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Evaluation Of CTLA-4 Blockage Therapy With Metronomic Chemotherapy For The Treatment Of Preclinical Breast Cancer

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EVALUATION OF CTLA-4 BLOCKAGE THERAPY WITH METRONOMIC CHEMOTHERAPY FOR THE TREATMENT OF PRECLINICAL BREAST CANCER

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Abstract

Background Although there are reports that metronomic cyclophosphamide can be immune stimulating, the impact of its combination with anti-CTLA-4 immunotherapy for the treatment of cancer remains to be evaluated.

Methods Murine EMT-6/P breast cancer, or its cisplatin or cyclophosphamide (CTX) resistant variants, or CT-26 colon, were implanted into Balb/c mice. Established tumors were monitored for relative growth following treatment with anti-CTLA-4 antibody alone or in combination with; a) metronomic CTX (ldCTX; 20mg/kg/day), b) Bolus (150mg/kg) plus ldCTX, or c) sequential treatment with gemcitabine (160mg/kg every 3 days).

Results EMT-6/P tumors responded to anti-CTLA-4 therapy, but this response was less effective when combined with Bolus plus ldCTX. Anti-CTLA-4 could be effectively combined with either ldCTX (without a bolus), or with regimens of either sequential or concomitant gemcitabine, including in orthotopic EMT-6 tumors, and independently of the schedule of drug administration. Tumor responses were confirmed with CT-26 tumors but were less pronounced in drug resistant EMT-6/CTX or EMT-6/DDP tumor models than in the parent tumor. A number of tumor bearing mice developed spontaneous metastases under continuous therapy. The majority of cured mice rejected tumor re-challenges.

Conclusion Metronomic CTX can be combined with anti-CTLA-4 therapy, but this therapy is impaired by concomitant bolus CTX. Sequential therapy of anti-CTLA-4 followed by gemcitabine is effective in chemotherapy naïve tumors, although tumor relapses can occur, in some cases accompanied by the development of spontaneous metastases.

Table of Contents

Abstract....iv

Table of Contents....v

List of Figures....vi

List of Illustrations....vii

Chapter 1: Introduction....1

1.1 Breast Cancer and Standard Therapies....1

1.1.2 Immunotherapy....5

1.1.3 Materials and Methods....8

1.1.4 Results....10

1.1.5 Discussion....33

Chapter 2: Alternative Therapies: Low Dose Chemotherapy....38

2.1 Low-dose Chemotherapy: Angiogenesis and the Immune System....39

2.1.1 Mechanisms of resistance to metronomic chemotherapy....40

2.1.2 Mechanisms of resistance to metronomic: Endothelial Resistance....44

2.1.3 Mechanisms of resistance to metronomic: Host-driven resistance....45

2.1.4 Overcoming Resistance to Metronomic Chemotherapy....46

2.1.5 Metronomic Chemotherapy versus Maximum Tolerated Dose....47

Chapter 3: Identification of New Anti-cancer Drugs for Combination Therapies 49

3.1.1 Introduction....49

3.1.2 Materials and Methods....50

3.1.3 Results and Discussion....54

Chapter 4: Summary....58

References....61

Appendix....76

Supplementary Figure 1....76

Supplementary Figure 2 and 3....77

Vita....80

List of Figures

Figure 1A-C. Impact of Bolus plus low-dose CTX combined with anti-CTLA-4 therapy on the growth of EMT-6/P tumors. 13
....14
Figure 1D. Impact of the different therapies as assessed by analysis of event-free survival (Kaplan-Meier analysis), where duration of event-free survival is defined as time to primary tumor progression beyond 1,200 mm^3 or >15% weight loss. 14
Figure 2. Effective combination of chemotherapy with anti-CTLA-4 therapy for the inhibition of the growth of EMT-6/P tumors. 18
....21
Figure 3. Combination of chemotherapy with anti-CTLA-4 therapy for the inhibition of the growth of EMT-6/CTX tumors, including gemcitabine administration (as indicated by the green and orange arrows). 23
....25
Figure 4. Combination of chemotherapy with anti-CTLA-4 therapy for the inhibition of the growth of EMT-6/DDP tumors. 26
....29
Figure 5. Anti-CTLA-4 therapy plus gemcitabine inhibits orthotopic EMT-6 tumors. 29
....30
Figure 6. Impact of anti-CTLA-4 therapies on CT-26 colon tumors. 30
....31
Figure 7. Evaluation of different anti-CTLA-4 combination therapies on EMT-6/P tumor growth. 31
....32
Figure 8. Analysis of CD31-positive staining in EMT-6 tumor treated with anti-CTLA-4 therapies. 32
....53
Figure 9. Identified HITS from PremiumSet and DIVERSet libraries tested on LM2-4-luc cells. 53
....55
....55
Figure 10. CC_{50} for compound 5935953. 55
....56
Figure 11. Compound 5935953 as first line therapy with sequential PTX. 56
Figure 11. Cell cycle profile for compound 5935953. 57
....59
Figure 12. Expression of proinflammatory immune response chemokines. 59
....76
Supplementary Figure 1. Examples of spontaneous metastasis under continuous therapy, from subcutaneously implanted EMT-6 tumor models 76
Supplementary Figure 2 and 3. Evaluation of CT-26 tumor with CTLA-4 therapy. 77

List of Illustrations

Illustration 1: Mechanisms of Resistance to Metronomic Chemotherapy. 42
Illustration 2: DNS Assay workflow adapted from Lema et al., 2011. 52

Chapter 1: Introduction

1.1 Breast Cancer and Standard Therapies

Cancer is a disease that causes uncontrolled growth of mutated cells that spread through the body. Breast cancer originates in either the lobules (i.e., milk production glands) or ducts (i.e., tubes carrying milk) of the breast tissue. Breast cancer progresses from an early stage to a more advanced stage by forming a lump or tumor made up of mutated cells, where the cells can enter the nearby blood vessels and spread to a secondary site (a process termed metastasis). It is projected that by 2018, there will be 266,120 new cases of invasive breast cancer and 63,960 cases of in situ breast lesions diagnosed in women in the United States[1]. In Texas alone, by 2018 there will be 18,260 new cases of invasive and in situ breast cancer combined. The predicted death rate for women diagnosed with breast cancer in Texas is 120.2 per every 100,000 cases reported for 2018. From the 18,260 new cases of invasive and in situ breast cancer combined in Texas, 2,880 will result in death[1].

The vast majority of breast cancers are sporadic in origin and are not restricted to women only, breast cancer can also develop in men, though men have a relatively lower risk (1 in 1,000) of getting the disease. The most common symptom of breast cancer is the appearance of a lump in the breast tissue. Other symptoms of breast cancer include inverted nipples, swelling, skin irritation, redness, and unusual nipple discharge. However, early breast cancer disease has no symptoms, therefore early detection via mammography screening is vital. Oncologists look for calcifications (i.e., white spots of calcium deposits) in the mammography screening that indicate a change in the breast tissue. Not all calcifications are an indication of breast cancer, some are due to changes caused by age or injuries (e.g., macrocalcifications). Smaller calcium deposits, called

microcalcifications, need to be checked via a biopsy to discard the possibility of breast cancer. Treatment for breast cancer often involves either surgical removal of the tumor (lumpectomy) or surgical removal of the breast (mastectomy) along with a chemotherapy or radiation regimen depending on the subtype of breast cancer.

There are different molecular subtypes (e.g. luminal A, luminal B, Her-2 positive, basal, and normal like) of breast cancer each with a distinct morphology and clinical implication[2]. For example, HER-2 positive breast cancer is a subtype of breast cancer that has an overexpression of the human epidermal growth factor receptor 2 (HER2) protein on cells. Mutation of the HER2 gene causes an overexpression of the HER2 protein therefore promoting the growth of cells at uncontrollable rates. This uncontrolled growth rate is the major driver of tumor development making HER2 an oncogene, or a gene that can cause cancer. HER2 overexpression has been observed in 20% of the breast cancers diagnosed in women, making it a crucial therapeutic target[3]. Targeted therapies against HER2 protein include humanized monoclonal antibodies (e.g., trastuzumab, pertuzumab) and tyrosine kinase inhibitors (e.g., lapatinib). Monoclonal antibodies (mAb) prevent the dimerization (outside of the cell) of HER2 receptors with other HER family members thus halting the continual expansion of cells. On the other hand, lapatinib is a drug that targets the tyrosine kinase domain of the HER2 (as well as HER1) receptor inside the cell thus halting HER activation.

Other subtypes of breast cancer require the use of chemotherapeutic regimens to halt the progression of the tumor. Chemotherapy regimens may be given either before (i.e., neoadjuvant chemotherapy) or after (i.e., adjuvant chemotherapy) the surgical removal of the tumor. A typical chemotherapy regimen consists in administering drugs at maximum tolerable doses (MTD) in a specific number of cycles over a set period of time with breaks in between each dose to allow for

recovery. For example, cyclophosphamide (a DNA alkylating agent) in combination with docetaxel (a drug that prevents microtubule disassembly) is administered as a standard adjuvant chemotherapy for HER2 negative breast cancers[4].

Cyclophosphamide (CTX) was synthesized as a chemotherapeutic agent derived from nitrogen mustards - it alkylates DNA (via intermediates that are generated by the liver) thus damaging DNA. The general mechanism and structure of nitrogen mustard compounds is that you have a nitrogen in the center and two chloroethyl groups attached to it. These chloroethyl groups are the main characteristic of nitrogen mustard compounds. In CTX you have a phosphorus atom double bonded to oxygen and a 6-member ring. Nitrogen mustard will attack guanine nucleotides in the DNA due to a series of events. The carbon from the ethyl group (in the nitrogen mustard group) binds to the double bond in the nitrogen center forming a positive charge (carbonium ion). This forms a very unstable 3-member ring, termed immonium ion and cleaves off the chloride atom (forming a chloride anion). The carbonium ion then attacks guanine organic base at the N7 position by nicking electrons. This transfers the positive charge from the carbonium ion to the nitrogen (N7) at the guanine organic base. An alkyl group has now been attached to the organic base, resulting in alkylating DNA. This process can keep going resulting in interstrand or intrastrand alkylating DNA. CTX requires enzymatic activation by hepatic cytochrome P-450 to form 4-hydroxycyclophosphamide, which is then able to diffuse within the cell as aldophosphamide and 4-hydroxycyclophosphamide (non-toxic metabolites). Aldophosphamide is later activated as a toxic metabolite and cleaved into nitrogen mustard resulting in interstrand and intrastrand DNA crosslinking. This crosslink is thought to result in the cytotoxic properties of CTX- which is primarily given in combination with other chemotherapeutic agents rather than as a monotherapy[5].

In the event that breast cancer transitions to metastatic disease (e.g., spread to distant sites) due to relapse or late stage diagnosis, different therapies are administered. Gemcitabine has been extensively studied and used an anticancer drug for the treatment of metastatic pancreatic cancer, non-small cell lung cancer, bladder and breast cancer[6–8]. Gemcitabine is a cytidine analogue that requires cellular uptake by human equilibrative nucleoside transporters (hENT) and intracellular pohosporylation by deoxycytidine kinase (dCK) and thymidine kinase 2 (TK2) in order to be converted into its two active metabolites, gemcitabine di- and triphosphate (dFdCDP, and dFdCTP)[9]. Integration of dFdCTP into DNA is considered to be the main method by which gemcitabine causes cell apoptosis. Furthermore, dFdCDP metabolite inhibits ribonucleotide reductase (RR) leading to a more efficient phosphorylation of gemcitabine. This enhancement of the overall inhibitory activities on cell growth is termed self-potentiation.

hENT1 transports gemcitabine into the cell; cells lacking this transporter are resistant to gemcitabine therapy[10]. The presence of NTs are a key factor for cell growth inhibition when treating with gemcitabine. Gemcitabine is then phosphorylated by dCK (deoxycytidine kinase) and TK2 (thymidine kinase 2) to produce gemcitabine monophosphate (dFdCMP). Gemcitabine is inactivated within the cell by deoxycytidine deaminase (dCDA) and converted into difluorodeoxyuridine monophosphate (dFdUMP) and subsequently to difluorodeoxyuridine (dFdU) [Heinemann]. These substrates are either excreted out of the cell due to the decreased affinity and competitive binding of deoxycytidine[11], or are kept as dFdCMP. If dFdCMP is not exported outside the cell, then it is converted to its two active metabolites; dFdCDP, and dFdCTP. After dephosphorylation, gemcitabine is transformed to gemcitabine triphosphate (dFdCTP) and starts accumulating in the cell. dFdCTP outcompetes deoxycytidine triphosphate (dCTP) to be incorporated into the DNA[12]. After incorporation of dFdCTP on the end of the elongating DNA

strand, an additional deoxynucleotide is added thus preventing DNA polymerases to continue. This mechanism (commonly termed as masked termination) prevents detection by the proofreading enzymes[13]. Despite the advances in therapy, breast cancer remains the second leading cause of cancer death among women. There is a current need to develop new therapies or combination of therapies to treat breast cancer. Several therapies available that are not precisely against breast cancer but have shown significant overall survival against other cancers, such as melanoma may have the potential to reduce breast cancer mortality.

1.1.2 IMMUNOTHERAPY

In 1984, Dr. Steven Rosenberg of the National Cancer Institute treated a woman diagnosed with metastatic melanoma with interlukin-2 (IL-2) infusion therapy. IL-2 helps different T-cell populations to proliferate and activate the immune system. Linda Taylor became the first patient to successfully respond to an immunotherapy. The FDA approved IL-2 therapy in 1992 and 1998 for the treatment of metastatic renal cancer and melanoma. On the same note, in 1996, Jim Allison's team reported that by blocking an immune system checkpoint, cytotoxic T lymphocyte antigen-4 (CTLA-4), the immune system was able to re-ignite an attack against tumors. The New England Journal of Medicine issued a publication revealing the effectiveness of ipilimumab therapy, a humanized antibody against CTLA-4, that delayed tumor progression and prolonged the lives of patients with metastatic melanoma. Immunotherapy has opened up new avenues for combination therapies. Nivolumab, an antibody targeting programmed cell death protein (PD-1) expressed on T-cells was approve in combination with ipilimumab for the treatment of metastatic, non-resectable melanoma in 2015 (DiGiulio S, 2015).

CTLA-4, is a receptor that restricts T-cell response after co-stimulatory activation. The activation of T-cells and requires two processes, (1) presentation of antigen and, (2) activation of co-stimulatory signals[14]. Antigen presenting cells (APCs) process foreign or abnormal antigens, and they present on their cell surface for display through a protein known as major histocompatibility complex class II (MHC-II). Helper T-cells (T_H) express specific T-cell receptors (TCR) on their surface that are able to recognize and engage the specific antigenic peptide bound to MHC-II. When engaged with MHC-II, T_H cells send co-stimulation signals through CD28 to induce activation of cytotoxic T cells (T_c) by binding to APCs via B7-1 and B7-2 receptors in addition to the TCR. CD28 is the most extensively studied co-stimulatory receptor on T-cells[15–17]. When TCR is bound to MHC-II and CD28 is bound to B7-1 (or B7-2), an immune response is stimulated inducing the proliferation of T-cells (due to IL-2 secretion). Preventing overstimulation of the immune system requires the synthetization and activation of CTLA-4 on activated T cells. Expression of CTLA-4 varies depending on the T-cell population – transient expression on CD8 T-cells but constitutive on T-regulatory (T_{Reg}) cells[18]. CTLA-4 checkpoint re-establishes homeostasis after activation of immune responses, which some tumors use for their advantage to resist immune-mediated killing. CTLA-4 is approximately 20% homologous to CD28 as they share similar structures and are 27% identical in mice and 31% in human[19]. Brunet et al, reported that the CTLA-4 receptor is a competing co-stimulatory inhibiting signal that binds to the same ligands (with higher avidity) as CD28. Blocking CTLA-4 receptor with antibodies restores T-cell expansion and proliferation by dephosphorylating TCR signals (e.g., CD3) thus inducing a heightened immune response[20].

The overall survival of patients diagnosed with breast cancer still depends on how far the cancer has spread to other organs. Patients who respond to immunotherapy (e.g., checkpoint

blockade therapy) seem to have a long-term benefit that can be further enhanced with the combination of traditional treatment modalities such as chemotherapy. Immunotherapy's success is mainly due to the known immunogenicity of melanoma and pancreatic tumors. Breast cancer has been reported to be not immunogenic therefore suggesting no response to immunotherapy. However, the proposed preclinical study in this thesis and other clinical studies now suggest that immunotherapy in combination with chemotherapy has improved the outcome for breast cancer patients. Recently, atezolizumab – an anti-programmed death-ligand 1 antibody (PD-L1) was approved in combination with paclitaxel for the treatment of metastatic triple-negative breast cancer in patients whose tumors express PD-L1. This thesis reports the effective combination of an anti- cytotoxic T lymphocyte antigen-4 (CTLA-4) antibody in combination with gemcitabine and/ or cyclophosphamide for the treatment of breast cancer in a preclinical mouse model.

In 2010, fourteen years after the report of CTLA-4 blockade causing tumor responses in preclinical models by blocking the immune suppressive functions of the CTLA-4 protein, the anti-CTLA-4 antibody ipilimumab was approved by the FDA for the treatment of non-resectable or metastatic melanoma. This approval was a pivotal event for cancer immunotherapy[21,22], a field now enriched by additional targets such as PD-1, PD-L1, and LAG-3[21–23]. Despite these successes, there remain several hurdles to be overcome in the quest for optimal anti-CTLA-4 based therapy regimens, including minimizing the likelihood of the development of autoimmune toxicity[21,24,25], or devising means to overcome the evolution of drug resistance to therapy[26]. Currently, there is a growing interest to improve anti-CTLA-4 therapy by exploiting the immunostimulating properties of some conventional chemotherapeutics[27,28]. In this study, we tested whether continuous low-dose (metronomic) chemotherapy, in this case cyclophosphamide (CTX), which has been reported to act in part by boosting the immune system[17,27,29,30], could be effectively combined with CTLA-4

antibody therapy for the treatment of breast cancer in a preclinical model. Surprisingly, we found that our previously designed protocol[31], consisting of bolus (high-dose) CTX injection combined with oral low-dose CTX, actually hindered the anti-tumor efficacy of anti-CTLA-4 therapy. Conversely, we noted that metronomic CTX (without an upfront bolus) can enhance anti-CTLA-4 therapy.

Furthermore, even more impressive tumor responses were obtained using a sequential regimen of CTLA-4 blockade followed by a previously described[32] metronomic gemcitabine chemotherapy (160mg/kg, every 3 days), irrespective of whether it was evaluated on the parent EMT6/P tumor or on variants selected for resistance to cisplatin or to CTX. We also noted that acquired drug resistance (at least in a subset of mice) was observed with all therapies evaluated in this study, as was the emergence of spontaneous metastases. Our results contribute to our understanding of the preclinical benefits of chemotherapy regimens in combination with CTLA-4 blockade[33–35]. They also serve as cautionary notes in that some regimens (e.g., high-dose CTX) may hinder the beneficial anti-tumor effects of CTLA-4 blockade-based therapies, and that the use of chemotherapy naïve tumor models[36].

1.1.3 MATERIALS AND METHODS

Drug Preparation: Gemcitabine Hydrochloride was purchased from Selleck Chemicals (Houston, TX) and made up in sterile phosphate buffered saline (PBS) immediately prior to i.p. administration. CTX was purchased from Sigma and made up in PBS prior to i.p. injection or prior to its addition to the mice's drinking water. Metronomic low-dose CTX (ldCTX) was administered at an estimated 20mg/kg/day as previously described (Man et al., 2002). Some regimens (termed B+ldCTX) included an upfront Bolus dose of CTX, administered on day 1 as a 150mg/kg i.p. injection of CTX [31,32].

Anti-CTLA-4 Antibody Preparation: Anti-mouse CD152 (CTLA-4), FG purified clone 9H10, purchased from Ebioscience (San Diego, CA), was diluted in PBS immediately prior to i.p. injection. Mice were administered 100μg of the antibody on day 1 of treatment, followed by a 35μg injection on day 6.

Cell lines: Murine EMT-6/P mammary carcinoma cells (ER+/PR+/HER2+, B. Teicher – personal communication, and as previously reported[37,38], and the CTX resistant EMT-6/CTX and cisplatin resistant EMT-6/DDP variants, were a gift from Beverly Teicher, and they were grown in RPMI supplemented with 10% fetal bovine serum and 2mM L-glutamine. Cells were grown in a humidified incubator at 37C and 5% CO2.

In vivo tumor growth assessment: Six-week-old female Balb/c mice were purchased from Harlan (Indianapolis, IN). Mice were allowed to acclimatize for 2 weeks before implantation of tumor cells. To prepare cells for injection, subconfluent plates were harvested with 1% trypsin-EDTA, and cells were then washed and resuspended in RPMI at 2 million cells per ml. Two hundred thousand EMT-6 cells were injected subcutaneously into the flank of the mice (for CT26 cells, 1 million cells per mouse were implanted). Mice were monitored twice weekly for fluctuations in body weight, and for tumor growth, as measured by Vernier calipers, and tumor volume was calculated by the formula (length x width^2)/2. Institutional guidelines were followed to determine when the experimental end points were reached. Results were also plotted as event-free survival (Kaplan-Meier analysis) over time, where duration of event-free survival is defined as time to primary tumor progression beyond 1,200 mm^3 or >15% weight loss, as per our previous study[39]. Primary tumor fragments, or established lung metastases, were isolated from selected euthanized mice, and used to derive cell cultures as previously described[40,41]. The orthotopic implant of EMT-6 and EMT-6DDP cells was carried out as previously described [40,41]; one hundred

thousand cells in 50 microlitres were implanted in the inguinal mammary fat pad of mice. All in vivo procedures and experiments were performed with the approval of the UTEP IACUC (IACUC reference #: A-201201-1).

Immunohistochemical Analysis: Paraffin embedded EMT-6 tumor sections were cut to 5 micron thickness and stained for anti-CD31 (Abcam 28364) used at a dilution of 1:400, using an antigen retrieval of citrate buffer pH6. Secondary antibody was goat anti-rabbit at a dilution of 1:200, using DAB for detection of positive staining, and counter stained with Hematoxylin for contrast.

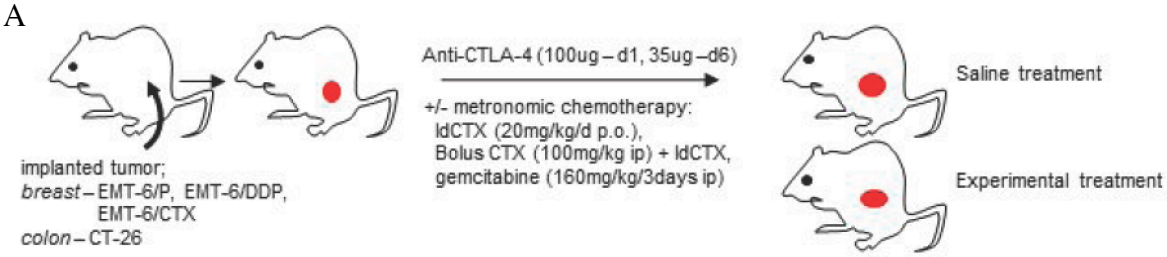
Statistical Analysis: The analysis of variance among groups (ANOVA), followed by the Student-Newman-Keuls test, was used to assess the statistical differences of data *in vivo*. Tumor therapy results are reported as mean ± SD. Survival curves were plotted by the method of Kaplan and Meier and were tested for survival differences using the log-rank test. The level of significance was set at $P<0.05$.

1.1.4 RESULTS

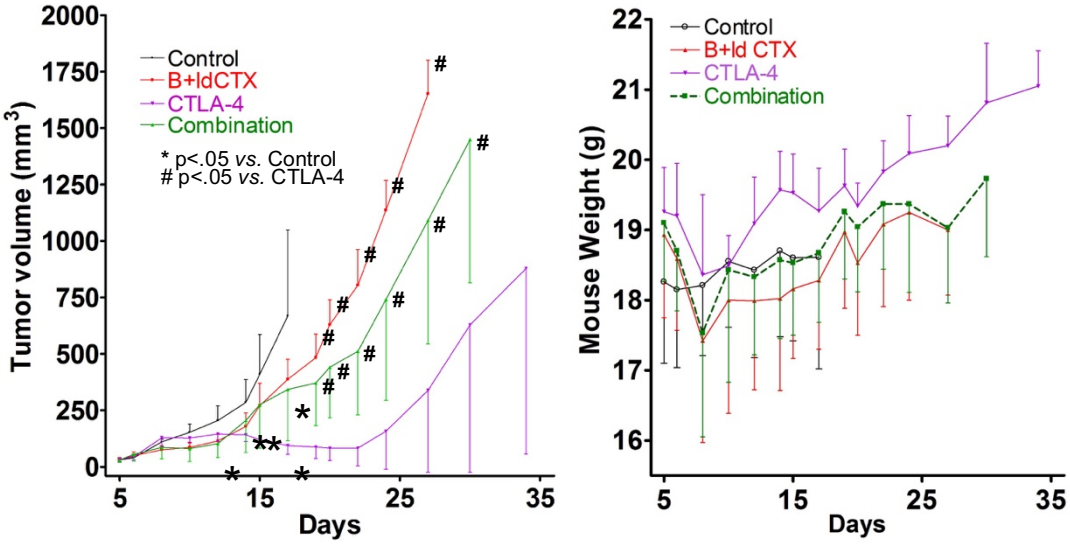
Anti-CTLA-4 therapy combined with bolus plus low-dose CTX: To evaluate whether metronomic CTX can be effectively combined with anti-CTLA-4 treatment, we tested a combination regimen on subcutaneously implanted EMT-6/P tumors (Fig. 1A and 1B). For the chemotherapy component, we sought to use a B+ldCTX protocol consisting of a Bolus CTX (given i.p. on day 1) plus low-dose CTX (20mg/kg/day, p.o.). Our choice was guided by our previous study (Shaked et al., 2005) in which the B+ldCTX protocol was shown to more effectively inhibit tumor growth than the sole low-dose CTX in the EMT6/P tumor, as well as in other tumor models. Mice (n=32) bearing EMT-6/P tumors were treated with saline (control), B+ld CTX, anti-CTLA-4 antibody, or with B+ldCTX plus anti-CTLA-4 antibody. Figure 1B shows the resulting impact

of the therapies on tumor growth. Thus, control treated tumors grew rapidly, the B+ldCTX treatment slowed down tumor growth, whereas anti-CTLA-4 antibody treatment caused tumor regressions over a 20 day period – followed by tumor relapses in the subsequent 15 days. Surprisingly, the B+ldCTX plus anti-CTLA-4 combination therapy did not produce tumor regressions and, furthermore, it produced a tumor growth rate that was only marginally slower than was observed with B+ldCTX alone. Thus, B+ldCTX significantly hinders the efficacy of antiCTLA-4 therapy in the EMT6/P tumor model. This was an unexpected finding, as we had recently reported that B+ldCTX could be effectively combined with an anti-VEGFR2 antibody[41], or with metronomic oral gemcitabine (LY2334737)[40]. We also noted that by day 22 the CTLA-4 antibody monotherapy resulted in complete tumor regression in 2 mice, one of which then began to show tumor regrowth a few days later. All anti-CTLA-4 treated tumors shrank after the therapy began, although tumor relapses were eventually observed in 6 out of 7 mice in this group.

1A



1B



1C

Statistical analysis of Event-free survival data (Fig 1c)

	Control	B+Id CTX	CTLA-4	Combination
Median Life	22	27	38	30
Control	1	0.0734	0.001	0.045
B+ Id CTX		1	<0.001	0.024
CTLA-4			1	0.010
Combination				1

Figure 1A-C. Impact of Bolus plus low-dose CTX combined with anti-CTLA-4 therapy on the growth of EMT-6/P tumors.

A) Schematic of evaluation of anti-CTLA-4 therapies with metronomic chemotherapy. EMT-6/P breast tumors, or CTX resistant (EMT-6/CTX) or DDP resistant (EMT-6/DDP) tumors were treated with anti-CTLA-4 antibody (administered on days 1 and 6). Chemotherapy regimens included low-dose metronomic CTX (ldCTX), Bolus plus ldCTX, or gemcitabine. Confirmatory studies were carried out with the murine CT-26 colon tumor. B) Murine EMT-6/P cells were implanted s.c. in female Balb/c mice. Therapies began when tumors were 50mm^3; the mice received control (n=8) saline (i.p.), anti-CTLA-4 (n=7), Bolus plus low-dose CTX (n=10), or the combination (n=7) of anti-CTLA-4 together with Bolus plus low-dose CTX. *P<0.05 vs. control, $^{\#}P$<0.05 vs. CTLA-4 (mean values ±SD). C) Mouse weights, as a measure of toxicity of the different treatments. D) Impact of the different therapies as assessed by analysis of event-free survival (Kaplan-Meier analysis), where duration of event-free survival is defined as time to primary tumor progression beyond 1,200 mm^3 or >15% weight loss. Significant event-free survival was observed with anti-CTLA-4 therapy, but this benefit was reduced by the addition of Bolus + low dose CTX. The sole survivor, by day 46, in the anti-CTLA-4 therapy group was still alive and tumor free at day 400 after tumor cell injection. *P<.05 was taken as statistical indication of difference vs. controls and between treated groups.

Therefore, anti-CTLA-4 therapy is effective in the EMT-6/P tumor model, but its therapeutic efficacy is significantly hampered by concurrent B+ldCTX treatment. To assess the relative toxicity of the therapies, we monitored body weights of the mice in the course of the experiment (as per our previous studies[32,39]. Figure 1C shows that the treatments that included a B+ldCTX component produced a short-term weight loss (as previously reported Shaked et al., 2005), followed by a gain in weight by the treated mice. We also plotted the tumor responses as a Kaplan-Meier plot (Fig. 1D), which shows that time to 50% event-free survival of CTLA-4 antibody treatment was 38 days, a significant increase compared to 22 days for the control group (P= 0.0011 CTLA-4 *vs* control). B+ldCTX had no significant impact on survival. Fig. 1D also shows that one anti-CTLA-4 antibody treated mouse, which had been bearing a palpable tumor in the first 2 weeks of this experiment, showed a tumor regression and remained tumor-free for the whole follow-up period. This mouse was still tumor-free 400 days later.

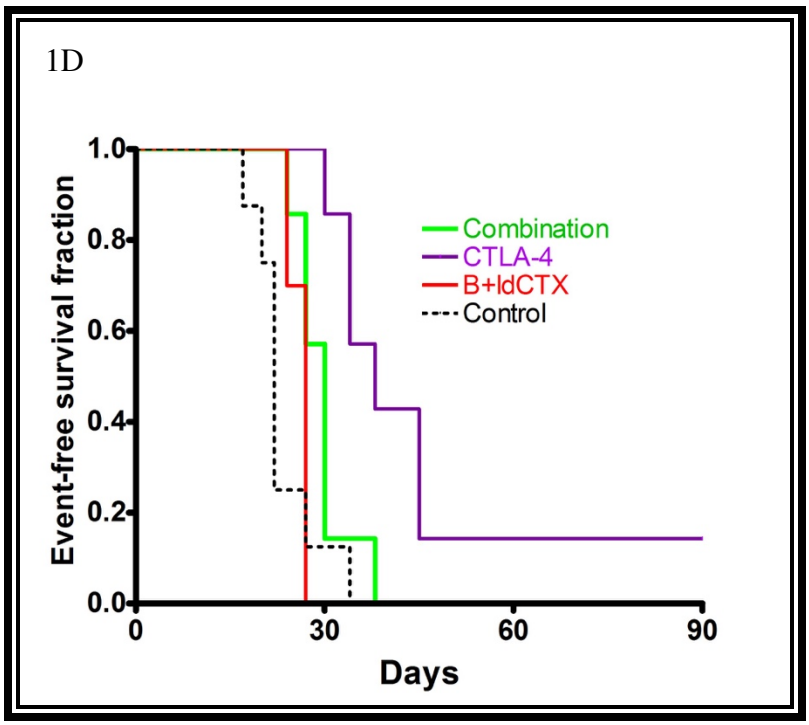


Figure 1D. Impact of the different therapies as assessed by analysis of event-free survival (Kaplan-Meier analysis), where duration of event-free survival is defined as time to primary tumor progression beyond 1,200 mm^3 or >15% weight loss.

Significant event-free survival was observed with anti-CTLA-4 therapy, but this benefit was reduced by the addition of Bolus + low dose CTX. The sole survivor, by day 46, in the anti-CTLA-4 therapy group was still alive and tumor free at day 400 after tumor cell injection. *P<.05 was taken as statistical indication of difference vs. controls and between treated groups.

Anti-CTLA-4 therapy combined with low-dose CTX, or with sequential gemcitabine therapy: We next decided to test whether we could incorporate other chemotherapy regimens, either in combination with or subsequent to the anti-CTLA-4 administration. We reasoned that since high-dose CTX can be immunosuppressive[5], the bolus CTX dose might impair the immune stimulating impact of the CTLA-4 blockade – thus negating its therapeutic benefit. Therefore, we either had to separate time of the dosing of chemotherapy from that of the anti-CTLA-4 antibody or omit the bolus CTX component. We chose to evaluate two additional strategies. One was to combine anti-CTLA-4 administration with low-dose CTX (i.e., without a bolus). This choice was based on reports that (in contrast to the high-dose CTX regimens) low-dose CTX can stimulate the

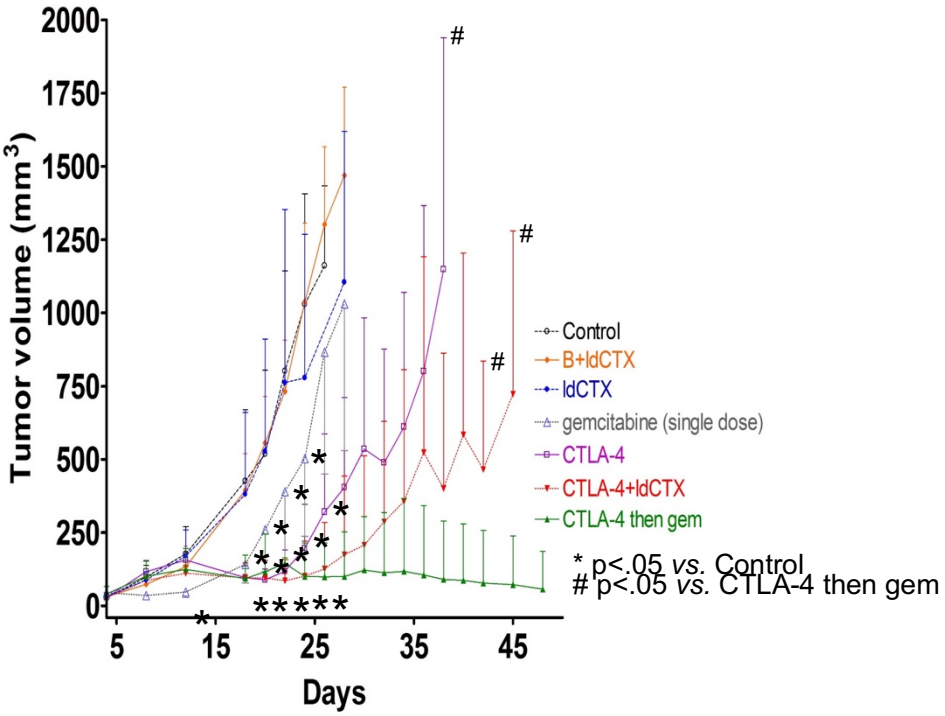
immune system[27,29,30]. Furthermore, in our previous studies, low-dose CTX effectively inhibited tumor growth without producing any obvious toxicity[40,42]. The second strategy involved the incorporation of a metronomic regimen of 160mg/kg gemcitabine given every 3 days, since we recently observed that it can produce remarkable tumor responses in a preclinical breast cancer model[32]. However, to avoid the possibility that gemcitabine might impair the therapeutic effect of the CTLA-4 blockade, we decided to administer the two drugs sequentially (i.e., after tumors began to regress following the anti-CTLA-4 treatment).

To evaluate these alternative therapies, we implanted EMT-6/P tumors into 43 mice, which were subsequently divided into 7 groups. Therapies began when all mice had established tumors and when the average tumor volume was approximately 50mm^3. We noted no or minimal impact of tumor growth, compared to controls, in the groups treated with ldCTX (Fig. 2A). We also noted that anti-CTLA-4 therapy led to a significant (initial) tumor regression in the first 2 weeks after therapy started, followed by tumor relapses. The combination of anti-CTLA-4 plus ldCTX (both co-administered from day 6 onwards) produced a greater inhibition of tumor growth that was observed with the anti-CTLA-4 monotherapy, although the results did not reach a statistical significance. The group treated with a single dose of gemcitabine unexpectedly showed weight loss in the days following treatment, and therefore this treatment was interrupted; the treatment caused initial inhibition of tumor growth, followed by tumor re-growth (Fig 2A), and a recovery of mouse body weight (Fig 2B). One group (treated with anti-CTLA-4 then Gem) initially received anti-CTLA-4 antibody, which again produced a tumor regression that lasted 2 weeks, followed by a tumor relapse (Fig. 2A). For this group, as soon as tumors started to relapse (i.e., around day 21), the second line therapy of gemcitabine was administered. In this group, we did not observe any weight loss after gemcitabine injection, and therefore gemcitabine treatment was continued. At the

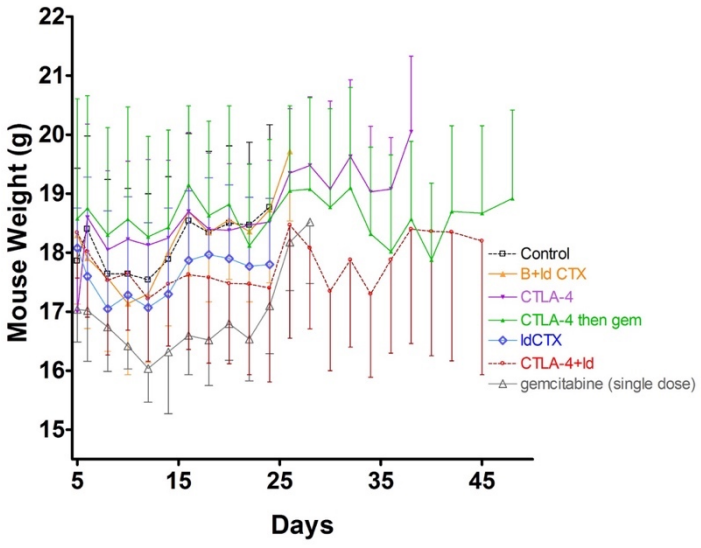
onset of the second-line gemcitabine therapy on day 21, all mice in this group had visible tumors and an average tumor volume of 145mm^3. Gemcitabine administration caused tumors to regress again, and by day 36 only two out of the six mice had any palpable tumors. These two mice eventually showed tumor re-growths, whilst under continuous gemcitabine therapy (Fig 2C). The remaining 4 mice showed significant (P<0.05) and complete tumor regression and are still tumor-free over 400 days later.

With regard to the relative toxicity of the tested therapies, other than the single gemcitabine treatment group as noted above, no significant changes in mouse weight were observed compared to controls (Fig. 2B). As noted in the subsequent studies (detailed below), we infrequently observed toxicity following gemcitabine injection, and this was typically resolved by allowing the mice a break from the treatment, as in our previous study[43]. No toxicity was observed in the CTLA-4 then gemcitabine group after the gemcitabine treatment started, and treatment continued for another 30 days without producing any obvious toxicity. We also performed a Kaplan-Meier analysis (Fig. 2C), which showed the observed event-free survival in 4 out of 6 mice in the CTLA-4 then gemcitabine group. We also noted long term event-free survival (>400 days) in 1 out of 6 mice for both the CTLA-4 monotherapy group and for the CTLA-4 plus ldCTX group. With regards to the CTLA-4 then gemcitabine treated group, we note that the 2 mice with visible tumors after day 36 eventually showed tumor relapses, and that one of these mice developed advanced lung metastases (whilst under gemcitabine therapy), and was sacrificed on day 107 (Supplementary Figure 1 – see appendix). These results show that although the anti-CTLA-4 then gemcitabine therapy is highly effective, tumor drug resistance eventually can develop in a subset of mice, and that metastatic disease (to the lungs) can develop, under therapy, in this tumor model.

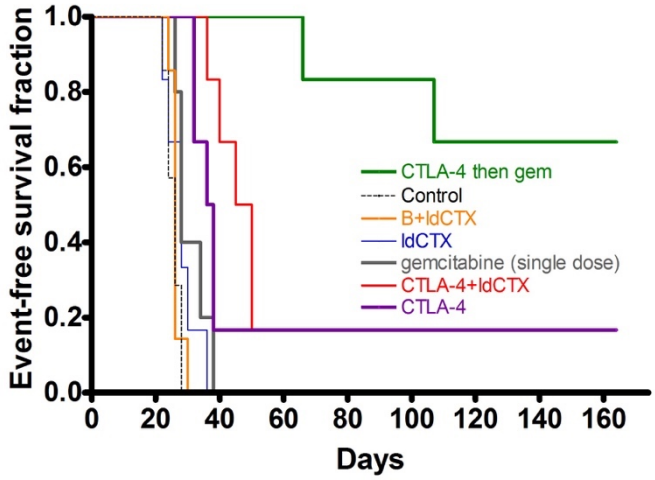
2A


Tumor volume (mm3)
2000
1750
1500
1250
1000
750
500
250
0
5
15
25
35
45
Days
Control
B+ldCTX
ldCTX
gemcitabine (single dose)
CTLA-4
CTLA-4+ldCTX
CTLA-4 then gem
* p<.05 vs. Control
# p<.05 vs. CTLA-4 then gem

2B


Mouse Weight (g)
22
21
20
19
18
17
16
15
5
15
25
35
45
Days
Control
B+ld CTX
CTLA-4
CTLA-4 then gem
ldCTX
CTLA-4+ld
gemcitabine (single dose)

2C



Statistical analysis of Event-free survival data (Fig 2c)

	Control	Id CTX	B+Id CTX	CTLA- 4 + Id CTX	CTLA-4	Gem	CTLA-4 + Gem
Median Life	26	28	26	47.5	37	28	>100
Control	1	**0.149**	**0.445**	<0.001	<0.001	0.036	<0.001
Id CTX		1	**0.363**	<0.001	0.008	**0.410**	<0.001
B+Id CTX			1	<0.001	<0.001	0.045	<0.001
CTLA- 4 + Id CTX				1	**0.242**	0.003	0.023
CTLA-4					1	**0.117**	0.024
Gem						1	<0.001
CTLA-4 + Gem							1

Figure 2. Effective combination of chemotherapy with anti-CTLA-4 therapy for the inhibition of the growth of EMT-6/P tumors.

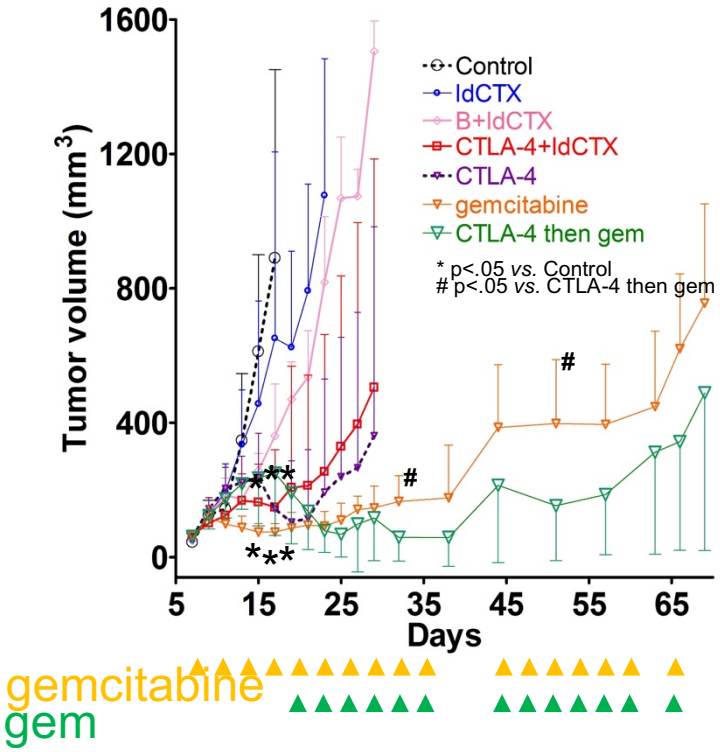
A) Murine EMT-6/P cells were implanted s.c. in female Balb/c mice. Therapies began when tumors were 50mm^3; the mice received control saline (i.p.; n=7), anti-CTLA-4 (n=6), Bolus plus low-dose CTX (n=7), a single dose of gemcitabine (160mg/kg; n=5), low-dose CTX (ldCTX; n=6), or the combination of anti-CTLA-4 plus ldCTX (n=6). One additional group received anti-CTLA-4 therapy as a first line treatment and then (when the tumors began to relapse around day 21) a second line therapy consisting of gemcitabine (160mg/kg every 3 days, i.p.; n=6), starting on day 21. *P<0.05 vs. control, $^{\#}P$<0.05 vs. CTLA-4 then gem (mean values ±SD). B) Mouse weights, as a measure of toxicity of the different treatments. C) Kaplan-Meier plot of event-free survival, where duration of event-free survival is defined as time to primary tumor progression beyond 1,200 mm^3 or >15% weight loss. *P<.05 was taken as statistical indication of difference vs. controls and between treated groups. Significant event-free survival was observed with anti-CTLA-4 therapy, and this benefit could be improved by the combination of anti-CTLA-4 therapy plus metronomic CTX, or by the sequential regimen using a first line of anti-CTLA-4 followed by gemcitabine chemotherapy on relapsing tumors.

Anti-CTLA-4 based therapies in EMT-6 tumors resistant to Cyclophosphamide (CTX): To evaluate the impact of selected CTLA-4 based therapies on drug resistant tumors, we implanted 50 mice with the EMT-6/CTX tumor, a population previously selected for in vivo resistance to CTX[44]. We noted a similar relative response to the different therapies as we had noted with the parental EMT-6/P tumor, although therapeutic benefit was reduced in the EMT-6/CTX drug resistant model (Fig. 3A). The initial administration of gemcitabine, either on its own or following CTLA-4 therapy, did not produce any immediate toxicity, and was continued. Only after more than 5 cycles of gemcitabine did we see some drop in mouse weights (particularly with gemcitabine alone), but this was readily resolved by adopting our previously reported strategy[43] of giving the mice short breaks in therapy (as shown by the arrows in Figures 3A/B). Gemcitabine monotherapy initially produced a significant ($P<0.05$) impact on tumor growth, but eventually all mice developed drug resistance (whilst under continuous therapy) as shown in Fig. 3C. Similarly, CTLA-4 therapy followed by gemcitabine treatment resulted in some tumors initially regressing, although in a number of mice drug resistance later developed under continuous gemcitabine therapy. 40% of mice in the CTLA-4 followed by gemcitabine therapy showed cures, whereas others developed drug resistance by day 80. The mice that did not develop resistance by day 80 were alive past day 200 – with no sign of tumor growth after cessation of therapy. Thus, although in drug resistant tumors (i.e., EMT-6-CTX) the CTLA-4 based therapies are less effective and drug resistance can readily emerge, we still noted a number of "cures" with the CTLA-4 based therapies. Furthermore, for those tumors that showed the development of drug resistance, CTLA-4 followed by gemcitabine therapy was the most effective regimen in delaying tumor re-growth although not statistically significant if compared to gemcitabine alone (with the exception of the volume measurement at day 50). Instead, Fig. 3C shows that CTLA-4 then gemcitabine treatment produced

a significantly longer survival compared to controls (P= 0.0002) and other treatments such as B+ldCTX (P= 0.0003) or gemcitabine alone (P= 0.0236). In contrast, metronomic CTX plus anti-CTLA-4 did not produce a significantly different response when compared to anti-CTLA-4 therapy alone.

Anti-CTLA-4 based therapies in EMT-6 tumors resistant to Cisplatin: Our results with the EMT-6/CTX model suggested that although metronomic CTX can be combined with CTLA-4 blockade, this regimen is not effective in tumors resistant to CTX. To further explore how drug resistant tumors respond to CTLA-4 based therapies, we evaluated their impact on the EMT-6/DDP model, a variant selected for resistance to cisplatin treatment in vivo[44]. Fig 4A shows that in this drug resistant tumor the CTLA-4 plus ldCTX had a greater anti-tumor effect than CTLA-4, although the difference was not statistically significant. The gemcitabine monotherapy was initially very effective, and did not produce toxicity, although all the mice eventually had tumors that became resistant to this therapy. The greatest anti-tumor effect was obtained with CTLA-4 followed by gemcitabine, and in this case, we also did not see significant toxicity with the gemcitabine therapy (Figures 4A/B). Moreover, also in terms of survival this combination determined a significant (P= 0.0018) benefit if compared to controls or CTLA-4 alone (P= 0.0010) (Figure 4C). In this aggressive EMT-6/DDP model, we did not observe any CTLA-4 blockade induced tumor regression, but only a growth delay. Consequently, the second line gemcitabine therapy started on day 19, when the data showed that tumors were no longer growth delayed. These results confirm the effectiveness of combining some chemotherapy regimens with anti-CTLA-4 therapy, but they also indicate that the use of chemotherapy naïve tumors (e.g. EMT-6/P) may produce overly optimistic results on the therapeutic benefit of some combination therapies.

3A


1600
1200
800
400
0
Tumor volume (mm3)
5
15
25
35
45
55
65
Days
Control
IdCTX
B+IdCTX
CTLA-4+IdCTX
CTLA-4
gemcitabine
CTLA-4 then gem
* p<.05 vs. Control
# p<.05 vs. CTLA-4 then gem
*
**
***
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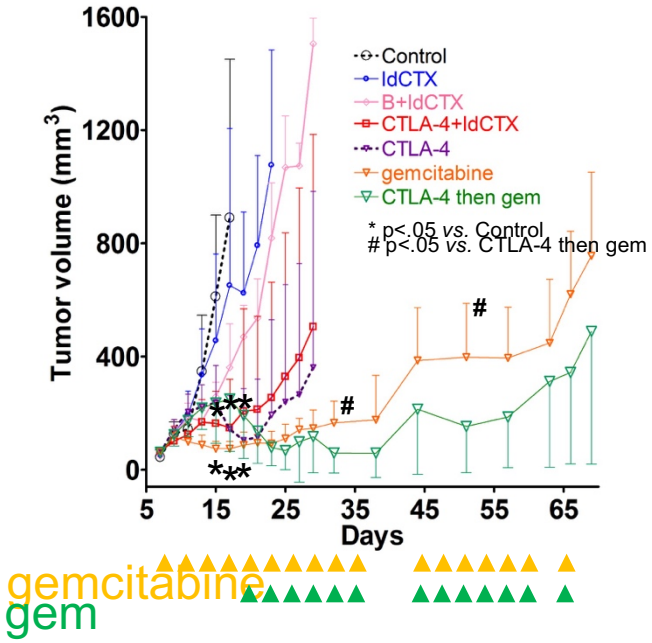
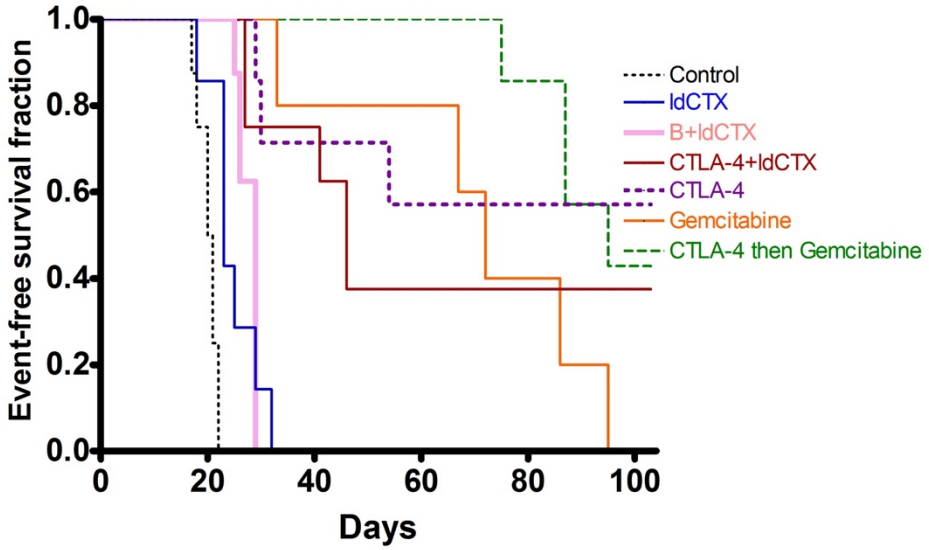
gemcitabine
gem

3B


1600
1200
800
400
0
Tumor volume (mm3)
5
15
25
35
45
55
65
Days
Control
IdCTX
B+IdCTX
CTLA-4+IdCTX
CTLA-4
gemcitabine
CTLA-4 then gem
* p<.05 vs. Control
# p<.05 vs. CTLA-4 then gem
#
#
**
**
gemcitabine
gem

3C



Statistical analysis of Event-free survival data (Fig 3c)

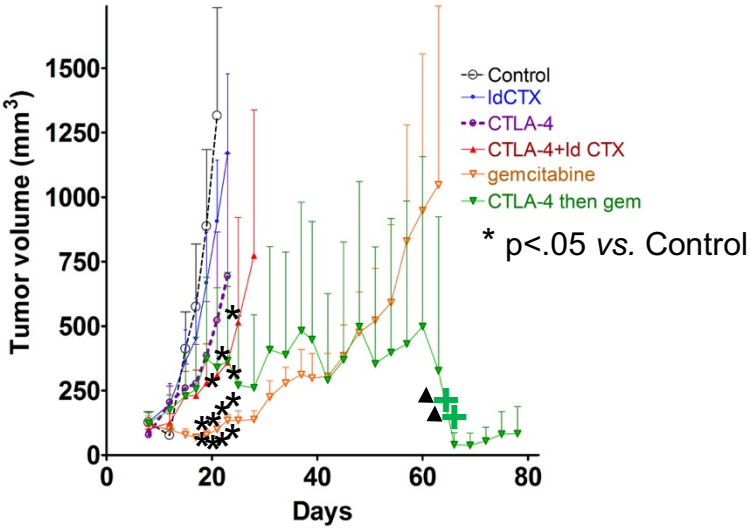
	Control	Id CTX	B+Id CTX	CTLA- 4 + Id CTX	CTLA-4	Gem	CTLA-4 + Gem
Median Life	20.5	23	29	46	>100	72	95
Control	1	0.003	<0.001	<0.001	<0.001	0.001	<0.001
Id CTX		1	**0.241**	0.001	0.002	0.001	<0.001
B+Id CTX			1	0.010	0.001	0.002	<0.001
CTLA- 4 + Id CTX				1	**0.444**	**0.612**	**0.379**
CTLA-4					1	**0.207**	**0.974**
Gem						1	0.024
CTLA-4 + Gem							1

Figure 3. Combination of chemotherapy with anti-CTLA-4 therapy for the inhibition of the growth of EMT-6/CTX tumors, including gemcitabine administration (as indicated by the green and orange arrows).

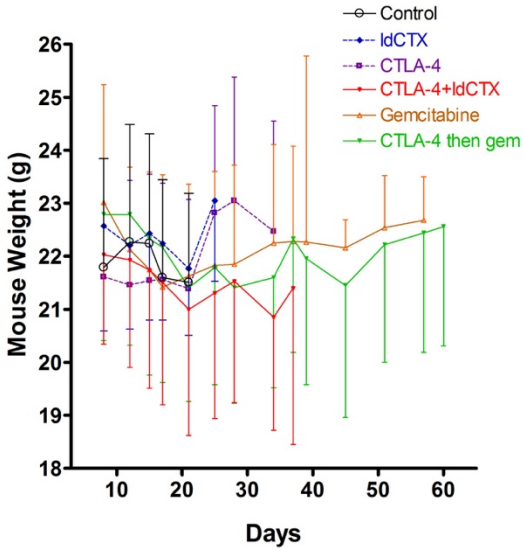
Murine EMT-6/CTX cells were implanted s.c. in female Balb/c mice. Therapies began when tumors were 50mm^3; the mice received control saline (i.p.; n=8), anti-CTLA-4 (n=7), gemcitabine (160mg/kg every 3 days, i.p.; n=5), Bolus plus low-dose CTX (n=8), metronomic CTX (n=7), or the combination of anti-CTLA-4 plus metronomic CTX (n=8). One additional group received anti-CTLA-4 therapy as a first line treatment and then a second line therapy consisting of gemcitabine (160mg/kg every 3 days, i.p.; n=7). *P<0.05 vs. control (on days 13, 15, and 17, P<0.05 for gemcitabine, CTLA-4+ldCTX and B+ldCTX; whereas for CTLA-4 and for CTLA-4 then gem, P<0.05 on days 15

and 17), $^{\#}P<0.05$ vs. CTLA-4 then gem (mean values ±SD). B) Mouse weights, as a measure of toxicity of the different treatments (θ indicates significant toxicity caused by gemcitabine treatment). C) Kaplan-Meier plot of event-free survival, where duration of event-free survival is defined as time to primary tumor progression beyond 1,200 mm^3 or >15% weight loss. $^{*}P<.05$ was taken as statistical indication of difference vs. controls and between treated groups. Significant event-free survival was observed with anti-CTLA-4 therapy, and this benefit could be improved by the sequential regimen using a first line of anti-CTLA-4 followed by gemcitabine chemotherapy on relapsing tumors. No significant difference was observed between antiCTLA-4 therapy and combination of anti-CTLA-4 therapy plus metronomic CTX.

4A


Tumor volume (mm3)
1500
1250
1000
750
500
250
0
0
20
40
60
80
Days
Control
ldCTX
CTLA-4
CTLA-4+ld CTX
gemcitabine
CTLA-4 then gem
* p<.05 vs. Control

4B


Mouse Weight (g)
26
25
24
23
22
21
20
19
18
10
20
30
40
50
60
Days
Control
ldCTX
CTLA-4
CTLA-4+ldCTX
Gemcitabine
CTLA-4 then gem

4C

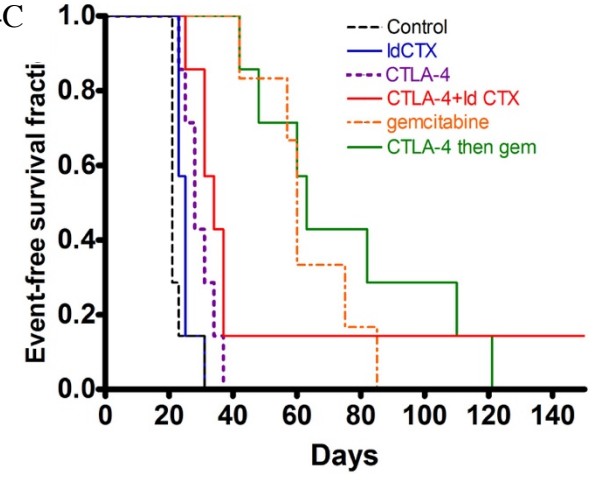

Event-free survival fracti
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
Days
Control
ldCTX
CTLA-4
CTLA-4+ld CTX
gemcitabine
CTLA-4 then gem

Figure 4. Combination of chemotherapy with anti-CTLA-4 therapy for the inhibition of the growth of EMT-6/DDP tumors.

A) Murine EMT-6/DDP cells were implanted s.c. in female Balb/c mice. Therapies began when tumors were 50mm^3; the mice received control saline (i.p.; n=7), anti-CTLA-4 (n=7), gemcitabine (160mg/kg every 3 days, i.p.; n=6), low-dose CTX (ldCTX; n=7), or the combination of anti-CTLA-4 plus ldCTX (n=7). One additional group received anti-CTLA-4 therapy as a first line treatment and then a second line therapy consisting of gemcitabine (160mg/kg every 3 days, i.p.; n=7). *P<0.05 vs. control (mean values ±SD). B) Mouse weights, as a measure of toxicity of the different treatments. C) Kaplan-Meier plot of event-free survival, where duration of event-free survival is defined as time to primary tumor progression beyond 1,200 mm^3 or >15% weight loss. *P<.05 was taken as statistical indication of difference vs. controls and between treated groups. Event-free survival was observed with anti-CTLA-4 therapy, and this benefit could be improved or by the sequential regimen using a first line of antiCTLA-4 followed by gemcitabine chemotherapy on relapsing tumors (note that, for this group, two green **+** signs indicate two mice with large tumors sacrificed around day 60, revealing a subgroup of mice with tumors that were very responsive to therapy).

CTLA-4 therapies on orthotopically implanted tumors: Our results raised a number of questions: 1) Are the tumor responses also observable in orthotopic tumors?, 2) can the results be replicated in a tumor model other than the EMT-6, and 3) is metronomic chemotherapy more effective when given in conjunction with anti-CTLA-4, or should it be given sequentially? To answer the first question, we implanted EMT-6/P and EMT-6/DDP in the inguinal mammary fat pad, as previously described[40,41], and then evaluated our most effective therapy (i.e., anti-CTLA4 with sequential gemcitabine). Anti-CTLA-4 produced a tumor growth delay in both models (Fig. 5A and 5B), and the sequential addition of gemcitabine produced an initial tumor regression. Due to the rapid growth of these orthotopic tumors, it was difficult to compare gemcitabine alone with anti-CTLA followed by gemcitabine, since the latter sequential treatment started with larger tumors (i.e., 400mm^3) following the end of CTLA-4 administration (i.e., around day 16 in both Fig 5A and 5B). Nonetheless, in spite of this challenge, the response obtained with the EMT-6/P and EMT-6/DDP models was consistent with our results with these tumors when grown subcutaneously (Figs. 3 and 5). Thus, EMT-6/P respond to CTLA-4 with sequential gemcitabine therapy, but the drug resistant EMT-6/DDP are less responsive to this regimen.

Evaluation of therapies in CT-26 tumors: Our preliminary studies with the murine CT26 colon tumor (Suppl. Figs. 2 and 3 – see appendix) showed that it responded to CTLA-4 therapies, and to gemcitabine. We therefore implanted CT-26 cells s.c. in Balb/c mice and evaluated our most effective combination therapy from our experiments with the EMT-6/P tumor (Fig. 2). As shown in Fig. 6, while CTLA-4 or gemcitabine monotherapies inhibited CT-26 tumor growth, the administration of CTLA-4 followed by metronomic gemcitabine led to tumor regression.

Evaluation of combination versus sequential treatment: To test whether gemcitabine is more effective than CTX as a drug partner for CTLA-4 therapies, and whether sequential chemotherapy is better than combination therapy, we implanted the highly responsive EMT-6/P tumor and evaluated different CTLA-4 based therapies. As shown in Fig. 7, anti-CTLA-4 is more effective when it is combined with gemcitabine - given either sequentially, or concomitantly - than when it is combined with metronomic CTX (Fig. 7). Therefore, our data suggest that the drug partner for CTLA-4 (i.e., gemcitabine) is more critical for effective anti-tumor response than is the schedule by which the drugs are given.

Intratumoral CD31 staining and Assessment of immune memory: To evaluate the impact of the different therapies on intratumoral blood vessel distribution (as a relative measure of angiogenic activity within treated tumors), we reassessed the data in Figures 1 and 2, and noted that maximal therapeutic response was noted 9-12 days after treatment started. This, taking note of the data in Fig. 2, we implanted EMT-6 tumors s.c., and then administered the different therapies (using 5-7 mice per group, following the same regimens shown in Figure 2) when tumors reached an average size of 200mm^3. Thereafter tumors were measured daily, and the mice were sacrificed, and the tumors excised after 7-12 days of therapy. At this termination point, tumors were 400-500mm^3 in size. Excised tumors were paraffin embedded and then evaluated for CD31 staining

(Fig. 8A). Analysis of the results (Fig. 8B) showed that, as expected, low-dose CTX caused a relative reduction in intratumoral CD31 staining compared to controls - but no significant differences in staining were observed in tumors that received CTLA-4 based therapies. In a number of our experiments (shown in Figures 2-5), a few mice were cured by antiCTLA-4 or combination therapies, and a number of mice survived for more than 500 days (some of the mice died of old age, after 400 or more days, without evidence of tumor regrowth). These results provided us with a very small pool of animals that had been cured of the implanted tumor, and these were used in tumor re-challenge experiments, where each mouse was given a second s.c. injection of originally implanted tumor cell line. Our results show that in 11/14 cases, the mice rejected the re-implanted tumor (injected between 60-500 days after the first tumor implant), suggesting that CTLA-4 combination therapies can produce tumor responses that are accompanied by the establishment of immune memory. These observations are consistent with previously published data on anti-CTLA-4 therapies[34].

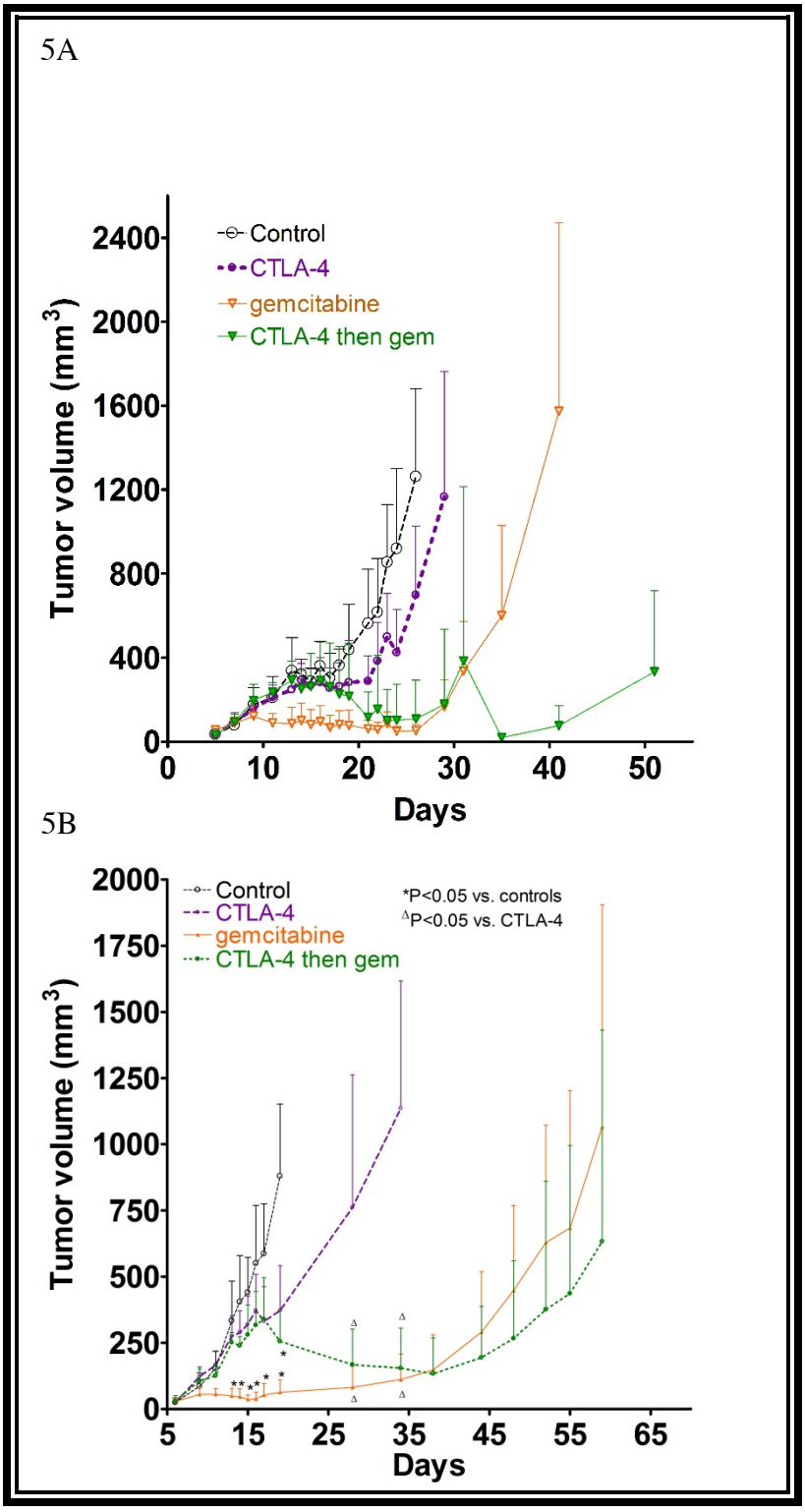


Figure 5. Anti-CTLA-4 therapy plus gemcitabine inhibits orthotopic EMT-6 tumors.

A) Murine EMT-6/P cells were implanted into the mammary fat pad of female Balb/c mice. The mice received control (n=4) saline (i.p.), anti-CTLA-4 (n=5), gemcitabine (n=5), or anti-CTLA-4 therapy as a first line treatment followed by second line therapy consisting of gemcitabine (160mg/kg every 3 days, i.p.; n=5). B) Murine EMT-6/DDP cells were implanted (n=10 mice per group) into the mammary fat pad of female Balb/c and treated with the same therapies described above. *P<0.05 vs. control, $^{\#}P$<0.05 vs. CTLA4 then gem (mean values ±SD).

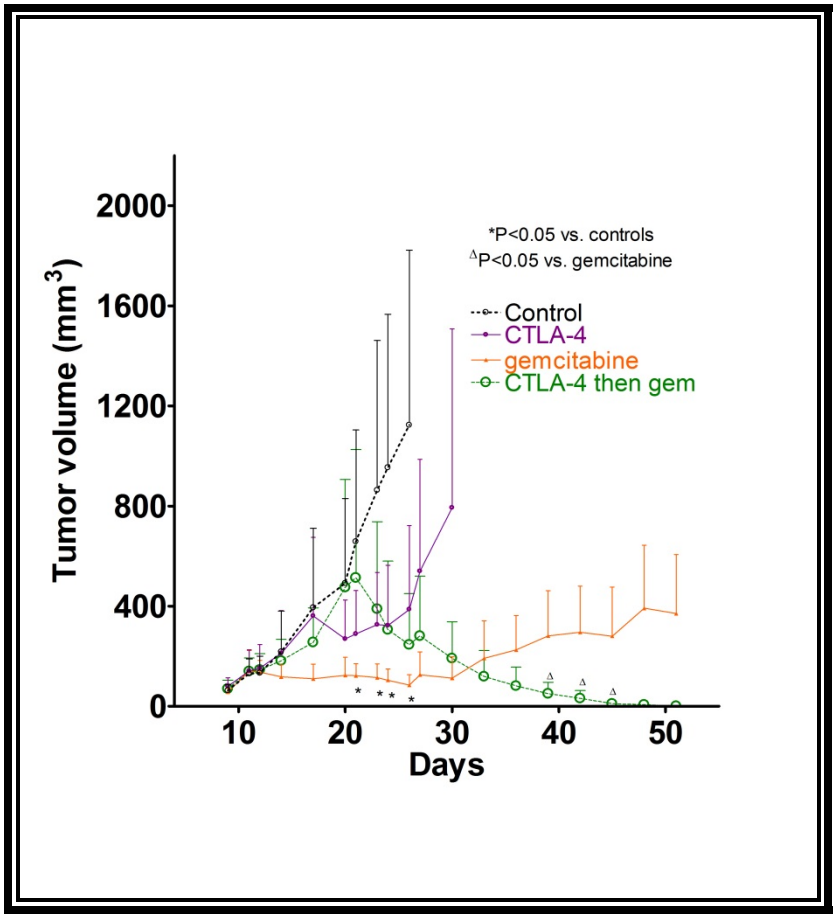


Figure 6. Impact of anti-CTLA-4 therapies on CT-26 colon tumors.

Murine CT-26 cells were implanted s.c. into female Balb/c mice. The mice received control saline (i.p.; n=7), anti-CTLA-4 (n=7), gemcitabine (n=8), or anti-CTLA-4 therapy as a first line treatment followed by second line therapy consisting of gemcitabine (160mg/kg every 3 days, i.p.) n=8). The anti-CTLA-4 treatment, and the gemcitabine treatment inhibited tumor growth, while anti-CTLA-4 followed by gemcitabine led to tumor regression. *P<0.05 vs. control, $^{\#}P$<0.05 vs. CTLA-4 then gem (mean values ±SD).

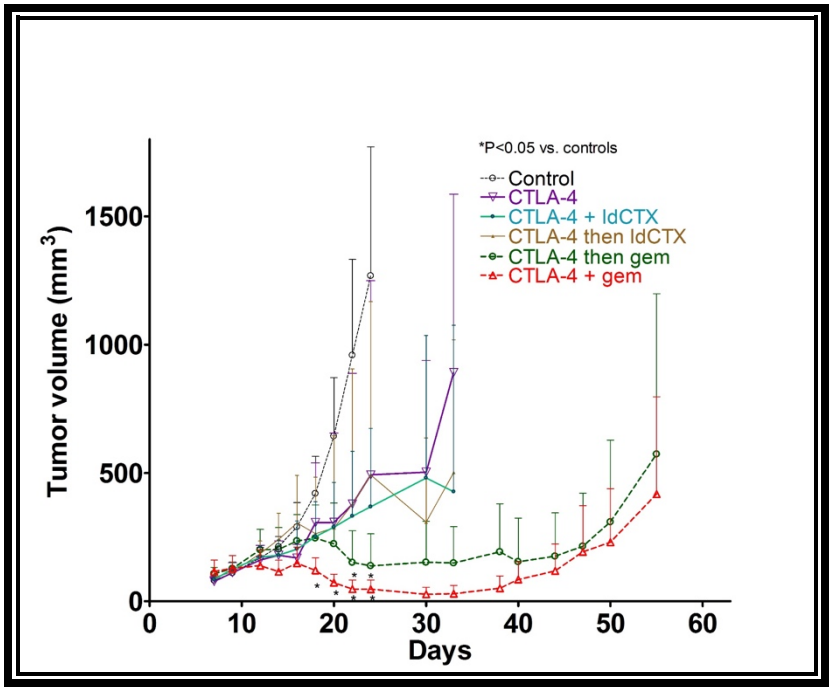


Figure 7. Evaluation of different anti-CTLA-4 combination therapies on EMT-6/P tumor growth.

Murine EMT-6/P cells were implanted s.c. into female Balb/c mice (n=7 per group). The mice received control saline (i.p.), anti-CTLA-4, or anti-CTLA-4 followed by second line therapy consisting of gemcitabine (160mg/kg every 3 days, i.p.; CTLA-4 then gem), or anti-CTLA-4 therapy given concomitantly to gemcitabine therapy (CTLA4 + gem). In addition, one group received anti-CTLA-4 followed by second line therapy consisting of CTX (20mg/kg/day, p.o.; CTLA-4 then CTX) or anti-CTLA-4 therapy with concomitant CTX therapy (CTLA-4 + CTX). The results show that gemcitabine is a more effective therapeutic partner for anti-CTLA-4 than CTX, irrespective of whether the administration of gemcitabine is sequential or concomitant to the anti-CTLA-4. $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. CTLA-4 then gem (mean values ±SD). Gemcitabine led to tumor regression.

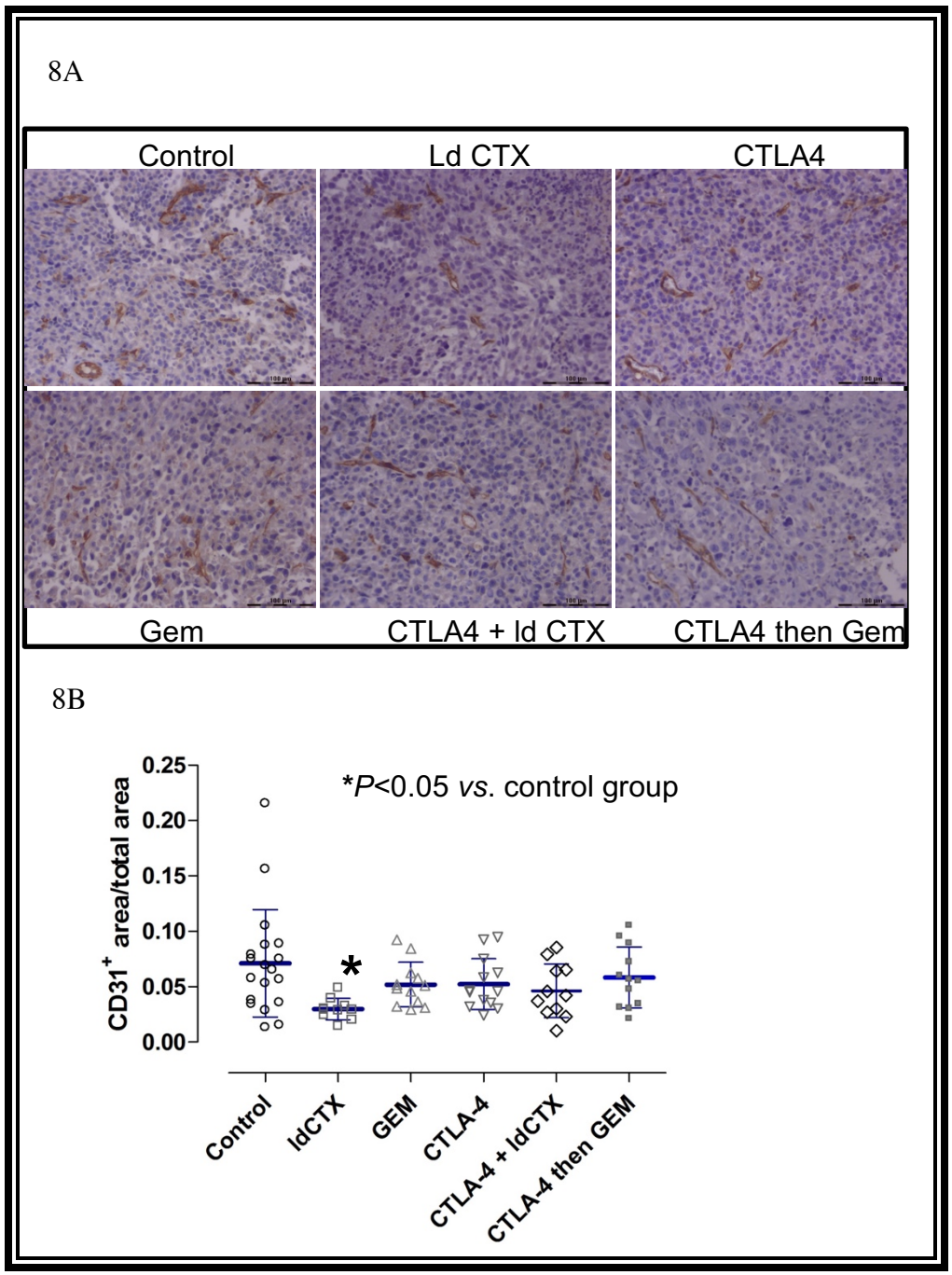


Figure 8. Analysis of CD31-positive staining in EMT-6 tumor treated with anti-CTLA-4 therapies.

EMT-6/P tumors were implanted s.c. into female Balb/c mice. Tumor bearing mice were treated with (i.e., a repeat of the experiment shown in Figure 2) saline control, CTLA-4, metronomic CTX (ld CTX), gemcitabine (Gem), or CTLA-4 plus ld CTX, or CTLA-4 with sequential Gemcitabine. The experiment was terminated as the tumors were starting to respond to the different therapies, as assessed by caliper measurements.

1.1.5 DISCUSSION

A number of immunotherapy approaches have in recent years translated into significant increased survival in patients with cancers such as melanoma. For example, targeting CTLA-4 using ipilimumab is used for the treatment of non-resectable metastatic melanoma, and clinical trials are ongoing to test its use for the treatment of other malignancies, including lung cancer, prostate cancer, and breast cancer (www.clinicaltrials.gov). Furthermore, a number of active clinical trials are evaluating different combinations of chemotherapy and ipilimumab in melanomas and other types of cancer[23]. We previously reported extensively on experimental therapeutic studies of metronomic chemotherapy[32,40,42,43,45–49], including the use of metronomic CTX chemotherapy[50] with an upfront bolus CTX dose[31], and the use of sequential chemotherapy regimens, as well as second line therapies[39,49]. The proposed mechanisms of action for metronomic chemotherapy are many[29,48,50,51], and they include inhibition of angiogenesis and inhibition of cancer stem cell growth. They also include activation of the immune system[27,29,30]. With regards to the latter, this has been documented for CTX (and for gemcitabine[33], and it remains to be determined the extent to which metronomic dosing of other clinically used chemotherapy drugs can also activate the immune system.

Since ipilimumab therapy is directed at immune activation (via inhibition of suppressor T-cells), and since combinations of ipilimumab with chemotherapy are being evaluated clinically, we sought to investigate whether the immune activation activity of CTLA-4 blocking could be augmented by the addition of metronomic CTX. Such combinations could provide data relevant to current clinical trials, such as the recently reported phase III trials of metronomic chemotherapy[52,53]. For example, treatment with CTLA-4 might be followed by a metronomic maintenance treatment, given the clinical low toxicity profile of metronomic chemotherapy[29,50] , as we have also reported[54–56]. We sought to use what, according to our previous studies, was the

most effective metronomic-type CTX regimen; a protocol[31] involving an upfront bolus (B) CTX dose, immediately followed by a metronomic CTX (ldCTX) regimen of adding CTX to the mice's drinking water[42]. To our surprise, this B+ldCTX approach actually caused a less effective tumor response than the anti-CTLA-4 monotherapy alone. Therefore, our results serve as a cautionary note against the use of a bolus plus metronomic CTX component in therapies involving a CTLA-4 blockade.

We previously reported that bolus plus metronomic CTX could be improved by the addition of a targeting agent such as the anti-VEGFR2 antibody DC101[41], or by the addition of metronomic LY2334737, an oral gemcitabine pro-drug[57].

However, we had hitherto not yet observed that the bolus plus metronomic CTX could hinder the anti-tumor efficacy of a targeted therapy, or of other anti-tumor strategies. One possible interpretation of our results is that the bolus (highdose) CTX, in our B+ldCTX regimen, is immunosuppressive thus blunting the therapeutic effect of anti-CTLA-4. We consequently sought to test two alternative strategies. One was to omit the bolus upfront CTX dose and administer anti-CTLA-4 together with metronomic CTX. The second was to adopt gemcitabine chemotherapy (160mg/kg every 3days) since we recently reported that it produces notable responses in a LM2-4 preclinical breast cancer model[57]. However, to avoid the possibility that gemcitabine administration would impair the CTLA-4 blocking strategy, we chose to separate its administration from that of the CTLA-4 antibody. Thus, we either combined a CTLA-4 with a sequential gemcitabine therapy, or we co-administered CTLA-4 antibody together with metronomic CTX. The results we obtained suggest that both strategies can improve a CTLA-4 monotherapy regimen, with the sequential gemcitabine therapy generating the more potent anti-tumor responses in the EMT-6/P model. Similar results were obtained with the CTX resistant EMT-6/CTX and cisplatin

resistant EMT-6/DDP models, although in the drug resistant models the benefits of these therapies were less evident and drug resistance to gemcitabine readily arose.

The reduced sensitivity of the EMT-6/DDP tumor to CTLA-4 with sequential gemcitabine therapy, compared to the response seen with the parent EMT-6/P tumor, was confirmed in orthotopically implanted tumors. In addition, the effectiveness of the same sequential therapy was confirmed in CT-26 tumors. Furthermore, our data shows that the effectiveness of CTLA-4 with sequential gemcitabine does not impact the relative intratumoral CD31 staining in EMT-6/P tumors, and we subsequently found that gemcitabine is equally effective irrespective of whether it is given sequentially or concomitantly with anti-CLTA-4 therapy. The intratumoral CD31 staining data we obtained are consistent with our previous study[57] showing that metronomic gemcitabine (and metronomic oral gemcitabine prodrug) can inhibit tumor growth without impacting systemic angiogenesis. Overall, there are five aspects of this work that deserve to be highlighted: 1) bolus plus low dose CTX can impair the antitumor efficacy of anti-CTLA-4 therapy. 2) Metronomic CTX, or metronomic gemcitabine, can effectively be combined with anti-CTLA-4 therapy and, 3) such combination therapies are also active against drug resistant tumors (e.g., EMT-6/CTX and EMT-6/DDP). 4) The efficacy of chemotherapy plus anti-CTLA-4 is not dependent on the schedule of drug administration and, 5) produces anti-tumor effect in the absence of significant changes in intratumoral blood vessel distribution.

Our results also suggest that alternative anti-tumor mechanisms are involved, including (as we previously suggested (Francia et al., 2012)), the direct targeting of tumor cells by the frequent (i.e., every 3 days) gemcitabine administration. Future studies will have to determine whether anti-CTLA-4 therapy impairs the induction of thromobospondin-1 by metronomic chemotherapy[51], and determine if such combinations can activate the immune system. That could be assessed by testing

whether such therapies result in an increase in intratumoral CD4 and CD8 staining, which would be consistent with studies showing activation of the immune system by metronomic chemotherapy[27]. These results may be of interest to clinicians and translational researchers that are studying means of improving anti-CLTA-4 therapy, and they also caution that these therapies may be less effective in drug resistant tumors. We did encounter a few cases of toxicity associated with gemcitabine administration, which may have been a consequence of tumor lysis syndrome, which has been reported by a few studies for this drug[58]. However, in our experience, these problems were easily overcome by giving the mice short drug-free breaks, a procedure that is not infrequent with cancer patients[59], as we previously reported with metronomic sorafenib in a preclinical model[43].

We had expected that B+ldCTX would increase the efficacy of anti-CTLA-4 therapy – but our results proved otherwise. Nonetheless, we also observed that some chemotherapy regimens can be effectively combined with anti-CTLA-4 therapy. Our results are in agreement with studies by other groups; such as those by Mokyr et al., who showed that low-dose melphalan can be effectively combined with antiCTLA-4 therapy, and of Lesterhuis et al., who recently reported that anti-CTLA-4 can be co-administered with gemcitabine to produce significant antitumor responses. Similarly, Jure-Kunkel et al., recently showed the effective combination of anti-CTLA-4 plus chemotherapy, involving the injection of the anti-CTLA-4 antibody one day after the administration of chemotherapy (including gemcitabine). We had not initially considered such a regimen, because of our disappointing results with the B+ldCTX. In contrast to the aforementioned studies, one evident difference in our work is the inclusion of tumor variants selected in vivo for resistance to alkylating agents[44], which we have previously used to study mechanisms of tumor drug resistance[60]. These variants can be used to model the clinical situation where patients are

eligible for immunotherapy following tumor relapses under standard chemotherapy regimens. In such cases, the response to immunotherapy might differ from that of chemotherapy-naïve tumors. In that regard, the EMT-6/CTX and EMT-6/DDP variants produced less pronounced therapeutic benefits than the EMT-6/P tumor, a result that highlights how the sole use of chemotherapy naïve tumors may exaggerate the potential preclinical benefit of a therapy. That is analogous to our observation that preclinical primary tumor models may in some cases exaggerate the impact of a therapy on the more clinically relevant metastatic disease[32]. In that respect, a number of mice in this study eventually succumbed to spontaneous metastases.

Overall, results obtained in this study show that although chemotherapy can augment the impact of anti-CTLA-4 therapy, caution is necessary in the design of such combinations, as some may be counterproductive.

Chapter 2: Alternative Therapies: Low Dose Chemotherapy

Conventional chemotherapy regimens are administered at maximum tolerated doses (MTD) which target all dividing cells and cannot differentiate between normal and cancerous cells. MTD require obligatory rest periods between doses to allow the patient to recover from the toxicity associated with such regimen. Cytotoxic agents used as chemotherapeutic drugs may be given intravenously or by mouth (before or after removal of primary tumor) as single agents or in combination. Gemcitabine and cyclophosphamide are some of the most common drugs used for the treatment of breast cancer (i.e., early and metastatic breast cancer), however, their efficacy is hindered by the lack of response to therapy either through acquired or inherent resistance (e.g., selection of resistant cell population due to a new event or pre-existing events) and often leads to dissemination of disease.

Resistance to *in vitro* models has also been seen where cancer cells are able to find alternative ways to maintain their proliferative profile. For example, upregulation of HER-2 receptor has been observed after treatment with cetuximab[61]. One way to study resistance to certain therapies *in vitro* is to established drug-resistant cell lines using dose-escalation methods. Different drug-resistant clones are then selected and tested against the parental cell line to determine the fold resistance[62]. One disadvantage of generating *in vitro* drug-resistant variants is the lack of clinical relevance (e.g., drug administration regimens, and drug levels not achievable)[63,64]. *In vivo* development of drug resistant variants can be done by implanting tumors into mice and treated them with therapy. After a couple of doses (and after the obvious emergence of resistance when therapy has failed), the tumors are resected and re-implanted into new animals thus becoming a more aggressive tumor variant when compared to the parental tumor. In this dissertation study, *in*

vivo selection of EMT-6 drug resistance variants (e.g., EMT-6/CDDP, and EMT-6/CTX) were obtained by repeated exposure to CDDP and CTX for 24hrs before passage of tumor into new animals[37,38]. The overall impact of establishing drug-resistant variants is that it can help researchers study any differences associated with different signaling pathways that are activated (or suppressed) particularly on the new derived variants. [Valenzuela, Parra et al unpublished data] Although tumors were resistant to therapy *in vivo*, they were not resistant when grown in monolayer cultures. This suggests that *in vivo* drug-resistance in mice is aided by the host microenvironment and interactions[37] such as endothelial cells. Paper published in Cancer Lett. 2017 Aug 1;400:311-318. – see appendix.

2.1 Low-dose Chemotherapy: Angiogenesis and the Immune System

In contrast to MTD, metronomic or continuous low-dose chemotherapy involves the administration of anti-cancer drugs at doses that are much lower (e.g., 1/20th of the MTD, or lower) and often given daily. Metronomic chemotherapy can be either used as a stand-alone therapy, or in combination with other chemotherapies. It has also been used as maintenance therapy. One of the main proposed mechanisms for metronomic chemotherapy is the elimination of dividing endothelial cells present in the blood vessels irrigating a tumor. The main assumptions driving the original development of metronomic chemotherapy were that a) targeting the tumor endothelial would starve the tumor of its blood supply and b) the targeting of genetically stable endothelial cells, these would not likely mutate into a drug resistant population[50]. Browder et al., demonstrated that the frequency by which chemotherapy was administered was a key factor to achieve an anti-angiogenic effect. They showed that endothelial cells were severely damage when CTX was administered at high doses with regular breaks.

However, the endothelial cells are able to recover (or repair) during the drug breaks that are part of MTD schedules, thus negating an overall anti-angiogenic effect. Browder then lowered the administration dose of CTX, and increased the frequency (to 3 times per week). This resulted in remarkable tumor regressions in vivo. Browder also demonstrated that metronomic CTX was able to regress tumor population that had been selected for resistance to CTX, thus suggesting that metronomic chemotherapy is effective in drug-resistant cancers (Browder et al., 2000). In a separate study, the combination of metronomic CTX and metronomic UFT, an oral 5-fluorouracil prodrug, given through gavage administration in immunodeficient mice also resulted in tumor eradication in mice that had established metastases[65]. These therapeutic benefits were also observed clinically in patients with non-small-cell lung cancer who stopped responding to conventional therapy and were switched to lower doses administered intermittently[66], and in breast cancer patients treated with metronomic CTX [52].

2.1.1 MECHANISMS OF RESISTANCE TO METRONOMIC CHEMOTHERAPY

Therapeutic resistance is amongst the major determinants of cancer mortality. Contrary to initial expectations, antivascular therapies are equally prone to inherent or acquired resistance as other cancer treatment modalities. However, studies into resistance to vascular endothelial growth factor pathway inhibitors revealed distinct mechanisms of resistance compared to conventional cytotoxic therapy. While some of these novel mechanisms of resistance also appear to be functional regarding metronomic chemotherapy, herein we summarize available evidence for mechanisms of resistance specifically described in the context of metronomic chemotherapy. Numerous preclinically identified molecular targets and pathways represent promising avenues to overcome resistance and enhance the benefits achieved with metronomic chemotherapy

eventually. However, there are considerable challenges to clinically translate the preclinical findings.

Twenty years after the late Judah Folkman had described the conceptual framework of antivascular tumor therapy, in the 1990's vascular endothelial growth factor (VEGF) pathway inhibitors (VEGFi), notably the monoclonal anti-VEGF antibody bevacizumab, entered clinical development with very high expectations[67,68]. In fact, antivascular tumor therapy was heralded as a promising way to overcome inherent or acquired therapeutic resistance, a key characteristic of malignant growth, and as a treatment modality potentially 'resistant to resistance'[69,70]. It was thought that diploid, genetically stable tumor endothelial cells were less prone to acquire mutational resistance than genetically unstable tumor cells.

Although VEGFi have become important components of standard treatment regimens for advanced stages of numerous tumor types over the last 15 years, a number of shortcomings of anti-vascular tumor therapy came to the fore: (i) most tumors are inherently resistant to VEGFi and other anti-vascular therapies used alone; (ii) even when used in combinations that increase the initial response rate, responsive tumors typically develop acquired resistance within a few months; and (iii) as opposed to life-prolonging applications of antivascular tumor therapies in advanced disease stages, the adjuvant use of these agents did not increase cure rates[71].

Resistance to antivascular tumor therapies is thought to be largely distinct from resistance to conventional cytotoxic treatment[72]. Based on mainly preclinical studies a number of mechanisms of resistance to VEGFi have been proposed, including evasive resistance due to angiogenic growth factor redundancy or HIF1α mediated overexpression of angiogenic factors, vascular remodeling resulting in more mature and VEGFi resistant tumor blood vessels, preferential expansion of VEGFi resistant vessel subtypes, the selection of hypoxia-resistant tumor

cell subpopulations with reduced vascular dependence, the integration of trans-differentiated tumor cells with endothelial cell properties into the tumor vasculature in a process named vasculogenic mimicry, vessel co-option by tumor cells capable of exploiting the abundant presence of pre-existing host vessels in organs such as liver and lungs, tumor infiltration by bone marrow derived leukocytes with proangiogenic properties, and stromal cell activation (Illustration. 1)[71,73–75]

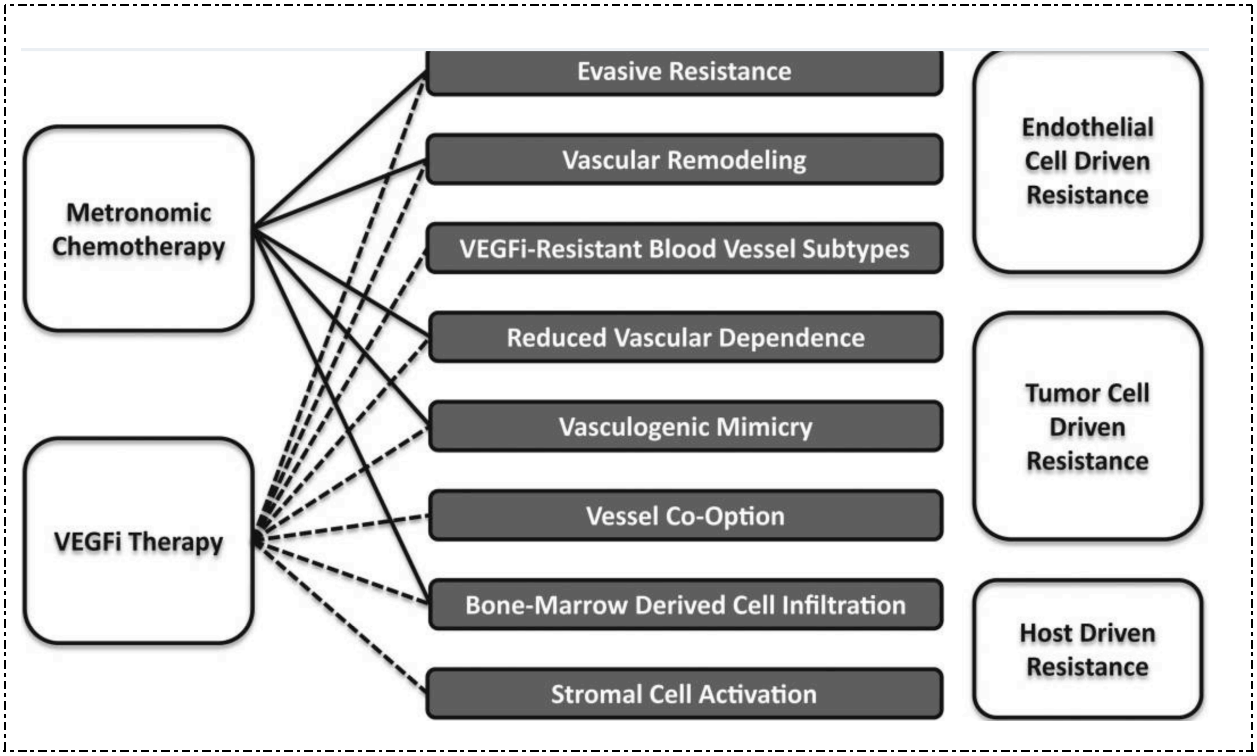


Illustration 1: Mechanisms of Resistance to Metronomic Chemotherapy.

Mechanisms of resistance to metronomic chemotherapy or vascular endothelial growth factor pathway inhibitors (VEGFi) – concepts. Numerous mechanisms of resistance to VEGFi have been described, involving endothelial cell, tumor cell, and host-driven mechanisms. Many of these mechanisms were also found to be functional when it comes to resistance to metronomic chemotherapy.

Research activities focusing on targeting the tumor vasculature revealed that many conventional chemotherapeutics and targeted agents exert collateral damage to tumor vessels. In the case of standard, maximum tolerated dose (MTD) chemotherapy (i.e. the cyclical

administration of high doses of chemotherapeutics with interspersed treatment-free breaks), the anti-vascular effects seen are similar in nature but also as short-lived as the vascular destruction inflicted by vascular disruptive agents[76,77]. On the other hand, the frequent and sustained use of low doses of conventional chemotherapeutics (i.e. low-dose metronomic chemotherapy; hereafter metronomic chemotherapy, MC) mimics the long-term antiangiogenic activities of VEGFi. Two seminal preclinical publications described key characteristics of the MC concept, which have been refined over time and largely validated in numerous clinical trials[56,77,78]. First, MC may overcome resistance to MTD chemotherapy. In other words, the mechanisms of resistance to metronomic versus MTD chemotherapy are at least partially distinct[45]. Second, inducing endothelial cell apoptosis is a main mechanism of action of MC, but MC may also affect other endothelial cell processes such as proliferation, migration, tube formation and sprouting[48,77,79,80]. Third, the majority of tumors are inherently resistant to MC alone, and even initially responding tumors eventually acquire resistance to MC, similar to what is seen with VEGFi[56]. Fourth, high levels of proangiogenic factors may contribute to resistance to MC, but such resistance may be overcome by combination with VEGFi amongst other strategies[78,81].

While initial publications on MC focused on the antiangiogenic activities of MC, there is emerging evidence that MC may also impair vasculogenesis[31,82], target tumor stem cells and their vascular niche[68,83], promote anti-tumor immunity[84,85], delay acquired chemoresistance compared to MTD chemotherapy[85,86], and may induce tumor dormancy[29]. This broad range of MC activities renders studies on mechanisms of resistance to MC challenging. Such studies also need to account for differential effects of distinct chemotherapeutics when used in metronomic manner[49]. Finally, MC is typically applied in combination with other treatment modalities that may affect the resistance phenotype and genotype seen[87]. Considering the complex anti-tumor activities of MC,

an integral understanding of resistance to MC not only involves endothelial cell-intrinsic mechanisms, but also tumor cell and host traits, as outlined in Illustration 1.

2.1.2 MECHANISMS OF RESISTANCE TO METRONOMIC CHEMOTHERAPY: ENDOTHELIAL RESISTANCE

When compared in vitro to tumor cells, endothelial cells are ultra-sensitive to the pro-apoptotic and anti-proliferative effects of low-dose, sustained chemotherapy administration[87]. This differential sensitivity is mediated in part by MC-induced expression of the endogenous angiogenesis inhibitor thrombospondin 1 by endothelial, tumor and/or stromal cells[51,88]. In contrast, proangiogenic factors such as VEGF and basic fibroblast growth factor impair the pro-apoptotic activities of chemotherapeutics towards endothelial cells; and MC was successfully combined with VEGFi in numerous preclinical studies, either upfront or to counteract acquired resistance[39,78].

Although normal diploid endothelial cells are considered genetically stable and hence less prone to therapeutic resistance than tumor cells, isolated tumor endothelial cells feature distinct functional properties when compared to normal endothelial cells, such as increased tolerance to serum starvation and resistance to chemotherapeutic drugs[89]. Several groups found tumor endothelial cells to harbor cytogenetic abnormalities suggestive of genetic instability, which in turn could result in conventional drug resistance[89]. For example, Akiyama et al. showed that VEGF-induced AKT activation increases the expression of the P-glycoprotein drug efflux pump[90] making tumor endothelial cells resistance to PTX in the presence of VEGF[91]. The differential expression of drug efflux pumps has been used to identify a vascular stem/progenitor cell side-population by flow cytometry[92]. Drug efflux pump positive endothelial stem/progenitor cells may contribute both to inherent or acquired resistance to VEGFi, and possibly to MC using chemotherapeutics that are efflux pump substrates. On the other hand, adding drug efflux pump

inhibitors such as verapamil or cyclosporine A decreased the colony formation of tumor-derived side-population endothelial cells exposed to vandetanib[93].

2.1.3 MECHANISMS OF RESISTANCE TO METRONOMIC CHEMOTHERAPY: HOST-DRIVEN RESISTANCE

While individual pharmacogenetic traits may affect active drug levels, there are other patient factors that may alter the benefit achieved with MC. The response of tumors to antiangiogenic and conventional cytotoxic therapies is not only shaped by characteristics of the intratumoral vasculature, but also depends on the frequency of intratumoral, bone-marrow derived circulating endothelial progenitor cells[82,94]. The number of circulating endothelial progenitor cells varies in different inbred mouse strains and is positively correlated with the robustness of tumor angiogenesis[31]. Thus, it is fair to assume that similar genetic heterogeneity may contribute to differential baseline or treatment-induced levels of circulating endothelial progenitor cells in patients. For example, in hepatocellular carcinoma patients undergoing treatment with the small molecule VEGFi sorafenib combined with metronomic tegafur-uracil, high baseline endothelial progenitor cell levels were associated with poor outcome in multivariate analysis[55]. Furthermore, in patients with heavily pre-treated advanced gastrointestinal malignancies who underwent treatment with metronomic tegafur-uracil and cyclophosphamide, combined with celecoxib, CD133 mRNA expression in peripheral blood mononuclear cells (a surrogate marker for circulating endothelial progenitor cells) increased in subjects with progressive disease[95]. Orlandi et al. analyzed VEGF single nucleotide polymorphisms in men with advanced castration-resistant prostate cancer patients treated with metronomic cyclophosphamide, plus celecoxib and dexamethasone[55]. The VEGF (-634CC) genotype was significantly associated with a shorter progression free survival.

2.1.4 OVERCOMING RESISTANCE TO METRONOMIC CHEMOTHERAPY

The response rate of metronomic monotherapy is moderate, as is typically also the case with other antivascular monotherapies[71]. On the other hand, MC is associated with a comparably low risk of severe side effects[56,96]. Hence, MC can be easily combined with other treatment regimens therefore some preclinical studies have focused on overcoming intrinsic rather than acquired resistance to MC. Preclinical studies have analyzed a wide range of combination therapies - to name a few, MC was successfully combined with standard anticancer treatment modalities such as VEGFi[78,97], conventional MTD chemotherapy, targeted therapies, and radiation[31,39,98,99]. The immunomodulatory activities of MC were exploited to enhance different types of cancer immunotherapy[36,100]. Other studies have capitalized on MC-induced tumor hypoxia and acidification by utilizing the hypoxic cell cytotoxin tirapazamine or the proton pump inhibitor lansoprazole in conjunction with MC[80,101].

To study resistance in a MC model, tumor xenografts of PC-3 (prostate cancer) were made resistant to metronomic cyclophosphamide in vivo which remained highly sensitive to MTD cyclophosphamide[45]. In addition, PC-3 tumor xenografts that progressed during metronomic cyclophosphamide therapy were responsive to MTD docetaxel, whereas parental PC-3 tumors (tumors not made resistant) were found to be largely resistant to MTD docetaxel[49]. These findings suggest that the use of below-MTD doses of conventional cytotoxic chemotherapeutic regimens does not necessarily promote acquired resistance to the same or other chemotherapeutic drugs used in MTD fashion. More importantly, by considering the seminal observations by Browder et al. (i.e. that MC can overcome resistance to MTD chemotherapy)[77] it becomes apparent that MC might be used to overcome resistance to MDT chemotherapy, as much as MTD chemotherapy might be able to conquer resistance to MC.

2.1.5 METRONOMIC CHEMOTHERAPY VERSUS MAXIMUM TOLERATED DOSE

Numerous combination strategies have already been tested clinically, including in a number of phase III trials[52,53,56,102–105]. However, only a few randomized phase II trials were specifically designed to study the efficacy of metronomic monotherapy versus MC combined with other treatment modalities, by combining MC with the poly (ADP-ribose) polymerase inhibitor veliparib[106,107], the tumor stroma modulating agents rofecoxib and pioglitazone[108], the antiangiogenic and immunomodulatory compound thalidomide[109], and the VEGFi bevacizumab. There is a numerically promising trend of improved overall survival of melanoma patients treated with metronomic trofosfamide combined with rofecoxib and pioglitazone compared to metronomic trofosfamide alone. However, none of the reported randomized studies reveals both a clinical relevant and at the same time statistically significant benefit of any of the combination regimens over MC alone. Chi et al. reported a 40% response rate and a 84% disease control rate by simultaneously adding the autophagy inducer rapamycin and the autophagy inhibitor hydroxychloroquine in 25 patients with various tumor types presenting with intrinsic resistance to numerous MC regimens[110]. Although available phase III clinical trial findings position MC as a promising anticancer treatment strategy, especially when used as maintenance or adjuvant therapy, typically the response rates to MC are moderate, and acquired resistance ensues within months[53,102–105,109].

Unfortunately, it is not unexpected that randomized trials comparing MC alone versus MC combined with other treatment modalities are rare. First, the most common MC regimens apply off-patent agents without associated commercial interest. Second, regulatory authorities are not expected to honor the metronomic use of conventional chemotherapeutics, which limits the interest of industry partners to conduct trials of MC combined with novel agents. Finally, partnering MC with other treatment modalities may compromise some of the advantages of MC used alone, such

as a low costs and low rates of severe side effects[56,111]. Nonetheless, MC is a very attractive treatment concept, especially in low and middle-income countries[112]. Maybe, such countries will show us the way, including with trials that combine MC with off-patent and affordable repurposed drugs such as metformin[113].

Chapter 3: Identification of New Anti-cancer Drugs for Combination Therapies

3.1.1 INTRODUCTION

One limitation of cancer research is the failure to successfully translate preclinical data to practical clinical applications despite researchers' efforts to improve the efficacy of therapies. One reason for this failure may occur during the development of study designs used to identify novel anti-cancer drugs along with the identification of their molecular mechanisms. During most preclinical study designs, novel anti-cancer drugs are tested by themselves and rarely in combination with already FDA approved treatments against cancer – how it is commonly done in early clinical trials for novel drugs[114–116]. Therefore, we are proposing an alternative drug screening approach to identify novel drugs and evaluate their efficacy on human breast cancer cells previously exposed to one of the most common chemotherapies used against breast cancer, paclitaxel. The rationale behind this approach is that the efficacy of novel drugs may exhibit antagonistic effects when combined with previously approved chemotherapies, an effect that may be negated when drugs are tested by themselves. Finding synergistic drug combinations that offer evidence of improved survival requires the understanding of the drugs individually and in combination therapy[36].

There is currently an evolving paradigm for drug screening collaboration models, such as the Differential Nuclear Staining (DNS) assay[117–121]. The DNS assay implements the use of two fluorescent dyes that bind to DNA; Hoeschst and Propidium Iodide (PI). Hoeschst is a bisbenzimide derivative that intercalates in adenine-thymine regions of dsDNA[122,123]. Hoeschst DNA binding ability stains both living and dead cells, making it a useful tool to label total number of cells in a sample[120]. PI also binds to dsDNA (e.g., intercalating between base pairs) but with an affinity for selecting dead cells due to compromised plasma membranes[124,125]. Both dyes will be used to identify potential drug candidates that can be used in addition to chemotherapy regimens. We hypothesize that by implementing this drug-screening method along with combination therapy we may find potential drug candidates to reduce tumor growth while increasing overall survival.

The innovation is to repurpose drugs that by themselves may have marginal effects on cancer cells, but when combined with paclitaxel (either as a first line or second line therapeutic regimen) the combination mitigates tumor burden. Paclitaxel (PTX) was chosen based on Dr. Urban Emmenegger's (oncologist) advice suggesting to be one of the chemotherapies most likely used for the treatment of breast cancer that will acquire resistance[126]. Therefore, PTX will be used to determine potential drug candidates that could be used in future application for the treatment of breast cancer as secondary line of therapies.

3.1.2 MATERIALS AND METHODS

Cell Culture: LM2-4-luc breast carcinoma cells were previously co-transfected with firefly luciferase[40] and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 4 mM L-glutamine. Cells were maintained in a humidified incubator at 37°C and 5% carbon dioxide. Phosphate Buffered Saline (PBS 1X) was used to wash cells prior Trypsin (1X) to prepare single-cell suspensions before plating cells as monolayer. MCF10A human mammary epithelial cells were used as normal control and grown in DMEM/F12 supplemented with 10% FBS, 10 μg/ml recombinant human insulin, 0.5 μg/ml hydrocortisone and 20 ng/ml epidermal growth factor[127].

Drug preparation: Paclitaxel (PTX) was made up in sterile PBS at a 1mM stock concentration. Gemcitabine hydrochloride was used as a positive control for cell death and made up in sterile PBS at a 4.5mM stock concentration. PTX was added at a final concentration of 1nM and Gemcitabine at a final concentration of 22.5uM. Chemical compounds were purchased from the PremiumSet and DIVERSet small molecule library (Code NT797; PS797; ChemBridge Corporation). Chemical compounds were received already dissolved in Dimethyl Sulfoxide (DMSO) at a 10mM stock concentration. Illustration 2 provides an overview of the methodology used to complete the identification of new drugs using the DNS assay along with the chemical structures of the compounds identified as HITS.

Differential Nuclear Staining Assay for Identification of Potential HITS - Dose Dependent Response and CC50 Values: To evaluate proof of principle, PremiumSet and DIVERSet small

molecule libraries were tested on LM2-4-luc cells that were previously exposed to PTX. Cells were seeded overnight in flat bottom 96-well plates at a cell density of 1,600 cells per well in 80µl. PTX was added after overnight incubation at a final concentration of 1mM and incubated for 24 hours in 80µl. After incubation, the media containing PTX was removed and 200µl of fresh complete media was added to each well. 1uL of the chemical compounds stock concentration from the libraries was added directly to individual wells and incubated for 72 hours. At 71 hours after incubation with chemical compounds, Hoechst and Propidium iodide (PI) fluorescent dyes stock solutions were added directly to individual wells for 1 hour at a final concentration of 1µg/ml for each dye. Montage (2x2) images from each well were captured with the multi-well plate reader IN Cell 2000 analyzer and analyzed with IN Cell Analyzer Workstation 3.2 software. Montage (3x3) images with the BD Pathway 855 bioimager system were captured using the IN Cell 2000 and analyzed with AttoVision v1.6.2 software. 1µl of Dimethyl Sulfoxide (DMSO) will be used as a vehicle control, 1µl of gemcitabine will be used as a positive control for cytotoxicity, and lastly a control for untreated cells.

Criteria for Identification of Potential HITS: Potential drug candidates reported as HITS will be based on the following criteria; (1) anti-proliferative efficacy similar to gemcitabine control (via fluorescent signal of stained cells with PI and Hoechst), and (2) using the equation below to obtain the death percentage similar to gemcitabine as well.

$$\left(\frac{\text{DMSO Dead Cell Count Average} - \text{Cell Count} + \text{Total Number of Dead Cells}}{\text{DMSO Dead Cell Count Average}}\right) \text{X } 100$$

The formula was used to normalize the percentage of dead cells against DMSO from the captured images after segmentation using both the IN Cell Analyzer Workstation 3.2 software and AttoVision v1.6.2 software[120,128,127].

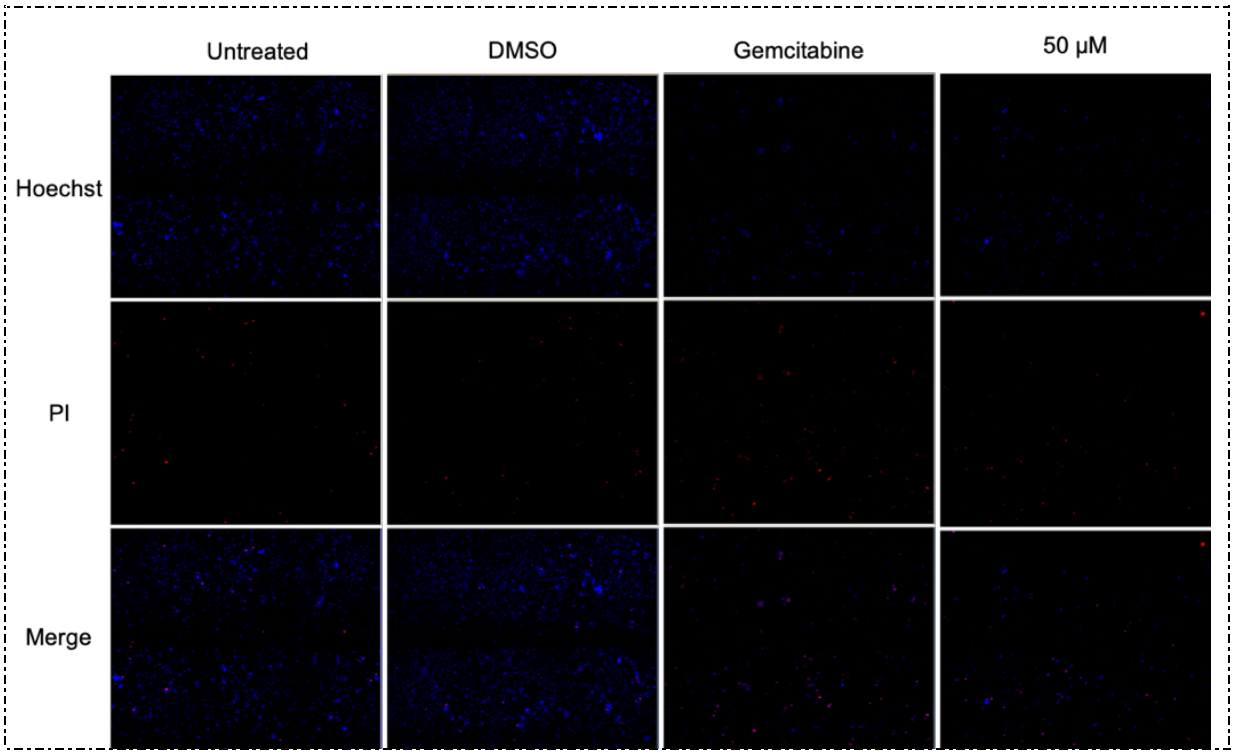


Illustration 2: DNS Assay workflow adapted from Lema et al., 2011.

LM2-4-luc cells were seeded in 96-well plates. PTX was added 24 hours after cells were seeded and incubated for an additional 24 hours. PTX was removed and compounds were added for 72 hours in fresh complete medium. Hoechst and PI were added at the 71 hour mark and incubated for one hour before image/data acquisition. Hoechst stains total population of cells as shown in blue color whereas PI stains dead cells shown in red color. 50uM of 5935953 was used as an experimenta. Data was quantified using both the IN Cell Analyzer Workstation 3.2 software and AttoVision v1.6.2 software[118–120].

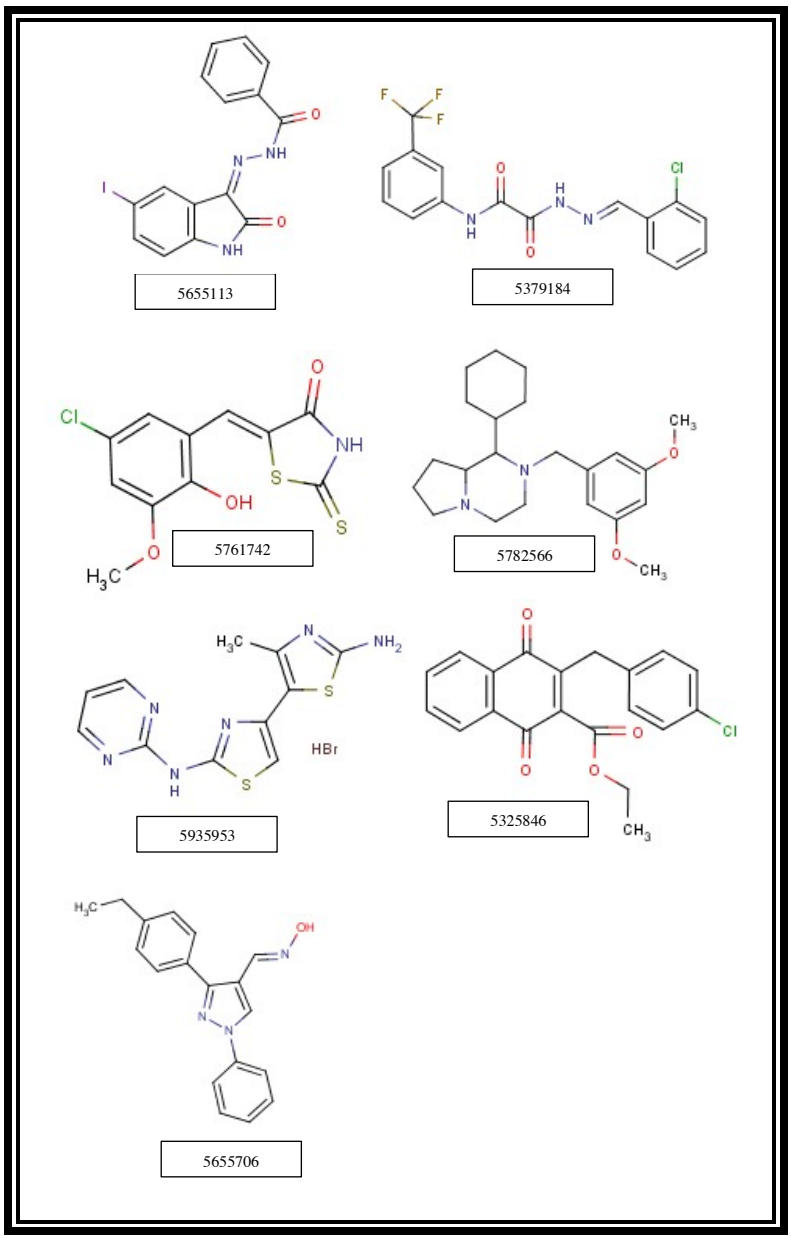


Figure 9. Identified HITS from PremiumSet and DIVERSet libraries tested on LM2-4-luc cells.

Potential drug candidates reported as HITS had an anti-proliferative efficacy similar to gemcitabine when comparing their death percentages.

Cell Viability Assay: LM2-4-luc cells were seeded in 6-well plates at 10^4 cells per well and treated as previously described (DNS assay). After 24 hour incubation with HITS, cells were exposed to trypsin and viability was assessed by trypan blue exclusion.

Cell Cycle Assay: LM2-4 cells were seeded in 24-well plates at 2 x 10^4 cells per well and treated as previously described. After 72 hours of incubation with HITS, the supernatant from each well was transferred into disposable/round bottom 12x75mm culture tubes. 200 microliters of Nuclear Isolation and Staining Solution (NPE Systems, Inc) and 100 microliters of PBS were added to each tube and analyzed in the Galliosflow cytometry machine (Beckman Coulture, Inc). The data collected from Gallios was analyzed with Kaluza Analysis Software (Beckman Coulture, Inc).

3.1.3 RESULTS AND DISCUSSION

Effects of various HITS on cell proliferation and viability of LM2-4-luc cells. Primary screening of 30,000 compounds resulted in 89 compounds as potential candidates for secondary screenings. Secondary screening of compounds (at 5uM and 1uM concentrations) led to the identification of 7 potential HITS. These HITS were further characterized by defining their cytotoxic concentrations 50% (CC_{50}) via DNS assay (data not shown for all compounds)[127,128]. Each experimental point was assessed in triplicate along with untreated and DMSO controls. In order to test proof of concept, HITS were further characterized and selected for having minimal effect as pre-treatment regimens and then as sequential regimens when combined with PTX on LM2-4-luc cells and compared to MCF10A epithelial cells. After DNS assay screening, compound 5935953 was further evaluated based on its consistent CC_{50} to determine their mechanism of action. Compound 5935953 when administered as a sequential regimen leads to higher cell death when compared to its administration as pre-treatment (Figs. 10 and 11). Pre-treatment was initialized following the same parameter as PTX (e.g., low drug concentration for 24 hours).

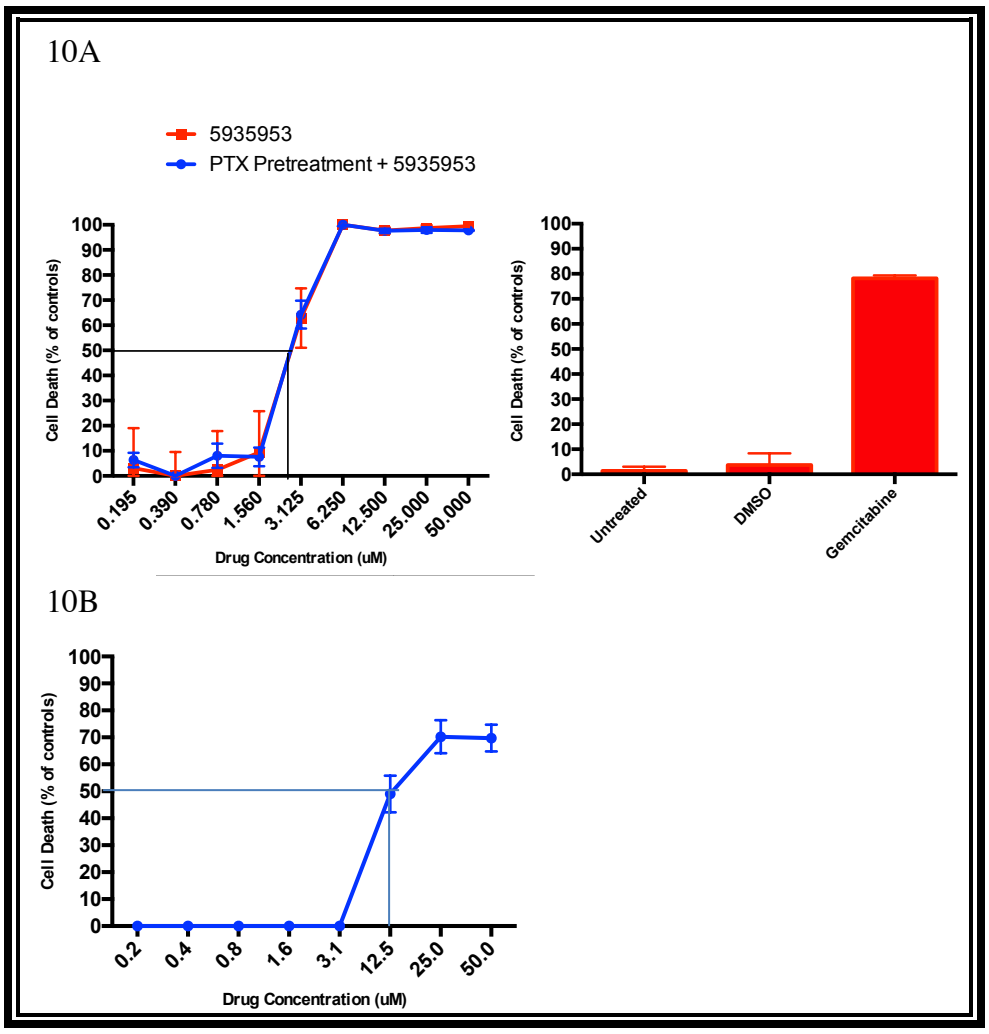


Figure 10. CC_{50} for compound 5935953.

A) LM2-4-luc cells were treated with different concentration of compound 5935953 alone or as a sequential treatment after PTX (1nM) for 72 hours. CC_{50} was determined via DNS assay. Cell death is represented on the Y-axis while drug dilutions are shown on the X-axis. Untreated, DMSO and gemcitabine (22.8uM) controls were used to compare the cytotoxicity of compound 5935953. Each point represents the mean and standard deviation of four replicas – CC_{50} for 5935953 is between 2.483 to 3.122 when administered alone or in combination (sequential therapy) with PTX. B) CC_{50} for 5935953 on MCF10-A cells is between 12.50 to 13.17 when administered as a monotherapy. Selective cytotoxicity (SCI) index value of 5935953 is 5.03 when compared against non-cancerous breast MCF-10A cells.

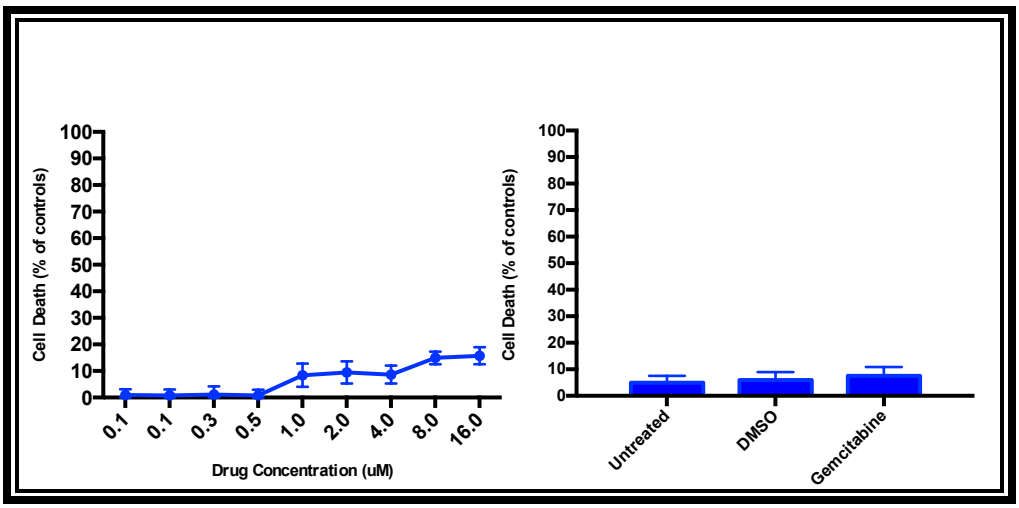


Figure 11. Compound 5935953 as first line therapy with sequential PTX.

LM2-4-luc cells were treated with compound 5935953 at 10nM as first line therapy followed by different concentrations of PTX (shown in graph) for 72 hours and analyzed via DNA assay. Cell death is represented on the Y-axis while PTX dilutions are shown on the X-axis. Untreated, DMSO and gemcitabine (22.8uM) controls were used to compare the cytotoxicity of compound PTX as a sequential treatment. Each point represents the mean and standard deviation of four replicas.

Compound 5935953 induces apoptosis. To assess a possible effect of 5935953 on the cell cycle distribution, LM2-4-luc cells treated with 5uM of 5935953 were examined by DAPI staining and flow cytometry after 72 hours incubation with and without PTX pretreatment (Fig. 11). Compound 5935953 arrested cells in the sub G0/G1 phase with minimal reduction of cells in the G2 phase – suggesting DNA fragmentation ($P<0.001$). DNA histograms were generated by staining cells incubated with compound 5935953 with nuclear isolation medium–4,6-diamidino-2-phenylindole dihydrochloride (NIM-DAPI)[118,127,129]. Compound 5935953 was chosen as a potential candidate for future studies based on its anti-proliferative property. Developing new study designs for drug evaluation such as the implementation of DNS and pre-treatments of FDA approved drugs is essential for the identification novel compounds for the treatment of breast cancer. Such compounds may be used as secondary line therapies once first line therapies fail.

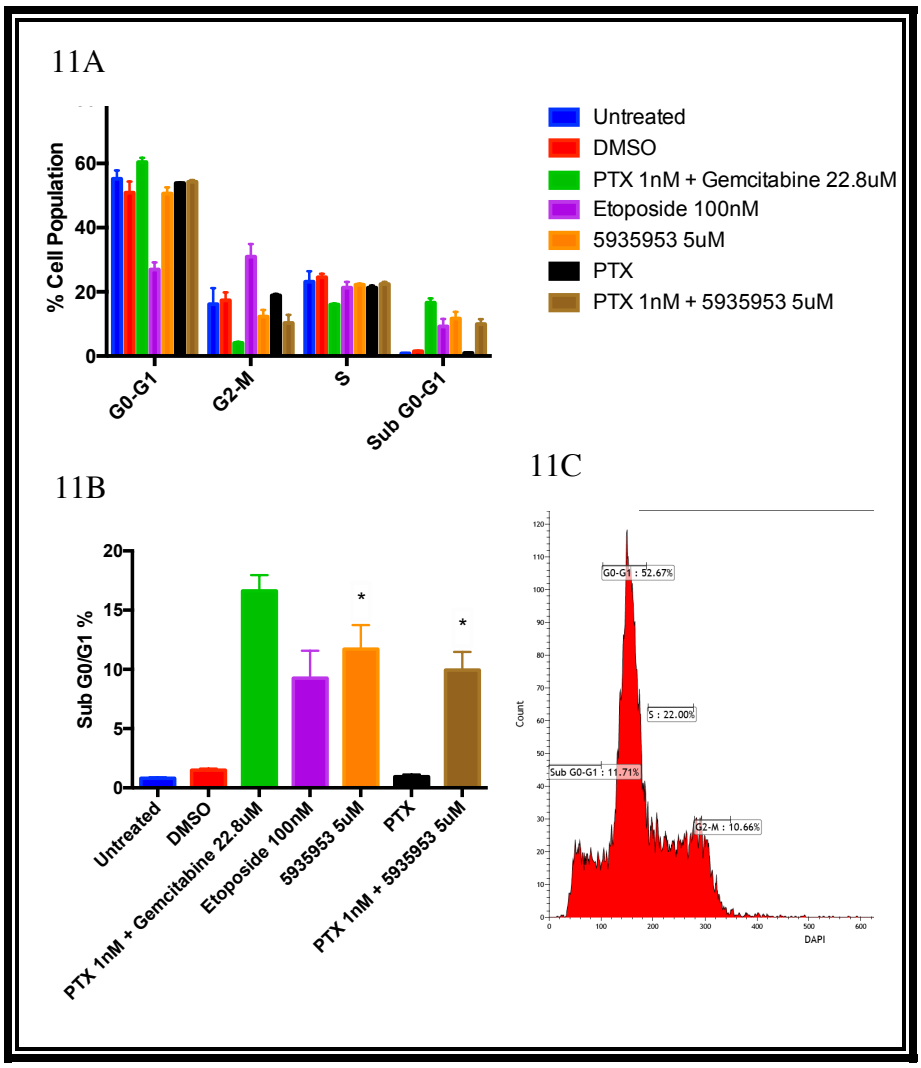


Figure 11. Cell cycle profile for compound 5935953.

LM2-4-luc cells were treated with 5935953 alone or as a sequential treatment after PTX (1nM) for 72 hours. A) Cell population is represented on the Y-axis – after 72 hours cells were stained with NIM-DAPI and analyzed via flow cytometry. Untreated, DMSO and gemcitabine (22.8uM) and etoposide controls were used to compare the cell cycle profile of compound 5935953. Each point represents the mean and standard deviation of 3 replicas. B) Compound 5935953 induces Sub G0/G1 when compared to untreated and DMSO controls (* P-value < 0.001). C) Cell cycle profile histogram.

Chapter 4: Summary

Our goal is to identify drug administration strategies that would improve the treatment of breast cancer, which is often hampered by the emergence of intrinsic or acquired resistance to anti-cancer therapies remains a major health problem. Intrinsic resistance pre-exists within the cell before receiving any type of chemotherapy. On the other hand, acquired resistance arises through mutations due to chemotherapy. Therefore, the focus of this thesis is to seek and evaluate new drug dosing strategies for the treatment of breast cancers which may prevent or contain drug resistant mechanisms. Using drug screening techniques, we will screen for chemotherapeutic agents that may bypass tumor drug resistance mechanisms. An innovative treatment concept called metronomic (continuous low-dose exposure) chemotherapy has been developed. In regards to drug dosing strategies, my mentor Dr. Giulio Francia, and his collaborator, Dr. Guido Bocci, recently reported that the continuous low dose exposure of a class of compounds called ceramide analogues may be an effective anti-cancer strategy for pancreatic cancer[48]. In addition, Dr. Bocci's group also found some similarly interesting anti-cancer properties of a new group of cyclic amide compounds in thyroid cancer[130].

There are currently many therapies being developed to treat breast cancer, however it is vital that we keep improving the efficacy of the available therapies. Chemotherapeutic agents often target the fast dividing cells with high doses of cytotoxic agents at cyclic schedules to allow restoration of peripheral leukocyte counts. In contrast to MTD, metronomic or continuous low-dose chemotherapy, involves the administration of anti-cancer drugs at doses that are much lower. Redirecting the conventional modality developed the emergence of new therapeutic approaches such as immunotherapy. In 1996, James Allison' lab reported that an antibody against Cytotoxic T lymphocyte antigen-4 (CTLA-4) could lead to activation of the immune system and a rejection

of implanted tumors[16]. CTLA-4 protein alters T-cell's response, thus down regulating the immune system while its cognate co-receptor CD28 stimulates it. This thesis combines low-dose administration of chemotherapeutic agents in combination with CTLA-4 immunotherapy in a Balb/c animal model along with the identification of new compounds for the treatment of breast cancer.

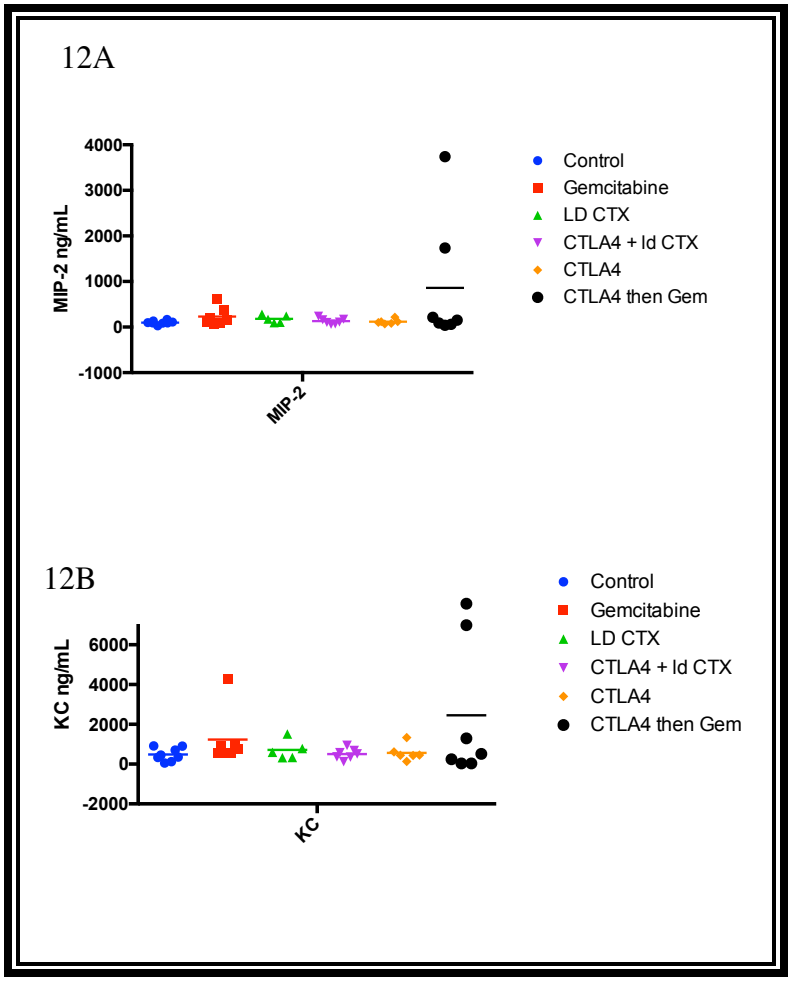


Figure 12. Expression of proinflammatory immune response chemokines.

Balb/c with EMT-6 tumors were divided into different groups (See Fig. 2). At the end of the experiment, blood was collected in tubes with lithium heparin and centrifuged at 6,500g for 15min for plasma collection. Samples were then analyzed for immune-related protein profiling using antibody-linked beads (MILLIPLEX Mouse Cytokine). Results were normalized based on total protein concentration.

One of the main proposed mechanisms for low-dose chemotherapy is the elimination of dividing endothelial cells present in the blood vessels irrigating a tumor[50]. We found that the efficacy of metronomic-like administration of gemcitabine in combination with anti-CTLA-4 immunotherapy may be attributed to the recruitment of chemokines (Fig. 12). Neutrophil activation in CTLA-4 then gemcitabine-treated tumors indicate an inflammatory response. Neutrophils are inflammatory cells considered to be first responders to infections[131]. Despite of these results, resistance to CTLA-4 then gemcitabine still emerged. In order to target resistance, new combination therapies are in dire need. In regards to this, we screened 30,000 compounds in hopes of finding small molecules that may have an anti-proliferative effect on cancer cells.

LM2-4-luc cells breast cancer cells were treated with such compounds to determine their efficacy at 5uM and 1uM. Gemcitabine was used as a comparative control – which will be later used to determine effective combination therapies with the proposed HITS. Compounds considered as HITS were screened using DNS assay[120] and had to follow 4 criteria; 1) similar death/ live cell ratio as gemcitabine, 2) low Hoechst positive cells, 3) high PI positive cells and 4) similar death percentage as gemcitabine based on the DNS assay formula. Compound 5935953 was further analyzed as a HIT alone or as a sequential treatment after PTX (1nM) for 72 hours. Future work will focus on characterizing compound 5935953 to determine its effect in combination studies with gemcitabine and other FDA approved drugs such as PTX for the treatment of breast cancer. The data obtained from these experiments will allow us to better comprehend how low dose regimens work as well as to understand the effects of new anti-cancer compounds. It may also help explain why some treatments are limited by drug resistance. We expect the results to be of translational value and contribute to the design of future clinical trials.

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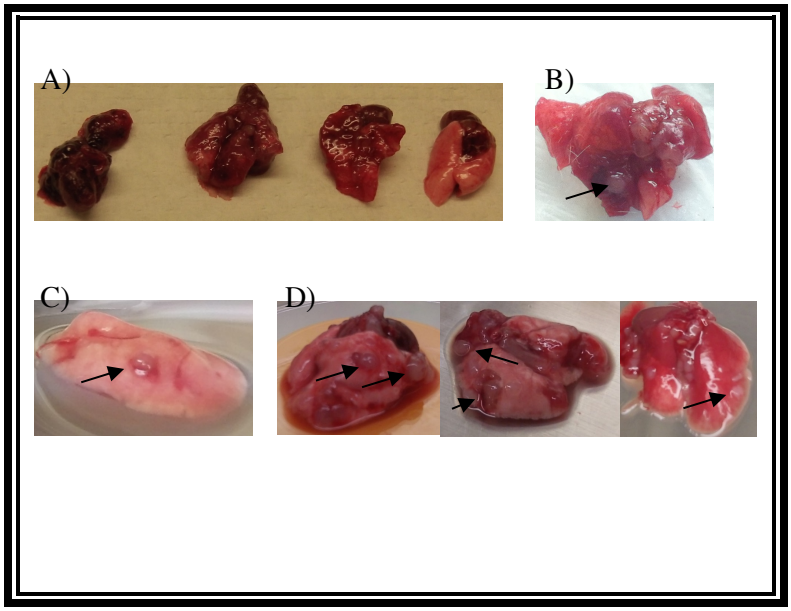
Appendix

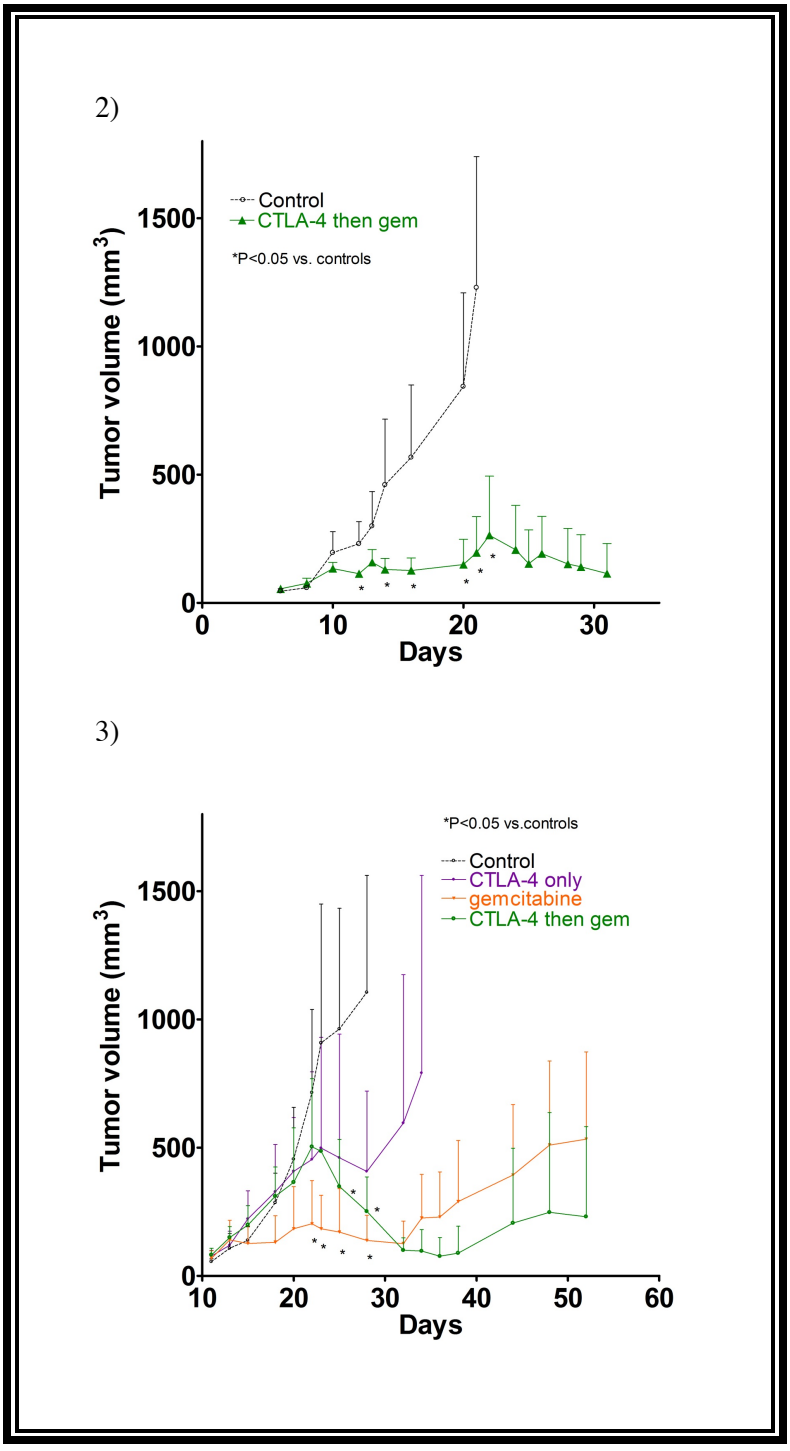
SUPPLEMENTARY FIGURE 1



Supplementary Figure 1. Examples of spontaneous metastasis under continuous therapy, from subcutaneously implanted EMT-6 tumor models

A) EMT-6/P tumor bearing mice treated with Bolus+ldCTX were sacrificed when they reached the experimental endpoint; upon autopsy three of the four mice were found to have lung metastases (i.e., the three leftmost lungs shown in this picture). B) Spontaneous metastases developed on after day 100 of therapy in a mouse with EMT-6/P tumor treated with the CTLA-4 then gemcitabine regimen. C) Similarly, we observed spontaneous lung metastases in mice bearing EMT-6/CTX tumors, in this case from a mouse treated with gemcitabine alone therapy and, D) with EMT-6/DDP tumor bearing mice (the leftmost lung was from a mice were treated with gemcitabine monotherapy, and the other two lungs were from mice treated with CTLA-4 then gemcitabine therapy; arrows point to individual lung metastases).

SUPPLEMENTARY FIGURE 2 AND 3



Supplementary Figure 2 and 3. Evaluation of CT-26 tumor with CTLA-4 therapy.

CT26 cells were implanted s.c. into Balb/c mice (n=4 per group) and treated with saline or with anti-CTLA-4 starting on day 6. On day 22, as the tumors were starting to regrow following CTLA-4 therapy, the mice were treated with gemcitabine (160mg/kg) given every 3 days i.p. Supplementary Figure 3 Evaluation of CT-26 tumor treated with

CTLA-4 combination therapy. CT26 cells were implanted s.c. into Balb/c mice (n=5-8 per group) and treated with saline, gemcitabine (160mg/kg every 3 days), anti-CTLA-4 starting on day 13 followed by gemcitabine starting on day 24. These results confirmed that CT-26 responds to anti-CTLA therapy and that such a response can be enhanced by sequential gemcitabine therapy. After day 30, some of the mice treated with anti-CTLA-4 followed by gemcitabine showed tumor regrowth.

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Impact of CTLA-4 blockade in conjunction with metronomic chemotherapy on preclinical breast cancer growth

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Background: Although there are reports that metronomic cyclophosphamide (CTX) can be immune stimulating, the impact of its combination with anti-CTLA-4 immunotherapy for the treatment of cancer remains to be evaluated.

Methods: Murine EMT-6/P breast cancer, or its cisplatin or CTX-resistant variants, or CT-26 colon, were implanted into Balb/c mice. Established tumours were monitored for relative growth following treatment with anti-CTLA-4 antibody alone or in combination with; (a) metronomic CTX (ldCTX; 20 mg kg^{-1} day^{-1}), b) bolus (150 mg kg^{-1}) plus ldCTX, or (c) sequential treatment with gemcitabine (160 mg kg^{-1} every 3 days).

Results: EMT-6/P tumours responded to anti-CTLA-4 therapy, but this response was less effective when combined with bolus plus ldCTX. Anti-CTLA-4 could be effectively combined with either ldCTX (without a bolus), or with regimens of either sequential or concomitant gemcitabine, including in orthotopic EMT-6 tumours, and independently of the schedule of drug administration. Tumour responses were confirmed with CT-26 tumours but were less pronounced in drug-resistant EMT-6/CTX or EMT-6/DDP tumour models than in the parent tumour. A number of tumour bearing mice developed spontaneous metastases under continuous therapy. The majority of cured mice rejected tumour re-challenges.

Conclusions: Metronomic CTX can be combined with anti-CTLA-4 therapy, but this therapy is impaired by concomitant bolus CTX. Sequential therapy of anti-CTLA-4 followed by gemcitabine is effective in chemotherapy-naive tumours, although tumour relapses can occur, in some cases accompanied by the development of spontaneous metastases.

In 2010, 14 years after the report of CTLA-4 blockade causing tumour responses in preclinical models (Mokyr *et al*, 1998), by blocking the immune suppressive functions of the CTLA-4 protein, the anti-CTLA-4 antibody ipilimumab was approved by the FDA for the treatment of non-resectable or metastatic melanoma (Hodi *et al*, 2010). This approval was a pivotal event for cancer immunotherapy (Pardoll, 2012; Li *et al*, 2013), a field now enriched by additional targets such as PD-1, PD-L1, and LAG-3 (Pardoll, 2012; Li *et al*, 2013; Postow *et al*, 2015). Despite these successes, there remain several hurdles to be overcome in the quest for optimal anti-CTLA-4-based therapy regimens, including minimising the likelihood of the development of autoimmune toxicity

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Mini-review

Resistance to metronomic chemotherapy and ways to overcome it

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ABSTRACT

Therapeutic resistance is amongst the major determinants of cancer mortality. Contrary to initial expectations, antivascular therapies are equally prone to inherent or acquired resistance as other cancer treatment modalities. However, studies into resistance to vascular endothelial growth factor pathway inhibitors revealed distinct mechanisms of resistance compared to conventional cytotoxic therapy. While some of these novel mechanisms of resistance also appear to be functional regarding metronomic chemotherapy, herein we summarize available evidence for mechanisms of resistance specifically described in the context of metronomic chemotherapy. Numerous preclinically identified molecular targets and pathways represent promising avenues to overcome resistance and enhance the benefits achieved with metronomic chemotherapy eventually. However, there are considerable challenges to clinically translate the preclinical findings.

Introduction

Twenty years after the late Judah Folkman had described the conceptual framework of antivascular tumor therapy, in the 1990's vascular endothelial growth factor (VEGF) pathway inhibitors (VEGFi), notably the monoclonal anti-VEGF antibody bevacizumab, entered clinical development with very high expectations [1,2]. In fact, antivascular tumor therapy was heralded as a promising way to overcome inherent or acquired therapeutic resistance, a key characteristic of malignant growth, and as a treatment modality potentially 'resistant to resistance' [3,4]. It was thought that diploid, genetically stable tumor endothelial cells were less prone to acquire mutational resistance than genetically unstable tumor cells.

Although VEGFi have become important components of standard treatment regimens for advanced stages of numerous tumor types over the last 15 years, a number of shortcomings of antivascular tumor therapy came to the fore: (i) most tumors are inherently resistant to VEGFi and other anti-vascular therapies used alone; (ii) even when used in combinations that increase the initial response rate, responsive tumors typically develop acquired resistance within a few months; and (iii) as opposed to life-prolonging applications of antivascular tumor therapies in advanced disease stages, the adjuvant use of these agents did not increase cure rates [5].

Resistance to antivascular tumor therapies is thought to be largely distinct from resistance to conventional cytotoxic treatment [6]. Based on mainly preclinical studies a number of mechanisms of resistance to VEGFi have been proposed, including evasive resistance due to angiogenic growth factor redundancy or HIF1α mediated overexpression of angiogenic factors, vascular remodeling resulting in more mature and VEGFi resistant tumor blood vessels, preferential expansion of VEGFi resistant vessel subtypes, the selection of hypoxia-resistant tumor cell subpopulations with reduced vascular dependence, the integration of trans-differentiated tumor cells with endothelial cell properties into the tumor vasculature in a process named vasculogenic mimicry, vessel co-option by tumor cells capable of exploiting the abundant presence of pre-existing host vessels in organs such as liver and lungs, tumor infiltration by bone marrow derived leukocytes with proangiogenic properties, and stromal cell activation (Fig. 1) [5,7–9].

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Vita

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Karla received her Bachelor's degree in Microbiology from the University of Texas at El Paso (UTEP), where she then continued her graduate studies by pursuing a PhD in Pathobiology. She has been working under the supervision of Dr. Giulio Francia and Dr. Renato Aguilera. Her projects consist in understanding immunotherapy with different chemotherapy regimens to improve preclinical breast cancer models. One of the main obstacles in cancer research is the low rate of success in reproducing encouraging preclinical therapeutic findings, using transplanted or spontaneous primary tumors in mice. The use of preclinical mouse tumor models, which mimic postsurgical adjuvant or metastatic therapy, may be a promising strategy to help improve our ability to predict subsequent clinical outcomes. Karla was funded by the RISE Research Scholarship NIH (HR25GM069611-11) which facilitates graduate student to conduct research and expand their professional development. Karla was also awarded the Outstanding Teaching Award to a Doctoral Student as a recognition for her commitment to teaching and ability to deliver excellence to undergraduate students.

This thesis/dissertation was typed by Karla Parra