

Metronomic Activity of CD44-Targeted Hyaluronic Acid-Paclitaxel in Ovarian Carcinoma

Sun Joo Lee¹, Sukhen C. Ghosh², Hee Dong Han^{1,4}, Rebecca L. Stone¹, Justin Bottsford-Miller¹, De Yue Shen¹, Edmond J. Auzenne², Alejandro Lopez-Araujo², Chunhua Lu¹, Masato Nishimura¹, Chad V. Pecot⁵, Behrouz Zand¹, Duangmani Thanappapasr¹, Nicholas B Jennings¹, Yu Kang¹, Jie Huang¹, Wei Hu¹, Jim Klostergaard², and Anil K. Sood^{1,3,4}

Abstract

Purpose: Most primary human ovarian tumors and peritoneal implants, as well as tumor vascular endothelial cells, express the CD44 family of cell surface proteoglycans, the natural ligand for which is hyaluronic acid. Metronomic dosing, the frequent administration of chemotherapeutics at substantially lower than maximum tolerated doses (MTD), has been shown to result in reduced normal tissue toxicity and to minimize "off-treatment" exposure resulting in an improved therapeutic ratio.

Experimental Design: We tested the hypothesis that hyaluronic acid (HA) conjugates of paclitaxel (TXL; HA-TXL) would exert strong antitumor effects with metronomic (MET) dosing and induce antiangiogenic effects superior to those achieved with MTD administration or with free TXL. Female nude mice bearing SKOV3ip1 or HeyA8 ovarian cancer cells were treated intraperitoneally (i.p.) with MET HA-TXL regimens (or MTD administration) to determine therapeutic and biologic effects.

Results: All MET HA-TXL-treated mice and the MTD group revealed significantly reduced tumor weights and nodules compared with controls (all *P* values < 0.05) in the chemotherapy-sensitive models. However, the MTD HA-TXL-treated mice showed significant weight loss compared with control mice, whereas body weights were not affected in the metronomic groups in HeyA8-MDR model, reflecting reduced toxicity. In the taxane-resistant HeyA8-MDR model, significant reduction in tumor weight and nodule counts was noted in the metronomic groups whereas the response of the MTD group did not achieve significance. While both MTD and metronomic regimens reduced proliferation (Ki-67) and increased apoptosis (TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling), only metronomic treatment resulted in significant reductions in angiogenesis (CD31, microvessel density). Moreover, metronomic treatment resulted in substantial increases in thrombospondin-1 (Tsp-1), an inhibitor of angiogenesis.

Conclusions: This study showed that MET HA-TXL regimens have substantial antitumor activity in ovarian carcinoma, likely via a predominant antiangiogenic mechanism. *Clin Cancer Res*; 18(15): 4114–21. ©2012 AACR.

Introduction

Ovarian cancer is the most common cause of death from a gynecologic malignancy (1). The standard first-line man-

agement approach for patients with advanced ovarian cancer currently consists of surgery followed by chemotherapy. Unfortunately, most patients will develop recurrent cancer and die as a result of progressive disease. Therefore, new approaches to treatment are needed to improve the outcome for these patients. Recent clinical trial results have provided compelling evidence that intraperitoneal or more frequent administration of drugs results in markedly improved survival in small volume disease patients (2–4).

In the quest for more molecularly targeted approaches for delivery of chemotherapy, a variety of approaches are being developed. Hyaluronic acid (HA), a linear polysaccharide of alternating D-glucuronic acid and N-acetyl-D-glucosamine units, is the only nonsulfated glycosaminoglycan and occurs primarily *in vivo* as sodium hyaluronate (5, 6). Hyaluronic acid plays important roles in cell adhesion, growth, and migration (7, 8). Hyaluronic acid also acts as a signaling molecule in cell motility, inflammation, wound

Authors' Affiliations: Departments of ¹Gynecologic Oncology and Reproductive Medicine, ²Molecular and Cellular Oncology, and ³Cancer Biology, ⁴Center for RNA Interference and Non-Coding RNA, and ⁵Division of Cancer Medicine, University of Texas M.D. Anderson Cancer Center, Houston, Texas

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J. Klostergaard and A.K. Sood are joint senior authors.

Corresponding Author: Anil K. Sood, Departments of Gynecologic Oncology and Cancer Biology, The University of Texas, M.D. Anderson Cancer Center, 1155 Herman Pressler, Unit 1352, Houston, TX 77030. Phone: 713-745-5266; Fax: 713-792-7586; E-mail: asood@mdanderson.org

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Translational Relevance

Most patients with advanced ovarian cancer eventually develop resistance to conventional chemotherapy despite maximum tolerated dose (MTD) regimens. Metronomic dosing of selected agents has been shown to result in reduced normal tissue toxicity and to minimize "off-treatment" exposure, resulting in an improved therapeutic ratio. In the present study, we have shown the efficacy of metronomic CD44-targeted, hyaluronic acid (HA)-conjugated paclitaxel (HA-TXL) to inhibit the growth of taxane-sensitive and -resistant ovarian carcinoma. These effects are thought to be mediated by antiproliferative and proapoptotic effects against the epithelial tumor cells, as well as the antivascular effects of metronomic dosing. Our findings suggest that MET HA-TXL is highly efficacious and should be considered for future clinical trials.

healing, and cancer metastasis (8). Most malignant solid tumors contain elevated levels of hyaluronic acid, and these high levels of hyaluronic acid production provide a matrix that facilitates invasion (6, 9). Hyaluronic acid internalization is mediated via matrix receptors, including the CD44 family, transmembrane receptors that can communicate cell-matrix interactions into cells and can alter the matrix in response to intracellular signals (6). Hyaluronic acid is also closely related to angiogenesis in many types of tumors, in which hyaluronic acid receptors, primarily CD44, are abundantly overexpressed on the cell surface (10). Thus, malignant cells with high metastatic potential often exhibit enhanced binding and uptake of hyaluronic acid (7, 10, 11). Using hyaluronic acid as a drug carrier should thus combine the advantages of both the passive (11-16) and active targeting ability of a polymeric prodrug (6). Moreover, coupling of antitumor agents to hyaluronic acid can provide advantages in drug solubilization, stabilization, localization, and controlled release (6, 12).

The CD44 proteoglycan family is expressed in as high as 90% of fresh samples from primary human ovarian tumors or peritoneal implants (5, 13-19), and a hydrophilic hyaluronic acid backbone can give paclitaxel (TXL) aqueous solubility without use of an excipient, such as cremophor in paclitaxel (5). Elimination of cremophor is particularly important as it may interfere with the antiangiogenic activity of taxanes (20, 21). Our earlier study indicated that HA-TXL, administered intraperitoneally in a single injection at near maximum tolerated dose (MTD) levels in peritoneally implanted CD44(+) human ovarian carcinoma mouse models, resulted in markedly reduced tumor burden (5). Standard chemotherapeutic regimens are designed to deliver the highest or MTD, which can be safely administered and is generally repeated in cycles. Usually, 3 to 4 weeks of rest periods are needed between treatments for recovery and to minimize additive toxicity, because of indiscriminate effects of chemotherapeutics on normal tissues. However,

recent studies indicate that tumor-associated endothelial cells continue to proliferate and promote cancer growth between treatments (22-24) and may also be accompanied by recruitment of endothelial cell progenitors from the bone marrow to the tumor (25); thus, the rest periods may allow repair and regrowth of the tumor vasculature. Moreover, unexpected delays ("treatment holidays") requiring dose reduction or concomitant bone marrow support occur frequently in dose-intensive strategies. Hence, as an alternative to current MTD-based chemotherapy dosing schedules, metronomic dosing is being evaluated. Metronomic dosing involves the frequent administration of chemotherapeutics at substantially lower doses than their MTDs. Ideally, the strategy would result in reduced normal tissue toxicity and minimize "off-treatment" exposure and risk of regrowth, resulting in an improved therapeutic ratio (22, 24). Metronomic dosing of cytotoxic agents is believed to function in an antiangiogenic manner, because the frequent, low-dose administration appears to target tumor associated endothelial cells (26, 27).

We have previously shown the superior efficacy/toxicity profile of single MTD HA-paclitaxel (HA-TXL) treatment compared with weekly dosing of paclitaxel in orthotopic human ovarian carcinomas (5, 28) and of weekly HA-TXL versus paclitaxel in head and neck squamous cell carcinoma models (29). The advantages of metronomic versus MTD dosing of free taxanes have also been shown in ovarian carcinoma models (30). Here, we show the superior antitumor activity of MET HA-TXL in multiple orthotopic mouse models of ovarian carcinoma.

Materials and Methods

Cell lines and culture

The human epithelial ovarian cancer cell lines SKOV3ip1, HeyA8, and HeyA8-MDR (taxane-resistant) have been described previously (31, 32). SKOV3ip1 and HeyA8 cells were cultured in RPMI-1640 medium supplemented with 15% FBS and 0.1% gentamicin sulfate (Gemini Bioproducts). HeyA8-MDR cell lines were maintained in RPMI-1640 medium supplemented with 15% FBS, 0.1% gentamicin sulfate, and 300 ng/mL of paclitaxel to sustain taxane resistance.

Cytotoxicity analyses of response to continuous or MET HA-TXL

SKOV3ip1 cells were cultured overnight in 96-well plates in 100 μ L of medium before drug treatment, as previously described (26). On the basis of preliminary experiments, cell numbers were adjusted to 1×10^4 cells per well to achieve subconfluent control cell monolayers at the end of the assay. The cytotoxic effects of HA-TXL were established using either continuous exposure for 144 hours to a dose range of drug up to 500 ng/mL (TXL equivalents) or to 3 cycles of 48-hour exposure to either 10 or 100 ng/mL HA-TXL (followed by washout each time), to mimic frequent, lower dose metronomic scheduling. Remaining viable cells were stained with MTT after 144 hours, and the percentage

of control survival as measured by optical density of incorporated dye was determined.

HA-TXL synthesis

Hyaluronic acid (~40 kDa) was provided by K3 Corporation. 1-Ethyl-3-[3V-(dimethylamino)propyl] carbodiimide (EDCI), diphenylphosphoryl chloride, adipic dihydrazide (ADH), succinic anhydride, *N*-hydroxysuccinimide (NHS), and triethylamine were purchased from Sigma-Aldrich Co. Paclitaxel (TXL) was purchased from HandeTech Development Co. All solvents were of reagent or high-performance liquid chromatography (HPLC) grade. HA-TXL was synthesized as previously described (5), using a modification of the previously published multistep synthesis (5, (6, 33)

Orthotopic mouse models of human ovarian cancer

Female athymic nude mice (NCr-*nu*) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. The mice were cared for in accordance with guidelines set forth by the American Association for Accreditation for Laboratory Animal Care and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the MDACC Institutional Animal Care and Use Committee.

The development and characterization of the orthotopic models of advanced ovarian cancer used in these experiments have been previously described by our laboratory (34). Ovarian cancer (SKOV3ip1) cells harvested in log-phase growth were injected intraperitoneally (i.p.) in 200 μ L of cell suspension. One week later, treatment was conducted by i.p. injection according to the following groups (10 mice/group): (i) control/PBS, (ii) MET HA-TXL, 10 mg/kg, (iii) MET HA-TXL, 20 mg/kg, (iv) MET HA-TXL, 30 mg/kg, and (v) MTD HA-TXL, single injection of 180 mg/kg, all HA-TXL doses being in TXL equivalents. Because the metronomic groups were to be given 9 injections, the 20 mg/kg group was total dose equivalent to the 180 mg/kg MTD group, and the lower dose was 50% of this level, and the higher, 150%. The HeyA8 and HeyA8-MDR groups were treated as follows: (i) control/PBS, (ii) MET HA-TXL, 5 mg/kg, (iii) MET HA-TXL, 10 mg/kg, (iv) MET HA-TXL, 20 mg/kg, and (v) MTD HA-TXL, single injection of 180 mg/kg. Thus, the total doses for the metronomic groups corresponded to 25%, 50%, and 100% of the MTD dose. To evaluate the effects of HA-TXL-based therapy on bulkier disease, we also carried out experiments where treatment was started 12 days following tumor cell injection (HeyA8-MDR). To assess the role of the HA/CD44 axis in the specificity of antitumor effects resulting from HA-TXL therapy, mice were pretreated with excess free hyaluronic acid immediately before injection of HA-TXL, both given i.p. For assessing effects on survival, treatment was continued until each animal became moribund (the experiment was terminated at 60 days posttumor implantation). To compare the effects of HA-TXL to free paclitaxel (TXL), the free TXL was

dosed either in metronomic (0.5 mg/kg) or MTD (10 mg/kg) fashion. Before injection, the initial weights of mice were measured and dose adjustments were carried out according to mean weight of each group. Metronomic therapy groups were injected i.p. every other day, beginning either on day 7 or 14. Body weights were measured every week, beginning on day 7. The aliquots of lyophilized HA-TXL were solubilized in PBS immediately before injection to ensure chemical stability. All animals were sacrificed and a necropsy conducted when mice from any of the groups became moribund (typically 3–6 weeks after therapy initiation depending on the model). The individuals conducting the necropsy were blinded to the different groups. Tumors were harvested and fixed in formalin for paraffin embedding or snap-frozen in optimum cutting temperature (OCT; Miles, Inc.) for immunohistochemical analyses.

Immunohistochemistry

Immunohistochemical analyses of CD31, Ki-67, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and thrombospondin-1 (Tsp-1) were conducted on 4- μ m thick frozen or formalin-fixed, paraffin-embedded tumor specimens. Primary antibodies used included: CD31 (rat monoclonal anti-mouse, 1:800 dilution; BD Bioscience, Pharmingen); Ki-67 (rabbit polyclonal anti-human, 1:200 dilution; Biocare Medical); Tsp-1 (1:25 dilution; Abcam); and TUNEL (Promega). Quantification of microvessel density (MVD, CD31), tumor cell proliferation (Ki-67), Tsp-1, and apoptosis (TUNEL) was conducted on slides from each treatment group, as previously described (34, 35). For assessment of endothelial cell apoptosis, frozen sections were stained for CD31 (red) followed by TUNEL (green), as described previously (36).

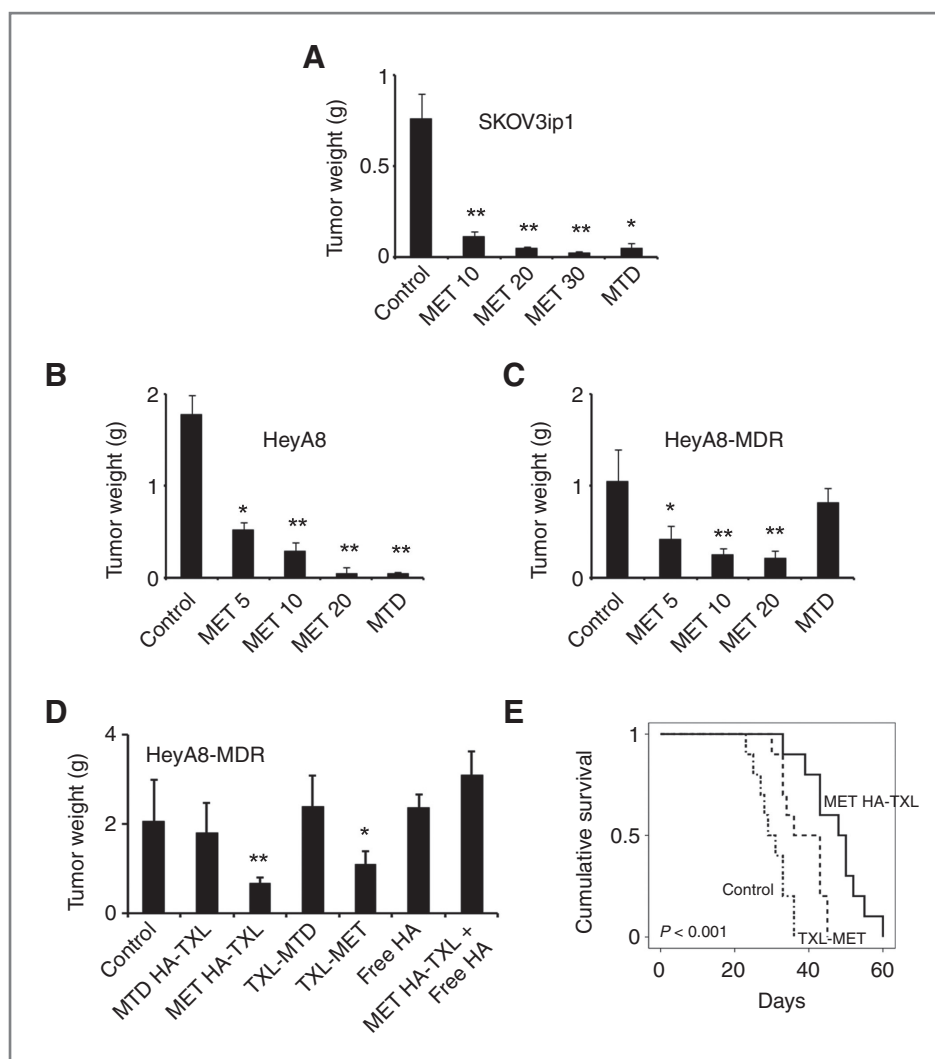
Determination of cytokines

Multiplex ELISA was carried out on the basis of the manufacturer's instruction for detecting both human and mouse interleukin (IL)-6, granulocyte colony-stimulating factor (G-CSF), and VEGF in plasma obtained from the control and treated groups. The MILLIPLEX MAG Human Cytokine/Chemokine panel (Millipore Corporation) allows us to quantitatively multiplex detection of dozens of analytes simultaneously, which can dramatically improve productivity. Mouse plasma was collected at day 7 (basal), 14 (7 days after starting treatment), and at the time of euthanasia. Blood sampling was carried out using a glass pipette coated with anticoagulant (EDTA) from the orbital sinus under anesthesia. Collected blood samples were centrifuged at 2,000 rpm for 10 minutes at room temperature. Tsp-1 levels were detected using an ELISA kit (R&D Systems).

Statistics

Continuous variables were compared with the Student *t* test (between 2 groups) or ANOVA (for all groups) if normally distributed, and the Mann-Whitney rank sum test or Kruskal-Wallis test (for all groups) if nonparametric. Pairwise differences in normally distributed variables in our

Figure 1. Effects of metronomic (MET) dosing and maximal tolerated dosing (MTD) of HA-TXL on tumor growth in SKOV3ip1 (A), HeyA8 (B), and HeyA8-MDR (C) models of ovarian cancer. Mice were randomly allocated to 5 groups and underwent treatment with intraperitoneal injection as follows for the SKOV3ip1 model: (i) control PBS, (ii) MET HA-TXL, 10 mg/kg, (iii) MET HA-TXL, 20 mg/kg, (iv) MET HA-TXL, 30 mg/kg, and (v) MTD HA-TXL 180 mg/kg, all HA-TXL doses being given as TXL equivalents. For the HeyA8 and HeyA8-MDR studies, the MET groups were lowered to 5, 10, and 20 mg/kg. For comparison of HA-TXL to TXL, the HeyA8-MDR model was used (D) according to the following groups: (i) control, (ii) MTD HA-TXL, 180 mg/kg, (iii) MET HA-TXL, 20 mg/kg, (iv) TXL-MTD, 10 mg/kg, (v) TXL-MET, 0.5 mg/kg, (vi) free HA, and (vii) MET HA-TXL + free HA. Effects of TXL-MET or MET HA-TXL were assessed on animal survival in the HeyA8-MDR model (E). Values are means + SEM. *, $P < 0.01$; **, $P < 0.001$.



treatment groups were compared by the Tukey–Kramer statistic for multiple comparisons. A Bonferroni adjustment to a (default value 0.05) was made based on the number of pairwise comparisons within a treatment experiment using the formula: $a(\alpha) = 0.05/k$, where k = number of comparisons against control. For *in vivo* therapy experiments, 10 mice in each group were used, as directed by a power analysis to detect a 50% reduction in tumor size (β -error, 0.2). A $P < 0.05$ on 2-tailed testing was considered significant.

Results

In vivo effects of HA-TXL therapy

To compare the effects of metronomic versus MTD HA-TXL therapy in taxane-sensitive ovarian cancer models, we first used the human SKOV3ip1 model. The metronomic regimens (10, 20, and 30 mg/kg) and MTD regimen (180 mg/kg) all reduced tumor weights by 88% to 96% as compared with PBS-treated mice ($P < 0.01$ for all, Fig. 1A). There were no significant differences in tumor growth among the metronomic and MTD dosing groups. As with

the tumor weights, the number of tumor nodules was reduced significantly when mice were treated with any of the metronomic regimens as well as with the MTD regimen compared with the PBS-treated control group ($P < 0.01$ for all; Supplementary Fig. S1). We also examined the effects of metronomic therapy in the HeyA8 model. Here, we also used a metronomic 5 mg/kg arm as there was substantial efficacy noted at the 10 mg/kg dose in the previous experiment and dropped the 30 mg/kg group. There was significant reduction in tumor growth in all metronomic treatment groups following 3 weeks of therapy (Fig. 1B).

Given the clinical reality of tumor resistance to chemotherapy in women with relapsed ovarian cancer, we also examined the effects of HA-TXL therapy in the taxane-resistant HeyA8-MDR model. While there was only non-significant, modest reduction in tumor growth in the MTD group, there was 60% to 78% reduction in tumor growth in the metronomic dosing groups (P values < 0.05 , Fig. 1C). The tumor nodule count generally mirrored the tumor weight pattern (Supplementary Fig. S1). There was approximately 15% reduction in body weight of mice in the MTD

group but no significant effects on body weight were noted in the HeyA8-MDR model with metronomic treatment (Supplementary Fig. S1D), suggesting that feeding habits and general health were not affected by highly efficacious metronomic therapy. Additional experiments were carried out to characterize the antitumor effects of HA-TXL compared with free paclitaxel (TXL). Moreover, to examine the effects on bulky, drug-resistant disease, treatment was started 12 days following tumor cell injection. MTD HA-TXL or TXL had no significant effect on HeyA8-MDR tumor growth (Fig. 1D). Metronomic dosing with either HA-TXL or TXL resulted in significant reduction in tumor growth. The reduction in tumor growth was greater (by 39%) in the HA-TXL metronomic group than in the TXL metronomic group ($P = 0.03$; Fig. 1D). To assess the specificity of the effects of HA-TXL and dependence on CD44 binding, we also used free hyaluronic acid alone or in combination with MET HA-TXL. Free hyaluronic acid completely blocked the efficacy of MET HA-TXL (Fig. 1D). Complete blood count analysis revealed that apart from a modest decrease in WBC with TXL-MTD, there were no significant effects on WBC or hemoglobin in the other groups (Supplementary Fig. S2).

Next, we examined potential effects of HA-TXL on animal survival in the HeyA8-MDR model. Because MTD regimens were ineffective, we focused on the metronomic regimens. Treatment was started 12 days following tumor cell injection and continued until each animal became moribund. The greatest improvement in survival was noted in the HA-TXL metronomic group (mean survival: control, 30.1 days; TXL-MET, 38.5 days; MET HA-TXL, 47.3 days; $P < 0.001$; Fig. 1E).

Effects of MET and MTD HA-TXL therapy on tumor microenvironment

We next assessed the effects of treatment on the tumor microenvironment. Tumors from the earlier experiments were examined for proliferation (Ki-67), apoptosis (TUNEL), MVD (CD31), and apoptosis of endothelial cells

(CD31/TUNEL). For the SKOV3ip1 model, there was a significant reduction in proliferation in all of the treatment groups ($P < 0.05$), metronomic and MTD (Fig. 2A), but significantly reduced MVD was noted only in the metronomic groups (Fig. 2B, $P < 0.05$). Increased TUNEL staining, indicating tumor cell apoptosis, was noted in all of the treatment groups (Fig. 2C). Because reduced MVD was noted primarily in the metronomic groups, we also examined the effects of metronomic versus MTD dosing on endothelial cell apoptosis. For this experiment, mice with established HeyA8-MDR tumors (17 days following tumor cell injection) were treated with either a single dose of MTD HA-TXL or with 3 doses of MET HA-TXL before resecting the tumors for fluorescence microscopy. There were significantly greater effects on endothelial cell apoptosis in the higher dose MET HA-TXL groups than in the MTD arm, reflecting an antiangiogenic effect (Supplementary Fig. S2E).

Some studies have reported increases in antiangiogenic factors, such as Tsp-1, following treatment with metronomic chemotherapy (37, 38). Tumors harvested from the SKOV3ip1 model were subjected to immunostaining for Tsp-1. There were marked increases in Tsp-1 expression following treatment with MET HA-TXL compared with the MTD group (Fig. 2D), which is consistent with the observed antiangiogenic effects. Similar effects of MTD versus metronomic dosing were noted in the HeyA8-MDR model (Supplementary Fig. S3). By ELISA, there was a 35% increase in tumoral Tsp-1 levels in the HA-TXL MTD group and a 275% increase in the MET HA-TXL group.

Effects of HA-TXL therapy on systemic cytokine production

Next, we examined the impact on selected angiogenic factors longitudinally in the treatment groups described earlier. Specifically, we examined plasma IL-6, G-CSF, and VEGF levels before the start of treatment and at days 7, 14, and 19, during treatment. As expected, there was a progressive increase in these cytokines in the untreated controls

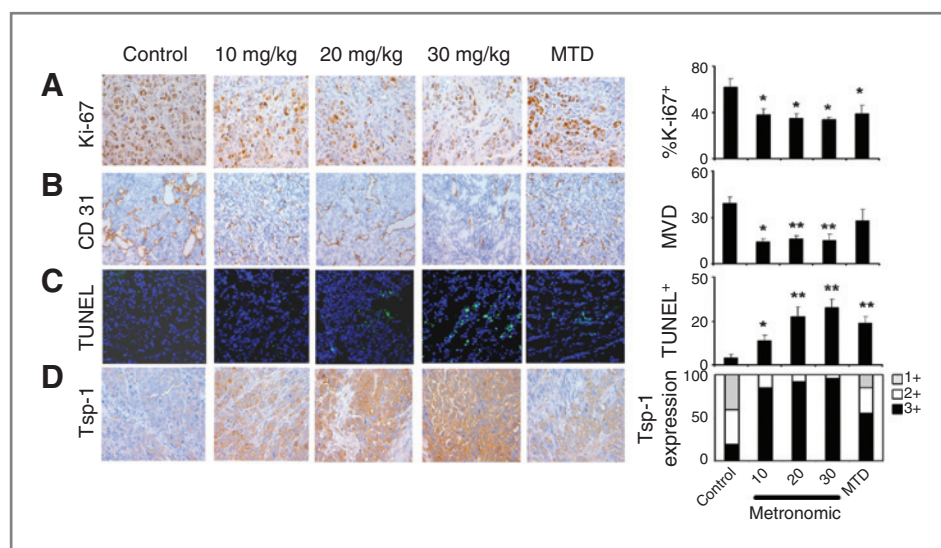


Figure 2. Effects of MET and MTD HA-TXL treatment in the SKOV3ip1 model on biologic endpoints, including (A) cell proliferation (Ki-67); MVD (CD31, B); apoptosis (TUNEL, C), and Tsp-1 (D). Tumors harvested following 3 to 4 weeks of therapy were stained for Ki-67, CD31, Tsp-1, and TUNEL. All photographs were taken at original magnification $\times 200$. The error bars represent SEM. *, $P < 0.05$; **, $P < 0.001$.

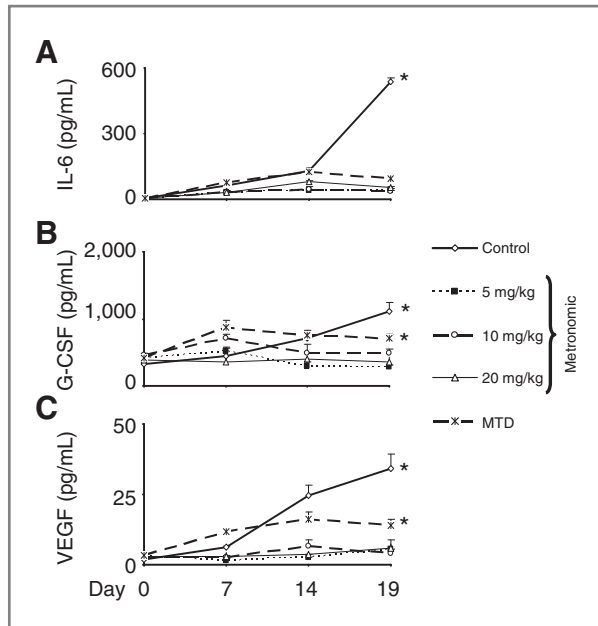


Figure 3. Effects of HA-TXL therapy on systemic cytokine production. Plasma was collected from HeyA8 model at day 7 posttumor implantation (basal), day 14 (7 days after starting treatment), and at the time of euthanasia. Multiplex ELISA was carried out based on the manufacturer's instruction for detecting human and mouse IL-6 (A), G-CSF (B), and VEGF (C) in plasma obtained from the control and treated groups. There was a progressive increase in these cytokines in the untreated controls; however, the cytokine levels remained lower throughout in the MET dosing groups than in the MTD treatment group. *, $P < 0.05$. Error bars represent SEM.

(Fig. 3). Interestingly, the cytokine levels remained lower throughout in the metronomic dosing groups than in the MTD treatment group.

In vitro cytotoxic effects of continuous versus pulsed HA-TXL treatment

We have previously shown that SKOV3ip1 cells are somewhat sensitive to brief (4 hours) treatment with HA-TXL, showing approximately 20% cytotoxicity (~80% survival) at a drug concentration of 500 ng/mL after further incubation for 72 hours (12). Here, SKOV3ip1 cells were either exposed continuously to a concentration range of up to 500 ng/mL of HA-TXL for a total of 6 days or were treated with 3 consecutive 48 hours pulses of HA-TXL, to mimic *in vivo* metronomic scheduling, at either 10 or 100 ng/mL HA-TXL. Results indicated that the lower MET HA-TXL concentration had minimal effects (>90% survival); however, the higher MET HA-TXL concentration decreased survival to approximately 20% at this time point, markedly lower than the approximately 55% survival with continuous exposure to 500 ng/mL HA-TXL (data not shown). These data suggest that while there are modest direct effects of metronomic therapy on cancer cells, the robust *in vivo* efficacy is likely to reflect both direct (tumor cell apoptosis) and indirect (anti-angiogenic) effects.

Discussion

The key findings of this study are that MET HA-TXL therapy was generally more effective than MTD HA-TXL in reducing tumor growth in murine xenograft models of advanced TXL-sensitive human ovarian cancer, as well as being less toxic, resulting in a higher therapeutic index. Moreover, MET HA-TXL therapy also showed such antitumor effects in a murine xenograft model bearing TXL-resistant human ovarian cancer, whereas the MTD arm was ineffective, both in terms of tumor volume and tumor nodule counts. All of the metronomic regimens were able to decrease cell proliferation and angiogenesis whereas increasing tumor cell apoptosis and markedly increasing expression of the inhibitor of angiogenesis, Tsp-1. We had hypothesized that MET HA-TXL therapy would be effective on both TXL-sensitive and TXL-resistant CD44(+) ovarian carcinoma cells, as drug resistance in the epithelial tumor compartment should not affect the susceptibility of the tumor endothelial cells to metronomic treatment. Both body weights and plasma IL-6, G-CSF, and VEGF levels indicated that metronomic schedules were less toxic than the MTD arm.

In a recent study (5), we used orthotopic (i.p.) human ovarian carcinoma xenografts and administration of MTD HA-TXL locoregionally (also i.p.). Hyaluronic acid could be regarded as a targeted backbone by which TXL might be delivered to CD44(+) tumor cells that would allow efficient and specific receptor-mediated prodrug uptake and internalization by CD44, as shown in *in vitro* binding studies (5). However, establishing the precise extent of the hyaluronic acid/CD44 interaction in the *in vivo* antitumor activity of HA-TXL is rendered complex, due to the absence of established CD44(-) human ovarian carcinoma xenograft models. It has been suggested that hyaluronic acid binds best to parental CD44 and that the additional amino acid sequences neighboring the hyaluronic acid-binding domain in the splice variants may interfere with this binding. This has been postulated to reside in electrostatic repulsions between negatively charged hyaluronic acid and sialic acid residues that tail the amino acids of the sequence. However, this is not a universal observation and remains rather controversial. For example, there is also evidence that such differences in rank order of binding efficiency are cell type-specific and may even be influenced by cellular exposure to cytokines and activation of other signaling networks. Ongoing clinical trials with hyaluronic acid-encapsulated irinotecan have not revealed such issues in patients with colorectal cancer (39).

Furthermore, by using free hyaluronic acid alone or in combination with MET HA-TXL, we found that free hyaluronic acid completely blocked the efficacy of MET HA-TXL, underscoring a key role for receptor-specific uptake and internalization of the conjugate. The hyaluronic acid backbone itself might have a direct role in the antitumor effect of HA-TXL; that is, hyaluronic acid may induce anoikis by disrupting CD44(+) tumor cell-extracellular matrix interactions (5, 40). However, we did not observe

any cytotoxicity from hyaluronic acid alone in *in vitro* assays (5), nor was a hyaluronic acid monotherapy arm active against orthotopic human head and neck squamous cell carcinoma xenografts (29), nor in the current study (Fig. 1E).

Metronomic dosing of chemotherapeutics has been shown to reduce normal tissue toxicity and minimize off-treatment exposure. Several clinical trials have documented that frequent lower dosing has low toxicity (41) and improved pathologic response rates in breast cancer patients (42). A previous preclinical study on metronomic taxane therapy reported superiority to MTD in both taxane-sensitive and -resistant ovarian cancer models (30). Another study on metronomic topotecan in ovarian cancer models showed the same effect as MTD in reducing tumor growth with tolerable toxicity (22). Of mechanistic significance, clinical studies in ovarian and breast cancer suggest that the major therapeutic impact of metronomic dosing may be on tumor endothelial cells and not on the tumor cells themselves (30, 42, 43). Metronomic regimens may have an inhibitory effect on the mobilization and viability of bone marrow-derived circulating endothelial precursor cells (25).

In the current study, we showed that all MET HA-TXL treatment groups reduced tumor weights and tumor nodule counts in the TXL-resistant HeyA8-MDR-bearing model. We designed the study so that the metronomic 5, 10, and 20 mg/kg HA-TXL groups had 25%, 50%, and 100%, respectively, of the TXL equivalents in the MTD 180 mg/kg group. Even the lowest dose MET HA-TXL (5 mg/kg) group was more effective than the MTD arm, indicating that MET HA-TXL treatment could overcome TXL-resistance in this ovarian carcinoma model, most likely due to the antivasular effects of MET HA-TXL chemotherapy. In that regard, the ability of even the lowest metronomic dose group to strongly induce Tsp-1 expression compared with the MTD arm is remarkable and aligns well with the patterns of reduced MVD, which is consistent with other reports (37, 38). A possible explanation might be that activated endothelial cells and circulating endothelial precursors are directly killed by metronomic chemotherapy, but not by MTD treatment. The induction of Tsp-1 by metronomic dosing is likely related to p53 upregulation by DNA-damaging activity (38). However, whether Tsp-1 could be a surrogate

marker to predict the response to metronomic treatment in patients with ovarian cancer is still controversial (44).

Other formulations of hyaluronic acid-bound paclitaxel are also under development (45, 46), including evaluation in ovarian tumor models (47–49), and reaching initial clinical testing in Bacillus Calmette–Guerin (BCG)-refractory bladder cancer (50). The latter, ONCOFID-P-B uses a longer hyaluronic acid chain (~200 kDa) than used in our studies (~40 kDa) and has a higher paclitaxel loading (~20% vs. 10%–12%), and all of the preclinical tumors studies with this formulation have used MTD or near-MTD dosing. This confounds direct comparisons to our results with HA-TXL.

In summary, we have shown the efficacy of MET HA-TXL to inhibit tumor growth using several orthotopic ovarian cancer models, and in particular, that MET HA-TXL could be effective against taxane-resistant ovarian carcinoma. These effects are thought to be mediated by antiproliferative and proapoptotic effects against the epithelial tumor cells themselves of the regimen, as well as the antivasular effects of metronomic dosing. Assessment of Tsp-1 may implicate a noninvasive biomarker of therapeutic response. Our data suggest that MET HA-TXL is highly efficacious and should be considered for future clinical trials.

Disclosure of Potential Conflicts of Interest

J. Klostergaard has ownership interest (including patents) for MD Anderson Cancer Center. No potential conflicts of interest were disclosed by other authors.

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