Discovery and Characterization of OC144-093, a Novel Inhibitor of P-Glycoprotein-mediated Multidrug Resistance


ABSTRACT

OC144-093 is a novel substituted diarylimidazole (M, 495) generated using the OntoBLOCK technology, a solid-phase combinatorial chemistry system, in combination with high-throughput cell-based screening. OC144-093 reversed multidrug resistance (MDR) to doxorubicin, paclitaxel, and vinblastine in human lymphoma, breast, ovarian, uterine, and colorectal carcinoma cell lines expressing P-glycoprotein (P-gp) with an average EC50 of 0.032 μM. Inhibition of MDR by OC144-093 was reversible, but the effect persisted for at least 12 h after removal of compound from the culture medium. OC144-093 had no effect on the response to cytotoxic agents by cells in vitro lacking P-gp expression or expressing a multidrug resistance-associated protein (MRP-1). OC144-093 was not cytotoxic by itself against 15 normal, nontransformed, or tumor cell lines, regardless of P-gp status, with an average cytostatic IC50 of >60 μM. OC144-093 blocked the binding of [3H]doxorubicin to P-gp and inhibited P-gp ATPase activity. The compound was >50% p.o. bioavailable in rodents and dogs and did not alter the plasma pharmacokinetics of i.v.-administered paclitaxel. OC144-093 increased the life span of doxorubicin-treated mice engrafted with MDR F388 leukemia cells by >100% and significantly enhanced the in vivo antitumor activity of paclitaxel in MDR human breast and colon carcinoma xenograft models, without a significant increase in doxorubicin or paclitaxel toxicity. The results demonstrate that OC144-093 is an orally active, potent, and nontoxic inhibitor of P-gp-mediated multidrug resistance that exhibits all of the desired properties for treatment of P-gp-mediated MDR, as well as for prevention of MDR prior to selection and/or induction of refractory disease.

INTRODUCTION

MDR2 is now recognized as one of the most common causes of failure of cancer chemotherapy. The MDR phenotype results from cross-resistance to a variety of structurally and functionally unrelated natural products used for cytotoxic antitumor therapy. These include anthracyclines, Vinca alkaloids, epipodophyllotoxins, and taxanes. Bing et al. (1) identified a M, 170,000 membrane glycoprotein (P-glycoprotein or P-gp), which was subsequently found to mediate ATP-dependent efflux of each of these cancer therapeutics from multidrug-resistant tumor cells (reviewed in Refs. 1 and 2). Additional mechanisms contributing to MDR have been described, including expression of the MRP class of transporters. MRP-related proteins appear to be able to transport certain anthracyclines, Vinca alkaloids, and epipodophyllotoxins but not taxanes. Members of this family are also involved in normal biliary transport (Ref. 3; reviewed in Ref. 4).

Significant progress has been made on the determination of the role of P-gp and related proteins in normal physiology. One P-gp gene (mdr1a) in humans and two genes (mdr1a and mdr1b) in rodents have been shown to play a significant role in drug resistance. Additional members of the P-gp gene family are involved in phospholipid and bile salt transport. Although simultaneous genetic knockout of mdr1a and mdr1b resulted in healthy mice, indicating that P-gp is not essential for basic physiological functions, the mice did exhibit significant alterations in the pharmacological handling of drugs. Blood-brain barrier function was decreased, and intestinal absorption of drugs was increased (reviewed in Ref. 5). These results are consistent with a role of P-gp in normal tissues to prevent uptake of xenobiotics. Recent evidence suggests that P-gp may also play a role in cholesterol trafficking (6).

Studies carried out over the last several years have demonstrated that intrinsic and acquired expression of P-gp plays a major role in clinical MDR. Tumor types that frequently express P-gp in the absence of exposure to chemotherapy include colorectal, renal cell, hepatocellular, and adrenocortical cancers, as well as chronic leukemia (reviewed in Ref. 7). Several additional tumor types express P-gp at diagnosis in approximately 10–50% of cases. Examples include breast carcinoma, acute myelogenous leukemia, and ovarian carcinoma (8–14). P-gp expression at diagnosis in these tumor types can play a significant role in treatment outcome. For example, patients with breast carcinomas expressing P-gp are three times more likely to fail to respond to chemotherapy than patients whose tumors are P-gp negative (15).

Chemotherapeutic agents such as doxorubicin can select for mutations leading to increased expression of P-gp and the appearance of MDR in tissue culture models. Coadministration of a P-gp inhibitor was found to suppress activation of mdr1 gene expression and decrease the mutation rate for resistance to doxorubicin (16, 17). More recently, transient activation of mdr1 gene expression has been observed in human metastatic sarcoma after in vivo exposure to doxorubicin (18). In light of these findings, it appears that the most effective way to use chemotherapeutic agents that are P-gp substrates will be in conjunction with a P-gp inhibitor at the time of tumor diagnosis (reviewed in Refs. 2, 19, and 20).

The first attempts to reverse P-gp-mediated MDR in cell lines, tumor-bearing animals, and in the clinic took advantage of the observation by Tsuruo et al. that calcium channel blockers such as verapamil are inhibitors of MDR (reviewed in Ref. 2). Similar observations were subsequently made with cyclosporin A (reviewed in Ref. 2). Although having some efficacy, these agents are relatively weak P-gp inhibitors (IC50, 2–10 μM), are often substrates for P-gp, and exhibit dose-limiting side effects that severely restrict their clinical utility. To address the problems described above, there has been considerable interest in second-generation P-gp inhibitors. VX-710 (21), PSC 833 (22), and XR9051 (23) are 3–100-fold more potent than...
the first-generation compounds and typically do not elicit significant toxicity at doses required for P-gp inhibition. Common dose-limiting toxicities for these types of compounds are ataxia and hyperbilirubinemia, which are reversible upon cessation of drug treatment.

An additional problem with most P-gp inhibitors is that they significantly alter the plasma PK of coadministered antitumor agents, increasing blood levels by reducing clearance and/or metabolism. For example, PSC 833 and VX-710 have been reported to produce significant PK interactions with agents such as paclitaxel (19, 24). This necessitates significant reductions in the dose of the chemotherapeutic agent to achieve target blood levels. Although feasible, interpatient variability in metabolism and PK interaction make this approach problematic. In addition, a requirement for dose reduction presents significant problems for the use of this approach in up-front therapy to kill cells expressing P-gp and/or to prevent appearance of MDR after first diagnosis (reviewed in Refs. 2, 19, and 20). Third generation P-gp inhibitors that exhibit nanomolar EC50’s and lack significant PK interaction with doxorubicin (GF120918) or with plasma levels of doxorubicin, etoposide, and paclitaxel (LY335979) have been reported (25–27).

The significant clinical use and utility of taxanes in a variety of major tumor types and the apparent lack of cross-resistance with the MRP family of transporters makes these agents highly attractive candidates for up-front therapy with a P-gp inhibitor. The properties likely to be required for safe and effective up-front therapy for treatment and prevention of P-gp-mediated MDR are low nanomolar potency, lack of nonspecific cytotoxicity, P-gp specificity, and relatively long duration of action with reversibility, good oral bioavailability, and lack of PK interaction with taxanes such as paclitaxel. To date, an inhibitor with all of these characteristics has not been described. In this report, we describe the discovery and preclinical characterization of a P-gp inhibitor (OC144-093) that appears to meet all of these criteria.

MATERIALS AND METHODS

Cell Lines, Animals, and Reagents. CCRF-CEM and CEM/VLB1000 human lymphoma and SKOV3 and SKVL1000 human ovarian carcinoma cells from V. Ling (British Columbia Cancer Agency, Vancouver, British Columbia, Canada) were grown in Alpha MEM with 2.0 mM glutamine and 10% FBS (Gemini BioProducts, Calabasas, CA), plus 1.0 mM g/ml vinblastine from Sigma Chemical Co. (St. Louis, MO). Alamar Blue was from BioSource International (Camarillo, CA) and was used according to the manufacturer’s instructions. [3H]Vinblastine sulfate (specific activity, 17 Ci/mmol) and [3H]azidopine (specific activity, 52 Ci/mmol) were from Amersham (Arlington Heights, IL). OC144-093 free base for in vivo studies was synthesized by IRIX Pharmaceuticals (Salt Lake City, UT). Chemochrom was 98% pure as judged by HPLC.

Cell Proliferation Assays. Cell proliferation IC50’s and MDR reversal EC50’s were determined from 3-day dose-response curves carried out in triplicate in 96-well plates essentially as described by Monks et al. (28). Cells were plated in standard growth medium at 2.5 or 5.0 × 10^4 cells/well (CCRF-CEM and CEM/VLB1000, respectively) or 5 × 10^5 cells (all other cell lines in a final volume of 100 μl). After a 2-h incubation for nonadherent cells and overnight incubation for adherent cells, the initial cell density was determined by fluorescence readout of Alamar Blue metabolism. OC144-093, cytotoxic agents, or compound vehicles were added to duplicate plates, and the incubation was continued for an additional 72 h. Final cell density was determined with Alamar Blue. In some experiments, a standard end point assay was used without analysis of initial cell density with Alamar Blue. Similar results were obtained with the two assays. EC50’s were derived by nonlinear regression analysis, assuming a sigmoidal dose-response using GraphPad Prism Software (San Diego, CA).

High-Throughput Vinblastine Cytotoxicity Assay. CEM/VLB1000 cells (2.5 × 10^4 cells/well in 96-well plates) were plated with 5.5 μM vinblastine and 10 μM test compounds in a final volume of 200 μl Alpha MEM, 10% FBS. The plates were incubated for 48 h, and then cell proliferation was determined with Alamar Blue.

[3H]Vinblastine Uptake Assay. Plates (96-well) were preblocked with PBS containing 1% BSA. CEM and CEM/VLB1000 cells were seeded at 1 × 10^5 cells/well and incubated with compounds or vehicle controls and 0.275 μM [3H]vinblastine in a final volume of 100 μl growth medium. Incubation was carried out for 3 h at 37°C. Cells were then washed and harvested onto glass fiber filtermats using a Tomtek Harvester-96 (EG&E Wallac, Gaithersburg, MD). Scintillant (Meltfix; EG&E Wallac) was added, and accumulation of labeled drug was measured by liquid scintillation counting.

[3H]Azidopine Photoaffinity Labeling of P-gp. CEM/VLB1000 membranes were prepared as described (29), and the experiment was carried out as described by Hyafil et al. (25). Membranes were incubated with OC144-093 for 25 min in the dark, followed by a similar incubation with 0.6 μM [3H]azidopine. After UV irradiation for 2 min, the photolabeled membranes were subjected to SDS-PAGE on a 7.5% gel, followed by fluorography. Identification of P-gp was performed via Western blotting using monoclonal antibody C219 (Signet Lab, Dedham, MA) and ECL detection.

ATPase Assay. CEM/VLB1000 membrane vesicles were prepared as described above for [3H]azidopine labeling of P-gp. Membranes (15 μg) were incubated with various concentrations of OC144-093 in 40 μl of assay buffer containing 2 mm DTT, 5 mm NaN3, 1 mm ouabain, 1 mm EGTA, 50 μM Tris ATP and ±100 μM sodium orthovanadate for 50 min (30). The reaction was stopped in the presence of 2.5% SDS, and liberated inorganic phosphate was quantitated immediately in comparison with a series of inorganic phosphate standards by use of a colorimetric method involving ammonium molybdate complication, as described (31). P-gp ATPase activity was defined as the orthovanadate-sensitive portion of the total ATPase activity observed in P-gp expressing cells.

Daunomycin Efflux. Studies were carried out essentially as described by Hyafil et al. (25). In brief, cells were incubated for 3 h at 37°C in glucose and phenol red-free α-MEM, 10% FBS containing 10 mM NaN3 and 17.7 μM daunomycin in the presence or absence of 2 μM OC144-093 (uptake). The cells were then washed and efflux was measured as the decrease in intracellular daunomycin concentration in the presence or absence of modulator for times varying from 0 to 8 h with 10^6 cells/time point. Daunomycin retention was determined by FACS analysis of washed cells. Flow cytometry was performed with a Becton

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the flow rate was 1.0 ml/min, 10% B; observed once per day (or more) for mortality and signs of ill health (weight measurement). At the indicated times after paclitaxel administration, three mice/time of 16 mg/kg in 200 ml of saline:Cremophor:ethanol (8:1:1 by volume) 25, 19, and 1 h prior to a single i.v. dose of PEG 400/Tween 20 (9:1 by volume) on days 4–6, 11–13, 18–20, and 25–27. Mean body weights were recorded at least every other day. Tumor weight was monitored approximately every other day by caliper measurements and calculated according to the formula [Tumor weight = (length × width)² ÷ 2]. This conversion formula was verified by comparing the calculation-derived tumor weights to excised and weighed tumors. Animals bearing ulcerated tumors or where tumor weight exceeded 10% of the animal’s body weight were sacrificed. The weights of the bilateral tumors were averaged for each mouse, and mean tumor weights for each treatment group ±SE were calculated. Statistical analysis was carried out by Mann-Whitney test using GraphPad Prism software (San Diego, CA).

Antitumor efficacy experiments with s.c. HCT-15 colon tumors were conducted essentially as described above, except that female athymic NCI-nu mice (eight/group) were implanted s.c. with 10⁶ HCT-15 cells on day 0. Paclitaxel (15 mg/kg/dose) was administered on days 2, 7, and 12 or days 5, 10, 15, and 20 for established tumors. OC144-093 (30 mg/kg/dose) was administered on days 1–3, 6–8, and 11–13 or days 4–6, 9–11, 14–16, and 19–21 for established tumors.

RESULTS

Discovery of OC144-093. Small molecule inhibitors of P-gp were initially identified by high-throughput cellular assays of the Ontogen combinatorial chemistry library, produced by the OntoBLOCK system (35). The assay was based on restoration of vinblastine-mediated cytotoxicity in P-gp-expressing, multidrug-resistant human lymphoma cells (CEM/VLB1000). Screening produced initial leads from a diaryl imidazole library with submicromolar potency. These compounds were optimized via structure-activity relationship studies, guided by in vitro potency and metabolic stability. The optimized lead compound, OC144-093, is a novel substituted diaryl imidazole with a molecular weight of M, 495 and log P of 5.0 (Fig. 1). The free base exhibited very low water solubility, but a water-soluble mesylate salt was prepared.

Modulation of P-gp-mediated Drug Resistance in Vitro. OC144-093 was able to reverse resistance to all classes of P-gp substrates in a wide variety of tumor cell types with EC₅₀s in the low
Table 1  Reversal of MDR by OC144-093 in P-gp-expressing cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doxorubicin (EC50 μM ± SD)</th>
<th>Vinblastine (EC50 μM ± SD)</th>
<th>Paclitaxel (EC50 μM ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM/VLB1000</td>
<td>0.09 ± 0.06</td>
<td>0.07 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>MES-SA/DX5</td>
<td>0.024 ± 0.006</td>
<td>0.034 ± 0.007</td>
<td>0.027 ± 0.007</td>
</tr>
<tr>
<td>SK/VLB1000</td>
<td>0.023 ± 0.008</td>
<td>0.015</td>
<td>0.033</td>
</tr>
<tr>
<td>MCF-7/ADR</td>
<td>0.038 ± 0.006</td>
<td>0.02 ± 0.01</td>
<td>0.031 ± 0.004</td>
</tr>
<tr>
<td>MDA/LCC6 MDR1</td>
<td>0.013</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>HCT-15</td>
<td></td>
<td></td>
<td>0.016</td>
</tr>
</tbody>
</table>

* Lymphoma.
* Uterine.
* Ovarian.
* Breast.
* Colorectal carcinoma.

nt range (Table 1). Similar results were obtained with etoposide in several models (data not shown). Complete reversal of MDR was typically seen with OC144-093 doses between 0.25 and 1.0 μM. Examples with cells expressing extremely high levels of P-gp as a result of drug selection (CEM/VLB1000), moderate levels of P-gp from gene transduction (MDA/LCC6 MDR1), and low (intrinsic) levels of P-gp (HCT-15) are illustrated in detail in Fig. 2. OC144-093 had no effect on doxorubicin or paclitaxel IC50s in non-P-gp-expressing CCRF-CEM and MDA/LCC6 cells, respectively, demonstrating the specificity of this compound for P-gp. OC144-093 retained full MDR reversal potency after incubation in human plasma, suggesting that protein binding-mediated inactivation will not be a problem in humans (data not shown).

MCF-7/VP cells that express MRP-1, but not P-gp (36), were used to further assess the specificity of OC144-093. The results presented in Fig. 3 demonstrate that OC144-093 was able to reverse paclitaxel resistance in P-gp-expressing MCF-7/ADR cells by almost four orders of magnitude. However, OC144-093 was unable to reverse resistance to the MRP-1 substrate etoposide in MCF-7/VP cells, suggesting that OC144-093 is not an inhibitor of MRP-1. OC144-093 had no effect on paclitaxel or etoposide IC50s in non-P-gp-expressing MCF-7 cells, further supporting the specificity of the compound for P-gp (data not shown).

**Nonspecific Toxicity of OC144-093.** Proliferation assays capable of measuring both cytostatic and cytotoxic responses were carried out as described in “Materials and Methods” with 15 nontransformed and transformed cell lines, including primary fibroblasts, nontransformed smooth muscle, leukemia, breast, colon, ovarian, and uterine carcinoma cells (± P-gp expression). Although OC144-093 reversed P-gp-mediated MDR in the low nanomolar concentration range, the compound was nontoxic by itself at doses up to 100 μM in all cell lines. Cytostatic IC50s ranged from 6 to 170 μM, with an average value of 60 μM (Table 2). IC50s for nonspecific cytotoxicity were similar in matched cell lines plus and minus P-gp expression. As described by Dantzig et al. (26), this type of result indicates that OC144-093 is probably not a P-gp transport substrate. If it were, some consistent degree of resistance would be expected in P-gp-expressing cells.

**Mechanism of Action Studies.** CEM/VLB1000 cells were used for mechanism of action studies because of the high levels of P-gp expression and the ease of working with cells that grow in suspension. OC144-093 restored [3H]vinblastine accumulation in CEM/VLB1000 cells and was found to be 25-fold more potent than cyclosporin A in this model system (Fig. 4). OC144-093 was able to restore [3H]vinblastine accumulation in CEM/VLB1000 cells to the level observed in parental CEM cells (data not shown). Direct interaction of OC144-093 with P-gp was assessed by examining the ability of the compound to prevent [3H]azidopine photoaffinity labeling of P-gp in CEM/VLB1000 membranes (25). Significant inhibition of azidopine binding to P-gp was observed with doses of OC144-093 as low as 0.05 μM (Fig. 5). OC144-093 was also found to be a potent inhibitor of P-gp-mediated ATPase activity, with an IC50 of 0.16 μM (primary data not shown).

**Duration of Drug Effect.** Many first-generation inhibitors of P-gp, such as cyclosporin A, are P-gp transport substrates. Second-
generation inhibitors, such as PSC 833, have been selected for lack of transport by P-gp and long duration of action (37, 38). We have used two well-established methods for indirect analysis of reversibility and duration of action. Cells expressing P-gp can be loaded with P-gp substrates if ATP synthesis is inhibited with a reversible metabolic poison (25). The effects of P-gp inhibitors can then be determined in washed cells under conditions that result in rapid restoration of ATP levels and P-gp function. Preloaded daunomycin was lost from CEM/VLB1000 cells within 10 min after release from metabolic block. In CEM cells that do not express P-gp, daunomycin was lost slowly by nonspecific diffusion, with significant retention of the drug 8 h after release from metabolic block (Fig. 6). Inclusion of OC144-093 in the uptake and efflux buffers completely prevented the rapid P-gp-mediated loss of daunomycin from the CEM/VLB1000 cells, producing a profile identical to that seen in CEM cells. When OC144-093 was included only in the uptake buffer, significant daunomycin retention was still observed in CEM/VLB1000 cells after 8 h. P-gp inhibitors that are transport substrates are rapidly effluxed after release from metabolic block, with subsequent rapid loss of daunomycin (25, 26). Our results provide additional indirect evidence that OC144-093 is not a P-gp transport substrate or is only a very weak substrate.

Persistence and reversibility can be assessed in a therapeutically relevant way by assaying MDR reversal after washout of a P-gp inhibitor (26). This is done very stringently by carrying out the P-gp inhibitor incubation and washout prior to exposure to the antitumor agent and initiation of the proliferation assay. The results presented in Table 3 demonstrate that reversal of MDR by OC144-093 in washed cells was the same without and with a 12-h compound-free incubation before addition of doxorubicin and initiation of the proliferation assay. The effects of OC144-093 were reversible after a 24-h washout. Table 3 also demonstrates that the MDR-reversing effects of verapamil are lost immediately upon cell wash.

**Pharmacokinetic Studies.** OC144-093 was p.o. bioavailable in rodents (mice and rats) and dogs (50–70%) and was well tolerated, with a terminal-half life of 4–7 h (Fig. 7; rodent data not shown).
Mean total plasma clearance (CL) in the dog was 1.37 l/h/kg, and the mean volume of distribution at steady-state (Vss) was 3.24 l/kg in the dog. The maximum tolerated dose for oral OC144-093 in the dog was 60 mg/kg/day. Ataxia was the only effect observed at this dose, with complete recovery within 24 h. There was no target organ toxicity observed during histopathological examination after i.v. administration at the maximum tolerated dose.

Blood levels > 1 µM OC144-093 were observed in the dog for ~3 h after an oral dose of 10 mg/kg (Fig. 7), demonstrating that therapeutically relevant levels can be attained with a wide safety margin. In mouse studies, administration of three 20 mg/kg oral doses of OC144-093 over a 24-h period produced blood levels of OC144-093 of 2–4 µM, which were sustained for an additional 24 h (data not shown). These results demonstrate that therapeutically relevant doses of this compound can be administered via the oral route.

**PK Interaction Studies.** Two enzymes primarily responsible for metabolism of paclitaxel are P450 CYP3A4 and CYP2C8 (39). Some P-gp inhibitors are also metabolized by P450 CYP 3A4, leading to inhibition of paclitaxel metabolism. This may contribute to PK interactions with paclitaxel (40). OC144-093 was not metabolized by P450 CYP3A4 or CYP2C8. The Kᵢ for OC144-093 inhibition of human CYP3A4-mediated paclitaxel metabolism was found to be 39.8 ± 5.1 µM. This is ~1000-fold higher than the EC₅₀₈₅ for reversal of MDR by OC144-093, suggesting that OC144-093 might not produce a significant PK interaction with paclitaxel in vivo. We investigated the effect of OC144-093 on plasma paclitaxel levels as described by Starling et al. (27) for LY335979. Fig. 8 demonstrates that pretreatment of mice with three oral doses of 30 mg/kg OC144-093 had no effect on i.v. plasma paclitaxel levels. It is important to note that this is the pretreatment dose and regimen of OC144-093 that was used for subsequent antitumor efficacy studies.

**Reversal of MDR in Vivo by OC144-093.** A standard i.p. P388 murine leukemia model of MDR was chosen for initial in vivo studies (32). OC144-093 (20 mg/kg) was found to almost completely reverse resistance to doxorubicin in this model. A dose-response for MDR reversal was observed at the lower doses (Table 4). On the bases of animal weight and mortality, no significant or reproducible enhancement of toxicity was observed when OC144-093 was combined with doxorubicin at the concentrations indicated. OC144-093 had no significant effect, by itself, on the survival of mice implanted with wild-type or MDR P388 ascites tumors (wild-type data not shown). In addition, OC144-093 did not enhance the life span of doxorubicin-treated mice implanted with wild-type P388 ascites tumors (Table 4).

The ability of p.o.-administered OC144-093 to reverse MDR in solid tumors in vivo was assessed with both the MDA/LCC6/mdr1 and HCT-15 models. Because OC144-093 produced no effect on paclitaxel blood levels, we have focused on up-front therapy or minimal residual disease models, as opposed to established tumors. MDA/LCC6/mdr1 cells that express P-gp were resistant to paclitaxel treatment in vitro, and this resistance was reversed by OC144-093 (Fig. 2B). The results presented in Fig. 9 demonstrate that treatment with paclitaxel or OC144-093 alone had no significant effect on in vivo tumor growth compared with the vehicle control. When OC144-093 and paclitaxel were combined, there was a statistically significant inhibition of tumor growth that persisted for at least 2 weeks after the last dose of paclitaxel. The growth delay produced by paclitaxel and OC144-093 in the MDA/LCC6/mdr1 xenografts was comparable with the growth delay produced by paclitaxel alone in MDA/LCC6 xenografts, suggesting complete reversal of MDR by OC144-093 (data not shown).

Combination treatment with 12 mg/kg paclitaxel and 30 mg/kg OC144-093 produced no significant increase in toxicity. The maxi-

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**Table 3 Persistence of MDR reversal in CEM/VLB1000 cells after incubation and washout of verapamil or OC144-093**

<table>
<thead>
<tr>
<th>Treatment schedule</th>
<th>Doxorubicin IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&gt;30</td>
</tr>
<tr>
<td>No wash</td>
<td></td>
</tr>
<tr>
<td>Verapamil (10 µM)</td>
<td>No wash</td>
</tr>
<tr>
<td>Wash</td>
<td>6.8</td>
</tr>
<tr>
<td>Wash, then add doxorubicin</td>
<td>&gt;30</td>
</tr>
<tr>
<td>OC144-093 (5 µM)</td>
<td>No wash</td>
</tr>
<tr>
<td>Wash</td>
<td>1.2</td>
</tr>
<tr>
<td>Wash, then add doxorubicin</td>
<td>1.8</td>
</tr>
<tr>
<td>Wash + 6 h inc. a before doxorubicin</td>
<td>2.1</td>
</tr>
<tr>
<td>Wash + 12 h inc. before doxorubicin</td>
<td>1.9</td>
</tr>
<tr>
<td>Wash + 24 h inc. before doxorubicin</td>
<td>25.6</td>
</tr>
</tbody>
</table>

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a inc., incubation.

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REVERSAL OF MULTIDRUG RESISTANCE BY OC144-093

In the experiment described in Fig. 10, no weight loss was observed in the combination treatment group that exceeded the maximum weight loss observed with the vehicle control group. In this figure and “Materials and Methods,” the average OC144-093 EC$_{50}$ for reversal of P-gp-mediated MDR, was $0.032 \pm 0.023 \mu M$, with complete reversal in the 0.25–1.0 $\mu M$ range. The average OC144-093 EC$_{50}$ for reversal of P-gp-mediated MDR was 0.032 ± 0.023 µM, with complete reversal in the 0.25–1.0 µM range.

### DISCUSSION

The characterization of GF120918 (25) and LY335979 (26, 27) established a paradigm for preclinical analysis of P-gp inhibitors, involving in vitro efficacy, nonspecific cytotoxicity, effect on P-gp substrate uptake and efflux, duration of action, direct binding to P-gp, effect on P-gp ATPase activity, PK, PK interaction, and in vivo efficacy. More recently, a similar approach was used to characterize XR9051 and XR9576 (23, 41, 42). These three different families of inhibitors represent the most potent and specific inhibitors described to date. We have used the same approach to characterize OC144-093, allowing for a relatively direct comparison of the key characteristics of these compounds.

The average OC144-093 EC$_{50}$ for reversal of P-gp-mediated MDR was 0.032 ± 0.023 µM, with complete reversal in the 0.25–1.0 µM range.
range. In terms of the EC_{50,8} and number of cell types and cytotoxic agents tested, OC144-093 is as least as potent as any other P-gp inhibitor currently in development (for examples, see Refs. 21–23, 26, and 42, 43). The average IC_{50} for nonspecific cytotoxicity mediated by OC144-093 against 15 cell types was >60 μM, demonstrating less nonspecific toxicity than any inhibitor described previously. For example, LY335979 was reported to exhibit IC_{50} of 6–15 μM against CCRF-CEM and MCF-7 cell lines (26). The complete lack of effect of OC144-093 on the response to cytotoxic agents by cells that do not express P-gp, or that express MRP-1, further demonstrates the specificity of this inhibitor (Figs. 2A, 2B, and 3).

Efflux of P-gp substrates from various P-gp-expressing cell lines after accumulation in the presence of XR9576 (23), GF120918 (25), or LY335989 (26) occurred with a half-life of 1–3 h, or significantly greater than 2 h for XR9576 (43). Using a similar procedure with highly resistant CEM/VLB1000 cells, we observed a half-life of ~6 h for daunomycin loss from cells preincubated with OC144-093. P-gp inhibitors that are transport substrates are rapidly effluxed after release from metabolic block, with subsequent rapid loss of daunomycin (25, 26).

The activity of LY335979 and XR9051 has been shown to persist for up to 24 h after compound washout (23, 26). The activity of OC144-093 persisted for at least 12 h after compound washout from CEM/VLB1000 cells. Taken together with the observation that the nonspecific cytotoxicity of OC144-093 was similar for drug-sensitive and drug-resistant cell lines, our efflux and persistence studies suggest that OC144-093 may not be a substrate of P-gp. In addition, our results indicate that OC144-093 activity persists in a manner similar to that of LY335979, XR9051, and XR9576.

OC144-093 inhibited [3H]azidopine photoaffinity labeling of P-gp at submicromolar doses, supporting a mechanism of action that involves direct binding. Additional studies will be required to determine whether OC144-093 interacts with other sites on P-gp. OC144-093 was also found to be a potent inhibitor of P-gp-mediated ATPase activity. The IC_{50} required for this effect was slightly higher than the EC_{50} for MDR reversal in the same cell line (160 versus 94 nM, respectively). Thus, inhibition of P-gp ATPase activity may be secondary to a mode of action that involves specific binding to another site on the protein. Further study will be required to determine whether ATPase inhibition plays a significant role in the inhibition of MDR by OC144-093. Regardless, it is highly unlikely that the compound is a general ATPase inhibitor, based on its specificity for P-gp-mediated MDR and lack of nonspecific toxicity.

P388/MDR ILS values of 60–80% have been reported for GF120918 (25), LY335979 (26), and XR9051 (41). We obtained values of 100 and 122% ILS in two independent experiments, demonstrating that OC144-093 is at least as potent as these compounds described previously in this type of model. Human tumor xenograft studies are considered the best predictors of potential clinical utility. Three p.o. treatments with XR9576 were shown to enhance the antitumor efficacy of paclitaxel against established MDR 2780AD ovarian carcinoma xenografts over a 10-day period (43). Five i.p. treatments with LY335979 were shown to enhance the antitumor efficacy of paclitaxel against established MDR U138-P3.03/VLB non-small cell lung carcinoma xenografts over a 14-day period (26).

Using three to four p.o. treatments with OC144-093, we obtained similar enhancements of paclitaxel antitumor efficacy, sustained for 16 days against established colon carcinoma xenografts and sustained for 27–34 days against non-established colon and breast carcinoma xenografts, respectively. Reversal of MDR by OC144-093 in vivo in the breast carcinoma model was observed at the highest possible nonlethal weekly dose of paclitaxel without any enhancement of paclitaxel toxicity. This result substantiates the lack of PK interaction observed via direct measurement of paclitaxel blood levels. Although differences in tumor type, growth rate, and regimen make direct comparison difficult, the magnitude and length of human tumor xenograft growth delays without toxicity obtained with OC144-093 are at least comparable with those published with P-gp inhibitors currently in development, such as PSC 833, LY335979, and XR9576.

On the other hand, there appear to be significant differences in the intrinsic PK profiles and drug interaction properties of OC144-093 and the above inhibitors. Only LY335979 has been shown previously to lack a PK interaction with plasma paclitaxel in preclinical models (27). However, LY335979 was administered i.p. and not p.o., as with OC144-093. Although definitive bioavailability studies for LY335979 have not been reported, steady-state plasma levels of only ~125 ng/ml were observed after oral administration of 300–400 mg/m^2 of LY335979 every 8 h to subjects during a Phase I study (44). This result suggests that LY335979 has low oral bioavailability. Recently reported human i.v. and p.o. PK parameters for XR9576 also suggest low oral bioavailability (45). In contrast, OC144-093 exhibited 50–70% oral bioavailability in rodents and dogs and 80% oral bioavailability in humans (46).

In summary, OC144-093 is a highly potent, specific, nontoxic, and reversible P-gp inhibitor with relatively long duration of action. Furthermore, it exhibits a favorable oral PK profile, lacks a PK interaction with respect to plasma paclitaxel, and does not inhibit MRP-1 or human P450 CYP3A4 at therapeutically relevant doses. OC144-093 appears to possess all of the desired properties for treatment of P-gp-mediated MDR, as well as for prevention of MDR prior to selection and/or induction of refractory disease. OC144-093 is currently undergoing clinical evaluation in humans.

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