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ORIGINAL ARTICLE

The microtubule-active antitumor compound TTI-237 has both paclitaxel-like and vincristine-like properties

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Abstract
Purpose To compare TTI-237 (5-chloro-6-[2,6-difluoro-4-[3-(methylamino)propoxy]phenyl]-*N*-[(1*S*)-2,2,2-trifluoro-1-methylethyl]-[1, 2, 4]triazolo[1,5-*a*]pyrimidin-7-amine butanedioate) with paclitaxel and vincristine in order to better understand the properties of this new anti-microtubule agent.
Methods Tubulin polymerization and depolymerization were followed by turbidimetric assays. Effects of compounds on the binding of [^{3}H]guanosine triphosphate ([^{3}H]GTP) to tubulin were studied by competition binding assays. Effects of compounds on the phosphorylation of a panel of intracellular proteins were determined by flow cytometry using phosphoprotein-specific antibodies.
Results At low molar ratios of TTI-237:tubulin heterodimer (about 1:30), TTI-237 enhanced depolymerization kinetics in response to low temperature, but stabilized the aggregates at higher ratios (about 1:4). Similarly, the aggregates induced in microtubule protein by TTI-237 were depolymerized by excess Ca^{++} at low TTI-237:tubulin-heterodimer molar ratios, but were stable at higher ratios. TTI-237 inhibited the exchange of [^{3}H]GTP at the exchangeable nucleotide site of the tubulin heterodimer, and was similar to vincristine in its effects on the phosphorylation of eight intracellular proteins in HeLa cells.
Conclusions TTI-237 has properties that distinguish it from typical vinca-site and taxoid-site ligands, and therefore it may exemplify a new class of microtubule-active compounds.

Keywords TTI-237 · Cevipabulin · Tubulin · Microtubule · Tubulin polymerizer · Antitumor agent

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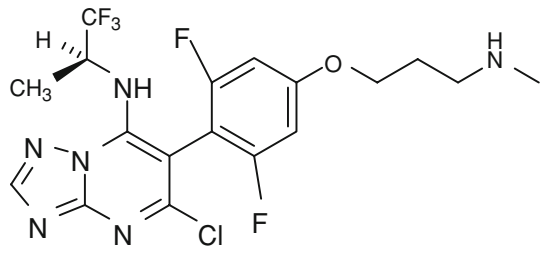
Introduction

The microtubule-active compound TTI-237 (5-chloro-6-[2,6-difluoro-4-[3-(methylamino)propoxy]phenyl]-*N*-[(1*S*)-2,2,2-trifluoro-1-methylethyl]-[1, 2, 4]triazolo[1,5-*a*]pyrimidin-7-amine butanedioate; USAN name cevipabulin; Fig. 1) [1] was recently described as a potent (IC_{50} of 18–73 nM on various tumor cell lines), fully synthetic, water-soluble compound of simple structure which is a poor substrate of the multi-drug resistance transporter P-glycoprotein and which shows in vivo anti-xenograft activity when administered to tumor-bearing mice either intravenously or orally [2]. However, the compound had some puzzling properties: it displaced [^{3}H]vinblastine (but not [^{3}H]colchicine or [^{3}H]paclitaxel) from tubulin, but it did not depolymerize microtubules as other vinca-domain ligands do. Rather, TTI-237 induced aggregation of both microtubule protein and purified tubulin, both in the presence and absence of added guanosine 5′-triphosphate (GTP), a property reminiscent of the taxanes paclitaxel and docetaxel.

There are three well-recognized drug binding sites on the tubulin heterodimer: the vinca domain, the taxoid site, and the colchicine site [3]. TTI-237 bears no similarities to colchicine-site ligands in its biochemical and cellular effects, but it has some properties that resemble vinca-domain ligands and some that resemble taxoid-site ligands. Since



TTI-237

Fig. 1 The structure of TTI-237

both classes of ligands (vincristine, vinblastine, vinorelbine; paclitaxel, docetaxel, ixabepilone) are used clinically in cancer treatment, it was of great interest to further characterize TTI-237. In this paper, some properties of TTI-237 are studied and compared with those of vincristine and paclitaxel in an effort to determine where this new compound falls on the spectrum of known tubulin ligands. Our results indicate that TTI-237 is both similar to and different from these reference compounds in certain ways, and therefore that it may be the prototype of a new class of microtubule-active compounds.

Materials and methods

Materials

TTI-237, as the free base or hydrochloride salt, was synthesized as described [1]. The compound was used as a stock solution in DMSO and was stored at −20°C. Paclitaxel, vincristine, and colchicine were obtained from Sigma (St Louis, Missouri, USA), and docetaxel was obtained from LKT Laboratories, Inc. (St Paul, Minnesota, USA); all were used as stock solutions in DMSO, with storage at −20°C. Microtubule-associated protein (MAP)-rich tubulin, also called microtubule protein, containing about 70% tubulin and 30% MAPs (#ML113), and highly purified tubulin (>99% pure, #TL238), both from bovine brain, were obtained from Cytoskeleton, Inc. (Denver, Colorado, USA). Both of these were lyophilized products and contained small amounts of GTP (approximately equivalent to 0.5 mM GTP when the protein concentration was 10 mg/mL). PEM buffer (80 mM piperazine-N,N′-bis[2-ethanesulfonic acid], pH 6.9, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM magnesium chloride), GTP, and the Advanced Protein Assay reagent were also obtained from Cytoskeleton. [8-^{3}H]GTP, NH_4^+ salt, specific activity 6.4 Ci/mmol, and MicroSpin G50 columns, were from Amersham Biosciences (Piscataway, NJ, USA); columns were prepared for use according to the manufacturer's instructions. HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and were cultured in Dulbecco's MEM with L-glutamine, supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Gibco, Grand Island, NY, USA). Cells were incubated at 37°C in humidified 5% CO_2 in air.

Tubulin polymerization: plate protocol

Immediately before use, microtubule protein or purified tubulin was dissolved in ice-cold PEM buffer. When used, GTP was present in PEM buffer at 1 mM. After holding the tubulin solution on ice for 10–15 min, it was centrifuged at top speed in an Eppendorf model 5415C microcentrifuge (Brinkmann Instruments, Westbury, New York, USA) for 10 min at 4°C to remove any particles or aggregates. The supernatant from this centrifugation was dispensed to wells of ½-area 96-well plates (Costar No. 3696, Corning, Inc., Corning, New York, USA) as described previously [2], with the following modifications. After recording the increase in absorbance at 340 nm, 30°C, for 60 min, the plate was removed from the SpectraMax Plus plate reader (Molecular Devices Corp., Sunnyvale, CA, USA) and immediately floated for 30 min in a large tray of water that was pre-cooled to 4°C. Control experiments demonstrated that this was 2–3 times longer than necessary to fully depolymerize control microtubules. During the 4°C incubation no absorbance readings could be taken. At the end of the 4°C incubation, the plate was rapidly dried and returned to the SpectraMax Plus at 30°C for another 60 min of recording. Changes in turbidity which occurred as a result of the low temperature incubation were revealed as sharp discontinuities in the absorbance curves over the 2 h recording. Every sample was run in duplicate in each experiment. Final concentrations of tubulin, compound, and DMSO are given in the legend to Fig. 2. Absorbance measurements were taken every min throughout the assay.

Tubulin polymerization: spectrophotometer protocol

Reactions were carried out in 1 cm path length quartz cuvettes in a Beckmann Model DU7400 spectrophotometer equipped with a six cuvette automatic sample changer and Peltier temperature controller. Each cuvette contained 270 μl of microtubule protein plus compound; final concentrations of protein, compound, and DMSO are given in the legend to Fig. 2. Polymerization reactions were recorded for 60 min at 24°C, then the temperature was shifted to 12°C and the depolymerization was followed for another 60 min. Absorbance at 340 nm was recorded every min for each sample.

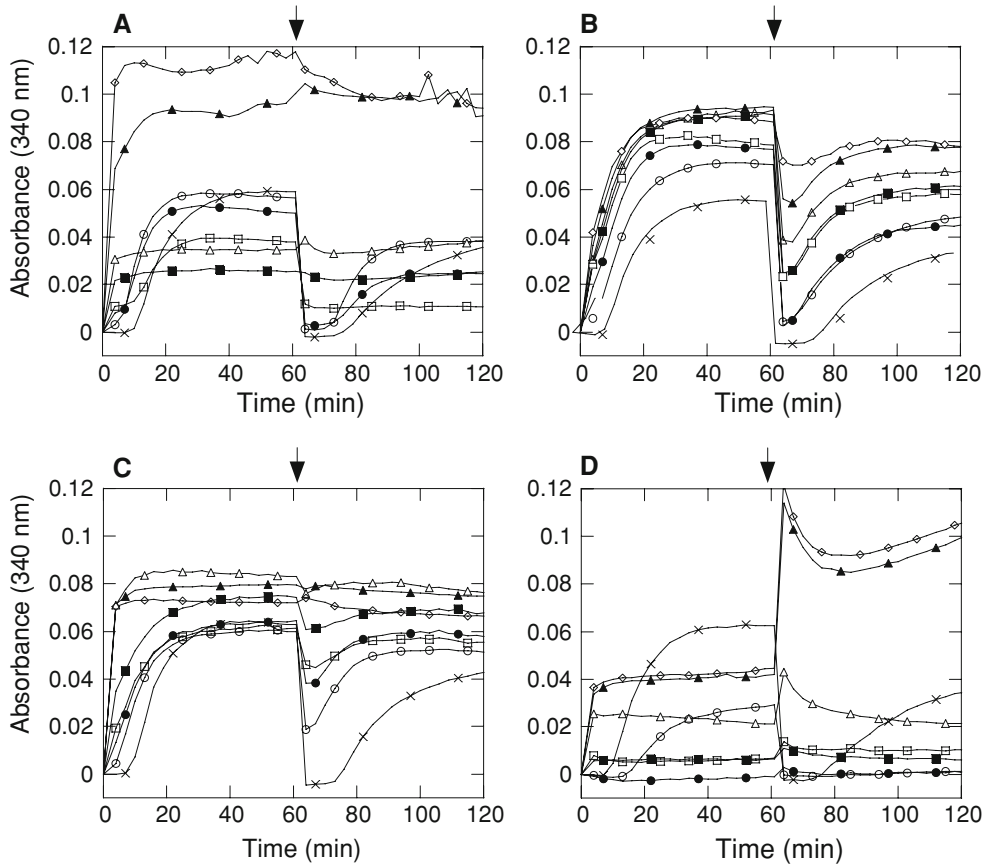


Fig. 2 Effects of temperature on the stability of adducts induced in microtubule protein by TTI-237 or reference compounds. A fixed concentration of microtubule protein (8.4 μM tubulin heterodimer, containing 1 mM GTP) was incubated with various concentrations of **a** TTI-237, **b** paclitaxel, **c** docetaxel, or **d** vincristine. The initial incubation was at 30°C for 60 min; then the plate was rapidly cooled to 4°C for 30 min, during which time no absorbance recording took place; finally, the plate was returned to the plate reader at 30°C for a further 60 min. The *arrow* above each graph indicates the start of the 4°C incubation. In all graphs, the concentrations of compound were: *X*, control (DMSO at the highest concentration used for experimental curves); *open circle* 0.3 μM, *filled circle* 0.6 μM, *open square* 1.2 μM, *filled square* 2.4 μM, *open triangle* 4.8 μM, *filled triangle* 9.6 μM, *open diamond* 19.2 μM. DMSO concentrations ranged from 0.006 to 0.38%

Tubulin polymerization: depolymerization induced by Ca^{++}

The spectrophotometer protocol given above was followed, except that after the first 60 min of recording at 25°C, all samples were made 3.6 mM in Ca^{++} by adding 10 μl of 100 mM $CaCl_2$. The solutions were rapidly mixed, and recording was continued at 25°C for 60 min. Data points were collected at 1 min intervals.

Binding of [^{3}H]GTP to purified tubulin

Reactions were carried out in standard, flat-bottom, 96-well plates. Highly purified tubulin was used at a final concentration of 11–14 μM heterodimer, and compounds were used at 100 μM final concentrations. In one set of experiments, tubulin was incubated first with compounds in quadruplicate for 30 min at room temperature before addition of [^{3}H]GTP (final concentration of 96.7 μM, specific activity 0.04 Ci/mmol). After a further 30 min incubation, aliquots of each sample were applied to MicroSpin G-50 columns which were centrifuged for 2 min at 3,000 rpm in an Eppendorf 5415C microfuge. An aliquot of each column effluent (containing tubulin and bound [^{3}H]GTP) was mixed with scintillation fluid and counted in a liquid scintillation spectrometer.

In a second set of experiments, tubulin was incubated first with [^{3}H]GTP (final concentration of 96.7 μM, specific activity 0.04 Ci/mmol) for 30 min at room temperature. Then compounds were added, in quadruplicate, and incubation continued for another 30 min. Aliquots of each sample were processed and counted as above.

In a third set of experiments, tubulin was incubated first with [^{3}H]GTP (final concentration of 10.2 μM, specific activity 0.376 Ci/mmol) for 30 min at room temperature; then compounds were added, in quadruplicate, and incubation continued for 15 min. Finally, a 10-fold excess of unlabelled GTP was added to all samples, incubation was continued for 30 min, and aliquots of each sample were processed and counted as above.

Flow cytometry

The antibodies used were: mouse monoclonal anti-phospho-Stat5 (Y694): AlexaFluor 647 conjugate (#612599); mouse monoclonal anti-phospho-ERK1/2 (T202/Y204): AlexaFluor 488 conjugate (#612592); mouse monoclonal anti-phospho-Stat3 (Y705): phycoerythrin conjugate (#612569); mouse monoclonal anti-phospho-p38 MAPK (T180/Y182): phycoerythrin conjugate (#612565), from BD Biosciences (San Jose, California, USA); and rabbit monoclonal anti-phospho-Akt (S473): AlexaFluor 647 conjugate (#2337); mouse monoclonal anti-phospho-SAPK/JNK (T183/Y185): AlexaFluor 647 conjugate (#9257); rabbit monoclonal anti-phospho-NF-κB p65 (S536): AlexaFluor 488 conjugate (#4886); rabbit monoclonal anti-phospho-S6 ribosomal protein (S235/S236): AlexaFluor 488 conjugate (#4854) from Cell Signaling Technology (Danvers, Massachusetts, USA).

Changes in the levels of phosphoproteins were determined by flow cytometry [4, 5]. HeLa cells were treated with no compound or with 125 nM TTI-237 or 12.5 nM paclitaxel or vincristine for 18 h. These concentrations were 4 times the IC_{50} values for inhibition of cell growth for each compound in a 3-day assay [2]. At the end of the culture period, adherent and non-adherent cells were collected, fixed with 1.9% formaldehyde (Ultra Pure, methanol-free, EM grade, Polysciences, Warrington Pennsylvania, USA) for 15 min at room temperature, and permeabilized overnight in methanol at −20°C. Each suspension was divided into thirds and one-third was stained (in PBS −0.1% bovine serum albumin) with anti-p-NF-κB, anti-p-Stat3, and anti-p-Akt; one-third was stained with anti-p-S6, anti-p-p38, and anti-p-Stat5; and one-third was stained with anti-p-ERK1/2 and anti-p-SAPK/JAK. Samples were analyzed with a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Changes in protein phosphorylation following compound treatment were estimated by calculating the $\log_2$ of the ratio of the median fluorescence intensity in the presence of the compound to the median fluorescence intensity of control cells in the absence of any compound.

Results

Cold-induced depolymerization of aggregates formed by TTI-237, paclitaxel, docetaxel, or vincristine

It was previously reported that TTI-237 enhanced microtubule protein aggregation at 24°C [2] but the effect of low temperature on these aggregates was not known. Therefore, after 60 min of aggregation at 30°C (to allow full polymerization of all samples), plates were incubated at 4°C for 30 min, and then returned to the plate reader at 30°C for another 60 min. The time at 4°C was more than sufficient to fully depolymerize the microtubules formed in control samples without compound, as judged by a decline of A_{340} to baseline. This procedure allowed the effects of compounds on the temperature stability of aggregates to be determined. Figure 2 shows the results for TTI-237, paclitaxel, docetaxel, and vincristine. For all four compounds, reactions were done at 7 compound:tubulin molar ratios ranging from tubulin in 28-fold excess, to compound in 2.3 fold excess, as well as a control without compound. In the presence of 0.3 and 0.6 μM TTI-237 (Fig. 2a) (tubulin in 14–28 fold molar excess) the initial increase in turbidity was faster than in the control reaction, as previously reported [2], and the plateaus reached were about the same as control. Both of these samples depolymerized almost completely (i.e., the A_{340} fell to nearly baseline) during the 4°C incubation and then re-polymerized faster than control during the second 30°C incubation. At 1.2 μM TTI-237 the plateau reached during the initial polymerization was lower than that of the control, but the aggregates showed some stability to the cold incubation. At TTI-237 concentrations of 2.4, 4.8, 9.6, and 19.2 μM, several changes in the aggregation curves were noted: the initial rise in absorbance was very rapid; plateaus increased with increasing compound concentration; and the aggregates were completely stable to cold, i.e., the plateaus reached during the first 30°C incubation did not change after exposure to 4°C for 30 min.

The corresponding curves for paclitaxel and docetaxel are shown in Fig. 2b and c. Both agents at all concentrations caused enhanced polymerization of microtubule protein compared to control. The initial polymerization rates in the presence of higher concentrations of docetaxel (4.8, 9.6, and 19.2 μM) (Fig. 2c) were especially fast, as was noted with the same doses of TTI-237. The polymers formed in the presence of low concentrations of paclitaxel (0.3 and 0.6 μM) (Fig. 2b) were almost completely depolymerized by cold exposure. As paclitaxel concentrations increased the polymers became more stable to cold, but even at the highest paclitaxel concentration examined (2.7-fold molar excess over tubulin heterodimer) the polymers were not completely stable. The results with docetaxel were similar, except that at each concentration this agent stabilized polymers (presumably microtubules) to a greater extent than the same dose of paclitaxel. At concentrations of 4.8, 9.6, and 19.2 μM docetaxel, the polymers were completely stable to cold exposure.

A quite different pattern was observed when vincristine was studied in analogous experiments (Fig. 2d). The initial polymerization reaction of microtubule protein was partially inhibited by 0.3 μM, and completely inhibited by 0.6 μM, vincristine. Note that the solution containing 0.3 μM vincristine failed to re-polymerize at all (as judged

by this turbidity assay) during the second 30°C incubation, in contrast to the corresponding TTI-237 solution. At higher vincristine concentrations (4.8, 9.6, and 19.2 μM), the initial rise in A_{340} was due to turbidity caused by the known formation of non-microtubule aggregates [6]. These aggregates were distinctive in that they increased during the cold incubation, in contrast to those induced by TTI-237, paclitaxel, and docetaxel.

Rate of cold-induced depolymerization of aggregate formed in the presence of TTI-237

The experiments just described showed that the aggregates induced by TTI-237 at a low compound:tubulin-heterodimer molar ratio were labile to cold, but they did not reveal the kinetics of cold-induced depolymerization. To examine this, microtubule protein was polymerized in a spectrophotometer that allowed recording at 12°C after polymerization at 24°C (Fig. 3). The control reaction without compound depolymerized substantially at 12°C as indicated by the fall in A_{340}. In the presence of 0.25 μM TTI-237 (TTI-237:tubulin-heterodimer molar ratio of 1:34), the initial aggregation reaction was faster than control (as seen previously), and the depolymerization reaction at 12°C was also faster than the control and proceeded to the same extent as control after 60 min. If the TTI-237 concentration was increased to 2.22 μM (TTI-237:tubulin-heterodimer molar ratio of 1:4), the initial aggregation rate at 24°C was even faster than at 1:34 ratio but the aggregates were stable at 12°C.

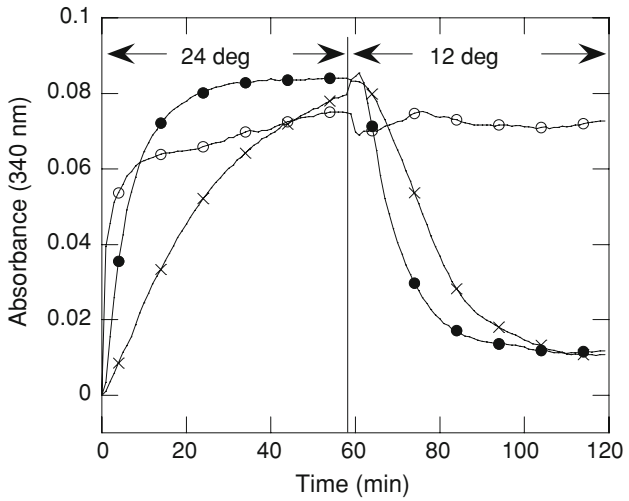


Fig. 3 Kinetics of polymerization and depolymerization of microtubule protein in the presence of TTI-237 at two temperatures. 1.18 mg/ml microtubule protein (8.3 μM tubulin heterodimer) plus 1 mM GTP contained TTI-237 at: *X*, control (no TTI-237), 0.4% DMSO; filled circle, 0.25 μM, 0.005% DMSO; open circle, 2.22 μM, 0.044% DMSO. After 1 h at 24°C, the temperature was shifted to 12°C for the final hour

Ca^{++}-induced depolymerization of aggregates formed by TTI-237, paclitaxel, docetaxel or vincristine

Control microtubules were completely depolymerized after the reaction solution was made 3.6 mM in Ca^{++}, sufficient to overcome the chelating capacity of EGTA in the buffer. It is known that paclitaxel and docetaxel stabilize microtubules and antagonize the effects of Ca^{++} [7]. We desired to determine the effects of TTI-237 on Ca^{++}-induced depolymerization. As shown in Fig. 4a, 3.6 mM Ca^{++} induced complete depolymerization of the aggregates formed in the presence of 0.25 μM TTI-237, and partial depolymerization of those formed in 0.74 μM TTI-237. At higher TTI-237 concentrations (6.67 and 20 μM) the aggregates were completely stable to 3.6 mM Ca^{++}. At an intermediate TTI-237 concentration (2.22 μM) there was some increase in A_{340} similar to what was seen with high concentrations of vincristine (Fig. 4d).

Under the same conditions, paclitaxel provided very little stabilization of microtubules to Ca^{++} when present in the reaction at 0.25 μM, but increasing concentrations of paclitaxel provided increasing, but still incomplete, stabilization (Fig. 4b). In contrast, docetaxel at 6.67 and 20 μM provided complete resistance to Ca^{++}-induced depolymerization whereas 0.25 μM docetaxel provided very little stabilization (Fig. 4c). In the presence of vincristine at 6.67 and 20 μM (Fig. 4d) there was a large increase in turbidity when Ca^{++} was added, suggesting that non-microtubule aggregate formation was enhanced.

TTI-237 inhibits nucleotide exchange

Vinblastine and other vinca-domain ligands are known to decrease the exchange rate of GTP at the exchangeable nucleotide site of the tubulin heterodimer [8]. In view of the fact that TTI-237 inhibited the binding of [^{3}H]vinblastine to tubulin [2], it was important to determine whether TTI-237 also inhibited nucleotide exchange. When purified tubulin was incubated with compounds (either paclitaxel, vincristine, colchicine, or TTI-237) before the addition of [^{3}H]GTP, vincristine and TTI-237, but not paclitaxel or colchicine, inhibited the binding of [^{3}H]GTP (Fig. 5a). This reduction could have been due to competition for the same or closely adjacent binding sites, or to an indirect or allosteric effect whereby the binding of vincristine or TTI-237 to one site reduced the rate or extent of binding of [^{3}H]GTP to another site. To distinguish between these possibilities additional experiments were done. When tubulin was incubated first with [^{3}H]GTP and then with compounds, none of the agents reduced the binding of the nucleotide (Fig. 5b). This indicated that vincristine and TTI-237 did not directly compete with [^{3}H]GTP, provided that the off-rate of bound [^{3}H]GTP was sufficient to allow competition to be seen. To

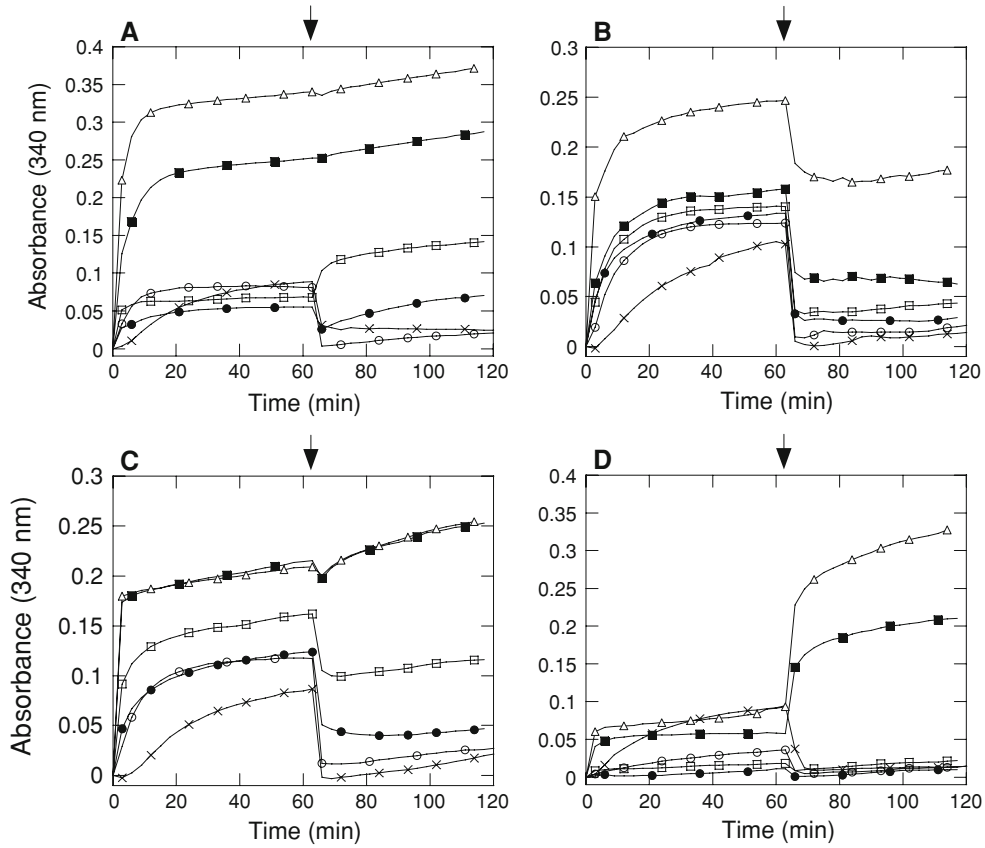


Fig. 4 Effects of Ca^{++} on the stability of adducts induced in microtubule protein by TTI-237 or reference compounds. A fixed concentration of microtubule protein (8.5 μM tubulin heterodimer, containing 1 mM GTP) was incubated for 2 h at 25°C with various concentrations of **a** TTI-237, **b** paclitaxel, **c** docetaxel, or **d** vincristine. At the times indicated by the arrows, all samples were made 3.6 mM in $CaCl_2$. In all graphs, the concentrations of compound were: *X*, control (DMSO at the highest concentration used for experimental curves); *open circle* 0.25 μM, *filled circle* 0.74 μM, *open square* 2.22 μM, *filled square* 6.67 μM, *open triangle* 20 μM. DMSO concentrations ranged from 0.005 to 0.4%

determine this, tubulin was again first incubated with [^{3}H]GTP, then with compounds, and then with an excess of unlabeled GTP (Fig. 5c). In this situation the binding of [^{3}H]GTP in the presence of vincristine or TTI-237 was greater than in the presence of paclitaxel, colchicine, or no compound. This indicated that the unlabeled GTP displaced [^{3}H]GTP in the presence of paclitaxel or colchicine, but displacement was inhibited in the presence of vincristine or TTI-237. Because TTI-237 tracked with vincristine in all these experiments, we conclude that TTI-237, like vincristine, inhibited GTP exchange on the tubulin heterodimer.

TTI-237 resembles vincristine in its effects on a group of cellular phosphoproteins

Microtubule-active agents cause many intracellular metabolic changes either consequent to or in addition to their classical effects on cellular microtubules [9–12]. One readily monitored change is the phosphorylation status of a group of proteins, many of which are important in various signaling pathways. The changes induced in these proteins, while complex and often difficult to interpret individually, in sum constitute a "signature" or characteristic pattern that can be used to classify new microtubule-active agents into groups having "paclitaxel-like" or "vincristine-like" effects. We sought to determine whether TTI-237 had a signature similar to one of these groups.

We measured the change in phosphorylation of a set of intracellular proteins (Fig. 6) using flow cytometric techniques and phosphoprotein-specific antibodies. If the change in intensity of a particular protein was expressed as log to base 2 of (median fluorescence intensity in the presence of compound/median fluorescence intensity in the absence of compound), then a value of 1 represented a 2-fold increase and a value of −1 represented a 2-fold decrease in median fluorescence intensity. Each compound was studied at 4 times its IC_{50} value for inhibition of cell growth (determined in a standard 3-day cell proliferation assay [2]) after a culture period of 18 h. In all cases, the 0 point on the abscissa represented the phosphorylation level of the protein in the absence of compound.

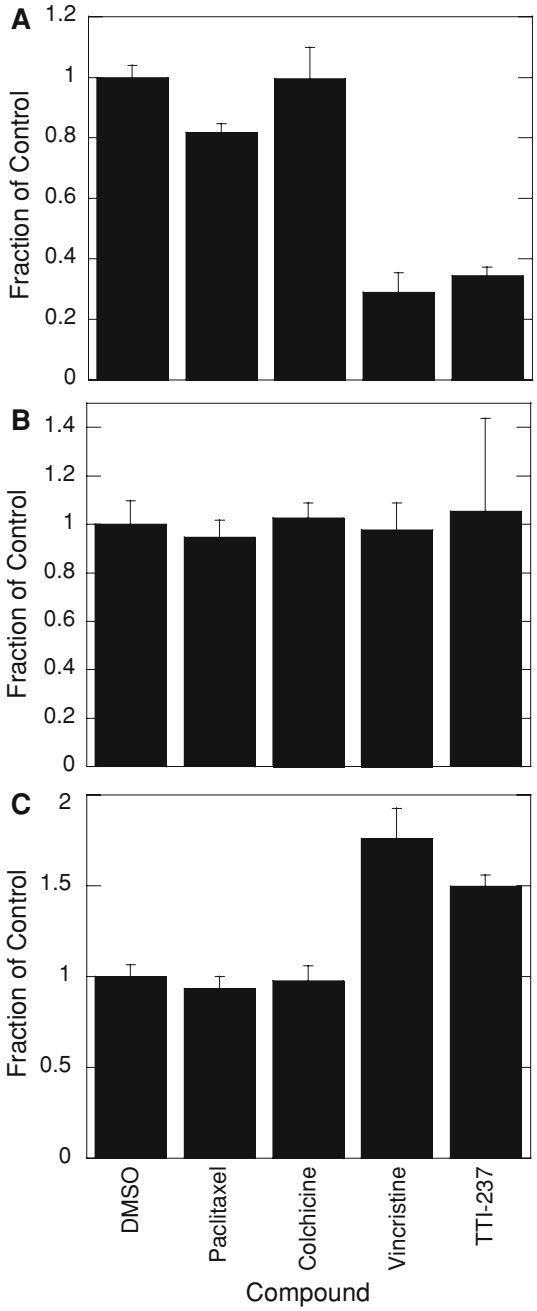


Fig. 5 Binding of [^{3}H]GTP to purified tubulin. **a** Tubulin incubated with compounds before addition of [^{3}H]GTP; **b** tubulin incubated with [^{3}H]GTP before the addition of compounds; **c** tubulin incubated first with [^{3}H]GTP, then with compounds, then with an excess of unlabeled GTP. Histograms show the means and standard deviations of quadruplicate assays. DMSO concentrations ranged from 0.51 to 1.0%; all controls contained 1.0% DMSO

As shown in Fig. 6, vincristine increased the phosphorylation level of every protein studied except ERK, whose level decreased slightly. Paclitaxel, on the other hand, had essentially no effect on the phosphorylation of any protein again except ERK. Thus vincristine and paclitaxel have quite different "signatures" in this analysis.

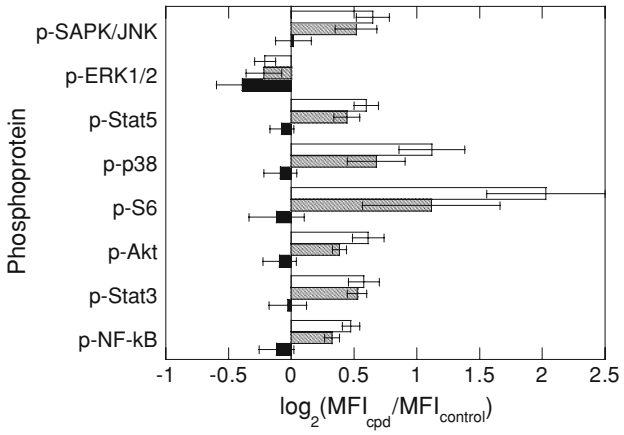


Fig. 6 Phosphoprotein signatures of three microtubule-active compounds in HeLa cells. The effects of TTI-237, paclitaxel, and vincristine on the phosphorylation of 8 intracellular proteins were compared by flow cytometry methods. Each compound was used at 4X its IC_{50} concentration for inhibition of cell growth, and cells were processed after 18 h of culture. The baseline for each protein was the phosphorylation level of that protein in untreated control cells. Data are the means and standard deviations of 4 (TTI-237) or 5 (paclitaxel and vincristine) independent experiments. *Clear bars* TTI-237, *hatched bars* vincristine, *filled bars* paclitaxel. *MFI* Median fluorescence intensity

The effects of TTI-237 resembled those of vincristine in every case.

Discussion

The effects of TTI-237 on the stability of tubulin aggregates to low temperature and Ca^{++} ions fell into two modes, which depended on the TTI-237:tubulin heterodimer molar ratio. At low ratios (i.e., heterodimer in excess by 20–30 fold), the polymerization turbidity curve was sigmoidal, like the control curve, but more rapid, and the depolymerization induced by low temperature was complete and also faster than control. We infer that normal microtubules were being formed in this mode, but that polymerization and depolymerization kinetics were increased by TTI-237. Similarly, the polymer formed at a low ratio of TTI-237:tubulin heterodimer was labile to Ca^{++}, consistent with the hypothesis that the polymers were "real" microtubules. The high excess of tubulin over TTI-237 in this mode implies that TTI-237 may act preferentially at microtubule + ends, as has been suggested for vinblastine itself [13]. But unlike vinblastine and some other microtubule-active agents, which suppress dynamicity parameters [14, 15], TTI-237 may enhance microtubule dynamicity, increasing the nucleation/growth and the catastrophe/shortening rates when present at low ratios to tubulin heterodimer. In this, it may mimic some actions of + end binding proteins which regulate microtubule dynamicity in vivo [16–18].

As TTI-237 concentration increased relative to tubulin, and the TTI-237:heterodimer molar ratio rose to ≈1:4 or greater, a second mode of tubulin aggregation became apparent. This mode was characterized by a rapid (within 0.5–3 min) jump in turbidity nearly to a concentration-dependent plateau level which did not increase substantially during a further 60 min of incubation. The aggregates formed during this mode were stable to cold and Ca^{++}, but aggregation did not increase in response to these agents as it did with vincristine. We suggest that the aggregates formed in this second mode are not microtubules but a tubulin polymer of anomalous structure as is known to be induced by other vinca-domain ligands [19, 20].

Also, the plateaus of the polymerization curves in the presence of TTI-237 (Fig. 2a) were dependent on compound concentration, whereas with paclitaxel and docetaxel the plateaus were largely independent of compound concentration once a certain level was reached (≈2.4 μM in Fig. 2b and c). This suggests that the aggregates induced by TTI-237 were different from those induced by paclitaxel and docetaxel (which were presumably "real" microtubules), and that the structure and/or amount of aggregates induced by TTI-237 were dependent on compound concentration.

At intermediate levels of TTI-237 (0.6 and 1.2 μM in Fig. 2a), the recordings revealed both a rapid but small jump in A_{340}, followed by a normal polymerization curve. Depolymerization by cold or Ca^{++} brought the absorbance reading down to about the initial "jump" level, which then remained stable. This implies that intermediate levels of TTI-237 (TTI-237:heterodimer ratios of about 1:10) produced both modes of tubulin polymerization.

Some anti-mitotic peptides and depsipeptides, such as tubulysin, phomopsin, cryptophycin, hemiasterlin, and dolastatins 10 and 15, inhibit vinblastine binding to tubulin and induce various kinds of abnormal tubulin polymers, including spirals, pinwheels, and small rings of various sizes, and also inhibit nucleotide exchange [21]. All of these agents inhibit tubulin polymerization and depolymerize pre-existing microtubules. TTI-237 seems to be distinct from these in that it causes more rapid polymerization of both microtubule protein and purified tubulin than control, it causes polymerization of purified tubulin even in the absence of GTP, and it does not induce depolymerization of pre-existing microtubules.

It was previously shown [2] that TTI-237 inhibited the binding of [^{3}H]vinblastine to the heterodimer, but it was not known if the inhibition was due to the binding of TTI-237 in the vinca domain, or was the result of an allosteric effect caused by binding at a novel site. The fact that TTI-237 inhibited GTP exchange is consistent with binding in the vinca domain, since only vinca-domain ligands have been shown to inhibit nucleotide exchange [8]. They do this by binding near the nucleotide site, and thereby partially interfere with nucleotide binding [8, 22].

Under the conditions used, paclitaxel had no effect on the phosphorylation state of any of the proteins studied except ERK1/2, where it caused a reduction in phosphorylation (Fig. 6). Vincristine, on the other hand, increased phosphorylation of all the proteins compared to the starting level without compound, again with the exception of ERK1/2. It is important to note that these changes were caused by low doses of each agent, only 4× their IC_{50} values for growth inhibition in a 3 day assay, and after 18 h of exposure to the compounds. We also examined compound concentrations of 2×, 8×, and 16× IC_{50} values (data not shown) and found that changes in phosphorylation were incomplete at 2×, but plateaued by 4× and in general were no greater at 8× and 16×. The changes in phosphorylation of this group of intracellular proteins induced by TTI-237 used at the same relative concentration (i.e., 125 nM or 4× its IC_{50} value) were quite similar to the pattern seen with vincristine and distinct from the pattern shown by paclitaxel. This is further support for the possibility that TTI-237 binds in the vinca domain of the heterodimer.

Table 1 summarizes some ways in which TTI-237 is similar to and different from vincristine and paclitaxel. It is clear that TTI-237, while having some similarities to each, does not match the profile of either compound. Chen and Horwitz [23] have described a difference between microtubule-stabilizing and -destabilizing compounds on mitotic response. At low concentrations, microtubule-stabilizing compounds, e.g., paclitaxel, epothilone B, and discodermolide, produced aneuploid populations of cells in the absence of mitotic block. This was due to the formation by these agents of mitotic cells with multipolar spindles which gave rise to multinuclear cells. Exactly this behavior was found for TTI-237 [2], suggesting that it be classified as a microtubule-stabilizing compound. This classification would be consistent with the ability of TTI-237 to promote aggregation of both microtubule protein and purified tubulin, even in the absence of added GTP, and with its ability to stabilize the aggregates formed to cold and Ca^{++}. However, TTI-237 inhibited the binding of [^{3}H]vincristine, but not [^{3}H]paclitaxel, and it inhibited nucleotide exchange, consistent with its being a vinca-domain ligand. Most significantly, in cells, it had a vincristine-like pattern of phosphoprotein activation.

The metabolic consequences of microtubule-active compounds, at least at low concentrations, may have more to do with the site at which they bind to tubulin than on effects on microtubule dynamicity or structure. It is likely that each pharmacologic binding site of tubulin has a different complement of natural interacting proteins that are perturbed as a result of compound binding, and that such perturbations are detected and ramified in distinct ways, giving rise to different profiles of metabolic changes in cells. Thus, TTI-237, which presumably binds in the vinca domain, has effects that are similar to those of vincristine on a panel of

Table 1 Similarities and differences between TTI-237 and vincristine or paclitaxel. Data from this paper and [2]

Compound	Similarities of TTI-237 to compound	Differences of TTI-237 from compound
Vincristine	Inhibits [^{3}H]vinblastine binding to tubulin heterodimer	Does not cause polymer disaggregation detectable by turbidity or sedimentation
	Inhibits GTP exchange	
	Similar phosphoprotein pattern	
Paclitaxel	Induces aggregation of both microtubule protein and purified tubulin, ± GTP	Does not produce tubulin bundles in interphase cells
	Stabilizes aggregates against depolymerization induced by cold or Ca^{++}	Does not inhibit [^{3}H]paclitaxel binding to microtubules
	Induces multiple centrosome-like clusters of microtubules in mitotic cells	
	Produces multinuclear cells at concentrations below those that cause mitotic block	

cellular proteins, yet its effects on cell division are more similar to those of paclitaxel.

Therefore, TTI-237, which is but one of many related compounds that have similar properties [1, 24], is the prototype of a new class of microtubule-active compounds which may complement known agents in studies of tubulin and microtubule function. TTI-237 also may have clinical applicability in the treatment of cancer, either as a single agent or more likely as a component of new combination regimens [25].

Conflict of interest statement None.

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