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Tumor progression and metastatic dissemination in ovarian cancer after dose-dense or conventional paclitaxel and cisplatin plus bevacizumab

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The efficacy of therapeutic regimens incorporating weekly or every-3-weeks paclitaxel (PTX) for ovarian cancer is debated. We investigated the addition of bevacizumab in regimens of chemotherapy with different PTX doses and schedules in preclinical models. Treatments were cisplatin (DDP) with weekly PTX (conventional), or dose-dense-equi (every other day to the conventional cumulative dose), or dose-dense-high (total dose 1.5 times higher), with or without bevacizumab. Treatment efficacy was evaluated analyzing tumor growth in different time-windows in two patient-derived ovarian cancer xenografts with different sensitivity to cisplatin. Tumor progression, metastasis and survival were studied in ovarian cancer models growing orthotopically and disseminating in the mouse peritoneal cavity. Short-term effects on cell cycle, tumor cell proliferation/apoptosis and vasculature were evaluated by flow cytometry and immunohistochemistry. PTX dose-dense (with/without DDP) was superior to the conventional scheme in a dose-dependent manner; the high efficacy was confirmed by the lower ratio of tumor to normal cells. All schemes benefited from bevacizumab, which reduced tumor vessels. However, DDP/PTX dose-dense-high (only chemotherapy) was at least as active as DDP/PTX conventional plus bevacizumab. DDP/PTX dose-dense-high plus bevacizumab was the most effective in delaying tumor progression, though it did not prolong mouse survival and the continuous treatment with bevacizumab was associated with a malignant disease. These findings indicate that the effect of bevacizumab in combination with chemotherapy may depend on the schedule-dose of the treatment and help to explain the unclear benefits after bevacizumab.

Introduction

Patients with ovarian cancer (OC) typically receive platinum- and taxane-based combination therapy;[1] recently bevacizumab, the monoclonal antibody against VEGF, has been incorporated into these regimens.[2] Therapeutic improvements might be obtained by more appropriate scheduling of anticancer agents, refining dose intensity or improving drug delivery. Dose-dense therapy is a strategy to boost antitumor

Key words: ovarian cancer, paclitaxel, bevacizumab, metastasis, xenografts

Abbreviations: AGD: absolute growth delay; B: bevacizumab; BEV: bevacizumab; BLI: bioluminescence imaging; BWL: body weight loss; D: cisplatin; DDP: cisplatin; DTregrowth: doubling time of tumor regrowth; ILS: increment of lifespan; i.o.: intraovarian; i.v.: intravenous; maint: maintenance; MST: median survival time; MTD: maximum tolerated dose; MVD: microvessel density; OC: ovarian cancer; OC-PDX: patient-derived ovarian cancer xenograft; OS: overall survival; P: paclitaxel; PCo: PTX conventional; PEq: PTX dose-dense-equi; PFI: platinum free interval; PFS: progression-free survival; PHi: PTX dose-dense-high; PTX: paclitaxel; s.c.: subcutaneous; TWnadir: tumor weight at nadir; V: vehicle

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What's new?

Patients with ovarian cancer typically receive platinum- and taxane-based therapies; recently, bevacizumab, the monoclonal antibody against VEGF, has been incorporated into these regimens. The benefit of bevacizumab in regimens of chemotherapy incorporating dose-dense paclitaxel (PTX) for ovarian cancer is still debated, however. Taking together the results of four preclinical trials in ovarian cancer xenograft models, here the authors found that the advantage of bevacizumab in combination regimens depends on the schedule-dose of chemotherapy. Furthermore, the quantitative analyses of tumor growth in different time-windows was linked to biological end-points and provided new insights into the different outcomes.

activity by giving the same or a higher cumulative dose of a drug and reducing the interval between doses, with the aim of minimizing the regrowth of tumor cells between chemotherapy cycles and – for agents that target tumor vasculature – maximizing the antiangiogenic effects.[3–6]

The literature on dosing and scheduling of taxanes is growing. Weekly paclitaxel (PTX) has been reported superior to 3-weekly PTX for metastatic breast cancer.[7–9] In ovarian cancer, the Japanese Gynecologic Oncology group (JGOG 3016 study) first showed that dose-dense weekly PTX prolonged progression-free survival (PFS) and overall survival (OS) compared to conventional every-3-weeks schemes.[10] However, in the Multicenter Italian Trials (MITO 7), assessing a weekly schedule of carboplatin and PTX, PFS was not prolonged compared to treatment every-3-weeks,[11] though the quality of life was better. In the Italian trial the PTX dose was lower than in the Japanese trial, suggesting the importance of the cumulative dose for the outcome.

The addition of bevacizumab to the standard combination of PTX and carboplatin every-3-weeks showed benefit in two large randomized clinical trials (GOG-0218- and ICON7) in ovarian cancer.[12,13] Recently, the GOG-0262 clinical trial reported that in combination with bevacizumab (and carboplatin), 3-weekly or weekly PTX regimens were equally effective (reported as PFS), with the former giving fewer adverse events.[14] This study also indicated similar PFS with weekly paclitaxel without bevacizumab (14.2 months) and with weekly or every-3-weeks PTX with bevacizumab (respectively 14.9 and 14.7 months),[14] thus questioning the advantage of adding bevacizumab to the treatment regimens.

A further issue in these trials is that although the delay in tumor progression (PFS) was a major achievement for women with ovarian cancer receiving bevacizumab-containing chemotherapy, this usually led to a scanty OS increase.[2,12,13] The escape to antiangiogenic treatments is well documented in preclinical studies, including in our models of patient-derived ovarian cancer xenograft (OC-PDX) treated with bevacizumab, which under certain circumstances increased survival but increased tumor dissemination.[15–17]

In our study we simulated the clinical trials with OC-PDX,[18] to study the efficacy of bevacizumab incorporated in chemotherapy regimens with different PTX doses and schedules.

To get insight into the effects of different treatment schemes on tumor growth, we used a set of parameters derivable with simple procedures from the growth curves, focusing on different time windows, and integrating this information with independent *ex vivo* measures of short-term effects by flow cytometry and immunohistochemistry.

To address the biological consequences of bevacizumab combined with chemotherapy incorporating different schedules/doses of PTX, we investigated the dissemination and metastasis into organs of the peritoneal cavity of OC-xenografts transplanted orthotopically in the mouse ovary, after the different treatment regimens.

All together, these data will enable us to compare the schemes of treatment under examination in an unprecedented way, far more detailed than achievable in clinical studies.

Materials and Methods

Animals

Six- to eight-week-old female NCr-nu/nu mice were obtained from Envigo Laboratories (Udine, Italy). Mice were maintained under specific-pathogen-free conditions, housed in isolated vented cages, and handled using aseptic procedures. Procedures involving animals and their care were conducted in conformity with institutional guidelines that comply with national (Legislative Degree 26, March, 2014) and international (EEC Council Directive 2010/63, August, 2013) laws and policies, in line with guidelines for the welfare and use of animals in cancer research.[19] Animal studies were approved by the Mario Negri Institute Animal Care and Use Committee and Italian Ministerial decree no. 84–2013.

Xenograft tumor models

Ectopic models. MNHOC18, platinum-sensitive, and MNHOC84, platinum-resistant, were two patient-derived high-grade serous ovarian carcinoma xenografts (OC-PDX). The former came from a primary ovarian tumor in a patient who had received epirubicin and cyclophosphamide in a neo-adjuvant setting, the latter from a patient at relapse who received adjuvant carboplatin and PTX.[18] OC-PDX recovered from frozen stocks were used within 5–6 mouse passages after establishment from patients, and transplanted subcutaneously (s.c.) as tumor fragments in the flank of nude mice. These OC-PDX models were molecularly, biologically and pharmacologically characterized and found similar to the original patient tumor.[18]

Orthotopic model. IGROV1-luc is a variant of the IGROV1 human ovarian cancer cell line[20] (obtained from the National Cancer Institute Tumor Repository in 1995), infected with lentiviral vector carrying the coding sequence of the synthetic firefly luciferase gene, *luc2* (*Photinus pyralis*).[21] Cell culture was maintained in RPMI 1640 (BioWest, Nuaillé, France) supplemented with 10% FBS (Euroclone, Pero, Italy). Master stocks of the cell line were stored frozen in liquid nitrogen; new ampoules were thawed as needed and cultures were maintained for no more than 6 weeks before use. IGROV1-luc cells were injected orthotopically in the ovary (i.o.), as previously described.[22] Briefly, in anesthetized mice the skin was disinfected with Betadine and a 1–2 cm lateral midline skin incision performed to access the left ovary and exteriorize it and the oviduct. 1×10^6 IGROV1-luc cells in 5–10 μL HBSS were injected under the bursa of the ovary, using a Hamilton syringe with a 26-gauge needle, followed by a slow removal of the needle to avoid spillage. The ovary was replaced in the peritoneal cavity, the incision closed with surgical thread and the skin with metal wound clips.

Drug preparation and treatment

Paclitaxel (PTX, Indena S.p.A., Milan, Italy) was dissolved in 50% Cremophor EL (Sigma-Aldrich, Milan, Italy) and 50% ethanol and further diluted with saline immediately before use. Cisplatin (*Cis*-diamminedichloroplatinum, DDP, Sigma-Aldrich, Milan, Italy) was dissolved in 0.9% NaCl. Bevacizumab (BEV, Avastin, Roche S.p.A., Milan, Italy) was diluted in saline before use.

Drugs were injected intravenously (i.v.) at different schedules and doses, as detailed in Table 1.

Drug doses and schedule were determined from previous studies,[15] taking 6 days in the mouse (conventional) as 3 weeks in the patient, and every other day in the mouse (dose-dense) as weekly in the patient. Drug doses have been translated from human to mouse using the U.S. FDA dose conversion guidelines (based on body surface area-BSA)[23] and adjusted to be safe in mice.

Briefly, PTX conventional was injected every 6 days for three courses at the dose of 20 mg/kg for MNHOC18 and 15 mg/kg for MNHOC84 and IGROV1-luc; PTX dose-dense-equi (total dose equal to conventional) was given every other day at the dose of 8 mg/kg (7 courses) for MNHOC18 and at 5 mg/kg (9 courses) for MNHOC84 and IGROV1-luc; PTX dose-dense-high (same schedule but 1.5 times higher) was used at the dose of 12 mg/kg for MNHOC18 or 7.5 mg/kg for MNHOC84 and IGROV1-luc. PTX dosages were reduced in the MNHOC84 and IGROV1-luc experiments after mild toxicity arose in the early experiment with MNHOC18. Cisplatin was given at the dose of 3 mg/kg every 6 days for three courses. Bevacizumab was injected at 5 mg/kg every 6 days for three courses or until disease progression in the maintenance regimen (treatment stopped when $\geq$50% of the mice had been euthanized). Vehicles were given with the same schedules as active compounds.

Evaluation of treatment

For the ectopic models, tumor growth s.c. was measured with a Vernier caliper, and weight (mg = mm^3) was calculated as [length (mm) $\times$ width^2 (mm^2)]/2. MNHOC18-bearing mice were randomized to treatment at a tumor weight of 100–300 mg (average 215 mg), and MNHOC84-bearing mice at 200–400 mg (average 332 mg). Tumor measurements were taken twice a week during and after treatments (6–7 mice per group). Each measure was normalized to the tumor weight of the same mouse at the start of treatment (Day 0) and weights were rescaled with the average absolute weight on Day 0 (i.e., all normalized weights were multiplied by a factor of 215 for MNHOC18 and 332 for MNHOC84). Tumor-free mice, confirmed by histological analysis 60 days after the last mouse had been euthanized, were considered cured.

Table 1. Treatment schedules

Treatment	I cycle			II cycle			III cycle														
DDP (D)	▲			▲			▲														
PTX conventional (PCo)	▲			▲			▲														
PTX dose-dense-equi (PEq)	▲	▲	▲	▲	▲	▲	▲	▲	▲												
PTX dose-dense-high (PHi)	▲	▲	▲	▲	▲	▲	▲	▲	▲												
BEV (B)	▲			▲			▲														
BEV maint (B maint)	▲			▲			▲			▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
Days from therapy	1	3	5	7	9	11	13	15	17	19	25	31	37	43	49	55	61	67	73	79	

Cisplatin (DDP) was injected i.v. at the dose of 3 mg/kg every 6 days for three cycles. Paclitaxel (PTX) conventional (PCo) was injected i.v. every 6 days for three cycles at the dose of 20 mg/kg (total dose 60 mg/kg) for MNHOC18 and 15 mg/kg (total dose 45 mg/kg) for MNHOC84 and IGROV1-luc; PTX dose-dense-equi (PEq) was injected i.v. every other day at the dose of 8 mg/kg (7 injections, total dose 56 mg/kg) for MNHOC18 or 5 mg/kg (9 injections, total dose 45 mg/kg) for MNHOC84 and IGROV1-luc; PTX dose-dense-high (PHi) was injected i.v. every other day at the dose of 12 mg/kg (7 injections, total dose 84 mg/kg) for MNHOC18 or at 7.5 mg/kg (9 injections, total dose 67.5 mg/kg) for MNHOC84 and IGROV1-luc. Bevacizumab (BEV, B) was injected i.v. at the dose of 5 mg/kg every 6 days for three cycles or continued in the maintenance regimen (BEV maint, B maint) until disease progression.

For the orthotopic model IGROV1-luc, bioluminescence imaging (BLI) was used to confirm the presence of tumor in the ovary, to randomize mice at the beginning of treatment and to follow tumor progression (6 mice per group).[21] Briefly, D-luciferin (150 mg/kg, i.p, Caliper Lifescience, Hopkinton, MA, US) injected mice were scanned after 10 min with ART (Advanced Research Technologies, Inc., Montreal, QC, Canada). Images were analyzed with the Optiview software (Advanced Research Technologies, Inc., Montreal, QC, Canada) and tumor burden was expressed as relative photon counts, dividing the number on any day of measurement by the count at the beginning of treatment for each single mouse. Macroscopic examination at autopsy and histological analysis confirmed that differences in photon counts reflected true differences in tumor burden (see below). Necrosis, which can alter photon counts, was not present.[21]

For survival analysis, animals were euthanized at the first signs of discomfort (the day of death being considered the limit of survival) and results were plotted as the percentage survival against days after tumor transplant. The increment of lifespan (ILS) was calculated as $100\times$[(median survival day of treated group – median survival day of control group)/median survival day of control group].[17]

A complete autopsy was done on each mouse to check the tumor burden in the peritoneal cavity. Pictures were taken with the MacroPath digital imaging system (Milestone S.r.l., Sorisole, Italy). The ovary bearing the primary tumor was harvested and weighed. Tumor dissemination into representative organs of the peritoneal cavity (liver, diaphragm, pancreas/omentum, contralateral ovary) was rated by two independent scientists using an arbitrary score: 0 = not infiltrated; 1 = small masses; 2 = evident masses; 3–4 = increasingly large masses; 5 = completely invaded, as previously described.[18]

Drug toxicity was estimated from the change of body weight, calculated as [(body weight on a given day/body weight on day treatment started)$\times$100] and expressed as relative body weight loss (BWL). BWL $\geq$10% was considered a toxic effect.

Analysis of the tumor growth curves

Treatment efficacy was evaluated from the tumor weight curve of individual mice using three independent parameters: the tumor weight at nadir (TWnadir), the absolute growth delay (AGD) and the doubling time of tumor regrowth (DTregrowth). TWnadir is the smallest tumor volume measured after treatment, and indicates the shrinkage observed in most treatment groups. In controls and less responding tumors, where no regression was seen, TWnadir was set as the weight at the start of treatment. AGD was calculated as the difference (in days) between the time to reach a target size (three times the size at the start of treatment for s.c. tumors, relative photon counts of 70 times for the orthotopic model) in a treated tumor and the average time to reach the same size in the control group. DTregrowth was calculated from the exponential fit of the last part of the growth curve when there was a frank and persisting increase in tumor size.

Flow cytometry

With MNHOC18, 3–4 additional mice were included in each treatment group and killed 2 days after the last drug dose in the first treatment week (i.e., after the first dose in the conventional and after the third in the dose-dense schemes), keeping the tumor for DNA measures and for histological analysis (see below).

Tumors were collected, minced and added to digestion medium (collagenase I, 200 U/mL + DNAse, 270 U/mL, Sigma). Single-cell suspensions were centrifuged, washed twice with serum-free medium and fixed in cold 70% ethanol for monoparametric staining of DNA content with a FACS Calibur (Becton Dickinson, San Jose, CA, US) flow cytometer. The relative numbers of tumor and "normal" cells (T/N) in the tumor specimens from treated and untreated mice were recorded (Supporting Information Methods).

Immunohistochemical analysis

Tumors (3–4 per group) excised at the end of one cycle of treatment for the MNHOC18 model or at the end of the whole cycle of treatments – 24 hr after the last dose for the MNHOC84 model – were formalin-fixed and paraffin-embedded. Immunohistochemical analysis was done with antibody against murine CD31 (PECAM, clone SZ31, Dianova, Hamburg, Germany) to assess microvessel density (MVD), with an antibody anti-Ki67 (Clone SP6, Thermo Scientific-Lab Vision, Monza, Italy) to assess cell proliferation, or an antibody against the active form of caspase (Asp175, Cell Signaling, Leiden, The Netherlands) to quantify apoptosis. Detailed procedures are described in the Supporting Information Methods. Analyses were done blind with an image analysis software (ImageJ, http://rsb.info.nih.gov/ij/).

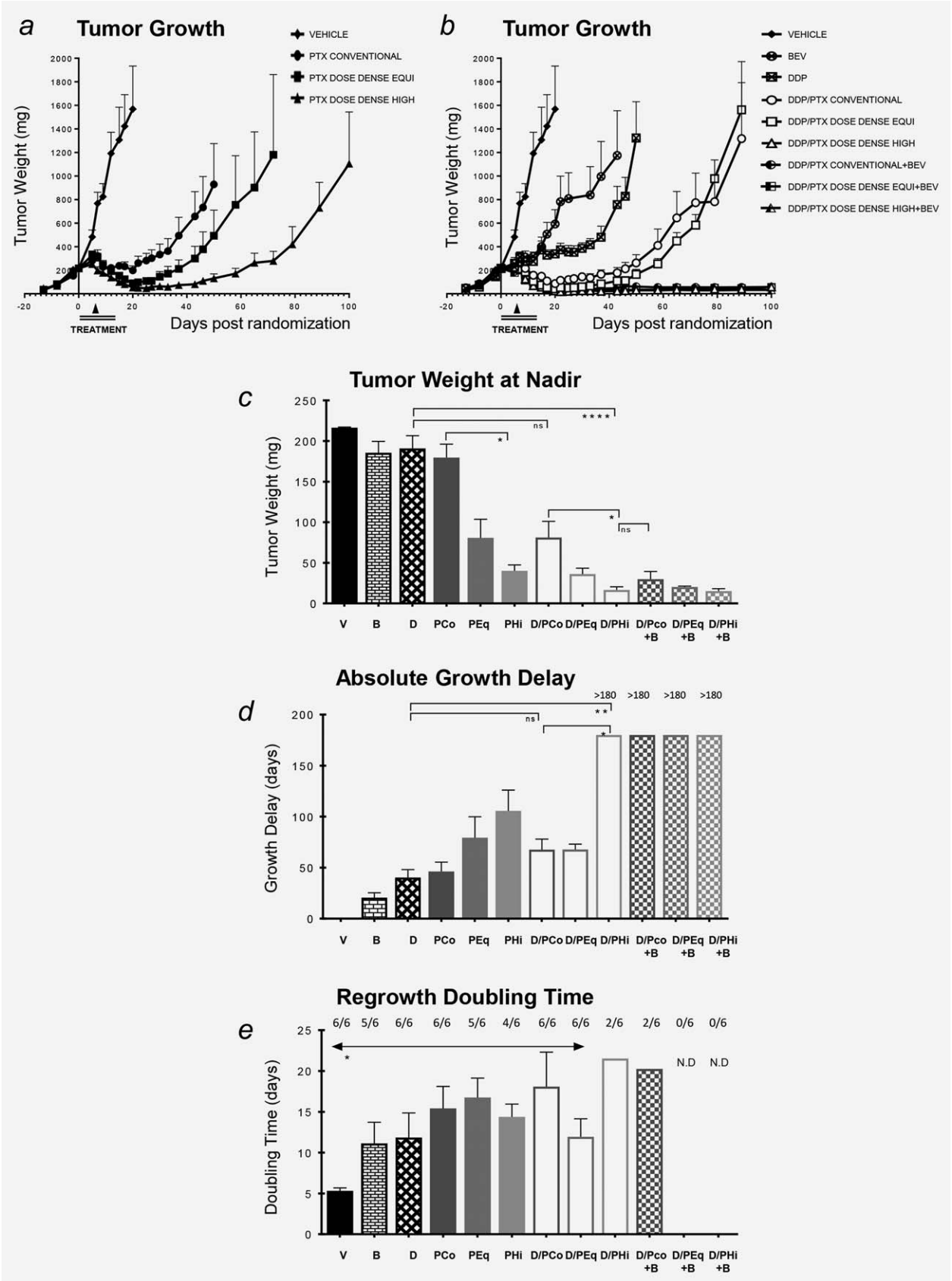
Statistical analysis

Statistical analyses were done using Prism 7 (GraphPad Software, La Jolla, CA, US). Differences in tumor weight at nadir (TWnadir), absolute growth delay (AGD), regrowth doubling time (DTregrowth), tumor/normal cells ratio, MVD, Ki67 and caspase 3 were analyzed by the Kruskal–Wallis test followed by Dunn's multiple comparison post-hoc test. Relative photon counts were analyzed by ordinary ANOVA followed by Tukey's post-hoc test. Kaplan-Meier survival curves were analyzed by the log-rank test.

Results

Chemotherapy with dose-dense paclitaxel is superior to conventional paclitaxel in a dose-dependent manner, but is equally effective as conventional paclitaxel when combined with bevacizumab

We investigated the effect of chemotherapy containing PTX at different schedules and doses, with or without bevacizumab, in two patient-derived ovarian cancer xenograft models:


a
Tumor Growth
VEHICLE
PTX CONVENTIONAL
PTX DOSE DENSE EQUI
PTX DOSE DENSE HIGH
Tumor Weight (mg)
TREATMENT
Days post randomization
b
Tumor Growth
VEHICLE
BEV
DDP
DDP/PTX CONVENTIONAL
DDP/PTX DOSE DENSE EQUI
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Regrowth Doubling Time
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Figure 1. MNHOC18 growth inhibition and parameters of activity. MNHOC18 ovarian cancer xenograft was transplanted subcutaneously in nude mice that were randomized to treatments at an average tumor weight of 215 mg. Chemotherapy regimens with/without bevacizumab and groups were as in Table 1. Response (6 mice per group) was shown as (*a*) tumor growth after different schemes of PTX monotherapy (arms of the same trial as in b, same vehicle) and (*b*) tumor growth after DDP/PTX with/without bevacizumab (arms of the same trial as in a, same vehicle) and evaluated as (*c*) tumor weight at nadir, (*d*) absolute growth delay and (*e*) regrowth doubling time. The mathematical modeling of the individual tumor growth curves of this experiment was described in Ref. 19. Δ sample collection after one cycle of therapy (48 hr from the last treatment), see Figure 2. Columns are mean and standard error $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$. Detailed statistics are in Supporting Information Table S1. V, vehicle; B, BEV, bevacizumab; D, DDP, cisplatin; P, PTX, paclitaxel; PCo, PTX conventional; PEq, PTX dose-dense-equi; PHi, PTX dose-dense-high.

MNHOC18, platinum-sensitive (Figs. 1 and 2), and MNHOC84, platinum-resistant (Fig. 3). Drug schedules and doses are described in Table 1.

The platinum-sensitive MNHOC18. In the MNHOC18 model, PTX monotherapy induced tumor reduction in all schemes (Fig. 1*a*), the dose-dense-high scheme giving the lowest TWnadir (Fig. 1*c*) and particularly delaying tumor growth (AGD > 100 days, Fig. 1*d*), with one mouse cured; regrowth was slow in all PTX-treated groups (three times the doubling time with vehicle) in a schedule-independent way.

DDP in the chemotherapy regimen (DDP/PTX) further inhibited tumor growth at all schedules (Fig. 1*b*). Despite an initial greater effect of DDP/PTX dose-dense-equi over DDP/PTX conventional (TWnadir; Fig. 1*c*), the two schemes caused similar growth delay (AGD; Fig. 1*d*) and all tumors regrew (DTregrowth; Fig. 1*e*). The DDP/PTX dose-dense-high was the most active with undetectable or very low tumor mass at nadir (4/6 cured mice) and only 1/6 regrowing tumor (Figs. 1*c*–1*e*).

Short-term effects were assessed 2 days after the first cycle of treatment by *ex vivo* DNA flow cytometric and histological analysis. A tenfold drop in the number of tumor cells compared to normal cells (T/N) was observed in mice given dose-dense DDP/PTX (Fig. 2*a*), against the twofold drop of the conventional scheme, suggesting important cytotoxic effects (Fig. 2*a* and Supporting Information Fig. S1).

The addition of bevacizumab to DDP/PTX significantly improved the response in all the regimens (Fig. 1*b*). TWnadir reached very low values (near the tumor detection limit) also in the conventional and dose-dense-equi schemes, improving the score for DDP/PTX without bevacizumab (Fig. 1*c*). Only 2/6 tumors in the conventional plus bevacizumab group regrew, reaching 220 and 250 mg at the last observation time (Day 180), while there were 0/6 regrowth in the dose-dense groups (Fig. 1*e*). The short-term measure (2 days after the first cycle) of T/N in the triple treatment groups distinguished differences between the three schemes, similarly to the double treatment (only DDP/PTX), with a negligible contribution of the single bevacizumab dose in the first cycle (Fig. 2*a*).

Analysis of the MNHOC18 tumor vasculature after the first cycle of treatment (one injection of bevacizumab) indicated a modest reduction in vessel density after bevacizumab single treatment, which became significant when combined with chemotherapy (DDP/PTX + BEV), though no difference emerged between the different PTX regimens (Figs. 2*b* and 2*c*).

All the PTX regimens were well tolerated. The maximum tolerated dose (MTD) was obtained in combination with DDP (BWL respectively 5%, 10%, 14% in conventional, dose-dense-equi and dose-dense-high). No additional toxicity was observed when adding bevacizumab (Supporting Information Figs. S3*a*–S3*c*).

This trial shows the best performance of PTX dose-dense and indicates that DDP/PTX dose-dense-high may be as efficacious as DDP/PTX conventional plus bevacizumab. Therefore in subsequent studies we focused on the comparison of conventional and dose-dense-high DDP/PTX schemes, with and without BEV.

The platinum-resistant MNHOC84. MNHOC84 is an OC-PDX with a low drug sensitivity profile,[18] as shown in Figures 3*a*–3*d* by the scant response to cisplatin. DDP/PTX dose-dense-high was more active than DDP/PTX conventional on MNHOC84 tumor growth (Fig. 3*a*), with a significantly lower TWnadir (34 mg in DDP/PTX dose-dense-high and 205 mg in DDP/PTX conventional), which was followed by a longer AGD (Figs. 3*b* and 3*c*).

The MNHOC84 tumor model is also not particularly responsive to bevacizumab monotherapy (Figs. 3*a*–3*c*), which only reduced the growth rate, with doubling time from 8.3 to 12.5 days (Fig. 3*d*). Bevacizumab combined with chemotherapy gives benefits in platinum-resistant ovarian cancer patients.[2,4] Figure 3*a* shows that incorporation of bevacizumab in the chemotherapy regimens affected tumor growth, with a reduction of the TWnadir and increase in AGD, particularly in the dose-dense-high regimen (3/6 cured mice) (Figs. 3*b* and 3*c*). It is worth noting that DDP/PTX dose-dense-high (without bevacizumab), which was better than DDP/PTX conventional, was not significantly different from DDP/PTX conventional plus bevacizumab. Regrowth was similarly delayed in all schemes (Fig. 3*d*).

Similarly to MNHOC18, in the MNHOC84 increased but tolerable toxicity was observed with the addition of DDP to PTX dose-dense-high (BWL 9%). No additional toxicity was observed when adding bevacizumab (BWL 8% in dose-dense-high) (Supporting Information Figs. S3*d*–S3*f*).

To confirm the efficacy of these schemes, we did an immunohistological analysis of Ki67 and caspase 3 in tumor samples at the end of the three cycles of therapy. There was a small decrease in proliferating cells (Ki67 positive) in

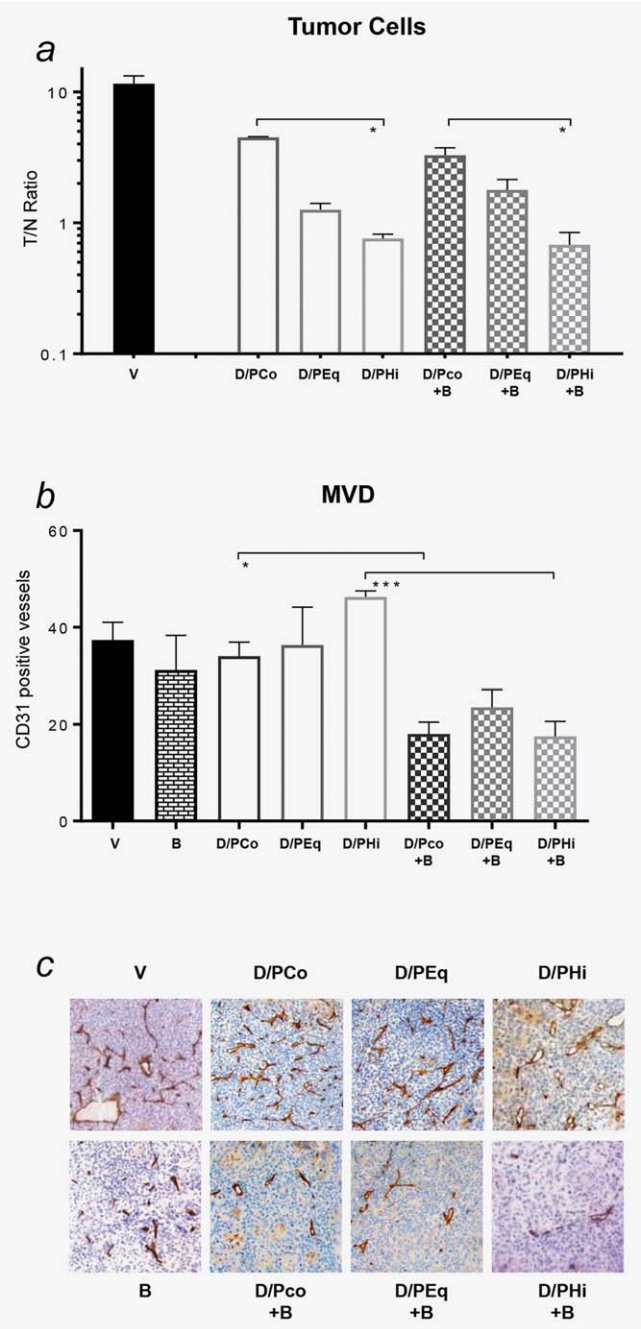


Figure 2. Short-term effects of treatments on MNHOC18. Experiment as in Figure 1. Representative mice (3–4 per group) were euthanized 48h after one cycle of therapy. (*a*) DNA histograms indicate the tumor/normal cells (T/N) ratio, (*b*) number of CD31-positive vessels in three (200x) fields and (*c*) representative images showing differences in vessel number by CD31 staining. T, tumor cells; N, normal cells; MVD, microvessel density; V, vehicle; B, bevacizumab; D, cisplatin; P, PTX, paclitaxel; PCo, PTX conventional; PEq, PTX dose-dense-equi; PHi, PTX dose-dense-high. Columns are mean and standard error $*p < 0.05$; $***p < 0.001$. Detailed statistics are in Supporting Information Table S1.

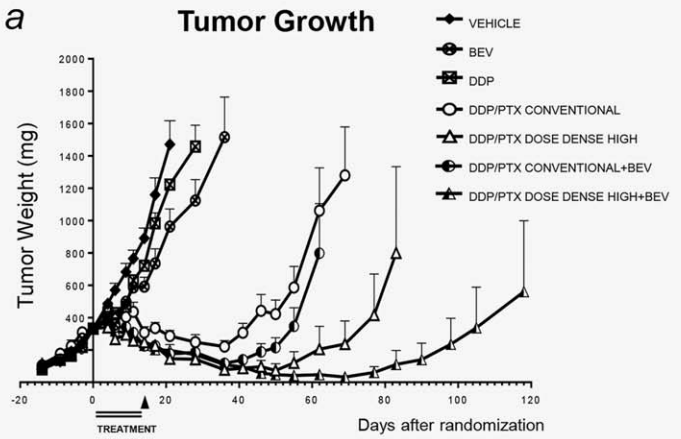
tumors treated with PTX dose-dense-high (Figs. 3*e* and 3*f*). The number of apoptotic cells increased with treatment, significantly more with the DDP/PTX dose-dense-high. The addition of bevacizumab to DDP/PTX conventional significantly increased apoptotic cells, but not differently from the group receiving DDP/PTX dose-dense-high with/without bevacizumab (Figs. 3*g* and 3*h*). On the whole, the parameters for MNHOC84 (tumor growth, TWnadir, AGD, caspase 3), showed no clear benefit of DDP/PTX conventional containing bevacizumab over DDP/PTX dose-dense-high (without BEV), though further improvement was observed on adding bevacizumab to the latter regimen.

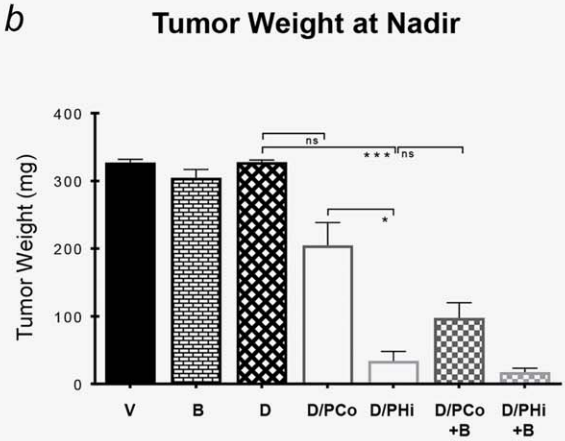
Bevacizumab combined with DDP/PTX delays tumor progression, prolongs survival and affects tumor dissemination and metastasis in mice

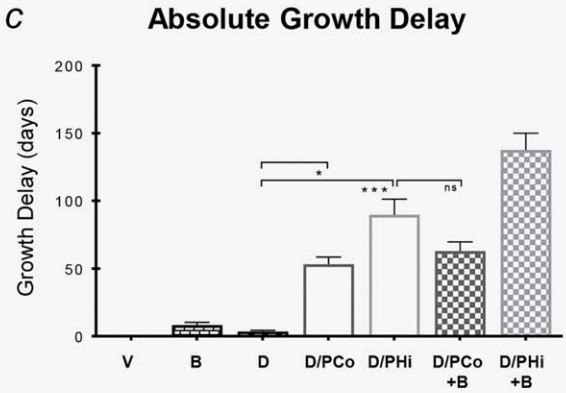
The site of tumor growth can affect the biology of the tumor, influencing the activity of treatments.[25,26] This prompted us to investigate our findings on ovarian tumor xenografts transplanted orthotopically and disseminating in the organs of the peritoneal cavity, thus mimicking the growth patterns of the disease (Figs. 4 and 5). Mice bearing IGROV1-luc in the ovary were treated with DDP/PTX conventional and DDP/PTX dose-dense-high regimens with/without bevacizumab; as in clinical trials, bevacizumab was also given in a maintenance regimen. Treatments as in Table 1. Tumor progression is indicated by the time course of the relative tumor burden, obtained by weekly BLI measures, shown in four time windows, at Day 20 (end of therapy), after 1 week (stable disease), 2 weeks (early progression) and 4 weeks (late progression) (Fig. 4*a*). At the end of treatment with DDP/PTX conventional and DDP/PTX dose-dense-high the tumor burden in the ovary was smaller than in the vehicle group; 1 week after the end of treatment the tumor burden remained almost stable in DDP/PTX conventional then increased at 2 and 4 weeks, while in DDP/PTX dose-dense-high tumor progression was slower (significantly different from conventional at 2 and 4 weeks), as shown by a longer AGD (Fig. 4*b*).

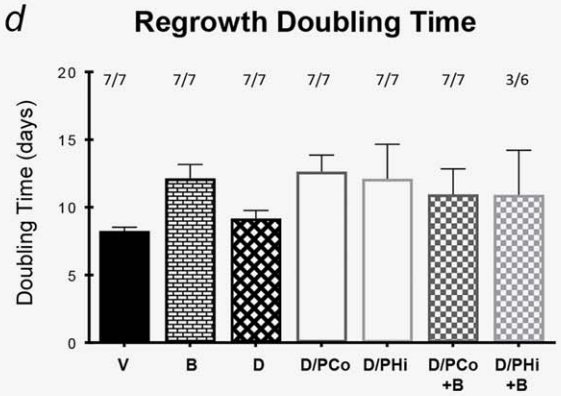
With the incorporation of bevacizumab in the treatment regimens tumor growth was already significantly inhibited at the end of treatment compared to the schemes without it. In the post-treatment period we observed a stable regressing tumor for at least 1 week, then regrowth from 2 weeks after treatment in the interrupted scheme and at 4 weeks in mice receiving bevacizumab in maintenance (Fig. 4*a*). In all groups receiving bevacizumab tumor progression was delayed (Fig. 4*b*); only in the group receiving DDP/PTX dose-dense-high plus bevacizumab in maintenance tumor progression was significantly further delayed (Fig. 4*b*).

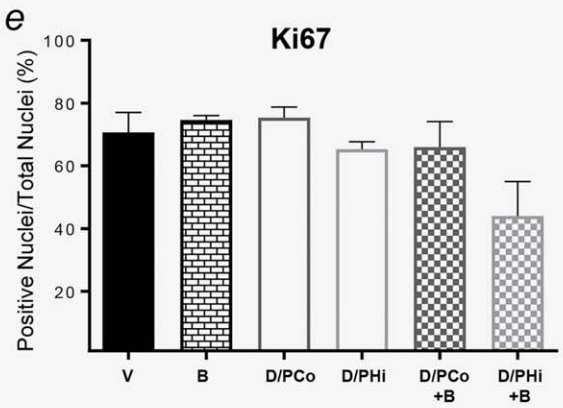
The final outcome of the study is shown by the Kaplan-Meier survival curves (Fig. 5*a*). The lifespan of mice receiving DDP/PTX conventional was significantly longer than with vehicle (ILS 53%) and more with DDP/PTX dose-dense-high (ILS 69%). The combination with bevacizumab improved survival in all schemes (ILS 122% and ILS 113% for DDP/

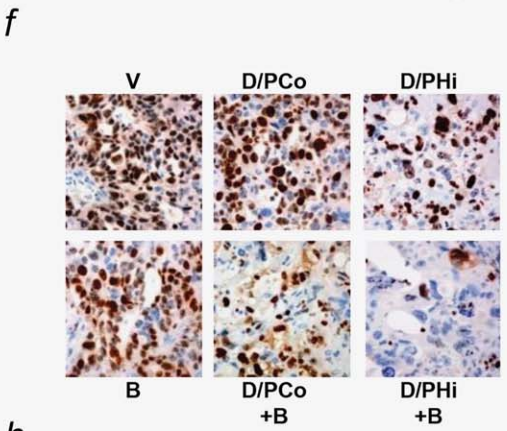

a
Tumor Growth
VEHICLE
BEV
DDP
DDP/PTX CONVENTIONAL
DDP/PTX DOSE DENSE HIGH
DDP/PTX CONVENTIONAL+BEV
DDP/PTX DOSE DENSE HIGH+BEV
Tumor Weight (mg)
Days after randomization
TREATMENT

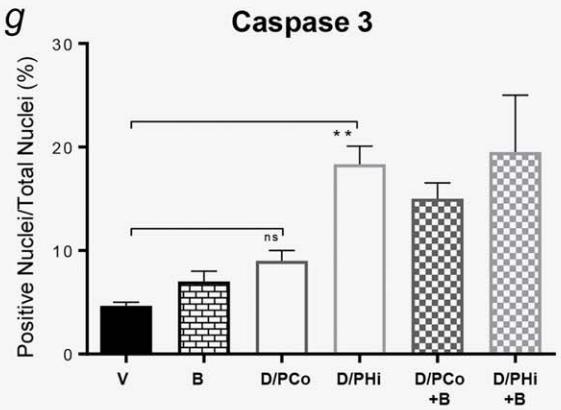

b
Tumor Weight at Nadir
Tumor Weight (mg)
V
B
D
D/PCo
D/PHi
D/PCo +B
D/PHi +B

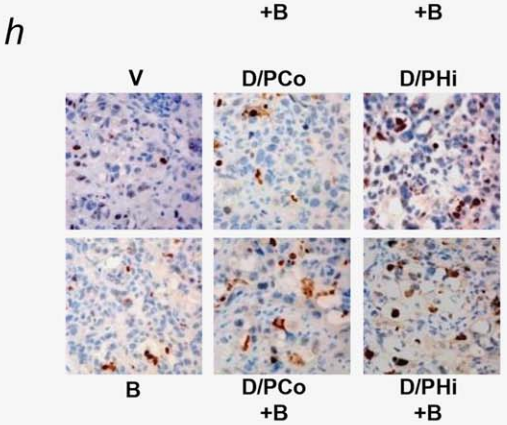

c
Absolute Growth Delay
Growth Delay (days)
V
B
D
D/PCo
D/PHi
D/PCo +B
D/PHi +B


d
Regrowth Doubling Time
Doubling Time (days)
7/7
7/7
7/7
7/7
7/7
7/7
3/6
V
B
D
D/PCo
D/PHi
D/PCo +B
D/PHi +B


e
Ki67
Positive Nuclei/Total Nuclei (%)
V
B
D/PCo
D/PHi
D/PCo +B
D/PHi +B


f
V
D/PCo
D/PHi
B
D/PCo +B
D/PHi +B


g
Caspase 3
Positive Nuclei/Total Nuclei (%)
V
B
D/PCo
D/PHi
D/PCo +B
D/PHi +B


h
V
D/PCo
D/PHi
B
D/PCo +B
D/PHi +B

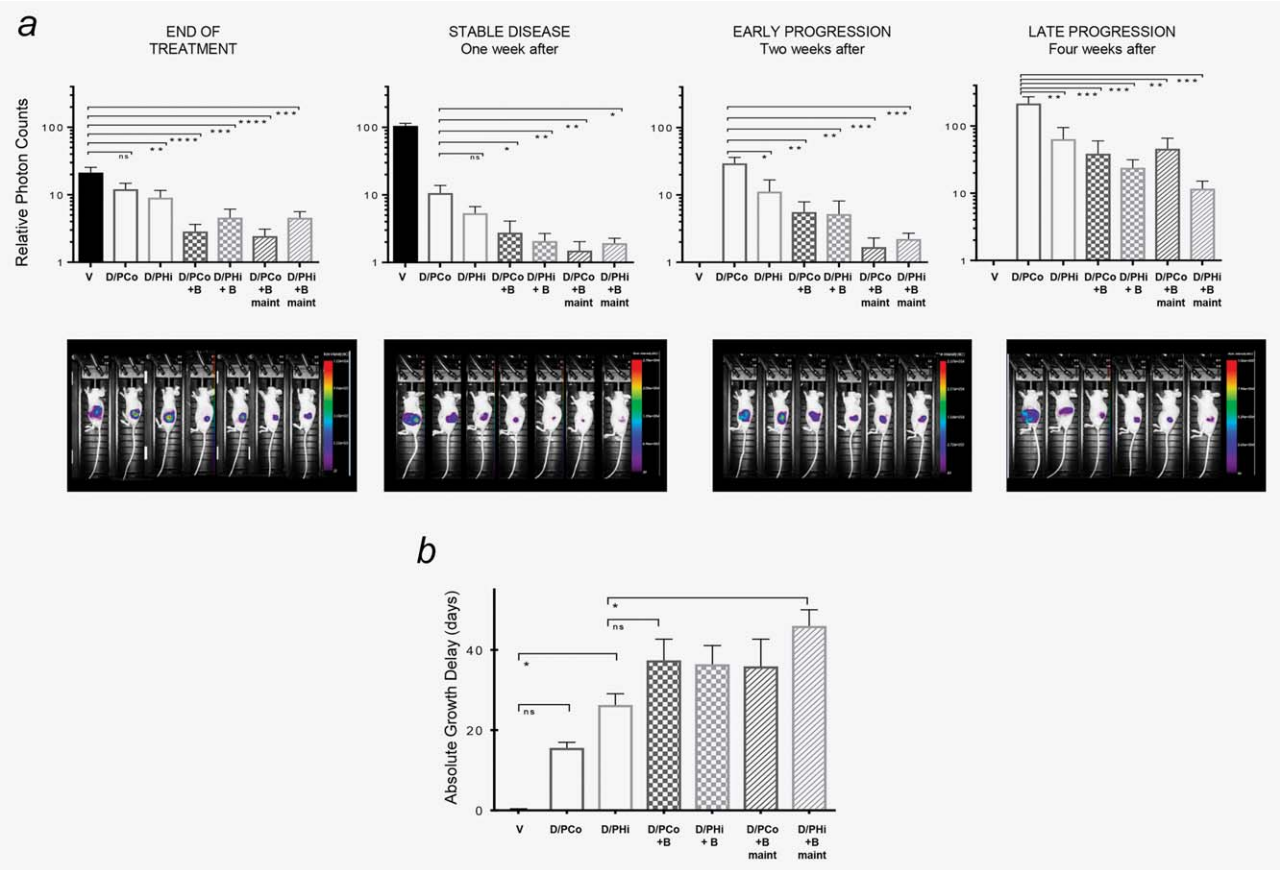


Figure 4. IGROV1-luc tumor progression and parameters of activity. IGROV1-luc was injected under the ovary bursa of nude mice (6 mice per group). Mice were randomized by photon counts 11 days after transplant, to receive the different treatment regimens (Table 1). (*a*) Tumor growth, detected by bioluminescence (BLI), was measured at different times after treatment and each measure was normalized to the bioluminescence of the same mouse at the start of treatment. Values are expressed as relative photon counts (mean and SD). Images are representative of each group at each time. (*b*) Absolute growth delay is the time (days) to reach relative photon counts 70 times the random. V, vehicle; B, bevacizumab; B maint, bevacizumab in maintenance; D, DDP, cisplatin; P, PTX, paclitaxel; PCo, PTX conventional; PHi, PTX dose-dense-high. Columns are mean and standard error $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. Detailed statistics are in Supporting Information Table S3.

PTX conventional and DDP/PTX dose-dense-high). Surprisingly – though expected from the progression results (Fig. 4*a*) – bevacizumab gave only a small advantage in maintenance (ILS 131% and ILS 140% for DDP/PTX conventional +BEV maint and DDP/PTX dose-dense-high +BEV maint). The BWL with DDP/PTX conventional was 4% and 9% with DDP/PTX dose-dense-high, but it did not worsen when bevacizumab was added.

To shed light on these findings, we studied the impact of the treatments on tumor dissemination. To compare the dissemination – without the bias of a different primary tumor burden – mice were euthanized at the same photon count (same primary tumor size), hence at different times (median survival time, MST, Fig. 5*b*). Mice treated with chemotherapy with/without bevacizumab had similar dissemination scores, slightly better in the groups receiving DDP/PTX dose-dense-

Figure 3. MNHOC84 growth inhibition and parameters of activity. MNHOC84 ovarian cancer xenograft was transplanted subcutaneously in nude mice that were randomized to treatments at an average tumor weight of 332 mg (7 mice per group). Chemotherapy regimens with/without bevacizumab and groups were as in Table 1. Tumor response is shown as (*a*) tumor growth after DDP/PTX at different regimens with/without bevacizumab and (*b*) tumor weight at nadir, (*c*) absolute growth delay and (*d*) regrowth doubling time. Additional mice (3–4 per group) were euthanized at the end of the therapy for immunohistological analysis, showing (*e*) total number of Ki67 positive cells as a percentage of the total number of neoplastic cells and (*f*) representative images of Ki67 positive cells, (*g*) total number of caspase-positive cells as a percentage of the total number of neoplastic cells and (*h*) representative images of caspase-3 stained cells. Cells were manually computed in three (400×) microscopic fields randomly selected within the bulk of the tumor using ImageJ software. [△] sample collection, at the end of therapy (Day 17). Columns are mean and standard error $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Detailed statistics are in Supporting Information Table S2. V, vehicle; B, BEV, bevacizumab; D, DDP, cisplatin; P, PTX, paclitaxel; PCo, PTX conventional; PHi, PTX dose-dense-high.

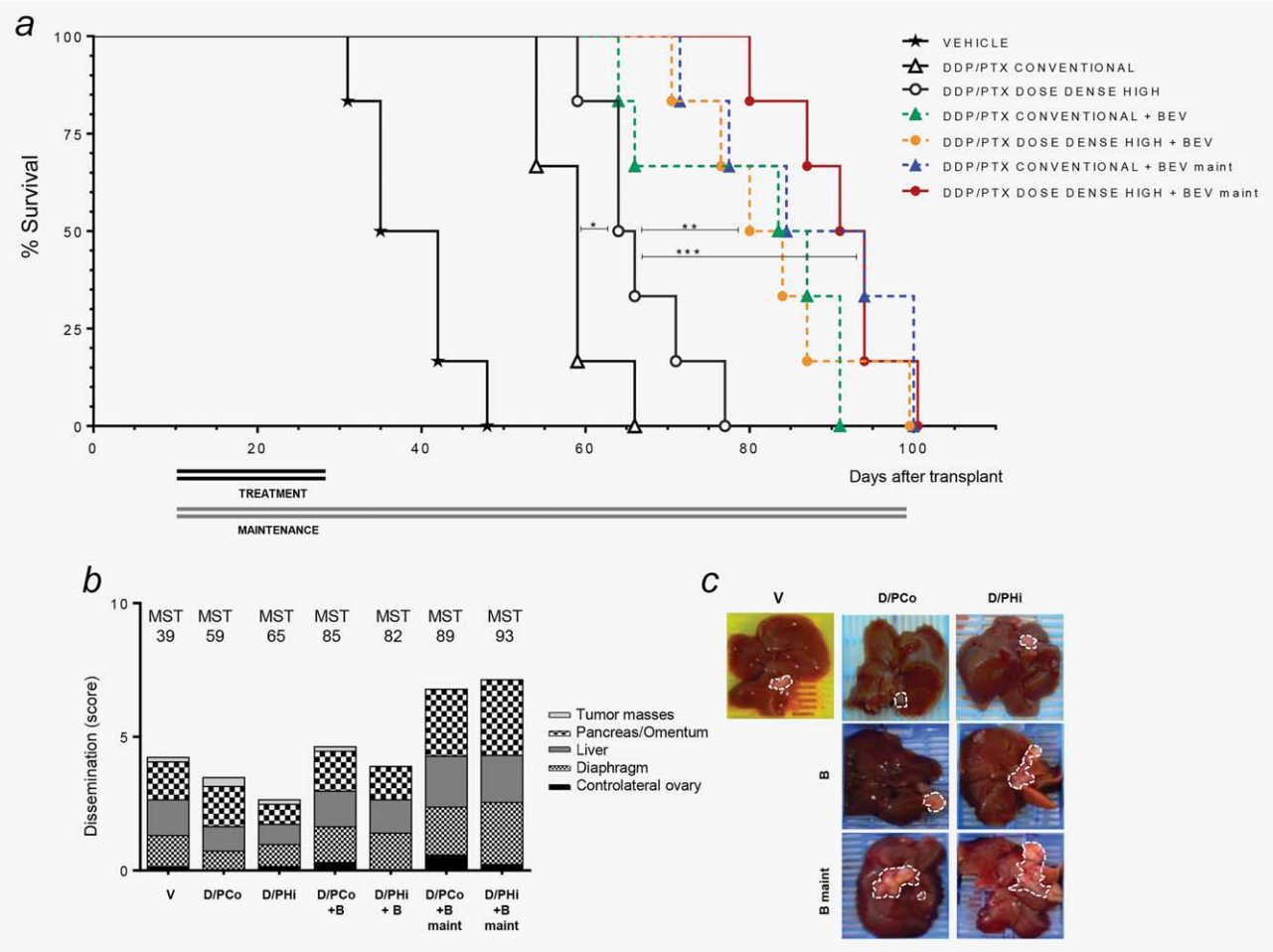


Figure 5. Effects on mouse survival and tumor dissemination. (*a*) Survival of mice bearing IGROV1-luc as from experiment described in Fig 4. (*b*) Tumor masses and tumor dissemination in representative organs of the peritoneal cavity (contralateral ovary, liver, diaphragm, pancreas/omentum) were rated using an arbitrary score for gross tumor dissemination as described in Materials and Methods. (*c*) Representative images of disseminated liver. V, vehicle; B, BEV, bevacizumab; B maint, BEV maint, bevacizumab in maintenance; D, DDP, cisplatin; P, PTX, paclitaxel; PCo, PTX conventional; PHi, PTX dose-dense-high; MST, median survival times (days). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Detailed statistics are in Supporting Information Table S3.

high (Fig. 5*b*), thus with similar primary and metastatic tumor burden at death. In contrast, both groups that received bevacizumab maintenance after chemotherapy (conventional and dose-dense-high) and showed limited survival advantage, scored higher in terms of tumor mass and metastases to the peritoneal cavity organs (Fig. 5*b*), as shown by representative images of disseminated livers (Fig. 5*c*). These findings suggest it could be beneficial to add bevacizumab to the chemotherapy regimens (to delay progression), but they may also explain the modest gains in long-term survival (high malignancy).

The results of our study were validated in a larger trial on 1A9luc, another ovarian cancer model growing orthotopically in the mouse peritoneal cavity (Supporting Information Fig. S2). Here DDP/PTX dose-dense-high (MST 58 days, ILS 83%) performed significantly better than DDP/PTX conventional (MST 48 days, ILS 50%), but was equally active to DDP/PTX conventional incorporating bevacizumab (MST 59 days, ILS 84%), and again not different from DDP/PTX dose-dense-high incorporating bevacizumab (MST 62 days, ILS 94%).

Discussion

We have a limited understanding of the antitumor effects of the different chemotherapy schedules in use in ovarian cancer treatment and incorporating bevacizumab. In our preclinical studies, we compared different PTX schemes of treatment in combination with DDP, with/without bevacizumab. Experimental models enable to analyze the response to treatment in a way unachievable in clinical setting, disclosing its dynamics with frequent measures, using complementary techniques and gaining information to optimize the treatment. Recently, we have shown the feasibility of decoding growth curves of treated tumors in terms of underlying cytostatic and

cytotoxic effects, exploiting *in silico* modeling.[27] In that study, by simulation of the growth curves of the MNHOC18 model, we demonstrated that DDP and PTX were both cytotoxic and cytostatic, while BEV was only cytostatic, and that more cells were killed by PTX dose-dense than with PTX conventional. Moreover, we showed that the cytotoxicity of the DDP/PTX combined treatment was similar to the single PTX treatment and considerably less than expected from the cytotoxicity of individual drugs, but the addition of BEV increased cell kill in all schemes. Here, we compared the efficacy of the treatment schemes using different parameters, directly derivable from the growth curve, additional flow cytometric and histological measures and biological endpoints in four ovarian cancer xenograft models, 2 growing subcutaneously and 2 growing orthotopically in the mouse peritoneal cavity.

Middle-time effects were assessed from the maximum tumor shrinkage (TWnadir) in the growth curve. Later, the animal either remained cured or the tumor started re-growing and AGD measures the interval before regrowth, somewhat like the disease-free survival in clinical studies. However, a re-growing tumor may be different or may arise in a different environment from the original one, and this long-term feature of the response was caught by the DTregrowth.

Short-term effects (a few days from the start of treatment), with a single cycle of therapy, cannot be appreciated on a growth curve and were studied by flow cytometry and/or histology.

From this sequence of analyses we found that i) dose-dense PTX (every other day treatment) was superior to conventional PTX (weekly treatment) and more when the cumulative dose was higher (dose-dense-high); ii) bevacizumab added to conventional chemotherapy delayed tumor progression and increased survival, but this regimen was no better than dose-dense-high PTX (only chemotherapy); iii) dose-dense-high PTX plus bevacizumab was the most efficacious regimen in delaying tumor progression, but did not improve significantly mouse survival; iv) the lack of survival benefit with therapy incorporating long-term treatment with bevacizumab was associated with an ambiguous increase in tumor dissemination and metastasis in the mouse peritoneal cavity.

The PTX dose-dense-high scheme was extremely effective, curing mice in combination with DDP. One could consider that these different effects are due to PTX pharmacokinetics. However pharmacokinetic measures indicate that a proportionally lower PTX exposure (plasma AUC) was achieved with the dose-dense scheme (Supporting Information Fig. S4), thus suggesting that the efficacy of this scheme cannot simply be ascribed to pharmacokinetics.

The flow cytometric analysis of the MNHOC18 model indicated a definite advantage of the dose-dense scheme over the conventional one for the combined DDP/PTX scheme, but also the importance of reaching a fairly high dosage in the dose-dense scheme. Our findings help explain the differences between clinical studies where the dose-dense schedule differed in terms of cumulative total dose administered.[10,11]

With the incorporation of bevacizumab in MNHOC18, an important improvement compared to the dual chemotherapy treatment without bevacizumab in the conventional group and consolidation of the response in the dose-dense ones were observed. This was reproduced in the orthotopically growing 1A9-luc model, where bevacizumab improved survival in the conventional but no further in the dose-dense scheme, so the triple treatments were almost as effective as the double dose-dense-high (Supporting Information Fig. S2). The superiority of the triple dose-dense-high (DDP/PTX dose-dense-high-+ BEV) over the triple conventional (DDP/PTX conventional + BEV) was more pronounced in the MNHOC84 model. However, in this model too, the double dose-dense-high (without bevacizumab) showed efficacy similar to the triple bevacizumab-containing conventional regimens.

In our study we used bevacizumab to target tumor-derived human VEGF, overlooking the possible role of host stromal VEGF in the disease progression. When the antibody B20 4.1.1, which neutralizes both murine and human VEGF, was tested on MNHOC18 a similar tumor growth impairment as with bevacizumab was observed (Supporting Information Fig. S5). It has been reported that the functional contribution of stromal VEGF varies among tumors, and consequently the efficacy of this cross-species VEGF-blocking antibody,[28] therefore we cannot exclude that in the other tumor models we have underestimated the response to bevacizumab.

PTX dose-dense-high significantly increased caspase 3-positive cells and a similar effect was obtained with DDP/PTX conventional plus bevacizumab. This is in line with studies showing that low continuous PTX exposure caused an abnormal mitotic block, inhibiting cell proliferation and inducing apoptotic cell death. In the orthotopic IGROV-1 model the addition of bevacizumab improved the efficacy of both schemes, but the gain in survival over chemotherapy alone was smaller in the dose-dense than in the conventional scheme, cancelling the superiority of the dose-dense scheme without bevacizumab. Thus, the conventional scheme appears to benefit more from the addition of bevacizumab. The benefit of bevacizumab in the dose-dense scheme is possibly more marked at lower doses, but it tends to be lost when sufficiently high dosages of chemotherapy are used and the maximum achievable effect without bevacizumab has already approached. This conclusion helps clarify the results of a recent clinical trial where the combination with bevacizumab 3-weekly or weekly PTX was equally effective.[14]

Clearly differences in response between patients' tumors and possible side effects need to be taken in account when selecting the best treatment scheme. We found all the combinations were well tolerated and with no change when bevacizumab was added.

Microtubule-binding agents such as PTX inhibit microtubule dynamics and impair endothelial cell functions at low

concentrations, an effect that is stronger in combination with antiangiogenic drugs.[29,30] We had previously reported that the antiangiogenic effect of PTX was related to its concentration,[4] within a range of activity *in vitro* against endothelial cells, and that PTX synergizes with VEGF blockades.[31]

Histological analysis of the xenografts after one cycle of chemotherapy only indicated no effect on vessel number with any of the PTX schemes. Although this suggests that the antitumor effect of dose-dense-high PTX was not primarily due to its antiangiogenic properties, the significant inhibition of MVD on adding bevacizumab upholds the anti-vascular effect of the combination. Antiangiogenic agents such as bevacizumab boost the efficacy of chemotherapy by multiple mechanisms which can affect different tumor compartments, and the vascular network.[26,32]

In clinical trials bevacizumab is also given as a maintenance regimen after chemotherapy. The IGROV-1 model transplanted in the mouse ovary (Figs. 4 and 5) with bevacizumab in maintenance enabled us to study the effect of the drug on tumor progression, survival and metastatic dissemination. Despite some delay in tumor progression, survival was prolonged only marginally in mice receiving bevacizumab in maintenance and they died with larger tumor burden disseminating to organs of the peritoneal cavity, which we think is the reason for the limited gain in survival over those with bevacizumab stopped. Increased malignancy after angiogenesis inhibitors has been reported in preclinical studies.[33] It depends on the tumor type and treatment modalities and can be counteracted by the combination with chemotherapy.[15,34,35] Recently the impact on ovarian cancer of bevacizumab incorporated in first-line chemotherapy was examined in a case–control study but despite a prolonged platinum free interval (PFI) it was often associated with more aggressive recurrent disease, less responsive to second-line chemotherapy.[36] This observation, together with our preclinical findings, may help explain the lack of clear benefit in terms of overall survival in trials containing bevacizumab.

In conclusion, these preclinical studies reinforce the therapeutic value of dose-dense PTX in the treatment regimen, but indicate generally that the addition of bevacizumab is not always more advantageous. The quantitative analyses of the response with multiple methodologies allowed to get insights into the outcome of the different schemes. Studies are needed to identify effective signature for patients who benefit from these type of combinations aimed at reducing the risk of resistance.

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Author Contribution

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Acquisition of data: F.B., F.F., E.D., E.E.

Conception and design: F.B., N.C., M.R.B., P.U., R.G.

Conduction of the animal experiment and establishing the different tumor models: F.B., E.D., A.D.

Histological analysis: L.M., E.S.

Data analysis and interpretation of data: F.B., F.A.P., L.M., M.R.B., P.U., R.G.

Writing the manuscript: F.B., P.U., M.R.B., R.G.

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