NPI-2358 is a tubulin-depolymerizing agent: in-vitro evidence for activity as a tumor vascular-disrupting agent

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The diketopiperazine NPI-2358 is a synthetic analog of NPI-2350, a natural product isolated from *Aspergillus* sp., which depolymerizes microtubules in A549 human lung carcinoma cells. Although structurally different from the colchicine-binding site agents reported to date, NPI-2358 binds to the colchicine-binding site of tubulin. NPI-2358 has potent in-vitro anti-tumor activity against various human tumor cell lines and maintains activity against tumor cell lines with various multidrug-resistant (MDR) profiles. In addition, when evaluated in proliferating human umbilical vein endothelial cells (HUVECs), concentrations as low as 10 nmol/l NPI-2358 induced tubulin depolymerization within 30 min. Furthermore, NPI-2358 dose dependently increases HUVEC monolayer permeability – an in-vitro model of tumor vascular collapse. NPI-2358 was compared with three tubulin-depolymerizing agents with vascular-disrupting activity: colchicine, vincristine and combretastatin A-4 (CA4). Results showed that the activity of NPI-2358 in HUVECs was more potent than either colchicine or vincristine; the profile of CA4 approached that of NPI-2358. Altogether, our data show that NPI-2358 is a potent anti-tumor agent which is active in MDR tumor cell lines, and is able to rapidly induce tubulin depolymerization and monolayer permeability in HUVECs. These data warrant further evaluation of NPI-2358 as a vascular-disrupting agent *in vivo*. Currently, NPI-2358 is in preclinical development for the treatment of cancer. *Anti-Cancer Drugs* 17:25–31 © 2006 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2006, **17**:25–31

Keywords: cancer, natural product, NPI-2358, tubulin-depolymerizing agent, vascular-disrupting agent

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Received 29 June 2005 **Accepted** 17 August 2005

Introduction

Solid tumors rely on a functional vasculature for their survival and growth [1]. Tumor vasculature differs from established vascular endothelium in normal tissues in that it has a reduced number of supporting pericytes and is highly permeable [2]. Furthermore, it is thought that a proportion of the highly proliferative tumor vascular endothelial cells lack a well-developed actin filament structure present in normal mature vasculature, resulting in an increased reliance on the microtubule network for structural integrity [1,3,4]. Similarly, proliferating human umbilical vein endothelial cells (HUVECs) typically lack a well-defined actin filament structure, making them more reliant on the microtubule network for maintenance of cell shape [5,6]. Consequently, proliferating HUVECs are used as an *in vitro* model of tumor vascular endothelial cells [4].

Tumor vascular collapse results in a reduction in the blood supply to the tumor leading to anoxia and subsequent tumor cell death in the core of the tumor [2,7,8]. There are two classes of compounds with tumor vascular-disrupting activity: ligand-directed vascular-disrupting agents (VDAs) and small molecule VDAs including selected microtubule-destabilizing agents [7,8]. Tubulin-destabilizing agents induce tumor vascular collapse by the rapid depolymerization of microtubules in the highly proliferating tumor vascular endothelial cells [3,4,9,10]. The earliest example of a tubulin-depolymerizing agent with vascular-disrupting activity is colchicine, which was shown to cause tumor vascular collapse when administered at or near its maximum tolerated dose (MTD) [11,12]. Similarly, the vinca alkaloid vincristine, which binds to a different site on tubulin, has been shown to cause vascular collapse at or near its MTD [12,13]. More recently studied tubulin-depolymerizing agents include the colchicine analogs combretastatin A-4-phosphate (CA4P) and ZD6126, which are the phosphate prodrugs of combretastatin 4A (CA4) and *N*-acetylcolchinol, respectively, and cause tumor vascular collapse *in vivo* within 10–30 min of administration at concentrations below their MTDs [3,4,14–16].

In addition to the tumor vascular-disrupting activity of microtubule-destabilizing agents, many also have direct cytotoxic activity [17]. However, the effectiveness of some chemotherapy agents is limited by the emergence of drug-resistant tumor cells. One common mechanism

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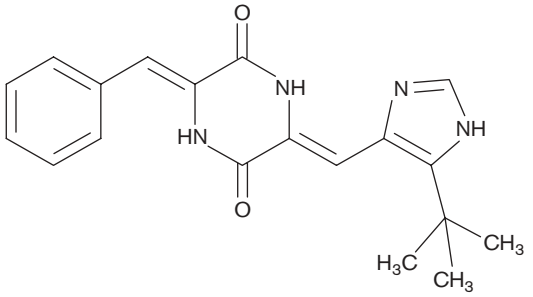
Fig. 1



Structure of the diketopiperazine NPI-2358

tumors employ to become resistant to chemotherapy agents is overexpression of the P-glycoprotein (P-gp) efflux pump [18]. Substrates of the P-gp pump include the microtubule-disrupting agents paclitaxel, vinblastine and vincristine [18]. An alternative mechanism of multidrug resistance (MDR) is the downregulation of topoisomerase (Topo) II which is thought to reduce the number of Topo II-mediated DNA strand breaks [18,19].

Here, we describe NPI-2358, a synthetic analog of the diketopiperazine phenylahistin (halimide). Phenylahistin is a natural product, which was discovered concurrently from a marine and a terrestrial fungus, *Aspergillis* sp., and is produced as a mixture of (+) and (−) enantiomers [20,21]. Early studies showed that the (−) enantiomer (NPI-2350) inhibited cell proliferation by binding at the colchicine-binding site on tubulin and disrupting the microtubule network, which resulted in G_2/M cell cycle arrest [22,23]. Additionally, compared to the (+) enantiomer, the (−) enantiomer exhibited elevated cytotoxic activity against various tumor cells including lung, colon, breast and leukemia with IC_{50} values in the low to submicromolar range [23]. To remove chirality and optimize biological activity, a series of synthetic analogs was generated, including NPI-2358 (Fig. 1).

NPI-2358 is structurally distinct from colchicine and its analogs CA4P and ZD6126, and binds to the colchicine-binding site of tubulin *in vitro* (Bishop *et al.*, manuscript in preparation). Data presented here further characterize NPI-2358, specifically the activity of NPI-2358 against a variety of human tumor cell lines (including MDR cell lines), the effect of NPI-2358 on HUVEC microtubule networks and the effect of NPI-2358 on HUVEC monolayer permeability.

Materials and methods

Reagents

The synthesis of NPI-2358 was accomplished by Nereus Pharmaceuticals in collaboration with Dr Hayashi. CA4 was purchased from Tocris Cookson (Ellisville, Missouri, USA). Colchicine, vincristine, paclitaxel, mitoxantrone, resazurin, BSA and FITC–dextran (38.2 kDa) were obtained from Sigma-Aldrich (St Louis, Missouri, USA). Anti-α-tubulin and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Eugene, Oregon, USA). Goat anti-mouse–FITC antisera and Vectashield were obtained from Jackson (West Grove, Pennsylvania, USA) and Vector (Burlingame, California, USA), respectively. Fibronectin was purchased from Chemicon (Temecula, California, USA). Unless stated otherwise, all other reagents were purchased from VWR (West Chester, Pennsylvania, USA).

Cell lines and cell culture

Human colon adenocarcinoma (HT-29), prostate adenocarcinomas (PC-3 and DU 145), breast adenocarcinoma (MDA-MB-231), non-small cell lung carcinoma (NCI-H292), T cell leukemia (Jurkat), uterine sarcoma (MES-SA) and the drug-resistant derivative (MES-SA/Dx5), and acute promyelocytic leukemia (HL-60) and the drug-resistant derivative (HL-60/MX2) cells were all purchased from ATCC (Manassas, Virginia, USA). HUVECs were obtained from Cambrex Bio Science (Walkersville, Maryland, USA), and were used between passages 2 and 6. All tumor cell lines were maintained in their respective ATCC recommended culture media at 37°C/5% CO_2/95% humidified air. HUVECs were grown in EGM-2 medium (Cambrex Bio Science) at 37°C/5% CO_2/95% humidified air.

In-vitro cytotoxicity assay

The cytotoxicity assays were performed essentially as described [24]. Briefly, the adherent cells were plated in 96-well flat-bottomed plates and allowed to attach for 24 h at 37°C. HL-60 and HL-60/MX2 cells were plated in 96-well plates on the day of compound addition. Serially diluted compounds were added in triplicate to cells at concentrations ranging from 2 pmol/l to 20 μmol/l (4 pmol/l to 40 μmol/l for mitoxantrone). Cells treated with a final concentration of 0.25% (v/v) DMSO served as the vehicle control. Cell viability was assessed 48 h later by measuring the reduction of resazurin with a fluorimeter (Perkin-Elmer, Torrance, California, USA). The IC_{50} values (the drug concentration at which 50% of the maximal observed cytotoxicity is established) were calculated in Prism (GraphPad, San Diego, California, USA) or XLFit 3.0 (ID Business Solutions, Emeryville, California, USA) using a sigmoidal dose–response model.

Tubulin visualization by indirect immunofluorescence

HUVECs were plated onto sterile tissue culture treated coverslips (Fisher, Hampton, New Hampshire, USA) in six-well plates. Following overnight incubation the proliferating HUVECs were treated with test compounds or vehicle [0.25% (v/v) DMSO]. The plates were returned to the incubator for 30 min. Following fixation

in 10% (v/v) neutral buffered formalin at room temperature, the cells were permeabilized in 0.2% (v/v) Triton X-100/dPBS before transferring to a humidified chamber and blocking for 2 h in antibody buffer [2% (w/v) BSA/0.1% (v/v) Tween-20/dPBS]. The coverslips were incubated with or without 0.1 μg/ml mouse anti-α-tubulin in antibody buffer for 1 h before washing and incubation with 1 μg/ml goat anti-mouse–FITC for 1 h in the dark. Finally, to visualize the nuclei, the cells were washed and treated with 2 μg/ml DAPI before mounting with Vectashield mounting media. The cells were imaged using a 60 × oil immersion objective on an upright microscope (Olympus BX51). The images were digitally captured using a CCD camera and Magnafire 2.0 software (Olympus, Melville, New York, USA). Post-image processing was performed in Photoshop Elements 2.0 (Adobe, San Jose, California, USA).

HUVEC monolayer permeability

The permeability experiments were performed essentially as described [9]. Briefly, following seeding, the HUVECs were incubated for 4 days to reach confluency. Subsequently, the test compounds were added and the plates were returned to the incubator 15 min before adding FITC–dextran to the upper chamber, resulting in a final concentration of 1 mg/ml. Thirty minutes later, the fluorescence of the lower chambers of the 24-well plates were read using a fluorometer with $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm filters. An increase in monolayer permeability was visualized by an increase in fluorescence signal in the lower chamber of the 24-well plate.

Results

NPI-2358: in-vitro anti-tumor activity

NPI-2358 was tested against six different human tumor cell lines representing a variety of tumor types (Table 1).

Table 1 NPI-2358 has potent anti-tumor activity against a variety of human tumor cell lines

Tumor cell line	Tumor type	IC_{50}(nmol/l)
HT-29	colorectal	9.8 ± 2.4
DU 145	prostate	18 ± 5
PC-3	prostate	13 ± 1
MDA-MB-231	breast	14 ± 2
NCI-H292	non-small cell lung	18 ± 1
Jurkat	leukemia	11[a]

IC_{50} values are the mean ± SD of three or more independent experiments.
[a]Mean value of two experiments.

The IC_{50} values of NPI-2358 ranged from 9.8 to 18 nmol/l. In addition, NPI-2358 retained equivalent cytotoxic activity when tested against MDR tumor cells overexpressing P-gp (MES-SA/Dx5) or with reduced nuclear Topo II activity (HL-60/MX2) (Table 2). The MDR phenotypes were confirmed by the observation that paclitaxel, a known substrate of P-gp, was over 1100-fold less active when tested against MES-SA/Dx5 cells relative to the parental MES-SA tumor cells. Furthermore, the Topo II-targeting agent mitoxantrone exhibited 16-fold less activity towards the drug-resistant derivative HL-60/MX2 cells relative to HL-60 tumor cells. Taken together, these data show that NPI-2358 has potent in-vitro anti-tumor activity and is active against tumor cell lines overexpressing P-gp or with reduced nuclear Topo II catalytic activity.

NPI-2358: dose-dependent microtubule depolymerization in HUVECs

Proliferating HUVECs were treated with various concentrations of NPI-2358 for 30 min and microtubules were visualized by indirect immunofluorescence. Results from these assays revealed that concentrations as low as 10 nmol/l NPI-2358 were able to rapidly induce microtubule depolymerization in proliferating HUVECs, as reflected by the lack of microtubule networks (Fig. 2). The known tubulin-depolymerizing agent colchicine was included as a positive control. Incubation with the secondary anti-mouse FITC antibody alone confirmed the specificity of the primary anti-α-tubulin antibody (Fig. 2). In addition, time-course experiments revealed that HUVEC microtubule networks were completely depolymerized within 20–30 min (data not shown). Taken together, these data show that low concentrations of NPI-2358 are able to rapidly induce tubulin depolymerization in HUVECs.

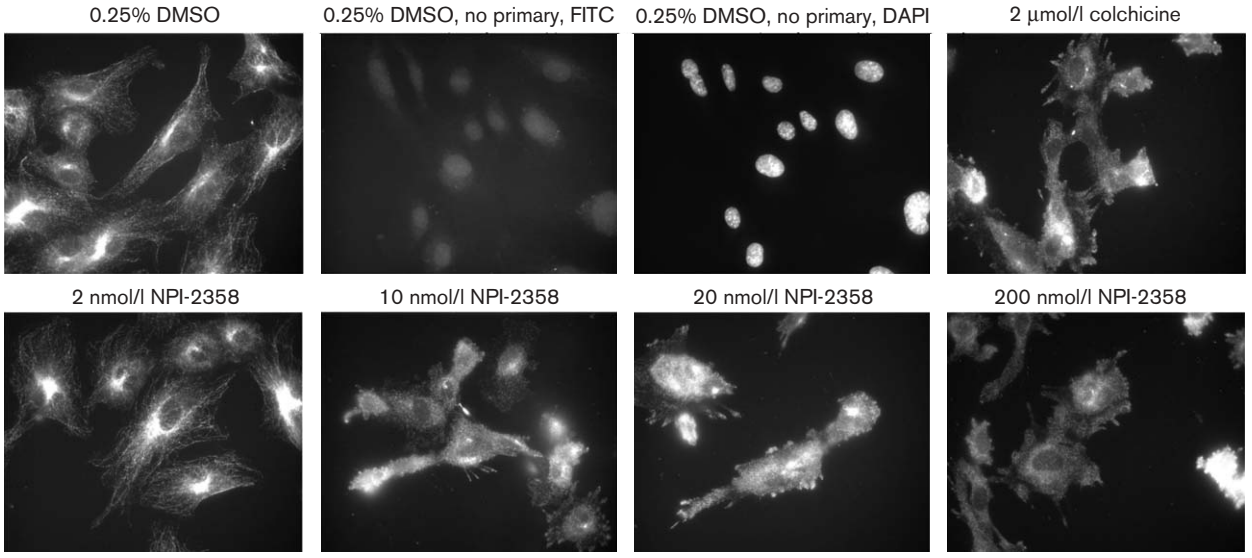
NPI-2358 was compared with the known tubulin-depolymerizing agents, colchicine, vincristine and CA4. High (2 μmol/l) concentrations of NPI-2358, colchicine or vincristine induced complete microtubule depolymerization within 30 min. In contrast to colchicine and vincristine, NPI-2358 was able to induce microtubule depolymerization when tested at 20 nmol/l (Fig. 3). In direct comparison with CA4, dose–response experiments revealed that concentrations as low as 10 nmol/l of NPI-2358 or CA4 were able to induce microtubule

Table 2 NPI-2358 has potent anti-tumor activity against MDR human tumor cell lines

Compound	IC_{50}(nmol/l)		Fold change	IC_{50}(nmol/l)		Fold change
	MES-SA	MES-SA/Dx5		HL-60	HL-60/MX2	
NPI-2358	11 ± 5	12 ± 5	1.1	4.3 ± 2.2	8.0 ± 1.2	1.9
Paclitaxel	4.2 ± 1.8	4800 ± 2400	1143	ND	ND	ND
Mitoxantrone	ND	ND	ND	270 ± 130	4400 ± 800	16

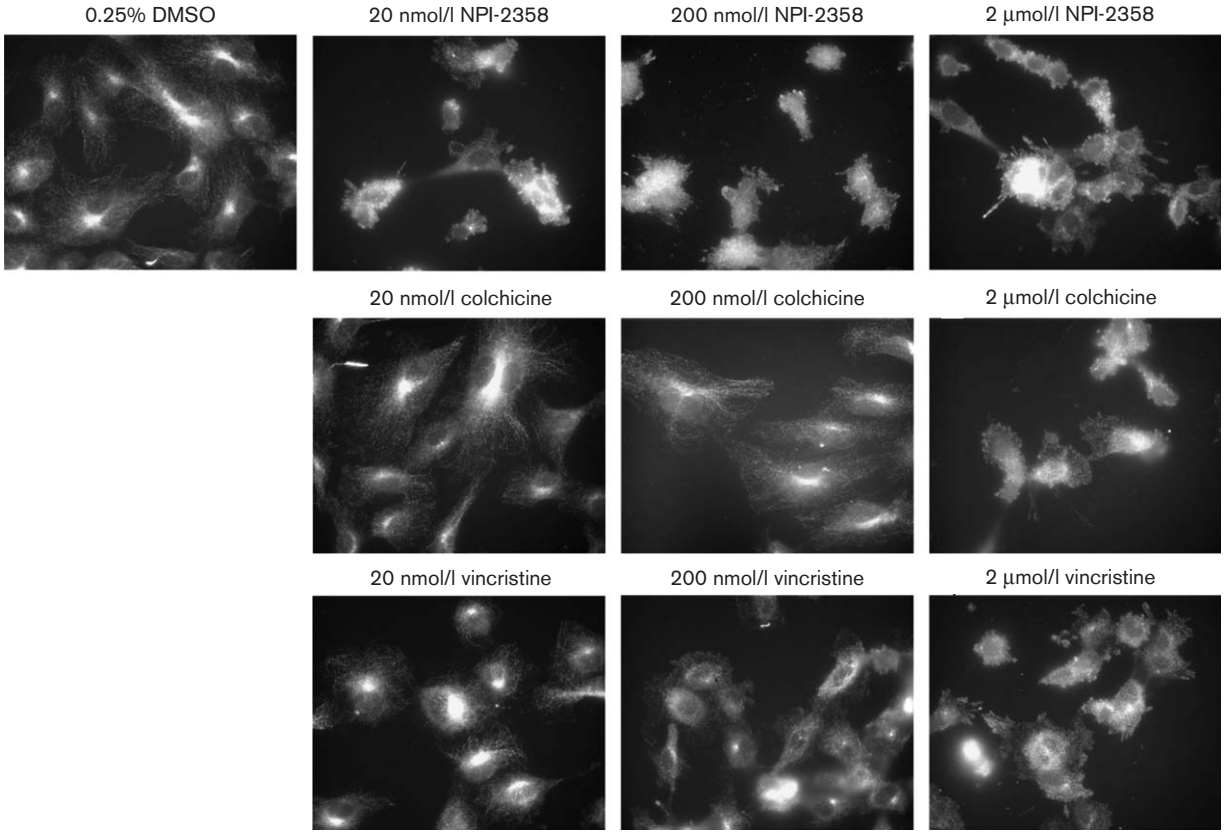
The in-vitro cytotoxic activity of NPI-2358 was determined against parental (MES-SA and HL-60) and their MDR derivative (MES-SA/Dx5 and HL60/MX2) tumor cell lines. Fold change represents the ratio of mean IC_{50} values (MDR: parental tumor cell line). Data presented are mean ± SD of three independent experiments. ND, not determined.

Fig. 2



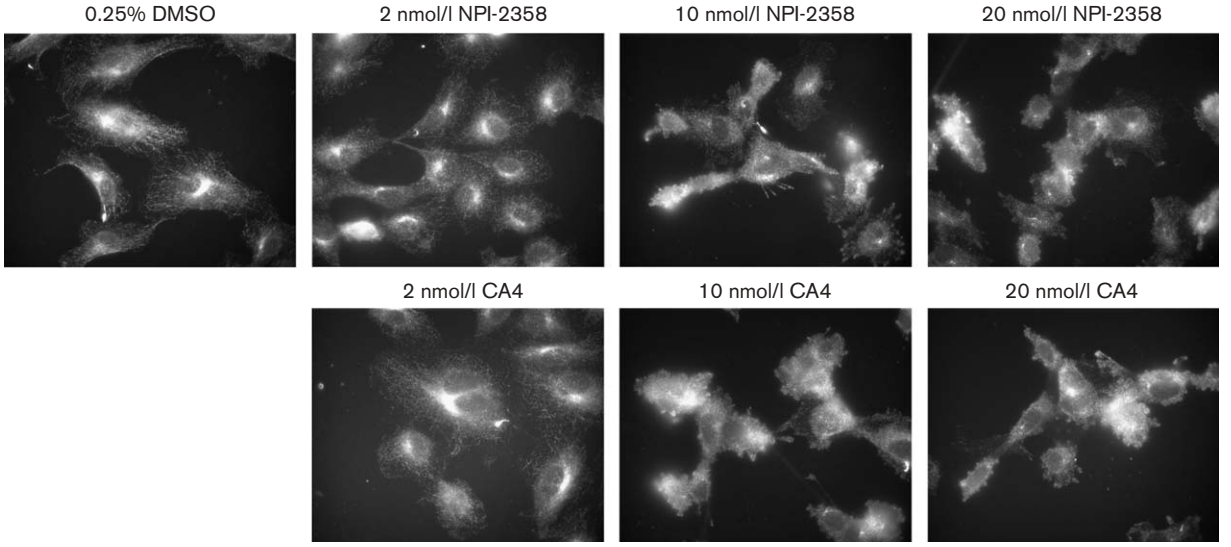
NPI-2358 at 10 nmol/l induces α-tubulin depolymerization in HUVECs. Proliferating HUVECs were treated with different doses of NPI-2358 for 30 min. The cells were then fixed and stained as detailed in Materials and methods. The '0.25% DMSO' represents the vehicle control-treated cells. For the secondary antibody alone (no primary) controls, the same field is shown during FITC (tubulin) and DAPI (DNA) fluorescence. Images shown are representative images of two or more experiments.

Fig. 3



In contrast to 20 nmol/l colchicine or 20 nmol/l vincristine, 20 nmol/l NPI-2358 induces α-tubulin depolymerization within 30 min in HUVECs. The '0.25% DMSO' represents the vehicle control-treated cells. Images shown are representative images of two or more experiments.

Fig. 4



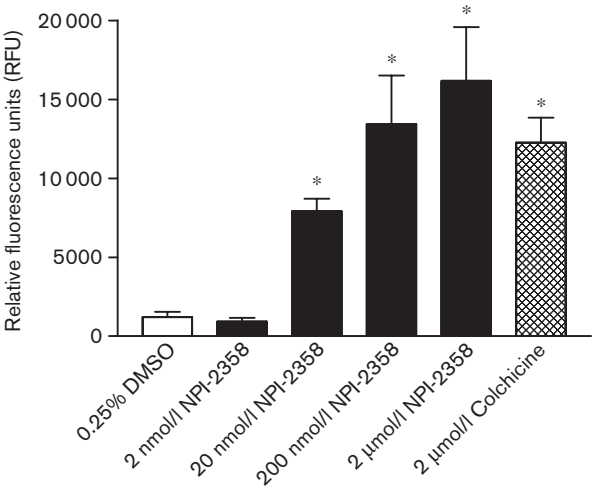
NPI-2358 and CA4 induce dose-dependent α-tubulin depolymerization in HUVECs within 30 min. The '0.25% DMSO' represents the vehicle control-treated cells. Images shown are representative images of two experiments.

depolymerization within 30 min (Fig. 4). Furthermore, similar morphological changes such as membrane blebbing and the rounding up of cells were observed in HUVECs treated with equimolar concentrations of NPI-2358 or CA4. Altogether, these data show that NPI-2358 is more potent than either colchicine or vincristine and that CA4 has a similar profile to NPI-2358.

NPI-2358: dose-dependent changes in monolayer permeability in HUVECs

Changes in HUVEC monolayer permeability provide an alternative *in vitro* method of assaying some of the events that occur during tumor vascular collapse *in vivo* [2]. Significant increases in endothelial cell permeability were observed when the confluent monolayers were treated with 20 nmol/l, 200 nmol/l or 2 μmol/l NPI-2358 (Fig. 5). Furthermore, high (2 μmol/l) and low (20 nmol/l) concentrations of NPI-2358, colchicine and vincristine were compared for their ability to induce HUVEC monolayer permeability (Fig. 6). These data revealed that, at 20 nmol/l, only NPI-2358, but not vincristine or colchicine, was able to induce monolayer permeability. When tested at 2 μmol/l, all three compounds exhibited similar activity.

Fig. 5

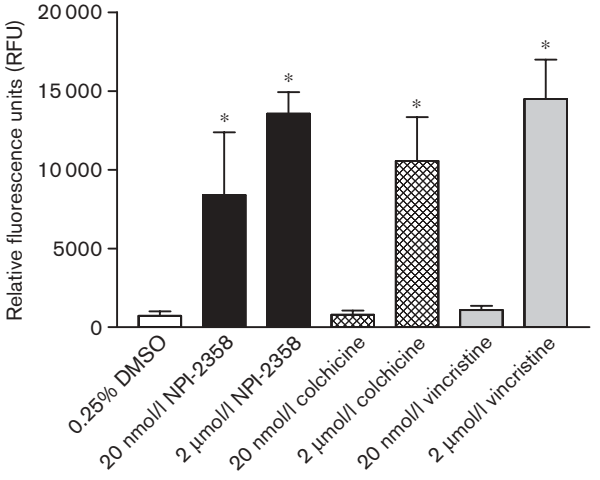


Dose-dependent induction of HUVEC monolayer permeability by NPI-2358. *$P<0.001$ (Student's *t*-test) compound treated versus vehicle control (0.25% DMSO)-treated cells. The data shown are the mean ± SD of four determinations (two experiments).

Discussion

To determine whether NPI-2358 has in-vitro anti-tumor activity, cytotoxicity assays were performed against a series of human tumor cell lines representing different tumor types (Tables 1 and 2). These data revealed that NPI-2358 is a potent anti-tumor agent with IC_{50} values ranging from 4.3 to 18 nmol/l. Furthermore, NPI-2358 maintained cytotoxic activity when tested against tumor cells overexpressing the P-gp efflux pump or with reduced nuclear Topo II catalytic activity. The in-vitro anti-tumor activity of NPI-2358 is consistent with the previously reported activity of the original natural product, NPI-2350, towards a variety of tumor cell lines [21,23]. However, NPI-2358 is considerably more potent than NPI-2350 [the IC_{50} value of NPI-2358 against HT-29 cells was 9.8 versus 580 nmol/l for NPI-2350 (data not shown)].

Fig. 6



A comparison of the ability of NPI-2358 relative to other tubulin-depolymerizing agents to induce HUVEC monolayer permeability. *$P<0.001$ (Student's *t*-test) compound treated versus vehicle control (0.25% DMSO)-treated cells. The data shown are the mean ± SD of six determinations (three experiments).

The depolymerization of the tubulin network in tumor vascular endothelial cells is responsible for the endothelial cell shape change and subsequent vascular disruption observed in tumors treated with VDAs such as CA4P and ZD6126 [3,4,10,14,15]. The original natural product, NPI-2350, binds to tubulin and induces microtubule depolymerization in A549 tumor cells [22]. Experiments performed with NPI-2358 have shown that NPI-2358 binds to tubulin at or near the colchicine-binding site and inhibits microtubule polymerization *in vitro* (Bishop *et al.*, manuscript in preparation). The present studies investigated the role of NPI-2358 in tumor vascular disruption. Specifically, HUVEC microtubule networks were visualized by indirect immunofluorescence following exposure to NPI-2358. Microtubule depolymerization was observed in proliferating HUVECs following a 30-min exposure to concentrations as low as 10 nmol/l NPI-2358 (Fig. 2). The tubulin depolymerization was complete within 30 min (data not shown). When the microtubule depolymerizing properties of NPI-2358 and CA4 (the active metabolite of CA4P) were directly compared, both 10 nM NPI-2358 and 10 nM CA4 induced microtubule depolymerization in HUVECs within 30 min (Fig. 4). Neither compound was able to induce microtubule depolymerization when tested at 2 nmol/l. In addition, colchicine and vincristine, two tubulin-depolymerizing agents which act as VDAs when used at or near their MTDs [11,13], were compared with NPI-2358 (Fig. 3). Taken together, our results indicate that NPI-2358 was more potent than either colchicine or vincristine and the profile of CA4 was similar to NPI-2358.

NPI-2358 rapidly induced morphological changes such as membrane blebbing and the rounding up of cells in HUVECs. These morphological changes are consistent with previously reported observations for CA4P and ZD6126 [3,10]. In addition, it was demonstrated that the early membrane blebbing observed in HUVECs following exposure to CA4P was an immediate response to microtubule depolymerization [9]. As such, it is likely that the membrane blebbing seen in HUVECs treated with NPI-2358 is a downstream effect of NPI-2358-mediated microtubule disruption.

HUVEC monolayer permeability assays were employed to mimic *in vitro* some of the events that occur during tumor vessel vascular collapse *in vivo* [2]. These experiments revealed that in agreement with the tubulin immunofluorescence images presented in Fig. 3, concentrations as low as 20 nmol/l NPI-2358 were able to induce HUVEC monolayer permeability (Fig. 5). Furthermore, when 20 nmol/l NPI-2358 was compared to equimolar concentrations of colchicine or vincristine, only 20 nmol/l NPI-2358 was able to induce HUVEC monolayer permeability (Fig. 6). The induction of monolayer permeability by NPI-2358 is similar to the HUVEC monolayer permeability data previously published for the combretastatins, where micromolar concentrations of CA1 and CA4P were shown to induce HUVEC monolayer permeability [9,25]. Importantly, both CA1 and CA4P have since been shown to function *in vivo* as tumor VDAs [9,14,15,25,26]. Taken together, these data show that 20 nmol/l NPI-2358 rapidly induces tubulin depolymerization and HUVEC monolayer permeability. In addition, preliminary results from two different models of tumor blood flow (Borgström, personal communication and Honess *et al.*, manuscript in preparation) strongly support the hypothesis that NPI-2358 is a selective tumor VDA.

In summary, NPI-2358 is a potent anti-tumor agent which is active in MDR tumor cell lines, and is able to rapidly induce tubulin depolymerization and monolayer permeability in HUVECs. Furthermore, these data show that in-vitro NPI-2358 has properties distinct from colchicine or vincristine. Moreover, the structure of NPI-2358 is different from the other colchicine-binding site molecules reported to date. Tumor VDAs have been shown to work effectively in preclinical models of cancer both as single agents and in combination with other chemotherapeutic agents or radiation [2,4,7,8,15,26,27]. Based on our encouraging in-vitro observations and studies in tumor models *in vivo* ([28] and Siemann *et al.*, manuscript in preparation), NPI-2358 is currently in preclinical development for the treatment of cancer.

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