Overcoming multidrug resistance of small-molecule therapeutics through conjugation with releasable octaarginine transporters

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Many cancer therapeutic agents elicit resistance that renders them ineffective and often produces cross-resistance to other drugs. One of the most common mechanisms of resistance involves P-glycoprotein (Pgp)-mediated drug efflux. To address this problem, new agents have been sought that are less prone to inducing resistance and less likely to serve as substrates for Pgp efflux. An alternative to this approach is to deliver established agents as molecular transporter conjugates into cells through a mechanism that circumvents Pgp-mediated efflux and allows for release of free drug only after cell entry. Here we report that the widely used chemotherapeutic agent Taxol, ineffective against Taxol-resistant human ovarian cancer cell lines, can be incorporated into a releasable octaarginine conjugate that is effective against the same Taxol-resistant cell lines. It is significant that the ability of the Taxol conjugates to overcome Taxol resistance is observed both in cell culture and in animal models of ovarian cancer. The generality and mechanistic basis for this effect were also explored with coelenterazine, a Pgp substrate. Although coelenterazine itself does not enter cells because of Pgp efflux, its octaarginine conjugate does so readily. This approach shows generality for overcoming the multidrug resistance elicited by small-molecule cancer chemotherapeutics and could improve the prognosis for many patients with cancer and fundamentally alter search strategies for novel therapeutic agents that are effective against resistant disease.

ovarian cancer | Pgp | Taxol | imaging | luciferase

Multidrug resistance (MDR) is a primary contributor to the failure of many cancer chemotherapeutic agents (1). It arises from increased expression of membrane proteins that mediate unidirectional energy-dependent drug efflux, thereby intercepting and exporting the drug before it reaches its intracellular target (2). This type of resistance is general, being observed for many cancer types, including those putatively of a stem-like cell origin (3, 4) and for a wide range of chemotherapeutic structural classes operating through a variety of targets and pathways (5). Moreover, resistance induced by one agent often results in cross-resistance to multiple agents (6).

To address this problem, much effort has been invested in the search for new agents that are less susceptible to MDR (7, 8) or that inhibit MDR efflux pumps (9). However, the efficacy of these inhibitors has been compromised by bioavailability problems, adverse interactions with the performance of the drug, and interference with drug clearance, resulting in elevated plasma concentrations and associated toxicity (10). An alternative approach to overcoming MDR has been to modify the drug itself (11, 12). Because drugs that elicit resistance often enter cells by passive diffusion, drug modifications that change the mode of entry offer a promising strategy for circumventing MDR. In recent years, we and others have shown that certain agents (generally referred to as molecular transporters, which include, e.g., cell-penetrating peptides) when attached to a drug or probe will facilitate cellular uptake through multiple mechanisms other than passive diffusion (13) (for recent reviews on transporters, including lead references to work on guanidinium transporters from the groups of Torchilin, Prochiantz, Langel, Futaki, Vives, Wender, Dowdy, Piwnica-Worms, Seebach, Gellman, Goodman, and others, see ref. 14). In principle this work provides a rationale for overcoming MDR as the transporter-drug conjugate would have a mode of entry and physical properties different from the drug itself. However, because the biological activity of the attached drug cargo would be influenced by the transporter, the general effectiveness of this approach would require release of the free drug after cellular entry.

The use of transporter conjugates to overcome resistance has received relatively little attention, being limited to nonreleasable conjugates of doxorubicin and doxorubicin-ester derivatives (15–19). Here we show that conjugation of an octaarginine peptide transporter through a bioactivatable linker to a representative small-molecule therapeutic agent (Taxol) produces conjugates with significantly improved activity against malignant cells that are resistant to the therapeutic agent alone. We further show that this approach is effective in animal models of ovarian cancer, and through the use of another known P-glycoprotein (Pgp) substrate, coelenterazine, this approach has potential general utility.

We selected Taxol for this study for several reasons. First, it is a widely used anticancer agent (20) that has markedly improved the survival of patients (21, 22). Notwithstanding this success, Taxol elicits resistance and, even more so than most chemotherapeutic agents, lacks significant activity against MDR tumors (10, 23, 24), thus providing a formidable and important challenge for evaluating strategies for overcoming MDR. Second, and of more immediate potential clinical utility, i.p.-injected Taxol recently showed promising clinical results for the treatment of ovarian cancer, including significant life extensions, prompting a recent National Cancer Institute clinical announcement on the merit of this procedure (25). i.p.-administered Taxol allows drug delivery directly to targeted tissue, thereby minimizing systemic exposure and off-target side effects. However, because Taxol is poorly water soluble ($\approx$0.4 μg/ml), it must still be formulated with Cremophor EL, a vehicle that often elicits hypersensitivity side effects and because of the formulated volume requires extended administration times (26, 27). In contrast, oligoarginine conjugates of Taxol are freely water soluble, thus precluding the need for Cremophor and allowing for smaller administration volumes and, thus, shorter administration times. Octaarginine conjugates, as illustrated herein with a luciferin probe, also preferentially remain at the site of administration as a result of enhanced cell adherence and uptake, thereby suppressing

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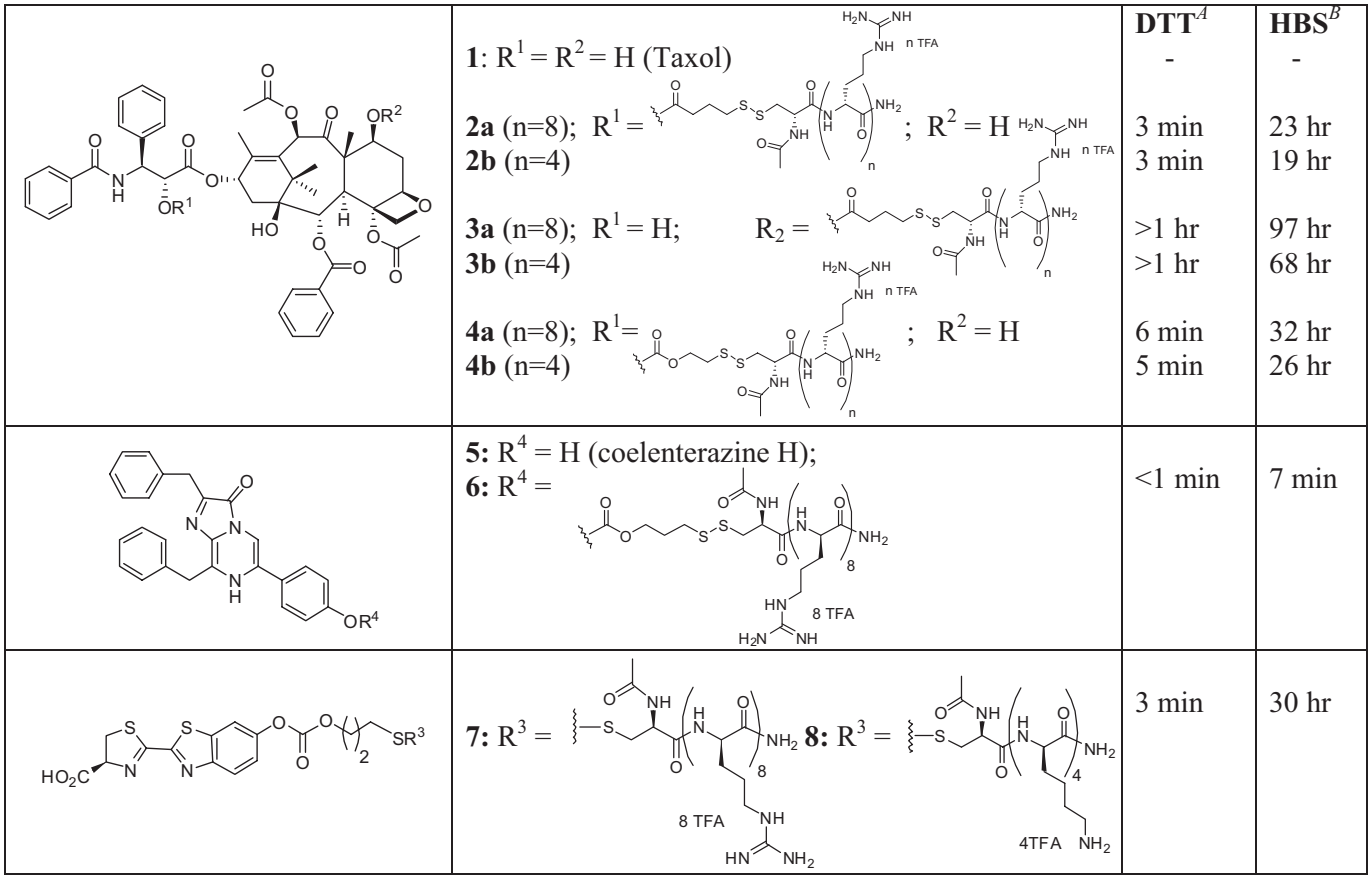

	DTT[A]	HBS[B]
1: $R^1 = R^2 = H$ (Taxol)	-	-
2a (n=8); R^1 = ; $R^2 = H$	3 min	23 hr
2b (n=4)	3 min	19 hr
3a (n=8); $R^1 = H$; R_2 =	>1 hr	97 hr
3b (n=4)	>1 hr	68 hr
4a (n=8); R^1 = ; $R^2 = H$	6 min	32 hr
4b (n=4)	5 min	26 hr
5: $R^4 = H$ (coelenterazine H);	<1 min	7 min
6: R^4 =		
7: R^3 =	3 min	30 hr
8: R^3 =		

Fig. 1. The structure, stability, and release characteristics of Taxol–, coelenterazine–, and luciferin–transporter conjugates. (*A*) Stability in reducing environment (10 mM DTT, HBS, pH = 7.4, 37°C). (*B*) hydrolytic stability (HBS, pH = 7.4, 37°C).

systemic distribution and, thus, off-target exposure. They also release drug cargo intracellularly and at a sustained rate dictated by linker cleavage, thus avoiding bolus effects associated with administration and uptake of the drug alone. The conjugate itself is not active. Finally and importantly, notwithstanding its general therapeutic value, Taxol was selected because it is not effective by itself against Taxol-resistant disease. These problems are not uncommon, being associated with many drugs with similar physical properties that render them substrates for Pgp-based resistance (1).

Results and Discussion

Design and Synthesis of Releasable Octaarginine–Taxol Conjugates. To explore the effect of oligoarginine transporter-assisted delivery of small-molecule therapeutics on cancer cells sensitive or resistant to the therapeutic agent alone, we attached an octaarginine transporter to the C2′ or C7 positions of Taxol by using a biocleavable disulfide linker (compounds **2**-**4**; Fig. 1). An octaarginine transporter was selected because of its demonstrated ability to enhance cellular uptake (13, 14) and its ease of synthesis (28). Preference was also given to a disulfide linker, because its cleavage would occur only after cellular entry of the conjugate after encountering a high glutathione concentration (typically 15 mM intracellular compared to 15 μM extracellular) (29). The thiol resulting from glutathione cleavage was expected to cyclize into the proximate carbonyl group of the linker, leading subsequently to the release of free Taxol at a rate controlled by linker design. The position of attachment of the linker to Taxol and the linking functionality were expected to be important for activity in both Taxol-sensitive and Taxol-resistant cell lines. Because modification of the C2′ alcohol of Taxol is known to result in considerable loss of activity (30), we set out to make one class of conjugates with a linker at C2′ (compounds **2a–b** and **4a–b**) that would be effective in cells only if the free drug were released. In addition, because the C7 position of Taxol can be modified without significant loss in activity (30) and is important in the interaction of taxanes with Pgp (31, 32), we also prepared a class of conjugates attached to C7 via the same disulfide-releasable linker (compounds **3a–b**). Tetraarginine–transporter conjugates were used as negative controls, because they possess all of the features of the octaarginine conjugates, including aqueous solubility and linking functionality, but they do not readily enter cells and, thus, would only exhibit activity if they cleaved extracellularly to produce free drug. The "accelerated" (37°C) hydrolytic half-lives of the tetraarginine and octaarginine conjugates ranged from 19 to 97 h, extending well beyond the incubation times (≤20 min) used for cell assays. Stabilities of the conjugates as solids at room temperature extend for months. In contrast, cleavage of the disulfide linker in the presence of DTT (analog of glutathione) occurred in seconds for all compounds and resulted in the subsequent release of active drug (half-lives indicated in Fig. 1). The activities of various conjugate-linker combinations were evaluated by using a panel of human cancer cells, leading to the identification of ester-linked conjugates **2a** and **3a** as preferred candidates (Table 1). Conjugates with a carbonate-based releasable disulfide linker (**4**) were significantly less active in cells (Table 1), whereas conjugates with longer ester and carbonate linkers did not show activity because of suppression of release (data not shown).

Cellular Assay of Taxol Conjugates. Cells from a panel of cancer cell lines (Table 1) were incubated for 20 min with Taxol or octaarginine or tetraarginine (r8 and r4) conjugates of Taxol and washed to

Table 1. IC_{50} values (nM)* and MDR factor (shown in parentheses)† of Taxol and its octaarginine‡ conjugates in selected cancer cell lines

Cell line	Taxol (**1**)§	C2′ ester r8 (**2a**)¶	C7 ester r8 (**3a**)¶	C2′ carbonate r8 (**4a**)¶
UCI–101 luc	250	60	170	95
UCI–107 luc	320	90	230	140
SCOV-3	29	11	24	18
OVCA 429	150	42	125	80
OVCA 429T	16,000 (107)	420 (10)	230 (2)	780 (10)
OVCA 429T×T	2,500 (17)	100 (2.4)	93 (0.7)	220 (3)
OVCA 433	290	21	62	44
OVCA 433T	6,200 (21)	130 (6)	95 (1.5)	210 (5)
OVCA 433T×T	12,000 (41)	3,000 (143)	1,300 (21)	8,000 (182)
MCF-7	170	40	150	110
MCF-7-Pgp	870 (5)	150 (3.8)	83 (0.6)	230 (2)

*IC_{50} values were determined by incubating the cells with the compound for 20 min and washing them twice with fresh medium, followed by 72 h of incubation in drug-free medium and determination of cell viability by MTS assay.

†MDR factor (shown in parentheses) = IC_{50} (MDR cell)/IC_{50} (sensitive cell).

‡Data for tetraarginine analogs (**2b**–**4b**) are not shown, because none of them displayed activity in this assay ($IC_{50} \gg 10\ \mu M$).

§Taxol was administered in 2% DMSO solution in PBS.

¶Conjugates were administered in 100% PBS. Results are of three separate experiments, each performed in triplicate with the average value of SE ± 12%.

remove any remaining agent that did not enter cells. After 72 h, the cytotoxicity of the relevant compound was determined by an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] based assay. Cell killing [expressed as IC_{50} values (the concentration at which the viability of the cells in culture is reduced by 50%)] mediated by several of the octaarginine conjugates occurred at significantly lower concentrations than those observed for free Taxol administered in DMSO or water. Only a 20-min exposure was required to differentiate the activities of Taxol and Taxol conjugates used in equimolar concentrations. No extracellular hydrolytic release of Taxol from the conjugate occurred during the incubation period, as evident from the dramatically different activities of the conjugate and the free drug and the lack of activity of the tetraarginine control conjugates (**2b**–**4b**), which, although similar in functionality to the octaarginine conjugates, do not readily enter cells. Within the panel of human cancer cells, there were three sets of related sensitive and resistant cell lines, the latter of which were based wholly or in part on Pgp-mediated efflux. These resistant lines were either created through stable transfection of MCF-7 cells with Pgp (MCF-7-Pgp) (33) or by selection through exposure of OVCA-429 and OVCA-433 cells to Taxol (OVCA-429T and OVCA-433T) or Taxotere (OVCA-429T×T and OVCA-433T×T), creating a complex MDR phenotype including Pgp up-regulation (34). Remarkably, for all resistant cells, Taxol conjugates **2a** and **3a** were both more effective than Taxol itself, and both displayed an ability to overcome drug resistance, with the C7 analog **3a** consistently being more effective.

Tubulin Depolymerization and Cell-Cycle Assays of Taxol Conjugates. To determine whether the mechanism of action of the r8-conjugated Taxols paralleled that of Taxol, *in vitro* (cell-free) tubulin depolymerization (35) and cell-cycle assays (36) were conducted (Fig. 2). As expected, in the tubulin depolymerization assay (Fig. 2*A*), only Taxol was active, indicating that the r8 conjugates are stable in the absence of disulfide-cleaving agents. A cell-cycle assay was also conducted, showing that the r8 conjugates were killing tumor cells through the same cell-cycle arrest mechanism as Taxol (Fig. 2*B*). As expected, conditions and conjugates that produced the greatest loss of cell viability correlated with increased accumulation of cells in the G_2/M interphase. Both C2′ and C7 r8 conjugates (**2a** and **2b**) produced a significantly greater percentage of OVCA-429 cells in the G_2/M phase than Taxol (P = 0.0045 for C2′ and 0.0051 for C7). The killing of the Pgp-up-regulated MDR cell line OVCA-429T by the r8 conjugates was similarly attributable to a block of cell-cycle entry into M phase. Relative to Taxol, only the C7 conjugate produced a significantly greater number of resistant cells in G_2/M arrest (P = 0.0034 for C7 and 0.073 for C2′). The activity of this conjugate against resistant cells was as effective as the activity of Taxol against Taxol-sensitive cells (OVCA-429), indicating that r8 conjugation to the C7 position of Taxol is capable of completely overcoming the MDR phenotype in this cell line.

Design, Synthesis, and Assay of an Octaarginine–Coelenterazine Conjugate. The mechanistic basis for the effect of the oligoarginine transporter was examined further by using the octaarginine conju-

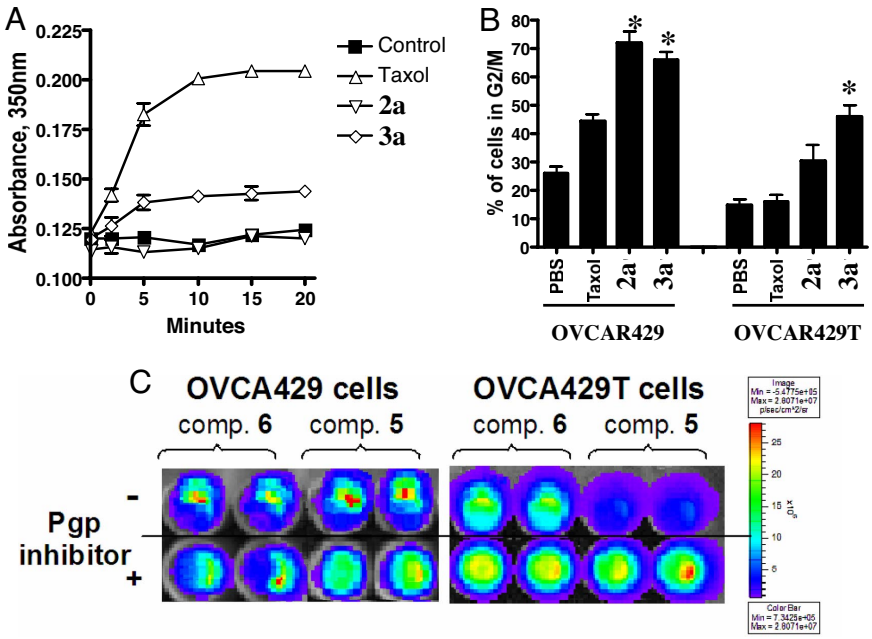

Fig. 2. Mechanisms of action of octaarginine–Taxol conjugates. (*A*) Tubulin polymerization assay. The ability of Taxol or octaarginine conjugates to polymerize free tubulin was determined by increase in turbidity (measured by absorbance at 350 nm). **2a**, C2′ octaarginine–Taxol conjugate; **3a**, C7 octaarginine–Taxol conjugate; squares: no drug control; triangles, Taxol; inverted triangles, conjugate **2a**; diamonds, conjugate **3a**. (*B*) Cell-cycle assay. Cells were treated for 15 min with 1 mM of the indicated compounds 24 h before analysis, stained with 7-AAD and analyzed by flow cytometry. Cell doublets were removed, and the percentage of single cells in the G_2/M interphase (displaying mitotic arrest) were determined for both **2a** and **3a** conjugates. *, conditions under which octaarginine conjugates produce a significantly greater percentage of cells in G_2/M than Taxol alone (OVCA-429 **2a**, P = 0.0045; **3a**, P = 0.0051; OVCA-429T **3a**, P = 0.0034). (*C*) Overcoming MDR through conjugation to octaarginine transporter. OVCA-429 (Taxol sensitive) and OVCA-429T (Taxol resistant through a complex mechanism including Pgp efflux pump up-regulation) cells expressing *Renilla* luciferase were treated with coelenterazine H (the substrate for *Renilla* luciferase and a substrate for Pgp), with or without pretreatment with the Pgp inhibitor cyclosporine A. Subsequent light output, as a measure of drug uptake, was determined by bioluminescence imaging.

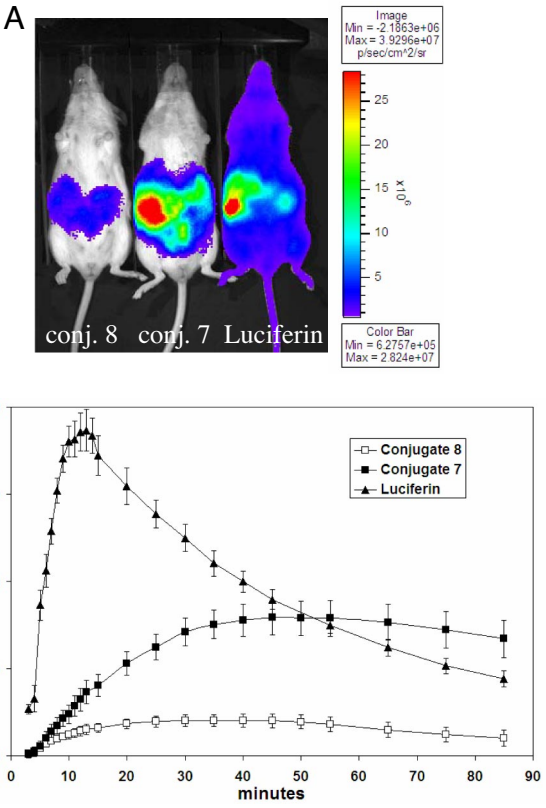

Fig. 3. Effect of octaarginine (r8) conjugation on biodistribution of drug. Luciferin, and luciferin conjugated to r8 (**7**) or k4 (**8**, tetralysine, which has similar water solubility to r8 but limited cellular uptake) were delivered at concentrations equimolar to those used in the studies with Taxol (5 mg/kg) via i.p. injection to transgenic mice ubiquitously expressing firefly luciferase. (*A*) Representative image of treated mice 40 min after injection. (*B*) Total signal per mouse at times after injection (average of two experiments, error bars represent range).

gate (**6**) of coelenterazine H (**5**). Coelenterazine H is a substrate for *Renilla* luciferase and, similar to Taxol, a substrate for Pgp-mediated efflux (37). Coelenterazines are made up of a lipophilic, amine-containing heterocycle with physicochemical properties similar to other substrates of Pgp. As expected, when coelenterazine H was incubated with multidrug-resistant OVCA-429T cells transfected with *Renilla* luciferase, the bioluminescence signal was reduced relative to the signal obtained with nonresistant OVCA-429 cells (Fig. 2*C*). It is significant that the r8 conjugate of coelenterazine H (**6**) overcame this efflux-mediated signal reduction, exhibiting similar luminescence in both cell lines. Preincubation of OVCA-429T cells with the Pgp-inhibitor cyclosporine A also overcame the signal reduction observed for coelenterazine H (**5**) but had little effect on the r8 conjugate-treated OVCA-429T cells. The ability of the r8 transporter to circumvent Pgp-mediated efflux of such varied structures (i.e., **1** and **5**) suggests that this approach could be extended to other small-molecule drugs and leads.

Biodistribution and Pharmacokinetics of an Octaarginine–Luciferin Conjugate. As a prelude to animal studies with Taxol conjugates, a third small-molecule system, luciferin, the substrate for firefly luciferase, was used as a drug surrogate to compare the biodistribution and pharmacokinetics of the r8 drug surrogate conjugate and the surrogate alone. Luciferin and luciferin conjugated to r8 (**7**) (38, 39), at concentrations equimolar to those used in the studies with Taxol (5 mg/kg), were delivered via i.p. injection into transgenic mice ubiquitously expressing firefly luciferase (Fig. 3). Because of the adherence and rapid cellular uptake of the r8 conjugate, it did not distribute throughout the body, remaining instead near the site of administration. For localized tumors, this transporter-based effect allows for greater sustained availability of drug in the vicinity of the tumor and correspondingly reduced systemic toxicity. Ovarian cancer with i.p. drug administration was chosen for this work, partially on the basis of the recent recommendation of the National Cancer Institute that approved treatments for advanced ovarian cancer (Taxol, cisplatin, carboplatin) include i.p. delivery. In addition, although uptake of the octaarginine conjugate is rapid, the sustained rate of release of the free drug allows for the maintenance of constant and controlled drug levels, thereby avoiding the bolus effect encountered when a free drug is administered. Pertinent to this point, comparison of the bioluminescence resulting from the r8 luciferin conjugate **7** and from luciferin alone showed that the latter produced a signal that peaked early (≈12 min after injection) and declined rapidly (Fig. 3*B*). The calculated area under the curve (total light production) was similar for both r8 conjugate **7** and luciferin alone, demonstrating that the drug surrogate (luciferin) was released efficiently from the conjugate but at a level sustained over a longer period relative to luciferin itself. The tetralysine luciferin conjugate **8**, possessing the same disulfide linker and cargo and similar polycation-based water solubility as the r8 conjugate, was used as a control. As expected, the k4 conjugate **8** produced 3.8-fold less total signal, confirming the important role of the enhanced cellular uptake of the octaarginine transporter in the activity of its conjugates. Overall, the Taxol conjugates are readily administered in aqueous solution, thereby avoiding prolonged administration of Taxol by using Cremophor EL, remain localized because of cell adherence and the rapid rate of cellular uptake, and, because of the sustained release of the free drug, avoid or minimize adverse bolus and peak-trough effects arising from the injection of free drug.

***In Vivo* Assay of Taxol Conjugates.** Several different mouse tumor models of ovarian cancer were examined next (Fig. 4). In an initial study, the human ovarian tumor cell line UCI-101 expressing luciferase was implanted into the peritoneal cavity. The animals were then treated with doses of 5 or 10 mg/kg (5 mg/kg is equivalent to the clinical dose of Taxol recommended to treat ovarian cancer) of either Taxol (administered in 10% DMSO PBS solution) or C2′ r8 Taxol (2a) (administered in PBS) delivered via i.p. injection. At both doses the r8 conjugate produced enhanced anticancer effects over Taxol alone ($P = 0.0039$ at 5 mg/kg and 0.047 at 10 mg/kg), with the 10 mg/kg dose resulting in 60% complete responses (compared with 12.5% complete responses for animals treated with Taxol at the same dose). Although the C7-conjugated Taxol (**3a**) was found to be as effective as Taxol against Taxol-sensitive tumors, mirroring the cell-line cytotoxicity assays, it was shown to overcome Taxol resistance in cells with Pgp-mediated MDR phenotypes (Table 1). This result was also found to be the case in the animal models for mice bearing peritoneal tumors formed from OVCA-429 (Taxol-sensitive) or OVCA-429T (Taxol-resistant) cell lines; although both C7 conjugate (**3a**) (administered in PBS) and free Taxol (administered in 10% DMSO) produced similar effects against the Taxol-sensitive OVCA-429 cell line, the C7 conjugate produced significantly enhanced benefits relative to Taxol in the Taxol-resistant OVCA-429T cells ($P = 0.0002$).

Conclusion

We have demonstrated that conjugation of octaarginine peptide transporters to a small-molecule drug (Taxol) and drug surrogates (luciferin or coelenterazine H) via disulfide linkers can provide a variety of benefits including improved administration as a result of enhanced aqueous solubility, altered (localized) biodistribution, lengthened pharmacokinetics, and, most importantly, the ability to overcome MDR elicited by the drug alone. In particular, it was found that r8 conjugated to the C2′ position of Taxol produces a highly water-soluble conjugate (thereby avoiding the need for

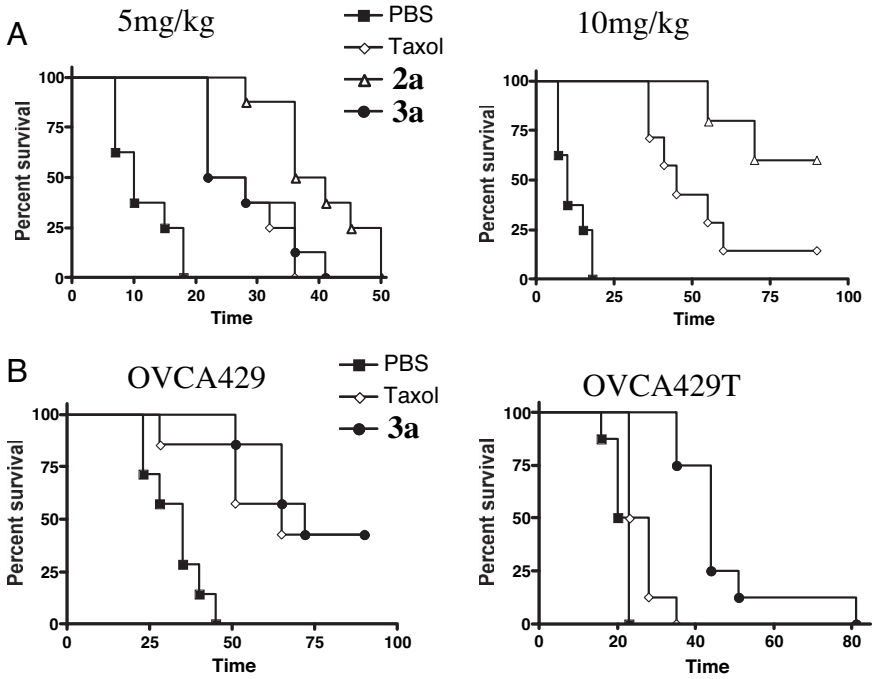

Fig. 4. Survival of tumor-bearing mice treated with Taxol or its derivatives. (*A*) 1×10^7 UCI-101 tumor cells expressing luciferase were implanted into the peritoneal cavity of athymic nu/nu mice 7 days before treatment. Mice were treated with i.p. injections of 5 mg/kg (*Left*) or 10 mg/kg (*Right*) of Taxol or equimolar amounts of its derivatives [octaarginine conjugated to C2′ (**2a**) or C7 (**3a**) positions] on days 0, 5, and 10. For the purpose of these experiments, trifluoroacetate (TFA^-) counteranions on both conjugates were exchanged to Cl^-. Subsequent tumor burden was followed by bioluminescence imaging ($n = 8$ per group). C2′ conjugate (**2a**) produces significantly greater survival than Taxol at both 5 mg/kg ($P = 0.0039$) and 10 mg/kg ($P = 0.047$). (*B*) Mice were implanted with 1×10^7 cells of OVCA-429 (Taxol-sensitive) or OVCA-429T (Taxol-resistant) cells expressing luciferase and subsequently treated (7 days later) with 5 mg/kg of Taxol or equimolar amounts of an octaarginine C7 conjugated derivative (**3a**) on days 0, 5, and 10. Tumor burden was followed by bioluminescence imaging ($n = 8$ per group). Kaplan-Meier survival curves are shown. The C7 octaarginine conjugate (**3a**) produces significantly better survival rates than Taxol in OVCA-429T cells ($P = 0.0002$).

Cremophor EL), allows for sustained release of the free drug (thereby minimizing peak-trough effects), enhances the cytotoxicity of the drug against a panel of cell lines, and provides significantly increased benefits in ovarian cancer mouse models relative to Taxol itself. It is significant that the C7 conjugate of Taxol (**3a**) overcomes the resistance exhibited by Taxol itself. This effect is observed for cell lines with overexpressed Pgp efflux pumps and for cells with complex MDR phenotypes in cell culture and in animal tumor models. Generally, if a cancer develops resistance to a drug, it is necessary to switch to a second drug to circumvent this resistance. The approach described here provides an alternative treatment strategy. Many drugs (e.g., etoposide, camptothecin, and doxorubicin) because of their hydrophobic nature are substrates for Pgp efflux pumps. Attachment of a transporter to these agents could dramatically change their physical properties and, therefore, mode of cell entry, thereby avoiding Pgp-based resistance. Although Taxol prodrugs with aqueous solubility as well as targeted delivery have been described previously (40–45), they often require the release of the solubilizing subunit of the conjugate to allow diffusion of the drug across the nonpolar plasma membrane. In contrast, the octaarginine transporter not only allows for solubilization of the conjugate in aqueous solution and the administration benefits derived therefrom, but it also enhances uptake and, significantly, with a suitable linker allows for controlled release of free drug after cell entry, factors that favor improved performance and minimize peak-trough effects. The ability to improve the administration and performance of a drug and especially to overcome resistance elicited by that drug through conjugation with an octaarginine transporter could improve the prognosis for the treatment of cancer with many therapeutic agents. More generally, this strategy could potentially extend to other diseases such as tuberculosis and malaria, for which enhanced efflux contributes to or determines resistance.

Materials and Methods

Synthesis of Reagents. Synthetic procedures for the synthesis of compounds listed in Fig. 1 are described in supporting information (SI).

Cell Lines. SKOV-3 cells were obtained from the American Type Culture Collection; UCI-101 and UCI-107 cell lines were obtained from P. DiSaia and A. Manetta (University of California); MCF-7 and MCF-7-Pgp were obtained from D. Piwnica-Worms (Washington University); and OVCA-429, OVCA-433, and the taxane-resistant derivatives of these cells (OVCA-429T, OVCA-429T×T, OVCA-433T, and OVCA-433T×T) were obtained from B. Sikic (Stanford University). All cells were grown in DMEM with 10% FBS.

Cell Cytotoxicity Assay. The cytotoxicity of relevant compounds was determined by IC_{50} assay. Cells were seeded overnight into 96-well plates and then incubated with a serial dilution of the indicated compound for 20 min before washing twice with fresh medium and incubation at 37°C for 72 h in drug-free medium. The IC_{50} values were determined as the concentration of compound required to inhibit the viability of the cell layer by 50% relative to untreated and cell-free control wells (100% and 0% viability, respectively) determined from semilogarithmic dose-response curves. Viability was assayed by CellTiter96 aqueous assay (MTS) according to manufacturer instructions (Promega). For Fig. 2*C*, bioluminescence was determined by using an IVIS 50 system (Caliper Life Sciences). Each compound and dilution was tested in triplicate per experiment, with each experiment reproduced three times.

Microtubule Assembly Assay. The tubulin depolymerization assay was performed according to an adaptation of the methods described by Mathew *et al.* (35). Briefly, 2 μM tubulin protein (Cytoskeleton Inc) was allowed to polymerize at 30°C in the presence of 10 μM Taxol, or derivative and 0.5 mM GTP in PEM buffer (80 mM Na-PIPES at pH 6.9, 1 mM $MgCl_2$, and 1 mM EGTA). Polymerization was determined as increasing turbidity, monitored by absorbance at 350 nm.

Cell-Cycle Analysis. Cell-cycle analysis was performed on cancer cell lines, or cells pretreated with Taxol, or its derivatives according to standard techniques (36). Briefly, cells were treated with Taxol or a derivative at 1 mM for 15 min and then washed twice before incubation for 24 h. Cells were then detached, stained with 7-amino-actomycin D (7-AAD), and analyzed by flow cytometry. Initially, doublets were removed by gating so that only single cells were analyzed. Then the FL3 channel was analyzed to divide the cells into G_2/M, S, and G_1/S subsets. The percentage of cells in G_2/M was recorded. Data from three separate experiments were combined.

Construction and Testing of OVCA-429 and OVCA-429T Cells Expressing Luciferase. Stable versions of the cell lines OVCA-429 and OVCA-429T were constructed by expressing firefly luciferase or firefly and *Renilla* luciferase enzymes. Two versions of the plasmid pCDNA3.1 were constructed; in the first construct, firefly luciferase was placed under the control of the CMV promoter, and the puromycin selection gene was inserted, and in the second construct, *Renilla* luciferase was placed under control of the CMV promoter, and the zeocin selection gene was inserted. Firefly luciferase expression was produced by Lipofectamine (Invitrogen) transfection of the appropriate plasmid according to manufacturer guidelines followed by selection on puromycin. A second transfection, into the firefly luciferase-expressing cells, and using the *Renilla* luciferase expression plasmid and zeocyin selection produced cell lines expressing both luciferase enzymes. Stability was determined by growth in medium without selection, and phenotypic properties (gross morphology, Taxol resistance, and growth rate) were determined to ensure that gene expression did not overtly alter the characteristics of the cells. In some experiments, cells expressing *Renilla* luciferase were treated with coelenterazine substrate at 50 μM (a known substrate of the Pgp transporter) either alone or in the presence of 5 μM cyclosporine A. Light output was measured as an indicator of Pgp function by using an IVIS 50 (Xenogen product line of Caliper Life Sciences). Luciferin and firefly luciferase bioluminescence were used to normalize the readings.

Mouse Biodistribution Studies. L2G85 transgenic mice, carrying the luciferase gene driven by the β-actin promoter, were treated with a single i.p. injection of 5 mg/kg luciferin or equimolar amounts of luciferin conjugates. Bioluminescence signal was determined at regular time points immediately after injection by using an IVIS 200 system (Caliper Life Sciences).

Mouse Tumor Models. Tumor xenografts were created by i.p. injection of female athymic CD1 nu/nu or SCID mice with 1×10^7 cells. Versions of UCI-101, OVCA-429, or OVCA-429T cells were used, expressing firefly luciferase. Tumor establishment and growth was verified by increasing bioluminescence signal (as determined by *in vivo* bioluminescence imaging (BLI) on an IVIS 100 system (Xenogen product line from Caliper Life Sciences) after i.p. injection with 30 mg/kg luciferin and anesthesia with 2% isoflurane). Mice were then treated with three i.p. injections (5 days apart) of 5 or 10 mg/kg Taxol or equimolar dose equivalents of the Taxol derivatives or PBS as a control. For the purpose of these experiments, TFA^- counteranions on both conjugates were exchanged to Cl^-. Subsequent tumor burden was followed by BLI. Once tumor burden reached 1×10^8 photons/sec per mouse, the mice were killed. All studies were run according to Institutional Animal Care and Use Committee-approved protocols.

Statistical Analyses. Comparisons of cell numbers in G_2/M phase were made by using Student's *t* test. Comparisons of survival (Kaplan-Meier) curves were made by the Wilcoxon rank test. Statistical significance was determined as $P < 0.05$.

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