High Levels of Expression of P-glycoprotein/Multidrug Resistance Protein Result in Resistance to Vintafolide

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Abstract

Targeting surface receptors overexpressed on cancer cells is one way to specifically treat cancer versus normal cells. Vintafolide (EC145), which consists of folate linked to a cytotoxic small molecule, desacetylvinblastine hydrazide (DAVLBH), takes advantage of the overexpression of folate receptor (FR) on cancer cells. Once bound to FR, vintafolide enters the cell by endocytosis, and the reducing environment of the endosome cleaves the linker, releasing DAVLBH to destabilize microtubules. Vintafolide has shown efficacy and improved tolerability compared with DAVLBH in FR-positive preclinical models. As the first FR-targeting drug to reach the clinic, vintafolide has achieved favorable responses in phase II clinical trials in FR-positive ovarian and lung cancer. However, some FR-positive patients in these clinical trials do not respond to vintafolide. We sought to identify potential biomarkers of resistance to aid in the future development of this and other FR-targeting drugs. Here, we confirm that high P-glycoprotein (P-gp) expression was the strongest predictor of resistance to DAVLBH in a panel of 359 cancer cell lines. Furthermore, targeted delivery of DAVLBH via the FR, as in vintafolide, fails to overcome P-gp-mediated efflux of DAVLBH in both in vitro and in vivo preclinical models. Therefore, we suggest that patients whose tumors express high levels of P-gp be excluded from future clinical trials for vintafolide as well as other FR-targeted therapeutics bearing a P-gp substrate. Mol Cancer Ther; 15(8): 1998–2008. ©2016 AACR.

Introduction

Vintafolide (EC145) relies on the folate receptor (FR, FRα, FR1, or FOLR1; referred to henceforth as FR) to gain entry into cancer cells. All cell types usually obtain their nutritional needs for folic acid through the reduced folate carrier (RFC), not through FR. A reduced form of folic acid, tetrahydrofolate, is a coenzyme for acid through the reduced folate carrier (RFC), not through FR. A cells. All cell types usually obtain their nutritional needs for folic acid through the reduced folate carrier (RFC), not through FR. A reduced form of folic acid, tetrahydrofolate, is a coenzyme for acid through the reduced folate carrier (RFC), not through FR. A

Once bound to FRs, folic acid enters the cell via endocytosis to be further metabolized. Numerous reports have shown that the FR, expressed at low levels in normal tissues, is overexpressed in many tissues including ovarian, lung, breast (1–6). Hence, FR is an attractive cancer-specific target to transport a cytotoxic drug into the cancer cell. To that end, we have been developing folic acid–linked drugs to specifically target tumor cells and reduce toxicity (7–9).

Vintafolide is an FR-targeting drug in which the microtubule destabilizing agent, desacetylvinblastine hydrazide (DAVLBH), is linked to a folate peptide (10, 11). Vintafolide is extremely potent in inhibiting the growth of FR-expressing tumor cell lines in vitro with IC50 values in the low nanomolar range (11). In vivo, vintafolide treatment of FR-expressing xenograft models results in complete tumor regression and cures with little toxicity. Importantly, when the same xenograft models were treated with the molar equivalent dose of DAVLBH, toxicity was observed and responses were not maintained after the treatment was halted (10). These results demonstrated that FR targeting is an advantageous strategy for cancer therapeutics and led to the initiation of several clinical trials involving vintafolide, the first FR-targeting agent of its kind to reach patients.

In phase I trials, vintafolide was well tolerated. Grade 1 or 2 constipation, nausea, vomiting, fatigue, and peripheral neuropathy were the most common reported side effects (12, 13). In the randomized phase II PRECEDENT trial, the combination of vintafolide with pegylated liposomal doxorubicin (PLD) had a statistically significantly better progression-free survival (PFS) rate than PLD alone in platinum-resistant ovarian cancer patients (14). Such promising clinical results warranted further

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Materials and Methods

Materials

Vintafolide (Endocyte, Inc.) clinical vial solutions in sterile water were used in all experiments. DAVLBH was synthesized at Endocyte, Inc. Calcein AM, zosuquidar HCl, elacridar, and verapamil were purchased from Sigma. Pegylated liposomal doxorubicin (PLD, trade name DOXIL) was obtained from the Purdue Pharmacy; paclitaxel was purchased from A.K. Scientific. Unless otherwise noted, all other common reagents were purchased from Sigma or Life Technologies. The 359 cell lines used in the drug response versus RNA panel were obtained and cultured directly from ATCC. The KB cell line was originally obtained from ATCC and cultured in reduced folate media. Genetica, NC-confirmed the authenticity of KB and its drug-resistant (DR) derivatives.

Bioinformatics and statistical methods

Gene expression data for the cell line panel were obtained from Affymetrix (Affy) microarray data available from the published source (15). Viability data for vinblastine and paclitaxel (Fig. 1A) were obtained from a Merck internal database, and viability data for DAVLBH were obtained from CellTiter-Glo proliferation assays described here (Fig. 1B and C). We calculated relative proliferation rates from measured luminescence values in control (dimethyl sulfoxide, DMSO) or drug-treated cells by evaluating proliferation rates from measured luminescence values in control supernatant for human folate receptor 1 (average concentration 10⁻⁷–10⁻⁶ M/l; NM_000802.2; GeneCopoeia cat. #LP-C0250-Lx-105-0205-S). Controls received polybrene only. Selection with puromycin at 3 μg/ml began 48 hours after transduction. After selection, cells were harvested with 0.05% trypsin, incubated with either mouse IgG-PE isotype control (R&D Systems #IC002P) or mouse monoclonal anti-human IgG-FOLR1-PE (R&D Systems #FAB5646P) antibody in Hank's balanced salt solution (HBSS) containing 1% BSA, and washed with HBSS. Surface expression of FR1 on live cells was measured in a BD flow cytometer and data were analyzed with FlowJo software (Treestar).

Chosen for its P-gp expression and resistance to P-gp substrates, the NCI/ADR-RES ovarian cancer cell line was obtained from ATCC and grown in complete RPMI1640 medium (containing 2.3 μmol/L folic acid) supplemented with 10% FBS (HyClone, GE Healthcare). Also obtained from ATCC, the KB cell line (2.5 × 10⁶ FRs/cell; ref. 16), a human nasopharyngeal cell line with markers of HeLa cervical cancer origin and high FR expression, was grown in folate-free RPMI1640 (FMRPMI, Gibco Life Technologies cat. #27016) supplemented with 10% FBS. Upper 5′/3′ primer cacgagcaggtgctcagcgagtagcaca and lower 5′/3′ primer agaggtagctgagcagcaca were used to generate the full-length human folate receptor alpha (FRα) from KB cells via PCR. The purified PCR product was cloned into an expression vector from Invitrogen (Life Technologies), pCDNA3.1D V5-His-TOPO, following the manufacturer’s instructions. The NCI/ADR-RES cells were transduced using Invitrogen’s LipofectaminePlus reagent following the supplied protocol. Transformants were selected by culturing in FMRPMI with 10% FBS and 1 mg/mL G418. Colonies were transferred to 24-well plates in duplicate, using sterile cloning cylinders (Fisher cat. #07-907-10), and grown to 50% confluence. Each duplicate was then incubated with EC17 (folate-fluorescein; Endocyte, Inc.) for 1 hour, washed with PBS, and then observed with a Diavert fluorescence microscope. The most highly fluorescent clone, NCI/ADR-RES-FR, was selected for further growth. Analysis by a previously described method (16) shows that these NCI/ADR-RES-FR cells express approximately 2.5 × 10⁶ FRs/cell as compared with the parental cells which express <1 × 10³ FRs/cell.

Generation of KB-DR150 and KB-DR300 cells

The drug-sensitive FR-expressing KB cell line served as a parental line to generate drug-resistant cell lines through exposure to increasing levels of DAVLBH. DAVLBH stock solutions were made

study regarding patient selection to increase probability of success.

To gain insight for future trials, we sought to better understand mechanisms of resistance to vintafolide in preclinical models. The P-glycoprotein (P-gp) pump binds several drugs including chemotherapeutic agents and transports them outside of the cell, leading to drug resistance. Here we show that high levels of P-gp expression predict resistance to DAVLBH and vintafolide in both in vitro and in vivo cancer models indicating that targeted delivery via FR does not overcome P-gp–mediated drug efflux. Our findings have implications for indication and patient selection for future clinical trials of vintafolide as well as other FR-targeting drugs with P-gp substrate warheads.

Generation of FR-positive cell lines

Kidney cancer cell lines were obtained from ATCC. A498 and A704 were grown in Eagle’s minimum essential medium (ATCC cat. no. 30–2003) supplemented with 10% FBS, and 786-O was grown in RPMI 1640 supplemented with 10% FBS. When cells reached approximately 70% confluence in a T25 flask, they were transduced with virus by adding polybrene and 40 μl lentiviral supernatant for human folate receptor 1 (average concentration 10⁻⁷–10⁻⁶ M/l; NM_000802.2; GeneCopoeia cat. #LP-C0250-Lx-105-0205-S). Controls received polybrene only. Selection with puromycin at 3 μg/ml began 48 hours after transduction. After selection, cells were harvested with 0.05% trypsin, incubated with either mouse IgG-PE isotype control (R&D Systems #IC002P) or mouse monoclonal anti-human IgG-FOLR1-PE (R&D Systems #FAB5646P) antibody in Hank’s balanced salt solution (HBSS) containing 1% BSA, and washed with HBSS. Surface expression of FR1 on live cells was measured in a BD flow cytometer and data were analyzed with FlowJo software (Treestar).

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Generation of KB-DR150 and KB-DR300 cells

The drug-sensitive FR-expressing KB cell line served as a parental line to generate drug-resistant cell lines through exposure to increasing levels of DAVLBH. DAVLBH stock solutions were made
in DMSO. Appropriate volumes of the sterile stock solutions were added to cell culture flasks to achieve the desired concentration of DAVLBH-supplemented media. DAVLBH concentrations were increased when cell death was no longer visible and growth rate had stabilized, usually every 2 to 4 weeks. Thus, drug-resistant KB cell lines were selected in vitro over the course of 6 months, through exposure to the following stepwise-increased concentrations of DAVLBH: 5, 10, 30, 50, 70, 100, 150, and 300 nmol/L. After approximately 26 passages to select for resistance, the KB-DR150 line was maintained in 150 nmol/L DAVLBH. After approximately 30 passages, the KB-DR300 line was maintained in 300 nmol/L DAVLBH.

Cell growth inhibition studies

KB and kidney cancer cell lines were seeded at 2,000–5,000 cells in 95 μL media per well in 96-well white-wall, clear-bottom plates. The parental line was seeded in complete medium (RPMI with 10% FBS, 10 mmol/L HEPES, 1X NEAA, and 1 mmol/L sodium pyruvate), and the line engineered to express FR1 was seeded in reduced folate medium (FFRPMI with 5% FBS, 10 mmol/L HEPES, 1X NEAA, and 1 mmol/L sodium pyruvate). The next day, cells were treated with either DAVLBH or vintafolide at the indicated concentrations. Two hours after treatment, media containing vintafolide were removed and wells were replenished with fresh, drug-free media. Untreated control plates were assayed by CellTiter-Glo on the same day as experimental cells were treated. For in vitro experiments with the P-gp inhibitor elacridar, cells were treated with a 9-point, 1:3 dilution series with a top concentration of 1 μmol/L of either DAVLBH or vintafolide (Fig. 2B; Supplementary Table S1), and each drug was combined with either 0.5 μmol/L elacridar or DMSO. Experimental plates were assayed by CellTiter-Glo and read on a Perkin Elmer Victor 72 hours after compound treatment. Cancer cell lines KB, KB-DR150, KB-DR300, NCI/ADR-RES, and NCI/ADR-RES-FR were maintained in FFRPMI containing 10% heat-inactivated FBS at 37°C in a 5% CO2/95% air-humidified atmosphere with no antibiotics. Exponentially growing cells were seeded in 24-well plates, 24 hours before treatment with graded concentrations of drugs. Cells receiving vintafolide were pulsed for 2 hours at 37°C, rinsed 3 times with 0.5 mL of medium, and then chased in 1 mL of fresh medium for up to 72 hours. Cells treated with DAVLBH or paclitaxel were treated continuously for 72 hours. Cells were then treated with fresh medium containing [3H]-thymidine for 2 hours at

Figure 1.

High ABCB1 mRNA is associated with resistance to microtubule-targeting agents in cell lines. A, P-gp expression strongly associates with lack of response to vinblastine (P = 0.004) and paclitaxel (P = 1E−29) in a panel of N = 359 solid cancer cell lines (15). Gene expression levels are color-coded from red (high expression) to green (low expression), with black indicating mean expression. Drug response levels are color-coded from red (resistance) to green (sensitivity), with black indicating no change. For drug response quantification, we chose cell line viability at the concentration maximizing the SD of the viabilities across cell lines (0.01 μmol/L for both considered drugs). The association between gene expression and drug response is quantified through a Fisher test using dichotomized gene expression (threshold = 2.3 AU) and dichotomized drug response (threshold = 70% viability). B, proliferation assays were performed in a subset of the cell line panel treated with DAVLBH. Each point represents one cell line. For these lines, ABCB1 expression levels within 1.5 AU above and below 1.9 correlate with DAVLBH IC50 (μmol/L) according to Pearson, Spearman, and Kendall analyses. C, sensitivity to DAVLBH in a proliferation assay was defined as IC50 < 0.75 μmol/L (red), and resistance was defined as IC50 > 0.75 μmol/L (blue). All sensitive cell lines show low ABCB1 expression, while resistant cell lines have both an ABCB1-high and low population.
37°C, washed with PBS, and then treated with ice-cold 5% trichloroacetic acid (TCA). After 15 minutes, TCA was aspirated and the cells solubilized by the addition of 0.25 N sodium hydroxide for 15 minutes at room temperature. Solubilized samples were transferred to scintillation vials containing Eco-Lume scintillation cocktail (MP Biomedicals, LLC) and counted in a liquid scintillation counter.

MRK-16 antibody analysis

P-gp on various cells was evaluated by flow cytometry using the anti-P-gp MRK-16 antibody (Enzo Life Sciences). One million cells were incubated with the primary antibody for 90 minutes at 4°C and then washed twice. Cells were incubated with secondary FITC goat anti-mouse antibody (Lifespan Biosciences) for 15 minutes in the dark and washed again. FITC signal was measured on a Beckman Coulter FC500 flow cytometer and results were analyzed with FlowJo. Negative controls were prepared in each experiment by replacing specific primary antibody with relevant immunoglobulin isotype. Results are expressed in mean fluorescence intensity (MFI) units.

Calcein efflux assay

Cells (5 × 10⁶) were washed twice in PBS and preincubated for 15 minutes at 37°C in RPMI containing 25 μmol/L verapamil or 0.5% DMSO. The cells were then incubated with 1 μmol/L calcein-AM (Molecular Probes) for 30 minutes at 37°C. The cells were washed with PBS containing 0.1% BSA, centrifuged (5 minutes, 3,000 g), and resuspended in 1% paraformaldehyde in PBS and incubated at room temperature for 30 minutes. The cells were washed with PBS containing 0.1% BSA, centrifuged (5 minutes, 3,000 g), resuspended in 0.1% BSA/PBS, stored at 4°C, and measured within 2 hours on a Gallios flow cytometer (Beckman Coulter). The activity of the multidrug transporters was expressed as MFI values measured in the presence and absence of verapamil (MFIv and MFIo, respectively). The % calcein efflux for each cell line was determined by using the formula 100 \((\frac{MFIv}{MFIo}) \times 100\) (17).

Xenograft studies

Four- to seven-week-old female nu/nu mice (Charles River) were maintained on a standard 12-hour light–dark cycle and fed

Figure 2.

FRI expression does not confer sensitivity to vintafolide. A, only cells transduced with lentiviral FRI show surface expression via flow cytometry. B, cells with and without FRI expression responded similarly to DAVLBH and vintafolide. Data from three separate experiments are plotted for each dose–response curve. C and D, DAVLBH (C) and vintafolide (D) dose–response assay in FR-positive KB cells. KB (●), KB-DR150 (○), or KB-DR300 (▲) cells were treated with increasing concentrations (10⁻⁸ mol/L to 10⁻⁵ mol/L) of DAVLBH for 72 hours (C) or vintafolide for 2 hours (D) followed by a 5-day chase in media. Cell viability was determined by 3H-thymidine incorporation into DNA. Data represent the average ± 1 SD. (n = 3).
ad libitum with a low-folate chow (TD.00434, Harlan Teklad) for the duration of the experiment. KB-DR150 cells were chosen for xenograft studies because they maintained some sensitivity to vintafolide and PLD, whereas KB-DR300 had become completely resistant. KB-DR150 cells (1 × 10^6 per nu/nu mouse) in 100 μL were injected in the subcutis of the dorsal medial area. Mice were divided into groups of 5, and freshly prepared test articles were injected through the lateral tail vein under sterile conditions in 200 μL PBS. Intravenous (i.v.) treatments of indicated compounds were typically initiated on day 7 after tumor cell implantation when the KB-DR150 tumors were approximately 95–184 mm^3 in volume. The mice in the control groups received no treatment. Growth of each subcutaneous tumor was followed by measuring the tumor 3 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 \[ V = \frac{1}{2} \times L \times W^2 \], 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. All animal housing, care, and procedures were followed according to Purdue Animal Care and Use Committee (PACUC)-approved protocols.

Patient-derived xenograft studies
Renal cancer patient-derived xenograft (PDX) models ST660, ST544, ST578, and CTG-0804 were propagated subcutaneously in 8–12 week old immunodeficient female mice at low passage numbers to maintain tumor heterogeneity. ST660, ST544, and ST578 efficacy studies were carried out at START (South Texas Accelerated Research Therapeutics), and CTG-0884 was tested at Champions Oncology, Inc.. Tumor fragments were implanted unilaterally in the flank. Animals were randomized into treatment groups of 10 and dosing initiated (day 0) when the mean starting tumor volumes reached approximately 150–250 mm^3. In vivo studies were performed in a similar manner as for KB-DR150, with the following exceptions. All compounds were administered by intraperitoneal injection (10 mL/kg dosing volume) for all treatments, and the vehicle was PBS, pH 7.2. Vintafolide was dosed at 1.5 and 3.8 mg/kg three times a week or 4.5 and 11.4 mg/kg once a week. DAVLBH was dosed at 0.77 mg/kg three times a week, which was the maximally tolerated dose (MTD). Animals were dosed for 3 weeks, and were monitored for up to 32 days thereafter relative to the start of dosing, depending upon the growth rate of the particular model. All animal housing, care, and procedures were followed according to Merck Institutional Animal Care and Use Committee (IACUC)-approved protocols.

FRI-positive PDX models were chosen for efficacy studies based on immunostaining of tissue microarrays (TMA) containing panels of PDX tumor fragments. TMAs originated from START or Champions Oncology, Inc. TMAs were stained with the Biocare Medical FRzl IHC Assay Kit (cat # BRI4006KAA), following the manufacturer’s protocol.

mRNA expression analysis via qRT-PCR
DNA tumor pieces of approximately 30 mg were homogenized in RLT buffer (QIAGEN) supplemented with 1% v/v β-mercaptoethanol on a TissueLyser. RNA extraction and on-column DNase digestion were performed with the QIAGEN RNeasy kit according to manufacturer’s protocol. cDNA was prepared using SuperScript VILO master mix. Quantitative real-time PCR was performed using TaqMan Gene Expression Master Mix and the following TaqMan assays (Applied Biosystems, Life Technologies): ABCB1 (assay Hs00184500_m1) and GAPDH (Hs99999905_m1). Assays were run in 384-well optical reaction plates in a 7900HT Applied Biosystems machine and results were analyzed with SDS software and Excel.

Figure 3.
P-gp accounts for innate and acquired drug resistance: anti-p-glycoprotein expression analysis. Cells were incubated with anti-p-glycoprotein MRK-16 primary antibody (■) or its isotype control (□) for 90 minutes at 4°C, washed, incubated with a fluorescein-labeled goat anti-mouse secondary antibody for 15 minutes, washed, and analyzed by flow cytometry.

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Results

High ABCB1 mRNA is associated with resistance to microtubule-targeting agents in cell lines

An unsupervised clustering of compound treatments on 359 solid tumor cell lines shows that, when cells express high levels of ABCB1 mRNA, they are resistant to microtubule-targeting agents such as vinblastine and paclitaxel (Fig. 1A). These data corroborate previous observations that P-gp, the drug resistance pump encoded by ABCB1, is a mechanism of resistance for this class of compounds (18). As vinblastine is closely related to DAVLBH, the warhead of vintafolide, we hypothesized that ABCB1 mRNA level would also predict resistance to DAVLBH. As expected, we found that levels of ABCB1 mRNA correlate with DAVLBH IC50 values (Fig. 1B), and cell lines sensitive to DAVLBH (IC50 < 0.75 µmol/L) all have low ABCB1 expression (Fig. 1C). However, many DAVLBH-insensitive lines do not express elevated P-gp, suggesting that high P-gp expression level is not the only mechanism of resistance to DAVLBH. Since transcriptional profiles, DNA copy number, and mutational analysis are available for all of these cell lines, we looked for additional biomarkers of resistance. In the available data, P-gp expression was the only marker that correlated significantly with vinblastine resistance in the 359 cell line panel.

High levels of expression of FR cannot overcome P-gp-mediated resistance in vitro

We hypothesized that increasing the expression of FR1 in intrinsically P-gp–expressing or DAVLBH-resistant cell lines may render the cells sensitive to vintafolide by increasing drug uptake (19). Our gene expression analysis of 359 cancer cell lines (Fig. 1) demonstrated that kidney cancer cell lines tend to express high levels of P-gp. Three kidney cancer cell lines with high P-gp and low FR1 surface expression, A498, A704, and 786-O, as confirmed by flow cytometric analysis (Supplementary Fig. S1), were chosen for transduction with lentiviral particles encoding FR1. Upon selection, a strong increase in FR1 expression in all three transduced cell lines was confirmed by flow cytometric analysis (Fig. 2A, Supplementary Fig. S2). All six cell lines (parental and FR-expressing) were treated with increasing concentrations of DAVLBH or vintafolide as indicated for 72 hours and the percentage of viable cells was determined by the CellTiter-Glo proliferation assay. FR1–overexpressing, P-gp–high cell lines did not show any significant increase in sensitivity to vintafolide over the parental lines (Fig. 2A and B and Supplementary Fig. S3; Supplementary Table S1). We conclude that when DAVLBH is linked to folate and brought into cancer cells through the folate receptor, it is still susceptible to efflux by P-gp. Therefore, high levels of expression of FR cannot overcome P-gp–mediated resistance.

To create an FR-expressing model of acquired resistance to DAVLBH, the DAVLBH- and vintafolide-sensitive KB cell line was treated with increasing concentrations of DAVLBH. KB-DR150 and KB-DR300 cells were derived from clones that survived final DAVLBH concentrations of 150 nmol/L and 300 nmol/L, respectively. These cells were then evaluated in vitro for their sensitivity to DAVLBH and vintafolide. Both KB-DR150 and KB-DR300 cells render the cells sensitive to vintafolide by increasing drug uptake (19). Our gene expression analysis of 359 cancer cell lines (Fig. 1) demonstrated that kidney cancer cell lines tend to express high levels of P-gp. Three kidney cancer cell lines with high P-gp and low FR1 surface expression, A498, A704, and 786-O, as confirmed by flow cytometric analysis (Supplementary Fig. S1), were chosen for transduction with lentiviral particles encoding FR1. Upon selection, a strong increase in FR1 expression in all three transduced cell lines was confirmed by flow cytometric analysis (Fig. 2A, Supplementary Fig. S2). All six cell lines (parental and FR-expressing) were treated with increasing concentrations of DAVLBH or vintafolide as indicated for 72 hours and the percentage of viable cells was determined by the CellTiter-Glo proliferation assay. FR1–overexpressing, P-gp–high cell lines did not show any significant increase in sensitivity to vintafolide over the parental lines (Fig. 2A and B and Supplementary Fig. S3; Supplementary Table S1). We conclude that when DAVLBH is linked to folate and brought into cancer cells through the folate receptor, it is still susceptible to efflux by P-gp. Therefore, high levels of expression of FR cannot overcome P-gp–mediated resistance.

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TCGA: Expression of ABCB1

Kidney renal papillary cell carcinoma
Adrenocortical carcinoma
Liver hepatocellular carcinoma
Pheochromocytoma and paraganglioma
Kidney renal clear cell carcinoma
Brain lower grade glioma
Colon adenocarcinoma
Rectum adenocarcinoma
Cholangiocarcinoma
Glioblastoma multiforme
Pancreatic adenocarcinoma
Prostate adenocarcinoma
Mesothelioma
Stomach adenocarcinoma
Thyroid carcinoma
Lymphoid neoplasm diffuse large B-cell lymphoma
Uterine corpus endometrial carcinoma
Esophageal carcinoma
Breast invasive carcinoma
Testicular germ cell tumors
Sarcoma
Lung adenocarcinoma
Skin cutaneous melanoma
Thymoma
Bladder urtheal carcinoma
Cervical squamous cell carcinoma and endocervical adenocarcinoma
Head and neck squamous cell carcinoma
Lung squamous cell carcinoma
Uterine carcinosarcoma
Kidney chromophobe
Ovarian serous cystadenocarcinoma
Uveal melanoma

B

Vehicle Control-TIW
DAVLBH 0.77 mg/kg-TIW
Vintafolide 1.5 mg/kg-TIW
Vintafolide 3.8 mg/kg-TIW
Vintafolide 4.5 mg/kg-QW
Vintafolide 11.4 mg/kg-QW

ST660
ST544
ST578
CTG0804

FOLR1
Isotype

C

ABCB1 mRNA expression in human renal carcinoma PDX Models

Mean quantity relative to GAPDH

A2780 A704 CTG0804 ST544 ST578 ST660

-2.5 -2 -1.5 -1 -0.5 0 0.5 1 1.5 2

ABCB1 expression (RPKM, log 10)
were found to be extremely insensitive to DAVLBH as well as to its folate conjugate, vintafolide, with IC_{50} values in the micromolar range or incalculable (Fig. 2C and D). In contrast, the parental FR-positive KB cells were highly sensitive to DAVLBH and vintafolide with IC_{50} values in the low nanomolar range.

P-gp accounts for innate and acquired drug resistance
To validate the role of P-gp in resistance to DAVLBH and vintafolide, we assessed surface P-gp expression and functionality in FR-expressing resistant cell lines using flow cytometry and a functional calcine efflux assay, respectively. Flow cytometry with the anti-P-gp MRK-16 antibody demonstrated that the surface expression of P-gp is much higher in all three drug-resistant cell lines tested (KB-DR300, NCI/ADR-RES, and NCI/ADR-RES-FR) than in the drug-sensitive KB cells (Fig. 3). Quantitative PCR analysis of ABCB1 mRNA expression also revealed significantly elevated levels in KB-DR300 versus KB, with respective RQ values of 591.7 ± 234.4 versus 1.1 ± 0.4. Similarly, in the calcine efflux assay all four drug-resistant cell lines tested (KB-DR150, KB-DR300, NCI/ADR-RES, and NCI/ADR-RES-FR) displayed high calcine efflux values as compared with KB cells, indicating the presence of functional P-gp (Supplementary Table S2). These data demonstrate that P-gp expression is a mechanism of both innate and acquired resistance to DAVLBH in vitro and this resistance is maintained even when the drug enters the cell through the folate receptor.

Inhibiting or overwhelming P-glycoprotein can enhance vintafolide activity
To provide further evidence that P-gp causes vintafolide resistance, we tested whether the P-gp inhibitor elacridar confers sensitivity to vintafolide and DAVLBH in a panel of kidney cancer cell lines expressing P-gp (Supplementary Table S1). Elacridar addition consistently enhanced response to DAVLBH in P-gp-expressing cells, regardless of FR status, by 2-fold or more. Elacridar increased sensitivity to vintafolide only in FR-expressing cell lines by 5-fold or more. KB cells served as a P-gp negative, FR-positive control where addition of elacridar did not further sensitize cells to either treatment. These results further suggest that high levels of P-gp expression cause resistance to DAVLBH and vintafolide in vitro, as inhibition of the P-gp pump abrogates resistance.

We went on to test the effect of combining a P-gp inhibitor with vintafolide in mice bearing P-gp-expressing FR-positive KB-DR150 tumors. Vintafolide and zosuquidar were administered, alone and in combination, at 2 μmol/kg and 25 mg/kg, respectively, three times a week for 2 weeks. As shown in Fig. 4A, single-agent vintafolide produced modest antitumor activity with 20% partial response (PR) while zosuquidar alone did not have any effect on these tumors by the end of the study. However, the vintafolide/zosuquidar combination generated greater antitumor activity: 20% of the animals displayed complete response (CR) and stable disease (SD) was observed in 40% of the animals. Addition of a P-gp inhibitor enhanced the efficacy of vintafolide in reducing tumor volume of the KB-DR150 model.

As inhibiting P-gp appears to enhance vintafolide’s activity, next we hypothesized that occupying P-gp with another substrate may also increase the efficacy of vintafolide. Doxorubicin is a well-established P-gp substrate and its clinical form, pegylated liposomal doxorubicin (PLD), is a standard of care for the treatment of ovarian cancer (19, 20). This indication may be an ideal candidate for treatment with vintafolide due to its typically high levels of FR expression. In the KB-DR150 xenograft tumor model, vintafolide and PLD were administered, alone and in combination, at 2 μmol/kg (three times a week) and 5 mg/kg (twice a week), respectively, for 2 weeks (Fig. 4B). Single agent vintafolide resulted in stable disease in only 40% of the mice (Fig. 4) while PLD itself did not have any antitumor effect. The variation of response to vintafolide alone ranging from PR to SD in the P-gp inhibitor combination experiment versus in the P-gp substrate combination experiment is expected experimental variation. Yet when vintafolide was combined with PLD, 100% of the animals in the combination group showed partial responses. These in vivo studies demonstrate that occupying the P-gp pump with a specific inhibitor (zosuquidar) or an additional substrate (doxorubicin) enhances the antitumor activity of vintafolide in FR-expressing resistant tumors.

Vintafolide is less likely to be an effective treatment for indications with high P-gp expression such as kidney cancer
Our in vitro cell line panel data suggested that kidney cancer tends to express high levels of P-gp and is resistant to microtubule-disrupting agents. These data together with our experiments demonstrating that high levels of FR expression cannot overcome P-gp-mediated resistance suggest that kidney cancer may not be the best indication for vintafolide in the clinic. To evaluate the potential clinical relevance of this hypothesis, we examined P-gp expression levels in cancer patient samples from The Cancer Genome Atlas (TCGA; Fig. 5A). Similar to cancer lines, kidney tumors from TCGA samples have high levels of ABCB1.

Next, the in vivo activity of vintafolide and DAVLBH was assessed in four renal cancer PDX models, ST660, ST578, ST544, and CTG-0804 (Fig. 5B). All four models expressed membrane FR1 as determined by IHC (Fig. 5B insets). Despite expression of FR, PDX models ST660, ST578, and ST544 were not responsive to vintafolide treatment. These three renal cancer models appear to
be inherently resistant to vinca alkaloids, as DAVLBH also had minimal antitumor activity. In contrast, CTG-0804 was responsive to both DAVLBH and vintafolide therapy. Quantitative real-time PCR analysis of RNA from these four PDX tumors revealed detectable levels of ABCB1 message relative to the negative control line A2780, but lower levels than that of the A704-positive control line (Fig. 5C). Notably, of the four PDX models tested, CTG-0804 expressed the lowest level of ABCB1. Together, these results provide further evidence that high ABCB1 expression may correlate with resistance to vintafolide, and provide a possible explanation for lack of efficacy in the FR-positive tumor models.

Platinum-resistant ovarian cancer patients may express high levels of P-gp

The initial clinical trials with vintafolide were conducted in platinum-resistant ovarian cancer (PROC). Moffitt Cancer Center ovarian cancer patients not involved in the trial have low ABCB1 expression levels (Fig. 6), with only a small subset of patients expressing high levels of ABCB1. Notably, the small number of ovarian cancer patients positive for ABCB1 expression stayed significantly less time on carboplatin treatment than their ABCB1-negative counterparts, which may indicate carboplatin resistance (Fig. 6). Furthermore, ABCB1-positive tumors tend to have lower FR1 expression (Supplementary Fig. S4), which may further prevent a favorable response to vintafolide. As carboplatin is not a P-gp substrate, we would not necessarily expect carboplatin-resistant patients or cell lines to express high levels of P-gp. However, this post hoc analysis suggests that PROC may be associated with high P-gp. Therefore, like kidney cancer, PROC may not be the optimal clinical indication for vintafolide, we sought to better understand the mechanisms of resistance to this first-in-class drug. Knowledge gained from the study of vintafolide may also shed light on possible resistance mechanisms for IMGN853, as DM4 is also a P-gp substrate.

Our drug sensitivity data in a large panel of cancer cell lines confirmed previous studies demonstrating that microtubule-disrupting agents, such as vinblastine and paclitaxel, are P-glycoprotein (P-gp, MDR1) substrates (18, 23, 24). Not surprisingly, high ABCB1 expression also predicts resistance to DAVLBH, a derivative of vinblastine (Fig. 1B). However, since the 1990s, the combination of P-gp inhibitors with chemotherapeutics has had marginal or no clinical success due to toxicities and unpredictable pharmacokinetic effects on the chemotherapeutic agents (18, 24, 25). While the use of P-gp inhibitors has not been clinically viable, they serve as great preclinical tools in the laboratory setting (18, 24). Using the P-gp inhibitors elacridar, zosuquidar, and verapamil, we were able to demonstrate that P-gp is responsible for both intrinsic and acquired resistance to DAVLBH in vitro and in vivo.

We hypothesized that the endocytic entry mechanism of vintafolide through FR could counteract DAVLBH resistance mediated by P-gp efflux at the plasma membrane (19). The data presented in this article derived from P-gp- and FR-expressing cell lines, as well as

Discussion

Chemotherapeutics have demonstrated significant benefit for cancer patients. However, their broadly toxic mechanisms of action, such as DNA intercalation or metabolic interference, routinely cause systemic toxicity due to lack of specificity for cancer cells. In contrast, so-called “targeted cancer therapeutics” exploit mutations or gene expression specific to cancer. Such targeted agents, for example, kinase inhibitors, mAbs, antibody–drug conjugates (ADCs), and small molecule–drug conjugates (SMDCs), all aim to bind a protein expressed preferentially in or on the surface of cancer cells to either shut down a critical pathway or signal for specific cell killing. As an SMDC linked to folate, vintafolide is the first drug of its kind to reach clinical trials. Its design of a ligand (folate) attached to a cytotoxic chemotherapeutic via a cleavable linker is similar to that of ADCs, which usually consist of an antibody attached to a toxin. Since 2013, two ADCs have had success in the clinic (21). IMGN853, a new ADC most closely related to vintafolide, also targets FR and is currently in phase I clinical development (22). Both IMGN853 and vintafolide take advantage of the higher levels of expression of the folate receptor (FR) on cancer cells to gain entry and deliver their chemotherapeutic warheads, DM4 or DAVLBH, respectively (22). In preclinical models, vintafolide showed improved safety and efficacy over untargeted DAVLBH. Early clinical trials confirmed a favorable safety profile for vintafolide. To aid in patient and indication selection for vintafolide, we sought to better understand the mechanisms of resistance to this first-in-class drug. Knowledge gained from the study of vintafolide may also shed light on possible resistance mechanisms for IMGN853, as DM4 is also a P-gp substrate.
PDX models, clearly demonstrate that folate conjugation with endocytic delivery does not reverse P-gp-mediated resistance to DAVLBH. In other words, drug entry via FR does not appear to protect the DAVLBH warhead from being removed from the cell. These results have important implications for future clinical trials of vintafolide and other FR-targeting drugs with P-gp substrate warheads. On the basis of these data and our analysis of P-gp expression in cancer patients (Fig. 5A), we would not recommend trials in kidney and colon cancer unless patients with high ABCB1/P-gp expression are excluded from such trials. Cancer indications with high FR and low ABCB1/P-gp, such as ovarian, breast, and lung, would be best suited for evaluation of resistance to vintafolide.

As shown in Fig. 1C, other factors are also responsible for drug resistance as over half of the cell lines in the resistant population have low levels of the ABCB1 mRNA encoding P-gp. With such a comprehensive dataset, we aimed to identify additional mechanisms of resistance, but these attempts were unsuccessful. Although we recommend excluding patients and indications with high levels of P-gp from vintafolide treatment, cell lines clearly display other determinants of sensitivity that we do not yet understand, which could also exist in patients. We would not expect to identify mechanisms of resistance related to vintafolide uptake using the cell line panel data. Preliminary work with KB cells in increasing concentrations of vintafolide suggests that cells may downregulate FR expression in addition to upregulating P-gp expression. KB cells have also shown decreased FR expression in response to treatment with methotrexate, an antifolate and P-gp substrate (26). Therefore, it is also possible that downregulation of FR could account, in part, for acquired vintafolide resistance observed in the clinic. Other unexpected effects may also occur due to FR’s recently identified role as a transcription factor with promoter activity that responds to intracellular folate levels (27). In addition, mRNA levels do not always reflect protein levels, so the relationship between ABCB1 mRNA expression and translation into P-gp would need to be examined in tumors. Further study is required to identify and characterize resistance mechanisms specific to vintafolide.

With high P-gp expression as our only confirmed vintafolide resistance mechanism at the time of this study, we analyzed ABCB1 levels in a population of ovarian cancer patients at the Moffitt Cancer Center (Fig. 6). Our analysis suggests that the platinum-resistant subset of patients may express high levels of P-gp. Despite promising phase II PRECEDENT trial results leading to a positive opinion from the Committee for Medical Products for Human Use (CHMP) of the European Medicines Agency (EMA) in March 2014, our Phase III PROCEED trial in platinum-resistant ovarian cancer (PROC) was suspended because efficacy in progression-free survival was not observed in an early interim analysis (22). The high P-gp level found in the subset of Moffitt ovarian cancer patients could explain why our phase III trial in PROC was unsuccessful. Caveats of this analysis include the following: (i) we are equating less time spent on carboplatin treatment with carboplatin resistance, which may not be equivalent; and (ii) the Moffitt Center patients (Fig. 6) are not the same as those treated in our trial, so their P-gp expression levels could be different. To provide stronger evidence that P-gp-mediated resistance contributed to the lack of efficacy in the phase III trial, we would need to analyze P-gp expression levels in patients from the trial. Circumventing P-gp-mediated resistance remains a challenge in the clinic.

The preclinical and clinical data with the FR-targeting drug vintafolide have demonstrated that folate conjugation is an effective method to specifically target FR-expressing tumors. Future development of FR-targeting drugs with non-P-gp substrate warheads, such as folate–tubulysin based SMDCs, may have broader therapeutic effects (28). Meanwhile, understanding resistance mechanisms to inform patient and indication selection may increase vintafolide’s success in the clinic moving forward.

Disclosure of Potential Conflicts of Interest
E.V. Schmidt has ownership interest (including patents) in Merck. C.P. Leamon has ownership interest (including patents) in Endocyte Inc. No potential conflicts of interest were disclosed by the other authors.

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