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ORIGINAL ARTICLE

Voreloxin, formerly SNS-595, has potent activity against a broad panel of cancer cell lines and in vivo tumor models

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Abstract
Purpose Voreloxin, formerly known as SNS-595 or AG-7352, is a novel naphthyridine analog currently under investigation for the treatment of ovarian and hematologic malignancies. Voreloxin mechanism of action includes DNA intercalation and inhibition of topoisomerase II that causes selective DNA damage. In this study, we describe the anti-proliferative activity of voreloxin in a wide range of in vitro and in vivo models of human cancers.
Methods The cytotoxicity of voreloxin in vitro was examined by MTT assay in 15 cell lines, including 4 drug-resistant lines. Activation of caspase in cell lines and tumors was evaluated by immunohistochemistry. Anti-tumor activity was assessed in 16 xenograft and 3 syngeneic tumor models in mice. Tumors were allowed to grow to approximately 150 mm^3 prior to treatment with voreloxin or comparator drugs. Activity of the anti-cancer agents was determined by calculating the inhibition rate (IR = [1 − (average tumor weight treated/average tumor weight control)] × 100%) and survival ratio (number surviving mice/number of mice per group at start of study) for each agent and dose and schedule tested.
Results In vitro studies demonstrated voreloxin has broad anti-proliferative activity in 11 tumor cell lines, with IC_{50} values ranging from 0.04 to 0.97 μM. Similar activity was observed in vitro in drug-resistant cell lines, including those that overexpress P-glycoprotein and have reduced topoisomerase levels. After a single intravenous dose, voreloxin concentrations in tumor were correlated with induction of the apoptosis marker caspase-3. The optimal dose and schedule was established using a KB nasopharyngeal carcinoma xenograft model. Administration of voreloxin at 20 mg/kg weekly for five doses effectively inhibited tumor growth (86%). Voreloxin demonstrated strong dose-dependent tumor growth inhibition (63–88%) in 10 of 11 solid tumor (breast, ovarian, colon, lung, gastric, and melanoma) xenograft models, 2 hematologic tumor xenograft models, 3 multidrug resistant tumor models and 3 murine syngeneic tumor models (Colon 26, Lewis Lung carcinoma, M5076 Ovarian Sarcoma).
Conclusions These data demonstrate that voreloxin is a broadly active anti-tumor agent in vitro and in vivo, with potent activity in aggressive and drug-resistant tumor models.

Keywords Voreloxin · Anti-cancer agent · Broad activity

Introduction

Cancer continues to be a leading cause of morbidity and mortality among humans worldwide and is the second leading cause of death in the USA [1]. There remains a large unmet need for new agents with novel mechanisms of action and potent anti-tumor activity. Voreloxin, formerly known as SNS-595 or AG-7352, is a novel naphthyridine analog, which is structurally related to the quinolone antibiotics, a chemical class not previously used for the treatment of cancer [2, 3]. Recently, quinolone analogs have been shown to mediate anti-tumor activity by targeting mammalian topoisomerases, and have demonstrated promising nonclinical activity [4–7]. Similarly, voreloxin intercalates

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DNA in the presence of topoisomerase II resulting in selective, replication-dependent DNA damage, irreversible G2 arrest and rapid apoptosis [8].

Voreloxin exhibits favorable pharmacologic properties, including low potential for drug–drug interactions, lack of efflux by P-glycoprotein (P-gp), dose proportional pharmacokinetics with low intra- and inter-patient variability [9]. In phase 1 and 2 studies, voreloxin was generally well tolerated [10]. The dose limiting toxicities (DLT) were myelosuppression in solid tumors and reversible oral mucositis in hematologic malignancies [11, 12]. Clinical activity, including objective responses, has been observed in ovarian cancer, small cell and non small cell lung cancers, as well as in advanced acute myeloid leukemia (AML) [11, 13, 14]. Clinical studies of voreloxin in AML, as a single agent and in combination with cytarabine, and as a single agent in ovarian cancer are ongoing.

In the current investigation, the activity of voreloxin was investigated in vitro and in vivo in a panel of tumor cell lines and in multiple tumor models including three that were multidrug resistant. The in vivo tumors included fourteen solid tumor human xenograft models, two hematologic models and three syngeneic mouse models. Three of the human xenograft models were drug-resistant due to one or more mechanisms including increased drug efflux, increased glutathione peroxidase activity, decreased activity of topoisomerases, and acquired cisplatin resistance. The data presented in this study demonstrate that voreloxin has broad in vitro anti-proliferative activity and potent anti-tumor activity in vivo and support the clinical investigation of voreloxin in multiple indications.

Materials and methods

Reagents

Voreloxin, (+)-1,4-dihydro-7-[(3*S*,4*S*)-3-methoxy-4-(methylamino)-1-pyrroli-dinyl]-4-oxo-1-(2-thiazoyl)-1,8-naphthyridine-3-carboxylic acid, was synthesized at Dainippon Sumitomo Pharmaceutical Co. Ltd Etoposide and cisplatin (CDDP) were purchased from Nippon Kayaku Co. Ltd (Tokyo, Japan), doxorubicin (ADM) and 5-fluorouracil (5-FU) were obtained from Kyowa Hakko Kogyo Co. Ltd (Tokyo, Japan), irinotecan hydrochloride was obtained from Yakult Honsha Co. Ltd, paclitaxel was obtained from Sigma–Aldrich (St Louis, MO, USA), and gemcitabine was obtained from Eli Lilly and Company (Indianapolis, Indiana). All culture media and supplements were purchased from ICN Biomedicals, Inc. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich.

Cell culture

The following human tumor cell lines were used: SK-BR-3 breast adenocarcinoma, HCT116 colon carcinoma, PA-1 ovarian carcinoma, Calu-6 lung carcinoma, NCI-H460 large-cell lung carcinoma, GT3TKB stomach adenocarcinoma (Riken Cell Bank, Japan), Hs746T stomach carcinoma, Panc-1 pancreatic carcinoma, SCaBER bladder carcinoma, KB nasopharyngeal carcinoma, MES-SA uterine sarcoma and its ADM resistant clone MES-SA/Dx5, SBC-3 small-cell lung carcinoma, and its ADM and etoposide resistant clones SBC-3/ADM, and SBC-3/ETP (Second Department of Medicine, Okayama University, Japan), PC-14 non-small cell lung carcinoma and its CDDP resistant clone PC-14/CDDP (Pharmacology Division of the National Cancer Research Institute in Japan) [15], SKOV3 ovarian adenocarcinoma, CCRF-CEM acute lymphoblastic leukemia, and LM-3 Jck lymphoma (Central Institute for Experimental Animals, Japan). Except as noted, cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

SK-BR-3, HCT116, MES-SA, and MES-SA/Dx5 cell lines were cultured in McCoy 5A media, supplemented with 10% fetal bovine serum (FBS). Calu-6, SCaBER, and KB cells were cultured in Eagle's Minimum Essential Media (EMEM) supplemented with 10% FBS and non essential amino acids, and sodium pyruvate for Calu-6 cells. PA-1 cells were cultured in EMEM, supplemented with 10% heat-inactivated FBS and NEAA. NCI-H460, SBC-3, SBC-3/ADM, and SBC-3/ETP cell lines were cultured in RPMI1640 media supplemented with 10% FBS. GT3TKB, HS746T, and PANC-1 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. SKOV3, PC-14, and PC-14/CDDP cell lines were cultured in RPMI1640 media supplemented with 10% heat-inactivated FBS. All cell lines were incubated at 37°C in a humidified atmosphere at 95% air and 5% CO_2.

Growth inhibition assays

Cell proliferation assays were performed using the MTT [(3-(4,5)-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cytotoxicity assay as previously described [16, 17]. Briefly, cells were seeded in 96 well plates at 500–5,000 cells per well and incubated for 24 h at 37°C. Cells were then exposed to various concentrations (twofold dilution series) of the indicated drugs for 72 h at 37°C. MTT solution was added and cells were incubated at 37°C for 4 h. The cells were lysed with DMSO (dimethyl sulfoxide) or 20% (w/v) SDS (sodium dodecyl sulfate) solution in 0.02 N HCl and absorbance at 570 nm was measured with a microplate reader. The drug concentration required for 50%

of growth inhibition (IC_{50}) was calculated by linear regression of the percentages of cell growth plotted against the natural logarithm of the compound concentrations.

Preparation of dosing solutions

Voreloxin was dissolved in 4.5% sorbitol containing 0.17% methanesulfonic acid and further diluted with 5% sorbitol containing 0.02% methanesulfonic acid to obtain dosing solutions with the required concentrations. Etoposide was diluted with distilled water and irinotecan, doxorubicin and gemcitabine were dissolved in saline to yield a dosing volume of 0.1 mL/10 g of body weight. Paclitaxel was formulated in a 1:1 mixture of ethanol:Cremophor EL and further diluted with saline to achieve a dosing volume of 0.15 mL/10 g of body weight. Injectable cisplatin was used without further dilution in a volume of 0.2 mL/10 g of body weight.

Animal experiments

All animal experiments were carried out according to the governing institutional animal use guidelines (Piedmont Research, Colo-26 model, Dainippon-Sumitomo, all other models). Animals were housed in cages and received pellet diet sterilized by γ-rays and autoclaved tap water *ad libitum*. Mice were weighed using a digital balance and tumor sizes were measured with digital electric calipers. Tumor weight was calculated from the tumor sizes according to the following formula: tumor weight (mg) = (tumor length in mm) $\times$ (tumor width in mm)^2/2.

Voreloxin plasma and tumor concentrations

HCT116 tumor cells were grown and implanted as described above. When tumor weights reached 300 mg, animals received a single intravenous dose of voreloxin. For determination of tumor and plasma levels, animals (n = 4/timepoint) received 20 mg/kg voreloxin. Animals were sacrificed and blood samples were obtained by cardiac puncture and tumors were harvested between 1 and 24 h postdose. Blood samples were collected into K_2-EDTA tubes and placed on ice until centrifugation to recover plasma. Plasma samples were stored frozen until analyzed by LC–MS/MS. A 50-mg tumor piece was homogenized with five volumes of ice-cold phosphate-buffered saline (PBS) (pH 7.4) and analyzed by LC-MS/MS.

Tumor IHC

For IHC analysis, animals received 20 mg/kg voreloxin. After euthanasia, tumor samples were harvested 4 h postdose, placed in Streck fixative and transported to BioPathology Sciences (South San Francisco, CA, USA) where they were paraffin embedded, sectioned, and transferred to slides. Tumor sections were then stained with hematoxylin and eosin (H&E) and a rabbit anti-caspase-3 antibody (Cell Signaling #9661, 1:200 diluted in Dako diluent), selective for cleaved caspase-3.

Quantification of intra-tumoral activation of caspase-3

Tumors from three animals were collected 4 h after a single dose of 40 mg/kg voreloxin for quantification of caspase-3 activation. Tumor samples were frozen on liquid nitrogen, and ground into a fine powder. Lysis buffer containing phosphatase inhibitors was added to the tumor powder followed by homogenization and a snap freeze cycle. The cellular debris was removed by centrifugation, and the protein concentration was measured using the BioRad DC Protein Assay. Caspase-3 was quantified using a capture ELISA. A mouse anti-human caspase-3 monoclonal antibody (BD610322) recognizing both procaspase-3 and active caspase-3 (capture antibody) was immobilized onto a MaxiSorp plate (Nunc) in 50 mM Na_2CO_3, pH 9.0, and blocked with 5% milk. An aliquot of tumor lysate [50 µg protein diluted in SuperBlock (Pierce)] was added to each well and incubated for 1 hr. Processed procaspase-3 was detected by a second rabbit polyclonal antibody that recognizes the large subunit of active caspase-3 (detection antibody, 1:2,000 in SuperBlock). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Chemicon #AP156P) and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Pierce) were used for detection according to the manufacturer's instructions.

LC–MS/MS analysis

For quantification of voreloxin, control mouse plasma was spiked with voreloxin to yield a standard solution containing 2,000 ng/mL. Serial twofold dilutions were prepared to yield concentrations as low as 1 ng/mL. Voreloxin was extracted from plasma standards, unknown plasma samples and tumor homogenates using an equal volume of internal standard solution (Verapamil in acetonitrile). An aliquot of the extract was analyzed using an API4000 mass spectrometer coupled to a turbo electro spray ionization source run in positive ionization mode. Chromatography was performed with an Agilent 1100 HPLC system using a Phenomenex Synergi Hydro-RP column (30 $\times$ 2 mm, 4 µm, 80A particle size), with a flow rate of 700 µL/min. The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following gradient was used: hold 90% solvent A for 0.5 min then increase to 95% solvent B over 1.5 min. Voreloxin and internal standard eluted between 1.1 and 1.5 min. The peak area of the product ion of voreloxin was measured

against the peak area of the product ion of the internal standard. Quantification was performed using a 1/× weighted linear least squares regression line generated from similarly extracted matrix calibration samples ranging from 1.0 to 2,000 ng/mL.

Optimization of voreloxin dosing schedule

The effective voreloxin dose and administration schedule in tumor-bearing mice was determined using female BALB/c/nu/nu mice that were intradermally implanted with 2.5×10^6 KB cells. When tumor weights reached 100–500 mg, groups of seven mice were pair matched. Animals received intravenous (IV) injections of vehicle or voreloxin starting on Day 1 according to one of the following dosing schedules: (1) 10–40 mg/kg as a single injection (qd × 1), (2) 1–4 mg/kg daily for 5 days (qd × 5), (3) 5–30 mg/kg every 4 days for five doses (q4d × 5) and (4) 10–40 mg/kg every 7 days for five doses (q7d × 5). Mice were weighed and tumor volumes were measured twice weekly. Activity of voreloxin was determined on Day 35 by calculating the inhibition rate (IR = [1 − (average tumor weight treated/average tumor weight control)] × 100%) and survival ratio (number surviving mice/number of mice per group at start of study) for each dose and schedule tested. Inhibition rate in the Colon 26 adenocarcinoma scheduling study was determined on Day 20. Complete regressions are defined as tumor volumes $\leq$13.5 mm^3 for three consecutive measurements.

Activity in solid tumor xenograft models

PA-1, SK-OV-3, WiDr, HCT116, NCI-H460, Calu-6, Hs746T, GT3TKB, and SK-MEL-5 cells were grown in vitro and transplanted subcutaneously (SC) or intradermally into female BALB/c/nu/nu mice. For RF-1 and MDA-MB-231 tumor models, 2–3 mm tumor pieces were transplanted SC into female BALB/c/nu/nu mice. When tumor weights reached 100–500 mg, animals received voreloxin 15 or 20 mg/kg or vehicle IV q7d × 5 doses. Mice were weighed and tumor volumes were measured twice weekly. The activity of voreloxin, as determined by IR, was compared to that observed with cisplatin (CDDP, 10 mg/kg IV qd × 1), etoposide (12 mg/kg IV qd × 5), doxorubicin (Dox, 12 mg/kg IV qd × 1), irinotecan (IRN, 100 mg/kg IV q4d × 3) and paclitaxel (28 mg/kg IV qd × 5).

Activity in leukemia and lymphoma tumor models

For CCRF-CEM and LM-3 Jck tumor models, 2–3 mm tumor pieces were transplanted SC into female BALB/c/nu/nu mice. When tumor weights reached 350–2,250 mg (CCRF-CEM) or 170–690 mg (LM-3 Jck), animals received voreloxin 20–30 mg/kg or vehicle IV q7d × 3 doses. Mice were weighed and tumor volumes were measured twice weekly. The activity of voreloxin, as determined by IR, was compared to that observed with cisplatin (CDDP, 10 mg/kg IV qd × 1), etoposide (12 mg/kg IV qd × 5), doxorubicin (Dox, 12 mg/kg IV q7d × 3 for CCRF-CEM and qd × 1 for LM-3 Jck), irinotecan (IRN, 100 mg/kg IV q4d × 3) and paclitaxel (42 mg/kg IV qd × 5).

Activity in multidrug resistant models

MES-SA/Dx5 cells were grown in vitro and transplanted SC into female BALB/c/nu/nu mice. For PC-14/CDDP and SBC-3/ETP tumor models, 2–3 mm tumor pieces were transplanted SC into female BALB/c/nu/nu mice. voreloxin (20 or 25 mg/kg) or vehicle was administered IV q7d × 5 and compared for effects on tumor growth inhibition. Similar comparisons were made following the administration of cisplatin (CDDP, 10 mg/kg IV qd × 1 and 7 mg/kg IV q4d × 3), etoposide (12 mg/kg IV qd × 5), doxorubicin (Dox, 12 mg/kg IV qd × 1), irinotecan (IRN, 100 mg/kg IV q4d × 3) and paclitaxel (42 mg/kg IV qd × 5).

Activity in syngeneic tumor models

Colon 26 adenocarcinoma (0.1 mL of a 2% tumor brei) was transplanted intradermally into the abdominal wall of CDF1 mice (Experiment A) or 1×10^6 cells were transplanted subcutaneously in the flank of BALB/c mice (Experiment B). Lewis Lung carcinoma (5×10^5 cells) was transplanted intradermally into the abdominal wall of BDF1 mice. M5076 sarcoma (1×10^6 cells) was transplanted intradermally into the abdominal walls of C57BL/6 mice. Tumor growth inhibition was determined following the administration of voreloxin (5–50 mg/kg IV q7d × 3, q14d × 3, q21d × 3), cisplatin (CDDP, 10 mg/kg IV qd × 1), etoposide (12 mg/kg IV qd × 5), doxorubicin (Dox, 24 mg/kg IV qd × 1), paclitaxel (28 mg/kg IV qd × 5), and gemcitabine (160 mg/kg IP q3d x 4). In addition to the inhibition rate, the inhibitory effect on pulmonary metastasis was estimated by calculation of the metastatic inhibition rate (IR = [1 − (average number of nodules on the lung surface in drug-treated group/average number of nodules on the lung surface in control group)] × 100%) in the Lewis Lung model.

Statistical analysis

Mean tumor size in vehicle- and drug-treated groups was compared using a two-tailed Student's t test, for comparison of two groups, or the two-tailed Dunnett's test, for comparison of more than two groups. A P value < 0.05 was considered to be statistically significant.

Results

Broad in vitro activity of voreloxin across multiple tumor cell lines

Voreloxin activity was profiled against the commonly used chemotherapeutic agents etoposide, doxorubicin, irinotecan, cisplatin, paclitaxel and 5-FU, in 11 tumor lines of diverse tissue origins (Table 1). Voreloxin was broadly active in these cell lines, inhibiting cell proliferation with IC_{50} values ranging between 0.039 and 0.968 μM. Paclitaxel IC_{50} values ranged between 0.001 and 0.003 μM, doxorubicin IC_{50} values ranged between 0.002 and 0.076 μM, cisplatin IC_{50} values ranged between 0.041 and 2.643 μM, etoposide IC_{50} values ranged between 0.042 and 5.595 μM, irinotecan IC_{50} values ranged 0.39–27.61 μM, and 5-FU IC_{50} values ranged between 1.28 and 100 μM. Voreloxin is, therefore, among the most potent agents tested.

Voreloxin retained potent anti-proliferative activity in four-cell lines that are multidrug resistant due to various resistance mechanisms (Table 1). In the P-glycoprotein (P-gp)-overexpressing cell line MES-SA/Dx5, the potencies of doxorubicin, etoposide and paclitaxel—all established P-gp substrates—were significantly diminished, with IC_{50} values that were 22-, 6- and >81-fold greater, respectively, relative to the parental MES-SA cells. In contrast, voreloxin inhibited cell proliferation with an IC_{50} of 0.237 μM, only a two-fold increase from that observed in parental MES-SA cells. Similarly modest increases in IC_{50} were observed for cisplatin, 5-FU or irinotecan, agents that are not substrates for the P-gp efflux pump. SBC-3/ADM and SBD-3/ETP cells are resistant to doxorubicin and etoposide, respectively, due to (1) increased efflux mediated by transporters such as P-gp and MRPs, (2) increased glutathione peroxidase activity and (3) decreased activity of topoisomerase I and II [18, 19]. The SBC-3/ADM cell line was 30-, 65-, and >180-fold more resistant to etoposide, doxorubicin and paclitaxel, respectively, but remained sensitive to voreloxin, irinotecan, cisplatin and 5-FU. Similarly, the SBC-3/ETP cell line, demonstrated 216-, 48-, and >180-fold increases in cross-resistance to etoposide, doxorubicin, and paclitaxel, respectively, but remained sensitive to voreloxin, irinotecan, cisplatin and 5-FU. The cell line PC-14/CDDP displays acquired cisplatin resistance which is likely due to a reduction in cisplatin accumulation in these cells [20, 21]. Compared to the PC-14 cell line, the resistant variant demonstrated an eightfold increase in IC_{50} when exposed to cisplatin, but remained sensitive to voreloxin, doxorubicin, etoposide, irinotecan, paclitaxel and 5-FU. Taken together, the data from these drug-resistant cell lines demonstrate that voreloxin retains potent anti-proliferative activity in cells which are multidrug resistant by distinct mechanisms.

Assessment of apoptosis and voreloxin pharmacokinetics in HCT116 xenografts

The potential of voreloxin to induce apoptosis in vivo was evaluated in HCT116 xenografts. This model was selected due to its favorable growth characteristics in vitro and its sensitivity to voreloxin in vitro and in vivo. Administration of a single IV dose of voreloxin resulted in activation of caspase-3, a key mediator of apoptosis. Examination of tumor sections showed a sevenfold increase in cells staining positive for cleaved caspase-3, Fig. 1a. A fivefold activation of caspase-3 was detected in lysates from tumor harvested 6 h after treatment with voreloxin (Fig. 1b). Plasma and tumor concentrations after single IV dose of 20 mg/kg are shown in Fig. 1c. Tumor concentrations exceeded plasma concentrations by 11- to 48-fold. While voreloxin plasma concentrations were below 10 ng/mL (the quantifiable limit) 16 h post-administration, tumors levels were still 726 ng/mL (1.8 μM) 24 h post-dose, resulting in 2 and 8 h terminal half-lives in plasma and tumor, respectively. The long tumor half-life supports intermittent dosing schedules for voreloxin.

Dose and schedule optimization

The tolerability and anti-tumor activity of various doses and schedules of voreloxin were evaluated using the human KB nasopharyngeal xenograft model. Administration of single IV doses voreloxin resulted in dose-dependent anti-tumor activity compared to vehicle, with IR of 39 and 43% at 30 and 40 mg/kg, respectively. However, the toxicity at 40 mg/kg was unacceptable as significant loss of body weight and some deaths were observed. Daily × 5 administration of voreloxin at 1 and 2 mg/kg lacked significant anti-tumor activity and higher doses (3 and 4 mg/kg) resulted in death of all animals. Potent anti-tumor activity was observed with 5 and 10 mg/kg doses administered q4d × 5, resulting in IRs of 55 and 74%, respectively. However, doses of 20 mg/kg and higher administered q4d × 5 were fatal to all the treated animals. Administration of voreloxin weekly × 5, at doses of 10, 20, and 30 mg/kg resulted in strong dose-dependent anti-tumor activity, yielding IRs of 61, 86, and 92%, respectively. Although all animals survived administration of 30 mg/kg weekly × 5, an unacceptable maximum body weight loss of 31% was observed, indicating that the maximum tolerated dose (MTD) on this schedule was approximately 20 mg/kg. Combined, these data indicate that the q7d × 5 was the least dose intensive schedule that exhibited effective anti-tumor activity with acceptable tolerability. This schedule was therefore selected for evaluation of voreloxin in subsequent in vivo studies.

Table 1 In vitro growth inhibition by voreloxin and other cytotoxic agents in human tumor cell lines

Tumor type cell line	IC_{50} values (μM)[a] (relative resistance)						
	Voreloxin	Etoposide	DOX	Irinotecan	CDDP	Paclitaxel	5-FU
Breast adenocarcinoma							
SK-BR-3	0.27	0.39	0.038	1.87	0.81	0.0011	38.46
Bladder carcinoma							
SCaBER	0.30	0.47	0.014	5.60	1.26	0.0006	1.51
Pancreas carcinoma							
PANC-1	0.97	2.72	0.076	27.61	2.40	0.0025	5.12
Nasopharyngeal epidermoid carcinoma							
KB	0.19	0.17	0.006	2.07	0.34	0.0009	10.00
Colon, carcinoma							
HCT 116	0.23	0.54	0.034	1.05	1.30	0.0010	3.90
Ovary, adenocarcinoma							
SKOV3	0.66	1.80	0.065	4.79	2.50	0.0032	6.49
Stomach adenocarcinoma							
GT3TKB	0.33	0.67	0.027	2.23	0.78	0.0018	7.35
Stomach carcinoma							
Hs746T	0.48	5.60	0.031	9.34	2.64	0.0032	100.00
Lung carcinoma							
Calu-6	0.75	0.73	0.053	7.33	0.34	0.0006	7.45
Large-cell lung carcinoma							
NCI-H460	0.10	1.30	0.006	1.75	0.41	0.0017	2.88
Ovarian carcinoma							
PA-1	0.04	0.04	0.002	0.39	0.04	0.0008	1.28
Average	0.39	1.31	0.032	5.82	1.16	0.0016	16.77
SD	0.29	1.62	0.025	7.76	0.95	0.0010	29.48
Drug resistant cell lines							
Uterus sarcoma							
MES-SA	0.096	0.223	0.013	1.576	0.109	0.00145	4.238
MES-SA DX5	0.237	1.418	0.285	4.633	0.340	>0.1	6.008
Doxorubicin resistant	(2)	(6)	(22)	(3)	(3)	(>81)	(1.4)
Small-cell lung carcinoma							
SBC-3	0.079	0.115	0.012	0.457	0.238	0.000648	1.823
SBC-3/ADM	0.110	3.418	0.795	2.059	0.257	>0.1	2.646
Doxorubicin resistant	(1.4)	(30)	(65)	(4)	(1.1)	(>181)	(1.4)
SBC-3/ETP	0.401	24.830	0.597	0.852	0.163	>0.1	1.962
Etoposide resistant	(5)	(216)	(48)	(2.0)	(0.7)	(>181)	(1.1)
Non-small-cell lung carcinoma							
PC-14	1.155	1.785	0.0856	5.367	2.447	0.00170	7.50
PC-14/CDDP	0.406	0.910	0.043	4.836	20	0.00499	2.038
Cisplatin resistant	(0.3)	(0.5)	(0.5)	(0.9)	(8)	(3)	(0.3)

Dox doxorubicin, *CDDP* cisplatin, *5-FU* 5-fluorouracil

[a] IC_{50} was determined by MTT assay as described in "Materials and methods". Each value is the mean of 2–5 independent experiments.

Voreloxin has potent activity in multiple human solid tumor xenografts

The anti-tumor activities of voreloxin, cisplatin, etoposide, irinotecan, doxorubicin, and paclitaxel, administered at their respective MTDs against 11 human tumors of diverse origins are summarized in Table 2. At 20 mg/kg, voreloxin had potent anti-tumor activity in 10 of the 11 models yielding IRs ranging from 55 to 88%. At a dose of 15 mg/kg (75% of the MTD), voreloxin still exhibited significant inhibition of tumor growth in most models with IRs ranging from 51 to 85%. The RF-1 (gastric) xenograft model was the only tumor cell line unresponsive to voreloxin.

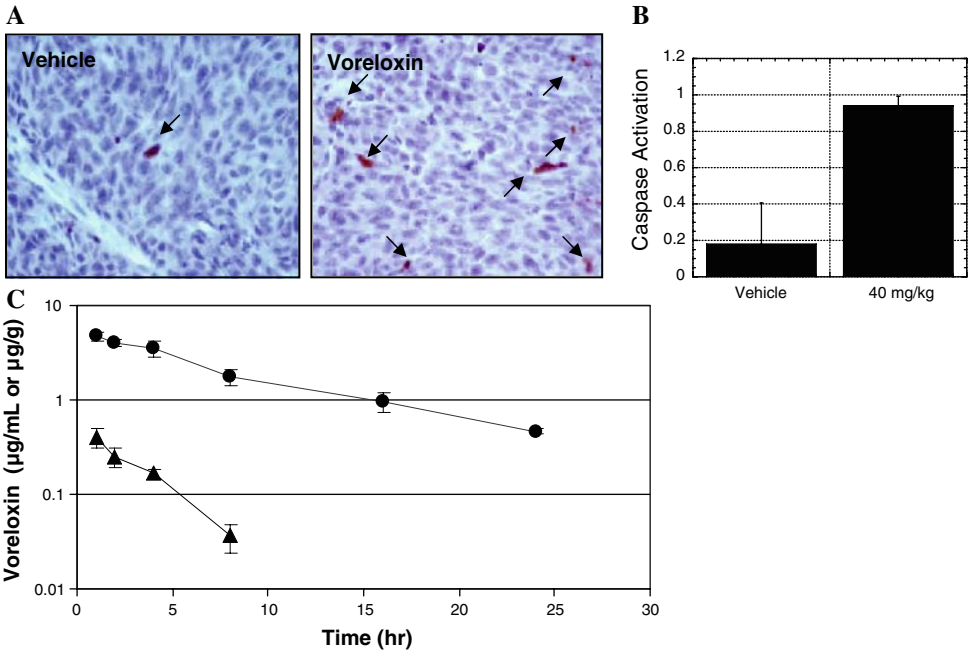

Fig. 1 Caspase-3 Activation and Pharmacokinetics in HCT116 Tumors after IV Treatment with voreloxin. **a** Tumor cross-section of vehicle and voreloxin treated tumors harvested 4 h after treatment with 20 mg/kg voreloxin. Voreloxin demonstrates 7 caspase-3 positive cells. **b** Quantitation of caspase-3 6 h after treatment with 40 mg/kg voreloxin. Voreloxin demonstrates a fivefold increase in caspase-3 compared to vehicle treated animals. **c** Plasma and tumor levels in HCT116 tumors after IV treatment with 20 mg/kg voreloxin; *filled circle* tumor concentration, *filled triangle* plasma concentration

Similar to voreloxin, irinotecan and paclitaxel exhibited significant inhibition of tumor growth in most models tested, with IRs ranging from 55–100 and 43–100%, respectively.

Cisplatin had significant anti-tumor activity in SK-OV-3, WiDr, NCI-H460, and Calu-6 models with IRs of 52, 33, 25, and 84%, respectively. Anti-tumor activity of cisplatin in the remaining models was modest. Etoposide demonstrated low, but significant, activity in the two lung models and the HCT116 colon carcinoma model, with IRs of 31, 45, and 26%, respectively. Doxorubicin showed significant activity in the two lung models, HCT116 colon carcinoma model and HS746T gastric tumor model with IRs of 49, 70, 40, and 99%, respectively. Therefore, consistent with its strong activity in vitro, voreloxin is among the most potent agents tested in vivo with significant activity in 10 of 11 tumor models. Only paclitaxel and irinotecan show comparable activity with significant anti-tumor effects in eight of eight and eight of nine tumor models, respectively. Doxorubicin, cisplatin, and etoposide had activity in less than half the models studied.

Voreloxin is active in hematologic tumor models

In addition to assessing the activity of voreloxin in solid tumor models, potency was also characterized in two hematologic tumor models (Table 2). In human CCRF–CEM acute lymphoblastic leukemia xenografts, voreloxin showed significant anti-tumor activity at 25 mg/kg, with an IR of 98%. Among the comparator drugs, doxorubicin, irinotecan, and paclitaxel showed significant anti-tumor activity with IRs of 50, 100, and 100%, respectively. Etoposide was only weakly active in this model (IR: 28%). In human LM-3 Jck lymphoma, voreloxin had significant anti-tumor activity at 20 mg/kg, with an IR of 96%. Among the comparator drugs, doxorubicin, irinotecan, and paclitaxel showed significant anti-tumor activity, with IRs of 57, 98, and 97%, respectively. Etoposide and cisplatin were not active in this tumor model.

Voreloxin is active in drug-resistant tumor models

Having demonstrated that voreloxin retained activity in drug-resistant cell lines in vitro, the activity of voreloxin in vivo was examined in three drug-resistant xenograft models. Administration of voreloxin at a dose of 25 mg/kg weekly × 5 showed significant anti-tumor activity in the multidrug resistant MES-SA/Dx5 (P-gp over-expressing) xenograft model with an IR of 87% (Fig. 2a). Irinotecan showed significant anti-tumor activity, with an IR of 47%. In contrast, the P-gp substrates doxorubicin and paclitaxel showed weak activity in this drug-resistant model. In the SBC-3/ETP model, voreloxin exhibited significant anti-tumor activity at a dose of 20 mg/kg, with an IR of 80% (Fig. 2b). Doxorubicin, etoposide, and paclitaxel lacked significant activity in this resistant tumor model. Finally, in the cisplatin resistant PC-14/CDDP tumor model, voreloxin had strong anti-tumor activity when administered at a dose of 20 mg/kg (IR of 79%) (Fig. 2c, d). Irinotecan and paclitaxel also exhibited anti-tumor activity, with IRs of 56 and 56%, respectively. In contrast, cisplatin, doxorubicin and etoposide showed weak or no anti-tumor activity in this tumor model.

Table 2 Tumor growth inhibition and survival in tumor xenograft models following voreloxin, irinotecan, doxorubicin, paclitaxel, cisplatin, and etoposide administration

Drug route, schedule dose (mg/kg)	Human Tumor Cell Line Type Percent Inhibition (%)[a,b]												
	Breast	Ovarian		Colon		Lung		Gastric			Melanoma	Leukemia	
	MDA-MB-231	PA-1	SK-OV-3	WiDr	HCT116	NCI H460	Calu-6	Hs746T	GT3TKB	RF-1	SK-MEL-5	CCRF-CEM	LM-3 Jck
Voreloxin IV, q7d × 5													
15	80* (6/6)	85* (6/6)	63* (7/7)	55* (7/7)	63* (7/7)	75* (7/7)	82* (7/7)	77* (7/7)	69* (6/6)	−13 (7/7)	51 (6/6)	ND	ND
20	85* (6/6)	85* (6/6)	71* (7/7)	63* (7/7)	82* (7/7)	84* (7/7)	88* (7/7)	83* (7/7)	65* (6/6)	−8 (7/7)	55* (6/6)	ND	96* (6/6)
25	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	98* (6/6)	ND
Cisplatin IV, Single dose													
10	25 (6/6)	33 (5/6)	52* (7/7)	33* (7/7)	13 (6/7)	25* (6/7)	84* (6/7)	55 (7/7)	45 (4/6)	40 (7/7)	ND	ND	3 (6/6)
Etoposide IV, qd × 5													
12	45 (6/6)	38 (5/6)	14 (7/7)	−1 (7/7)	26* (7/7)	31* (7/7)	45* (7/7)	1 (7/7)	37 (6/6)	−37 (6/7)	30 (6/6)	28 (6/6)	3 (6/6)
Irinotecan IV, q4d × 3													
100	88* (7/7)	94* (7/7)	70* (7/7)	55* (7/7)	71* (7/7)	64* (7/7)	90* (7/7)	100* (7/7)	55 (7/7)	ND	ND	100* (5/6)	98* (6/6)
Doxorubicin IV, qd × 1													
12	44 (7/7)	47 (7/7)	20 (7/7)	26 (7/7)	40* (7/7)	49* (7/7)	70* (7/7)	99* (7/7)	46 (7/7)	ND	ND	50* (6/6)	57* (6/6)
Paclitaxel IV, qd × 5													
28	ND	99* (7/7)	97* (7/7)	97* (7/7)	96* (7/7)	43* (7/7)	100* (7/7)	100* (7/7)	98* (7/7)	ND	ND	ND	ND
42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	100* (6/6)	97* (6/6)

[a] Values represent (1 − T/C) × 100%

[b] (number surviving animals/number animals at beginning of study)

* Statistical significance was evaluated by comparing the mean-tumor size of vehicle-treated groups to drug-treated groups using a two-tailed Dunnett's test. *P* values of less than 0.05 were considered statistically significant

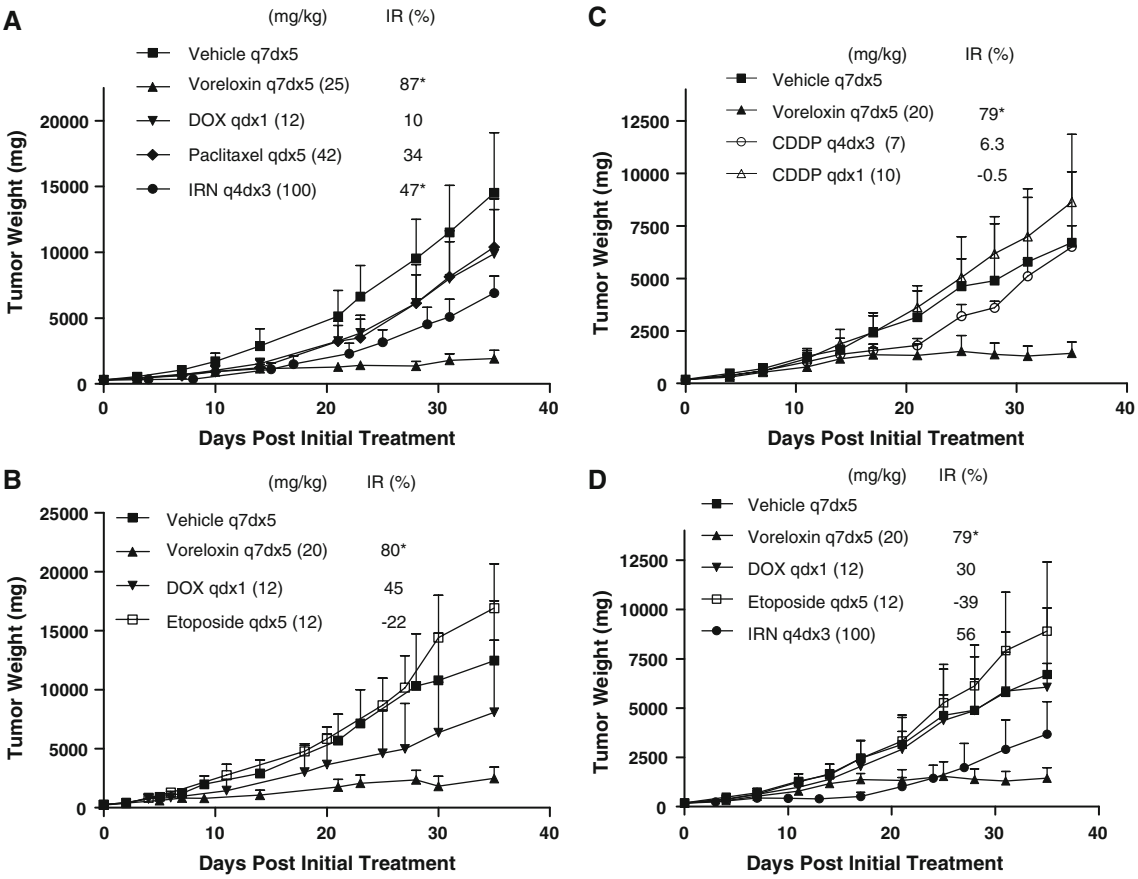

Fig. 2 Activity in multidrug-resistant tumor models. **a** MES-SA/Dx5; **b** SBC-3/ETP; **c** PC-14/CDDP: comparison of voreloxin against CDDP. **d** PC-14/CDDP: comparison of voreloxin against other marketed therapeutics. All agents were administered intravenously using the schedules and doses indicated above. Inhibition rate (*IR*) represents $(1 - T/C) \times 100$. Values marked with * are statistically significant compared to vehicle-treated groups. Each schedule and agent had its own vehicle control group; only the voreloxin vehicle group is shown. *Error bars* indicate one standard deviation. Abbreviations: *CDDP* cisplatin, *Dox* doxorubicin, *IRN* irinotecan

Voreloxin is active in syngeneic tumor models

The anti-tumor activity of voreloxin was next examined in three aggressive syngeneic murine tumor models, Colon 26 adenocarcinoma, Lewis lung, and M5076 and compared to that of doxorubicin, cisplatin, etoposide, irinotecan, paclitaxel, and gemcitabine. Shown in Fig. 3a and b, voreloxin had significant anti-tumor activity against Colon 26 adenocarcinoma with IRs between 70 and 99%. In the 20 and 10 mg/kg groups all animals showed tumor regressions, and two out of six animals had complete regressions (CR). Among the comparator anti-tumor agents, only doxorubicin showed significant anti-tumor activity with an IR of 73% at a dose of 24 mg/kg. All other agents showed weak activity or were inactive at their MTD. In a separate study using this model, at 20 mg/kg voreloxin, there were complete regressions that persisted through day 60, the end of the experiment, in eight out of eight animals (data not shown). Administration of the comparator drug in that study, irinotecan, at its MTD of 100 mg/kg q4d × 3, resulted in progressive tumor growth in all animals and a tumor growth delay of only 7.2 days (Experiment A, CDF1 mice, see "Materials and methods").

The activity of voreloxin in the Colon 26 adenocarcinoma model was further investigated using a more intermittent dosing interval. voreloxin was administered weekly (q7d), every two weeks (q14d) or every three weeks (q21d) for three doses. Shown in Fig. 3c and d, at 20 mg/kg voreloxin, IRs ranged between 90 and 99%, and long term complete remissions were observed in eight out of ten animals when administered on the three schedules. When administered at 10 mg/kg, IRs ranged between 82 and 98%, and there were nine out of ten long term CR on the weekly schedule, eight out of ten on the q14d schedule and two out of ten CR on the q21d schedule (Experiment B, Balb/c mice, see "Materials and methods").

In the Lewis lung carcinoma model voreloxin at doses of 6.25–50 mg/kg demonstrated significant anti-tumor activity at the implantation site resulting in dose-dependent

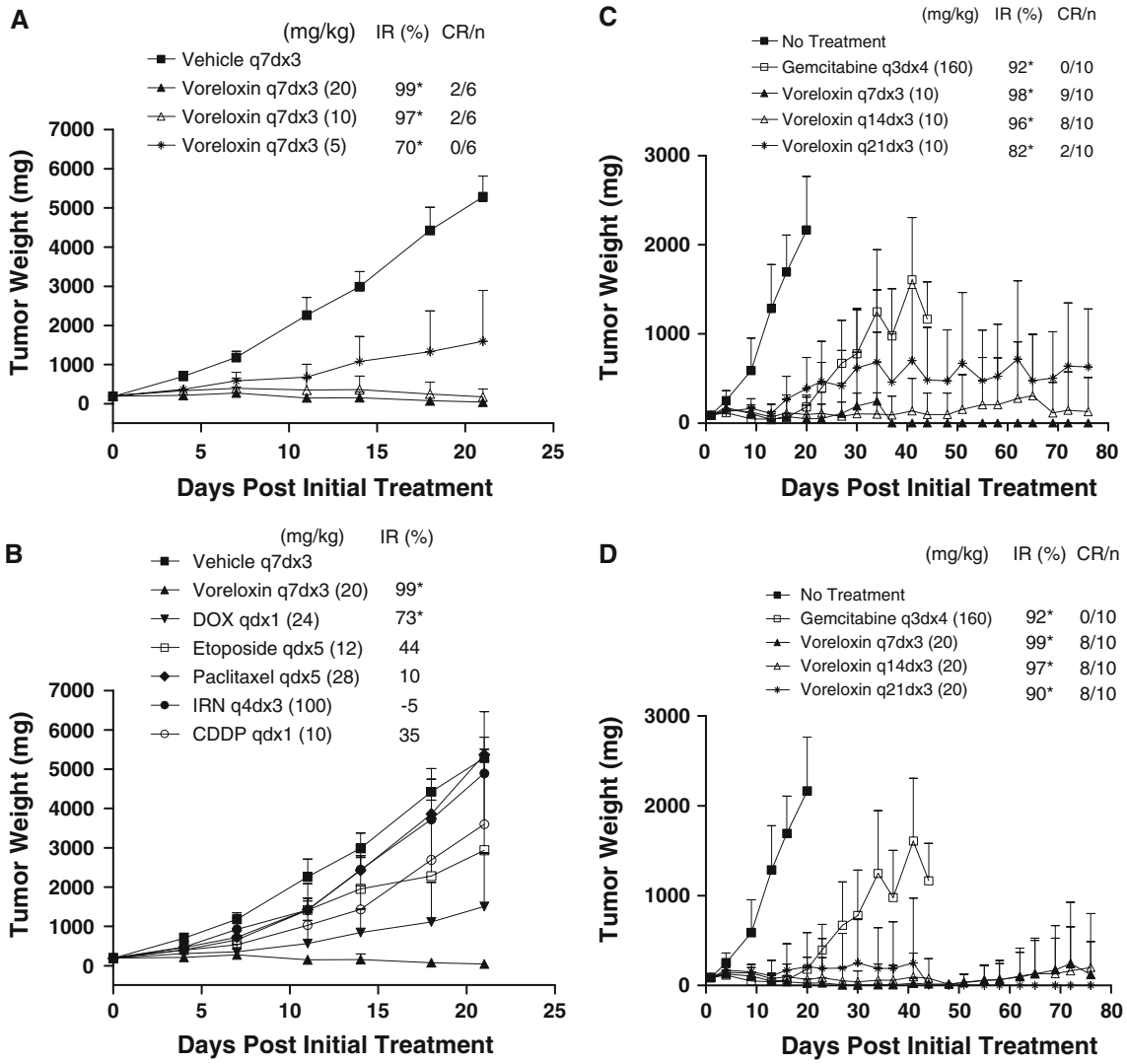

Fig. 3 Activity in Colon 26 syngeneic mouse tumor model. **a** voreloxin dose response. **b** Comparison of voreloxin against marketed therapeutics. **c** Schedule dependent activity in Colon 26 syngeneic mouse tumor model at 10 mg/kg voreloxin. **d** Schedule dependent activity in Colon 26 syngeneic mouse tumor model at 20 mg/kg voreloxin. All agents were administered intravenously using the schedules and doses indicated above. Inhibition rate (*IR*) represents (1 − T/C) × 100. Values marked with *asterisk* are statistically significant compared to vehicle-treated groups. Each schedule and agent had its own vehicle control group; only the voreloxin vehicle group is shown. *Error bars* indicate one standard deviation. Abbreviations: *CDDP* cisplatin, *Dox* doxorubicin, *IRN* irinotecan

IRs ranging from 29 to 99% (Fig. 4a, b). Doxorubicin (24 mg/kg) also showed anti-tumor activity, with an IR of 58%. Etoposide, cisplatin, paclitaxel and irinotecan were ineffective at all tolerated doses. In addition to tumor growth inhibition of the primary tumor, voreloxin also completely inhibited formation of pulmonary metastasis at all doses tested (data not shown). Similarly, voreloxin had potent anti-tumor activity against M5076 ovarian sarcoma with IRs between 91 and 96% (Fig. 4c, d). Among the comparator drugs, only cisplatin and doxorubicin showed significant anti-tumor activity, with IRs of 80 and 56%, respectively. Thus, voreloxin has potent anti-tumor activity in all three of these aggressive syngeneic tumor models.

Discussion

Voreloxin is a novel naphthyridine analog, a class of compounds not previously used in cancer treatment. The structurally related quinolone analogs, which mediate their anti-tumor activity by targeting mammalian topoisomerases, have recently been investigated and have demonstrated promising nonclinical activity [4, 6, 7, 22]. Voreloxin is a replication-dependent DNA damaging agent that causes double-strand DNA breaks, irreversible G2 arrest, and rapid apoptosis, acting via intercalation into DNA and inhibition of topoisomerase II.

In the present study, voreloxin showed potent growth inhibition in vitro in a broad range of tumor cell lines, with

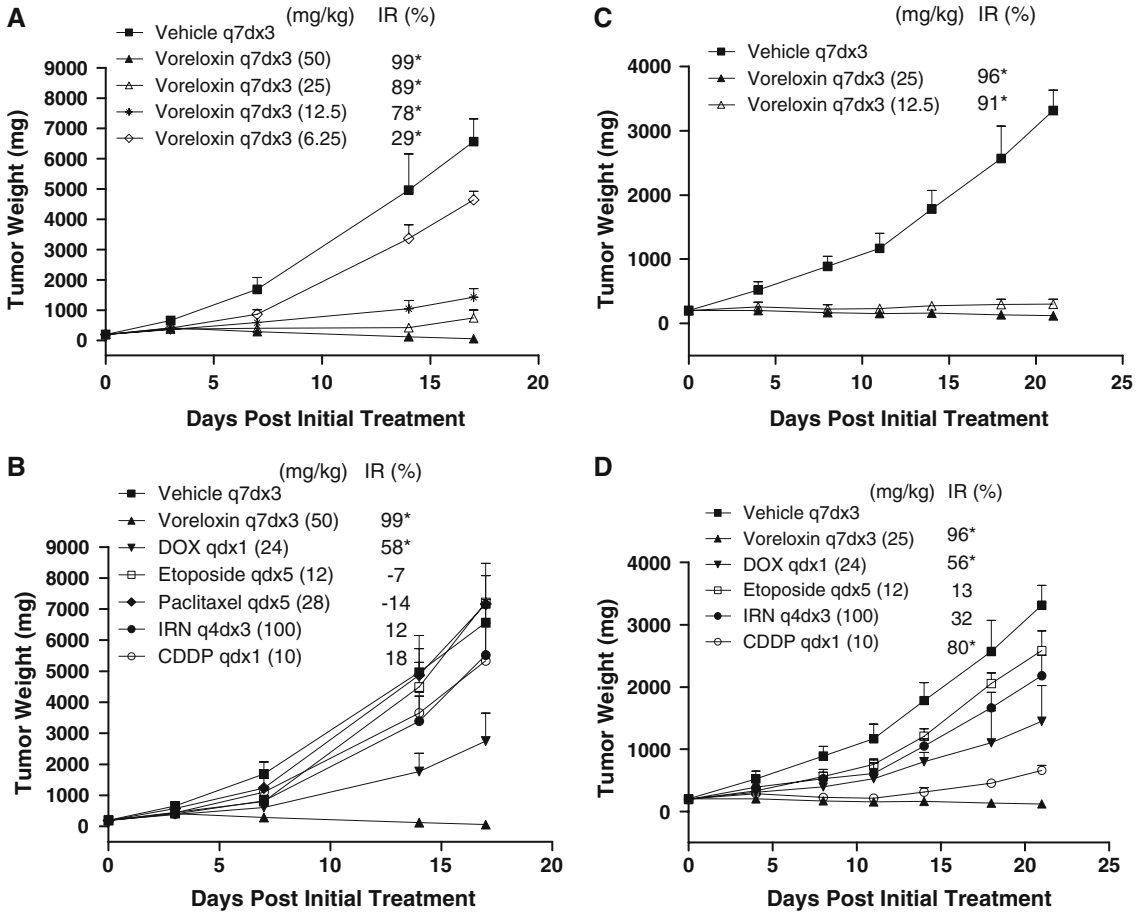

Fig. 4 Activity in Lewis lung syngeneic mouse tumor model (**a**, **b**) and M5076 syngeneic mouse tumor model (**c**, **d**). All agents were administered intravenously using the schedules and doses indicated above. Inhibition rate (*IR*) represents (1 − T/C) × 100. Values marked with *asterisk* are statistically significant compared to vehicle-treated groups. Each schedule and agent had its own vehicle control group; only the voreloxin vehicle group is shown. *Error bars* indicate one standard deviation. Abbreviations: *CDDP* cisplatin, *Dox* doxorubicin, *IRN* irinotecan

an average IC_{50} of 0.392 μM. Among the comparator agents, doxorubicin and paclitaxel are widely used to treat an array of cancers, including breast, ovarian, lung, head-and-neck, gastric, prostate, lymphomas and leukemias [23]. Similar to voreloxin, both agents were broadly active; average IC_{50} values were 0.032 and 0.002 μM, respectively. Although widely used, the efficacy of doxorubicin and paclitaxel may be limited by their activities as substrates for the efflux transporter P-glycoprotein, which is associated with multidrug resistance in tumors [24, 25]. Resistance ratios of >20 were observed for both doxorubicin and paclitaxel in the P-gp overexpressing MES-SA/Dx5 cell line. In contrast, the potency of voreloxin was similar in cells overexpressing P-gp. Moreover, voreloxin is highly cell permeable and is neither a substrate nor an inhibitor of the P-gp efflux pump when tested in polarized human colon carcinoma cells (Caco-2) and MDR1-transfected MDCKII cells (data not shown).

Consistent with its activity in drug-resistant cell lines, voreloxin retained potent anti-tumor activity in drug-resistant xenograft models. The resistant tumor models studied were refractory to doxorubicin, etoposide, and cisplatin and showed cross-resistance to several other cytotoxic agents commonly used to treat cancer. Especially interesting is the activity of voreloxin in the drug-resistant PC-14 NSCLC xenograft tumor model. In addition to resistance to cisplatin, this model showed cross-resistance to doxorubicin, etoposide, irinotecan, and paclitaxel, agents widely used to treat lung cancer. The lack of cross-resistance with commonly encountered resistance mechanisms indicates that voreloxin has the potential to remain active in tumors that are refractory to frequently used classes of anti-cancer drugs.

In addition to the drug-resistance models, the in vivo activity of voreloxin was evaluated in a broad range of human tumor models in mice. Dosing schedule studies revealed that voreloxin administered intravenously achieved up to 90% tumor growth inhibition using several dosing schedules, including single injection, q4d × 5, and weekly × 5. The q7d × 5 dosing regimen displayed the

best balance of anti-tumor activity and tolerability, as measured by survival and body weight loss, and was used for the subsequent in vivo studies. Voreloxin showed potent anti-tumor activities against 12 of the 13 human tumor xenografts examined including breast, ovarian, colon, lung, gastric, melanoma and leukemias. This activity of voreloxin was comparable to that of irinotecan and paclitaxel. In contrast, the activity of doxorubicin, cisplatin, and etoposide was more modest.

Voreloxin intercalates DNA in the presence of topoisomerase II. The resulting DNA damage is highly selective, and shows greater selectivity for proliferating cells compared to other commonly used DNA-damaging drugs. These targeted DNA-protein interactions may contribute to the broad therapeutic window observed with voreloxin. Previous studies in synchronized HCT116 cells demonstrated rapid activation of caspase-3 after voreloxin treatment with maximum activation observed 5 h after treatment [26]. Voreloxin distributes to tumor tissue in which it has a terminal half-life of 8 h following a single administration of 20 mg/kg. Tumor drug levels in excess of the average cellular IC_{90} of 3.92 μM (1.57 μg/mL) are thus maintained for at least 10 h and result in the rapid activation of the Caspase mediated apoptotic cascade.

The broad activity observed with voreloxin in human xenograft tumors was extended in three highly invasive and metastatic syngeneic tumor models, Colon 26 adenocarcinoma, Lewis Lung and M5076 ovarian carcinoma. These highly invasive and metastatic tumor models are often refractory to established chemotherapeutic drugs. Voreloxin showed dose-dependent tumor growth inhibition with inhibition rates >95% in these aggressive syngeneic tumor models. Voreloxin proved to be highly effective in the Colon 26 adenocarcinoma model when given intermittently (q7d, q14d, q21d) resulting in complete remissions and cures at and below the MTD. In addition to effects on the primary tumor, voreloxin prevented formation of lung metastases in the Lewis Lung model (data not shown). Doxorubicin was the only other agent active in all three models, although with inferior inhibition rates that did not exceed 73% at the MTD.

The demonstrated potent and broad anti-tumor activity, uncompromised by multidrug resistance, and the consistent potency in aggressive syngeneic tumor models, support the clinical study of voreloxin as a novel and potentially broadly active anti-cancer agent.

In phase 1 and 2 studies, voreloxin was generally well tolerated. The dose limiting toxicity in solid tumor indications was myelosuppression, and in advanced hematologic malignancies the DLT was reversible oral mucositis. Clinical activity, including objective responses, have been observed in ovarian cancer, small cell and non small cell lung cancers, as well as in advanced acute myeloid leukemia (AML) [10–13]. Clinical studies of voreloxin in AML, alone and in combination, and in ovarian cancer are ongoing.

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