

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

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ABSTRACT

OC144-093 is a novel substituted diarylimidazole (M_r 495) generated using the OntoBLOCK system, a solid-phase combinatorial chemistry technology, 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 EC_{50} of 0.032 μ M. 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 IC_{50} of >60 μ M. OC144-093 blocked the binding of [³H]azidopine 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 P388 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

MDR⁶ 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. Ling *et al.* (1) identified a M_r 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 (*mdr1*) 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 (EC_{50} s, 2–10 μ M), 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

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⁶ The abbreviations used are: MDR, multidrug resistance; ILS increase in life span; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; PK, pharmacokinetics; ATCC, American Type Culture Collection; FACS, fluorescence-activated cell sorter; HPLC, high-performance liquid chromatography; BID, twice per day; QD, once per day; PEG, polyethylene glycol; SCID, severe combined immunodeficient; Veh, vehicle.

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 EC₅₀s 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 SKVLB1000 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 μg/ml vinblastine sulfate for maintenance of drug resistance. MCF-7 and MCF-7/ADR (National Cancer Institute, Developmental Therapeutics Program Tumor Repository) and MCF-7/VP human breast carcinoma cells (E. Schneider, Wadsworth Center, Albany, NY) were grown in RPMI 1640 with 10% FBS. MES-SA and MES-SA/DX5 human uterine carcinoma cells (ATCC) were grown in McCoy's 5A with 10% FBS, plus 500 ng/ml doxorubicin for maintenance of drug resistance. MDA435/LCC6 and *mdr1* transduced MDA435/LCC6^{MDR1} human breast carcinoma cells from R. Clarke (Georgetown University, Washington, DC) were grown in IMEM with 5% FBS. HCT-15 human colon carcinoma cells (ATCC) were grown in RPMI 1640 with 10% FBS. P-gp expression or lack thereof in cell lines was confirmed by FACS analysis using the monoclonal antibody MRK16 (Kamiya Biomedicals, Berkeley, CA). Non-transformed HISM human smooth muscle and primary CCD-986SK human skin cells (ATCC) were grown in DMEM with 10% FBS and Iscove's modified Dulbecco's medium with 10% FBS, respectively.

Female BDF₁ and SCID/RAG2 mice, 6–8 weeks of age, were obtained from Charles River Laboratories of Canada and the Joint Animal Care Facility at the British Columbia Cancer Research Center, respectively. For *in vivo* studies, P388 and P388/ADR murine leukemia cells (National Cancer Institute, Developmental Therapeutics Program Tumor Repository) were propagated in the peritoneum of BDF₁ mice by weekly transfer of 0.5 ml of peritoneal fluid containing 10⁶ cells. MDA435/LCC6 and MDA435/LCC6^{MDR1} cells (10⁷ in 0.5 ml) were similarly propagated every 2–3 weeks in SCID/RAG2 mice. Cells were used between the third and twentieth passages. Animal studies were

carried out with protocols approved by the British Columbia Cancer Agency/University of British Columbia Institutional Animal Care Committee and were performed in accordance with the Canadian Council on Animal Care Guidelines. The *in vivo* HCT-15 study was conducted by Serquest, a division of Southern Research Institute (Birmingham, AL), with young, adult female athymic NCr-nu mice.

Vinblastine, doxorubicin, daunomycin, and verapamil were purchased from Fluka (Ronkonkoma, NY). Paclitaxel and cyclosporin A were 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, Inc. (Florence, SC). The material was 98% pure as judged by HPLC.

Cell Proliferation Assays. Cell proliferation IC₅₀s and MDR reversal EC₅₀s 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 × 10⁴ cells/well (CCRF-CEM and CEM/VLB1000, respectively) or 5 × 10³ cells/well (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. EC₅₀s 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 × 10⁴ 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 × 10⁵ 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 (Meltalix; 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 Na₂S₂O₈, 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 complexation, 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 Na₂S₂O₈ and 17.7 μM daunomycin in the presence or absence of 2 μM OC144-093 (uptake). The cells were then washed and efflux was carried out in 0.5 ml phenol red-free growth medium in the presence or absence of modulator for times varying from 0 to 8 h with 10⁶ cells/time point. Daunomycin retention was determined by FACS analysis of washed cells. Flow cytometry was performed with a Becton

Dickinson FACSscan (BD Immunocytometry Systems, San Jose, CA). A minimum of 10,000 events were counted for each datapoint. The fluorescence data are expressed as mean arbitrary fluorescence units and were corrected for autofluorescence by subtracting the fluorescence of control samples.

Duration of MDR Reversal. The experiment was carried out as described by Dantzig *et al.* (26). In brief, 8×10^5 CEM/VLB1000 cells/ml were incubated for 24 h with or without the P-gp inhibitor before being washed 0 or 3 times with growth medium. The cells were then incubated for 0, 6, 12, or 24 h before the addition of varying concentrations of doxorubicin or vehicle. The incubation was continued for 24 h. The cells were then washed two times by centrifugation, resuspended at 5×10^4 cells/well in 0.2 ml growth medium, and grown for an additional 3 days prior to Alamar Blue analysis and IC₅₀ determination.

PK of OC144-093 in the Dog. For i.v. infusion, OC144-093 was dissolved in PEG 400:5% dextrose (7:3) at a concentration of 5 mg/ml. The solution was stable at room temperature. The compound was infused via the cephalic vein in the dog's front leg over a period of 30 min at a rate of 2 ml/kg/h. Healthy female beagle dogs weighing from 8 to 10 kg were used for these studies. Blood samples (~5 ml) for pharmacokinetic analysis were collected from a jugular vein over EDTA anticoagulant at selected times over 24 h. Plasma was separated and frozen at -20°C to await analysis. For oral administration, OC144-093 was dissolved in PEG 400:Tween 20 (9:1) at a concentration of 25 mg/ml and loaded into hard gelatin capsules immediately prior to dosing. The dosing volume was 0.32 ml/kg. The oral dose was followed by 10 ml of water. OC144-093 was quantitated in plasma at MDS Analytical Solutions, Inc. (Sunnyvale, CA) using a validated HPLC procedure. The method used reverse-phase chromatography with UV detection at 310 nm. The limit of quantitation was validated at 25 ng/ml of OC144-093 in plasma. Pharmacokinetic analysis was performed using standard noncompartmental methods.

Paclitaxel PK in SCID/RAG2 Mice Treated with OC144-093. Plasma paclitaxel levels were determined in SCID/RAG2 mice after pretreatment with three p.o. gavage doses of 30 mg/kg OC144-093 (free base) or vehicle (100 μ l of PEG 400:Tween 20; 9:1 by volume) 25, 19, and 1 h prior to a single i.v. dose of 16 mg/kg paclitaxel in 200 μ l of saline:Cremophor:ethanol (8:1:1 by volume). At the indicated times after paclitaxel administration, three mice/time point were anesthetized with CO₂, and blood was removed by cardiac puncture into Microtainer tubes containing EDTA. Plasma samples generated by centrifugation were extracted with acetonitrile, and the analysis for paclitaxel content was carried out by HPLC using a Waters 600E multisolvent delivery system, 717 plus autosampler, and 996 photodiode array detector. Baccatin III (0.8 nmol/200 μ l of plasma) was used as the internal standard and added to plasma samples during extraction with acetonitrile. Standard curve samples as well as quality control samples were also prepared from spiked control plasma to verify the accuracy of the HPLC analysis. Paclitaxel and the internal standard were resolved on a Nova-Pak C₁₈ column (4 μ m, 150 \times 3.9-mm inside diameter; Waters, Milford, MA) with double-distilled water (A) and acetonitrile (B), using the following gradient profile: $t = 0$ min, 10% B; $t = 5$ min, 10% B; $t = 30$ min, 65% B; $t = 40$ min, 65% B; $t = 45$ min, 10% B; $t = 50$ min, 10% B. The gradient was formed using a high pressure mixer, and the flow rate was 1.0 ml min⁻¹. A Waters 996 Photo Diode Array Detector was used to scan at multiple wavelengths, and chromatograms were processed for traces obtained at 230 nm.

In Vivo Antitumor Efficacy Models. Three different preclinical models of MDR were used to characterize the chemosensitizing properties of OC144-093: (a) a P-gp overexpressing murine P388/ADR ascites tumor that was derived from drug-sensitive P388 lymphocytic leukemia by long-term doxorubicin exposure (32); (b) an orthotopic MDA435/LCC6^{MDR1} human breast carcinoma xenograft that overexpresses P-gp as a result of *mdr1* gene transduction (33); and (c) a s.c. HCT-15 human colon carcinoma xenograft that intrinsically expresses P-gp (34).

For the P388 ascites tumor model, female BDF1 mice (five/group) were injected i.p. with 10⁶ ascites-propagated P388 or P388 ADR cells on day 0. OC144-093 mesylate (5, 10, or 20 mg/kg/dose) was administered BID i.p. in 100 μ l of PEG 400:dextrose (7:3 by volume) on days 0, 1, 4, 5, 8, and 9. Doxorubicin (3 mg/kg/dose) was administered QD i.p. in 200 μ l of normal saline on days 1, 5, and 9, 1 h after the first and 6 h before the second dose of OC144-093. Mean body weights were recorded daily. All animals were observed once per day (or more) for mortality and signs of ill health (weight loss, change in appetite, or behavioral changes). Animals were terminated if

moribund or exhibiting severe toxicity. Time of death was then logged on the following day. Therapeutic comparisons were made between control and treatment groups by determining the median survival times (MST) and calculating the percent increase in life span (%ILS) as: %ILS = [(MST_{treatment}/MST_{control}) - 1] \times 100. Statistical significance between the various groups was determined by log rank analysis using a significance criterion of $P < 0.05$.

Antitumor efficacy experiments with orthotopic MDA435/LCC6 and MDA435/LCC6^{MDR1} tumors were conducted in female SCID/RAG2 mice. Ascites-propagated cells (2×10^6 in 50 μ l) were injected into mammary fat pads bilaterally on day 0 before randomization into groups of five mice/group. Paclitaxel (12 mg/kg/dose) was administered QD i.v. (tail vein) in 200 μ l of saline:Cremophor:ethanol (8:1:1 by volume) on days 5, 12, 19, and 26. OC144-093 free base (30 mg/kg/dose) was administered BID p.o. by gavage in 100 μ l 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 \times width²) \div 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 \pm 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 NCr-nu mice (eight/group) were implanted s.c. with 10⁷ 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 diarylimidazole 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 diarylimidazole with a molecular weight of M_r 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

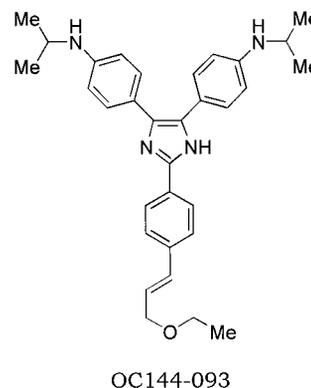


Fig. 1. Chemical structure of OC144-093.

Table 1 Reversal of MDR by OC144-093 in P-gp-expressing cell lines

The IC₅₀s for the indicated antitumor agents were determined in the presence of various concentrations of OC144-093 as described in "Materials and Methods." The OC144-093 EC₅₀ is the concentration that produced half-maximal reversal of antitumor agent resistance. Each experiment was carried out two to four times.

Cell line	OC144-093 EC ₅₀ (μM ± SD)		
	Doxorubicin	Vinblastine	Paclitaxel
CEM/VLB1000 ^a	0.09 ± 0.06	0.07 ± 0.01	
MES-SA/DX5 ^b	0.024 ± 0.006	0.034 ± 0.007	0.027 ± 0.007
SK/VLB1000 ^c	0.025 ± 0.008	0.015	0.033
MCF-7/ADR ^d	0.038 ± 0.006	0.02 ± 0.01	0.031 ± 0.004
MDA/LCC6 ^{MDR1d}	0.013		0.009
HCT-15 ^e			0.016

^a Lymphoma.

^b Uterine.

^c Ovarian.

^d Breast.

^e Colorectal carcinoma.

nm 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 noncytotoxic 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-

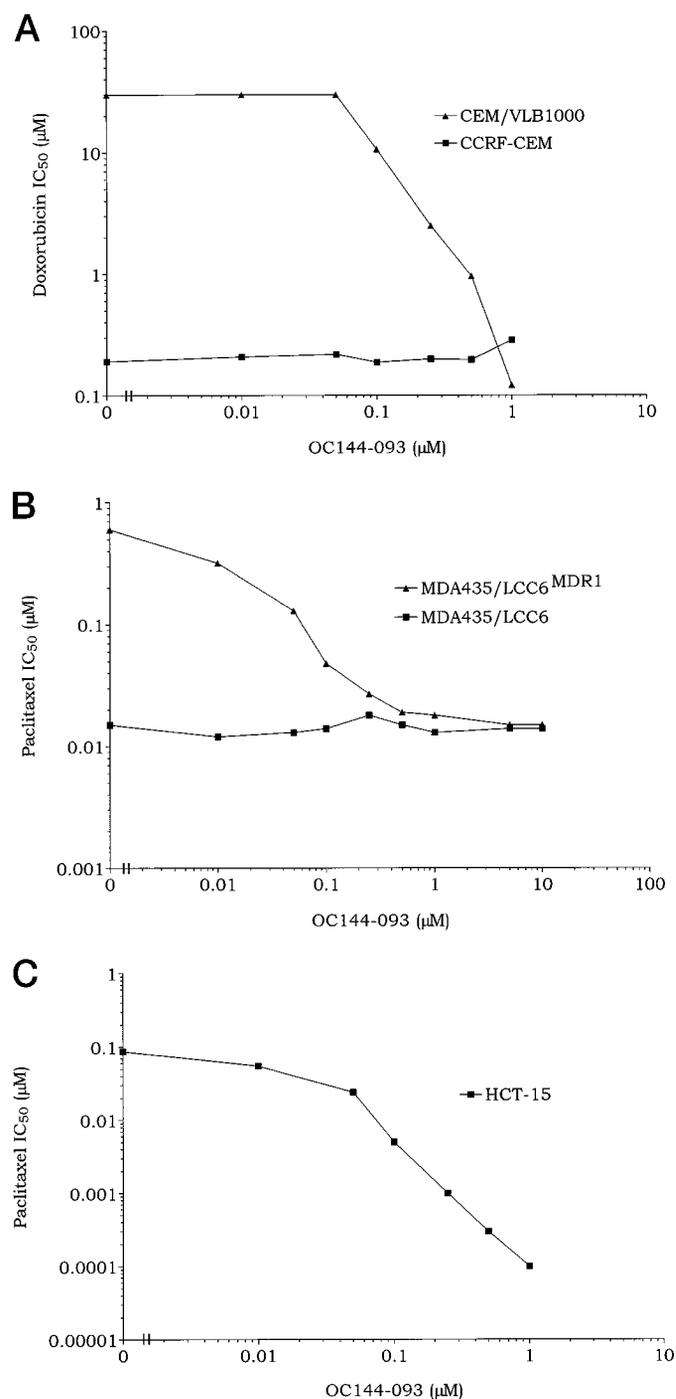


Fig. 2. Complete and highly specific reversal of MDR by OC144-093. A, effect of OC144-093 on CCRF-CEM and P-gp-expressing CEM/VLB1000 human lymphoma response to doxorubicin. B, effect of OC144-093 on MDA435/LCC6 and P-gp-expressing MDA/LCC6^{MDR1} human breast carcinoma response to paclitaxel. C, effect of OC144-093 on P-gp-expressing HCT-15 human colon carcinoma response to paclitaxel. Treatment with OC144-093 alone at the concentrations indicated had no effect on the proliferation of the cell lines. Similar results were obtained in two to five independent experiments.

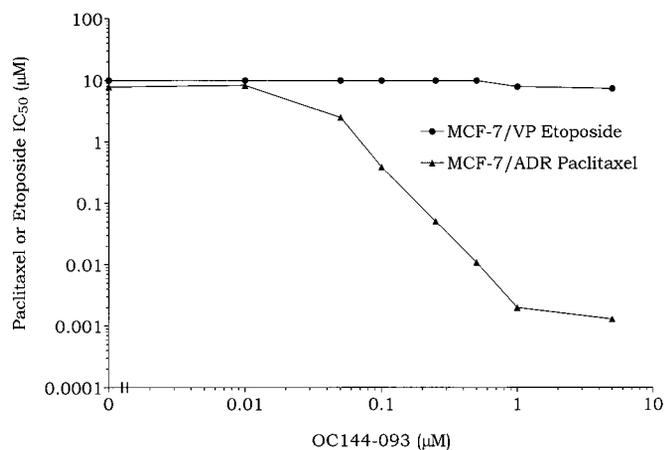


Fig. 3. Effect of OC144-093 on P-gp-expressing MCF-7/ADR and MRP-1-expressing MCF-7/VP human breast carcinoma response to paclitaxel or etoposide. Paclitaxel or etoposide IC_{50} s were determined with cell monolayers in the presence of various concentrations of OC144-093. Treatment with OC144-093 alone at the concentrations indicated had no effect on the proliferation of the cell lines. Similar results were obtained in three independent experiments.

Table 2 Effect of OC144-093 on the proliferation of various cell lines

The IC_{50} (μM) for inhibition of the growth of cell lines by OC144-093 was determined as described in "Materials and Methods." Each experiment was carried out at least two times with similar results.

Human cell type/line	P-gp expression ^a	OC144-093 IC_{50}
Primary fibroblast CCD-986SK		>100
Smooth muscle HISM		>100
Lymphoma		
CEM	Negative	38
CEM/VLB1000	Positive	32
Ovarian carcinoma		
SKOV3	Negative	48
SK/VLB1000	Positive	15
Uterine carcinoma		
MES-SA	Negative	51
Breast carcinoma		
MCF-7	Negative	30
MCF-7/ADR	Positive	68
MDA/LCC6	Negative	>100
MDA/LCC6 ^{MDR1}	Positive	>100
Colorectal carcinoma		
SW480	Positive	>100
SW620	Negative	>100
HT-29	Negative	12
HCT-15	Positive	6

^a Direct analysis and/or from the literature.

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).

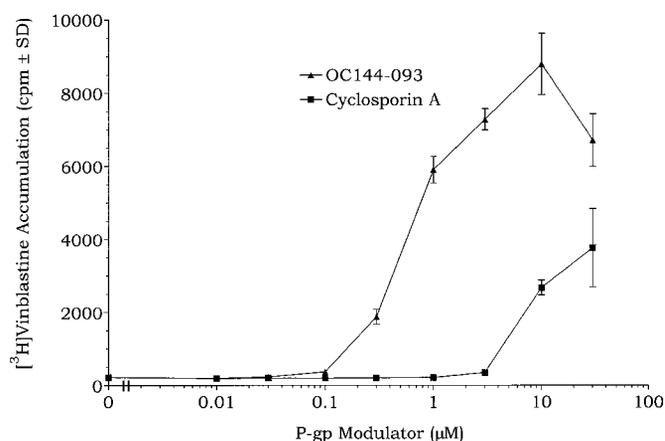


Fig. 4. Effects of OC144-093 and cyclosporin A on [³H]vinblastine uptake in P-gp-expressing CEM/VLB1000 human lymphoma cells. Vinblastine accumulation was determined with cell suspensions in the presence of various concentrations of OC144-093. Similar results were obtained in five independent experiments.

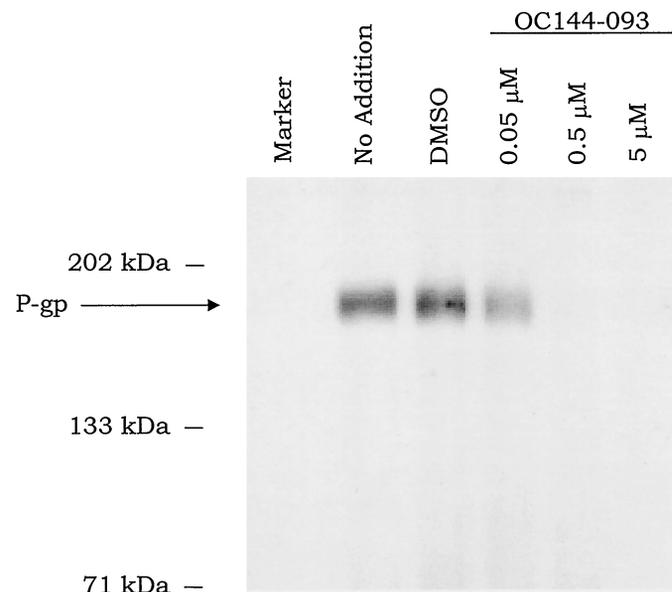


Fig. 5. Effect of OC144-093 on [³H]azidopine photoaffinity labeling of P-gp in CEM/VLB1000 human lymphoma membranes. Identification of P-gp was performed via Western blotting using a monoclonal antibody (C219) and ECL detection. No staining was observed with membranes from parental CCRF-CEM cells. Similar results were obtained in three independent experiments.

Mean total plasma clearance (CL) in the dog was 1.37 l/h/kg, and the mean volume of distribution at steady-state (V_{ss}) 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 $>1 \mu\text{M}$ 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 K_i for OC144-093 inhibition of human CYP3A4-mediated paclitaxel metabolism was found to be 39.8 ± 5.1

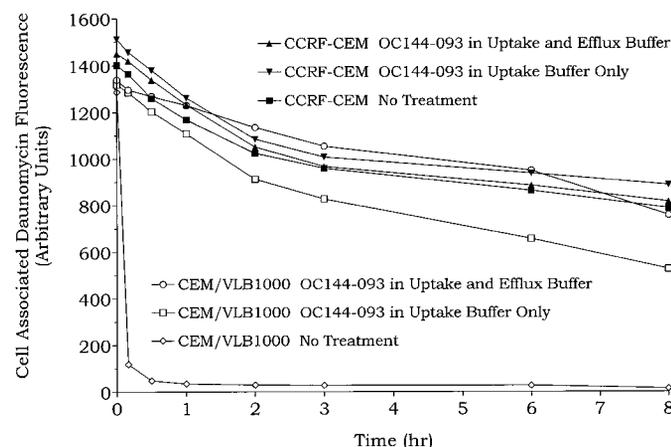


Fig. 6. Effect of OC144-093 on daunomycin efflux from metabolically poisoned and washed CCRF-CEM and CEM/VLB1000 cell suspensions. Metabolically poisoned cells were loaded with daunomycin for 3 h in the presence and absence of OC144-093. The cells were then washed, and daunomycin efflux was monitored in the presence and absence of OC144-093. Daunomycin retention was determined by FACS analysis of intrinsic fluorescence. Similar results were obtained in four independent experiments.

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

CEM/VLB1000 cells were incubated for 24 h with or without P-gp modulator before being washed 0 or three times with growth medium. Cells were then incubated for 0, 6, 12, or 24 h in the absence of modulator or doxorubicin. Subsequently, various concentrations of doxorubicin were added to the cells and incubated for 24 h. After this treatment, cells were washed and incubated in growth medium for 3 days prior to Alamar Blue (IC_{50}) determination. The experiment was carried out twice with similar results.

Treatment schedule	Doxorubicin IC_{50} (μM)
Control	
No wash	>30
Verapamil (10 μM)	
No wash	6.8
Wash, then add doxorubicin	>30
OC144-093 (3 μM)	
No wash	1.2
Wash, then add doxorubicin	1.8
Wash + 6 h inc. ^a before doxorubicin	2.1
Wash + 12 h inc. before doxorubicin	1.9
Wash + 24 h inc. before doxorubicin	25.6

^a inc., incubation.

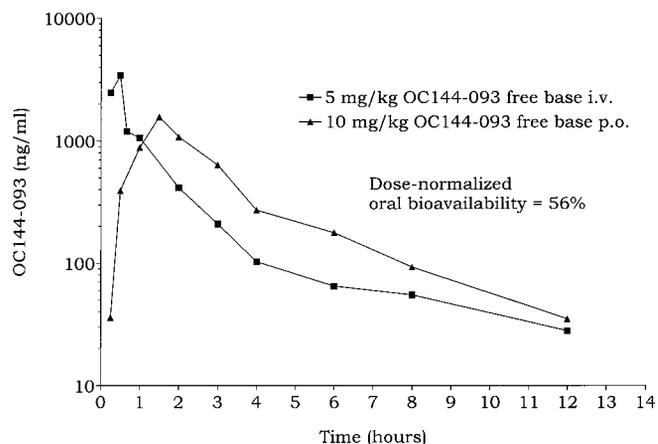


Fig. 7. PK of OC144-093 free base in the dog. Male beagle dogs (four/dose route) received 5 mg/kg OC144-093 free base in PEG 400:D5W (7:3 by volume) as a 30-min i.v. infusion or 10 mg/kg OC144-093 free base in PEG 400:Tween 20 (9:1 by volume) by oral gavage. Plasma levels of OC144-093 were determined by HPLC. Representative data are presented.

μM .⁷ This is ~ 1000 -fold higher than the EC_{50} s 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. P338 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-

⁷ E. Guns, P. L. Bullock, M. Reimer, R. Dixon, and L. D. Mayer, unpublished data.

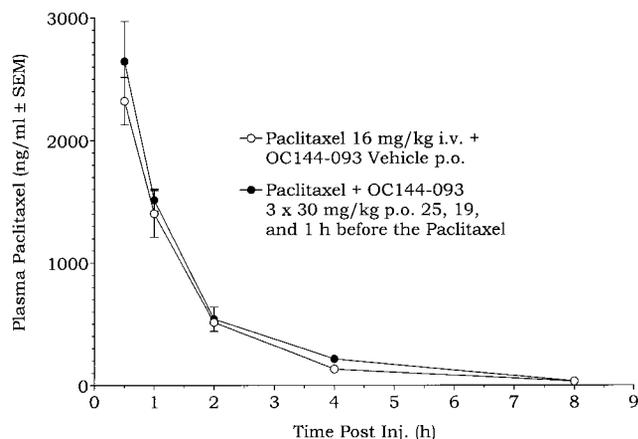


Fig. 8. Effect of oral OC144-093 on plasma levels of i.v.-administered paclitaxel in SCID/RAG2 mice. Mice were pretreated with three p.o. gavage doses of 30 mg/kg OC144-093 (free base) or vehicle (100 μ l of PEG 400:Tween 20; 9:1 by volume) 25, 19, and 1 h prior to a single i.v. dose of 16 mg/kg paclitaxel in 200 μ l of saline: Cremophor: ethanol (8:1:1 by volume). At the indicated times after paclitaxel administration, three mice/time point were anesthetized with CO₂, and blood was removed by cardiac puncture for HPLC analysis of paclitaxel. Bars, SE.

Table 4 Effect of OC144-093 in combination with doxorubicin on life span of mice bearing wild-type (WT) P388 or MDR P388 ascites tumors

The experiment was carried out as described in "Materials and Methods." Similar results were obtained in two independent experiments.

Tumor type	OC144-093 ^a (mg/kg)	Doxorubicin ^b (mg/kg)	Median survival time (days)	% ILS
WT	Veh	Veh	9	0
WT	Veh	3	22	144
WT	20	3	18	100
MDR	Veh	Veh	9	0
MDR	20	Veh	10	11.1
MDR	Veh	3	9	0
MDR	5	3	13	44.4
MDR	10	3	15	66.7
MDR	20	3	20	122

^a OC144-093 mesylate administered BID i.p. on days 0, 1, 4, 5, 8, and 9. Veh, vehicle.

^b Doxorubicin administered QD i.p. on days 1, 5, and 9.

imum average body weight loss in the vehicle, OC144-093, and paclitaxel groups was 4%. Maximum average weight loss in the OC144-093 plus paclitaxel combination group transiently exceeded this figure by another 1–4% on only 4 days during the experiment. There were no compound-related deaths. When paclitaxel alone was increased by 33% to 16 mg/kg, two of six mice died after four treatments (data not shown). Thus, a relatively small effect of OC144-093 on paclitaxel area under the curve would be expected to produce significant toxicity.

The HCT-15 human colon carcinoma cell line was derived from a patient not previously exposed to antitumor agents. This cell line expresses P-gp and is considered a model system for intrinsic P-gp-mediated MDR (34). OC144-093 was able to significantly reverse resistance to paclitaxel in this cell line *in vitro* (Fig. 2C). p.o.-administered OC144-093 was also able to reverse resistance to paclitaxel in s.c. HCT-15 xenografts. Similar results were obtained with non-established and pre-established 100 mm³ tumors (Fig. 10). 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.

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 EC₅₀ for reversal of P-gp-mediated MDR to doxorubicin, vinblastine, and paclitaxel in six different cell lines was 0.032 \pm 0.023 μ M, with complete reversal in the 0.25–1.0 μ M

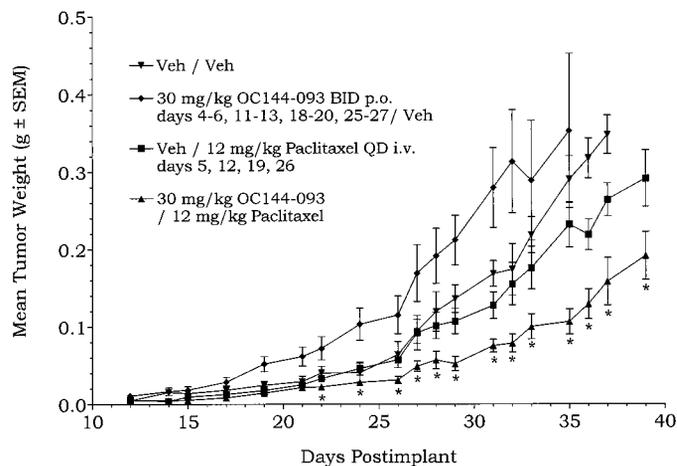


Fig. 9. Effect of combination treatment with OC144-093 and paclitaxel on growth of orthotopic MDA/LCC6^{MDR1} human breast carcinoma in SCID/RAG2 mice. Ascites-propagated cells (2×10^6 in 50 μ l) were injected into mammary fat pads bilaterally in groups of five female SCID/RAG2 mice on day 0. Paclitaxel (12 mg/kg/dose) in 200 μ l of saline: Cremophor: ethanol (8:1:1 by volume) and OC144-093 free base (30 mg/kg/dose) in 100 μ l of PEG 400:Tween 20 (9:1 by volume) were administered as indicated in the figure and "Materials and Methods." *, statistically significant difference in mean group tumor size compared with paclitaxel alone by Mann-Whitney analysis ($P < 0.05$). Similar results were obtained in two independent experiments. Bars, SE.

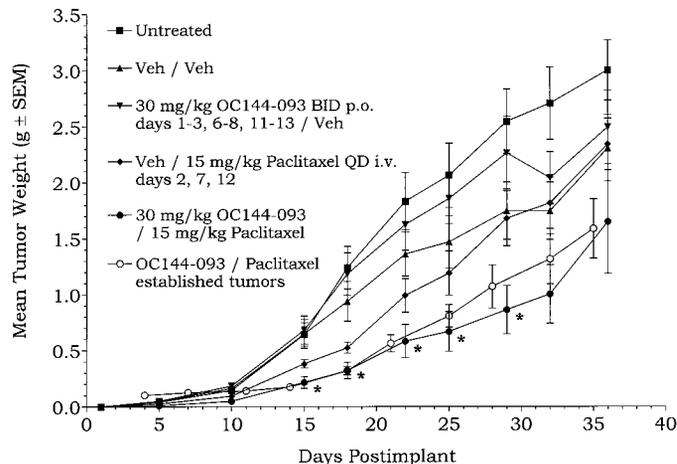


Fig. 10. Effect of combination treatment with OC144-093 and paclitaxel on growth of s.c. HCT-15 human colon carcinoma in athymic mice. The experiment was carried out with female athymic NCr-nu mice (eight/group) implanted s.c. with 10^7 HCT-15 cells on day 0. Paclitaxel (15 mg/kg/dose) in 200 μ l of saline: Cremophor: ethanol (8:1:1 by volume) and OC144-093 free base (30 mg/kg/dose) in 100 μ l of PEG 400:Tween 20 (9:1 by volume) were administered as indicated in the figure and "Materials and Methods." *, statistically significant difference in mean group tumor size compared with paclitaxel alone by Mann-Whitney analysis ($P < 0.05$). Similar results were obtained in a separate experiment when treatment with OC144-093 plus paclitaxel was initiated with established (0.1 g) tumors (curve with \circ). Paclitaxel treatment was carried out on days 5, 10, 15, and 20. Control and paclitaxel-treated tumor growth was similar to the illustrated experiment (data not shown). Bars, SE.

range. In terms of the EC₅₀s and number of cell types and cytotoxic agents tested, OC144-093 is at least as potent as any other P-gp inhibitor currently in development (for examples, see Refs. 21–23, 26, and 42, 43). The average IC₅₀ 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 IC₅₀s 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 XR9051 (23), GF120918 (25), or LY335979 (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 IC₅₀ required for this effect was slightly higher than the EC₅₀ 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 UCLA-P3.003VLB 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/m² 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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