

HTI-286, a Synthetic Analogue of the Tripeptide Hemiasterlin, Is a Potent Antimicrotubule Agent that Circumvents P-Glycoprotein-mediated Resistance *in Vitro* and *in Vivo*¹

Frank Loganzo,² Carolyn M. Discafani, Tami Annable, Carl Beyer, Sylvia Musto, Malathi Hari, Xingzhi Tan, Carolyn Hardy, Richard Hernandez, Michelle Baxter, Thiruvikraman Singanalore, Gulnaz Khafizova, Marianne S. Poruchynsky, Tito Fojo, James A. Nieman, Semiramis Ayral-Kaloustian, Arie Zask, Raymond J. Andersen, and Lee M. Greenberger

Oncology Research [F. L., C. M. D., T. A., C. B., S. M., M. H., X. T., C. H., R. H., M. B., L. M. G.] and Chemical Sciences [T. S., G. K., S. A.-K., A. Z.], Wyeth Research, Pearl River, New York 10965; The National Cancer Institute Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892 [M. S. P., T. F.]; and Department of Chemistry, University of British Columbia, Vancouver, Canada V6T 1Z1 [J. A. N., R. J. A.]

ABSTRACT

Hemiasterlin is a natural product derived from marine sponges that, like other structurally diverse peptide-like molecules, binds to the *Vinca*-peptide site in tubulin, disrupts normal microtubule dynamics, and, at stoichiometric amounts, depolymerizes microtubules. Total synthesis of hemiasterlin and its analogues has been accomplished, and optimal pharmacological features of the series have been explored. The biological profile of one analogue, HTI-286, was studied here. HTI-286 inhibited the polymerization of purified tubulin, disrupted microtubule organization in cells, and induced mitotic arrest, as well as apoptosis. HTI-286 was a potent inhibitor of proliferation (mean $IC_{50} = 2.5 \pm 2.1$ nM in 18 human tumor cell lines) and had substantially less interaction with multidrug resistance protein (P-glycoprotein) than currently used antimicrotubule agents, including paclitaxel, docetaxel, vinorelbine, or vinblastine. Resistance to HTI-286 was not detected in cells overexpressing the drug transporters MRP1 or MXR. In athymic mice implanted with human tumor xenografts, HTI-286 administered i.v. in saline inhibited the growth of numerous human tumors derived from carcinoma of the skin, breast, prostate, brain, and colon. Marked tumor regression was observed when used on established tumors that were >1 gram in size. Moreover, HTI-286 inhibited the growth of human tumor xenografts (e.g., HCT-15, DLD-1, MX-1W, and KB-8-5) where paclitaxel and vincristine were ineffective because of inherent or acquired resistance associated with P-glycoprotein. Efficacy was also achieved with p.o. administration of HTI-286. These data suggest that HTI-286 has excellent preclinical properties that may translate into superior clinical activity, as well as provide a useful synthetic reagent to probe the drug contact sites of peptide-like molecules that interact with tubulin.

INTRODUCTION

A number of tubulin-binding agents, constituting a large family of structurally distinct compounds (mostly natural products), inhibits microtubule function and tumor growth in animal models (1). At low concentrations, all antimicrotubule agents disrupt microtubule dynamics in a similar manner (2). Two classes of molecules are distinguished based on their interaction with microtubules at high, stoichiometric concentrations; agents such as the taxanes (e.g., paclitaxel and docetaxel) stabilize the microtubule against depolymerization, whereas agents such as the *Vinca* alkaloids (vinorelbine, vinblastine, and vincristine) bind to the tubulin dimer, block the formation of new microtubules, and lead to the depolymerization of existing microtubules.

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² To whom requests for reprints should be addressed, at Wyeth Research, Oncology Research, 401 North Middletown Road, 200/4709, Pearl River, NY 10965. E-mail: loganzf@wyeth.com.

Despite the ability of taxanes and *Vinca* alkaloids to inhibit the progression of some cancers, inherent resistance to antimicrotubule agents is encountered in many tumor types, and acquired resistance usually occurs during multiple cycles of therapy (1, 3). Beyond this, side effects are significant and can be attributed to the compound itself and/or the vehicle required for administration. Therefore, there has been great interest in identifying novel antimicrotubule drugs that overcome various modes of resistance and have improved pharmacology profiles. Experimental antimitotic drugs in clinical trials include novel taxanes, epothilones, and peptide-like agents (4).

This study focuses on a synthetic hemiasterlin analogue, HTI-286 (Fig. 1). Originally identified as natural products from marine sponges (*Cymbastella* sp., *Hemiasterella minor*, *Siphonochalina* sp., and *Auletta* sp.), hemiasterlins comprise a small family of naturally occurring tripeptides containing three highly modified amino acids (5–7). Hemiasterlin is a potent inhibitor of cell growth that, like vinblastine, depolymerizes microtubules and arrests cells in the G₂-M phase of the cell cycle (8). However, like other peptide-like antimicrotubule agents, such as dolastatin-10 and cryptophycin-1, hemiasterlin non-competitively inhibits the binding of vinblastine to tubulin (9). Because hemiasterlin competitively inhibits the binding of dolastatin-10 to tubulin, it has been proposed that these molecules bind to the *Vinca*-peptide-binding site of tubulin (9).

Extremely limited quantities of hemiasterlins hampered development of this series of compounds, particularly in animal models where limited testing has been reported (5). However, synthetic methods for producing hemiasterlin and its analogues (10, 11) have allowed the evaluation of many related compounds³ for antitumor properties and a better understanding of the binding site of peptide-like molecules with tubulin. The following data show that a hemiasterlin analogue, HTI-286, is an antimicrotubule agent that retains potency in cellular models resistant to several chemotherapeutics, including taxanes and *Vinca* alkaloids. The agent, formulated only in saline and given i.v. or p.o., inhibits the growth of human tumors in xenograft models that are either sensitive or resistant to currently approved antimicrotubule drugs.

MATERIALS AND METHODS

Compounds. HTI-286 (*N*, β , β -trimethyl-L-phenylalanyl-L-N¹-[(1*S*,2*E*)-3-carboxy-1-isopropylbut-2-enyl]-N¹,3-dimethyl-L-valinamide, also known as SPA110) was synthesized at Wyeth based on methods reported previously (11) that were modified and will be reported elsewhere.³ Hemiasterlin was purified from an extract of *Cymbastella* sp. as described previously (5). Paclitaxel, vincristine, vinblastine, colchicine, and doxorubicin were obtained from Sigma (St. Louis, MO). Docetaxel and vinorelbine were obtained from MedWorld

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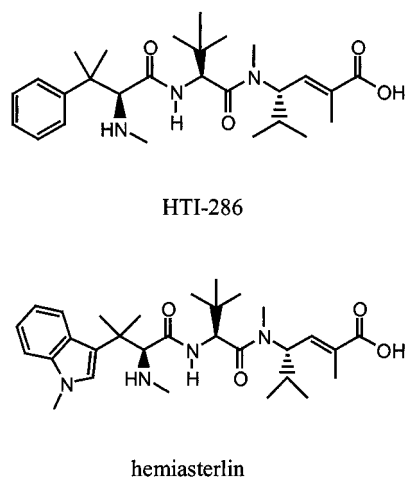


Fig. 1. Chemical structures of HTI-286 and hemiasterlin.

Pharmacy (Valley Cottage, NY). Mitoxantrone and CL-329,753 (12) were manufactured by Wyeth. Epothilone A and B were obtained from Calbiochem (San Diego, CA). Dolastatin-10 was generously provided by the National Cancer Institute. Compounds were prepared as 1 or 10 mM stocks in DMSO for *in vitro* studies.

Cell Lines. Cell lines were obtained from the American Type Culture Collection (Rockville, MD), except as indicated. S1 and S1-M1-3.2 cells have been described previously (13). The parental and resistant cell counterparts were generously provided as follows: (a) KB series (Dr. M. Gottesman, National Cancer Institute; Ref. 14); and (b) HL60 and HL60/ADR cells (Dr. M. Center, University of Kansas; Ref. 15). The PC3MM2 prostate carcinoma cell line was generously provided by Dr. I. J. Fidler (M. D. Andersen Cancer Center). MX-1-Wyeth, designated MX-1W, was a subclone of the breast carcinoma MX-1 that was originally obtained from the National Cancer Institute. MX-1W cells were obtained by adapting the growth of these fragments to tissue culture. For *in vivo* experiments, MX-1W tumors were maintained by passage of tumor xenograft fragments between athymic mice. Cell lines were maintained in either DMEM or RPMI 1640, supplemented with 10% FCS and 10 units/ml penicillin and 10 μ g/ml streptomycin, with the exception of the KB series, where 20% FCS was used. KB-8-5 and KB-V1 were maintained in 25 nM colchicine and 1.1 μ M vinblastine, respectively, except during growth assays. Cells were maintained at 37°C in a 5% CO₂/humidified incubator.

Tubulin Polymerization Assays. For *in vitro* tubulin polymerization assays, bovine MAP-rich⁴ tubulin, PEM buffer [80 mM Na-PIPES (pH 6.9), 1 mM MgCl₂, and 1 mM EGTA], and GTP were purchased from Cytoskeleton (Denver, CO). MAP-rich tubulin (final concentration 1.5 mg/ml) was dissolved in cold PEM buffer containing 1 mM GTP (GPEM) and centrifuged at 12,000 \times g for 10 min at 4°C. The tubulin solution (100 μ l/well) was added rapidly to wells of a low-volume, 96-well plate already containing duplicate aliquots (10 μ l) of test compounds in GPEM. Final compound concentrations were 0.1 or 1 μ M. Control wells contained the same final concentration of DMSO (0.3%). After initiation of the reaction, absorbance at 340 nm was measured every minute for 60 min at 24°C using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA).

Immunofluorescence Microscopy. KB-3-1 epidermoid carcinoma cells were plated on coverslips (No. 1 thickness), placed in 6-well dishes, and cultured overnight. Compounds diluted in media were added to the wells. After 20 h of incubation with compounds, cells were washed in PBS, fixed for 10 min in methanol at -20°C, rehydrated in PBS, and incubated for 20 min at 37°C with a 1:500 dilution of anti- α -tubulin antibody (clone DM 1A; Sigma) in PBS. After a PBS wash, cells were incubated with 1:100 dilution of FITC-conjugated F(ab')₂ fragment of goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA) and 50 μ g/ml propidium iodide (Roche, Indianapolis, IN) for 20 min at 37°C in PBS. After extensive washing, cells were rinsed once

in water and mounted in one drop of Slowfade Light Antifade (Molecular Probes, Eugene, OR). Cells were examined with an Olympus BX61 microscope (Olympus America, Inc., Melville, NY) and \times 60 objective using epillumination. Images were obtained with a Cooke Sencicam QE cooled CCD camera (Cooke Corp., Auburn Hills, MI) and Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO).

Proliferation Assays. Cells were plated in 96-well plates in 100- μ l media at densities predetermined to produce 60–90% confluence at the time of analysis. Compounds, which were serially diluted into media as 2 \times stocks, were added to cells in duplicate. After 3 days of incubation, cell survival was assessed by the SRB assay as described (13). For nonadherent cells, proliferation was assayed with the CellTiter 96 Aqueous nonradioactive cell proliferation assay according to the manufacturer's directions (Promega, Madison, WI).

Cell Cycle Analyses. KB-3-1 cells were seeded into 6-well dishes and treatments initiated when cells were 60–70% confluent. Drugs were diluted in media as 4 \times stocks and added to cells to provide the specified final concentrations. Cells were processed for cell cycle analyses as described previously (16).

Analysis of P-Glycoprotein (MDR1) Expression. The level of P-glycoprotein in tumor cell lines was determined by isolating membranes from cells as described previously (13), followed by immunoblot analysis using a primary antibody specific for P-glycoprotein (Ab-1; Oncogene Science, Uniondale, NY) and a goat-antirabbit IgG horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). Detection was done by the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

In Vivo Efficacy Studies. Athymic *nu/nu* female mice, 5–6 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA). Mice were implanted s.c. with 1.5 \times 10⁶ Lox melanoma cells, 2.5 \times 10⁶ KB-3-1 cells, 7 \times 10⁶ KB-8-5 cells, 6 \times 10⁶ DLD-1 cells, 5 \times 10⁶ HCT-15 cells, or five fragments of MX-1W. When tumors attained a mass of between 80 and 120 mg (day 0), animals were randomized into treatment groups. In some experiments, tumors were allowed to grow \leq 2.5 grams in size before drug treatment was initiated. After randomization, animals were treated i.v. with one or more doses of HTI-286 prepared in saline, 60 mg/kg/dose paclitaxel, or a saline vehicle control (0.2 ml of bolus injection except 0.5 ml for paclitaxel). In some experiments, animals were treated i.p. with 1 mg/kg/dose vincristine prepared in saline. Paclitaxel was formulated by suspension in 100% ethanol, followed by mixing with Cremophor EL (Sigma) to yield a 25 mg/ml stock (50% ethanol/50% Cremophor EL), which was diluted into saline immediately before administration. The final dosing formulation was 6% ethanol/6% Cremophor EL/88% saline. The doses chosen for both paclitaxel and vincristine were between 80 and 95% of the maximum tolerated dose for each drug. Tumor mass [(length \times width²)/2] was determined once a week for \leq 35 days. The data were analyzed by a one-sided Student *t* test. A drug dose was considered toxic if there was >20% lethality or if animals lost \geq 20% of their initial body weight.

RESULTS

Effect of HTI-286 on Microtubule Protein Polymerization. Purified bovine tubulin, in the presence of MAPs and GTP, polymerizes into microtubules producing turbidity of the assay solution that can be measured as an increase in absorbance over time (Fig. 2, DMSO control). Because agents that depolymerize microtubules inhibit this process, we compared the effect of these compounds with that of HTI-286. When added at 0.1 μ M, colchicine, vincristine, and HTI-286 inhibited polymerization, as detected by a lower absorbance of the samples with the drugs compared with controls, by 19, 30, and 41%, respectively, after 60 min. At 1 μ M, HTI-286 and vincristine completely inhibited microtubule formation, whereas colchicine had a partial effect. Although colchicine appears to be a weaker inhibitor of tubulin assembly under these conditions, this may be attributable to slow binding of colchicine to tubulin. In contrast to depolymerizing agents, microtubule-stabilizing drugs, such as paclitaxel, produced an increase in turbidity with a reduced lag time and faster initial rate

⁴ The abbreviations used are: MAP, microtubule-associated protein; SRB, sulforhodamine B; MDR1, multidrug resistance protein-1.

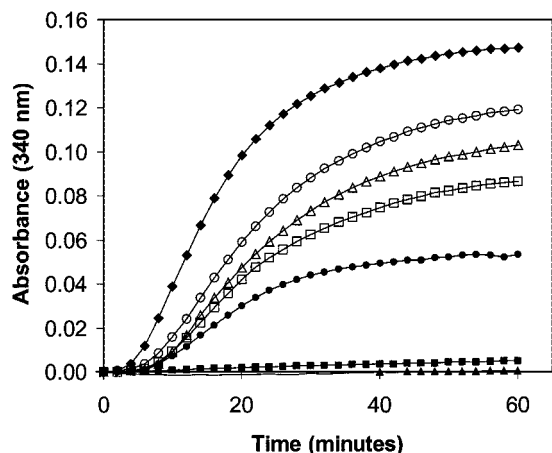


Fig. 2. Inhibition of microtubule formation by HTI-286 and other agents. The ability of drugs to inhibit microtubule formation of MAP-rich tubulin was assessed by a spectrophotometric assay. DMSO control (◆); HTI-286, 0.1 μM (□), 1 μM (■); vincristine, 0.1 μM (△), 1 μM (▲); colchicine, 0.1 μM (○), 1 μM (●). Data shown are the mean of duplicate reactions at 2-min intervals and representative of numerous experiments.

(data not shown). These data demonstrate that HTI-286 inhibits tubulin polymerization in a cell-free system.

Effect of HTI-286 on Microtubules in Cells. The effect of HTI-286 on cellular microtubule structure was examined by immunofluorescence microscopy using an antibody specific for α -tubulin. Untreated KB-3-1 epidermoid carcinoma cells in interphase contained a dense and complex network of microtubules evident in the cytoplasm, generally originating in the perinuclear region and extending into the cell periphery (Fig. 3A). Cells in metaphase in the untreated samples displayed a bipolar mitotic spindle (Fig. 3A, inset). In dividing cells, condensed chromosomes lined up between the spindle poles to form the metaphase plate (data not shown). KB-3-1 cells were exposed to 1, 4, and 16 nM HTI-286 for 16 h, because the concentration of drug needed to inhibit cell growth by 50% (IC_{50}) after 3-day continuous exposure was 1 nM. Under these conditions, HTI-286 disrupted the appearance of microtubules in a concentration-dependent manner. Exposure of KB-3-1 cells to 1 nM HTI-286 produced rounding of cells

with accumulation of $\sim 50\%$ of cells in metaphase (Fig. 3B). When 4 nM HTI-286 was used, the density of the cytoplasmic network was decreased, and a diffuse staining was observed (Fig. 3C, right-hand inset). In addition, multipolar spindles were detected (Fig. 3C, left-hand inset). When cells were treated with 16 nM HTI-286, few microtubules were observed, and most cells had diffuse staining of tubulin (Fig. 3D, inset). Diffuse cytoplasmic staining was also observed when cells were treated with 8 nM vinblastine (data not shown). These data confirm that HTI-286 permeated cells and modified microtubule structure, consistent with the effects of depolymerizing agents.

Effect of HTI-286 on Cell Cycle Distribution. Hemiasterlin, like other antimicrotubule agents, arrests cells in mitosis and induces apoptosis (8). To confirm that HTI-286 operated by a similar mechanism, cells treated with the drug were analyzed by flow cytometry. KB-3-1 epidermoid carcinoma cells were treated with HTI-286, paclitaxel, or vinblastine for 24, 48, or 72 h at doses that were ~ 1 -, 3-, or 10-fold higher than the IC_{50} needed to inhibit cell growth after 3 days. Because the data for the 48- and 72-h time points were similar, only the 24- and 48-h results are shown in Table 1. HTI-286 treatment induced a dose-dependent increase in cells accumulated in mitosis (G_2 -M) after 24 h of exposure. Approximately 90% of the cell population was detected in this phase when exposed to 10 nM HTI-286. After 2–3 days of treatment with each dose of HTI-286, a greater percentage of cells underwent apoptosis. This pattern was similar to that observed for cells treated with vinblastine. Pronounced G_2 -M accumulation also occurred in cells treated with the high dose (30 nM) of paclitaxel. However, more apoptosis was observed at the lower dose of paclitaxel compared with HTI-286 or vinblastine. These data are consistent with reported cell cycle effects of taxanes, *Vinca* alkaloids, and hemiasterlin (2, 8).

Effect of HTI-286 on Cell Proliferation. The ability of HTI-286 to inhibit tumor cell growth compared with other antimicrotubule agents was examined. Data for paclitaxel are shown. Cell growth was assessed by 3-day continuous exposure to candidate agents. HTI-286 inhibited the growth of 18 tumor cell lines with an average IC_{50} of 2.5 ± 2.1 nM (mean \pm SD) and a median value of 1.7 nM (Table 2). The activity of HTI-286 was independent of tumor origin. For all cell

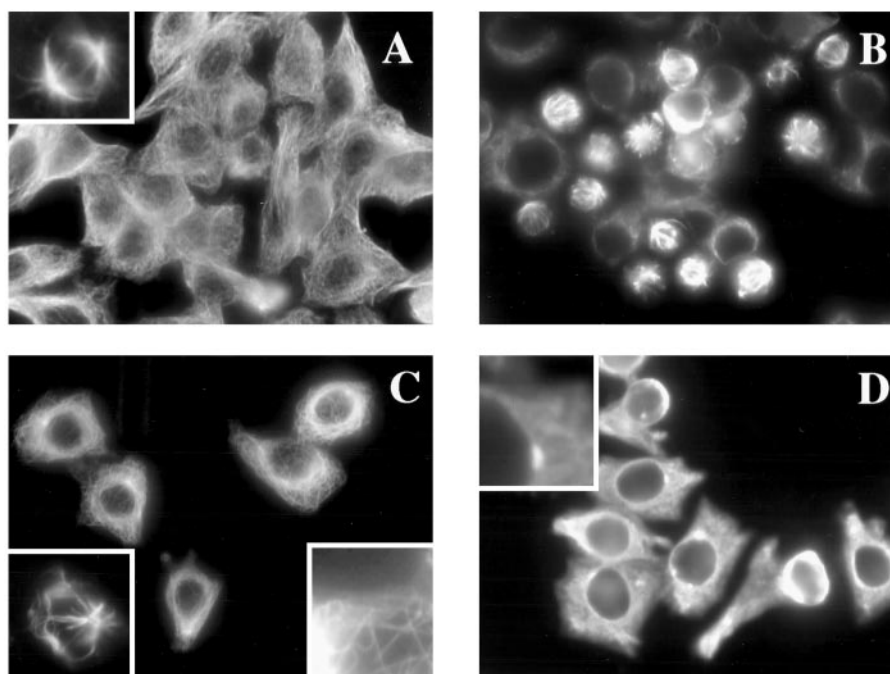


Fig. 3. Microtubule structure in KB-3-1 cells treated with HTI-286 and assessed by immunofluorescence microscopy. Cells were untreated (A) or treated for 16 h with HTI-286 at 1 nM (B), 4 nM (C), or 16 nM (D). Cells were then fixed and incubated with antibody specific for α -tubulin, followed by FITC-conjugated secondary antibody. Magnification is $\times 60$, except for insets, which are magnified further to show morphological details.

Table 1 Comparative effects of HTI-286, vinblastine, and paclitaxel on the cell cycle in KB-3-1 cells^a

Treatment	24 h				48 h			
	Apop	G ₀ -G ₁	S	G ₂ -M	Apop	G ₀ -G ₁	S	G ₂ -M
Untreated	1	53	17	29	1	60	17	23
HTI-286								
1 nM	2	54	16	29	6	61	15	18
3 nM	10	26	14	49	24	20	26	30
10 nM	3	1	4	92	10	8	31	53
Vinblastine								
1 nM	2	50	17	32	4	65	12	19
3 nM	8	28	17	47	27	24	22	28
10 nM	3	1	7	89	9	9	49	36
Paclitaxel								
3 nM	20	43	18	19	16	47	20	17
10 nM	23	18	16	43	48	19	23	11
30 nM	4	2	1	93	12	5	10	73

^a KB-3-1 cells were treated for 1–3 days with the indicated drugs at concentrations representing ~1-, 3-, or 10-fold the 3-day IC₅₀ for each drug. Cells were then fixed, stained with propidium iodide, and analyzed by flow cytometry. Assays were performed two to three times; data are from representative experiments.

lines tested, HTI-286 was more potent than paclitaxel. The mean IC₅₀ for paclitaxel in these cell lines was 128 ± 369 nM and had a median value of 14 nM.

It is well established that paclitaxel is an excellent substrate for the drug efflux pump known as P-glycoprotein (3). We attempted to correlate the response to paclitaxel with P-glycoprotein expression and then determine whether cells that express P-glycoprotein were resistant to HTI-286. In cells that had very little or no detectable expression of P-glycoprotein (“0” value in Table 2), the IC₅₀ value for HTI-286 and paclitaxel was 1.0 ± 0.4 and 5.3 ± 1.9 nM, respectively. In cells that had slight to moderate expression of P-glycoprotein (levels equal to or greater than “+” in Table 2), the IC₅₀ value for HTI-286 and paclitaxel was 3.5 ± 2 and 105 ± 165 nM, respectively. Therefore, the IC₅₀ for HTI-286 and paclitaxel was 3.5- and 20-fold greater, respectively, in cells with detectable to high P-glycoprotein expression than in nonexpressing cells. These data suggest that cells that overexpress P-glycoprotein in the absence of drug selection were inherently resistant to paclitaxel. Such cells retained nearly complete sensitivity to HTI-286, although there was a minor increase in resistance (*P* = 0.03, Student’s two-tailed *t* test). Consistent with these data, MX-1W, HCT-15, and DLD-1 cells were sensitized to paclitaxel

but not HTI-286 when they were coincubated with a P-glycoprotein-specific inhibitor (5 μM CL-329,753; Refs. 12 and 13; data not shown). The inhibitor was used at the concentration that maximally reversed resistance but had no significant inhibition on the growth of cells by itself.

Effect of HTI-286 on Growth of Cells that Have Acquired Overexpression of Drug Efflux Pumps. Cell lines selected for expression of drug efflux pumps by chronic drug exposure were also used to compare the activity of HTI-286 with other chemotherapeutics (Table 3). KB-8-5 cells were selected for low-level resistance to colchicine (14) and overexpress moderate levels of P-glycoprotein (approximately equivalent to levels observed in DLD-1 cells; data not shown). These cells were moderately resistant to paclitaxel (19-fold), docetaxel (18-fold), vinblastine (37-fold), vinorelbine (52-fold), colchicine (9.8-fold), dolastatin-10 (7.1-fold), and doxorubicin (15-fold) compared with parental cells. In contrast, KB-8-5 cells had only 2.4-fold resistance to HTI-286. Resistance to HTI-286 can be mediated by P-glycoprotein in extreme circumstances, because 81-fold resistance to HTI-286 was found in the KB-V1 cell line that expresses very high levels of MDR1 (14). This line had profound resistance to paclitaxel, vinorelbine, and vinblastine (~1400–1800-fold) and was also highly resistant to docetaxel (669-fold) and colchicine (382-fold). Resistance to paclitaxel and HTI-286 in the KB-V1 cell line was likely attributable to P-glycoprotein itself and not a coselected factor, because resistance to these drugs was completely reversed with an MDR1-specific inhibitor, CL-329,753 (12, 13). In particular, in this experimental series, the IC₅₀ of HTI-286 in KB-V1 cells was 56.6 ± 9.2 nM in the absence of CL-329,753 but 1.3 ± 0.6 nM in the presence of 5 μM CL-329,753. This closely matched the IC₅₀ in the parental KB-3-1 cells, which was 1.2 ± 0.3 or 0.9 ± 0.5 nM in the absence or presence of 5 μM CL-329,753, respectively. Therefore, resistance to HTI-286 in KB-V1 cells was reversed nearly to wild-type levels. In contrast, the IC₅₀ in KB-V1 cells for paclitaxel was >3000 nM and 15 ± 11.3 nM in the absence and presence of 5 μM CL-329,753, respectively.

HTI-286 was selected for further study from among numerous analogues of hemiasterlin. Selection was based on several criteria, including the ability to inhibit growth of cell lines expressing moderate to high levels of P-glycoprotein. Hemiasterlin showed minimal resistance in KB-8-5 cells (3.2-fold) but moderate resistance in the high P-glycoprotein-expressing KB-V1 cells (239-fold; Table 3). Compared with hemiasterlin and some other synthetic analogues,³ HTI-286 consistently showed a lower level of resistance in KB-8-5 and KB-V1 cells, as well as in other cell lines expressing P-glycoprotein.

In addition to analogues of hemiasterlin, we studied the effective-

Table 2 Inhibition of proliferation by HTI-286 and paclitaxel in a panel of tumor cell lines^a

Cell line	Tumor origin	P-glycoprotein expression ^b	IC ₅₀ (nM)	
			HTI-286	Paclitaxel
A549	NSCLC ^c	0	1.1 ± 0.5	8.8 ± 3.6
HCT-116	Colon	0	0.7 ± 0.2	5.4 ± 1.3
KB-3-1	Epidermoid	0	1.0 ± 0.5	3.9 ± 1.8
1A9	Ovarian	0	0.6 ± 0.1	5.1 ± 0.9
SK-Mel-2	Melanoma	0	1.7 ± 0.5	3.2 ± 1.8
MX-1W	Breast	+	1.8 ± 0.6	15.4 ± 2.9
SW620	Colon	+	3.6 ± 0.8	14.3 ± 4.0
NCI-H1299	NSCLC	+	6.8 ± 6.1	37.7 ± 12.6
S1	Colon	++	3.7 ± 2.0	92 ± 100
DLD-1	Colon	+++	1.1 ± 0.4	30.8 ± 12.7
HCT-15	Colon	++++	4.2 ± 2.5	438 ± 248
MCF-7	Breast	nd	7.3 ± 2.3	8.1 ± 1.9
Colo205	Colon	nd	1.5 ± 0.6	6.9 ± 2.3
KM20	Colon	nd	1.8 ± 0.6	8.9 ± 1.9
Moser	Colon	nd	5.3 ± 4.1	1594 ± 1989
CCRF-CEM	Leukemia	nd	0.2 ± 0.03	3.3 ± 1.3
A375	Melanoma	nd	1.1 ± 0.8	14.1 ± 7.3
Lox	Melanoma	nd	1.4 ± 0.6	17.3 ± 5.6
Average			2.5 ± 2.1	128 ± 369

^a Cells were grown in the presence of HTI-286 or paclitaxel for 3 days, and the growth was assessed by SRB staining or, for the leukemia line, CellTiter 96 assay. Data are mean IC₅₀ (nM ± SD) (*n* ≥ 2).

^b P-glycoprotein levels were estimated by immunoblot analysis and compare favorably with MDR1 mRNA levels as determined by quantitative PCR analysis. Scoring code: 0, not detected; + to +++++, low- to high-level expression; nd, not determined.

^c NSCLC, non-small cell lung cancer.

Table 3 Effect of HTI-286 and other agents on the growth of cells that overexpress the drug efflux pump P-glycoprotein^a

Compound	KB-3-1	KB-8-5	KB-8-5	KB-V1	KB-V1
	IC ₅₀ , nM	(P-glycoprotein +++) ^b IC ₅₀ , nM	RR ^c	(P-glycoprotein +++++) ^b IC ₅₀ , nM	RR
HTI-286	0.96 ± 0.5	2.3 ± 1.2	2.4	77.4 ± 44	81
Hemisterlin	0.319 ± 0.095	1.0 ± 0.5	3.2	76.1 ± 13.8	239
Paclitaxel	3.9 ± 1.8	63.3 ± 29	19	5484 ± 2780	1406
Docetaxel	0.55 ± 0.45	9.7 ± 6.6	18	368 ± 257	669
Vinblastine	0.79 ± 0.5	29.2 ± 22	37	1464 ± 1022	1848
Vinorelbine	2.4 ± 1.7	125.1 ± 50.3	52	>3000	>1250
Colchicine	6.4 ± 1.3	62.7 ± 9.2	9.8	2442 ± 2176	382
Dolastatin-10	0.037 ± 0.02	0.263 ± 0.05	7.1	21.2 ± 1.4	573
Doxorubicin	43.3 ± 32	658 ± 483	15	11489 ± 9672	265

^a Data are mean IC₅₀ (nM) ± SD for the indicated agents based on two or more independent experiments.

^b P-glycoprotein +++, moderate level expression; P-glycoprotein +++++, very high-level expression as determined by immunoblot analysis.

^c RR, relative resistance = ratio of IC₅₀ of the resistant cell line to IC₅₀ of the corresponding sensitive cell line. Lower relative resistance values indicate greater sensitivity of cells to the drug.

ness of another peptidic antimicrotubule agent. Dolastatin-10 is a highly potent microtubule-depolymerizing agent, which, like hemisterlin, interacts at the peptide-binding site of tubulin (9). However, two differences between dolastatin-10 and HTI-286 were found: (a) dolastatin-10 was ~25-fold more potent than HTI-286; and (b) resistance to dolastatin was 3- and 7-fold higher compared with HTI-286 in KB-8-5 and KB-V1 cells, respectively. These data are consistent with a report that dolastatin-10 is a highly potent molecule that interacts with P-glycoprotein (17).

The ABC-transporter MRP1 and ABC half-transporter MXR are members of two other classes of drug transporters known to mediate resistance to chemotherapeutics, although they do not effectively mediate resistance to most antimicrotubule agents (18). It was unknown whether the hemisterlin family of peptides interacted with these drug pumps. No resistance to HTI-286 was detected in cells selected for overexpression of MRP1 (IC₅₀ in HL60 and HL-60/ADR cells was 0.21 ± 0.05 and 0.25 ± 0.45 nM, respectively) or MXR (IC₅₀ in S1 and S1-M1-3.2 cells was 3.7 ± 2 and 4.8 ± 2.1 nM, respectively), although these cell lines were highly resistant to both doxorubicin (HL-60/ADR cells: >124-fold; S1-M1-3.2 cells: 52-fold) and mitoxantrone (HL-60/ADR cells: 32-fold; S1-M1-3.2 cells: 535-fold), compared with parental cells.

In Vivo Efficacy in Xenograft Tumor Models of HTI-286 Given i.v. Initial *in vivo* studies were performed with tumors known to be sensitive to paclitaxel or vincristine. HTI-286, paclitaxel, or vincristine were given on days 1, 5, and 9 to athymic mice bearing small established tumors (~100 mg) derived from Lox melanoma (Fig. 4A) or KB-3-1 epidermoid carcinoma (Fig. 4B). When given the maximum tolerated dose of HTI-286 (1.6 mg/kg i.v.), paclitaxel (60 mg/kg i.v.), or vincristine (1 mg/kg i.p.), growth of Lox tumors was inhibited by 96–98% on day 12 with each agent, compared with vehicle-treated controls. Similar results were obtained with the KB-3-1 tumor model (Fig. 4B). The minimum effective dose of HTI-286 in the Lox melanoma model was ~0.2 mg/kg (data not shown). Any weight loss attributable to HTI-286, paclitaxel, or vincristine on a 1-, 5-, and 9-day schedule was greatest by the second dose (maximally 12% versus controls) but recovered within 1–2 days after the completion of treatments. HTI-286 also inhibited the growth of other paclitaxel-sensitive tumors in xenograft assays, e.g., 1 mg/kg HTI-286 given on days 1, 5, and 9 inhibited tumor growth on day 14 by 73 ± 9% (*n* = 2 independent experiments) in PC3-MM2 prostate carcinomas, 96 ± 5% (*n* = 3) in SW620 colon carcinomas, 83% in U87 gliomas, 94% in MCF-7 breast carcinomas, and 65% in LoVo colon carcinomas.

HTI-286 also inhibited the growth of large, established tumors derived from Lox, KB-3-1, LoVo, and PC3MM2. For example, mice bearing Lox tumors (2.5 grams) were given HTI-286 (1.6 mg/kg)

weekly for four cycles (Fig. 4A). HTI-286 inhibited further growth of these larger tumors within 4 days after the first dose of the agent and, on subsequent dosing, led to a 93% reduction in tumor size. Paclitaxel also induced regression of large tumors in this taxane-sensitive model (data not shown). Excellent efficacy was also observed against several xenografts with intermittent dosing, including multiple cycles of 1, 2, or 3 weeks (data not shown). Collectively, it is concluded that HTI-286 effectively inhibits the growth of taxane-sensitive human tumor models *in vivo*.

The activity of HTI-286 was also studied in tumors derived from cell lines that either have acquired or inherent resistance to paclitaxel. KB-8-5 tumors, which had acquired ~20-fold resistance to paclitaxel in tissue culture (Table 2) as a result of P-glycoprotein overexpression, were not responsive to 60 mg/kg paclitaxel or 1 mg/kg vincristine (Fig. 4C). The response of tumors derived from KB-8-5 cells was in sharp contrast to KB-3-1 parental cells where 60 mg/kg paclitaxel caused >95% inhibition of tumor growth (Fig. 4B). HTI-286 given at 1.6 mg/kg on days 1, 5, and 9 significantly inhibited the growth of KB-8-5 tumors by 84% on day 14, compared with the vehicle control group, although growth resumed by day 21.

The ability of HTI-286 to inhibit the growth of tumors that had inherent resistance to paclitaxel or vincristine was tested in MX-1W human breast carcinoma (Fig. 4D), DLD-1 human colon carcinoma (Fig. 4E), and HCT-15 human colon carcinoma (Fig. 4F). These tumors were chosen because they were relatively resistant to paclitaxel *in vitro* and overexpressed P-glycoprotein (Table 2). In tissue culture, compared with the KB-3-1 cell line, which was among the most sensitive cell line to paclitaxel, the MX-1W, DLD-1, and HCT-15 cell lines were ~4-, 8-, and 112-fold resistant to paclitaxel, respectively (Table 2). Tumors derived from these three cell lines were resistant to paclitaxel or vincristine therapy *in vivo* when these agents were given at their maximum tolerated dose. However, 97% tumor growth inhibition was achieved in the MX-1W model with 1.6 mg/kg HTI-286. The minimum effective dose for inhibition of MX-1W tumors was found to be ~0.7 mg/kg. Although tumor growth resumed on day 28 after HTI-286 therapy was initiated, complete inhibition of tumor growth was maintained for ≥8 weeks when HTI-286 continued to be administered weekly (data not shown). Furthermore, the tumors derived from DLD-1 and HCT-15 were also significantly inhibited (maximum of 80 and 66%, respectively) by tolerated doses of HTI-286 by day 13–14, ~5 days after the last dose was administered. However, growth resumed after this period (Figs. 4, E and F). In general, higher doses of HTI-286 were needed to achieve efficacy in paclitaxel-resistant tumors, compared with paclitaxel-sensitive tumors, although these doses were still well tolerated.

In Vivo Efficacy of HTI-286 by p.o. Administration. MDR1 is expressed by gastrointestinal epithelial cells, and this significantly

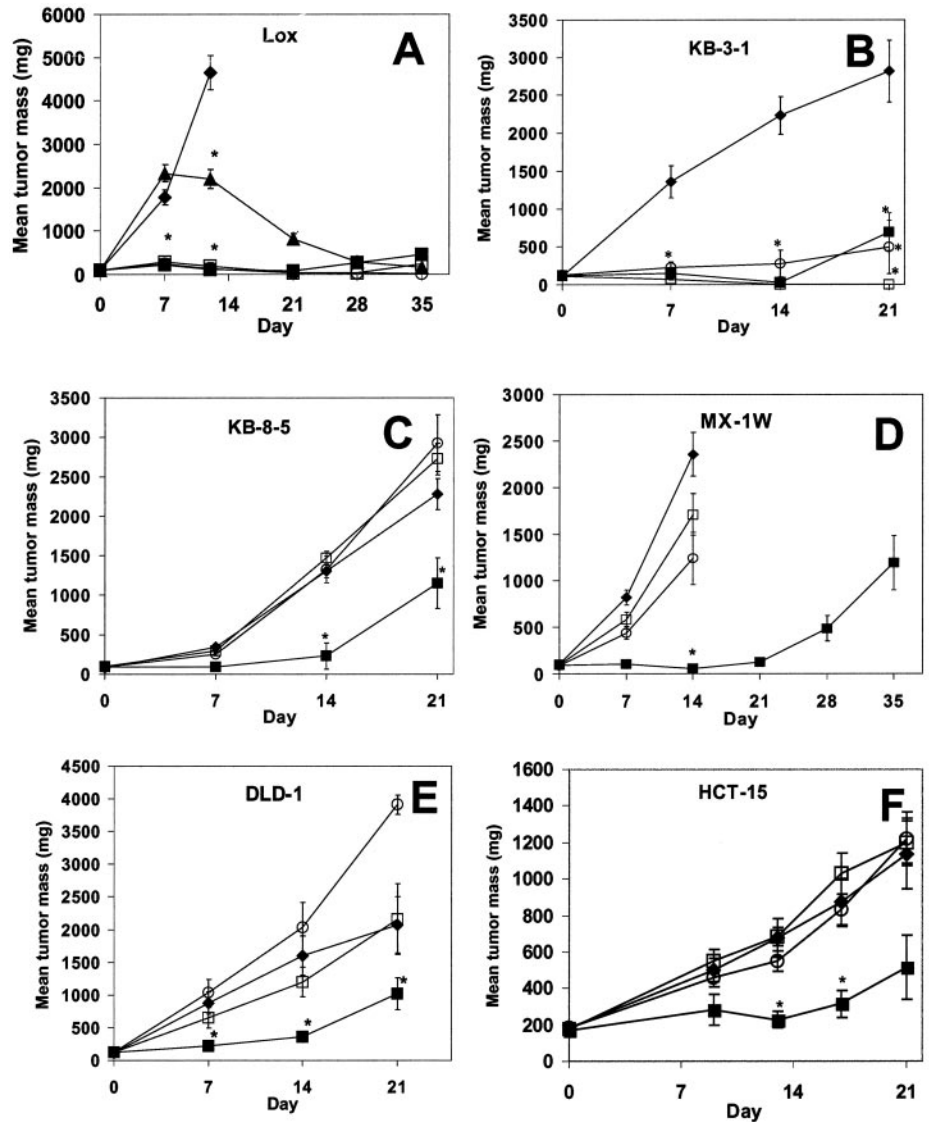


Fig. 4. Effect of HTI-286 on the growth of human tumor xenografts. Groups of 5 or 10 female athymic mice were injected s.c. with the indicated tumor cell lines. A, Lox melanoma; B, KB-3-1 epidermoid; C, KB-8-5 epidermoid; D, MX-1W breast; E, DLD-1 colon; F, HCT-15 colon. Animals bearing established tumors were treated with vehicle or drugs at the doses and times indicated. Relative tumor growth was determined weekly. Mice were treated on days 1, 5, and 9 with saline vehicle control i.v. (◆), 1.6 mg/kg HTI-286 i.v. (■), 60 mg/kg paclitaxel i.v. (□), or 1 mg/kg vincristine i.p. (○). For the Lox experiment (A), one set of tumors was allowed to grow to a large size (2.5 grams) before treatment with 1.6 mg/kg HTI-286 i.v. on days 7, 14, 21, and 28 (▲). Data are the mean \pm SD of tumor size (milligram) at each time point. *, $P < 0.01$.

limits p.o. absorption of drugs that interact with MDR1, including paclitaxel (3). Because HTI-286 overcomes MDR1-mediated resistance in most cells, this suggested that the drug might be efficacious by p.o. administration. Consistent with this, 3 mg/kg HTI-286 administered by p.o. gavage on days 1, 5, and 9 inhibited growth on day 14 by $97.3 \pm 2.5\%$ ($n = 3$ independent experiments) in Lox melanoma xenografts. Growth inhibition (82%) was also observed on day 22 in the KB-3-1 epidermoid xenograft model when 3 mg/kg HTI-286 was given p.o. on days 1, 8, and 16.

DISCUSSION

HTI-286 is a synthetic analogue of a small family of naturally occurring peptides known as hemiasterlins, which were originally derived from extracts of marine sponges (5–7). Before this study, hemiasterlin was known to inhibit cell growth (8) and interact with the peptide-binding site of tubulin (9). Further exploration of the utility of hemiasterlin has been severely hampered because this natural product was available in extremely limited quantities. This study demonstrates that a synthetic analogue of hemiasterlin, HTI-286, is a potent inhibitor of cell growth that depolymerizes microtubules. It is distinct from paclitaxel and vincristine because HTI-286 is a poor substrate for

P-glycoprotein and inhibits tumor growth in human tumors grown in mice that are sensitive or resistant to vincristine or paclitaxel. Beyond this, HTI-286 is one in a large series of hemiasterlin analogues (11) that, it is anticipated, will help define structure–activity relationships with tubulin, as well as probe the peptide-binding site within tubulin. Recently, the interaction of radioactive photoaffinity analogues of HTI-286 with tubulin purified from bovine brain has been studied (19). On the basis of initial analyses, these probes exclusively photolabel α -tubulin. Consistent with this, mutations in α -tubulin have been found in cells selected for resistance to HTI-286 (20). Interaction with α -tubulin is uncharacteristic of most antimicrotubule agents examined to date.

The initial clinical assessment of novel antimicrotubule drugs, such as HTI-286, will depend on their effectiveness in those patients who have inherent or acquired resistance to existing therapies. Although taxanes and *Vinca* alkaloids have been widely used in the clinic, it is well documented that certain tumors (e.g., colon) are refractory to these agents, whereas other tumors acquire resistance during the course of repeated therapy (1). The basis of resistance to antimicrotubule agents is complex (21). In experimental systems, taxane resistance has been attributed to high expression of MDR1, tubulin muta-

tions, differential expression of tubulin isoforms, and most recently, alterations of proteins that regulate cell death via apoptosis, such as survivin (22).

From the data in the current report, we conclude that HTI-286 overcomes P-glycoprotein-mediated resistance to paclitaxel or vincristine both in xenograft models and most cell lines that overexpress the protein. The clinical significance of this observation awaits further study. The correlation between MDR1 expression in patients and resistance to substrates of P-glycoprotein (e.g., *Vinca* alkaloids, paclitaxel, docetaxel, and doxorubicin) is poorly defined in most cancer types. A positive correlation between MDR1 expression and poor survival or response has been established in leukemias and myelomas (3), but the correlation in solid tumors is controversial. Elevated levels of MDR1 have been found in breast, renal, and colon cancers. However, since there is substantial heterogeneity in expression within tumor types and between individual reports, the association with lack of response to chemotherapy is unclear (3). On the basis of meta-analysis in breast cancers, Trock *et al.* (23) concluded that patients whose tumors express MDR1 were three times more likely to fail to respond to chemotherapy than patients who were MDR1 negative.

The data shown here demonstrate that the KB-8-5, MX-1W, and DLD-1 cell lines have 4–20-fold resistance to paclitaxel in tissue culture and very low to moderate P-glycoprotein levels, compared with parental cells or other paclitaxel-sensitive cells that have no P-glycoprotein detected by immunoblot analysis. Remarkably, tumors derived from all these cell lines are highly resistant to paclitaxel or vincristine in animal xenograft models. These data suggest that even small changes in MDR1, perhaps within the limits of detection in clinical assessment, may mediate paclitaxel resistance in patients. Consistent with this hypothesis, double knockout of *mdr1a* and *mdr1b* in murine fibroblasts, which have low P-glycoprotein expression in wild-type cells, caused a 15–25-fold increased sensitivity to paclitaxel (3). Because HTI-286 can overcome paclitaxel resistance in tumors derived from such cell lines, HTI-286 may have utility in taxane-refractory tumors or in patients who have failed paclitaxel therapy caused by MDR1 overexpression.

It should be noted that 80-fold resistance to HTI-286 was detected in KB-V1 cells that have been selected for very high levels of P-glycoprotein and are ~570–1800-fold resistant to dolastatin-10, docetaxel, paclitaxel, vinorelbine, and vinblastine. However, the expression of P-glycoprotein in these cells is higher than what is typically found in clinical samples from a variety of tumors (24). Therefore, we predict that identifying molecules having the least interaction with P-glycoprotein, as assessed by lack of resistance in KB-V1 cells, will have the best chance of avoiding detoxification and excretion by P-glycoprotein-mediated mechanisms in the liver and small intestine, as well as penetrating tumors that have high expression of P-glycoprotein in patients. A similar argument has been made with the epothilones, which also have low interaction with P-glycoprotein (25).

Hemiasterlin competes with the binding of dolastatin-10 to purified tubulin (9). Hence, these molecules may be expected to act in cells by similar mechanisms. However, this may not be true because dolastatin-10 is a better substrate for P-glycoprotein and was ~20-fold more potent ($IC_{50} = 50$ pmol) than HTI-286 in cytotoxicity assays reported here and elsewhere (17, 26). To date, the clinical experience with dolastatin-10 (4) and a dolastatin-15 analogue, cemadotin (27, 28), has not been favorable; granulocytopenia or cardiovascular toxicities have been observed with little evidence of antitumor efficacy. Clinical trials with HTI-286 are required to determine the utility of this molecule for the treatment of cancer.

In tissue culture, resistance to paclitaxel or another polymerizing agent, epothilone B, has been associated with point mutations in β -tubulin (29, 30). These studies, along with crystallographic methods

(31), have helped define the binding sites of taxanes within tubulin. We have examined if ovarian carcinoma cells selected for paclitaxel or epothilone resistance (29) or an A549 lung carcinoma line selected for epothilone resistance (30) were cross-resistant to HTI-286. These cell lines have five distinct point mutations in β -tubulin and do not express MDR1. No resistance to HTI-286 was found.⁵ These data are consistent with the prediction that HTI-286 does not bind to the taxane/epothilone binding site within tubulin. Because mutations in tubulin have not been reliably detected in cancer patients (32), the clinical significance of this observation remains undefined.

In conclusion, the data suggest that the synthetic analogue of hemiasterlin, HTI-286, is an antimicrotubule agent that is highly effective at inhibiting the growth of tumors, including those where resistance to paclitaxel and vincristine is associated with P-glycoprotein overexpression. Because HTI-286 is a synthetic molecule, numerous analogues can be made that may further the understanding of the interaction of peptide-like molecules with tubulin. On the basis of the efficacy and safety assessment of HTI-286 in animals, HTI-286 is currently undergoing Phase I clinical evaluation in cancer patients.

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