrary units relative to the control group.

**Figure 6. Treatment with gemcitabine following the metronomic and the chemo-switch schedules reduced progenitor and pancreatic CSCs subpopulation in tumours.**

A) Dot-plot of CD133+ and CD133- populations for different treatments and control tumour samples. The CD133+ live population is illustrated in red.

B) Progenitor CD133+ cell subpopulation tumour quantification by flow cytometry. Results are expressed as the mean  $\pm$  SE in four independent tumours of the percentage of CD133+ cells relative to total living cells.

C) CD133+CD24+ dot-plot of CD44+ live (Hoecht-) population for different treatment and control tumour samples.

D) CD133+CD44+CD24+ pancreatic CSC quantification by flow cytometry. Results are expressed as the mean  $\pm$  SE of the percentage of CD133+CD44+CD24+ triple-positive cells relative to total living cells in four independent tumours from each group.