

# **ANTI-ANGIOGENIC EFFECT OF GEMCITABINE FOLLOWING METRONOMIC ADMINISTRATION IN A PANCREAS CANCER MODEL**

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**Running title: Metronomic gemcitabine in a pancreas cancer model**

**Key words: Gemcitabine, metronomic chemotherapy, xenograft pancreatic model, anti-angiogenesis.**

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## **ABSTRACT**

Gemcitabine shows a marked anti-tumor effect as a result of its cytotoxic action toward proliferative cells. In this article we aim to investigate the potential anti-tumor and anti-angiogenic effect of Gemcitabine following a metronomic schedule, that involves the regular administration of cytotoxic drugs at doses lower than standard treatment. *In vitro* results showed that human endothelial cells are more sensitive to Gemcitabine (IC<sub>50</sub> 3 nM) than pancreatic tumor cells (IC<sub>50</sub> 20 nM). For *in vivo* studies we used an orthotopic implantation model of human pancreatic carcinoma in nude mice. Gemcitabine was administered intra-peritoneally following a low-dose schedule (1 mg/Kg/day for a month) compared with the conventional schedule (100 mg/Kg days 0, 3, 6 and 9 post-implantation). Metronomic treatment effect on established tumor was equivalent to standard administration. Measure of CD31 endothelial marked area allowed us to demonstrate an *in vivo* anti-angiogenic effect of this drug that was further enhanced when using metronomic administration. This effect correlated with an induction of thrombospondin-1 (TSP-1), a natural inhibitor of angiogenesis. Our results allow us to hypothesize that, in addition to a direct anti-proliferative or cytotoxic anti-endothelial cell effect, a secondary effect involving TSP-1 induction might provide an explanation for the specificity of the effects of metronomic gemcitabine treatment.

**205 words**

## INTRODUCTION

Adenocarcinoma of the exocrine pancreas is the fifth leading cause of cancer death in Western countries and it is estimated that only 1-4% of patients with pancreatic cancer will be alive for 5 years after diagnosis. This is largely attributable to difficulties in diagnosis, the progressive growth and metastasis even after extensive surgery, and the lack of effective systemic therapies (1). Effective treatment is not available and in most patients (more than 80%) surgical resection is not feasible.

This tumor is less chemo-sensitive than other commonly occurring solid malignancies. Gemcitabine is a pyrimidine anti-metabolite (2). It is actively transported into the cells using specific transport systems such as the Equilibrative Nucleoside Transporter 1 (hENT1) protein. After metabolization, it is incorporated into DNA resulting in chain termination. Gemcitabine is active during S phase of the cell cycle against actively dividing cells and was approved for symptom management and prolonging survival in advanced pancreatic cancer (3).

Metronomic chemotherapy is a novel and promising therapeutic strategy that involves regular administration of cytotoxic drug at doses that are low enough to avoid myelosuppression and other dose-limiting side effects that otherwise obligate rest periods. A number of pre-clinical studies have implicated angiogenesis blockade in the anti-tumor effects of metronomic chemotherapy (4). Metronomic chemotherapy continuously exposes the activated but slowly proliferating tumor endothelial cells to the damaging actions of the cytotoxic drug, thereby limiting their opportunity to repair and recover (5). In this context targeting of the growing neo-vasculature is believed to be responsible of the anti-tumor effect observed. The basis for this endothelial cell

selectivity and sensitivity is unknown. However, a possible clue was provided on the basis of gene (cDNA microarray) profiling studies undertaken on endothelial cells exposed to protracted low-dose chemotherapy drugs *in vitro*. A marked induction of thrombospondin 1 (TSP-1) a well known, highly specific and potent endogenous inhibitor of angiogenesis, was observed (6).

In this article, we have aimed to investigate the anti-tumor effect of low-dose metronomic dosage of Gemcitabine in an orthotopic model of Gemcitabine-sensitive pancreatic carcinoma (NP18) and compare it to standard Gemcitabine administration. We have combined *in vivo* studies with *in vitro* experiments that evaluated the drug effect on the tumor and endothelial cells. Our results have shown that metronomic is as effective as conventional Gemcitabine administration and confirm that an anti-angiogenic mechanism of action is involved.

## **MATERIAL AND METHODS**

### ***IN VITRO STUDIES***

#### Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from Advancell (Barcelona) and Cambrex (Baltimore), and were cultivated in M199 (Biowhittaker) supplemented with 20% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin sulphate, 150 µg/ml endothelial cell growth supplement, 100 µg/ml heparin, 2 mM sodium pyruvate, 1 mM Hepes pH 7.4.

Human Micro-Vessel Endothelial cells (HMEC-1), provided by Dra. Ofelia Martinez (UB, Barcelona), were maintained in MCDB 131 medium (Invitrogen) supplemented with 20% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin sulphate, 10 ng/ml epidermal growth factor (EGF), 1 µg/ml hydrocortisone, 2 mM sodium pyruvate, 1mM Hepes pH 7.4.

The p53 mutant NP18 cell line was derived from a poorly differentiated liver metastasis of a human adenocarcinoma of the pancreas which had been perpetuated as a xenograft in nude mice (7-9). NP18 cells were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum and penicillin (100 U/mL) and streptomycin (100 µg/mL). All cells were incubated in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

#### Western blots

HUVEC or NP18 cells were washed twice in cold phosphate-buffered saline (PBS) and lysed for 15 min at 4°C in RIPA lysis buffer (0.1% SDS, 1% NP-40, 0.5% sodium deoxicholate, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 µM

sodium orthovanadate, 100  $\mu$ M phenylmethylsulfonyl fluoride, 1  $\mu$ M pepstatin A, 1  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml aprotinin). NP 18 tumors were lysed in laemmli sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, pH 6.8). Insoluble material was removed by centrifugation at 12000 X g for 5 minutes at 4°C. Western blots were performed as described (10). The blots were incubated with polyclonal rabbit anti-PARP antibody (Cell Signaling), polyclonal anti-caspase-3 antibody (Cell Signaling), monoclonal anti-caspase-9 antibody (Upstate), monoclonal anti-thrombospondin-1 antibody (Ab11, NeoMarkers, Fremont, CA, kindly provided by Dr. Mónica Feijoo, Madrid, Complutense University) or monoclonal anti-tubulin antibody (Sigma) in blocking solution overnight at 4°C.

#### Cell viability assessment

HUVE cells were grown to 80% of confluence in a 24-well plate. They were harvested with M199 medium 0.5% FCS during 24 hours. Then, they were stimulated with M199 medium 10% FCS in the absence or presence of increasing concentrations of gemcitabine, and with 600 nM staurosporine. After 24 hours of incubation at 37°C, dead cells were identified by staining with 0.4  $\mu$ g/ml propidium iodide during 10 minutes at 37°C. The fluorescence images were captured with a Leica inverted phase-contrast microscope DMIRBE equipped with digital capture software.

#### [<sup>3</sup>H]Thymidine Incorporation Assay

HUVEC and HMEC-1 were seeded in 24-well plate. They were grown to confluence and then, they were harvested with M199 or MCDB 131 medium without FCS. After 24 hours, cells were stimulated with 10 ng/ml VEGF (Oncogene Research Products) in the absence or presence of increasing concentrations of gemcitabine and

with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ] thymidine ([6- $^3\text{H}$ ]thymidine) (0.5 Ci/mmol, Amersham Pharmacia Biotech). NP18 cells were grown in 24-well in RPMI 1640 medium supplemented with 10% fetal bovine serum for 24 hours. Thymidine incorporation was measured as described (10).

## ***IN VIVO STUDIES***

### Orthotopic models

NP18 is a pancreatic cancer cell line established from a poorly differentiated and metastatic adenocarcinoma of the pancreas, which have been previously perpetuated in our laboratory as xenografts in nude mice by orthotopic implantation (8, 9). Four week old male nu/nu Swiss mice weighing 20-25 g (Iffa-Credo Animaux de Laboratoire, L'Abresle, France) were used for tumor expansion. After sacrifice, thirty mg solid tumor fragments of human pancreatic NP18 xenografts were used for implantation at the pancreas (orthotopic implantation) of the distinct animals. Nude mice were anaesthetized with 2,2,2-tribromoethanol (Sigma-Aldrich). Implantation was performed anchoring tumor pieces with prolene 7.0 suture at the pancreas after left sub-costal incision. Incision was closed with staples or silk suture. All animal studies described were approved by the local committee for animal care.

### Drugs and Therapeutics Schedules

Gemcitabine was purchased by Eli Lilly (Spain) and dissolved in buffered saline before administration.

### **Dose-finding experiments:**

#### Standard dose:

Treatment with 100-120 mg/kg of Gemcitabine, injected four times at 3-day intervals (days 0, 3, 6 and 9 after implantation) was the more effective in all the reviewed studies (11-13) and was chosen as the standard dose.

#### Metronomic dose:

The pharmacokinetics of Gemcitabine has been examined following single parenteral doses of Gemcitabine hydrochloride in mice, rats, and dogs. The elimination of Gemcitabine is rapid in all species, with mean values for primary half-life ranging from approximately 0.3 hours in mice to 2.1 hours in rats (Lilly). We designed a small toxicity study, using immune-competent Swiss mice where Gemcitabine was administered intra-peritoneally (ip) in a daily schedule for a month, at increasing doses of 1 mg/kg, 2 mg/kg and 5 mg/kg (five animals per group). Mice were weighed twice per week. Animals were sacrificed 4 weeks after the first administration of the drug. At sacrifice, animals were bled from cardiac puncture and blood was collected for studying haematological parameters. Gemcitabine caused the death of all mice at 5 mg/kg. Deaths were attributed to adverse intestinal enteropathy. Histopathology of liver and kidney demonstrated hydropic degeneration and congestion respectively. No deaths were observed in the remaining groups. Dose-related clinical observations included rough hair, perineal soiling, and decreases in values of haematological parameters (hematocrit and platelets). All these side effects were minimal at 1 mg/kg. With this toxic good profile and the previous pre-clinical studies from Lilly, we chose 1 mg/kg as the “optimal” metronomic dose.

#### **Gemcitabine treatment starting on implantation day**

**Standard Gemcitabine schedule:** Xenografted animals were randomly distributed to control (n=13) and experimental (Gemcitabine) (n=13) groups. Four of the 26 animals



died -three belonging to the control group and one to the treatment group- within the first week after implantation. Death was attributed to the surgical procedure and mice were excluded from the analysis. Treatment began at the day of implantation using Gemcitabine 100 mg/kg ip on days 0, 3, 6 and 9. Control group received sham injections with vehicle alone (saline serum).

**Metronomic gemcitabine schedule:** Once implanted, nu/nu Swiss mice were randomly distributed to control (n=15) and experimental group (n=15, low-dose Gemcitabine). One of the 30 animals died -belonging to the treatment group- within the first week after implantation. Death was attributed to the surgical procedure and mouse was excluded from the analysis. Treatment began at the day of implantation following Gemcitabine schedule: 1 mg /kg ip daily for a month. Control group received sham injections with vehicle alone (saline serum). Four weeks after implantation animals were sacrificed. Control of the animals and tumor processing was performed as described above.

**Gemcitabine treatment starting when tumor was established.**

Once implanted, forty nu/nu Swiss mice were randomly distributed to control (n=10; saline) and two experimental groups, one with low-dose Gemcitabine (n=15, 1 mg/Kg per day for one month) and the other group with “standard” Gemcitabine dose, as described above (n=15, 100 mg/Kg on days 0, 3, 6 and 9 assuming day 0 as the day of palpable tumor volume). In this experiment, in one of the forty mice there was no tumor growth. Two mice (one in the control group and one in the metronomic group) died of bowel obstruction after the surgical procedure and were excluded from the analysis. Treatment started when the tumor volume reached roughly 0.5-1cm<sup>3</sup> (palpable tumor volume) occurring between day +8 and +18 post-implantation.

## **Time course experiment with gemcitabine treatment starting with established tumor**

Once NP18 tumours were palpable, twenty four nu/nu Swiss mice were randomly distributed to control (n=8; saline) and two experimental groups, one with metronomic schedule of Gemcitabine (n=8, 1 mg/Kg per day) and the other group with “standard” Gemcitabine dose (n=8; 100 mg/Kg on days 0, 3, 6 and 9 assuming the day 0 as the day of palpable tumor). Two time points, days 10 and 20 after initiation of treatment, were selected. Three animals were excluded from the analysis (1 post-surgical death and two animals with no apparent tumor growth). At each time point, 3 or 4 mice from each group were sacrificed and tumor processing was performed as described above.

### **Immunofluorescence staining**

Five-micrometer-thick sections were used for CD31 (BD Pharmigen) (endothelial marker)/ Ki-67 (NeoMarker) double staining for quantification of micro-vessel density and proliferating cells respectively. Micro-vascular Density Average Vessel was quantified as the mean number of CD31 structures observed in 5 high power (40 x) fields of vision per tumor (central area); 4 independent tumors per experimental group were analyzed. Quantification of the CD31 staining area ( $\mu\text{m}^2$ ) was calculated using the Leica Confocal Software .

### **Assessment of human tumor VEGF levels and circulating mouse VEGF levels by Enzyme Immunoassay:**

Expression levels of human VEGF in tumor (given the human origin of the implanted tumor) were examined by ELISA following the manufacturer’s instructions (Quantikine Immunoassay Kits: R&D Systems). We examined lysed tumors from each treatment

group at each time point of the time course experiment. Circulating mouse plasma VEGF from the blood collected at the end of the experiment corresponding to each group of treatment (n=5 per each group), was also examined by ELISA. All experiments were repeated twice with at least two replicates per sample.

### Statistical Analysis

We estimated the equal sample size for the study groups with the help of a statistical software (UCLA Department of Statistics <http://calculators.stat.ucla.edu/powercalc>). The study was designed to be able to detect a 0.35 difference in a continuous variable with 0.90 power and an alpha error of 0.05. Statistical analysis was carried out by SPSS packed program. Differences in tumours volume, weight and VEGF levels were compared by the Mann-Whitney *U* test based on a two-tailed test. Kruskal-Wallis test was made for comparison between the three groups.  $P < 0.05$  was considered statistically significant.

## **RESULTS**

### *In vitro results*

In order to evaluate a putative anti-angiogenic role of Gemcitabine, we performed *in vitro* experiments using endothelial cells in culture. We measured thymidine incorporation to DNA in HUVEC (primary cultures of endothelial cells from human umbilical vein) and HMEC-1 (immortalized endothelial cell line from human microvasculature) and compared with incorporation in NP18, a tumoral pancreatic cell line. Endothelial cells were harvested for 24 hours and incubated in the presence of 10

ng/ml VEGF (vascular endothelial growth factor) and different concentrations of Gemcitabine (from 5 nM to 500 nM) measuring thymidine incorporation for 24 hours. As observed in figure 1A, Gemcitabine caused a dose-response decrease of thymidine incorporation with a maximal inhibition obtained at 25 nM, and an IC<sub>50</sub> of 3 nM. HUVE cells are a primary culture that under an incubation period without growth factors died. In order to rule out that the high sensitivity of endothelial cells to Gemcitabine was caused by harvesting, we repeated this experiment with exponentially growing HUVEC. In this condition results were comparable, with an IC<sub>50</sub> for Gemcitabine of 3 nM (data not shown). In contrast, exponential growing NP18 cells presented an IC<sub>50</sub> for Gemcitabine of 20 nM (**Fig 1A**).

To study a possible effect of Gemcitabine inducing apoptosis of endothelial cells, cell viability by staining dead cells with propidium iodide was measured in HUVEC cultures after an incubation of 24 hours with different gemcitabine concentrations (**Fig. 1B**). Apoptosis was only observed at high doses of Gemcitabine (500 nM), but not at low doses of Gemcitabine when proliferation was already affected. We observed apoptosis at low Gemcitabine doses only when the drug was added for at least 48 h (data not shown). To confirm these results, we examined by Western Blot activation of a typical apoptotic signaling pathway (active caspase-3 and -9 and PARP cleavage). Only gemcitabine at high doses was associated with a slight activation of caspase-3 activity (**Fig. 1C**).

### **In vivo results**

Given the high sensitivity of endothelial cells to gemcitabine, next we analyzed the effect of this drug on a xenograft pancreatic carcinoma (NP18) growth using a

putative *in vivo* anti-angiogenic administration schedule, the metronomic dosing. After a toxicity study (Table 1, described in Material and methods section) a metronomic dose of 1 mg/kg/day was chosen and compared it with the standard schedule.

#### Gemcitabine treatment starting on implantation day

Using the conventional mice dosing gemcitabine significantly inhibited NP18 local tumor weight and volume (**Fig 2A**). No distal metastases were observed in control and standard gemcitabine treated groups. No significant differences were observed regarding mice weight gain between both groups: 5 g for standard Gemcitabine *versus* 7 g in the control group (p=0.44).

Using the optimal metronomic dose similar results were obtained, observing a significant reduction in tumor weight and volume (**Fig 2B**). Using this schedule the mice weight gain rate was significantly diminished in the metronomic Gemcitabine group: 3.9 g. in metronomic Gemcitabine *versus* 5.9 g in the control group (p= 0.026).

#### Gemcitabine treatment starting when NP18 tumours were established.

Given the results obtained, next we assayed if metronomic schedule was also effective blocking growth of NP18 established tumor. We observed that both standard and metronomic schedules similarly inhibited tumor growth compared to the control group (**Fig. 3A**). No distal metastases were observed in control or treated groups.

In this setting, no significant differences were observed regarding mice weight between the control, standard and metronomic groups. Thus, the diminished mice weight gain observed in the first experimental setting can be attributed to the combined stress of the surgical procedure and the immediate initiation of Gemcitabine treatment.

### NP18 time course and tumor micro-vessel study

In order to better analyse the relative contribution of the anti-tumoral and anti-angiogenic action of gemcitabine, we performed a time-course with established NP18 tumours. As shown in **Figure 3B** and **C**, no differences in tumor growth between treated and control groups were observed at day 10 of treatment. This tendency changed dramatically at the second time point (20 days of treatment), where a greater and significant anti-tumoral effect was observed in experimental groups compared with saline group. Differences were significant between metronomic and control ( $p=0.034$ ) and close to significance between standard and control groups ( $p=0.05$ ). In spite of the fact that median tumor volume in the metronomic group was usually half of the standard treatment group, differences observed among treated groups did not reach statistical significance ( $p=0.29$ ) (**Fig 4A**). Thus we conclude that metronomic Gemcitabine blocked tumor growth as effectively as the standard Gemcitabine schedule being this action evident at day 20 after the initiation of treatment.

The time course experiment performed, with and earlier (10 days of treatment) and medium time points (20 days of treatment), allowed us to study more deeply the tumor histology and by immune-fluorescence staining the vessels patterns of the three groups. Macroscopically, at day 20 we found that control tumours were more vascular than treated ones, as Fig 4A shows. Microscopically, while great vessels forming tubular structures were apparent in control group, only isolated endothelial cells were observed in both treated groups (**Fig. 4B**). While no differences in the number of vessels were observed among the two treated groups, a marked and significant decrease in CD31 staining area was observed at day 20 in metronomic treated tumors (**Fig. 4C**), suggesting that vascular structures were altered by metronomic gemcitabine treatment.

### Assessment of human and mouse VEGF and thrombospondin-1 levels

To explore the association between the anti-angiogenic effect suggested by immunofluorescence in the metronomic group and a putative molecular mechanism of action, first we explored a possible effect of gemcitabine on VEGF expression. We measured human VEGF levels from tumor samples collected at each time point in the time course experiment. For both explored time points there were no differences in tumor VEGF median levels between the three groups (data not shown). In addition, no differences were observed in circulating mouse VEGF levels at the end of the experiment with gemcitabine starting when NP 18 tumor was established (five mice per each group): metronomic group 58 pg/ $\mu$ l (range 52-64), 66.5 pg/ $\mu$ l (range 58-82) in standard gemcitabine group and 63 pg/ $\mu$ l (range 58-93) in control group.

Finally we explored the role of thrombospondin-1 (TSP-1) on the metronomic effect of gemcitabine. We measured TSP-1 protein levels in the three groups of established tumors treated with saline, standard or metronomic gemcitabine for 10 and 20 days. At day 10, metronomic gemcitabine was associated with high TSP1 protein levels that decreased at day 20 (**Fig. 5A** and data not shown). In order to identify the origin of TSP-1, we performed additional *in vitro* experiments treating HUVEC and NP18 cells with gemcitabine for 24 h and measured TSP-1 protein levels by western blot. As we show in **Fig. 5B**, increased TSP-1 levels were observed in treated HUVEC without any effect on NP18.

## **DISCUSSION**

In the present study we have shown that the anti-tumor efficacy with metronomic low dose Gemcitabine schedule is equivalent to that of conventional dosing

in an orthotopic model of human pancreatic carcinoma. Our *in vitro* and *in vivo* studies point to a combined cytotoxic action on tumor cells and cytostatic action on endothelial cells.

Low-dose metronomic chemotherapy is a promising therapeutic cancer strategy thought to have an anti-angiogenic basis (5) (14). It involves the regular administration of cytotoxic drugs at doses low enough to avoid myelosuppression and other dose-limiting side effects that otherwise obligate rest period. Although a number of alternative schedules and chemotherapy combinations of gemcitabine have been evaluated (11, 15-17), no attempt has been made to explore a low-dose well-tolerated administration schedule for gemcitabine in preclinical models of pancreatic carcinoma. This is especially needed when metronomic-like scheduling of Gemcitabine has been initially evaluated in the clinical setting with encouraging results (18, 19) and its putative efficacy has to be tested in phase III clinical trials.

Our results indicate that, at total doses that are 12 times lower than the conventional gemcitabine mice schedule, metronomic Gemcitabine showed the same efficacy observed with the conventional schedule, in the absence of toxic effects. Gemcitabine not only was effective inhibiting tumor growth when administered immediately after tumor implantation, but in exponentially growing tumors as well. The latter results suggest that gemcitabine may be active in the proliferating tumor vessels. More experiments need to be performed to confirm or discard this hypothesis.

The corresponding tumor cell line NP18 is sensitive to gemcitabine at doses [our present results and (7)] that are well below the previously reported peak plasma concentration (100  $\mu$ M) of gemcitabine at maximum tolerated dose (17). These results suggest that following a metronomic schedule, gemcitabine may have an anti-tumor effect. Sensitivity of human pancreatic tumor cell lines derived from primary or



metastatic pancreatic tumours has been previously explored (13, 20-26). Some of these cell lines are sensitive to gemcitabine at similar doses that we use, suggesting that our findings may be relevant to a significant subset of pancreatic tumors.

A number of pre-clinical studies have implicated angiogenesis blockade in metronomic anti-tumor effects, where targeting of the growing neo-vasculature led to a secondary anti-tumor effect. Human endothelial cells of normal and tumor vasculature express hENT-1, the nucleotide transporter for gemcitabine, necessary to exert its putative cytostatic action (27). On the other hand, several studies have been undertaken to test the anti-proliferative, migration-inhibitory and sometimes cytotoxic effects of pico-molar concentrations of chemotherapeutic drugs on various human cell types, including micro-vascular or macro-vascular endothelial cells (4, 28, 29). Initially, cyclophosphamide, when administered at metronomic (one third of the MTD) schedule, showed impressive anti-angiogenic and anti-tumor effects on mice tumor cell lines subcutaneously grown in the syngenic mice. A detailed temporal analysis showed that endothelial cells were the first cell type within the tumor to undergo apoptosis (4). Other studies have shown similar effect of various microtubule inhibitors, such as vinblastine, paclitaxel and docetaxel (28, 30-33). In these experiments, ultra-low concentrations of these drugs were reported to inhibit proliferation or migration of endothelial cells. In our study, gemcitabine induces cell-cycle arrest in human endothelial cells in line with previous reports (34). Interestingly, gemcitabine did not affect their ability to induce *in vitro* tubular structures (data not shown), suggesting a lack of effect on endothelial cell migration.

Concerning *in vivo* experiments, the time course experiment performed allowed us to study more deeply the tumor histology and, by immune-fluorescence staining, the vessels patterns of the three groups. Differences in CD31 staining area indicate a clear

effect of gemcitabine on the architecture of vascular structures, decreasing the number of tubular vessels and increasing the number of isolated and non structured endothelial cells. These results confirm the previous *in vitro* observation of gemcitabine on endothelial cells. Moreover, this effect is enhanced by metronomic administration of the drug, supporting anti-angiogenesis as a relevant mechanism of action of metronomy.

Thrombospondin 1 (TSP-1), a component of the extracellular matrix that can also be secreted and found in the circulation, is a well known endogenous inhibitor of angiogenesis (35). In our model system, TSP-1 protein induction might provide an explanation for the specificity of the effects of metronomic chemotherapy treatment. In addition to or perhaps even instead of a direct anti-proliferative or cytotoxic anti-endothelial cell effect TSP-1 may be the potential mediator of the effects of metronomic chemotherapy (6, 36). In a previous study, 5 days of exposure to low concentrations of various chemotherapeutic drugs caused a marked increase in *TSP-1* mRNA and protein levels in vascular endothelial cells *in vitro* (other cells were not tested) (6). In our experimental system, NP18 cells in culture express low levels of TSP1 even after gemcitabine treatment; in contrast, HUVEC treated with gemcitabine express increased TSP-1 protein levels supporting the vascular origin of TSP-1 in our xenograft pancreatic carcinoma model. This hypothesis could explain the decrease in TSP-1 levels in 20 days metronomic treated tumors, when vascular structures were clearly affected. Future studies will help us to confirm or discard this hypothesis.

TSP-1 can also bind and sequester VEGF, and therefore block its pro-angiogenic activity (37). In our work, we could not show a variation in human tumor VEGF expression between groups but we found a diminished circulating mouse plasma VEGF level in metronomic group comparing with standard group, although this difference was

not statistically significant. In this regard, Colleoni *et al.* have reported that serum VEGF levels declined in patients who responded to metronomic therapy (38).

Altogether, our results are consistent with the evidence, mostly *in vitro*, indicating that the "activated" endothelial cells of newly forming blood-vessel capillaries are highly sensitive to very low doses of various chemotherapeutic drugs. While it has been postulated that this effect is due to a limited ability to repair the induced damage, this issue remains largely unknown.

Some promising preliminary results have begun to emerge in small clinical studies using mostly orally administered metronomic chemotherapy-based regimens (38-41), including those in the adjuvant setting for early-stage cancer. A recent prospective randomized study by Sakamoto *et al.* comparing a low-dose Gemcitabine regimen and standard-dose regimen in pancreatic adenocarcinoma patients, showed a reduced toxicity and an improved safety profile without survival differences between groups (19). Moreover, Takahashi *et al.* describe a metronomic-like weekly dosing of Gemcitabine in metastatic pancreatic cancer looking for an optimal biological and nontoxic dose of Gemcitabine (18). However, these results clearly need to be supported by pre-clinical studies and validated in well designed prospective randomized phase III clinical trials. Our results (both, *in vivo* using an orthotopic pancreatic model and *in vitro* using human endothelial cells) are an additional argument to continue exploring alternative Gemcitabine administration schedules in pancreatic cancer patients, such as a low-dose regimen based on an additional anti-angiogenic mechanism of action.

In summary, our study emphasizes that exploration of the anti-angiogenic and anti-tumor effects of metronomic chemotherapy in pre-clinical animal models is an important part in the continuous quest of developing regimens that can be used in the

clinic. Clearly, more pre-clinical studies are needed in order to elucidate drug-specific features and to optimize the metronomic dose and treatment schedule in different animals' models of different tumor types.

## **ACKNOWLEDGMENTS**

We thank F.J. Pérez Martí for help in statistical analysis, Mónica Feijoo (Universidad Complutense, Madrid) for thrombospondin-1 antibody, Vanessa Soto-Cerrato and Ricardo Pérez-Tomás (Universitat de Barcelona) for active caspase-3 and caspase-9 antibodies and Benjamín Torrejón for technical support. CL is a recipient of a pre-doctoral fellowship from IDIBELL. This work was supported by research grants from Ministerio de Educación y Ciencia (Programa Ramón y Cajal, BFI2001-2987 and SAF2004-01350 to Francesc Viñals and AGL2004-07579-04 to Gabriel Capellà) and Lilly Spain.

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## FIGURE LEGENDS

### **FIG. 1 *In vitro* gemcitabine effect on endothelial cell proliferation and apoptosis.**

**A)** Thymidine incorporation measured in the absence or presence of the indicated concentrations of gemcitabine for 24 h in HUVEC and HMEC-1 stimulated with VEGF (10 ng/ml) and NP18 in the presence of FCS (10%). Results are an average of three different experiments.

**B)** Immunofluorescence microscopy of depleted HUVEC stimulated with 10% FCS in the absence or presence of the indicated concentrations of gemcitabine for 24 h. Staurosporine was used as positive control of apoptosis.

**C)** HUVEC incubated as in B) were lysed and PARP, inactive caspase-9, active caspase-3 and tubulin (as a loading control) were detected by immunoblotting.

### **FIG.2 Gemcitabine treatment starting on implantation day.**

**A)** Tumor volume median in standard Gemcitabine group after a month of treatment was  $0.007 \text{ cm}^3$  (range 0-0.028) *versus*  $0.29 \text{ cm}^3$  in control group (range 0.079-2.28) (M=Median) ( $p < 0.001$ ); tumor weight median Gemcitabine  $0.014 \text{ g}$  (range 0- 0.035) *versus* control  $0.39 \text{ g}$  (range 0.12-1.7) ( $p < 0.001$ ).

**B)** Tumor volume median in metronomic Gemcitabine group after a month of daily treatment was of  $0.007 \text{ cm}^3$  (range 0-0.5) *versus*  $0.28 \text{ cm}^3$  in control group (range 0.01-1.06). ( $p < 0.001$ ). Tumour weight in gemcitabine treated was  $0.009 \text{ g}$  (range 0-0.31) *versus*  $0.4 \text{ g}$  (range 0.03-1.81) in the control group ( $p < 0.001$ ).

**FIG. 3 Gemcitabine treatment starting with established tumor and time course.**

A) At day 28 both treated groups were better than control group as figure 2 represents (Kruskall-Wallis Test  $P < 0.001$ ). Data corresponded to the medians of the three groups (Median in metronomic group 0 cm<sup>3</sup>, range 0-0.03, M 0.003 in standard group with range 0-0.63 and M 2.35 in control group, range 0.01-4.73). Tumor weight was 0 g (range 0-0.3) in metronomic vs 0.01 g (range 0-0.59) in standard gemcitabine vs 1.8 g (range 0.07-3.28) in control group ( $p < 0.001$ ). Metronomic gemcitabine administration significantly inhibited tumor growth, compared with standard gemcitabine group ( $p = 0.01$ ), although these results reflect the same biological effect.

B) Time course effect: We did not find significant differences concerning tumor volume between three groups at day 10. Data corresponded to the medians of the three groups (Median in metronomic group 0.53 cm<sup>3</sup>, range 0.38-0.68, M 0.44 in standard group with range 0.39-0.60 and M 0.69 in control group, range 0.49-1.07). We observed greater and significant anti-tumoral effect in metronomic and standard groups comparing with saline group ( $p = 0.034$  and  $p = 0.05$  -respectively) at day 20. Data corresponded to the medians of the three groups (Median in metronomic group 0.25 cm<sup>3</sup>, range 0.11-0.67, M 0.51 in standard group with range 0.19-0.94 and M 1.1 in control group, range 1.05-3.11).

C) Representation of the median tumor volume values at days 10, 20 of treatment and at the end of the experiment (day 28 of treatment).

**Fig.4 *In vivo* anti-angiogenic effect of gemcitabine**

A) Representative images of macroscopic aspect of NP18 pancreatic tumor at 2<sup>nd</sup> time point (20 days of treatment).

B) Immunofluorescence expression of CD31 on endothelial cells (red), Ki67 nuclear antigen (proliferation marker, green) in the three groups after 20 days of treatment.

C) Median CD31 area values ( $\mu\text{m}^2$ ) from five fields (40x) of vision per tumor from four different mice belonging to the three groups of treatment.

**Fig. 5. Effect of gemcitabine on Thrombospondin-1 protein levels in tumors and cell cultures**

A) Thrombospondin-1 (TSP-1) and tubulin (as a loading control) were measured by immunoblotting from lysates from established control tumors (C), and from established treated tumors following the standard (S) and metronomic schedule (M) for 10 days. Two independent tumors for every treatment are shown.

Quantification of TSP-1 protein levels measured by western blot at day 10 corrected by tubulin levels. Mean of two independent tumors is showed.

B) HUVEC and NP18 cells were treated with gemcitabine IC<sub>50</sub> doses (3 nM and 20 nM respectively) for 24 h. After lysis, TSP-1 protein levels were measured by western blot, quantified by densitometry and corrected by tubulin levels. Mean of two independent experiments is showed.