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Translational Development of A Tumor Junction Opening Technology

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Abstract

Our goal is to overcome treatment resistance in ovarian cancer patients, which occurs in most cases after an initial positive response to chemotherapy. A central resistance mechanism is the maintenance of desmoglein-2 (DSG2)-positive tight junctions between malignant cells, which prevents drug penetration into the tumor. We generated JO4, a recombinant protein that binds to DSG2, resulting in the transient opening of junctions in epithelial tumors. Here, we present studies on the clinical translation of JO4 in combination with PEGylated liposomal doxorubicin/Doxil® for ovarian cancer therapy. A manufacturing process for cGMP-compliant production of JO4 was developed. GLP toxicology studies using material from this process in DSG2 transgenic mice and cynomolgus macaques showed no treatment-related toxicities after intravenous injection at doses reaching 24 mg/kg. Multiple cycles of intravenous JO4 plus Doxil® (4 cycles, 4 weeks apart, simulating the treatment regimen in the clinical trial) elicited antibodies against JO4 that increased with each cycle and were accompanied by elevation of pro-inflammatory cytokines IL6 and TNF. Pretreatment with steroids and cyclophosphamide reduced the anti-JO4 antibody response and blunted cytokine release. Our data indicate acceptable safety of our new treatment approach if immune reactions are monitored and counteracted with appropriate immune suppression.

Introduction

Epithelial junctions that link cancer cells create physical barriers to the intratumoral penetration of therapeutic agents. Most drugs - and in particular nanoparticle- or liposome-based drugs (diameters ~/>100 nm) - do not diffuse more than a few cell layers from blood vessels, implying that more distant tumor cells receive only subtherapeutic drug exposure [1–3]. A major junction protein associated with this resistance is desmoglein-2 (DSG2). We have previously reported that the degree of DSG2 expression correlated with ovarian cancer grade and treatment resistance [4].

DSG2 is also used as a receptor by species B human adenoviruses [5]. Among DSG2-targeting viruses is serotype 3 (Ad3). Ad3 is able to efficiently breach the epithelial barrier in the airway tract and infect airway epithelial cells. We found that this is achieved by the binding of an Ad3 fiber capsid protein to DSG2 and subsequent intracellular signaling that results in transient opening of tight junctions between epithelial cells [6]. We have capitalized on this mechanism and created recombinant proteins that contain the minimal structural domains from Ad3 that are required for junction opening [7, 8]. We have shown that intravenous injection of junction opener proteins increases intratumoral penetration and the efficacy of monoclonal antibodies and chemotherapeutic drugs in a broad range of human xenograft models [9, 10].

JO4 is a rationally designed protein with an artificial dimerization domain and specific mutations that greatly increase the affinity to DSG2 in a way that triggers junction opening more efficiently than the parental Ad3 virus [11]. Disruption of junctions by JO4 is transient, and the junction structure is completely restored in a short time period after JO4 is eliminated. JO4 action is tumor-specific because DSG2 in normally polarized epithelial tissues is trapped in lateral junctions and not accessible to JO4.

We plan to clinically test JO4 in combination with PEGylated liposomal doxorubicin (PLD) in ovarian cancer patients. PLD is marketed under the names Doxil®, Caelyx® or Lipodox®. The liposomal formulation is formed of a polyethylene glycol (PEG) coat on the exterior protruding from an amphipathic bilayer. The core is comprised of a single aqueous nanocrystal in the liposome core. The size of the liposome is approximately 100 nm, which precludes it from capillary junctions such as those found in the heart, further decreasing the possibility of cardiotoxicity and fatal side effects [12]. As such, Doxil® has been approved for use in platinum-resistant ovarian cancer [13]. However, response rates to Doxil® are low, the response duration is short, and the toxicity is significant [14–16]. There is a great need for improvement of both the efficacy and the safety of Doxil® therapy in ovarian cancer patients.

Here, we performed toxicology studies for JO4 + Doxil® combination therapy as part of the preparation of an Investigational New Drug (IND) submission. The studies were performed with JO4 drug substance that was manufactured based on a cGMP-compliant protocol and that had clinical-grade quality. We used two adequate animal toxicology models: DSG2 transgenic mice and cynomolgus monkeys. The homology between the human and mouse DSG2 genes is 77.1%, and neither Ad3 nor JO4 binds to mouse cells [17]. We therefore generated transgenic mice that contained the 90 kb human DSG2 locus, including all regulatory regions. These mice express human DSG2 in a pattern and at a level similar to humans [17]. In the past, we accumulated a substantial amount of efficacy and safety data in hDSG2 transgenic mice [6, 7, 11, 17]. JO4 triggers junction opening in hDSG2-expressing epithelial mouse tumor cells, indicating that hDSG2 interacts with mouse signaling and cytoskeletal proteins, thus overriding a potential function of mouse DSG2 in the maintenance of junctions in transgenic mice. Nonetheless, a DSG2 animal model with homologous downstream signaling machinery would be preferable. The DSG2 gene homology between humans and macaques is 96.6%. The biodistribution in cynomolgus monkeys (*Macaca fascicularis)* is similar to that in humans [17]. JO4 binds to monkey DSG2 and triggers junction opening at a level that is comparable to its effect on human cells [17].

Overall, our studies showed that JO4 + Doxil® treatment is well tolerated. In addition to defining the highest nonsevere toxic dose (HNSTD) of JO4 in NHPs, we used the NHP model to assess JO4 immunogenicity after multiple treatment cycles. The data accumulated will be included as part of the preclinical sections of an IND application.

Results

Bridging from Research Material to Industrial cGMP-compliant product. Previously produced JO4 research material (“r-JO4”) had problems with yields, endotoxin, and aggregation and would not be suitable for clinical development[7]. We therefore worked with a cGMP-compliant manufacturing organization, Gennova Biopharmaceuticals (Pune, India), which developed a new purification protocol that could be used for cGMP-compliant production of c-JO-4. The protocol involved affinity resin purification followed by a refolding step to yield partially purified, active c-JO-4. The protein was then put through a size exclusion step to remove high molecular weight aggregates and polished using an ion

exchange resin to remove residual endotoxins and other host cell contaminants, such as proteins and nucleic acids. The final product was then buffer exchanged into PBS, 5% D-sorbitol, pH 7.4 buffer and sterile filtered to yield the infusible product at a yield of approximately 0.7 grams per liter of fermented bacterial bulk.

This c-JO4 product had a purity of >99% with endotoxin levels of <200 EU/ml. c-JO4 was characterized by biophysical methods (Fig. 1). In agreement with previous r-JO4 data [7, 8], even in the presence of 0.1% SDS, the c-JO4 dimer trimer migrates in a polyacrylamide gel at approximately 65 kDa, likely as an assembled trimer (Fig. 1A). Boiling in the presence of a reducing agent resulted in dissociation into monomers with a molecular weight of ~25 kDa. The new refolding technology used for manufacturing c-JO4 resulted in a product with only minimal aggregation. Both SEC (Fig. 1B) and electron microscopy (Fig. 1C) showed uniform c-JO4 dimers of trimers of 11 nm in length with a maximum diameter of 7 nm.

We performed a series of potency studies with c-JO4 in comparison to r-JO4 to bridge the large amount of efficacy data that we previously obtained with r-JO4 (Fig. 1D-H). The first assay measured the ability of JO4 to block the infection of 293 cells by a GFP-expressing Ad5/3 adenovirus vector that has the same tropism to DSG2 as JO4 (Fig. 1D). This assay did not show differences between the two JO4 preparations. It also showed that c-JO4 did not lose potency after storage at 4°C for up to two years (last time point analyzed) (Fig. 1E).

Furthermore, we used 3D spheroid cultures formed by colon cancer T84 tumor cells to analyze the effect of c-JO4 on Doxil uptake. T84 spheroids form typical epithelial junctions that stain for DSG2 (Fig. 1F, G). Doxil was added to the spheroid culture medium in the presence or absence of c-JO4. One hour later, the spheroids were washed and digested with trypsin to obtain a single cell suspension. The amount of Doxil (based on its mean autofluorescence intensity – MAFI) inside cells (Fig. 1F) and the amount of extracellular Doxil (present in the supernatant of digested and pelleted cells) (Fig. 1G) were significantly higher for c-JO4-treated spheroids, indicating better penetration and uptake of Doxil due to c-JO4-mediated epithelial junction opening. Finally, we confirmed the activity of c-JO4 *in vivo* in mice with subcutaneous T84 xenograft tumors that were intravenously injected with Doxil or c-JO4+Doxil (Fig. 1H). While all mice treated with Doxil reached the endpoint by 50 days, animals treated with the c-JO4+Doxil combination survived significantly longer, with 40% of mice being tumor-free at the end of the observation period (day 140).

Taken together, these data demonstrate that c-JO4 is functionally active.

GLP toxicology studies in DSG2-transgenic mice. These studies were performed at the GLP test site, Experimur Toxicology and Research. The objectives of this study were to evaluate the potential toxicity of c-JO4 when administered intravenously alone and in combination with Doxil to female hDSG2-transgenic mice weekly for 4 consecutive weeks with 2 weeks of recovery. The study consisted of one control and five treated groups with 0, 4, and 20 mg/kg c-JO4 and a constant Doxil dose (1.1 mg/kg) (Fig. 2A). This dose was obtained by allometric scaling of the therapeutic dose in humans, which is $^{40\ mg/m2}$. Each

group had six core female mice (undergoing standard evaluations such as clinical observations, body weight, clinical pathology, organ weights and histopathology). In addition, 4 female mice per group in groups 1, 3, and 6 were designated recovery animals. The recovery animals were handled exactly as the Core mice but were held for at least 14 days of rest after the last dose to determine the reversibility of potential treatment-related effects. Treatment-related observations were limited to the mice that received 20 mg/kg c-JO4 (Groups 3 and 6). Hypoactivity was observed for 10 of 10 mice in Group 3. Hypoactivity, cold to touch, convulsions, ptosis, and lethargy were noted for females given 20 mg/kg c-JO4 + 1.1 mg/kg Doxil (Group 6). Evaluation of group mean body weight, body weight gains and hematology revealed no test article-related changes (Suppl. c-JO4- or c-JO4+Doxil-related changes in clinical chemistry were limited to females given 20 mg/kg JO4 (Group 3) and included decreased serum albumin globulin, total protein, calcium, and cholesterol and an increased serum A/G ratio at termination (Suppl. No test article-related changes in organ weight parameters were noted.

In summary, weekly administration of c-JO4 intravenously (with and without Doxil) for 4 weeks to female hDSG2- transgenic mice caused no test article-related critical toxicity. The NOAEL for the study was the highest dose, i.e., 20 mg/kg/injection.

Intravenous dose range finding study in female cynomolgus monkeys. These studies were also performed in GLP compliance at Experimur Toxicology and Research. The objective of this study was to find the highest nonsevere toxic dose (HNSTD) of c-JO4 when administered intravenously to cynomolgus monkeys. The study consisted of a single intravenous administration of c-JO4 with a 7-day follow-up. Three c-JO4 doses (4, 12, and 24 mg/kg) were tested, and each group had 2 female monkeys (Fig. 2B). The dose range was based on efficacy studies in xenograft tumor models [10, 18–20], safety studies in DSG2 transgenic mice (see above), and pilot studies in cynomolgus monkeys that were performed with 2 mg/kg r-JO4 [11].

c-JO4 was well tolerated when administered intravenously to female monkeys at dose levels up to 24 mg/kg. No treatment-related observations were noted. Evaluation of body weight, body weight gain, clinical chemistry and hematology values revealed no test article-related effects. Gross necropsy observations were limited to adhesions of the cecum and rectum for one high-dose female. Adhesions appear to be a pre-existing condition and are not thought to be test article related. In histopathology analyses, mild hemorrhage and chronic inflammation were noted at the injection site for one animal given 12.0 mg/kg/day. There were no other treatment-related microscopic findings.

Blood samples were collected pretreatment and at 6, 24, 46, 72 hours, day 5 and day 7. c-JO4 Serum clearance and correlative pharmacokinetics showed a half-life of 10-12 hours for all three doses (Fig. 3A, B). Animals showed a consistent clearance ranging from 1.5 – 2.5 L/h/kg for all animals except one, which had received the 4 mg/kg dose. Peak concentrations of JO4 in serum increased accordingly, as expected, with dosage. Serum IL-6 levels were elevated in the animals treated with 24 mg/kg, with a peak at 6 hours (Fig. 3C). None of the animals had pretreatment anti-cJO4 IgM or IgG antibodies (Fig. 3D). As

expected, c-JO4 injection triggered the development of antibodies as early as day 5 in an animal from the 4 mg/kg group and in an animal from the 12 mg/kg group.

In conclusion, no systemic treatment-related findings were noted for animals administered 4.0, 12.0 and 24.0 mg/kg/day. Injection of 24 mg/kg triggered a transient elevation of serum IL-6 not lasting more than 24 hours. We used these data to determine that the HNSTD was 24 mg/kg.

Non-GLP 4-cycle tox/PK/immunogenicity study in cynomolgus monkeys. Because c-JO4 is a viral protein, adaptive immune responses are likely to develop, particularly after repeated injection. Furthermore, Doxil contains a polyethylene glycol (PEG) coat, which can potentially trigger anti-PEG antibody responses [21]. To assess the safety of repeated c-JO4/Doxil treatment, we designed a study in two female cynomolgus monkeys that closely reflected the treatment regimen planned in ovarian cancer patients (Fig. 2C). c-JO4 (2 mg/kg) was given intravenously one hour before IV Doxil at a dose of $^{40\ mg/m2}$. The treatment was repeated 3 times with an interval of 4 weeks. Twelve hours after the 4th treatment cycle, animals were euthanized, and a necropsy was performed. As c-JO4 immunogenicity has already been previously noted, a common immunosuppression regimen was tested in a second animal for potential implementation in a clinical study. Immunosuppression consisted of cyclophosphamide given 2 days before c-JO4 and methylprednisolone plus dexamethasone given 30 min before c-JO4.

Physical health and hematological parameters

Both animals tolerated treatment well. Daily monitoring of the infusion site, food and water intake, and feces/urine did not show any abnormalities. There was no weight loss. Electrocardiograms were taken prior to each injection of c-JO4+Doxil, and the results were normal (data not shown). Overall, no unexpected test article-related changes were found in hematological analyses. Immunosuppression in the IS+Tx animals resulted in low white blood cell/lymphocyte counts (Suppl. As expected, glucocorticoids triggered a transient dip in cortisol levels (Suppl. In both animals, c-JO4+Doxil injection resulted in a small, temporary decline in C3 complement levels and red blood cell parameters (Suppl. Fig. 2B), which could be multifactorial. No treatment-related pathological or histopathological findings were listed in audited necropsy reports (see Suppl. Information).

c-JO4 pharmacokinetics and biodistribution

Serum samples were taken immediately before each c-JO4 injection and then at 3, 6, and 24 hours and 2, 7 and 14 days after each injection. c-JO4 concentrations were measured by ELISA (Figs. 4A, B). Overall, peak c-JO4 concentrations declined from cycle 1 to cycle 4. The decline was less pronounced for the animal that received immunosuppression (“IS+Tx”). After the first cycle, the c-JO4 serum half-life was 4.83 and 5.46 hours for the “Tx” and “IS+Tx” animals, respectively (Fig. 4B). Six hours after the 4th c-JO4 injection, c-JO4 was mainly found in the spleen and liver (Fig. 4C). Immunofluorescence studies on liver sections showed that anti-JO4 antibody staining colocalized with staining for the macrophage/Kupffer cell marker CD163 (Fig. 4D). c-JO4 biodistribution was similar for both animals. c-JO4 was detected in

urine at necropsy 2 hours after the last administration of c-JO4+Doxil® at concentrations of 1.15 ng/mL and 1.37 ng/mL for the IS+Tx and Tx animals, respectively.

Anti-cJO4 serum antibodies

Serum anti-cJO4 IgG titers increased with each cycle (Fig. 5A, B). Immunosuppression was partially effective. Intravenous injection of 2 mg/kg c-JO4 was sufficient to completely saturate anti-JO4 serum antibodies (Suppl. Fig. 3). Notably, anti-cJO4 IgM was detectable in serum before treatment, most likely due to early exposure of the animals to an adenovirus that carried c-JO4 antigens (e.g., Ad3 virus). This "priming" might explain the strong humoral response that led to the faster-than-expected clearance of c-JO4 from serum and the increasing speed of clearance with successive injections.

To study the potential consequences of the transient formation of immune complexes between c-JO4 and antibodies, we measured preinflammatory cytokines in serum samples using an inflammatory Th1/Th2 cytokine array. The cytokines tested included interferon gamma, tumor necrosis factor (TNF), IL-6, IL-5, IL-4, and IL-2. Of these, IL-2 was not detected in any sample at any time point. Spikes in the other cytokines were detected shortly after each injection, peaking at the 2-hour postcJO4/Doxil administration time point for each injection and increasing thereafter for subsequent injections (Fig. 5C). The highest levels were found for IL-6 and TNFa. Notable differences were seen between the two animals in IL-6 and TNF levels. In the "Tx" animal, which received no immunosuppression, IL-6 and TNFa levels reached 10,000 pg/mL and 220 pg/ml, respectively. In the "IS+Tx" animal, cytokine levels were 5-10-fold lower and in the noncritical range. As before, the scatter in response could be due to differential exposure to related adenoviruses.

Doxil® concentration and antibody response: *The* Doxil® concentration in serum was measured using ELISA with anti-PEG monoclonal antibodies [21] (Fig. 6A). Pharmacokinetics of Doxil® in serum followed a peak within 2 hours of administration, declining until approximately day 14 when it was no longer detectable. There were no discernible differences between the two animals in Doxil® kinetics, suggesting that immunosuppression had no significant effect on the clearance of Doxil® from serum. Additionally, subsequent administrations did not significantly affect the PK of Doxil. The half-life of Doxil® in the serum after the first injection was calculated to be 2.08 and 2.36 days for animal "IS+Tx" and animal "Tx", respectively (Fig. 6B). When compared to published parameters for Doxil®, clearance is a magnitude slower than in human patients (approximately 0.04 $L/h/m^2$) [22]. Notably, antibody responses against PEG were not detected in serum in either animal, thus suggesting that the administration of Doxil does not trigger a humoral immune response.

Pre-existing anti-cJO4 and anti-PEG antibodies in ovarian cancer patients. Despite the fact that approximately one-third of humans have neutralizing antibodies against human adenovirus serotype 3, most of these antibodies are directed against the main capsid protein, called hexon [23], and not the fiber protein, from which c-JO4 is derived. High-titer, pre-existing serum anti-cJO4 antibodies could trigger anaphylactic reactions after intravenous cJO4 protein infusion. To assess the seroprevalence of anti-

cJO4 antibodies in a relevant target population, we obtained serum samples from women with progressive, persistent or recurrent ovarian/fallopian tube cancer who had previously received standard therapies. This would be the cohort of patients considered for our clinical trial. Materials were provided by the TOR Biorepository at the FHCRC. We determined that an IC50 IgG titer of greater than 800 neutralized c-JO4 in our *in vitro* blocking assay (Fig. 7A). With this threshold, 10% of patient samples would have neutralizing IgG anti-c-JO4 titers. These patients would have been excluded from the trial. Serum antibody titers against PEG were below the threshold in all patient samples (Fig. 7B). Clearly, an important task in the clinical trial would be to monitor the development of anti-drug antibodies along with the analysis of cytokines - and other potentially anaphylactic - responses after each treatment cycle.

Discussion

Our goal is the clinical translation of c-JO4 in combination with Doxil for ovarian cancer therapy. A major step toward this goal was the establishment of a cGMP-compliant protocol for c-JO4 manufacturing. As part of an IND application, we performed toxicology studies with this material along with additional studies to assess immunological and biochemical parameters in animals in response to the administration of the clinical candidate protein. We also assessed and confirmed the potency of the material in a series of *in vitro* studies to ascertain its equivalency to previously produced r-JO4 material.

A potential concern was the safety of intravenously injected c-JO4 because the c-JO4 target receptor DSG2 is expressed in most epithelial tissues. However, DSG2 in normal epithelial tissues displays strict apical basal polarization and is trapped in lateral junctions and thus is not readily accessible to intravenously injected ligands. We believe that this is a compounding factor for the good safety profile we demonstrated here. Dose-escalation studies in both relevant animal models, DSG2 transgenic mice and cynomolgus monkeys, did not trigger adverse clinical side effects in single-cycle and multicycle treatment regimens in combination with a clinically relevant dose of Doxil.

Intravenous c-JO4 injection is expected to trigger antibody responses, which could affect the safety and efficacy of the approach. In the multicycle treatment study, we confirmed the development of an antibody response. This resulted in the formation of immune complexes, as suggested by the transient disappearance of anti-cJO4 IgG from the circulation immediately after cJO4 injection (Fig. 5A). This affected the pharmacokinetics, leading to a faster clearance of c-JO4 from the blood circulation. Our immunofluorescence studies suggest that immunocomplexes containing JO4 were taken up by macrophages of the liver and spleen. A consequence of macrophage activation is the release of proinflammatory cytokines. In our study, we found elevated IL-6 and TNFa levels, and these levels increased with each treatment cycle in concordance with increased humoral immune responses. Clearly, the formation of immune complexes and downstream reactions pose a safety risk [24]. We therefore implemented a prophylactic regimen consisting of pretreatment with cyclophosphamide/Cytoxan and glucocorticoids. Cyclophosphamide has been used as a single agent before the development of platinum-based medications [25] and was investigated as a potential cotherapeutic with paclitaxel or cisplatin in ovarian cancer patients [26, 27]. Glucocorticoids mediate temporary immunosuppression, decreasing the

function and/or numbers of neutrophils, lymphocytes (including both B cells and T cells), monocytes, and macrophages. In our studies, the combination of cyclophosphamide and glucocorticoids, given before c-JO4 injection, greatly decreased anti-JO4 antibody levels and blunted cytokine reactions even after the 4th cycle. In an additional upcoming GLP toxicology study, we plan to add pentostatin to the immunosuppressive regimen to further block anti-JO4 antibody development. Previous reports demonstrated that pentostatin plus cyclophosphamide chemotherapy safely prevented anti-immunotoxin antibody formation with uniform efficacy [28]. Another potential solution to the immunogenicity of c-JO4 would be PEGylation. PEGylated drugs (e.g., PEGylated factor VIII) are widely used clinically and have good safety profiles [21]. Notably, anti-protein drug immune responses are common for immunotoxins, and there is substantial clinical experience to counteract them.

Although it was not found to be an issue in our NHP studies, immune responses against PEGylated drugs such as Doxil are of concern in humans. Many humans have been exposed to PEGylated medicines [21]. In a clinical trial with Doxil, up to 7% of participants developed hypersensitivity reactions [29,] and after the recent widespread use of PEGylated liposomal mRNA COVID-19 vaccines, this percentage would probably be now even higher. The potential for emergence of anti-PEG responses will be closely monitored in our future clinical trial.

In summary, we evaluated the safety profile of c-JO4 plus Doxil in two adequate animal models under GLP-compliant conditions. We found no critical clinical side effects. The study also allowed us to determine HNSTD for additional preclinical and clinical studies. Mild toxicity caused by anti-cJO4 antibody responses was observed in a multicycle treatment regimen. These reactions appear to be manageable, as our data with glucocorticoid+cyclophosphamide pretreatment indicate. Clearly, in the clinical trial, pre-existing anti-JO4 antibodies and treatment-induced immune reactions would be monitored, especially after multiple cycles, so that they can be counteracted with appropriate immune suppression.

Currently, there are no epithelial junction openers used clinically for cancer therapy. Our work could lead to a novel therapeutic approach requiring lower dosages of therapeutic agents while increasing their efficacy and tumor penetration. While we focused on Doxil in this study, successful completion of these clinical studies could have a profound impact on cancer therapy across numerous solid tumors with a number of different therapeutics, including other nanoparticle-based chemotherapy drugs, monoclonal antibodies, oncolytic viruses, and CAR T-cells.

Methods

Protein Production. cGMP compliance (“c-JO4”) was produced by Gennova Biopharmaceuticals Ltd. (Pune, India). JO-4 is expressed in *E. coli* and accumulates intracellularly in the form of inclusion bodies (insoluble aggregates of misfolded protein lacking biological activity). c-JO4 was isolated from the cultured cells using a pressure-based cell homogenizer. Cell mass containing c-JO4 was resuspended in 10× lysis buffer containing Tris, EDTA, urea, NaCl, and β mercaptoethanol (BME), pH 8.0, and subjected to

cell lysis at 1000 bar. After cell disruption, the crude inclusion bodies (IBs) were washed with 1% nonionic surfactant (Triton X100) to remove contaminating host cell impurities such as DNA and proteins, followed by a water wash to remove excess surfactant and impurities. Purified IBs were recovered as settled pellets after centrifugation at 10000 × g. Even after multiple washing steps, IBs were mainly composed of an aggregated mass of overexpressed heterologous proteins, which further needed to be solubilized in solubilization buffer containing strong denaturants (8 M urea, Tris base, NaCl and BME, pH = 10.0) for 2 hours. The pH of the solubilized IBs was adjusted to 7.4, and the solubilized sample was clarified by centrifugation at 15,000 × g for 30 minutes followed by 0.45 μm filtration.

Clarified and solubilized samples were subjected to immobilized metal affinity chromatography (IMAC) for further purification. IMAC was performed under denaturing conditions. This column utilizes an immobilized nickel metal chelate resin, which binds to His-tagged c-JO4 protein with a high degree of specificity. The column was equilibrated with buffer containing 8 M urea at pH 7.4. To remove process- and product-related impurities, a low concentration imidazole wash was developed, and the target protein was eluted in purified and concentrated form using a step gradient to a buffer with a higher concentration of imidazole.

After solubilization and primary capture using IMAC, the resulting protein needed to be refolded to its native conformation. Pooled elution fractions from the capture chromatography were reduced for 30 minutes using 5 mM cysteine, followed by 10× dilution in refolding buffer containing Tris, 5% D-sorbitol, and 2 M urea, pH 8.5. Cysteine and cystine were used as redox reagents in refolding buffer to obtain refolded and active protein. Dimerized, refolded c-JO4 was next subjected to gel filtration chromatography to remove high- and low-molecular-weight impurities based on their size. Phosphate buffer containing D-sorbitol was used as a working buffer for this step. The elution peak of the purified product was collected and subjected to anion exchange chromatography for polishing.

The final anion exchange chromatographic step was incorporated in the process to reduce host cell proteins, residual DNA, and bacterial endotoxins. A strong anion exchange resin was packed in a glass chromatographic column. Under optimized loading and washing conditions, c-JO4 was obtained in the flow through and then buffer exchanged using Tangential Flow Filtration (TFF) to yield c-JO4 in its final buffer for infusion. The TFF system consisted of a 10 kDa nominal molecular weight cutoff cassette primed with a buffer containing PBS and 5% D-sorbitol at pH 7.4 ± 0.2. The anion exchange chromatography flow-through fraction containing c-JO4 was taken and concentrated to 1.0 ± 0.2 mg/ml. The concentrated protein solution was terminally filtered through a 0.2 μm sterilization grade filter. Purified bulk c-JO4 was then stored at 2 to 8°C in a sterile PETG container. Final endotoxin levels were <200 EU/ml.

Therapeutics and Dosing. PEGylated liposomal doxorubicin/Doxil® was purchased from the University of Washington Medical Center, manufactured by Dr. Reddy's Laboratories (Hyderabad, India). Methylpredisolone, dexamethasone, ceftadizime, acyclovir, cyclophosphamide (Cytoxan®), and mesna were obtained from the University of Washington Medical Center in generic forms. c-JO4 was injected at

the concentration indicated per animal. Doxil® was dosed at 40 mg/m^2 using the Mosteller formula: $SurfaceArea\left(m^2\right) = \frac{\sqrt{Height(cm)*Weight(kg)}}{60}$. Drugs were diluted in physiological saline immediately prior to administration.

Animals. All experiments were conducted in accordance with the institutional guidelines set forth by the University of Washington. The experimental protocols were approved by the University of Washington IACUC (protocols #3108-01 and 3108-05).

Xenograft tumor model: Immunodeficient (CB17) mice (strain name: NOD. CB17-Prkdcscid/J) were obtained from The Jackson Laboratory (Bar Harbor, ME). Human colon cancer T84 cells (ATCC, CCL-248) were established by injection of the corresponding tumor cells into the mammary fat pad (1:1 with Matrigel) of CB17 mice. c-JO4 was intravenously injected one hour before the application of PEGylated liposomal doxorubicin/Doxil® Tumor volumes were measured three times a week. Each treatment group consisted of a minimum of five mice. Mice were euthanized when tumors reached a volume of 1000 mm^3 or displayed ulceration.

DSG2 transgenic mice: These mice are homozygous for the human DSG2 locus (two copies). Husbandry and genotyping are described elsewhere [17]. Mice were housed in specific pathogen-free facilities. All mouse experiments were conducted in accordance with the institutional guidelines set forth by the University of Washington under IACUC protocol #3108-01.

NHP studies at Experimur

Female naïve Chinese origin cynomolgus monkeys *Macaca fascicularis* (purpose-bred cynomolgus monkey) from Primate Products, Inc. (Immokalee, FL) or Envigo Global Services, Inc. were selected for use in this study. The animals were approximately 2-5 years old and weighed 2.4-4.4 kilograms at the time of first test article administration.

NHP studies at the Washington National Primate Research Center (WaNPRC). Female *M. fascicularis* were obtained from Altasciences Inc. (Everett, WA, USA) and at the WaNPRC at the University of Washington. The studies were performed by the WaNPRC Research Support Team. c-JO4 and Doxil® were filtered through 0.2 µm filters before administration into the arm vein of sedated animals. Upon completion of the study, animals were sedated before euthanasia and extraction of organs for histological analysis.

Size Exclusion Chromatography (SEC). Samples were loaded onto a Superdex 200 Increase 10/300 GL column (Cytiva Life Sciences) and flowed through an Agilent 1260 series HPLC using 1× Tris-buffered saline (TBS) at a flow rate of 1 mL/min to obtain the elution profiles.

SDS-PAGE/Western Blot. Mini-Protean precast gels (Bio-Rad, Hercules, CA) with 4 to 15% gradient polyacrylamide were used. A total of 1 µg protein mixed with 2× loading buffer (10 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol [DTT], 4% SDS, 20% glycerol, 0.2% bromophenol blue) was loaded per lane.

Samples were either boiled (B) for 5 min or loaded unboiled (UB). The following running buffer was used: 25 mM Tris, pH 8.3, 0.192 M glycine, 0.1% SDS. After electrophoresis, proteins were transferred to nitrocellulose and incubated with recombinant human DSG2 protein and anti-DSG2 antibodies as described previously [5].

Transmission Electron Microscopy (TEM). Recombinant c-JO4 protein was visualized by negative-stain EM to assess its assembly status. The standard mica-carbon preparation was used with protein at 0.1 mg/ml. The sample was stained using 1% (wt/vol) sodium silicotungstate (pH 7.0) and visualized on a JEOL-1200 electron microscope at 100 kV.

Potency assay – Viral inhibition. A 293 cell suspension was confirmed to be >98% viable, and their concentration was adjusted to 1×10^5 cells/ml and then plated in black, flat clear bottom 96-well plates at 200 µL per well (Corning, Inc., Corning, NY). Following 18 hours of incubation at 37°C in 5% CO_2, growth media was discarded, and 62.5 µL of protein or control diluted in complete DMEM (DMEM containing 10% FBS, 1x Pen/Strep, 1 mM Glutamax) was added to each well in quadruplicate. A total of 11 half log dilutions were tested in quadruplicate for each protein. Following a 1 h incubation, 50 µL of Ad3-GFP virus (25 pfu/cell) in complete DMEM was added. Two hours later, the medium was removed and replaced with fresh complete DMEM, and the plates were further incubated for 16 – 18 hours at 37°C and 5% CO_2. The following day, GFP fluorescence was measured from the bottom read orientation at 475 nm excitation and 505 nm emission using a SpectraMax i3 plate reader (Molecular Devices, Inc., Sunnyvale, CA) utilizing SoftMax Pro software. Data were plotted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA), and the $IC_{50\ was}$ determined using a 5-parameter nonlinear fit of the sigmoidal curves using Softmax Pro software.

Potency assay – Spheroids. T84 cells were seeded in Aggrewell 400 (Stemcell Technologies, Vancouver, Canada) at a seeding density of 200 cells per microwell (equivalent to 2.4×10^5/well). Spheroids were allowed to grow in cell media (DMEM containing 10% FBS, 1× Pen/Strep, 1 mM Glutamax) for at least 4 days to allow sufficient spheroid growth and tight junction formation. Spheroids were then collected by pipetting media through a reversible 70 µm cell strainer; trapped spheroids were flushed for further use. Approximately 1000 spheroids (1 mL) were transferred into a 24-well plate for incubation with JO4 and Doxil® combinations and incubated at 37°C at 5% CO_2 for the indicated time points. At the time point, spheroids were collected and dissociated with 0.05% trypsin solution (Gibco). Cells were then collected for flow cytometry measuring either Doxil® fluorescence (DOX+) or mean autofluorescence intensity (MAFI) using wavelengths of 470/560 nm, corresponding to the phycoerythrin (PE) profile on a BD FACSCanto flow cytometer.

Antibodies. Polyclonal rabbit antibodies directed against the Ad3 fiber knob and mouse monoclonal anti-Ad3 fiber knob antibodies (clone 2-1) were described earlier [17]. DSG2 antibodies were obtained from Invitrogen (Carlsbad, CA, USA). Anti-PEG antibodies were kindly provided by Dr. Steve Roffler (Academia Sinica, Taipei, Taiwan).

Preparation of tissues for ELISA. One hundred milligrams of tissue or organ in PBS-0.05% Tween 20 was homogenized using the TissueRuptor system (Qiagen), sonicated for 20 seconds, and subjected to three freeze-thaw cycles. Cell debris was spun down, and supernatants from lysed tissues were used in c-JO4 and Doxil ELISAs at 1:5, 1:20, and 1:100 dilutions.

Serum JO4 antibody ELISA. Animal blood obtained in serum separation tubes (SST) at the indicated time points was centrifuged at 5000 RPM for 10 minutes, after which the serum was kept at -20°C before use. ELISA plates were coated with c-JO4 protein (0.3 mg per well) at 4°C overnight and then blocked with 5% nonfat milk/PBS for 1 hour. Serum or tissue homogenate serial dilutions (starting at 1:50, 3× subsequent dilutions) were added for 1 hour. After washing, HRP-conjugated secondary antibodies against NHP IgG or IgM (Invitrogen PA1-84631 and 62-6820, respectively) were added at 1:10,000 for 1 hour. After washing, Thermo 1-Step Ultra TMB Solution (Thermo Fisher Scientific) was added, and the color was allowed to develop for 7 minutes before stopping with 2 N sulfuric acid. Absorbance readings at 450 nm were taken with a plate reader. Antibody reactivity curves were plotted with GraphPad Prism with a 4-parameter curve, and EC50 values were accordingly calculated.

Antibodies in patients. Serum samples from ovarian cancer patients were made available by the Fred Hutchinson Research Center (Seattle, WA) without any confidential information which would serve to identify a patient. The samples were collected in accordance with relevant guidelines and regulations set forth by the Fred Hutchinson Cancer Research Center, under FHCRC Institutional Review Board protocol # 4563. In the context of this protocol, informed consent was obtained from all subjects.

Serum samples were assessed for anti-JO4 antibody titers by using the protocols for JO4 ELISA and Doxil® ELISA as outlined below. EC50 measures were calculated using Prism Software and plotted.

JO4 ELISA. The ELISA consisted of a polyclonal rabbit antibody directed against the Ad3 fiber knob as the capture antibody and a mouse monoclonal anti-Ad3 fiber knob antibody (clone 2-1) as the detection antibody, using the common ELISA protocol as stated above, incorporating a standard curve using the c-JO4 protein at concentrations ranging from 100 ng/mL to 0.0064 ng/ml. The sensitivity of the ELISA was 0.5 ng/ml.

Serum Doxil ELISA. For Doxil detection, plates were coated with anti-PEG antibodies (rAGP6 from anti-PEG, Academia Sinica, 250 ng per well) overnight at 4°C. Plates were then blocked with 5% nonfat milk for 1 hour, and serum dilutions were added after washing (starting at 1:10, 10× subsequent dilutions) and incubated at room temperature for 1 hour. This was followed up with another anti-PEG antibody with different specificities (15-2b-biotin) and incubated for 1 hour. After washing, HRP-streptavidin (Jackson ImmunoResearch #016-030-084, West Grove, PA, USA) was added to the wells at 0.5 mg/mL and incubated for 1 hour. Doxil standard concentration curves were generated by plotting data points and using a 4-parameter curve fit (GraphPad Prism) and were used to determine Doxil concentrations in serum.

DSG2 immunofluorescence. Organs were placed in optimal cutting temperature compound (OCT, Sakura Finetek, Torrance, CA, USA) and flash frozen in liquid nitrogen. Sections were sliced at 6 mm thickness and fixed in 5% paraformaldehyde at room temperature for 15 minutes. Slides were then blocked and permeabilized in 5% nonfat milk in 0.05% Tween-20 PBS overnight at 4°C. After washing, the slides were incubated with the primary antibody diluted in 2% nonfat milk/PBS for 1 hour, followed by the secondary antibody diluted in 2% nonfat milk/PBS for 1 hour. Slides were then mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Labs, Burlingame, CA, USA) before visualization using fluorescent filters under an EVOS M5000 microscope (Invitrogen). Images were overlaid using ImageJ software (NIH, Bethesda, MD, USA). The following antibodies were used on 4% paraformaldehyde-fixed OCT sections of monkey organ/tissue: anti-HAdV3 fiber knob mAb-clone 2-1; goat-α-human DSG2 (AF947, R&D Systems), mouse-α-CD163 (Biolegend #333602) with the following secondary antibodies: Alexa Fluor 594-conjugated chicken anti-rabbit IgG (Invitrogen A-21442), Alexa Fluor 488-conjugated donkey anti-goat IgG (Abcam #6881), and Alexa Fluor 488-conjugated goat anti-mouse IgG (Biolegend #405319).

Cytokine Cytometric Bead Array. Animal sera were taken and assessed for cytokines (Th1/Th2) using the Nonhuman Primate Th1/2 cytokine kit/Cytometric Bead Array (CBA) (BD Biosciences, San Jose, CA, USA). Flow cytometry data were obtained using a BD LSR II flow cytometer. The results were quantified using BD CBA FCAP Array Software.

Hematological analyses. Blood samples were collected into EDTA-coated tubes, and analysis was performed on a HemaVet 950FS (Drew Scientific, Waterbury, CT).

Statistical analyses. For comparisons of multiple groups, 1- and 2-way analyses of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons were used. Statistical analysis was performed with Prism, version 6.01 (GraphPad Software Inc, La Jolla, CA). For GLP toxicology studies, continuous data were analyzed for homogeneity of variance using Levene's test. If the variances were homogeneous ($p > 0.001$), the data were further analyzed by one-way ANOVA. If a significant F value was observed ($p \leq 0.05$), each treatment group was compared to the vehicle control group using Dunnett's two-tailed t-test (significant at $p \leq 0.05$). If Levene's test was significant ($p \leq 0.001$), an appropriate transformation was applied to the data (e.g., log-transformation or rank-transformation), and the analyses were performed on the transformed data.

Declarations

Data Availability

All data can be found within the article (Figs. 1-7), Supplementary Figures (Figs. S1-S3), and Supplementary information (Pathology reports). Audited reports from the GLP test site, Experimur, are available from the corresponding author on request.

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Author Contributions

D.C., C.D., S.S., and A.L. provided the conceptual framework for the study. J.K., C.D., S.K. and A.L. designed the experiments. J.K., L.S., A.R., H.W., C.L., A.G., B.C., P.F. performed the studies. S.R., C.D., D.C., and S.K. provided critical comments on the manuscript. A.L. wrote the manuscript.

Ethical approval

All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington and in accordance with ARRIVE guidelines. The University of Washington is an Association for the Assessment and Accreditation of Laboratory Animal Care International–accredited research institution, and all live animal work conducted at this university is in accordance with the Office of Laboratory Animal Welfare Public Health Assurance policy, US Department of Agriculture Animal Welfare Act and Regulations, the Guide for the Care and Use of Laboratory Animals, and University of Washington's Institutional Animal Care and Use Committee protocols 3108-01 and 3108-05. All animal experiments were ethically approved by the University of Washington IACUC.

Serum samples from ovarian cancer patients were made available by the Fred Hutchinson Research Center (Seattle, WA) without any confidential information which would serve to identify a patient. The samples were collected in accordance with relevant guidelines and regulations set forth by the Fred Hutchinson Cancer Research Center, under FHCRC Institutional Review Board protocol #4563. In the context of this protocol, informed consent was obtained from all subjects. All experiments involving human serum samples were ethically approved by the FHCR IRB.

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Competing Interests

D.C. is founder of and holds an equity interest in HDT Bio Corp. a company that licensed JO-4 technology and is assisting in its clinical development.

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Figures

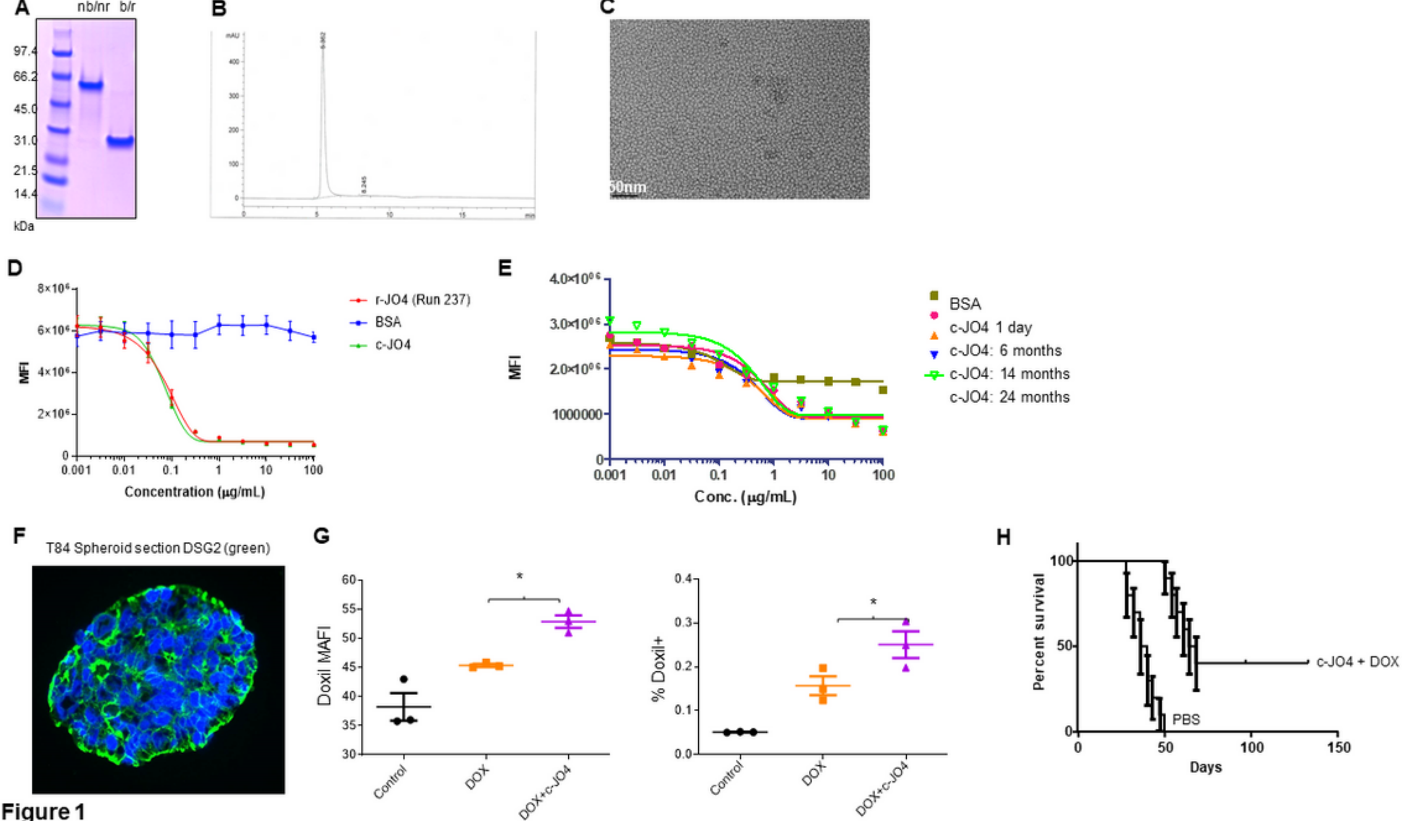
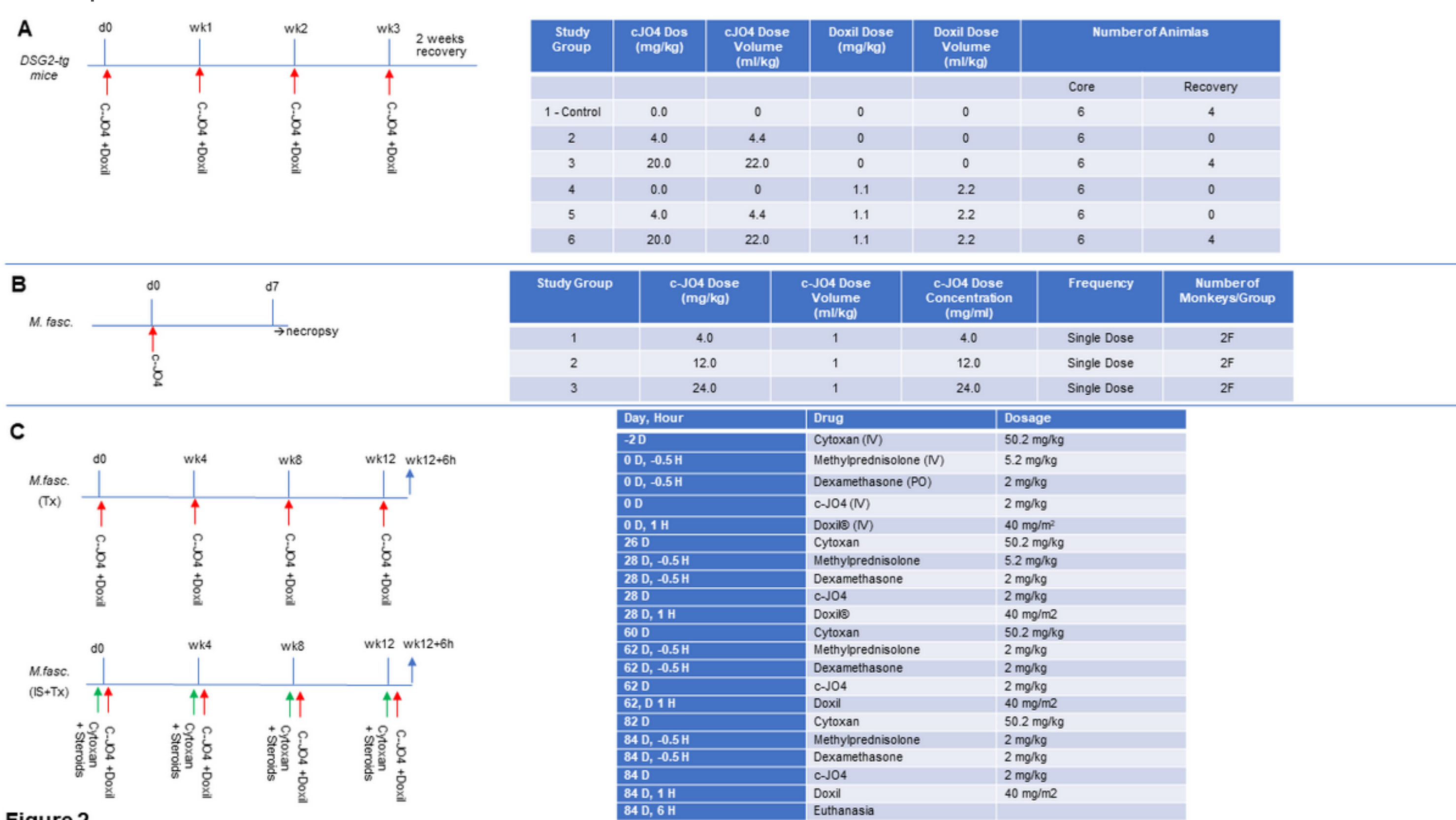


Figure 1

Characterization of c-JO4. A) Polyacrylamide gel electrophoresis. Gennova's preparation of JO4 (c-JO4) was treated as indicated. b/r – boiled and reduced; nb/nr – not boiled and not reduced. 1 μg protein was run on the gel. B) Size exclusion chromatography (SEC) profile of c-JO4 run through a Superdex 200 increase 10/300 column with TBS. C) Electron microscopy of c-JO4 negative staining with SST. D-H) Potency assays. D) Ad3-GFP Infection competition. Research r-JO4 run (#237) was compared with c-JO4. 293 cells were incubated in 96-well plates with JO4 proteins at the indicated concentrations for 1 hour before the addition of Ad3-GFP virus for competition. GFP expression was measured 18 hours after virus addition to measure the extent of JO4 competition with Ad3-GFP. E) c-JO4 was stored at 4°C for 1 day (red dot), 6 months (orange triangle), 14 months (blue triangle), and 24 months (empty green triangle) and then tested in an infection competition assay. F and G) c-JO4-enhanced Doxil penetration in epithelial tumor spheroids. F) Confocal immunofluorescence images of T84 spheroids stained for DSG2 (green) and DAPI (blue). G) Left panel: Uptake of liposomal doxorubicin (Doxil®) (DOX) in T84 cell spheroids after treatment with c-JO4. The amount of Doxil (mean autofluorescence intensity - MAFI) in tumor cells was measured 1 hour after adding it to tumor sphere cultures. Right panel: Doxil released from spheroids into the supernatant after trypsin digestion of spheroids. * $p<0.05$. H) c-JO4-enhanced antitumor activity. CB17 immunodeficient mice bearing epithelial tumors derived from T84 human colon adenocarcinoma cells were used for testing. When tumors reached a volume of 100 mm3, mice were intravenously injected with either PBS or c-JO4 (2 mg/kg) followed by Doxil (1 mg/kg) one hour later. Mice received three injection cycles 3 days apart. Tumor volumes were measured twice a week until reaching a tumor volume of 1000 mm3 when they were sacrificed. The day of sacrifice served as the

endpoint in Kaplan-Meier survival studies. In the c-JO4/Doxil group, tumor growth was significantly delayed ($p<0.0001$), and 45% of the animals were still tumor-free at day 140 after tumor cell inoculation. N=10. $p <0.001$ for Doxil vs c-JO4 + Doxil.



Study Group	cJO4 Dos (mg/kg)	cJO4 Dose Volume (ml/kg)	Doxil Dose (mg/kg)	Doxil Dose Volume (ml/kg)	Number of Animlas	
					Core	Recovery
1 - Control	0.0	0	0	0	6	4
2	4.0	4.4	0	0	6	0
3	20.0	22.0	0	0	6	4
4	0.0	0	1.1	2.2	6	0
5	4.0	4.4	1.1	2.2	6	0
6	20.0	22.0	1.1	2.2	6	4

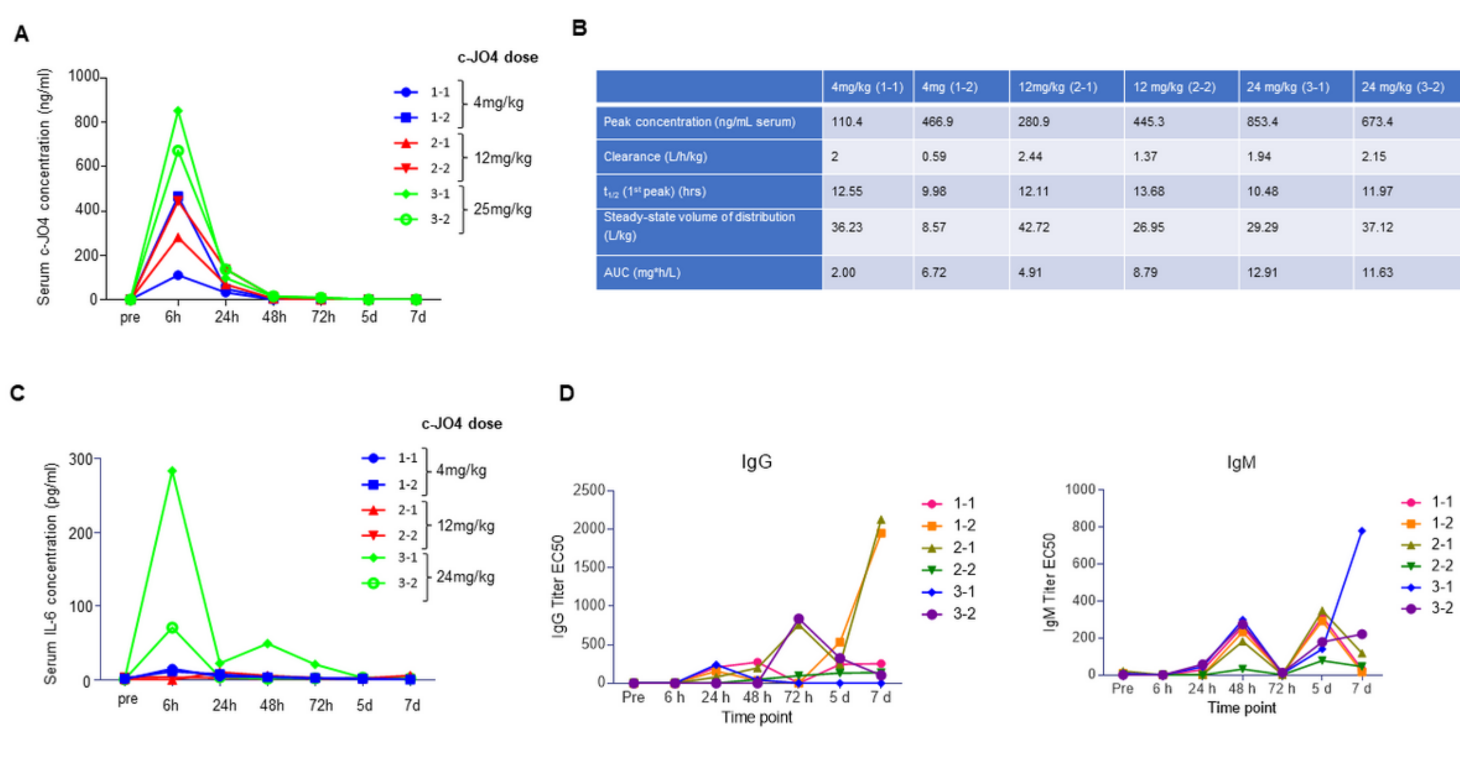
Study Group	c-JO4 Dose (mg/kg)	c-JO4 Dose Volume (ml/kg)	c-JO4 Dose Concentration (mg/ml)	Frequency	Number of Monkeys/Group
1	4.0	1	4.0	Single Dose	2F
2	12.0	1	12.0	Single Dose	2F
3	24.0	1	24.0	Single Dose	2F

Day, Hour	Drug	Dosage
-2 D	Cytoxan (IV)	50.2 mg/kg
0 D, -0.5 H	Methylprednisolone (IV)	5.2 mg/kg
0 D, -0.5 H	Dexamethasone (PO)	2 mg/kg
0 D	c-JO4 (IV)	2 mg/kg
0 D, 1 H	Doxil® (IV)	40 mg/m²
26 D	Cytoxan	50.2 mg/kg
28 D, -0.5 H	Methylprednisolone	5.2 mg/kg
28 D, -0.5 H	Dexamethasone	2 mg/kg
28 D	c-JO4	2 mg/kg
28 D, 1 H	Doxil®	40 mg/m2
60 D	Cytoxan	50.2 mg/kg
62 D, -0.5 H	Methylprednisolone	2 mg/kg
62 D, -0.5 H	Dexamethasone	2 mg/kg
62 D	c-JO4	2 mg/kg
62, D 1 H	Doxil	40 mg/m2
82 D	Cytoxan	50.2 mg/kg
84 D, -0.5 H	Methylprednisolone	2 mg/kg
84 D, -0.5 H	Dexamethasone	2 mg/kg
84 D	c-JO4	2 mg/kg
84 D, 1 H	Doxil	40 mg/m2
84 D, 6 H	Euthanasia	

Figure 2

Summary of animal studies. A) Four-week intravenous GLP toxicology study of c-JO4 in female hDSG2-transgenic mice. The objectives of this study were to evaluate the potential toxicity of c-JO4 when administered intravenously together with Doxil to hDSG2-transgenic mice weekly for 4 consecutive weeks with 2 weeks of recovery. The animals were approximately 5-11 weeks old and weighed 19.9-26.0 grams at the time of the first dose administration. The study consisted of one control and five treated groups. Each group had 6 core female mice (undergoing standard evaluations such as clinical observations, body weight, clinical pathology, organ weights and histopathology). In addition, 4 female mice per group in groups 1, 3, and 6 were designated recovery animals. The recovery animals were handled exactly as the Core mice but were held for at least 14 days of rest after the last dose to determine the reversibility of potential treatment-related effects. B) Intravenous dose range finding study in female cynomolgus monkeys. Dose range study plan of c-JO4 in female cynomolgus monkeys. Three groups of monkeys (N=2) were administered escalating doses of c-JO4 (4, 12, 24 mg/kg). Group 3 was dosed after no toxicity was observed at the previous dose level. Necropsy was performed at day 7 after administration of the c-JO4 dose to collect organ and tissue samples. C) Non-GLP four-cycle toxicity/pharmacokinetics/immunogenicity study with c-JO4 + Doxil in cynomolgus monkeys. One animal received c-JO4 and, one hour later, Doxil® as indicated (“Tx”). The second animal additionally received immunosuppressive drugs (“IS+Tx”). Immunosuppression consisted of cyclophosphamide given

2 days before c-JO4/Doxil) and methylprednisolone + dexamethasone (0.5 before c-JO4 injection). The timing and dosage of drug injection are shown on the right side.



	4mg/kg (1-1)	4mg (1-2)	12mg/kg (2-1)	12 mg/kg (2-2)	24 mg/kg (3-1)	24 mg/kg (3-2)
Peak concentration (ng/mL serum)	110.4	466.9	280.9	445.3	853.4	673.4
Clearance (L/h/kg)	2	0.59	2.44	1.37	1.94	2.15
$t_{1/2}$ (1st peak) (hrs)	12.55	9.98	12.11	13.68	10.48	11.97
Steady-state volume of distribution (L/kg)	36.23	8.57	42.72	26.95	29.29	37.12
AUC (mg*h/L)	2.00	6.72	4.91	8.79	12.91	11.63

Figure 3

Intravenous dose range finding study in female cynomolgus monkeys. A-B) Pharmacokinetic parameters of c-JO4 in cynomolgus monkeys. A) c-JO4 concentrations in serum collected at the indicated time points subsequent to c-JO4 administration were measured by ELISA. The standard curve used to quantify c-JO4 is shown on the right side. B) Pharmacokinetic data calculated by using serum concentration curves of each animal. Units are as indicated. C) Cytokines IL-2, IL-4, IL-5, IL-6, TNF, and IFN-gamma were measured by cytometric bead array (CBA) (NHP th1/Th2 cytokine kit from BD Biosciences). Only IL-6 was detectable. D) Antibody titers present in serum against c-JO4 were measured by ELISA using recombinant c-JO4 protein for capture and anti-NHP-IgG-HRP and anti-NHP-IgM-HRP for detection. Shown are IgM and IgG titers as IC50.

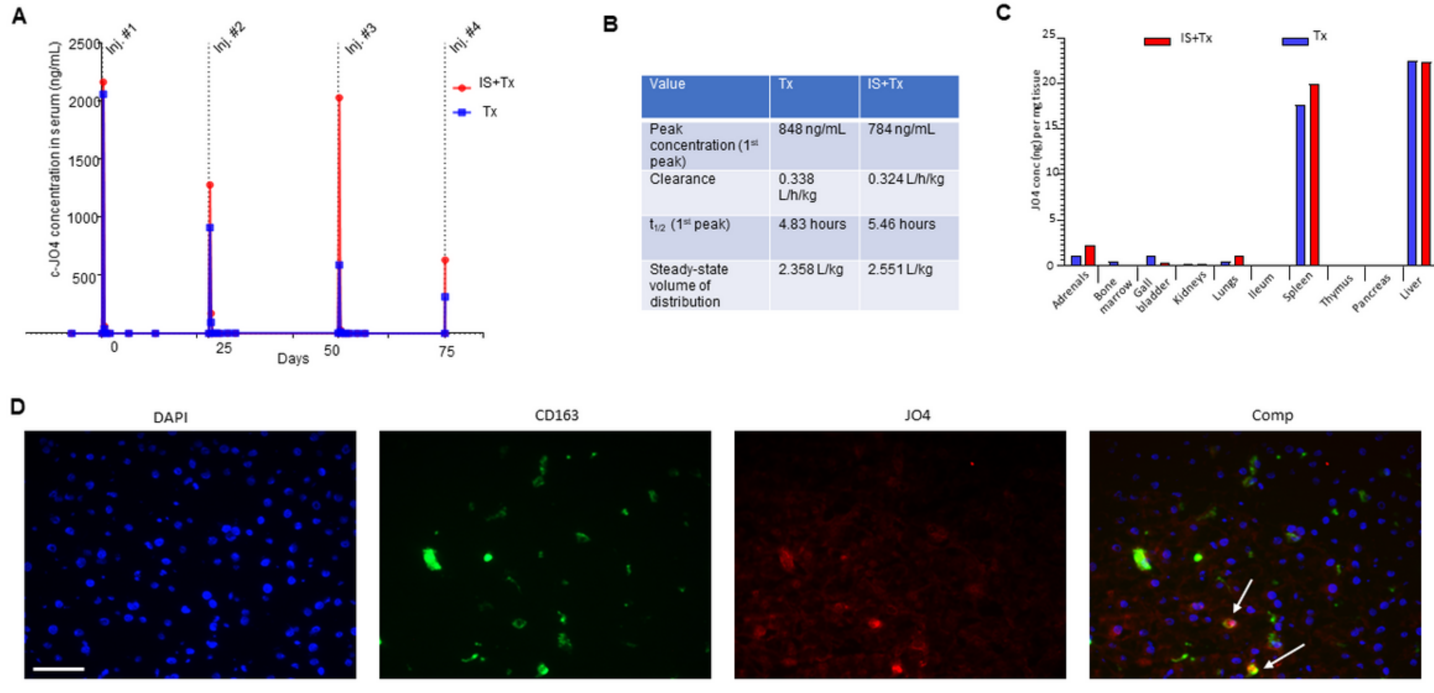
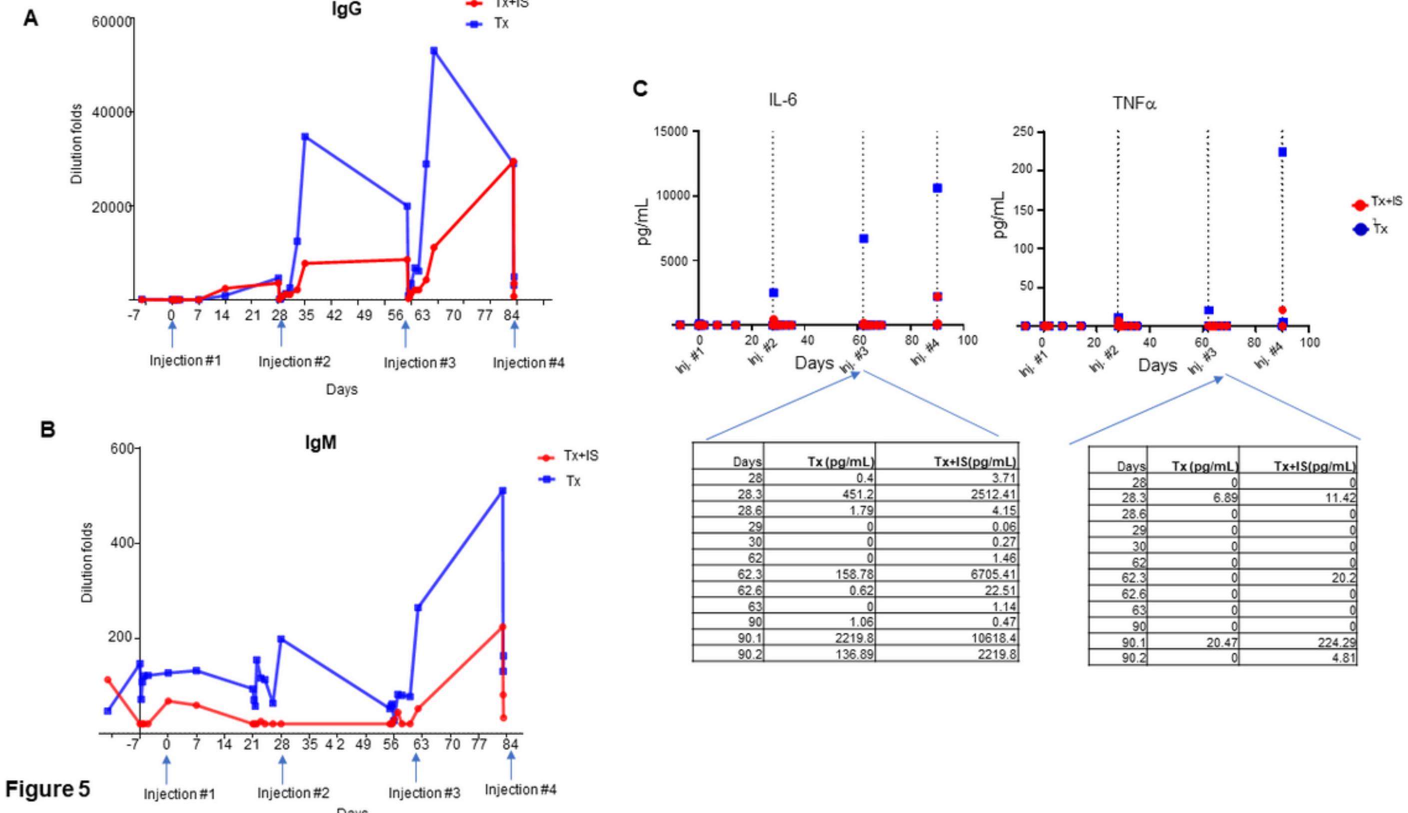


Figure 4

Non-GLP four-cycle toxicity/pharmacokinetics/immunogenicity study with c-JO4 + Doxil in cynomolgus monkeys. A, B) c-JO4 pharmacokinetics. A) c-JO4 concentration in serum collected at the indicated time points as measured by ELISA. B) PK parameters. C) c-JO4 concentration in it issues. Tissues were ground with QiaShredder, and the resulting tissue lysates were measured by ELISA for JO4. c-JO4 levels in tissue lysates were normalized to mg tissue. D) Immunofluorescence with anti-CD163 and anti-cJO4 antibodies on liver sections of the “Tx” animal. Nuclei are stained blue with DAPI. The scale bar is 20 mm. In the composite panel (right), costaining is marked by arrows.

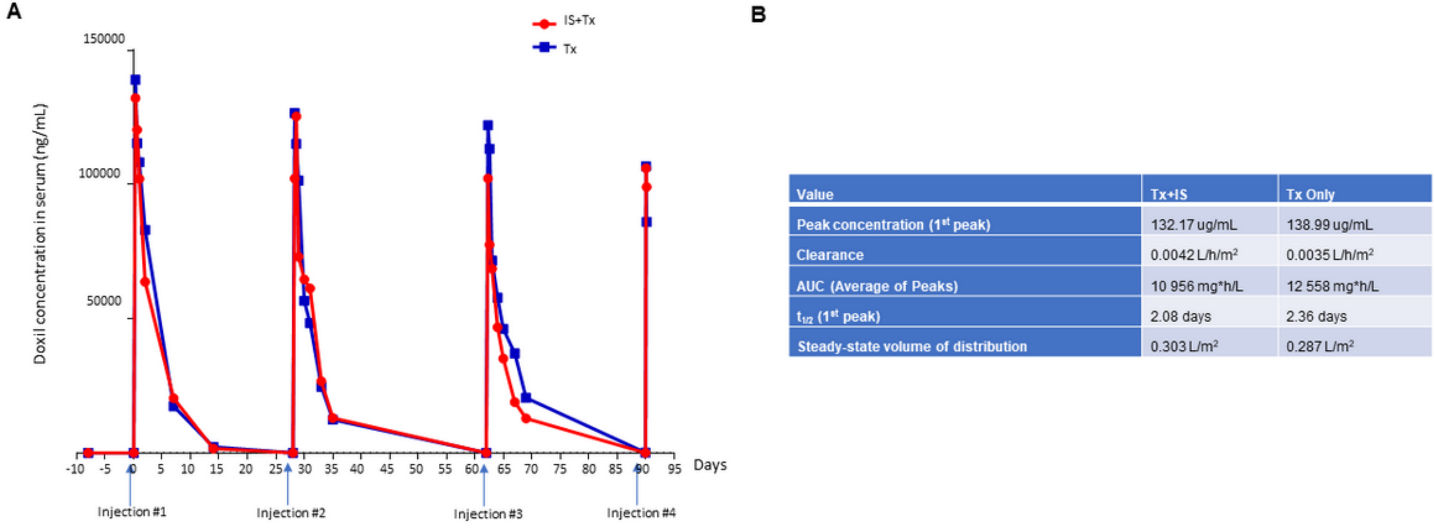


Days	Tx (pg/mL)	Tx+IS(pg/mL)
28	0.4	3.71
28.3	451.2	2512.41
28.6	1.79	4.15
29	0	0.06
30	0	0.27
62	0	1.46
62.3	158.78	6705.41
62.6	0.62	22.51
63	0	1.14
90	1.06	0.47
90.1	2219.8	10618.4
90.2	136.89	2219.8

Days	Tx (pg/mL)	Tx+IS(pg/mL)
28	0	0
28.3	6.89	11.42
28.6	0	0
29	0	0
30	0	0
62	0	0
62.3	0	20.2
62.6	0	0
63	0	0
90	0	0
90.1	20.47	224.29
90.2	0	4.81

Figure 5

Non-GLP four-cycle toxicity/immunogenicity study with c-JO4 + Doxil in cynomolgus monkeys. A, B) Serum anti-JO4 antibody titers. Anti-JO4 IgG and IgM titers were measured at the indicated time points by ELISA. C) Serum cytokines. Serum samples were run on a Th1/Th2 inflammatory cytokine array using the CBA flow panel (BD Biosciences). The only cytokines above the limit of detection were IL-6 and TNFα. Other cytokines tested included IL-2, IL-4, IL-10 and IFNγ. Inlet shows the values at and around each injection time point.

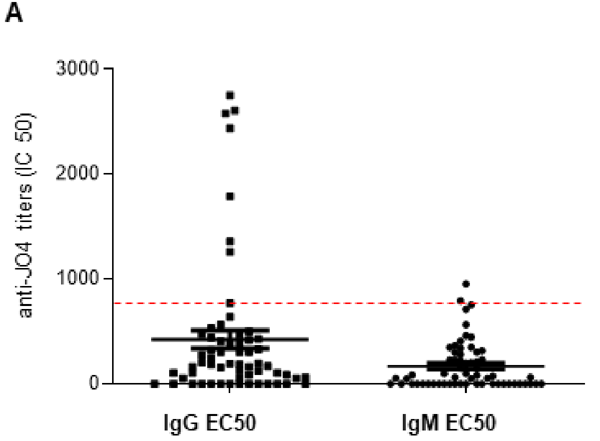


Value	Tx+IS	Tx Only
Peak concentration (1st peak)	132.17 ug/mL	138.99 ug/mL
Clearance	0.0042 L/h/m^2	0.0035 L/h/m^2
AUC (Average of Peaks)	10 956 mg*h/L	12 558 mg*h/L
$t_{1/2}$ (1st peak)	2.08 days	2.36 days
Steady-state volume of distribution	0.303 L/m^2	0.287 L/m^2

Figure 6

Figure 6

Pharmacokinetics of Doxil® in cynomolgus monkeys. Doxil® was detected in serum using anti-PEG antibodies and an HRP-conjugated secondary antibody. A) PK after each cycle. B) PK parameters after the first cycle.



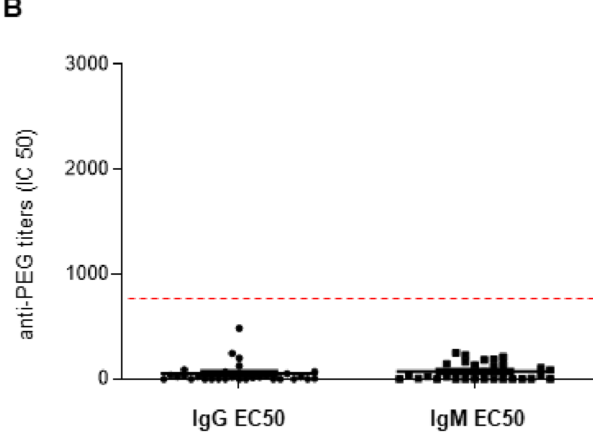


Figure 7

Figure 7

Serum antibodies against c-JO4 and PEG/Doxil in ovarian cancer patients A titer below 1:800 is not neutralizing in in vitro infection studies. Serum samples from the Fred Hutchinson Cancer Research Center were taken for analysis of antibodies against A) c-JO4 and B) PEG/Doxil using anti-Ad3 and anti-PEG antibodies, respectively, by ELISA. EC50 values are shown.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplinfo.pdf