

Deepak Sampath, Carolyn Discafani, Carl Beyer, Maria Nunes, Malathi Hari, Hao Liu, Tami Annable, Sylvia Musto, Patricia Gallagher, Carol Rios, Frank Loganzo and Lee M. Greenberger.

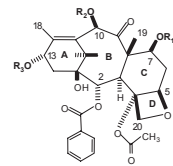
Wyeth Research, Department of Oncology, Pearl River, NY.



Abstract

The anti-microtubule agents, paclitaxel (PTX) and docetaxel (DTX), are two approved taxanes that have been used to treat a wide variety of solid tumors. Since resistance to these taxanes is frequently observed, new anti-microtubule agents, in particular stabilizing agents, have been sought. We have previously identified a novel taxane, known as MAC-321, that that overcomes PTX- and DTX-resistance *in vitro* and *in vivo*. We now report a structurally distinct taxane compared with MAC-321 or marketed taxanes, designated as MST-997 [5β,20-epoxy 1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate - ester with (2R,3S)-N-isopropoxycarbonyl-3-(2-thienyl) isoserine], that has similar properties as MAC-321 (1). MST-997 was a potent microtubule polymerizing agent (EC₅₀ = 0.9 μM) that induced the bundling of microtubules and induced G₂/M arrest in cells. MST-997 was a potent inhibitor of PTX and DTX-sensitive tumor cell lines that did not have detectable P-glycoprotein (IC₅₀ = 2.8 ± 1.5 nM). In addition, minimal (1- to 3-fold) resistance to MST-997 was found in cell lines in which acquired (KB-8-5, KB-D-15 and KB-P-15) and inherited (DLD-1 and HCT-15) resistance to PTX and DTX associated with over expression of P-glycoprotein (MDR1). Moreover, in a cell line that had very high level of MDR1 over expression, much less cross-resistance to MST-997 (44-fold) was detected whereas > 425 or 821- fold resistant to DTX and PTX, respectively, was observed. Less or no resistance to MST-997 was also observed in two MDR1 negative cell lines that were resistant to PTX and harbored point mutations in β-tubulin. Most notable, MST-997 displayed superior *in vivo* efficacy since: 1) a single 70 mg/kg IV dose eliminated the detection of tumors that were partially responsive to a single dose of PTX, 2) MST-997 either partially or completely inhibited tumor growth in 3 models that over expressed P-glycoprotein and were resistant to PTX and 3) unlike PTX or DTX, MST-997 was highly effective when given orally. Taken together, MST-997 represents a novel and potent microtubule-stabilizing agent that has greater pharmacological efficacy *in vitro* and *in vivo* than the currently approved taxanes. Our findings suggest that MST-997, which has entered phase I evaluation, may have broad therapeutic value.

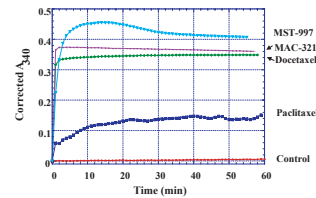
Figure 1 - Chemical Structures of MST-997 compared to MAC-321, paclitaxel and docetaxel.



Compound	R ₁ =	R ₂ =	R ₃ =
Paclitaxel	H		
Docetaxel	H	H	
MAC-321		H	
MST-997	H		

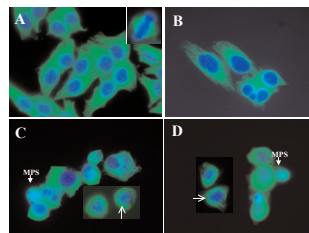
Modifications of the baccatin III core structure (denoted as A, B, C and D) are indicated as R₁, R₂ and R₃.

Figure 2 - MST-997 is a Potent Inducer of Tubulin Polymerization



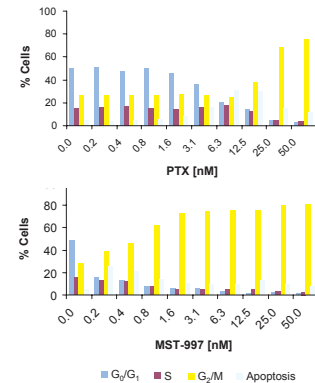
Tubulin polymerization assays were conducted at 24°C using 1.5 mg/ml of purified bovine brain MAP free-tubulin in the presence of vehicle control or 24.3 μM of MST-997, MAC-321, docetaxel or paclitaxel. Turbidity was measured by absorbency (340 nm) for up to 60 minutes.

Figure 3 - MST-997 Induces Bundling of Microtubules in Cells



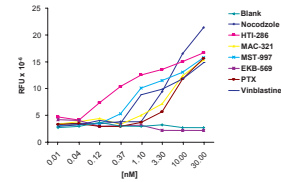
Microtubule structure in KB-3-1 cells treated with MST-997 and assessed by immunofluorescence microscopy. Cells were untreated (A) or treated for 16 hrs. with MST-997 at 1 nM (B), 10 nM (D), or 10 nM MAC-321 (C). After MeOH fixations cells were incubated with anti α-tubulin followed by FITC-conjugated F(ab')₂ fragment of goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). DNA was visualized by staining with DAPI. Cells were examined with an Olympus BX61 microscope (Olympus America, Inc., Melville, NY) and 60x objective using epi-illumination. Open arrows denote bundling of microtubules in cells treated with MST-997 or MAC-321. Multipolar spindles (MPS) are also observed after treatment with 10 nM MAC-321 (C) or MST-997 (D) (closed arrows).

Figure 4 - MST-997 Induces G₂/M Cell Cycle Arrest



Cell Cycle Analysis of MST-997 vs PTX. KB epidermoid cells were plated at 10,000 cells/well and treated in triplicate at the indicated doses for 16 hrs. Cells were detached by trypsinization and fixed with 70% EtOH for 12 h at -20°C. Fixed cells were resuspended in 25 μg/ml propidium iodide (PI) and analyzed using a PCA 96 Cell Sorter (Guava Technologies). The resulting DNA histograms were collected from at least 2000 PI stained cells at an emission wavelength of 690 nm. The number of cells in each phase of the cell cycle and in apoptosis phase were determined.

Figure 5 - MST-997 Induces Phospho-Nucleolin; A Biochemical Marker of G₂/M Arrest



Induction of Phospho-Nucleolin by MST-997 as a Marker for G₂/M Arrest. KB epidermoid cells were plated at 2000 cells/well and treated with serial dilutions of the indicated compounds for 16 hrs. After fixation and blocking with 3% milk, cells were incubated with a combination of primary antibody to phospho-nucleolin (TG-3; Molecular Genetics Corp., Vernon Hills, IL) and anti-mouse IgM secondary antibody at 1:8000 and 1:20,000 dilution, respectively for 1 h at room temperature. Absorbance (A₄₅₀) was read on a Victor V multi-label plate reader and data collected using Wallac 1420 Workstation software. Other antimicrotubule agents tested include nocodazole, HTI-286 (2), MAC-321 (1), PTX and vinblastine. EKB-569 is an EGFR kinase inhibitor known to induce G₁/G₁ arrest (3) and was used as a negative control.

Table 1 - MST-997 is a Potent Inhibitor of Paclitaxel and Docetaxel Sensitive Tumor Cell Lines

Cell Line	Tumor Origin	IC ₅₀ [nM] ^a			
		MST-997	MAC-321	DTX	PTX
KB-3-1	epidermoid	0.8 ± 0.2	1.3 ± 0.5	1.1 ± 0.6	3.9 ± 1.3
HCT-116	colon	1.5 ± 0.3	3.1 ± 0.7	5.4 ± 0.8	8.2 ± 1.4
NCI H838	NSCLC ^b	3.2 ± 0.6	3.3 ± 0.3	2.1 ± 0.9	6.3 ± 0.7
A549	NSCLC	1.8 ± 0.9	1.1 ± 0.6	1.9 ± 0.7	7.5 ± 1.3

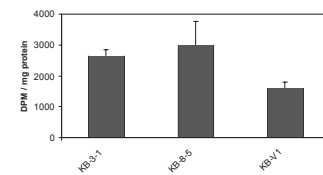
^aCell toxicity was measured using the Cell-Glo™ method as described by the manufacturer (Promega Inc., Madison, WI) to assess the levels of ATP after a 72 hour incubation with MAC-997, MAC-321, PTX (paclitaxel) or DTX (docetaxel). IC₅₀ ± standard deviation
^bNSCLC: Non Small Cell Lung Cancer

Table 2 - MST-997 Overcomes PTX and DTX Resistance Associated with β-tubulin Mutations or P-glycoprotein Over Expression

Cell Line	Tumor Origin	Resistance Phenotype	P-gp Levels ^a	Relative Resistance ^b			
				MST-997	MAC-321	DTX	PTX
KB-8-5	epidermoid	P-gp Overexpression	++	2	1	18	8
KB-P-15	epidermoid	P-gp Overexpression	++	3	3	16	30
KB-D-15	epidermoid	P-gp Overexpression	++++	8	6	62	145
KB-V1	epidermoid	P-gp Overexpression	++++	45	80	425	821
DLD-1	colon	P-gp Overexpression	+++	1	1	3	4
HCT-15	colon	P-gp Overexpression	++++	2	1	10	53
KB-PTX099	epidermoid	β-tubulin mutation (26 ^{Met}) ^c	0	1	2	8	19
A549 (EpoB40)	NSCLC ^c	β-tubulin mutation (292 ^{Met}) ^c	0	7	8	15	17

^aAverage relative resistance is defined as the ratio of the IC₅₀ of the resistant cell model to that of the respective sensitive/parental cell counterpart (Table 1).
^bP-glycoprotein expression was determined by western blotting and quantitative real time RT-PCR using MDR1 specific antibodies and probes, respectively. Scoring code based on western blotting: 0, not detected; + to +++, low to high
^cNSCLC: Non Small Cell Lung Cancer

Figure 6 - Lower Accumulation of [¹⁴C]-MST-997 Only in Cells that Express High Levels of P-glycoprotein



Accumulation of MST-997 in MDR - and + KB epidermoid cells. The intracellular accumulation of MST-997 in KB-3-1, KB-8-5 (P-gp++) and KB-V1 (P-gp++) cells was performed by incubating cells in the presence of 0.025 μCi of [¹⁴C]-MST-997 (59 μCi/mmol) at a final concentration of 1.5 μM for 2 hrs at 37°C. Radioactivity in the cell lysates were determined by liquid scintillation counting and was normalized for protein content. Data are reported as mean DPM/mg of total protein from triplicate wells. We have previously demonstrated that 50 and 90% less accumulation of PTX is observed in KB-8-5 and KB-V1 cells, respectively, when compared to the parental KB-3-1 cells (1).

Table 3 - MST-997 has Superior Efficacy at a Single Dose in PTX-Sensitive and Resistant Tumor Xenografts

Paclitaxel-sensitive models (P-glycoprotein-negative)	Tumor Origin	MST-997		Paclitaxel	
		260 mg/kg Single IV dose	60 mg/kg qd4 x 3 or 36-100 mg/kg Single IV dose	60 mg/kg qd4 x 3 or 30 mg/kg qd4 x 5 IV	60 mg/kg qd4 x 3 or 30 mg/kg qd4 x 5 IV
MV1	Breast	+++	+	+++	+++
Pano-1	Pancreatic	+++	+	+++	+++
LOX	Melanoma	+++	ND	+++	+++
KB	Epidermoid	+++	+++	+++	+++
HE-29	Colon	+++	++	+++	+++

Paclitaxel-resistant models (P-glycoprotein-positive)	Tumor Origin	MST-997		Paclitaxel	
		70-100 mg/kg Single IV dose	36-100 mg/kg Single IV dose	60 mg/kg qd4 x 3 or 30 mg/kg qd4 x 5 IV	60 mg/kg qd4 x 3 or 30 mg/kg qd4 x 5 IV
KB-8-5	Epidermoid	+++	0	0	0
DLD-1	Colon	+++	+	0	0
HCT-15	Colon	++	+	0	0

^aScoring: +++ = "cures" (no tumor detected 30 to 60 days after the onset of dosing), ++ = good response but excellent response with tumor growth inhibition greater than approximately 80%; + = good response with tumor inhibition less than 80% but more than 50%; = = statistically significant but partial response; 0 = no different than control group.
^bMST-997 was prepared in an Intralipid™ containing vehicle; a non-cremophor and non-tween based formulation.

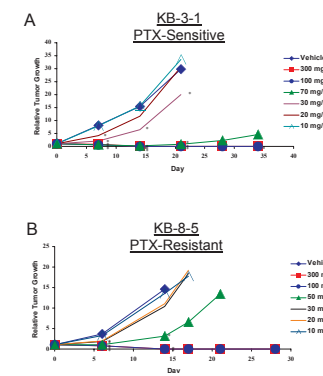
Table 4 - MST-997 has a Wide Therapeutic Window in PTX-Sensitive KB Epidermoid Tumor Xenografts

Drug	N ^a	Dose (mg/kg)	Schedule	% of MTD	RTG			
					Day 7	Day 14	Day 21 ^b	Day 28 ^c
Control	6				6 ± 1.1	15 ± 3	20 ± 2	
Paclitaxel ^d	2	60	q4d x 3	ND	0.1 ± 0.01	0.4 ± 0.2	1 ± 1	
Paclitaxel ^e	1	60	qd x 1	100	0.3	0.2	0.5	2
HTI-286 ^f	3	1.3-2	q4d x 3	100	0.4 ± 0.1	0.4 ± 2	5 ± 6	-
MAC-321 ^g	3	70	qd x 1	100	0 ± 0	0 ± 0	0 ± 0	1 ± 1
MST-997 ^h	2	70	qd x 1	70	0.3 ± 0.3	0.1 ± 0.1	0 ± 0	0 ± 0
MST-997 ^h	2	10	qd x 1	10	1 ± 0.2	8 ± 0.6	13.9	20.1

^aRTG = Relative tumor growth is the size of the tumor on 1 day indicated divided by the size of the tumor at the onset of therapy. Tumors had an established size of approximately 100 mg prior to onset of dosing. Values are mean ± SD. Zero indicates no tumor detected. Values above and below 1.0 indicate tumor growth and regression, respectively.

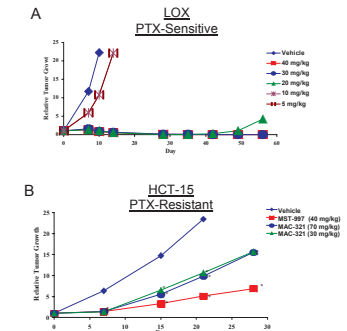
^bN = number of independent experiments.
^cIf the average tumor size exceeded 3 grams, the value in this grid could not be determined. Some experiments were terminated on day 21 or 28. Therefore the grid value, indicated by "-" was not determined.
^dHTI-286, Paclitaxel and MAC-321 was prepared in 0.9% saline, Cremophor-containing formulation Liposyn™-containing vehicle, respectively and all were given IV.
^eMST-997 was prepared in an Intralipid™-containing vehicle.

Figure 7 - MST-997 is Orally Efficacious in PTX-Sensitive And Resistant Tumors *In Vivo*



The effects of single dose MST-997 administered PO in PTX-sensitive (A) and resistant (B) tumor xenografts. Nude mice (n=5/group) bearing staged KB-3-1 (A) or KB-8-5 (B) tumors were treated with a single PO dose of MST-997 and relative tumor growth was determined. Data are presented as a mean fold increase in tumor volume in each group. *p < 0.05 by Student's two tailed t-test. The response of KB-3-1 and KB-8-5 tumors to PTX has been previously reported (1). Note that MST-997 is highly effective at 70 mg/kg when given either PO or IV (see Table IV).

Figure 8 - Multiple Low IV Dosing of MST-997 are Well Tolerated And Efficacious in Highly Resistant Xenografts *In Vivo*



The effects of multiple IV doses of MST-997 on PTX-sensitive (A) and resistant (B) tumor growth *in vivo*. A) Female nu/nu mice (n=5/group) bearing staged LOX melanoma tumors were treated with MST-997 at the doses indicated on days 1, 5 and 9 post staging and relative tumor growth was determined. B) Groups of 5 or 10 female nu/nu mice bearing HCT-15 colon staged tumors were treated with either vehicle control or 40 mg/kg MST-997 on days 1, 5 and 9 or a single IV dose of MAC-321 at the doses indicated. Relative tumor growth was determined every 7 days for a period of 21 days. Data are presented as a mean fold increase in tumor volume in each group. No significant weight loss was observed at any of the doses tested. * p ≤ 0.01 and by Student's two tailed t-test.

MST-997 Preclinical Pharmacology Summary

- Microtubule-polymerizing drug with similar potency vs. MAC-321 and Taxotere[®].
- Overcomes Taxol[®] & Taxotere[®] resistance *in vitro* and *in vivo*.
- Low resistance in Taxol[®] and epothilone B-selected cells containing β-tubulin mutations.
- Exceptional *in vivo* activity achievable; tumors undetectable after single IV dose.
- High oral efficacy in Taxol[®]-sensitive and resistant models.
- Multiple low doses are well tolerated IV.
- Efficacious in non-cremophor vehicle (IV)

Conclusion: MST-997 is a potent third generation taxane that has a superior preclinical profile compared with paclitaxel. Phase I trials are under way (see www.clinicaltrials.gov/ct/show/NCT0088647patients)

References

- Sampath, D. *et al.* MAC-321, a novel taxane with greater efficacy than paclitaxel and docetaxel *in vitro* and *in vivo*. Mol Cancer Ther 2(9): 873-884, 2003.
- Loganzo, F. *et al.* HTI-286, a synthetic analog of the tripeptide hemisterin, is a potent anti-microtubule agent that circumvents P-glycoprotein mediated resistance *in vitro* and *in vivo*. Cancer Res 63: 1838-1845, 2003.
- Wissner A. *et al.* Synthesis and structure-activity relationships of 6,7-disubstituted 4-aminoquinoline-3-carbonitriles. The design of an orally active, irreversible inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor-2 (HER-2). J Med Chem 46(1):49-63, 2003.