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ORIGINAL ARTICLE

Hyaluronic acid–paclitaxel: effects of intraperitoneal administration against CD44(+) human ovarian cancer xenografts

Ilaria De Stefano · Alessandra Battaglia · Gian Franco Zannoni · Maria Grazia Prisco · Andrea Fattorossi · Daniele Travaglia · Silvia Baroni · Davide Renier · Giovanni Scambia · Cristiano Ferlini · Daniela Gallo

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Abstract
Purpose Hyaluronan (HA)-receptors (mainly CD44 and RHAMM) are overexpressed in a wide variety of cancers including ovarian tumors, and HA-bioconjugates have been developed to enhance selective entry of cytotoxic drugs into HA receptor-expressing cancerous cells. Here, we evaluated the potential application of a new HA-paclitaxel bioconjugate, ONCOFID-P, for intraperitoneal (IP) treatment of ovarian cancer.
Methods In vitro cytotoxic effect of ONCOFID-P was first assessed on CD44(+) OVCAR-3 and SKOV-3 human ovarian cancer cell lines. Studies were performed in female Balb/c athymic mice IP implanted with OVCAR-3 or SKOV-3 and treated with IP ONCOFID-P, and IP and intravenous (IV) free paclitaxel, at their maximum tolerated dose (MTD 168, 80 and 80 mg/kg, total dose, respectively). The potential detrimental effect of the IP ONCOFID-P and IP free paclitaxel on hematopoiesis was also assessed on peripheral blood, bone marrow and spleen.
Results Results show that ONCOFID-P cytotoxicity against both OVCAR-3 and SKOV-3 cell lines was somewhat less effective than free paclitaxel. Conversely, in in vivo experiments, IP treatment with ONCOFID-P was overall more effective than IV and IP free paclitaxel in inhibiting intra-abdominal tumor dissemination, abrogating ascites, prolonging survival and curing mice. ONCOFID-P and IP free paclitaxel were equivalent in terms of myelotoxicity, although the former was administered at a two-fold higher dose.
Conclusions Present data strongly support the development of ONCOFID-P for locoregional treatment of ovarian cancer.

Keywords Hyaluronan · Paclitaxel · CD44 · Mice · Ovarian cancer

I. De Stefano · A. Battaglia · M. G. Prisco · A. Fattorossi · D. Travaglia · G. Scambia · C. Ferlini · D. Gallo (✉)
Department of Obstetrics and Gynaecology,
Catholic University of the Sacred Heart,
Largo A. Gemelli, 8, 00168 Rome, Italy
e-mail: d.gallo@rm.unicatt.it

G. F. Zannoni
Department of Histopathology,
Catholic University of the Sacred Heart,
Largo A. Gemelli, 8, 00168 Rome, Italy

S. Baroni
Institute of Clinical Biochemistry,
Catholic University of the Sacred Heart,
Largo A. Gemelli, 8, 00168 Rome, Italy

D. Renier
Fidia Farmaceutici S.p.A, Via Ponte della Fabbrica 3/A,
35031 Abano Terme (PD) Padova, Italy

Introduction

Epithelial ovarian cancer is the leading cause of death for gynecological cancer in most of the Western world. It is the ninth most common cancer and the fifth leading cause of cancer death among women in the United States [1]; in European women, it accounts for 41,900 cases (about 3.9% of all female cancers) [2]. Ovarian cancer is recognized to be one of the most chemotherapy-sensitive malignancies, with 70–80% of newly diagnosed patients being anticipated to exhibit a response to primary platinum plus taxane chemotherapy; despite this fact, the majority of women who present with advanced ovarian cancer will experience recurrence, and ultimately die of complications associated

with progressive disease [3]. The peritoneum is the predominant site of failure, and intraperitoneal spread of disease is present in patients with early recurrence. In this regard, the regional delivery of chemotherapeutic agents into the peritoneal cavity may provide a pharmacologic advantage, by directly exposing tumor to greater concentrations of anti-neoplastic drugs, and for a longer period, compared to systemic therapy [4]. It is still unknown, however, whether the antitumor effect is mainly related to direct drug tumor penetration, prolonged drug exposure, higher serum drug levels due to absorption, or whether IP chemotherapy modifies the immune response against tumor cells in the peritoneal cavity [5]. Patients with microscopic or small volume residual disease seem to be most suitable for this approach, while, in more bulky disease, IP chemotherapy is unlikely to be beneficial because drug penetration is limited [6].

Cisplatin has been most frequently used for IP chemotherapy in ovarian cancer patients, with Phase-III-trial data revealing, in small-volume residual advanced ovarian cancer, a superior efficacy of this treatment modality compared with systemic platinum delivery. However, this approach has been associated with greater toxicity owing to both the use of cisplatin (rather than carboplatin) and the requirement for IP catheter placement for drug delivery [7]. Paclitaxel and docetaxel are also promising drugs for IP chemotherapy in ovarian cancer, for several reasons. They have a considerable cytotoxic activity against ovarian cancer and show favorable pharmacokinetics after IP administration: in addition, since they have a dose-dependent response, the high locoregional drug concentrations should theoretically lead to increased cytotoxicity. Recently, feasibility and efficacy of this treatment have been demonstrated in a number of clinical studies [8].

An additional strategy to improve antitumor efficacy of traditional treatments is based on the use of delivery systems, as liposomes or other carriers, which allow selective drug accumulation in tumor tissues, tumor cells, or even compartments of tumor cells. By increasing bioavailability of drugs at sites of action, this approach may even enhance efficacy against resistant tumors [9]. Hyaluronan (HA) is a linear polymer, composed of repeating disaccharides of glucuronic acid and N-acetyl-d-glucosamine that can reach a molecular mass of several million Daltons [10]. So far, cell receptors that have been identified for HA fall into three main groups: CD44, RHAMM (receptor for HA-mediated motility), and ICAM-1 (intracellular adhesion molecule-1) [10, 11]. HA receptors (CD44, RHAMM) are overexpressed in a wide variety of cancers including ovarian tumors [12–14]. Targeting of anti-cancer agents to tumor cells and tumor metastases can thus be accomplished by receptor-mediated uptake of bioconjugates of anticancer agents and HA, followed by the release of free drugs through the degradation of HA in cell compartments. HA-drug bioconjugates may present a markedly enhanced selectivity for cancerous cells, while exhibiting improved water solubility relative to the parent drug [15]. However, since CD44 is also expressed by hemopoietic stem/progenitor cells and on cells of the hemopoietic microenvironment [16–18], it is important to ascertain whether HA-bioconjugates exert a more toxic effect than their parent compounds on the hemopoietic tissue.

The present study extends previous findings on the development of a new HA-paclitaxel bioconjugate (ONCOFID-P) for IP treatment of ovarian cancer [19]. Our research was aimed at evaluating drug activity at the maximum tolerated dose (MTD) identified in preclinical toxicological studies for regulatory submission (210 mg/kg/day, Fidia Farmaceutici, internal data). We also verified the potential effect of the tested substance on peripheral blood, bone marrow cellularity and spleen morphology. Results obtained confirmed that ONCOFID-P has a significant therapeutic effectiveness, and distinct advantages respect to conventional paclitaxel therapy (standard intravenous treatment). We also showed that ONCOFID-P proved to be more effective than free IP paclitaxel, and exhibited similar side effects at a two-fold higher dose. Importantly, the relatively high water solubility of the bioconjugate eliminates the requirement of Cremophor EL as a solvent, with its recognized irritating and toxic properties [20].

Materials and methods

Drugs

The preparation of HA-paclitaxel bioconjugate (ONCOFID-P, Fidia Farmaceutici, Abano Terme, Italy) with ~20% w/w of paclitaxel loading has been previously described [21]. Briefly, HA (Mw ~ 200 kDa) was employed to produce the tetrabutylammonium-hyaluronic salt used for the reaction with the paclitaxel ester generated by activation with 4-bromo butyric acid. For in vitro studies, ONCOFID-P® and paclitaxel (donated by Fidia Farmaceutici, Padova, Italy) were diluted with DMSO. Solutions were further diluted at each experimental day in order to achieve a 0.1% final DMSO concentration. All reagents were purchased from Sigma (Sigma-Chemical Co. St. Louis, Mo.), unless indicated.

Cell lines

OVCAR-3 and SKOV-3 cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). OVCAR-3 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% non-essential

amino acids mixture, 1% Kanamycin. SKOV-3 were grown in McCoy's medium supplemented with 10% fetal bovine serum, 1% MEM, 1% Kanamycin. Cells, propagated as a monolayer culture, were trypsinized twice weekly and plated at a density of 10×10^4 cells per ml. All cultures were incubated at 37°C under 5% CO_2 in a high-humidity atmosphere. On the day of tumor inoculation, cells were trypsinized, collected in RPMI 1640 medium without supplements, and a suspension of 8×10^6 cells were injected intraperitoneally in each animal (0.2 ml per mice).

Growth inhibition assay

Cells were seeded (20,000 cells/well) in 96-well flat-bottom plates (Viewplates, Perkin Elmer Life Science, Waltham, MA). After 24 h, media were replaced and, after one washing, media containing the test compounds were added. Three independent experiments were performed in quadruplicates. After 72 h of culture in the presence of the compounds, plates were harvested and the number of viable cells was estimated by ATP dosing, using the ATPlite kit (Perkin-Elmer Life Science), and the automated luminometer Topcount (Perkin-Elmer Life Science). For each compound, a dose–response curve was plotted, and the IC50 values were then calculated by fitting the concentration–effect curve data obtained in the three experiments with the sigmoid-Emax model using non-linear regression, weighted by the reciprocal of the square of the predicted effect [22].

In vivo studies

Animals and treatments

Female athymic mice (Balb/c nu/nu 4–5 weeks old) were purchased from Charles River, S.r.l. (Calco, Lecco, Italy) and housed under controlled conditions. Procedures and facilities followed the requirements of Commission Directive 86/609/EEC concerning the protection of animals used for experimental and other scientific purposes. Italian legislation is defined in the Decreto Legislativo No. 116 of 27 January 1992. In addition, the UKCCCR guidelines for the welfare of animals in experimental neoplasia were followed [23]. Studies were approved by the Animal Care and Use Committee of the Catholic University of the Sacred Heart (Rome, Italy), and by the Italian Ministry of Health.

Efficacy studies (OVCAR-3 and SKOV-3)

On the day of inoculation, a suspension of OVCAR-3 or SKOV-3 (8×10^6 cells) was injected intraperitoneally in athymic mice. ONCOFID-P was formulated daily by dissolution in glucosate 5% (w/v in physiological saline) at a concentration of 15 mg/ml. A stock solution of paclitaxel (concentration of 20 mg/ml) was prepared by dissolving it into equal volumes of absolute ethanol and Cremophor. The stock solution was prepared once per week and stored at 4°C. Immediately before administration, the stock solution was further diluted with physiological saline to give the concentration required for dosing (i.e., 2 mg/ml). ONCOFID-P and its vehicle were administered by the IP route at a dose volume of 14 ml/kg; paclitaxel was administered either IV or IP at a dose volume of 10 ml/kg. Dose volume was calculated on the basis of the most recently recorded body weight. For each experimental model, a total of 4 experimental groups were used: control (glucosate 5%, days 8, 15, 30, 37, IP), IP ONCOFID-P (210 mg/kg/injection, days 8, 15, 30, 37), IP free paclitaxel (20 mg/kg/injection, days 8, 15, 30, 37) and IV free paclitaxel (20 mg/kg/injection, days 8, 11, 15, 18). ONCOFID-P regimen was chosen according to preliminary experimental data (unpublished results). IV paclitaxel was administered at the MTD, and according to a conventional schedule treatment able to achieve a 60% cure rate in the sensitive A2780 ovarian cancer model [24]. IP paclitaxel was administered at its MTD determined in preliminary studies (unpublished results), with the same regimen as ONCOFID-P.

During the study, mice were checked daily for any adverse clinical reactions, and body weights were measured twice a week. Each experimental group included 15 mice. In the OVCAR-3 study, 10 mice/group were used for evaluating survival, while the remaining five were killed 6 weeks following tumor inoculation (day 42 of the study) to assess rate of tumor progression by measurement of tumor size at necropsy and possible evidence of ascites. As IP ovarian xenografts disseminate throughout the peritoneal cavity, all macroscopic (visible) nodules were excised harvested and weighed, followed by fixation in neutral buffered 10% formalin, and subsequent dehydration and embedding in paraffin. The paraffin block was cut into 3- or 5-μm sections and processed for light microscopy (hematoxylin/eosin staining, H&E) or immunohistochemistry for evaluation of CD44 expression (see below). Blood was collected from the caudal vena cava and subsequently used for assessment of CA 125 levels. Liver, kidneys and spleen were weighed in all mice.

CD44 immunohistochemistry

Three-micrometer-thick paraffin sections were mounted on Superfrost coated slides and dried overnight. The sections were deparaffinized in xylene and rehydrated in graded solutions of ethanol; the endogenous peroxidase was blocked with 3% H_2O_2 for 5 min. The antigen retrieval procedure was performed by microwave oven heating in 10 mM citric acid, pH 6 (3 times for 5 min). To reduce

non-specific binding, the sections were incubated with 20% normal goat serum for 30 min, at room temperature. To detect expression of CD44, sections were stained overnight at 4°C, with rabbit monoclonal CD44, clone EPR1013Y, (Abcam Inc., Cambrige, MA, UK) at 1:100 dilution; sections were incubated with the secondary, anti-rabbit EnVision System-HRP (DakoCytomation, Carpinteria, CA, USA) for 30 min, at room temperature. The slides were developed with diaminobenzidine (DAB substrate System, DakoCytomation), counterstained with Mayer's Haematoxylin, dehydrated in ethanol and xylene, and finally mounted.

CA 125 measurement

The serum levels of CA 125 antigen were measured by Access® OV MONITOR (Beckman Coulter) non-competitive immunochemiluminescent assay, using the Beckman Coulter UniCel DxI 800 analyzer. The OV Monitor assay uses alternative antibodies (OVK95 and OV185) rather than the traditional OC125 and M11 antibodies for the detection of CA 125 [25, 26]. The minimum detectable concentration (MDC) is calculated to be 0.1 U/ml [25]. All studied samples were assayed in duplicate; moreover, all mouse sera were measured undiluted and appropriately diluted for evaluating the possible presence of circulating protein interferents.

Safety study

In this study, we compared the hematological toxicity of ONCOFID-P and IP free paclitaxel by assessing changes in peripheral blood, bone marrow, and spleen. To this end, female athymic mice (Balb/c nu/nu, $n = 5$ per group) were allocated to three experimental groups: control untreated, IP ONCOFID-P (210 mg/kg/injection, days 8, 15, 30, 37), and IP free paclitaxel (20 mg/kg/injection, days 8, 15, 30, 37). Mice were killed one week after the last dosing (day 42, see above). Blood was collected at necropsy from the caudal vena cava, and bone marrow cells were isolated by flushing the femoral bones with phosphate-buffered saline (PBS). Spleens were weighed and processed for light microscopy (H&E staining).

For peripheral blood assessment, at least 300µl of blood sample was drawn from caudal vena cava. Total leukocyte and differential count were performed. Briefly, leukocytes were incubated with buffered NH_4Cl (blood/NH_4Cl ratio 1:20) at room temperature for 10 min. Leukocytes were then centrifuged at 350*g* for 10 min, washed once with PBS and resuspended in 2 ml of the same buffer. Polymorphonuclear (PMN) cells, lymphocytes and monocytes were measured using the forward and side scatter signals in a Beckman Coulter XL flow cytometer. Forward and side scatter signals were collected in linear mode and served also to exclude unwanted events (i.e., not viable cells, debris and cell clumps) from cell evaluation. A minimum of 5×10^3 up to 15×10^3 cells of interest were acquired for each sample. List mode data were analyzed using Expo 32™ (Beckman Coulter) software. Total white blood cell (WBC) count per ml was obtained using a Z2 Coulter Particle Count and Size analyzer (Beckman Coulter). Leukocyte subset proportions were converted to the absolute number per ml multiplying each cell percentage by the number of leukocyte per ml derived from the WBC count.

The marrow was passed through a 26-gauge needle to generate single cell suspensions and erythrocytes lysed using buffered NH_4Cl. BM cells were then counted using a Z2 Coulter Particle Count and Size analyzer.

Statistical methods

To determine significant differences in survival curves between groups, the Kaplan–Meier survival analysis was used, followed by log rank test. Body weight data were analyzed by the repeated-measures ANOVA, followed by the Bonferroni method as post-test. Tumor incidence in the satellite groups was compared using a Chi-square test. All the remaining data were analyzed for homogeneity of variance using Bartlett's test. If the group variance appeared homogenous, a parametric ANOVA was used, followed by Tukey's multiple comparison test. If the variances were heterogeneous, log or reciprocal transformations were made in an attempt to stabilize the variances. If the variances remained heterogeneous, a non-parametric test such as the Kruskall-Wallis test, followed by Dunn's multiple comparison test, was used. Statistical analysis was carried out with GraphPad Prism5 Software (San Diego, CA, USA). Values in the text are means ± SEM. $P < 0.05$ was used as the critical level of significance.

Results

In vitro tumor growth inhibition

ONCOFID-P was tested against OVCAR-3 and SKOV-3 ovarian cancer cell lines, and its activity was compared with that of free paclitaxel. ONCOFID-P yielded IC50 values of 642 ± 284, and 820 ± 330 ng/ml, following 72 h drug exposure of OVCAR-3 and SKOV-3, respectively (corresponding to 128 ± 57 and 164 ± 66 ng/ml in paclitaxel equivalent). Unconjugated paclitaxel produced IC50 values of 41.1 ± 24.4 and 2.8 ± 0.7 ng/ml, for OVCAR-3 and SKOV-3, respectively. Collectively results showed that the bioconjugate exhibited obvious cytotoxicity against

both OVCAR-3 and SKOV-3 ovarian cancer lines, i.e., paclitaxel is released from the tested conjugate without losing cytotoxicity, although its antitumor activity in vitro was lower than that of pure paclitaxel.

In vivo anti-tumor efficacy—OVCAR-3 study

Treatment with 210 mg/kg/injection ONCOFID-P (corresponding to 42 mg/kg/injection in paclitaxel equivalent) had a significant therapeutic activity, resulting in a 2.5-fold increase in survival in comparison with control mice: specifically, median survival was 113.5 vs. 45.5 days in controls ($P < 0.001$) (Fig. 1a). IP treatment with free paclitaxel (20 mg/kg/injection) also produced a significant therapeutic benefit (median survival, 89 days, $P < 0.001$ vs. controls), with a 2.0-fold increase in survival over control. Notably, on day 189 of the study (i.e., 5 months after the end of treatment), there were two tumor-free cures in the ONCOFID-P group, and one in the IP free paclitaxel group. On the other hand, the conventional IV paclitaxel (20 mg/kg/injection) did not significantly increase survival in comparison with controls (median survival 67.5 days, $P = 0.23$). Body weight curves during the study are reported in Fig. 1b. Administration of ONCOFID-P did not significantly affect body weight of mice throughout the study period. Conversely, animals receiving IP free paclitaxel lost weight upon treatment, this change achieving statistical significance on weeks 2 and 3 ($P < 0.05$). Similarly, paclitaxel IV-treated mice had a group mean body weight significantly lower than control ($P < 0.05$) from week 2 until week 7 (end of treatment period, and last statistical analysis, surviving control mice = 5).

Interim killing—Satellite groups

Figure 1c shows representative pictures of mice in the different treatment groups 6 weeks after tumor inoculation, and Fig. 1d shows histological features and CD44 expression of OVCAR-3 tumors collected at the interim killing. At this time, tumor was evident in all mice from control, IP free paclitaxel and IV free paclitaxel (100%), while only 2 out of 5 ONCOFID-P-treated mice (40%) showed evidence of tumor ($P < 0.05$ vs. control, Fig. 1e). Mean tumor weight in ONCOFID-P and IP paclitaxel group was significantly lower than control ($P < 0.05$); a trend toward a reduction in tumor burden was also observed in the IV free paclitaxel group, although this change did not reach statistical significance (Fig. 1f).

The presence of ascitic fluid was found in all mice in the control group (100%), with a mean volume per mouse of 1.5 ± 0.6 ml; conversely, there was no evidence of ascites either in ONCOFID-P- or in IP free paclitaxel-treated mice ($P < 0.05$). In the IV free paclitaxel group, only one mouse showed ascites at necropsy (Fig. 1g).

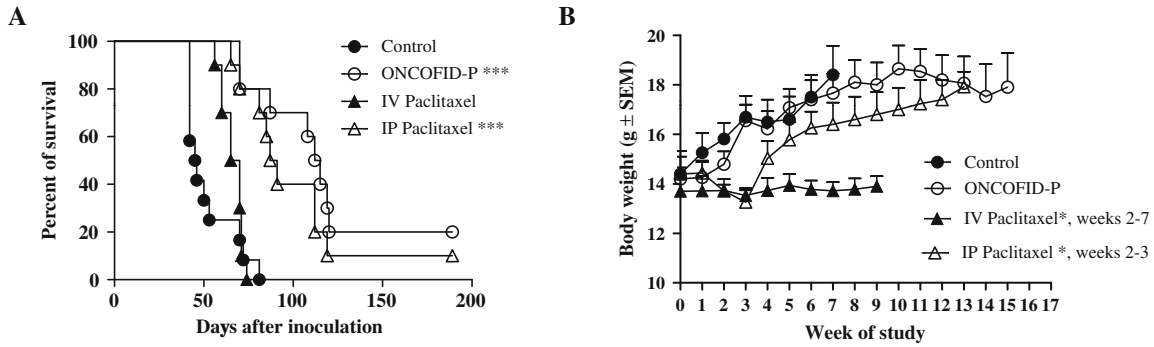
The serum CA 125 level of tumor-bearing control mice was 145.9 ± 90.9 units/ml. By comparison, it was extremely lower in all treated groups, with mean levels of 0.2 ± 0.2, 0.0 ± 0.0, and 0.9 ± 0.9, for ONCOFID-P, IV free paclitaxel and IP free paclitaxel, respectively (Fig. 1h). The differences observed in CA 125 levels among treatment groups did not closely mirror changes measured in tumor burden, as a likely consequence of a low stage of the disease, at which the peritoneal barrier is efficient in controlling the release of tumor antigens in the circulation [27]. According to this, although elevated CA 125 levels occur in about 80% of patients with advanced ovarian cancer, the frequency of positivity is quite lower in low-stage disease [28].

Liver, spleen, and kidneys from all animals were weighed at necropsy. Results obtained showed no effect of the different treatments on both absolute and relative liver and kidney weights. Conversely, there was a significant increase in absolute and relative spleen weight of animals receiving ONCOFID-P and IP free paclitaxel when compared to controls ($P < 0.05$, Fig. 1i,j).

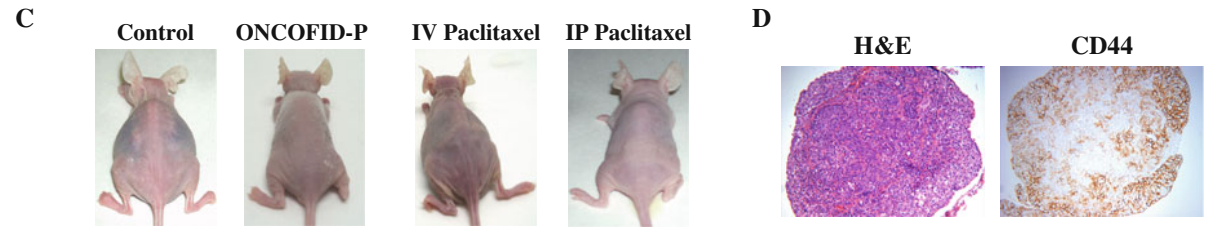
In vivo anti-tumor efficacy—SKOV-3 study

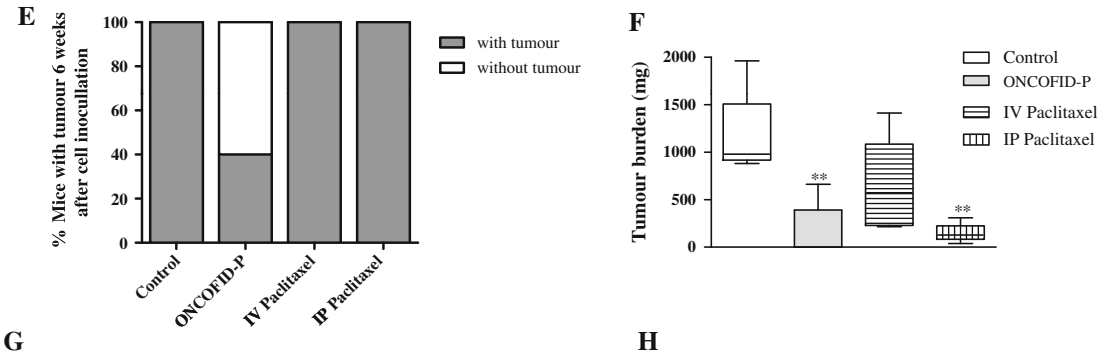
The therapeutic activity of ONCOFID-P was confirmed using SKOV-3, although the effects observed were overall reduced, as a likely consequence of a fast tumor growth rate. Specifically, treatment with 210 mg/kg/injection ONCOFID-P (corresponding to 42 mg/kg/injection in paclitaxel equivalent) had a significant therapeutic activity, resulting in an increase in survival in comparison with control mice, with a median survival of 50 versus 35 days in controls ($P < 0.001$) (Fig. 2a). IP treatment with free paclitaxel (20 mg/kg/injection) also produced a slight increase in survival over control, although to a lesser extent (median survival, 39 days, $P < 0.05$ vs. controls). Notably, differences between ONCOFID-P and IP free paclitaxel approached statistical significance ($P = 0.057$). IV free paclitaxel (20 mg/kg/injection) did not affect survival (median survival 31 days, $P = 0.8$). On day 189 of the study (i.e., 5 months after the end of treatment), there were two tumor-free cures in ONCOFID-P group. Body weight curves during the study are reported in Fig. 2b. Administration of either ONCOFID-P or IP free paclitaxel did not significantly affect the body weight of treated mice throughout the study period. Conversely, IV paclitaxel-treated mice had group mean body weight significantly lower than controls on weeks 3 and 4 ($P < 0.05$). Figure 2c shows histological features and CD44 expression of SKOV-3 tumors.

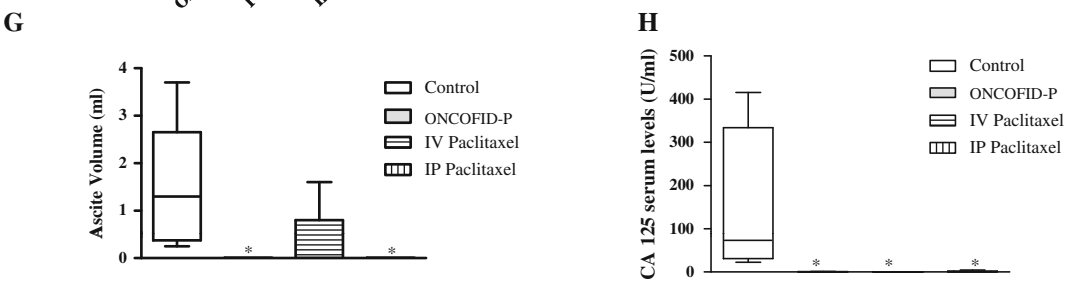
Main groups

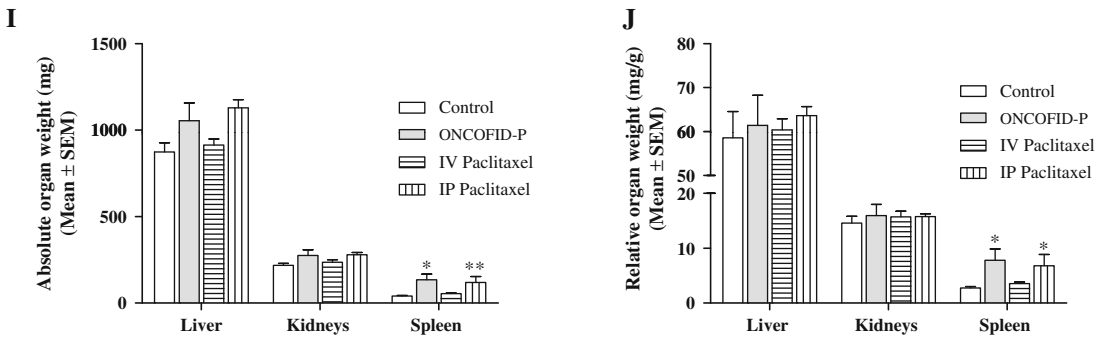


Satellite groups









◀ **Fig. 1** Effect of ONCOFID-P on tumor growth, dissemination, and ascites formation. Female athymic mice were inoculated IP with OVCAR-3 cells. One week after inoculation, the mice were randomized into four treatment groups: Controls (IP glucosate 5%, days 8, 15, 30, 37), IP ONCOFID-P (210 mg/kg/injection, days 8, 15, 30, 37), IP free paclitaxel (20 mg/kg/injection, days 8, 15, 30, 37), and IV free paclitaxel (20 mg/kg/injection, days 8, 11, 15, 18). Six weeks after tumor inoculation, five mice from each treatment group were killed to evaluate tumor progression. **a, b** Tumor growth and body weight curves in the main experimental groups. Values are means ± SEM, $n = 10$. $^{*}P < 0.05$, and $^{***}P < 0.001$ vs. Controls. **c** Representative images of mice in the different treatment groups, 6 weeks after tumor inoculation. **d** Histological features and CD44 expression of OVCAR-3 tumors (magnification 10×). **e–h** Percent of mice with tumor, tumor burden, ascite volume, and CA 125 serum levels, in the different treatment groups, at the 6-week interim killing. The box extend to the 25th and 75th percentiles; the horizontal bars indicate median values, and whiskers minimum and maximum values ($n = 5$). **i–j** Absolute and relative organ weight in the same animals. Columns represent mean ± SEM, $n = 5$. $^{*}P < 0.05$, and $^{**}P < 0.01$ vs. Controls

Safety evaluations

WBC count in control mice averaged around $2.83 \pm 0.37 \times 10^6$ cells/ml. Both treatments significantly reduced WBC counts to a similar extent ($P < 0.01$, Fig. 3a). This effect was accounted for by a significant decrease in both lymphocyte and polymorphonuclear cell counts, whereas monocyte count was not modified by either treatment (Fig. 3b). ONCOFID-P and free paclitaxel by the same route of dosing did not affect BM cellularity (Fig. 3c).

When compared with controls, mice receiving IP free paclitaxel showed a significant increase in both absolute and relative spleen weights ($P < 0.001$ for both parameters), while ONCOFID-P- treated mice only showed a significant increase in the absolute organ weight ($P < 0.05$) (Fig. 3d). Noteworthy, both absolute and relative spleen weights of IP free paclitaxel-treated mice were significantly higher than those measured in ONCOFID-P-treated mice ($P < 0.01$). Histological evaluation showed that when compared with controls, treated mice showed a modest but consistent red pulp expansion, with a more prominent extramedullary hematopoiesis, which is typical of mice spleen [29]. Changes induced by ONCOFID-P (Fig. 3e) and IP free paclitaxel (not shown) were comparable.

Discussion

Results from the present study are in line with and extend data of previous reports demonstrating that ONCOFID-P affords a potent in vivo therapeutic activity when tested in preclinical models of ovarian cancer [19]. We purposely evaluated bioconjugate activity in two different in vivo experimental models closely reflecting disease progression in human ovarian cancer, with tumor dissemination throughout the peritoneum and ascites production [30]. Results obtained showed that, in both experimental models, IP treatment with ONCOFID-P at the MTD inhibited intra-abdominal tumor dissemination and production of ascites, resulting in prolonged survival and cured mice. Conversely, therapy at the MTD with free paclitaxel by the IV route, which is still the standard administration route of the drug, was completely devoid of efficacy, while exerting toxicity, as demonstrated by the body weight loss observed.

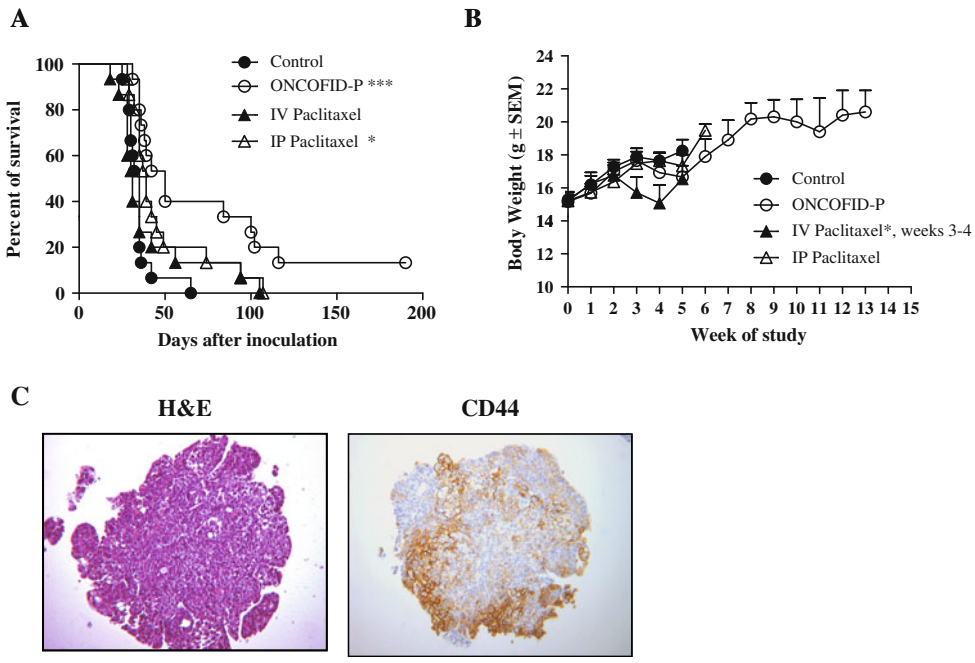


Fig. 2 Effect of ONCOFID-P on tumor growth in female athymic mice inoculated IP with SKOV-3 cells. One week after inoculation, the mice were randomized into four treatment groups: Controls (IP glucosate 5%, days 8, 15, 30, 37), IP ONCOFID-P (210 mg/kg/injection, days 8, 15, 30, 37), IP free paclitaxel (20 mg/kg/injection, days 8, 15, 30, 37), and IV free paclitaxel (20 mg/kg/injection, days 8, 11, 15, 18). **a, b** Tumor growth and body weight curves in experimental groups. Values are means ± SEM, $n = 15$. $^{*}P < 0.05$, and $^{***}P < 0.0001$ vs. Controls. **c** Histological features and CD44 expression of SKOV-3 tumors (magnification 10×)

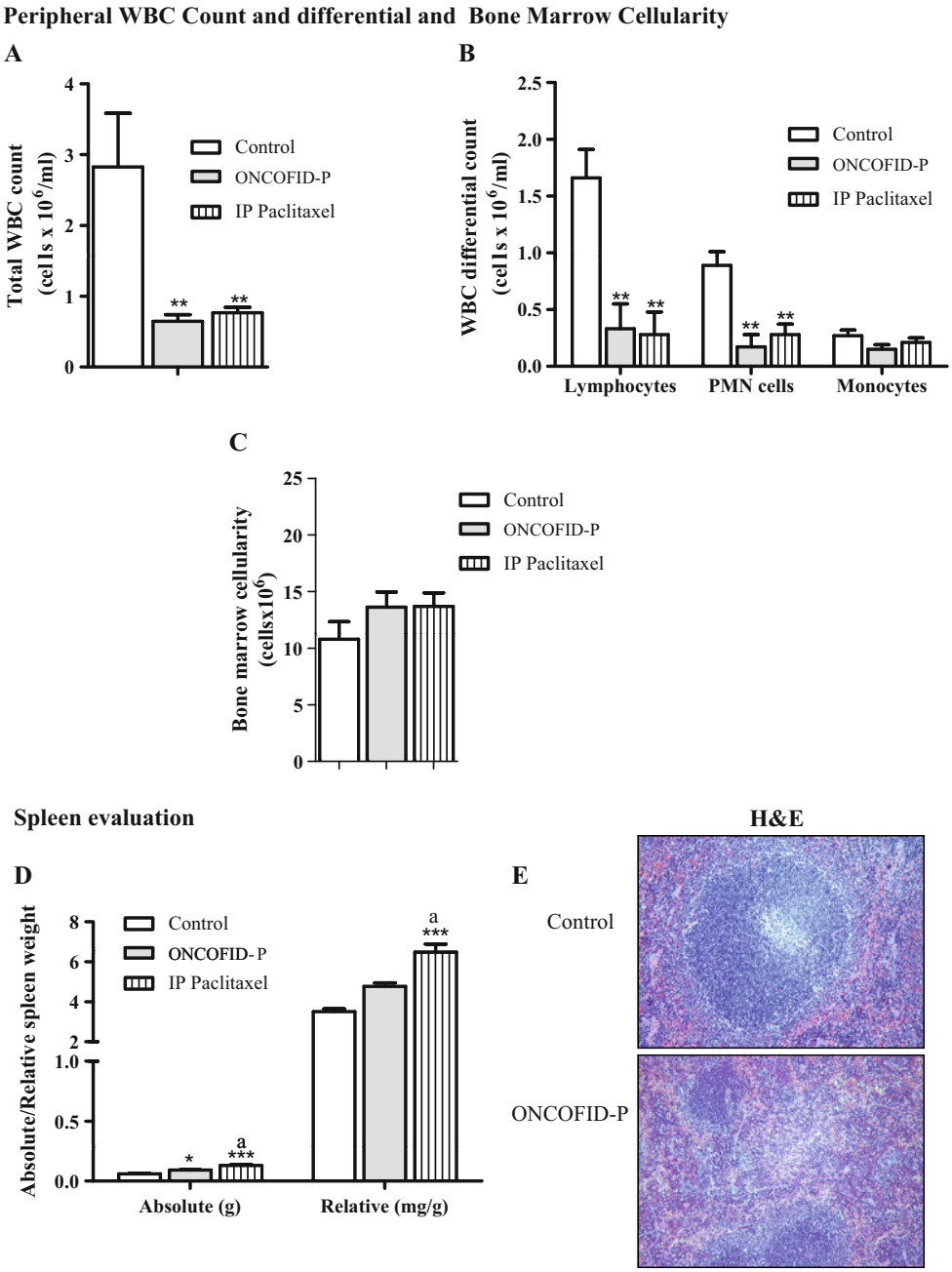


Fig. 3 Effect of ONCOFID-P on hematopoiesis in female athymic mice. Mice were randomized into three treatment groups: Controls (IP glucosate 5%, days 8, 15, 30, 37), IP ONCOFID-P (210 mg/kg/injection, days 8, 15, 30, 37), and IP free paclitaxel (20 mg/kg/injection, days 8, 15, 30, 37). **a, b** WBC count and differential and **c** bone marrow cellularity in the different treatment groups. **d** Absolute and relative spleen weight in the different treatment groups. Values are means ± SEM, $n = 5$. $^{*}P < 0.05$, $^{***}P < 0.0001$ vs. Controls, and a $P < 0.01$ vs. ONCOFID-P. **e** White and red pulp distribution in the spleen of controls (*upper panel*) and ONCOFID-P-treated mice (*lower panel*) (magnification 10×). IP free paclitaxel induced architectural changes (*not shown*) comparable to those induced by ONCOFID-P

Notably, administration of free paclitaxel by the IP route although increased effectiveness in comparison with free drug given by the IV route was however still less efficient than ONCOFID-P administration, in both experimental models used. These in vivo findings are seemingly not in line with our in vitro data showing that, in our experimental conditions, antiproliferative activity of ONCOFID-P was lower than that of pure paclitaxel. This, however, is not a surprising result, as the advantages of ONCOFID-P over paclitaxel are likely to be more prominent in vivo than in vitro. Among the valuable factors, a significant role is certainly played by the favorable pharmacokinetic behavior of the bioconjugate compared to that of the free drug, with different critical outcomes in terms of distribution pattern. Indeed, pharmacokinetic studies indicated that while following IP free drug administration, the plasma concentrations peaked in 6 h and were almost negligible at 24 h, after IP ONCOFID-P there was a striking increase in drug plasma levels which plateaued within 24 h, lasted up to 48 h, and declined slowly in the following days, returning to basal levels only at 120 h [19]. Thus, it is very likely that the IP-injected bioconjugate remains in the peritoneal cavity allowing a direct, selective, and long-lasting (several days) interaction with ovarian cancer cells widely expressing

the CD44 receptor [14, 31], so maximizing drug uptake and therapeutic effectiveness. Further studies on tumor drug accumulation are needed to definitively confirm this advantage. It is worthy to note that previous investigations have already demonstrated an important pharmacokinetic advantage for free paclitaxel after IP regional delivery, when compared to the standard IV route [8], and other studies showed that conjugation with hyaluronic acid further extend the retention time of paclitaxel to the abdominal cavity [32]. However, for free IP paclitaxel, effective dosing is impaired by the local dose-limiting toxicity [8]. In this respect, the relatively high water solubility of the bioconjugate, eliminating the requirement of Cremophor EL as a solvent, with its recognized irritating and toxic properties [20], allows the administration of higher dosages. Collectively, the increased injection dose, the longer duration, and the enhanced local retention of the bioconjugate give support to the higher in vivo efficacy showed by ONCOFID-P even compared with IP paclitaxel.

It is worth mentioning that one of the advantages of ONCOFID-P over free paclitaxel resides in its enhanced capability to enter tumor cells via cell surface CD44. Paradoxically, the CD44 binding capability of ONCOFID-P might entail a modification of the side-effect profile compared to free paclitaxel, due to the expression of CD44 also on the cell surface of leukocytes and hematopoietic stem/progenitor cells. This possibility seems remote however as ONCOFID-P, although administered at a two-fold higher dose, proved not to be more myelotoxic than free IP paclitaxel. Interestingly enough, we observed that while at the time of killing bone marrow cellularity had already recovered in both treatment groups, a modest expansion of the spleen red pulp was still present in treated mice, likely reflecting the occurrence of an enhanced and long-lasting extra medullary hematopoiesis attempting to control the myelotoxic effect induced by both treatments. This observation is well in line with the notion that in mice red pulp is physiologically involved in hematopoiesis [29], and overall, our data are in keeping with previous results showing that the bone marrow and the spleen display different time-courses of functional recovery in murine hematopoiesis post paclitaxel injury [33, 34]. On the whole, changes observed imply that ONCOFID-P does not perform significantly worse than its comparator in terms of hematological toxicity. In this context, it is worth pointing out that myelosuppression is the dose-limiting toxicity for most chemotherapeutic drugs, including paclitaxel, and infection remains a common cause of death in chemotherapy-treated patients. Thus, besides improvement in efficacy, the attainment of a reduction in toxicity represents a goal in the development of new taxanes or taxane analogues. Notably, macromolecular drug delivery systems have been developed as one approach to improve the therapeutic index and possibly overcome drug resistance.

In conclusion, results from our study, in keeping with previous literature data, strongly support the development of ONCOFID-P for locoregional treatment of ovarian cancer. This novel paclitaxel formulation shows to be a more tolerable, and safer method for drug administration, capable of providing higher dosages without increasing adverse effects. Furthermore, this delivery system demonstrates greater therapeutic efficacy in two different experimental models of human ovarian cancer. Importantly, a phase I study is now ongoing in our institution to investigate the MTD and the safety profile of ONCOFID-P following IP infusion in patients affected by intraperitoneal carcinosis due to ovarian, breast, stomach, bladder, and colon cancer. Further studies should be also envisaged to investigate the feasibility of using a metronomic rather than an MTD schedule for this drug.

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