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Taxol-Oligoarginine Conjugates Overcome Drug Resistance *in-vitro* in Human Ovarian Carcinoma

Paul A. Wender^a, Wesley C. Galliher^a, Neelima M. Bhat^b, Thomas H. Pillow^a, Marcia M. Bieber^b, and Nelson N. H. Teng^b

^aDepartment of Chemistry and Chemical and Systems Biology, Stanford University, CA 94305, USA

^bDepartment of Obstetrics and Gynecology, Division of Gynecologic Oncology, Stanford University School of Medicine, Stanford, CA 94305, USA

Abstract

Objective—Multidrug resistance is the major cause of failure of many chemotherapeutic agents. While resistance can arise from several factors, it is often dominated by drug efflux mediated by P-glycoprotein (P-gp), a membrane-bound polysubstrate export pump expressed at high levels in resistant cells. While co-administration of pump inhibitors and a drug could suppress efflux, this two-drug strategy has not yet advanced to therapy. We recently demonstrated that the reversible attachment of a guanidinium-rich molecular transporter, polyarginine, to a drug provides a conjugate that overcomes efflux-based resistance in cells and animals. This study is to determine whether this strategy for overcoming resistance is effective against human disease.

Methods—Tumor samples from ovarian cancer patients, both malignant ascites cells and dissociated solid tumor cells, were exposed to Taxol-oligoarginine conjugates designed to release free drug only after cell entry. Cell Viability was determined via propidium-iodide uptake by flow cytometry. To analyze bystander effect, toxicity of the drug conjugates was also tested on peripheral blood leucocytes.

Results—Human ovarian carcinoma specimens resistant to Taxol *in vitro* demonstrated increased sensitivity to killing by all Taxol-transporter conjugates tested. These studies also show that the drug conjugates were not significantly more toxic to normal human peripheral blood leukocytes than Taxol.

Conclusions—These studies with human tumor indicate that oligoarginine conjugates of known drugs can be used to overcome the efflux-based resistance to the drug, providing a strategy that could improve the treatment outcomes of patients with efflux-based drug-resistance.

Keywords

human; ovarian carcinoma; drug resistance; taxol; and oligoarginine

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Corresponding Author: Nelson N. H. Teng, MD, PhD, Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, 300 Pasteur Drive, room HH333Stanford, CA 94305-5317, USA, Teng@stanford.edu, Phone #: 650-723-7334, Fax #: 650-736-0902.

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Introduction

Ovarian carcinoma (OVCA) is commonly diagnosed at a late stage, two thirds of patients eventually relapse and a subpopulation develops resistance to Taxol drugs [1,2,3,4,5]. A significant problem in the treatment of OVCA is multidrug resistance (MDR). Chemotherapeutic agents initially produce a cytoreductive response, but the development of MDR leads to failure of the drug and frequently cross-resistance to other therapeutics. While many factors contribute to MDR, including drug target mutation, drug metabolism and sequestration [6], a major contributor is the active export of drugs by transmembrane polysubstrate efflux pumps that prevent drugs from reaching their intracellular targets. The recently reported X-ray crystal structure of the mouse P-gp efflux pump, which has 87% sequence identity to human P-gp [7], along with earlier binding studies on the interactions of P-gp with its substrates [8,9], provides a rationale for how this protein recognizes and mediates the unidirectional efflux of numerous drugs. Hydrophobic drugs with high membrane solubility can enter the internal cavity of the pump through two portals positioned on the inner leaflet of the membrane after which an energy-dependent conformational change in the pump results in drug efflux into the extracellular milieu. Drug efflux by P-gp represents one of the best-studied mechanisms of resistance to hydrophobic anticancer drugs [10].

Much effort has been invested in the development of new drug candidates that are less susceptible to efflux, often requiring synthesis and evaluation of numerous analogs to identify a candidate that maintains target efficacy but is not a P-gp substrate. An alternative approach has focused on using a second agent, to suppress pump expression, accelerate the post-translational degradation of the pump, or inhibit pump efflux by the co-administered drug. However, because export pumps are ubiquitously expressed and required for normal function and because the co-administration of two agents can produce highly variable and often undesired pharmacokinetic responses, this approach has not advanced beyond clinical trials.

We have designed a method that involves the attachment of a drug to oligoarginine, a guanidinium-rich molecular transporter through a releasable linker [11]. The guanidinium-rich molecular transporters, which include oligoarginine and other cationic peptides, have been shown in recent years to facilitate the delivery of attached cargo into cells by rendering them water-soluble [12,13]. Significantly, because the drug-transporter conjugate is not a substrate for P-gp export and passes rapidly through the cell membrane, it evades efflux-mediated resistance. After cell entry the conjugate is cleaved and the free drug is released at a rate controlled by the linker design.

From a clinical perspective, this approach offers a number of advantages. Taxol, is water-insoluble and must be administered with solubilizing agents like Cremephor-EL that often elicit an acute hypersensitive reaction [14,15,16,17]. In contrast, Taxol conjugated to the transporter can be formulated in minimal volumes (as little as 1-2 ml) of saline. Furthermore, the conjugate is designed to be inactive until it enters cells, thereby minimizing off-target toxicity. Upon entering cells, the conjugate releases free drug over time at a pre-determined rate controlled by linker design, thus avoiding peak-trough effects often associated with a bolus administration. Additionally, the conjugates can be given intraperitoneally (IP), taking advantage of a mode of administration recommended in a National Cancer Institute announcement indicating that IP-administered paclitaxel, when used with intravenously (IV)-administered paclitaxel and cisplatin, provides a 16-month extension to median survival in ovarian cancer patients [18]. Previous biodistribution studies in transgenic mice where the bioluminescent probe luciferin is conjugated to an oligoarginine transporter provide evidence that transporter conjugates injected IP remain

localized within the cavity [11]. IP administration of the Taxol-oligoarginine conjugates in larger volumes of saline will minimize localized toxicity associated with the transporter and maintain uniform distribution confined to the IP region. Thus, the balance between drug delivery/maintenance in cancer cells and the drug toxicity profile can be regulated in this drug-conjugate linker system to achieve maximum efficacy and manageable side effects.

To determine if the drug-conjugate linker system is applicable to the complexity and heterogeneity of human disease, we examined the effectiveness of our approach in an in-vitro study using human specimens' from ovarian cancer patients. By testing the cancer cells without selecting for growth advantages or expanding particular subpopulations, the assay emulates the heterogeneity of the clinical disease. The growth rate of primary tumor samples (ascites cells and dissociated solid tumor cells) in vitro replicates the physiological environment where cancer cells grow more slowly than established cell lines [19,20]. The diverse set of transporter-drug conjugates evaluated includes prodrugs that exhibit extended stabilities under assay conditions, allowing them to be administered and remain intact until cell entry, after which they are rapidly cleaved in the intracellular reducing environment, releasing the free drug at tunable rates ranging from minutes to hours and controlled by linker design.

Materials and Methods

Synthesis of Conjugates

Synthesis of compounds is described in the supporting information.

Determination of the Prodrug Half-Lives

To evaluate the half-lives of the synthesized conjugates under the assay conditions, each conjugate (0.3 mg) was dissolved in 200 μ l HEPES buffered saline, pH 7.4 and incubated at 37°C containing 10 μ l of a solution of 1-naphthalenemethanol in methanol (0.42 mg/ml), which served as an internal standard. At appropriate intervals 20 μ l of the solutions were removed and analyzed by reverse-phase HPLC. The percent decomposition was calculated from the integrated peak areas of the conjugate, the internal standard, and the various decomposition products. To evaluate the half-lives under reducing conditions, the conjugates were incubated as described above with 10 mM dithiothreitol.

In-vitro Cytotoxicity Assay

All tissue samples were obtained with IRB certified informed patient consent, and the study approved by the Stanford Research Compliance Office. Ascites cells were centrifuged, filtered through sterile gauze, cryopreserved or tested immediately. For frozen ascites samples, cells were thawed, washed twice with media, assessed for >80% cell viability and resuspended and incubated at 37°C for 90 minutes before plating. Freshly harvested solid tumor specimens were processed overnight with Accutase (Sigma), washed twice before plating.

For cytotoxicity assays, 200 μ l of a 2×10^5 cells/ml in Iscove's media with 10% FCS was distributed into each well of 48-well polypropylene plates. Conjugates and Taxol were added to the wells in duplicate at appropriate concentrations. Control wells were established in parallel and the plates were incubated at 37°C for 72 hours (unless otherwise noted). Cells were analyzed by flow cytometry. Due to limited quantities of the patient samples, results are given as the mean \pm SEM of duplicate wells unless otherwise noted.

Human Peripheral Blood Leukocyte Assay

Neutrophils were obtained by a modified technique of Boyum [21]. In brief, a double gradient was prepared by layering an equal volume of HISTOPAQUE®-1077 over HISTOPAQUE®-1119 (Sigma). Freshly obtained heparinized blood sample (10 ml) diluted in 20 ml PBS was carefully layered on top of the double gradient. After centrifugation at 800g for 20 minutes, granulocytes were recovered at the 1077/1119 interphase and mononuclear cells at the plasma/1077 interphase. Recovered cells were washed twice with PBS and re-suspended to 1×10^6 cells/ml in medium with FCS. 200 μ l of the solution was distributed into each well of 48-well plates. Conjugates and Taxol were added to the wells in duplicate at appropriate concentrations. Control wells were run in parallel and the plates were incubated at 37°C overnight and then analyzed by flow cytometry.

Flow Cytometry Analysis

200 μ l of a propidium-iodide (PI) solution (10 μ g/ml) was added to each well, mixed and transferred to tubes with the cells. Analysis was performed on a FACScan or LSR flow cytometer with data management using Cell Quest (Becton-Dickinson) and FlowJo (TreeStar Inc., Oregon). In some experiments cells were stained with CD45-FITC (Caltag) according to the manufacturer's protocol to exclude hematopoietic cells. However, electronic gates on forward and side scatter were sufficient to exclude hematopoietic cells for most samples. Live cells were determined by PI exclusion. EC₅₀ values were calculated from semi-logarithmic dose response curves (GraphPad Prism, CA).

Results

Design, Synthesis and Properties of Releasable Taxol-Octaarginine Conjugates

An octaarginine (r8) transporter was attached to either the C2' or C7 position of Taxol using different biocleavable disulfide linkers. At the outset, it was expected that C2' derivatives would be inactive until free drug was released, because C2'-modification of Taxol is known to attenuate or eliminate activity [22]. C7 conjugates were also prepared because this position in Taxol can often be modified without loss of activity [22]. Eight conjugates were prepared and evaluated for *in-vitro* activity (Figure 1, C2'-conjugates: 2a-d, C7-conjugates: 3a-d). Conjugates 2a and 3a contain unhindered ester linkages attached directly to Taxol at the C2' or C7 position, respectively. Conjugates 2b and 3c incorporate linkers with two or one methyl groups, respectively, attached at the alpha carbon of the ester linkage. Conjugate 2c extends the linker by an additional carbon resulting in additional flexibility. Finally, conjugates 2d, 3b, and 3d connect the transporter through carbonate linkers of varying length and functionality. The linkers are designed to provide shelf-stable conjugates that would release drug only after cell entry and at tunable rates. For example, installation of methyl groups adjacent to an ester linkage (compounds: 2b, 3c) increases the stability of the prodrugs (due to increased steric hindrance at a potential hydrolysis site) under assay conditions while retaining rapid kinetics of induced release of free Taxol. Replacement of the ester linkage with a carbonate moiety (compounds: 2d, 3b, 3d) also improves the intrinsic stability of the prodrug to hydrolytic degradation.

In-vitro Human Ascites and Primary Tumor Assay

Tumor spread via survival and proliferation in body cavity fluids, including the peritoneal fluid, is an important route of cancer metastasis [19]. As a prelude to clinical evaluation, the efficacy of the Taxololigoarginine conjugates against eight different OVCA ascites (patients-A-H) and one solid tumor sample (patient-I) was examined (Table 1). All but two patient samples were derived from serous papillary tumor, the most common subtype of OVCA. Patient-G presented with clear cell carcinoma, a rare subtype that is often refractory

to standard therapies. This 7-to-1 ratio of serous papillary to clear cell approximates the actual percentage of patients who normally present with each subtype of OVCA. Patient-H presented with an unusual carcinosarcoma (formerly called malignant-mixed mesodermal tumor).

Taxol was inactive in vitro against eight of the nine tissue samples tested. Five of nine patients succumbed to disease in less than 19 months post sample collection. The only sample in which Taxol displayed modest activity was the fresh solid-tumor (patient-I). In contrast, each of the Taxol-oligoarginine conjugates displayed good cytotoxic activity against both ascites cells and solid tumor samples. The dose-response curves in Figure 2 demonstrate the efficacy of the drug-conjugates against both clear cell carcinoma (Figure 2A) and serous papillary carcinoma (Figure 2B) ascites cells. Demonstrating efficacy against clear cell carcinoma is significant as the prognosis is extremely poor for patients with this highly resistant subtype [23]. EC_{50} values for each of the conjugates and Taxol are provided in Table 2. The activity of the conjugates is independent of histology or treatment prior to sample collection. Furthermore, the doses at which the conjugates are effective against the primary OVCA samples (1-20 μ M) are in the pharmacokinetic range for IP-administered Taxol (60 mg/m²) [24]. The fact that micromolar concentrations are required for cytotoxicity is expected in patient ascites and cell-lines grown as multicellular clumps or spheroids [25,26,27], unlike adherent OVCA cell-lines where EC_{50} is achieved by nanomolar concentrations of Taxol [28].

Toxicity Profile Control Assays

To confirm that ascites cells-killing activity was the result of intracellular delivery and release of free Taxol rather than transporter-associated toxicity, the transporter by itself was also tested for toxicity. Octaarginine alone showed very little toxicity against all three different OVCA subtypes at concentrations up to 2.5 μ M (Figure-3A). At concentrations higher than 2.5 μ M, octaarginine transporter was toxic but the conjugates consistently yielded 2-4X more cell death than the transporter by itself. Previous studies have shown that changes in the length or stereochemistry of a transporter can be used to modulate its toxicity and activity as well as its lifetime through protease degradation [29].

As part of the preclinical development of Taxol-oligoarginine conjugates as therapeutic agents, the toxicity of the conjugates against normal human neutrophils was assessed (Figure 3B). Cells were incubated for 48 hours with Taxol, octaarginine alone, or Taxol-r8 conjugate. Taxol dilutions were made from a DMSO stock whereas octaarginine and Taxol-r8 conjugate were from PBS stock. It is encouraging that the octaarginine transporter alone (r8, blue bars) showed very little toxicity towards neutrophils, even at the highest administered dose. Notably, none of the Taxol- r8 conjugates (2a-red bars, 2b-gray bars, 3c-green bars, and 3d-purple bars) were significantly more toxic to neutrophils compared to Taxol (black bars). Toxicity to mononuclear cells was also assessed and again the Taxol-r8 conjugates demonstrated similar toxicity profiles to Taxol (data not shown).

Discussion

Efflux-based resistance is a significant contributor to the failure of drugs. It has been demonstrated in cancer cell lines and animal models that the attachment of clinically tested molecular transporter to a known drug (Taxol) affords a conjugate that can overcome efflux-based multidrug resistance in vitro. Here we demonstrate that this strategy is applicable to human disease in an assay that evaluates efficacy against tumor samples from ovarian cancer patients in-vitro. The readily prepared conjugates utilize known drugs with validated pathways, are highly water-soluble thereby improving formulation and administration, and are taken up rapidly by cells.

Considering the heterogeneous and complex environment surrounding tumors and our intent to identify preferred compounds for preclinical advancement, we designed and synthesized several Taxol-octaarginine conjugates differing by site of transporter attachment to Taxol and by linker composition. Overall, the conjugates exhibit a wide range of stabilities (with half-lives ranging from hours to weeks) far exceeding the time required for administration and cell entry but they release free drug in the presence of a reducing environment in minutes to hours. Shelf life of the conjugates as solids at room temperature extends for months. The rates of both intrinsic linker degradation as well as designed instability in a reducing environment can be tuned and appropriately selected based on the application or mode of administration.

While various bioactivatable linker-cleavage strategies (e.g., protease, esterase, or phosphatase) are compatible with this approach, all conjugates in this initial in-vitro study incorporated a cleavable disulfide linker. Cleavage of such linkers would occur only after entry of the conjugate into cells where a high glutathione concentration is encountered (generally 1-10 mM intracellular compared to 10 μ M extracellular) [30]. Glutathione is reported to be an important stimulus for cancer cell proliferation and thus disulfide linkers were selected in part to exploit the elevated levels of glutathione in cancer cells [31] and even higher levels in resistant disease [32,33]. The specific structure of the linker was varied to determine whether the stability of the conjugate under assay conditions or the rate of release of free drug would influence activity. Although all eight conjugates tested in this in-vitro study gave identical results, further studies will determine which conjugate has the necessary stability and release rates essential for in-vivo efficacy.

In conclusion, the Taxol-oligoarginine conjugates contain bioreleasable disulfide linkers and exhibit a number of beneficial physical properties, including prolonged shelf stability, prodrug stability during administration, and tunable release rates of the free drug in the intracellular reducing environment. Significantly, in all the human tumor samples, the conjugates outperform Taxol, which was inactive in vitro against the ascites cells. Furthermore, when evaluated against normal human leukocytes, neutrophils and monocuclear cells, the conjugates were comparable in toxicity to the parent drug alone. The ability to improve both the administration and efficacy of a therapeutic drug, particularly against resistant disease, by attaching a molecular transporter could significantly improve the prognosis of OVCA patients

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

‘Taxol-Oligoarginine Conjugates Overcome Drug Resistance *in-vitro* in Human Ovarian Carcinoma’ by Paul A. Wender, Wesley C. Galliher, Neelima M. Bhat, Thomas H. Pillow, Marcia M. Bieber, Nelson N. H. Teng

1. Overcome efflux-based resistance of Taxol in tumor samples from OVCA patients
2. Reversible attachment of polyarginine to Taxol allows for easy drug delivery
3. Tunable release rates of active drug in intracellular reducing environment

	Prodrug half-life under assay conditions (37°C) ^A	Prodrug half-life under reducing conditions (37°C) ^B
2a: X = -CH ₂ -	23 hrs	3 min
2b: X = -C(CH ₃) ₂ -	43 hrs	4.5 min
2c: X = -CH ₂ CH ₂ -	55 hrs	19 min
2d: X = -OCH ₂ -	22 hrs	105 min
3a: X = -CH ₂ -	114 hrs	> 24 hrs
3b: X = -O-	425 hrs	2.3 min
3c: X = -CH(CH ₃)-	271 hrs	> 24 hrs
3d: X =	36 hrs	1 min

Figure 1. Structures of Taxol-transporter conjugates and their half-lives under assay or reducing conditions: (A) Prodrug half-life under assay conditions (HBS, pH=7.4, 37°C). (B) Prodrug half-life under reducing conditions (10mM DTT, HBS, pH=7.4, 37°C).

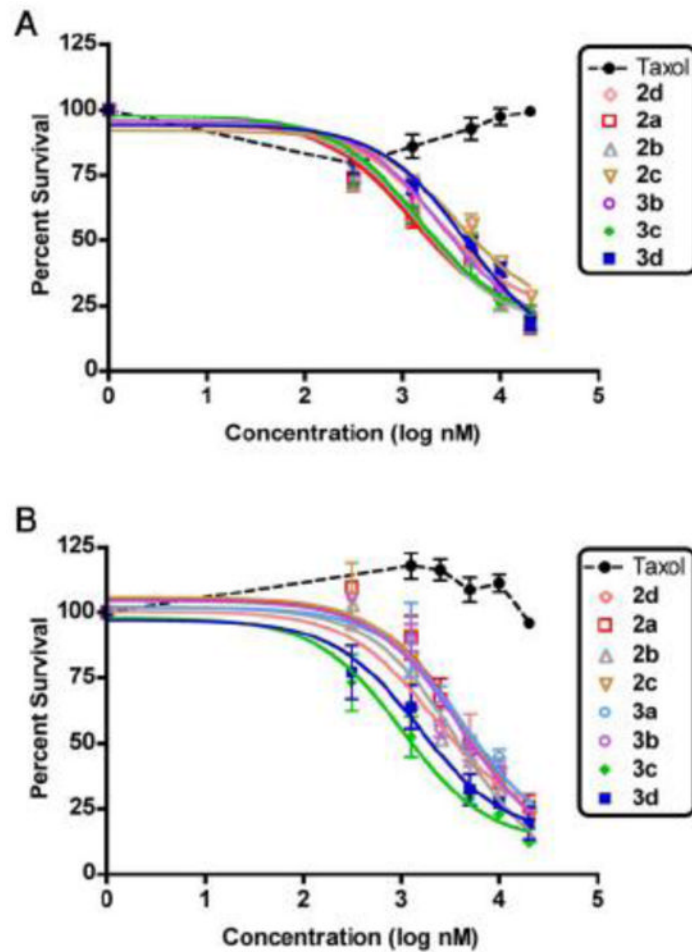


Figure 2. Dose-response curves of Taxol and Taxol-r8 conjugates against two subtypes of ovarian carcinoma (mean \pm SEM of two independent experiments each performed in duplicate), (A) clear cell carcinoma (patient G) and (B) serous papillary carcinoma (patient F).

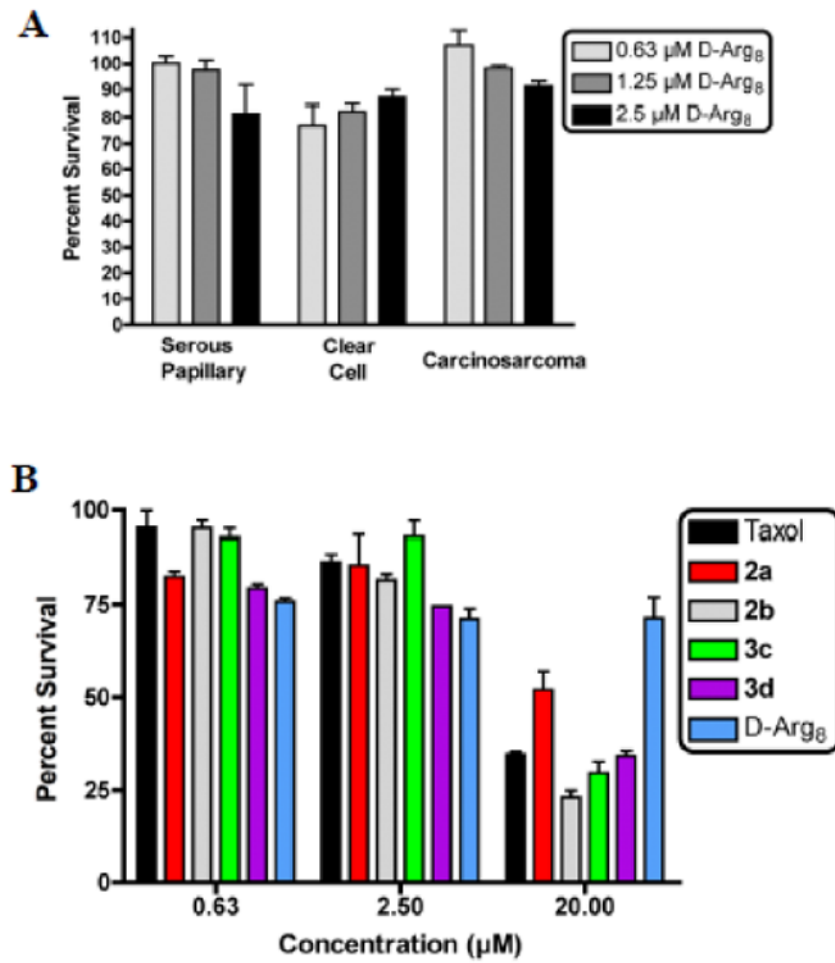


Figure 3. A. Toxicity of D-Arg₈ towards OVCA tumor specimens. B. Toxicity of Taxol, Taxol-r8 conjugates and D-Arg₈ towards normal human neutrophils; Taxol (black), conjugates 2a (red), 2b (gray), 3c (green), 3d (purple), and D-Arg₈ alone (blue).

Table 1

Patient* A	Age (yrs) [†] 60	Histology Serous/high grade OVCA	Stage [‡] /grade IIIC/3	Chemotherapy Treatment Post surgery: 8 cycles taxane/carboplatin and etoposide	Specimen Type Ascites at surgery, frozen	Patient Status [§] DOD, 19 months
B	65	Serous/papillary OVCA	NIC/3	Pre specimen collection: 3 cycles taxane/cisplatin, recurrence. Post specimen: 4 cycles taxane/cisplatin	Ascites from paracentesis, frozen	DOD, 11 months
C	65	Serous/high grade OVCA	IIIC/3	Neoadjuvant pre specimen: 3 cycles taxane/carboplatin. Post surgery: 6 cycles paclitaxel/carboplatin IP	Ascites at surgery, frozen	DOD, 10 months
D	51	Serous/papillary OVCA	IIIC/3	Post surgery: 6 cycles taxane/cisplatin	Ascites at surgery, frozen	DOD, 13 months
E	54	Serous/high grade OVCA	IIIC/3	Post surgery: 6 cycles taxane IV, cisplatin IP	Ascites at surgery, frozen	AWD, 11 months
F	74	Serous/papillary OVCA	IIIC/3	Post surgery: 6 cycles taxane/cisplatin	Ascites at surgery, frozen	AWD, 11 months
G	69	Clear cell OVCA	IIIC/3	None	Ascites at surgery, fresh	DOD, 2 months
H	86	Carcinoma [Malignant Mixed Mesodermal]	IIB/3	None	Ascites at surgery, fresh	AWD, 6 months
I	46	Serous/high grade OVCA	IIIC/3	Post surgery: 8 cycles taxane/carboplatin	Metastasis to omentum, fresh	NED, 10 months

* All patients were chemo naive at the time of surgery, except Patients B and C, who received standard taxane/platinum combination chemotherapy.

[†] At the time of diagnosis.

[‡] International Federation of Obstetrics and Gynecology (FIGO) System.

[§] Post-Diagnosis; DOD = died of disease; AWD = alive with disease; NED = alive with no evidence of disease.

Table-2

EC₅₀ values of Taxol

A: First generation Taxol-r8 conjugates

Patient	EC ₅₀ (μM)		
	Taxol	2a	3a
A ascites *	≫20	1.4	1.3
B ascites	≫20	7.7	4.6
C ascites	≫20	15	11
D ascites [‡]	≫20	17	24
E ascites ^{‡,¶}	≫20	14	17
F ascites *	≫20	4.2	4.4
G ascites ^{*,§}	≫20	1.2	---
H ascites ^{‡,¶}	≫20	21	---
I solid tumor [‡]	16.2	3.9	3.1

B: Second generation Taxol-r8 conjugates

Patient	EC ₅₀ (μM)					
	2b	2c	2d	3b	3c	3d
A ascites *	1.0	---	---	2.2	---	1.1
B ascites	8.0	---	---	1.2	---	---
F ascites *	3.4	4.2	2.3	3.7	1.5	1.0
G ascites ^{*,§}	1.5	3.7	2.3	2.5	4.3	1.5
H ascites ^{‡,¶}	23.0	---	---	---	20.0	---

Refer to Figure 1 for chemical structures of Taxol-r8 conjugates (2a, 3a) and (2b, 2c, 2d, 3b, 3c, and 3d)
 Values calculated from 5 different compound concentrations ranging from 0.625 μM to 20 μM

* Results are the mean of two independent experiments

[‡] 48-hr incubation.

[§] Clear cell carcinoma.

[¶] Carcinosarcoma