I-387, a Novel Antimitotic Indole, Displays a Potent \textit{In Vitro} and \textit{In Vivo} Antitumor Activity with Less Neurotoxicity

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Abstract

(3-(1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl)methanone (I-387) is a novel synthetic compound that inhibits tubulin action and exhibits potent antitumor activity in various preclinical models. I-387 inhibited the in vitro growth of a number of human cancer cell lines with IC₅₀ values in the range of 15 to 39 nM. Nanomolar concentrations of the compound induced apoptosis and caused phosphorylation of the anti-apoptotic protein Bcl-2. I-387 induced a strong and concentration-dependent G₂M arrest in PC-3 cells by constitutive activation of Cdc2/cyclin B1 complex and destabilized polymerization of purified tubulin in vitro by binding to the colchicine-binding site. In vivo, I-387 treatment effectively inhibited tumor growth in mice bearing PC-3 tumor xenografts. In vitro studies of nerve growth factor (NGF)-dependent neurite outgrowth in PC12 pheochromocytoma cells and in vivo studies of mouse behavior showed that I-387 was less neurotoxic than vinblastine and vincristine, tubulin destabilizers with known neurotoxicity. Interestingly, multidrug resistant cell lines that over-expressed P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP) were rendered resistant to docetaxel, vinblastine, SN-38, and doxorubicin, but not to I-387. I-387 dosed at 10 mg/kg was equally effective with 76% tumor growth inhibition in xenograft models using MES-SA uterine sarcoma cells and MES-SA/DX5 cells over-expressing P-gp. In contrast, docetaxel and vinblastine were not effective in MES-SA/DX5 xenograft models. The potent in vitro and in vivo antitumor activity of I-387 suggests that it may represent a new antimitotic agent for management of various malignancies, particularly for patients with drug resistant cancer.

Introduction

Microtubules, which are comprised of α- and β-tubulin dimers, are one of the most useful subcellular targets in chemotherapy. It is generally accepted that the antiproliferative properties of tubulin inhibitors result from their interference with microtubule dynamics, since microtubules are critically important components in cell mitosis and cell signaling [1]. In animal cells, mitosis requires the formation of a unique structure, the mitotic spindle, which is essential for the maintenance of euploidy. The proper assembly and function of the mitotic spindle is necessary for adequate chromatid segregation [2].
There are two major groups of antimitotic agents consisting of microtubule-stabilizing agents (i.e., taxanes) and microtubule-destabilizing drugs (i.e., vinca alkaloids and colchicines). They interact physically with tubulin by binding to one of the three main binding sites: colchicine-, vinblastine-, or paclitaxel-binding site [3]. Tubulin binding agents alter the dynamic behaviors of microtubules and arrest mitotic cells in the M-phase of the cell cycle, thus leading to apoptotic cell death. Despite the clinical success of tubulin inhibitors, agents currently used for chemotherapy are known to have limitations such as peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug resistance due to multidrug resistance (MDR) transporters and tubulin mutation [4-7]. These limitations have led to the search for new agents that inhibit tubulin activity and circumvent these limitations. (3-(1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl)methanone (hereafter referred to as I-387, Fig. 1A) was identified during studies to discover new pharmacophores for treatment of cancer. We examined the tubulin binding properties as well as the in vitro and in vivo efficacy and toxicity of I-387.

MATERIALS AND METHODS

Chemicals and Animals. Monoclonal antibodies to phospho-Bcl-2 (pBcl-2), cyclin B1, Cdc25C, Cdc2, phospho-Cdc2 (pCdc2), and horseradish peroxidase conjugated secondary antibodies were purchased from Millipore Corporation (Billerica, MA). Bovine brain tubulin protein was purchased from Cytoskeleton, Inc (Denver, CO). [3H]Vinblastine and [3H]podophyllotoxin were purchased from Moravek, Inc (Brea, California). Sephadex G25 column and Cell Death Detection ELISA (anti-histone ELISA) were purchased from Roche Applied Science (Indianapolis, IN). Murine 2.5S nerve growth factor was purchased from Promega (Madison, WI). All other chemicals were purchased from Sigma (St. Louis, MO).

Four to five week old male ICR mice and male nu/nu nude mice were purchased from Harlan Biosciences (Indianapolis, IN). All animal protocols were approved by the Animal Care and Use Committee at The Ohio State University or the University of Tennessee Health Science Center.

Cell culture. LNCaP, PC-3, DU-145, PPC-1, TSU-Pr1, HT-29, MCF-7, K562, PC-12, HEK-293, MES-SA, and MES-SA/DX5 were originally obtained from ATCC (Rockville, MD). All cells obtained from ATCC were
immediately expanded and frozen down such that all cell lines could be restarted every 2-3 months from a frozen vial of the same batch of cells. K562/DOX, HEK293-pcDNA3-10, and HEK293-482R2 were kindly provided by Dr. Duxin Sun in 2007 (College of Pharmacy, The Ohio State University). PcDNA3-10 vector and pcDNA3-10 vectors containing human MRP1 and MRP2 cDNAs were obtained from Dr. Susan P. C. Cole (Department of Pharmacology & Toxicology, Queen’s University) and transfected into HEK-293 cells in 2007 [8, 9]. For the in vivo xenograft studies, PC-3, MES-SA, and MES-SA/DX5 were authenticated at Research Animal Diagnostic Laboratory (Columbia, MO) within four months before studies. Inter-species contamination was tested by PCR and the identity of the cell lines was verified by generating a genetic profile. For all other cell lines, authentication was not performed other than what was done by ATCC. MES-SA and MES-SA/DX5 were maintained in McCoy's 5A Medium containing 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS). PC12 was maintained in RPMI-1640 medium with 5% FBS and 10% heat-inactivated horse serum. All other cells were maintained in RPMI-1640 medium with 2 mM L-glutamine and 10% FBS.

**Growth Inhibition Assay.** The cytotoxic or anti-proliferative activity of test compounds was investigated in several cell lines using the sulforhodamine B (SRB) assay [10]. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was also used for the leukemia cell lines [11]. Cultured cells were plated into 96-well plates and incubated with medium containing different concentrations of the test compounds for 96 h. Cells were stained with SRB solution or MTT solution. The optical density was determined at 540 nm on a microplate reader (Dynex Technologies, Chantilly, VA). Plots of percent inhibition of cell growth versus drug concentration were constructed, and the concentration that inhibited cell growth by 50% relative to the untreated control (IC50) was determined by nonlinear least squares regression using WinNonlin software (Pharsight Corporation, Cary, NC). WinNonlin was provided by a Pharsight Academic License to The Ohio State University.

**Determination of DNA Fragmentation by ELISA.** Apoptosis was measured by quantitation of cytoplasmic histone-associated DNA fragments using the cell death detection ELISA kit. Cells were seeded in 6-well plates and exposed to I-387 for 24 h at different concentrations. The quantitation of DNA fragments was performed according...
to the manufacturer’s instructions. Results are expressed as the enrichment factor (i.e., the ratio of the optical density in treated cells to the optical density in control cells).

**Cell Cycle Analysis.** Cell cycle distribution was determined by propidium iodide (PI) staining. Treated cells were washed with PBS and fixed with 70% ice-cold ethanol overnight. Fixed cells were then stained with 20 μg/ml of PI in the presence of RNase A (300 μg/ml) at 37°C for 30 min. Cell cycle distribution was analyzed by fluorescence-activated cell sorting (FACS) analysis core services at The Ohio State University, Columbus, OH.

**Western Blot Analysis.** Treated cells were lysed in cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton x-100, and freshly added Na₃VO₄ (2 mM), NaF (20 mM) and complete protease inhibitor cocktail). For each sample, an aliquot (20 to 40 μg) of total protein was loaded and run on a SDS-Page gel. Protein was transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), blocked with 5% non-fat milk, incubated with primary antibody overnight at 4°C, and then incubated with secondary antibody at room temperature for 1 h. ECL™ (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was used for detection. The band intensity was measured by densitometry using TotalLab TL100 Software v2006 (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

**In Vitro Tubulin Polymerization Assay.** Tubulin polymerization kits (Cytoskeleton, Denver, CO) were used to study tubulin depolymerization. The reaction contained 50 μL of 4 mg/mL tubulin in PEM buffer (80 mM PIPES, pH 7.0, 0.5 mM EGTA, 2 mM MgCl₂, 1 mM GTP, 10% glycerol). The procedure was done according to the manufacturer’s protocol. Tubulin polymerization was monitored in a UV spectrophotometer for 40 minutes at 340 nm at 37°C.

**In Vitro Binding Assay by Spin Column.** A spin column binding assay was done as reported previously [12] using bovine tubulin, size-exclusion Sephadex G25 columns, and ³H-labeled ligands. Briefly, tubulin (0.1 mg/mL) was incubated in PEM buffer either with 3 μM vinblastine containing [³H]vinblastine (4×10^4 dpm/nmol) in the presence
of different concentrations of vincristine or I-387 or with 3 μM podophyllotoxin containing [3H]podophyllotoxin (4×10⁴ dpm/nmol) in the presence of different concentrations of colchicine or I-387 at 37 °C for 1 h. Samples were then loaded onto size-exclusion Sephadex G25 columns and centrifuged at 200 g for 1 min, and radioactivity in the flow-through was analyzed by scintillation counting.

**Indirect Immunofluorescence Microscopy.** PC-3 cells were plated on poly-D-lysine coated glass cover slips in 6-well plates (5×10⁴ cells/well) the day before treatment. After cells were incubated with cytotoxic agents at 37°C for 24 h, cells were fixed on the cover slips with 4% formaldehyde in PBS for 10 min and permeabilized by 2% Triton-X in PBS for 5 min at room temperature. Fixed cells were blocked with 3% bovine serum albumin in TBST for 30 min and incubated with anti-α-tubulin-FITC antibody (1:100 dilution) at 4°C overnight. Cover slips were mounted on slides using mounting medium with 4',6-diamidino-2-phenylindole (DAPI), followed by microscopic analysis with a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss, Thornwood, NY). Images were acquired with a Zeiss Axiocam HRc, using Zeiss AxioVision.

**In Vivo Antitumor Efficacy Study.** PC-3 cells (2.5×10⁶ cells/site), MES-SA cells (2×10⁶ cells/site), and MES-SA/DX5 (2×10⁶ cells/site) plus Matrigel (BD biosciences, San Jose, CA) were injected subcutaneously into flanks of male nu/nu mice. Tumor size was measured using calipers every 3-4 days and calculated as V = π/6 × (length) × (width)² [13]. When tumors reached a volume of approximately 100~150 mm³, drug treatment was initiated. The control group was treated with vehicle (10% DMSO in PEG300). During the treatment, tumor size and body weights were measured every 2-4 days.

**In Vitro Neurite Outgrowth.** PC-12 cells were seeded in 6-well plates coated with poly-D-lysine (BD, Franklin Lakes, NJ) (10000 cells/well). After 6 h, cells were pretreated for 3 days by adding 100 ng/ml murine 2.5S β-NGF (Promega, Madison, WI) to induce neuronal differentiation and neurite outgrowth. Differentiated cells were then treated for 24 h with compounds at various concentrations in the presence of NGF (100 ng/ml). Vinblastine and docetaxel were used as positive controls. After compound treatments, approximately 100 cells in each well were
randomly chosen and the numbers of cells with no neurites, short neurites (<2 × cell body), and long neurites (> 2 × cell body) were counted (n=3).

**Rotarod Performance.** The training and rotarod test was conducted as described previously [14]. Briefly, during the training phase for 2 consecutive days (3 trials/day), mice (10 mice/group) were trained to run in a rotarod apparatus without falling at a constant speed (12 rpm) for a maximum of 2 min. For the rotarod performance test, duration of each trial was less than 5 min. During this time, the rotation speed was constantly increased from 5 rpm to 40 rpm. Tests were performed for 2 weeks (2 days/week). The animals were tested in three trials per day with an intertrial interval of 30 min. Animal performance was recorded as the average elapsed time for the mice to fall off.

**Statistical Analysis.** The results (mean values ± SD) were subjected to statistical analysis by single-factor ANOVA. The level of significance was set at $P < 0.05$.

**RESULTS**

**The Effect of Indoles on Cell Proliferation.** To explore the effect of I-387 on cancer cell proliferation, we treated human cancer cell lines from prostate, colon, breast, and bladder with different concentrations of compounds using vinblastine and docetaxel as positive controls. Cell proliferation was measured by SRB assay. Proliferation of all cell lines was inhibited by I-387 in a concentration-dependent manner with IC$_{50}$ values ranging from 15.1 to 39.2 nM (Table 1). Vinblastine and docetaxel confirmed the assay validity by exhibiting high potency in cancer cell lines with IC$_{50}$ values ranging from 1.1 nM to 6.3 nM.

Since ABC transporters are thought to be one of the major causes of MDR of anticancer drugs [7, 15], the antitumoral efficacy of I-387 was compared with other anticancer drugs in a cytotoxicity assay using MDR cell lines with high levels of P-gp, MRP1, MRP2, or BCRP (Table 1) [8, 16-19]. As judged from the resistance factor (RF, the ratio of the IC$_{50}$ values in cells over-expressing MDR transporters relative to the IC$_{50}$ values in their control cells), cells with P-gp were 13- to 203-fold resistant to vinblastine and docetaxel. RFs for vinblastine, SN-38, and docetaxel were 4.9, 2.1 and 7.2 in cells with high MRP1 expression and 3.7, 1.5, and 7.6 in cells over-expressing
MRP2. Cells transfected with the vector including BCRP gene were also 33-fold resistant to SN-38. In contrast, the cytotoxicity efficacy of I-387 against cancer cells was unaltered by the MDR phenotypes.

**Apoptotic Effect Induced by I-387.** To determine whether I-387 induces apoptosis of cancer cells, we examined DNA fragmentation by using a cell death detection ELISA kit. Vinblastine was used as a positive control to confirm assay validity. Vinblastine and I-387 increased the enrichment factor (absorbance of treated cells/absorbance of vehicle control cells) in a concentration-dependent manner in PC-3 cells with EC$_{50}$ values of 8.1 and 42.7 nM, respectively (Fig. 1B). We also assessed whether I-387 induced the phosphorylation of Bcl-2, thus inactivating the protein. Following 24 h treatment in PC-3 cells, immunoblots of pBcl-2 showed that 5 nM vinblastine and 50 nM I-387 induced phosphorylation (Ser70) of Bcl-2. As a whole, these studies provide persuasive evidence that I-387 potently induces apoptosis in PC-3 cells that is at least partially mediated by inactivation of Bcl-2.

**Effect of I-387 in Cell Cycle Distribution.** The potent antiproliferative activity and apoptosis of I-387 on cancer cells prompted us to test its effects on the cell cycle. We selected single cells excluding debris and aggregates in the study and the percentage of sub-G1 phase increased from 0.2 (control) to 0.5, 0.7, and 1.3% at I-387 concentration of 10, 50, and 100 nM, respectively. The percentage of PC-3 cells in G$_{2}$M phase increased (Fig. 2A) from 19 (control) to 24, 63, and 77% at I-387 concentrations of 10, 50, and 100 nM, respectively, indicating that PC-3 cells were significantly arrested in the G$_{2}$M phase in a concentration-dependent manner, a pattern that is commonly observed with taxanes and vinca alkaloids. When the percentages of cells in G$_{2}$M phase were plotted against different concentrations of the compounds (0.1 nM to 1 μM), vinblastine, docetaxel, and I-387 arrested the cell cycle with EC$_{50}$ values of 7, 14, and 34 nM, respectively (Fig. 2B). The effect of these drugs arresting cells in the G$_{2}$M phase is closely related to the IC$_{50}$ values of cytotoxicity in PC-3 cells (1, 6, and 28 nM, respectively; Table 1), suggesting that this is a direct link to the mechanism of action. Therefore, changes of expressed and phosphorylated levels of key mitotic regulators following I-387 treatment were evaluated. Cyclin B1 accumulation, Cdc25C dephosphorylation (Ser216), and Cdc2 dephosphorylation were observed at I-387 concentrations of higher than 50 nM (24 h treatment) in PC-3 cells (Fig. 2C).
The Effect of I-387 on Microtubule Polymerization. Since I-387 caused cell arrest in G2M phase, we investigated whether I-387 affects microtubule organization. In order to visualize the microtubule changes in the cells, immunofluorescence microscopy was used. As expected, vehicle-treated control cells demonstrated a variety of fluorescence patterns showing different phases of the cell cycle. On the other hand, vinblastine treated cells demonstrated the appearance of short microtubules due to fragments in the cytoplasm. In contrast, treatment with docetaxel resulted in stabilization of microtubules illustrated by an increase in the density of microtubules with brighter fluorescence. I-387 treatment showed fluorescence similar to vinblastine-induced microtubule changes (Fig. 3A). Therefore, vinblastine and I-387 were compared for their ability to inhibit tubulin polymerization in a cell free system using tubulin polymerization kits. As expected, vinblastine decreased the microtubule formation in a concentration-dependent manner with an IC50 of 283 nM, and I-387 mimicked the effects of vinca alkaloids with an IC50 of 381 nM (Fig. 3B). It is generally observed that antimitotic drugs interact with tubulin either at the colchicine-, vinblastine-, and paclitaxel-binding sites. Binding studies using a spin column assay (Fig. 3C) showed that I-387 inhibited [3H]podophyllotoxin binding to tubulin as colchicine did, whereas I-387 was not able to displace [3H]vinblastine. This suggests that I-387 directly interacts with tubulin by binding to the colchicine-binding domain.

Efficacy of I-387 in Tumor Xenograft Models. We next investigated examined the ability of I-387 to inhibit growth in PC-3, MES-SA, and MES-SA/DX5 xenograft models after i.p. injection. Docetaxel (5 mg/kg) treated mice were included as a positive control. In twice weekly treatments against PC-3 xenograft models, doses of 5 and 10 mg/kg I-387 were well tolerated. Tumor growth was inhibited 36% and 68% for the 5 mg/kg and 10 mg/kg I-387, respectively. The 10 mg/kg I-387 group showed comparable efficacy to the 5 mg/kg docetaxel group in this regimen (Fig 4A) without any general toxicity. In contrast, only 70% of the mice survived in the docetaxel treated group after 3 weeks with an end of study survival rate less than 30%. In the second xenograft study, the efficacy of I-387 against tumors with ABC transporter related resistance was tested using MES-SA/DX5 cells over-expressing P-gp [16]. Doses of 5 mg/kg docetaxel and 0.5 mg/kg vinblastine showed tumor growth inhibition in MES-SA xenografts with TGIs of 54% and 48%, respectively (Fig 4B). These control compounds, however, were not...
effective in MES-SA/DX5 xenografts with much lower TGIs of 9% (5 mg/kg docetaxel, q2d) and 24% (0.5 mg/kg vinblastine, q2d) (Fig 4C). In contrast, I-387 inhibited tumor growth significantly in MES-SA/DX5 as well as in MES-SA xenografts with the q2d regimen (Fig 4B, 4C). In the MES-SA/DX5 xenograft model, I-387 showed similar TGIs of 47% and 76% in the 5 and 10 mg/kg treatment groups, respectively, as compared to the MES-SA model with 47% and 74%. I-387 treatment did not induce any general toxicity or significant body weight loss in either the 5 or 10 mg/kg treatment groups.

**Neurotoxicity studies of I-387**

NGF-dependent neurite outgrowth is commonly used as a *in vitro* model to study the neurotoxic effects of drugs [20, 21]. NGF-dependent neurite outgrowth assay showed dose-dependent reduction of PC12 neurite extensions in the vinblastine and I-387 treatment groups. 88% of vehicle treated cells expressed neurite elongation (Fig. 5A). Twenty-one percent of cells had elongated neurites when treated with 5 nM vinblastine (IC$_{70}$ value of vinblastine in PC-12 cell growth inhibition), but long neurites were only observed in 1.5% of cells. I-387, however, resulted in higher percentages of neurite forming cells than vinblastine with 59% of cells showing neurite elongation at concentration of 50 nM I-387 (IC$_{70}$ value of I-387 in PC-12 cell growth inhibition) ($P<0.01$). Further, 50 nM I-387, a more toxic dose than IC$_{50}$ value, did not reduce neurite growth showing 60% of cells with neurites (Fig. 5A). To evaluate *in vivo* neurotoxicity, mouse performance on the accelerating rotarod was examined following exposure to vehicle, vinblastine, vincristine, or I-387. Vehicle-treated mice stayed on the rotating rod for 151 s at the end of 2 weeks, whereas 0.5 mg/kg vinblastine- and vincristine-treated mice showed significant reduction in their ability to stay on the rotating rod with mean times on the rod of 121 ($P=0.0006$) and 104 s ($P=0.00006$), respectively. I-387-treated mice (10 mg/kg) did not show impaired rotarod performance as compared to the vehicle control ($P=0.6$) (Fig. 5B).
DISCUSSION

Microtubules are considered to be one of the major therapeutic targets of anticancer drugs. In spite of clinical success of tubulin inhibitors, some limitations remain, such as high peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug resistance conferred by multidrug resistance (MDR) transporters and tubulin mutations [4-7]. The structural complexity of naturally-derived tubulin inhibitors such as vinca alkaloids, taxanes, maytansin, and halichondrin B analogs limits the use of conventional approaches to characterize structure-activity relationships and to isolate active compounds [5]. Drug resistance is also a significant problem in clinical chemotherapy. The expression of multidrug resistance proteins, such as P-gp and MRPs, and mutations of the target protein are known to result in drug resistance. In addition, peripheral neurotoxicity is considered to be a major side effect in the clinical application of antimitotic agents because they bind to beta-tubulin which is the primary component of the axonal microtubules [22].

Here, we identified a structurally novel compound, I-387, \((3-(1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl)methanone\), which inhibits cell growth in a variety of human tumor cells and arrests cell cycle progression in G2M phase. We, therefore, examined the influence of I-387 on changes of key mitotic regulators. I-387 treatment induced abnormal cyclin B1 accumulation and Cdc2 dephosphorylation in PC-3 cells. Phosphorylation of Cdc25C (Ser216) is known to induce nuclear export and reduction of activity of Cdc25C by allowing the binding of 14-3-3 to pCdc25C, while abundant phosphorylation of Cdc25C (Ser191, Ser198, etc) activates Cdc25C [23]. Phosphorylation in Cdc25C (Ser216) decreased significantly after I-387 treatment, resulting in dephosphorylation of Cdc2 (Thr14 and Tyr15) that is required for activation of Cdc2/cyclin B1 and subsequent entry into mitosis [24]. These data suggested that I-387 regulated the cell transition from G2 to mitosis by constitutive activation of Cdc2/cyclin B1 complex. Since I-387 caused cell arrest in G2M phase, further in vitro studies were conducted to investigate whether I-387 affects microtubule organization. These studies showed that I-387 destabilizes microtubules in human tumor cells by binding to the colchicine-binding site. In the xenograft models using PC-3 cell line, 10 mg/kg I-387 (2 days/week, i.p.) showed comparable tumor growth inhibition to 5 mg/kg docetaxel (2 days/week, i.p.) without signs of toxicity. Furthermore, I-387 induced potent cell growth inhibition against drug-resistant cell lines over-expressing ABC transporters and showed potent efficacy in the...
animal xenograft models with MES-SA/DX5 as well as MES-SA. In contrast, 0.5 mg/kg vinblastine and 5 mg/kg docetaxel were not effective in MES-SA/DX5 resistance model as compared to sensitive MES-SA. These in vivo results suggest I-387 may be effective in tumors that have obtained resistance by over-expressing P-gp. There was no apparent macroscopic toxicity in terms of body weight loss, hunched posture, diarrhea, or hematological toxicity in mouse xenograft models.

Mitotic spindle poisons leading to dynamic instability of microtubules induce tumor cell death. However, since microtubules fulfill important functions in interphase, resting, and differentiated cells for the maintenance of cytoskeletal functions and intracellular transport processes, microtubule inhibitors are known to exhibit unwanted side effects including peripheral neuropathies. The peripheral neurotoxicity might result from a disruption of microtubule mediated axonal flow. Microtubule inhibitors such as taxanes and vinca alkaloids are also known to induce myelosuppression and neutropenia due to the inhibition of the proliferation of non-transformed cells such as hematopoietic precursor cells [25, 26]. A neurite outgrowth assay in PC12 cells has been proposed as an in vitro model to investigate chemotherapy-induced neuropathy [20, 21]. In this neurite outgrowth study, I-387 caused less neurotoxic damage with higher percentages of neurite forming cells compared to vinblastine. Furthermore, since in vivo behavioral assays are also recommended to detect neuropathic pain, rotarod performance was examined. The rotarod is one of the widely used tests for neuromotor performance since 1968 [14] [27]. The status of the motor system of ICR mice was tested on the accelerating rotarod. We tested equi-efficient doses of the compounds, based on tumor growth inhibition studies in mouse xenograft models. 0.5 mg/kg Vinblastine and 10 mg/kg I-387 were effective in MES-SA xenografts and 0.4–1.6 mg/kg vincristine showed efficacy in numerous xenograft models [28-30]. Vinblastine (0.5 mg/kg, q2d, i.p.) and vincristine (0.5 mg/kg, q2d, i.p.) showed impaired performance from day 4 to day 14. I-387 (10 mg/kg, q2d, i.p.), in comparison, showed no loss of function, thus indicating that I-387 had no effect on motor performance. Although studies are needed to elucidate the effects of I-387 in the central as well as peripheral nervous system, our rotarod studies provide initial but compelling comparison of the peripheral neurotoxicity of I-387 to the vinca alkaloids, due to the limited access of vinca alkaloids to the central nervous system. Additional electrophysiologic and nerve biopsy studies would be useful to understand how I-387 affects neuronal cell bodies, Schwann cells, myelins, axons, and/or nerve roots [31]. In addition, other animal behavior
studies using hot plate and von Frey fiber could be considered [14]. However, it is important to note that the clinical neuropathy in humans is not always predictable by the results in animal or in vitro models [32]. Neuropathic pain in humans is usually spontaneous with individual variance making it very difficult to quantify or predict from animal models.

In addition to their ability to inhibit tumor cell growth, tubulin-binding agents have generated considerable interest due to potential tumor-selective antivascular activity including antiangiogenic and vascular-disrupting properties [33]. Low molecular weight drugs with rapid tumor selective vascular disrupting properties are categorized as vascular disrupting agents (VDAs). Microtubule destabilizers are considered as the largest family of VDAs. Although the cellular and molecular mechanisms for these actions are not clearly defined, the disruption of endothelial adherent junctions is considered as one of the main mechanisms of VDAs. Studies to examine the vascular disrupting activity of I-387 are underway in our laboratory.

In conclusion, a number of studies are ongoing in the field of tubulin-binding agents to improve the safety profile and overcome other limitations (e.g., drug resistance) of conventional agents. In this study, I-387 exhibited potent anticancer activity by targeting the colchicine-binding site in a broad spectrum of human cancer cells including those that express ABC transporters without any detrimental effects on animal motor performance. Our study indicates that I-387 has potential against various malignancies including drug-resistant tumors.
# REFERENCES


Table 1. Anticancer efficacy of I-387 in different cancer cell lines and MDR cell lines with different resistance phenotypes

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>I-387 (nM)</th>
<th>Vinblastine (nM)</th>
<th>SN-38 (nM)</th>
<th>Docetaxel (nM)</th>
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<tr>
<td>LNCaP</td>
<td>30.9 ± 3.5</td>
<td>3.4 ± 0.9</td>
<td>ND</td>
<td>4.7 ± 1.3</td>
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<td>PC-3</td>
<td>28.2 ± 2.0</td>
<td>1.4 ± 0.3</td>
<td>ND</td>
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<td>DU-145</td>
<td>22.8 ± 4.1</td>
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<td>5.2 ± 1.0</td>
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<td>PPC-1</td>
<td>18.7 ± 1.5</td>
<td>1.1 ± 0.4</td>
<td>ND</td>
<td>2.7 ± 1.0</td>
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<tr>
<td>TSU-Pr1</td>
<td>17.6 ± 2.1</td>
<td>1.6 ± 0.1</td>
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<td>2.6 ± 0.9</td>
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<tr>
<td>HT-29</td>
<td>15.1 ± 1.3</td>
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<tr>
<td>MCF-7</td>
<td>39.2 ± 2.9</td>
<td>1.4 ± 0.4</td>
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<td>3.8 ± 0.8</td>
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<tr>
<td>K562</td>
<td>23.6 ± 2.4</td>
<td>1.4 ± 0.4</td>
<td>ND</td>
<td>2.7 ± 1.0</td>
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<td>K562/Dox</td>
<td>18.0 ± 4.4 (0.8)</td>
<td>268 ± 48 (191)</td>
<td>ND</td>
<td>548 ± 76 (203)</td>
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<td>MES-SA</td>
<td>31.5 ± 2.9</td>
<td>2.3 ± 0.8</td>
<td>ND</td>
<td>5.9 ± 1.1</td>
</tr>
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<td>MES-SA/DX5</td>
<td>32.3 ± 8.2 (1.0)</td>
<td>45.7 ± 5.3 (20)</td>
<td>ND</td>
<td>76.4 ± 8.7 (13)</td>
</tr>
<tr>
<td>HEK293-pcDNA3.1</td>
<td>23.8 ± 1.7</td>
<td>5.0 ± 1</td>
<td>8.4 ± 1</td>
<td>4.4 ± 2</td>
</tr>
<tr>
<td>HEK293-MRP1</td>
<td>30.4 ± 6.4 (1.3)</td>
<td>24.3 ± 2 (4.9)</td>
<td>17.7 ± 1 (2.1)</td>
<td>31.3 ± 5 (7.2)</td>
</tr>
<tr>
<td>HEK293-MRP2</td>
<td>31.5 ± 8 (1.3)</td>
<td>18.1 ± 4 (3.7)</td>
<td>12.4 ± 1 (1.5)</td>
<td>33.1 ± 4 (7.6)</td>
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<tr>
<td>HEK293-pcDNA3-10</td>
<td>13.5 ± 1.6</td>
<td>ND</td>
<td>2.9 ± 0.3</td>
<td>ND</td>
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<tr>
<td>HEK293-MR2</td>
<td>13.6 ± 1.3 (1.0)</td>
<td>ND</td>
<td>94.3 ± 20 (32.5)</td>
<td>ND</td>
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</tbody>
</table>

NOTE: P-gp, MRP1, MRP2, BCRP were over-expressed in K562/DOX, MES-SA/DX5, HEK293-MRP1, HEK293-MRP2 and HEK293-482R2. The resistance factor (in parentheses) was calculated as the ratio of IC₅₀ values for the resistant cell subline to that of the parental cell line. All experiments were performed at least in three replicates. ND not determined.
Fig. 1. Chemical structure of I-387 and apoptosis induced by I-387 treatment. A, Chemical structures of I-387, vinblastine, and docetaxel. B, I-387 induces DNA fragmentation in PC-3 cells. The extent of apoptosis (i.e., the enrichment factor) was determined using a commercially available anti-histone ELISA after 24 h treatment. C, I-387 induced the Bcl-2 phosphorylation in PC-3 cells (24 h) and actin was used as a loading control.

Fig. 2. Effect of I-387 on cell cycle. PC-3 cells were treated with different concentrations (0 to 1 μM) of I-387, vinblastine, or docetaxel for 24 h, and DNA content of the cells was analyzed by FACS. A, I-387 arrested cells in G2M phase. B, The percentage of cells in G2M phase of the cell division cycle was quantified and dose response curves are shown. C, Changes in expressed and phosphorylated status of G2-M regulators including cyclin B1, Cdc25C, pCdc25C, Cdc2 and pCdc2 by I-387 were evaluated in PC-3 cells (24 h). Actin was used as a loading control and relative band intensity was shown as the mean ± SD (n=2-3). * P < 0.05; # P < 0.01.

Fig. 3. Inhibition of microtubule formation by I-387 and other agents. A, PC-3 cells were treated with antimitotic agents for 24 h. Fixed cells were incubated with anti-α-tubulin-FITC antibody and the cellular microtubules were observed with a Zeiss Axioplan 2 fluorescent microscope. B, the microtubule polymerization was monitored by measuring the turbidity at 340 nm in the absence or presence of drugs. Representative experiment. Control (■); I-387, 0.1 μM (●); I-387, 1 μM (○); I-387, 5 μM (▲); I-387, 10 μM (△). Data shown are the mean of duplicate reactions. The percentage of microtubule polymerization at 40 min was quantified and dose response curves are shown (vehicle control set at 100%). C, [3H]Vinblastine was incubated with tubulin in the presence of different concentrations of vincristine or I-387. [3H]Podophyllotoxin was incubated with tubulin in the presence of different concentrations of colchicine or I-387. Tubulin-bound [3H]podophyllotoxin and [3H]vinblastine were plotted against the concentrations of the competitors.

Fig. 4. Efficacy and tolerability of I-387 in xenograft models after i.p. injection. A, PC-3 xenografts were treated with vehicle (2 days/week), docetaxel (5 mg/kg, 2 days/week), or I-387 (5 and 10 mg/kg, 2 days/week) for 4 weeks. B, Xenograft models using MES-SA cells were treated with vehicle (q2d), vinblastine (0.5 mg/kg, q2d), docetaxel (5
mg/kg, q2d), or I-387 (10 mg/kg, q2d). C. Xenograft models bearing MES-SA/DX5 cells over-expressing P-gp were treated as same as the MES-SA xenograft model. The tumor volumes (mm³) were plotted against time and are the means ± SD from eight animals. The tumor volumes were shown in left panel and survival rates or body weights were shown in right panel.

Fig 5. In vivo and in vitro neurotoxicity of I-387. A, PC-12 cells pretreated with 100 ng/ml murine 2.5S β-NGF were exposed to vehicle control, vinblastine, or I-387 at various concentrations in the presence of 100 ng/ml NGF for 24 h. The numbers of cells with no neurites, short neurites (<2 × cell body), and long neurites (> 2 × cell body) were counted (left panel). All determinations were confirmed using three independent experiments. PC-12 cell survival was quantitated by the SRB assay (right panel). Each value represents the mean ± SD of three independent experiments. B, ICR mice were dosed with vehicle control, vinblastine (0.5 mg/kg, i.p., q2d), vincristine (0.5 mg/kg, i.p., q2d), or I-387 (10 mg/kg, i.p., q2d) for 2 weeks. Mice were placed on an accelerating rotating rod and the performance on the rotarod was monitored 2 days/week (n=10). * P < 0.02; # P < 0.01.
Figure 1

A

B

C

I-387
Vinblastine
Docetaxel

[Chemical structures]

Enrichment factor

Concentration (nM)

pBcl-2 (Ser70)

Actin

[Graphs showing concentration vs. enrichment factor and Western blot for pBcl-2 and Actin]
Figure 2

A

B

C

Figure 2
Figure 3

A

Control 10 nM Vinblastine 30 nM Docetaxel 50 nM I-387 100 nM I-387

B

C

Microtubule polymerization (%)

Concentration (M)

Concentration (M)

Concentration (M)

Time (min)

Concentration (M)

Microtubule polymerization (%)

Concentration (M)

Concentration (M)

Concentration (M)
Figure 4

A. PC3

B. MES-SA

C. MES-SA/DX5

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
**Figure 5**

### A

#### Cell growth inhibition assay

<table>
<thead>
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<th>IC50 (nM)</th>
<th>Vinblastine</th>
<th>I-387</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3.2 ± 1</td>
<td>36.9 ± 6.9</td>
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#### % of Cells with Neurites

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<th>50</th>
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<tbody>
<tr>
<td>I-387 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- no neurite
- short neurite
- long neurite

### B

#### Performance of mice on rotating rod

- Control
- 0.5 mg/kg Vinblastine
- 0.5 mg/kg I-387
- 10 mg/kg I-387

#### Body weight (g)

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<th>Time (day)</th>
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<th>2</th>
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<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
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</thead>
<tbody>
<tr>
<td>Vinblastine</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>I-387</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>6</td>
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</tbody>
</table>
Molecular Cancer Therapeutics

I-387, a Novel Antimitotic Indole, Displays a Potent In Vitro and In Vivo Antitumor Activity with Less Neurotoxicity


Mol Cancer Ther Published OnlineFirst September 9, 2010.