Clinical Cancer Research

Rational Combination Therapy of Vintafolide (EC145) with Commonly Used Chemotherapeutic Drugs

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Abstract

Purpose: When evaluated in patients with ovarian and other cancer, vintafolide (EC145), a potent folate-targeted vinca alkaloid conjugate, displayed a toxicity profile that seemed to be nonoverlapping with many standard-of-care cancer therapeutics. It was, therefore, hypothesized that combining vintafolide with certain approved anticancer drugs may afford greater therapeutic efficacy compared with single-agent therapy. To explore this concept, vintafolide was evaluated in combination with pegylated liposomal doxorubicin (PLD; DOXIL), cisplatin, carboplatin, paclitaxel, docetaxel, topotecan, and irinotecan against folate receptor (FR)–positive models.

Experimental Design: FR-expressing KB, M109, IGROV, and L1210 cells were first exposed to graded concentrations of vintafolide, either alone or in combination with doxorubicin (active ingredient in PLD), and isobologram plots and combination index values generated. The vintafolide combinations were also studied in mice bearing various FR-expressing tumors.

Results: Vintafolide displayed strong synergistic activity against KB cells when combined with doxorubicin, and no less-than-additive effects resulted when tested against M109, IGROV, and L1210 cells. In contrast, when either desacetylvinblastine hydrazide (DAVLBH; the vinca alkaloid moiety in vintafolide) or vindesine (the vinca alkaloid most structurally similar to DAVLBH) were tested in combination with doxorubicin, less-than-additive antitumor effects were observed. *In vivo*, all vintafolide drug combinations produced far greater antitumor effect (complete responses and cures) compared with the single agents alone, without significant increase in overall toxicity. Importantly, these benefits were not observed with combinations of PLD and DAVLBH or vindesine.

Conclusions: On the basis of these encouraging preclinical results, clinical studies to evaluate vintafolide drug combination therapies are now under way. *Clin Cancer Res; 20(8); 2104–14. ©2014 AACR.*

Introduction

The vitamin folic acid (FA; folate) is a high-affinity ligand to the folate receptor (FR; $K_d \sim 0.1$–1 nmol/L) that maintains its strong binding property when conjugated to other molecules. The FR is functionally expressed in high quantities by many primary and metastatic cancers (2–8). As a result, "folate targeting" has been successfully applied toward the delivery of a wide variety of therapeutic- and imaging-based agents to tumors that express the FR protein (9–12). Compared with their nontargeted counterparts, folic acid–bearing drugs and delivery systems have repeatedly shown greater cancer cell specificity and selectivity in numerous preclinical studies (13–20). Hence, this targeting strategy leads to improvements in the safety and efficacy of anticancer agents, resulting in an increased therapeutic advantage.

We have recently described the biologic activity of vintafolide (EC145), a folate conjugate of the microtubule-destabilizing agent, desacetylvinblastine hydrazide (DAVLBH; a derivative of the natural product, vinblastine; refs. 18, 19). DAVLBH was chosen as the drug moiety for vintafolide because it contains a modifiable hydrazide functional group to which one can attach a hydrophilic folate–peptide compound (e.g., FA–Asp–Arg–Asp–Asp–Cys) via a disulfide linker. Vintafolide was determined to have high affinity for the FR, and it is extremely potent *in vitro* against FR-expressing cells with IC$_{50}$s in the low nanomolar range (19). When evaluated against FR-expressing tumor xenograft models, vintafolide produces impressive antitumor activity, with CRs or cures in 100% of the tumors under conditions that produce little to no toxicity.

Clinically, single-agent vintafolide was shown to be well tolerated by patients, with constipation and mild peripheral neuropathy being the dose-limiting toxicities, especially in patients with lower vintafolide clearance (21). Importantly, vintafolide did not cause appreciable hematopoietic

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Translational Relevance

Our report highlights the potential for safely combining vintafolide (a folate-targeted vinca alkaloid) with pegylated liposomal doxorubicin (PLD), platinum-based agents, topoisomerase inhibitors, and taxanes to yield enhanced antitumor effects, including complete responses (CR) and cures against human tumor xenografts. The observed therapeutic benefits of these combinations were specific for folate receptor (FR)–positive tumor models, likely because vintafolide remains associated with tumor cells for multiple M phases of the cell due to its natural high-affinity binding to the FR and the slow recycling rate of that receptor. On the basis of the disclosed preclinical data, a randomized phase II trial (PRECEDENT) was conducted with the vintafolide/PLD combination. Final results showed a statistically significant progression-free survival rate in patients with platinum-resistant ovarian cancer (PROC; ref. 1). Importantly, a randomized phase III study has now been initiated to further evaluate the vintafolide/PLD combination in women with PROC (PROCEED trial).

toxicity, which is very common among standard-of-care chemotherapeutic agents.

Current practice in clinical oncology is to use multidrug combinations instead of single-agent chemotherapy. Such regimens are designed to take advantage of nonoverlapping tissue toxicity and antitumor activity against differentially resistant cancer cell clones. In an attempt to prioritize and guide future vintafolide clinical studies, we assessed the therapeutic effects of various vintafolide-based combination regimens relative to single-agent therapies against FR-expressing cells and tumor models. Pegylated liposomal doxorubicin (PLD; which causes stomatitis and palmar-plantar syndrome), paclitaxel, docetaxel, topotecan, and irinotecan (neutropenia), cisplatin (neurotoxicity), and carboplatin (myelosuppression) were all chosen for study due to their nonoverlapping dose-limiting toxicities compared with vintafolide. Herein, we present the results of binary combinations of vintafolide with each of these seven clinically relevant anticancer agents.

Materials and Methods

Materials

Vintafolide (Endocyte Inc.) clinical vial solutions were used in all the experiments. Doxorubicin, carboplatin, and cisplatin were purchased from Sigma; PLD (DOXIL) and docetaxel was obtained from the Purdue Pharmacy; paclitaxel, topotecan, and irinotecan were purchased from A.K. Scientific. All other common reagents were purchased from Sigma or other major suppliers.

Estimation of surface FRs on various cell lines

To quantify the number of cell surface FRs, 1×10^6 cells were harvested and transferred in 1 mL of folate deficient (FD)-RPMI + 10% fetal calf serum (FCS) to a 5 mL polypropylene tube in triplicates. Either 100 nmol/L ^{3}H-folic acid or 100 nmol/L ^{3}H-folic acid + 10 μmol/L folic acid was then added to the cells and the cells were incubated for 15 to 30 minutes on ice. Cells were then washed twice with PBS and solubilized in 0.25 N NaOH. The samples were then mixed with a scintillation cocktail and counted in a liquid scintillation counter. The number of molecules per cell was determined from the radioactivity in each sample treated with ^{3}H-folic acid minus the average radioactivity found in the ^{3}H-folic acid + excess folic acid samples.

Cell growth inhibition studies

FR-positive human nasopharyngeal carcinoma KB cells (a human cell line containing markers of HeLa cervical cancer origin; 6.0×10^6 FRs/cell), mouse lung carcinoma Madison 109 (M109, 1.1×10^6 FRs/cell) cells generated from M109 tumors in mice, mouse leukemic monocyte macrophage RAW cells (3.3×10^6 FRs/cell), mouse lymphoma J6456 cells (2.7×10^6 FRs/cell), and human lung A549 cells (0.008×10^6 FRs/cell) were maintained in FDRPMI containing 10% heat-inactivated FCS (HIFCS) at 37°C in a 5% CO_2/95% air-humidified atmosphere with no antibiotics. KB cell (American Type Culture Collection, ATCC) integrity was confirmed (Genetica DNA Laboratories) with cells being similar to reference cells from the ATCC. Exponentially growing cells were seeded in 24-well plates 24 hours before treatment with drugs. Cells were exposed to graded concentrations (four replicates) of doxorubicin and vintafolide (or DAVLBH or vindesine) either alone or in combination. Cells receiving vintafolide ± doxorubicin were pulsed for 2 hours at 37°C, rinsed four times with 0.5 mL of medium, and then chased in 1 mL of fresh medium or fresh medium with doxorubicin for up to 72 hours. Cells treated with single-agent doxorubicin, DAVLBH, or vindesine (or in combination) were treated continuously for 72 hours. Cells were treated with fresh medium containing ^{3}H-thymidine for 2 hours at 37°C, washed with PBS, and then treated with ice-cold 5% trichloroacetic acid (TCA). After 15 minutes, the TCA was aspirated and the cells solubilized by the addition of 0.25 N sodium hydroxide for 15 minutes at room temperature. For L1210 cells, the TCA precipitation step was skipped and the cells were directly treated with 0.25 N NaOH. Each solubilized sample was transferred to scintillation vials containing EcoLume scintillation cocktail and counted in a liquid scintillation counter. Chemosensitivity values were expressed as the drug concentration that inhibited cell growth by 60% (IC_{60}) determined from concentration-effect relationships using GraphPad prism.

Analysis of combined drug effects

Drug synergy was determined by the isobologram and combination index methods derived from the median-effect principle of Chou and Talalay (22). Data obtained from the growth inhibitory experiments were used to perform these analyses. The isobologram is a graphical representation of the drug interaction and is formed by selecting a desired cell kill fraction (60% in this case) and

plotting the individual drug doses required to generate that fraction on the respective x- and y-axes. A straight line is then drawn to connect the points. Multiple combinations of corresponding drug concentrations that achieved the 60% growth inhibition effect are plotted in the isobologram. Combination data points that fall on the line represent an additive drug–drug interaction, whereas data points that fall below or above the line represent synergism or antagonism, respectively. The combination index method is a quantitative representation of a two-drug interaction. For each combination of drug concentrations [e.g., vintafolide + doxorubicin ($D_{EC145\ (60)}$, $D_{doxorubicin\ (60)}$)] producing an IC_{60} effect, whereas in combination, a combination index (CI) value was calculated as follows: $CI = (D_{EC145\ (60)}/IC_{60},\ vintafolide) + D_{doxorubicin\ (60)}/IC_{60},\ doxorubicin)$, where "$IC_{60}$, EC145" and "$IC_{60}$, doxorubicin" are the concentrations of each individual drug that would produce the IC_{60} effect if given alone. Importantly, a CI of 1 indicates an additive effect between two agents, whereas a CI < 1 or CI > 1 indicates synergism or antagonism, respectively.

Cell-cycle analysis

KB Cells were seeded in 6-well plates, 24 hours before treatment with drugs. Cells were then treated with or without EC145 and doxorubicin, or their combination in varying sequential order. The cells were harvested at 48 and 72 hours after first drug treatment and washed twice with cold PBS. The cells were then resuspended in 200 μL of cold PBS, and fixed by drop wise addition of 800 μL of ice-cold 100% ethanol and gentle vortexing. The ethanol-fixed cells were stored at −20°C for 1 to 3 days, washed twice with cold PBS, and resuspended in 500 μL propidium iodide staining solution [50 μg/mL propidium iodide (Sigma), 0.2 mg/mL RNase A (Sigma), and 0.1% Triton X-100 (Sigma) in PBS], and incubated at 37°C for 20 minutes. Subsequently, the cells were transferred on ice and data acquisition performed on Gallios Flow Cytometer (Beckman Coulter Inc.). Samples were analyzed in triplicates and a minimum of 50,000 events per sample were recorded. Two independent experiments were conducted. The collected data were analyzed using Kaluza Flow Cytometry Software (Beckman Coulter Inc.).

In vivo antitumor experiments

Four- to 7-week-old female nu/nu mice (Charles River Laboratories), female Balb/c mice, or female DBA mice (Harlan Sprague Dawley, Inc.) were maintained on a standard 12-hour light-dark cycle and fed ad libitum with a low-folate chow (Harlan diet # TD.00434; Harlan Teklad) for the duration of the experiment. KB Cells (1×10^6 per nu/nu mouse), M109 cells (1×10^6 per Balb/c mouse), IGROV cells (1.7×10^6 FRs/cell, 4×10^6 per nu/nu mouse), or L1210 cells (2.1×10^6 FRs/cell, 2×10^5 per DBA mouse) in 100 μL were injected in the subcutis of the dorsal medial area. Mice were divided into groups of five, and freshly prepared test articles were injected through the lateral tail vein under sterile conditions in a volume of 200 μL of PBS.

Intravenous treatments were typically initiated on day 7 posttumor cell implantation (PTI) when the KB tumors were approximately 100 to 212 mm^3 in volume, on day 7 PTI when the M109 tumors were approximately 67 to 90 mm^3 in volume, on day 6 PTI when the IGROV tumors were approximately 95 to 147 mm^3 in volume, and on day 8 PTI when the L1210 tumors were approximately 64 to 175 mm^3 in volume. The mice in the control groups received no treatment. Growth of each subcutaneous tumor was followed by measuring the tumor three times per week during treatment and twice per week thereafter, until a volume of 1,500 mm^3 was reached. Tumors were measured in two perpendicular directions using Vernier calipers, and their volumes were calculated as $V = 0.5 \times L \times W^2$, where L, measurement of longest axis in mm and W, measurement of axis perpendicular to L in mm. As a general measure of gross toxicity, changes in body weights were determined on the same schedule as tumor volume measurements. Maximum percentage of weight loss on any given day due to treatment was determined for each mouse and the average for the group is reported as average of maximum percentage of weight loss (AMWL%). Survival of animals was monitored daily. Animals that were moribund (or unable to reach food or water) were euthanized by CO_2 asphyxiation. All animal housing, care, and procedures were followed according to the Purdue Animal Care and Use Committee–approved animal care and use protocols.

Tumor response criteria

Individual tumor response endpoints were reported in terms of tumor volume change. A partial response (PR) was defined as volume regression >50% but with measurable tumor (>2 mm^3) remaining at all times. CR was defined as a disappearance of measurable tumor mass (<2 mm^3) at some point within 90 days after tumor implantation. Cures were defined as CRs without tumor regrowth within the 90-day study time frame. For all the noncured mice, gross log cell kill values (LCK) were calculated by dividing the delay of tumor growth value (T–C) by the tumor volume doubling time multiplied by the exponential function 3.32. Delay of tumor growth is defined as the difference in days required for the treated tumors (T) to reach the predetermined target size of 1,500 mm^3 compared with those of the control group (C).

Results

Doxorubicin shows synergistic cell growth inhibitory effect when combined with vintafolide but antagonizes with nontargeted DAVLBH or vindesine

To investigate the effect of combining doxorubicin with the antiprolific activity of vintafolide, FR-expressing cells (KB, M109, RAW, and J6456) were exposed to increasing concentrations of individual drugs, alone and in combination. Cell viability was later assessed and IC_{60} values used to perform isobologram analysis. The isobologram and combination index methods developed by Chou and Talalay were used to determine and quantify the synergism observed between the two agents. Isobolograms were
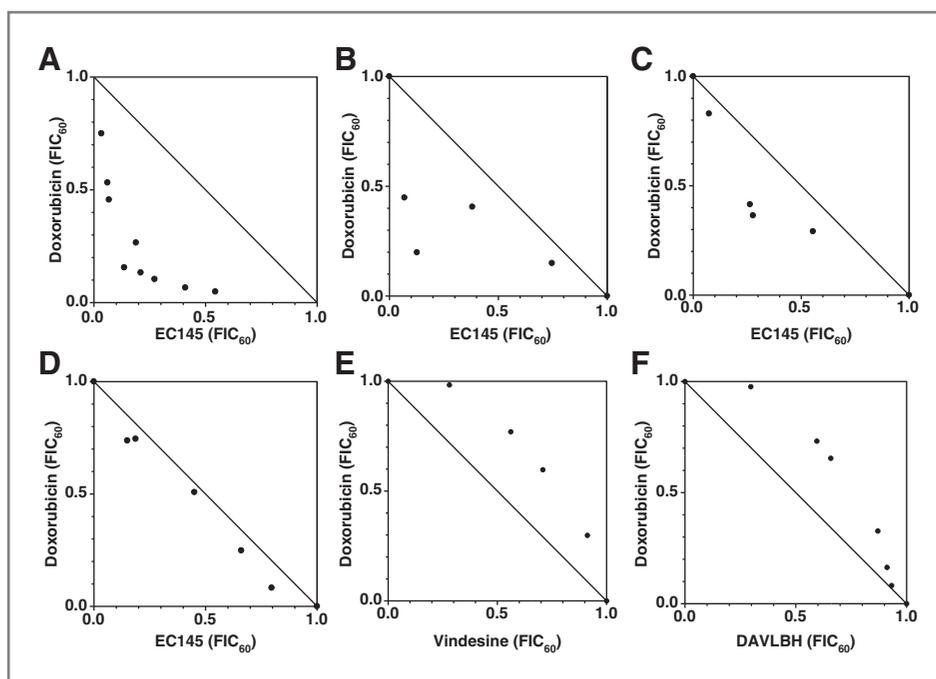
Figure 1. A, isobolograms for the interaction between doxorubicin and (A) vintafolide in KB cells, mean $FIC_{60} = 0.510$; B, vintafolide in M109 cells, mean $FIC_{60} = 0.632$; C, vintafolide in RAW cells, mean $FIC_{60} = 0.767$; D, vintafolide in J6456 cells, mean $FIC_{60} = 0.914$; E, vindesine in KB cells, mean $FIC_{60} = 1.28$; F, DAVLBH in KB cells, mean $FIC_{60} = 1.20$. Cells were exposed to vintafolide (for first 2 hours of incubation) and doxorubicin, vindesine, or DAVLBH (for 72 hours). The IC_{60} values for each of the drugs in every combination were used to define a normalized drug concentration, set to the value of 1, and is represented on the x- and y-axis with the diagonal line connecting the two points (line of additivity). Experimental data points represented by dots (●) are the combinational IC_{60} concentrations (expressed relative to the IC_{60} concentrations of each drug as single agent) of the two agents, which, when combined, inhibit cell growth by 60%. Points located below, on, or above the line indicate synergy, additivity, or antagonism between the two combination drugs, respectively.

constructed by plotting IC_{60} unit values for doxorubicin against corresponding IC_{60} unit values for vintafolide (Fig. 1). The distribution of points along the diagonal connecting the values of 1 suggests an additive effect of the two drugs, whereas the points below or above the line indicate their synergism and antagonism, respectively.

Experimental combination data points for the vintafolide/doxorubicin analysis plotted well below the expected additive line (Fig. 1A), indicating strong synergism (CI = 0.510) to produce increased growth inhibition against the KB cells. The vintafolide/doxorubicin combination also displayed good synergy against M109 cells (Fig. 1B; CI = 0.632), and to a lesser extent against RAW cells (Fig. 1C; CI = 0.767) and J6456 cells (Fig. 1D; CI = 0.914).

The vintafolide/doxorubicin combination effect was also tested against FR-positive IGROV and L1210 cells, and no more than an additive effect was observed in both cell lines. This is presumably because both of these cell lines are less sensitive to the vinca alkaloid base drug causing vintafolide's IC_{50} to be greater than 1 μmol/L. At such higher concentrations of vintafolide, we have observed that the level of competition with folic acid is reduced, indicating that the untargeted portion of the molecule's antimitotic activity is higher. As receptor-specific activity of vintafolide is reduced, similar to the nontargeted DAVLBH, synergistic activity between vintafolide and doxorubicin is compromised.

Propidium iodide cell-cycle analyses were also performed on KB cells treated with single-agent vintafolide, doxorubicin, or their combination. Compared with untreated cells (21.9% ± 2.8%), accumulation of cells in the G_2–M phase was significantly increased after incubation with 16 nmol/L doxorubicin (42.0% ± 0.6%; Fig. 2B). Treatment with 16 nmol/L vintafolide resulted in an even greater (56.8% ± 0.7%) number of cells in the G_2–M phase. However, combination of the two drugs at the same concentrations resulted in a further increase (62.7% ± 1.2%) of G_2–M-arrested cells. These results again confirm that vintafolide combines extremely well with doxorobucin to produce a greater level of cellular mitotic activity as compared with the single agents alone.

To determine whether the observed synergy with the vintafolide/doxorubicin combination was unique to the cell-targeting property of vintafolide, the active vinca alkaloid part of this conjugate (i.e., DAVLBH), as well as a clinically used vinca alkaloid that is structurally similar to DAVLBH (vindesine), were also tested in combination with doxorubicin. As shown in Fig. 1, all of the isobologram points for vindesine/doxorubicin (Fig. 1E; CI = 1.28) and DAVLBH/doxorubicin (Fig. 1F; CI = 1.2) combinations lay above the diagonal line. These results indicate that an antagonistic, or less-than-additive response, occurs when untargeted vinca alkaloids are combined with doxorubicin *in vitro*.
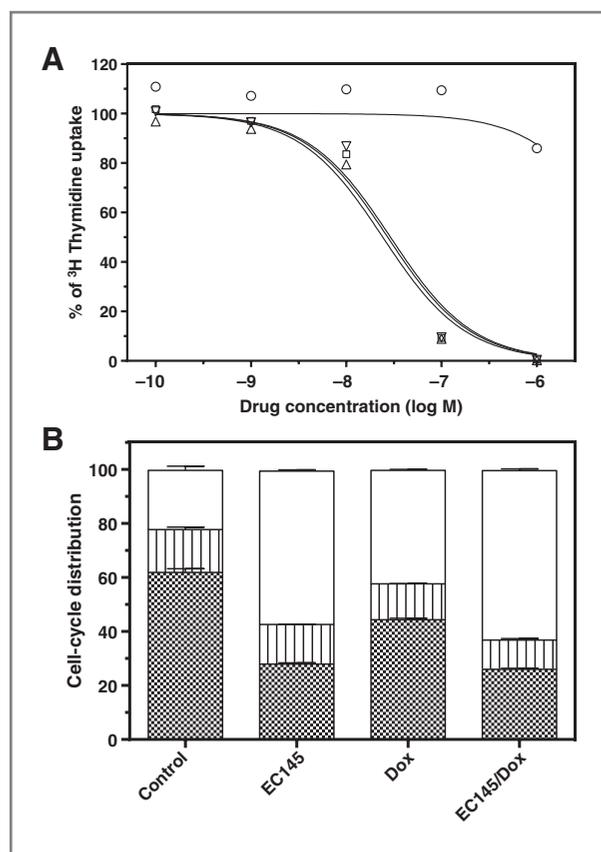
Figure 2. Vintafolide/doxorubicin combination effect. A, FR-negative A549 cells were treated with increasing concentrations of vintafolide (for 2 hours, ○) or doxorubicin (for 72 hours, □) alone or in combination (vintafolide 0.5 μmol/L, △; 1 μmol/L, ▽). B, KB cells were treated with or without vintafolide (16 nmol/L), doxorubicin (16 nmol/L), or their combination. The cells were harvested at 48 hours, fixed, and stained with propidium iodide after first drug treatment. Data acquisition was performed on Gallios Flow Cytometer and analyzed using Kaluza Flow Cytometry Software. (O, G_2–M phase; III, S phase; ▨ G_0–G_1 phase)

Finally, to confirm the importance of FR-targeting ability of vintafolide to synergize with doxorubicin, the vintafolide/doxorubicin combination was also tested against the FR-negative A549 cell line. Here, vintafolide alone was not found to be active up to 1 μmol/L, whereas doxorubicin by itself was active against these cells with an IC_{50} of 27 nmol/L. When combined with even high concentrations of vintafolide (500–1,000 nmol/L), the dose–response curves looked identical to doxorubicin alone with no reduction in the IC_{50} values of the combination groups (Fig. 2A), thus indicating that the aforementioned combination drug effects are dependent on FR expression.

FR-targeted vintafolide displays synergistic *in vivo* antitumor effect when combined with PLD

The observed *in vitro* synergistic activity between vinta-folide and doxorubicin prompted a similar investigation using tumor-bearing mice. Owing to the unfavorable tox-icity profile of doxorubicin (23), PLD was used in combi-nation with vintafolide for *in vivo* studies involving FR-

positive KB, M109, IGROV, and L1210 tumor models. Because each of these tumor types will respond to single-agent vintafolide therapy, suboptimal noncurative dosing regimens of vintafolide were used to better determine the benefits of combining with PLD. Thus, vintafolide was administered using vintafolide regimen-A [1 μmol/kg, three times a week (TIW) for 2 weeks] or vintafolide regimen-B (2 μmol/kg, TIW for 2 weeks). PLD was also administered at 1 of 2 regimens [PLD-A, 4 mg/kg twice a week (BIW) for 2 weeks; or PLD-B, 4 mg/kg once a week for 3 weeks] that represent 50% to 75% of the maximum tolerated dose range for the murine species used in these studies. Importantly, among the combination groups, if both agents had to be administered on the same day, PLD was always dosed 4 hours following vintafolide administration.

Athymic nude mice with established subcutaneous KB xenografts were treated with vintafolide regimen-A, PLD-A, or the respective drug combination. As shown in Table 1 and Fig. 3A, single-agent vintafolide produced minor anti-tumor activity with 20% PR and LCK of 1.2. Single-agent PLD was more active against this model, with 20% PRs, 40% CRs, and LCK value of 3.5; plus, 20% of the mice in this cohort were cured. However, the vintafolide/PLD combi-nation generated greater antitumor activity, in which 20% of the animals displayed CRs, and more impressively, 80% of the animals were cured. The uncured tumor did have an LCK value of 4.8, indicating strong antitumor effect. Some weight loss occurred in the combination cohort (AMWL% 8.2), which was mostly attributed to PLD because the single-agent PLD cohort had an AMWL of 6.6%. In contrast, the single-agent vintafolide cohort had an AMWL of only 0.7% (Table 1).

When tested against the M109 tumor Balb/c mouse model, vintafolide regimen-B produced 60% cures (LCK of 0.7), whereas PLD-B generated 60% cures and 20% CRs (LCK of 2.2; Table 1, Fig. 3B). However, the combination of these two drugs resulted in an impressive 100% cure rate. Furthermore, when tested against the IGROV tumor *nu/nu* mouse model, vintafolide regimen-B produced 80% PRs and 20% cures (LCK of 1.3), PLD-A produced 60% PRs and 40% cures (LCK of 1.9), and the combination demonstrated enhanced antitumor activity with 60% cures, 20% CRs, and 20% PRs (LCK of 2; Table 1; Fig. 3C). A similar study was repeated using the L1210 tumor/DBA mouse model. Here, vintafolide regimen-B produced LCK of only 0.4, and PLD-A produced LCK of only 0.3. Although results were not as impressive as those observed in other models, the drug combination did show greater antitumor effect compared with either single-agent therapy, with LCK of 0.7 (Table 1; Fig. 3D). In all three models the AMWL% for the com-bination groups was in the 3.5% to 4% range (Table 1), whereas the single-agent PLD cohorts were in the 1.6% to 3.8% range, and the single-agent vintafolide cohorts were in the 1% to 2.4% range. Vintafolide/PLD combinations were also tested in *nu/nu* mice implanted with RAW tumors. Unfortunately the RAW tumors were found to be far less sensitive than their cellular form, with vintafolide at 2 μmol/kg, TIW × 2, DOXIL at 4 mg/kg, BIW × 2, and

Table 1. Activity and response of each treatment in various FR-positive mouse tumor models

Tumor model	Treatment regimen	PR%	CR%	Cures%	LCK	AMWL %	PTI (d)
KB	Controls	0	0	0	0	1.5 ± 2.1	10
	Vintafolide (1 µmol/kg TIW × 2)	20	0	0	1.2	0.7 ± 0.9	9–11
	PLD (4 mg/kg BIW × 2)	20	40	20	3.5	6.6 ± 2.0	17–24
	Vintafolide (1 µmol/kg TIW × 2) + PLD (4 mg/kg BIW × 2)	0	20	80	4.8	8.2 ± 4.0	17–19
M109	Controls	0	0	0	0	5.3 ± 5.2	28–30
	Vintafolide (2 µmol/kg TIW × 2)	0	0	60	0.7	1.0 ± 1.3	9–14
	PLD (4 mg/kg q7d × 3)	0	20	60	2.2	2.7 ± 2.6	16–28
	Vintafolide (2 µmol/kg TIW × 2) + PLD (4 mg/kg q7d × 3)	0	0	100	—	3.5 ± 0.9	16–23
IGROV	Controls	0	0	0	0	0.7 ± 1.5	11
	Vintafolide (2 µmol/kg TIW × 2)	80	0	20	1.3	1.0 ± 1.3	15
	PLD (4 mg/kg BIW × 2)	60	0	40	1.9	3.8 ± 3.5	15
	Vintafolide (2 µmol/kg TIW × 2) + PLD (4 mg/kg BIW × 2)	20	20	60	2.0	3.6 ± 1.0	15–18
L1210	Controls	0	0	0	0	1.3 ± 1.2	10
	Vintafolide (2 µmol/kg TIW × 2)	0	0	0	0.4	2.4 ± 1.2	11–15
	PLD (4 mg/kg BIW × 2)	0	0	0	0.3	1.6 ± 2.0	10
	Vintafolide (2 µmol/kg TIW × 2) + PLD (4 mg/kg BIW × 2)	0	0	0	0.7	4.0 ± 3.1	13–22
KB	Controls	0	0	0	0	3.0 ± 2.6	16
	PLD (4 mg/kg BIW × 2)	80	0	0	3.9	4.6 ± 1.4	15–25
	DAVLBH (0.75 µmol/kg TIW × 2)	0	0	0	0.5	0.2 ± 0.2	13–15
	DAVLBH (0.75 µmol/kg TIW × 2) + PLD (4 mg/kg BIW × 2)	0	0	0	3.4	8.0 ± 2.9	27
	Vindesine (1 mg/kg TIW × 2)	0	0	0	0.7	1.4 ± 1.7	13–15
	Vindesine (1 mg/kg TIW × 2) + PLD (4 mg/kg BIW × 2)	0	0	0	2.5	5.1 ± 3.7	22–27

NOTE: CR, disappearance of measurable tumor mass (<2 mm^3) at some point in the study; cures, CRs without tumor regrowth; PR, volume regression >50% but with measurable (>2 mm^3) tumor.

their combination producing 0 PRs in all the three groups. We have seen similar results with the J6456 tumors, i.e., unlike their cellular counterparts, which are far more sensitive to vintafolide and doxorubicin, thus making it impossible to determine the advantage of a combination treatment *in vivo*.

Weight losses in each group of every study were observed during the dosing periods, or within a week after the last dose, but the mice recovered to their pretreatment weights soon thereafter. The weight loss seen on days 28 to 30 in the M109 control group was due to the tumors reaching a volume greater than 1,000 mm^3. Collectively, the enhanced antitumor effect observed in all vintafolide/PLD combination cohorts was accompanied by only minimal change in the animals' health as compared with animals treated with single-agent PLD.

DAVLBH and vindesine in combination with PLD displays *in vivo* antitumor effect that is inferior to single-agent PLD

Because drug "targeting" to the FR was shown to be an essential component of the *in vitro* synergistic activity observed between the vinca alkaloid–containing agent vintafolide and doxorubicin (Fig. 1), the validity of this drug delivery approach was tested *in vivo* by determining the antitumor activity of nontargeted vinca alkaloids in combination with PLD. Here, athymic nude mice with established subcutaneous xenograft KB tumors were treated with DAVLBH, vindesine, PLD, or their respective combinations. DAVLBH and vindesine were administered at dose levels 20% below those that would cause 5% weight loss following a schedule similar to that of vintafolide (i.e., TIW for 2 consecutive weeks). DAVLBH dosed at 0.75 µmol/kg[1] and vindesine dosed at 1 mg/kg both produced 0% PRs with LCKs of 0.5 and 0.7, respectively (Table 1; Fig. 3E and F). In this experiment, single-agent PLD (regimen A) produced 80% PRs with LCK of 3.9. Interestingly, the combination of PLD with either untargeted DAVLBH or untargeted vindesine produced antitumor responses that were inferior to that of PLD alone (0% PRs with LCKs of 3.4 and 2.5, respectively), with accompanying AMWL of 8.0% ± 2.9% and 5.1% ± 3.7%, respectively. Hence, the improved antitumor activity observed with the vintafolide/PLD combination was not reproduced when vintafolide was replaced with its nontargeted drug counterpart (DAVLBH) or its close structurally related vinca alkaloid, vindesine. These results con-

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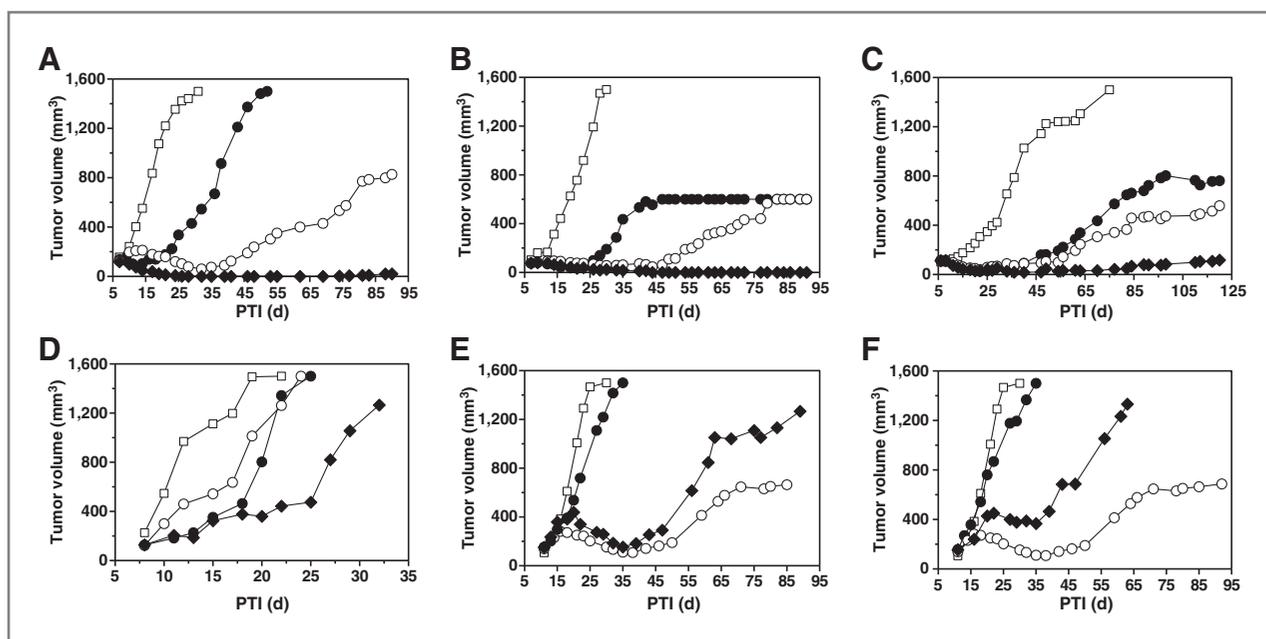[1]1 µmol/kg of DAVLBH caused weight loss of up to 14%; see Ref. 18.

Figure 3. Antitumor effects of PLD in combination with vintafolide or DAVLBH or vindesine. One million tumor cells (A, E, and F: KB; B: M109; C: IGROV; D: L1210) were inoculated subcutaneously into mice (A, C, E, and F: *nu/nu*; B: Balb/c; D: DBA) and therapy started on randomized mice with tumors in various ranges (A, 100–162 mm^3; B, 67–90 mm^3; C, 95–147 mm^3; D, 64–175 mm^3; E, 122–192 mm^3; F, 123–205 mm^3). Each curve shows the average volume of five tumors. □, control; ●, TIW × 2, A, vintafolide, 1 μmol/kg; B–D, vintafolide, 2 μmol/kg; E, DAVLBH, 0.75 μmol/kg; F, vindesine, 1 mg/kg; ○, PLD; A, C–F, 4 mg/kg, BIW × 2; B, 4 mg/kg, q7d × 3; ◆, combination of PLD + vintafolide (A–D) or DAVLBH (E) or vindesine (F) at their respective single-agent doses and schedules.

firm that the added therapeutic benefits observed with the vintafolide/PLD combination regimen are dependent on the FR-targeted drug delivery mechanism.

Vintafolide combines synergistically with anticancer agents of varying mechanisms of action

The potential of vintafolide to combine with other clinically relevant anticancer agents was evaluated using drugs with differing mechanisms of action. For this study, we selected agents commonly used in treating patients with advanced ovarian cancer, such as DNA crosslinking agents (cisplatin and carboplatin), microtubule inhibitors (paclitaxel and docetaxel), and topoisomerase inhibitors (topotecan and irinotecan). *In vitro*, combination results of vintafolide varied from strong synergistic effect with topotecan (CI = 0.49), to slightly less than additive effects with paclitaxel (CI = 1.15).

All of these approved drugs were tested in combination using vintafolide regimen-A (1 μmol/kg, TIW for 2 consecutive weeks) against the KB tumor *nu/nu* mouse model. Single-agent vintafolide produced some antitumor activity ranging from 0% PRs to 20% cures, and LCK ranging from 0.6 to 1.7 (Table 2; Fig. 4). When tested at 3 mg/kg BIW for 2 weeks, the DNA crosslinker cisplatin yielded a modest antitumor effect of 40% PRs and LCK of 1.8, whereas combining it with vintafolide generated a striking 100% cure rate (Table 2, Fig. 4A). Similarly, single-agent carboplatin dosed at 50 mg/kg TIW produced 60% PRs and LCK of 2.0; its combination with vintafolide resulted in an 80%

cure rate, 20% CR, and LCK of 4.4 (Table 2; Fig. 4B). Importantly, the weight loss seen with both of these crosslinking agents (10.5% vs. 5.9% for cisplatin and carboplatin, respectively) was not exacerbated by the addition of vintafolide (cisplatin: 11.9% vs. 5.3% for vintafolide/cisplatin and vintafolide/carboplatin, respectively).

As single agents, the microtubule inhibitors paclitaxel (40 mg/kg, once a week (SIW) for 3 weeks) and docetaxel (10 mg/kg, TIW for 1 week) produced nominal antitumor activity, with 40% and 0% PRs, respectively. When combined with vintafolide, paclitaxel produced stronger antitumor effect, with 40% cures, 60% CRs, and LCK of 2.5 (Table 2, Fig. 4C). Both the single-agent and the combination cohorts were not accompanied by significant weight loss (AMWL of 2.3% and 2.8%, respectively). Interestingly, the vintafolide/docetaxel combination produced superior efficacy, in which 100% of the treated mice were cured (Table 2, Fig. 4D). In addition, weight loss in the combination group seemed to be much less severe (10.5%) compared with the single-agent docetaxel group (16.3%), a finding that was reproducibly observed in subsequent vintafolide/docetaxel studies (data not shown).

Significantly greater antitumor effect was also observed when vintafolide was combined with topoisomerase inhibitors. Thus, as single agents, topotecan (5 mg/kg, TIW for 2 weeks) and irinotecan (20 mg/kg, BIW for 2 weeks) produced minimal antitumor activity (0% PRs) against KB tumors. In contrast, the vintafolide/topotecan combination produced 40% CRs and 60% PRs, with LCK of 2.4; the

Table 2. Activity and response of each combination treatment in the KB tumor *nu/nu* mouse tumor model

Treatment regimen	PR%	CR%	Cures%	LCK	AMWL %	PTI (d)
Control mice with KB tumors 1	0	0	0	0	3 ± 2.2	9–11
Control mice with KB tumors 2	0	0	0	0	1.3 ± 1.7	7–17
Vintafolide (1 μmol/kg TIW × 2)	0	0	20	0.6	0.8 ± 1.3	9–11
Cisplatin (3 mg/kg BIW × 2)	40	0	0	1.8	10.5 ± 4.5	12–19
Vintafolide (1 μmol/kg TIW × 2) + Cisplatin (3 mg/kg BIW × 2)	0	0	100	—	11.9 ± 4.4	12–24
Vintafolide (1 μmol/kg TIW × 2)	0	0	0	1.1	0.4 ± 0.9	8–15
Carboplatin (50 mg/kg TIW)	60	0	0	2	5.9 ± 1.7	13–15
Vintafolide (1 μmol/kg TIW × 2) + Carboplatin (50 mg/kg TIW)	0	20	80	4.4	5.3 ± 2.7	11–15
Vintafolide (1 μmol/kg TIW × 2)	80	0	20	1.7	1.3 ± 1.5	6–20
Paclitaxel (40 mg/kg SIW × 3)	40	0	0	2.3	2.3 ± 2.2	6–11
Vintafolide (1 μmol/kg TIW × 2) + Paclitaxel (40 mg/kg SIW × 3)	0	60	40	2.5	2.8 ± 2.9	6–15
Vintafolide (1 μmol/kg TIW × 2)	0	0	20	0.6	0.8 ± 1.3	9–11
Docetaxel (10 mg/kg TIW)	0	0	0	1.2	16.3 ± 4.4	16–23
Vintafolide (1 μmol/kg TIW × 2) + Docetaxel (10 mg/kg TIW)	0	0	100	—	10.5 ± 3.2	16–21
Vintafolide (1 μmol/kg TIW × 2)	0	0	20	0.6	0.8 ± 1.3	9–11
Topotecan (5 mg/kg TIW × 2)	0	0	0	0.6	11.8 ± 5.7	9–22
Vintafolide (1 μmol/kg TIW × 2) + Topotecan (5 mg/kg TIW × 2)	60	40	0	2.4	6.7 ± 5.5	20
Vintafolide (1 μmol/kg TIW × 2)	0	0	0	1.1	0.8 ± 1.0	10–12
Irinotecan (20 mg/kg BIW × 2)	0	0	0	1.0	1.2 ± 1.0	8–9
Vintafolide (1 μmol/kg TIW × 2) + Irinotecan (20 mg/kg BIW × 2)	0	40	60	4.3	1.4 ± 1.5	8–14

vintafolide/irinotecan combination produced 60% cures and 40% CRs with LCK of 4.3 (Table 2; Fig. 4E and F). Once again, compared with the single-agent groups, in which AMWL was 11.8% versus 1.2% for topotecan and irinotecan, respectively, the health of the mice in the combination groups was not worsened by the addition of
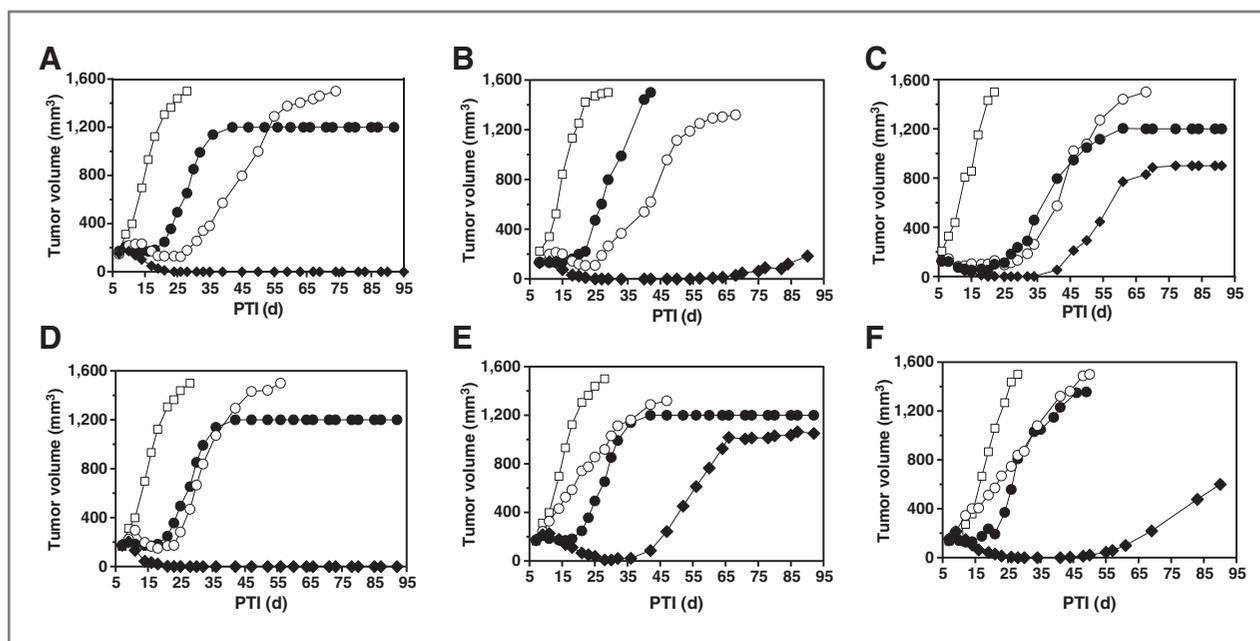

Figure 4. Antitumor effects of vintafolide in combination with cisplatin (A), carboplatin (B), paclitaxel (C), docetaxel (D), topotecan (E), and irinotecan (F). KB tumor cells (1 × 10^6) were inoculated subcutaneously into *nu/nu* mice and therapy started on randomized mice with tumors in the 108 to 212 mm³ range. Each curve shows the average volume of five tumors. □, Control; ●, vintafolide, 1 μmol/kg, TIW × 2; ○, A: cisplatin, 3 mg/kg, BIW × 2; B: carboplatin, 50 mg/kg, TIW; C: paclitaxel, 40 mg/kg, SIW × 3; D: docetaxel, 10 mg/kg, TIW; E: topotecan, 5 mg/kg, TIW × 2; F: irinotecan, 40 mg/kg, BIW × 2; ◆, combination at their respective single-agent doses and schedules.

vintafolide (AMWL 6.7% vs. 1.4% for topotecan and irinotecan, respectively). Together with the aforementioned combination data, these results show that vintafolide can combine with various anticancer drugs, of diverse mechanisms of action, to produce superior antitumor effect over the single agent under conditions that produce little to no added toxicity.

Discussion

Because most standard chemotherapy regimens in the clinic use multidrug combinations, the goal of these studies was to determine whether vintafolide, an FR-targeted vinca alkaloid conjugate, could favorably be combined with clinically approved agents, of various mechanisms of action, to improve therapeutic efficacy. The combination effect of vintafolide was first investigated with PLD because this agent is a widely used second-line treatment of recurrent ovarian cancer, and vintafolide was recently found to be active against this indication in a single-agent, phase II clinical trial (24). The mild toxicity profile of PLD renders it an ideal partner and comparator arm for evaluating combination regimens.

For cell-based studies, we chose to use doxorubicin (the active drug component in PLD) instead of PLD because the liposomal formulation has no cytotoxic activity *in vitro*. Under these conditions, we found that vintafolide combined extremely well (additive to synergistic effects) with doxorubicin in each of four FR-positive cell lines tested. When evaluated *in vivo*, a suboptimal dose of vintafolide was commonly used because chances of observing an enhanced therapeutic effect of any combination regimen would have been limited because of the potential curative activity of "optimal" single-agent vintafolide therapy (18, 19, 25). Our results showed that when combined with PLD, vintafolide produced superior antitumor activity compared with the individual drugs administered as single agents. In addition, the vintafolide/PLD combination displayed significantly enhanced therapeutic efficacy, and possible synergy, when tested against difficult-to-treat FR-positive tumor models such as M109, IGROV, and L1210. Such positive improvements in efficacy were directly correlated with the ability of vintafolide to target FR-expressing tumor cells because nontargeted vinca alkaloids (DAVLBH or vindesine) failed to improve the cytotoxic activity against the KB model when combined with either doxorubicin (*in vitro*) or PLD (*in vivo*). Overall, effective combination of any tested drug with vintafolide against a cell line or tumor model seems to be dependent on the FR expression levels of the cell and its sensitivity to DAVLBH. We have observed greater synergy against cell lines that express higher levels of FR and are more sensitive to DAVLBH. Because vindesine and DAVLBH do not use the FR pathway to enter the cells, there was no observed synergy with that combination.

As a vinca alkaloid, DAVLBH is a cell-cycle–specific (M phase) agent, which means that it would be most active when delivered into the cells during the M phase of cell division. Because at any given time a relatively small pro-

portion of proliferating tumor cells are in mitosis, transient exposure to a bolus-administered, nontargeted vinca alkaloid (i.e., DAVLBH or vindesine) would not likely be able to harm every cancer cell within a solid tumor. But with an FR-targeted drug like vintafolide, specific high-affinity binding to the FR allows it to dock to the cancer cell surface for prolonged retention within the tumor tissue[2]. When combined with its moderately slow FR-recycling rate[3], it is likely that vintafolide remains associated with the tumor cells for multiple M phases of the tumor cell cycle. In other words, unlike the untargeted vinca alkaloids, vintafolide therapy may increase drug exposure to the tumor and promote greater therapeutic effects, including in the context of combination therapy.

After initial tumor debulking surgery, a majority of patients with ovarian cancer receive a first-line combination regimen that comprises a platinum drug (cisplatin or carboplatin; ref. 26) and a taxane (paclitaxel or docetaxel; refs. 27, 28). To address the potential of vintafolide to replace one of these drugs, we evaluated the antitumor effect of combinations in which either the taxane or the platinum agent was substituted with vintafolide. When a taxane was replaced with vintafolide, the combinations with cisplatin and carboplatin produced remarkable antitumor activity in our models, with up to 100% cure rates. When a platinum agent was replaced with vintafolide, the combinations with paclitaxel and docetaxel also produced notable antitumor activity, also with up to 100% cure rates. In all four combinations, the antitumor activity of the combinations was far superior to that observed with any of the individual drugs tested alone. In addition, combining vintafolide with any of these agents was found not to increase the inherent toxicity/weight loss above that produced by the untargeted single agents.

Topoisomerase I inhibitors, such as topotecan and irinotecan, have also been shown to be effective treatment options for patients with advanced or relapsed ovarian cancer (29, 30). These agents are generally tolerated, with associated hematologic toxicities being manageable, reversible, and noncumulative. When combined with vintafolide, both topotecan and irinotecan produced superior activity over single-agent therapy. In fact, CRs and cures were only observed in the combination cohorts. Surprisingly, the health of the treated mice in the combination cohorts was slightly better as compared with the single-agent cohorts. Although the reason for this effect is not understood at this time, it is reproducible and warrants further study into this apparent drug–drug interaction.

Combination therapy may be especially useful when deployed in a cell-targeted therapy scenario because (i) tumor cells can express varying levels of the cell surface receptor, and (ii) some cancer cells may not express the receptor at all. This

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[2]Greater than 90% of ^{111}In-DTPA-folate tumor uptake was retained in the tumor at a 24 h time point, when compared to a 30 minute time point (26).

[3]Folate conjugate internalization has a rate of $1–3 \times 10^5$ molecules/h, with 10–25% internalized in 6 h across various cell lines (27).

can be challenging for targeted agents such as vintafolide that are constructed using cytotoxic moieties that must bind to and be internalized within receptor-expressing cells to exert their effect. FR "heterogeneity" within tumor lesions is a concern, but it may be overcome by the use of vintafolide in a noncompetitive combination setting using drugs that have different mechanisms of action. Other possible mechanisms of reduced activity, such as emergence of resistant cell populations, or FR-negative clones that cannot be effectively treated with single-agent therapy, may also be obviated by using a rational combination approach.

The vintafolide/PLD combination therapy provided an enhanced therapeutic benefit and seemed to be synergistic in various FR-positive tumor models in our laboratory. In the clinic, vinca alkaloids, such as vinorelbine (31), have shown good activity against cisplatin-resistant ovarian cancer, with a favorable toxicity profile. PLD is also well tolerated with nonoverlapping toxicity to vinca alkaloids, and it has well-documented clinical activity against ovarian cancer. On the basis of the preclinical results presented above, together with positive phase II single-agent results of vintafolide for treatment of advanced ovarian cancer (24), our team initiated a randomized phase II trial (PRECEDENT) comparing PLD versus a vintafolide/PLD combination in women with platinum-resistant ovarian cancer (PROC). The trial was completed in 2012, and results confirmed that the vintafolide/PLD combination provided, for the first time, a statistically significant higher progression-free survival rate in FR-positive patients when compared with PLD alone (1) On the basis of these promising results, the vintafolide/PLD combination is currently being evaluated in the PROCEED trial (sponsored by Merck and Endocyte), which is a global, randomized, phase III study in women with PROC.

Conclusions

The high percentage of CRs and 90-day survivors (i.e., cures) in seven preclinical, binary treatment regimens highlighted the potential of vintafolide to yield enhanced clinical therapeutic effect with these agents in a combination setting. Our preclinical findings, thus, provide a rationale for the design of clinical studies combining vintafolide with approved chemotherapeutic agents such as PLD, platinum agents, topoisomerase inhibitors, and taxanes, with the goal of achieving improved efficacy, acceptable safety, and reduced long-term complications to patients.

Authors' Contributions
Conception and design: J.A. Reddy, C.P. Leamon
Development of methodology: J.A. Reddy, R. Dorton, J. Guan, C.P. Leamon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.A. Reddy, R. Dorton, A. Bloomfield, M. Nelson, M. Vetzel, J. Guan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.A. Reddy, R. Dorton, M. Vetzel, J. Guan
Writing, review, and/or revision of the manuscript: J.A. Reddy, C.P. Leamon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.A. Reddy, M. Vetzel
Study supervision: J.A. Reddy

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