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Research paper

Antitumor activity of tricyclic pyrone analogs, a new synthetic class of microtubule de-stabilizing agents, in the murine EMT-6 mammary tumor cell line *in vitro*

Elisabeth M Perchellet, James B Ladesich, Yi Chen,[1] Hong-Sig Sin,[1] Duy H Hua,[1] Susan L Kraft[2] and Jean-Pierre Perchellet

Anti-Cancer Drug Laboratory, Division of Biology, Ackert Hall, [1]Department of Chemistry and [2]Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA. Tel: (+1)785 532-7727; Fax: (+1)785 532-6653.

Novel tricyclic pyrone (TP) analogs synthesized in Hua's laboratory (code names H10, H14 and H16) were tested against a spectrum of known antimitotic drugs for their ability to disrupt microtubule (MT) dynamics, alter the mitotic index, and prevent murine EMT-6 mammary sarcoma cells from synthesizing DNA and proliferating *in vitro*. At 2–10 μM, H10 inhibits DNA synthesis, tubulin polymerization and tumor cell growth to a greater degree than H14, whereas H16 has no effect. A linear skeleton with a pyridyl ring at C-3 of the A-ring, a pyran B-ring and no alkylation at C-7 of the C-ring is required for the antitumor activity of these TPs. Since H10 mimics the effect of vincristine (VCR), but not that of paclitaxel, on tubulin polymerization, TPs may represent a novel synthetic class of MT de-stabilizing anticancer drugs. H10 is less potent than VCR against tubulin polymerization (IC_{50}: 1.5 μM versus 0.15 μM) and tumor cell proliferation (IC_{50}: 1.5 μM versus 5 nM) but inhibits DNA synthesis (IC_{50}: 10 μM) more effectively than all other MT-disrupting agents tested, except tubulozole-C. Although TPs disrupt DNA synthesis and might affect several phases of the cell cycle, the ability of H10 to increase the percentage of mitotic cells indicates that these novel compounds may be cell cycle-specific anticancer drugs useful for arresting mammalian cells in M-phase. [© 1998 Lippincott-Raven Publishers.]

Key words: DNA synthesis, mammary sarcoma, mitotic index, tricyclic pyrones, tubulin polymerization, tumor cell growth.

Introduction

Pyripyropenes and arisugacin are natural microbial products with closely related tetracyclic structures, which, respectively, inhibit acyl-CoA:cholesterol acyltransferase and acetylcholinesterase activities, and may be useful in the treatment of atherosclerosis and Alzheimer's disease.[1-10] Since the anticancer potential of different portions of the molecular structures of pyripyropenes and arisugacin is unknown, novel tricyclic pyrone (TP) analogs have been synthesized in Hua's laboratory, using a simple one-pot coupling reaction of pyrones and enals, and evaluated for their antitumor effects *in vitro*.[11-15]

Condensation of various 6-substituted 4-hydroxypyrones with 1-cyclohexenecarboxaldehydes in the presence of L-proline in ethyl acetate gives high yields of 3,5a,6-substituted 1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyrans.[11,13,14] When tested at 50 μM, a tetracyclic pyripyropene analog is totally inactive but several new TP derivatives possessing aryl groups, such as 3,4-dimethoxyphenyl or 3-pyridyl, decrease the ability of murine L1210 leukemic cells to synthesize DNA and grow *in vitro* by 79–100%.[12] Introduction of a methyl group at C-5a and a formyloxy or hydroxy group at C-6 does not alter the antitumor effects of the 3,4-dimethoxyphenyl benzopyrans but reduces those of the 3-pyridyl benzopyrans.[12] Moreover, TPs lacking the above aryl groups have no or very little inhibitory effects, suggesting that a greater conjugation is required for the antitumor activity.[12] At concentrations of 25 μM or below, which are required to assess the structure-activity relationships for this class of compounds, the most effective antileukemic TP synthesized so far is the 3-pyridyl benzopyran H10 (Figure 1).[15]

Although somewhat less potent, the TPs can inhibit macromolecule synthesis and L1210 cell growth to the same degree as a representative spectrum of clinically important anticancer drugs.[12,15] Their short-term

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Correspondence to J-P Perchellet

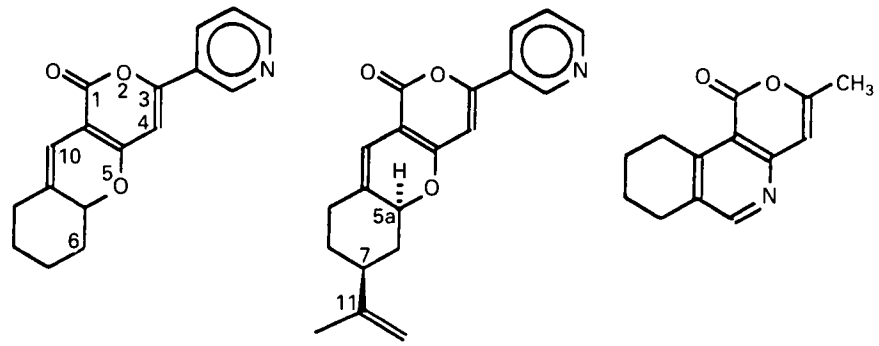

Figure 1. Chemical structures and code names of the TP analogs tested for their effectivess against EMT-6 mammary tumor cells *in vitro*.

inhibition of nucleic acid synthesis is reversible following drug removal but their long-term inhibition of tumor cell growth is irreversible and precedes the decline in cell viability, suggesting that the TPs are cytostatic rather than cytotoxic.[15] The discrepancy between the abilities of various TPs to inhibit macromolecule synthesis and leukemic cell growth suggests that other molecular targets may be involved in the antitumor action of these drugs.[15]

Because synthetic TPs exhibiting antileukemic activity *in vitro* might be valuable to develop a new class of anticancer drugs, further studies must be undertaken to characterize their molecular mechanism of action and demonstrate their effectiveness against different tumor cell lines capable of forming solid malignant neoplasms. Preliminary findings suggest that the antiproliferative activity of our most promising TP might be linked to its ability to interfere with the polymerization of purified tubulin.[15] Since they might work as novel spindle poisons, therefore, H10 and other TP analogs were tested in the present study against a spectrum of known antimitotic drugs for their ability to disrupt microtubule (MT) dynamics, alter the mitotic index, and prevent murine EMT-6 mammary sarcoma cells from synthesizing DNA and proliferating *in vitro*.

Materials and methods

Cell culture and drug treatments

All solutions of synthetic TPs and colchicine, nocodazole, podophyllotoxin, paclitaxel, tubulozole-C (all from Sigma, St Louis, MO) and vincristine (VCR) were dissolved and diluted in dimethyl sulfoxide (DMSO). Murine EMT-6 mammary sarcoma cells were maintained in continuous exponential growth by twice-a-week passage in Eagle's minimum essential medium with L-glutamine, Earle's salts, non-essential amino acids, 1 mM sodium pyruvate, bovine insulin (10 μg/ml), 10% fortified bovine calf serum (HyClone, Logan, UT) and penicillin (100 IU/ml)-streptomycin (100 μg/ml). Monolayer cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2 and adherent cells were harvested with 0.25% trypsin. All drugs were supplemented to the culture medium in 1 μl aliquots and the concentration of vehicle in the final incubation volume (0.5 ml) never exceeded 0.2%. Such low concentration of DMSO does not affect the rates of DNA synthesis and growth in EMT-6 cells. Control cells incubated in the absence of drugs were similarly treated with vehicle only and, in every experiment, all incubates received the same volume of solvent.

DNA synthesis and cell proliferation assays

EMT-6 cells (1×10^5 cells/0.5 ml/well) were plated for 24 h at 37 C in 48-well Costar cell culture plates (Costar, Cambridge, MA), incubated for 2 h in the presence or absence of drugs and then pulse-labeled for an additional 60 min with 1 μCi of [methyl-^{3}H]thymidine (50 Ci/mmol; Amersham, Arlington Heights, IL) to estimate the rate of DNA synthesis. After removing the radioactive medium by suction, monolayer cultures were fixed in 0.5 ml of 100% methanol for 10 min, dried, washed with 0.5 ml of Dulbecco's phosphate-buffered saline (PBS) and held on ice for 10 min in the presence of 0.5

ml of 10% trichloroacetic acid (TCA). The acid-insoluble material was washed twice with 0.5 ml of 5% TCA, dissolved in 0.5 ml of 1% sodium dodecyl sulfate in 0.3 N NaOH for 30 min at room temperature and transferred to liquid scintillation vials. The radioactivity bound to the acid-precipitable material was counted in 5 ml of Bio-Safe II (Research Products International, Mount Prospect, IL). For tumor cell growth, EMT-6 cells were plated at an initial density of 1×10^4 cells/0.5 ml and incubated at 37°C in 48-well Costar cell culture plates. Cell monolayers were grown for 4 days in the presence or absence of drugs, harvested by trypsinization and their density was monitored every 24 h using a Coulter counter (Coulter Electronics, Luton, UK).

MT assembly

The polymerization of purified tubulin protein from bovine brain in the presence or absence of glycerol was analyzed using the Tubulin/Microtubule Biochem kit purchased from Cytoskeleton (Denver, CO).[16] The polymerization reactions contained, in a final volume of 0.2 ml, either tubulin minus glycerol or tubulin plus 10% glycerol (2.2 mg protein/ml) in 80 mM PIPES buffer, pH 6.8, supplemented with 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP and either 0 or 10% glycerol. The TP analogs and known MT-disrupting agents under study were all added to the assay mixture in 1 µl aliquot of DMSO:tubulin buffer (40:60) to obtain the final concentrations of 0.0065–25 µM tested. This vehicle did not affect the rates of tubulin polymerization in drug-untreated control reactions incubated in the presence or absence of glycerol. Samples were immediately incubated at 35°C in quartz microcells and the rate of tubulin polymerization was followed over 20 min by recording the increased absorbance of the solution at 340 nm, using a Shimadzu UV-160 spectrophotometer equipped with dual-beam optics and a thermostatically controlled cell holder.

Mitotic index

EMT-6 cell monolayers were plated in triplicate at an initial density of 0.5×10^6 cells/0.5 ml, grown at 37°C for 24 h in the presence or absence of TP or known antimitotic drug, harvested by trypsinization and collected by centrifugation for 10 min at 100 *g*. For hypotonic treatment, cells were resuspended in 1 ml of 75 mM KCl for 20 min at 4°C. After addition of 1 ml of methanol:glacial acetic acid (3:1), the cells stood for 10 min at room temperature and were centrifuged. Cell pellets were fixed for another 1 h in 0.5 ml of methanol:acetic acid (3:1) on ice. Final cell pellets were collected by centrifugation, resuspended in 100 µl of methanol:acetic acid (3:1), dispensed onto glass slides and air-dried. Samples were stained by spreading 50 µl of 0.1% crystal violet under a coverslip. The percentage of cells in mitosis was determined microscopically by counting 500 cells/slide. The mitotic index was calculated as the percentage of mitotic cells in drug-treated cultures

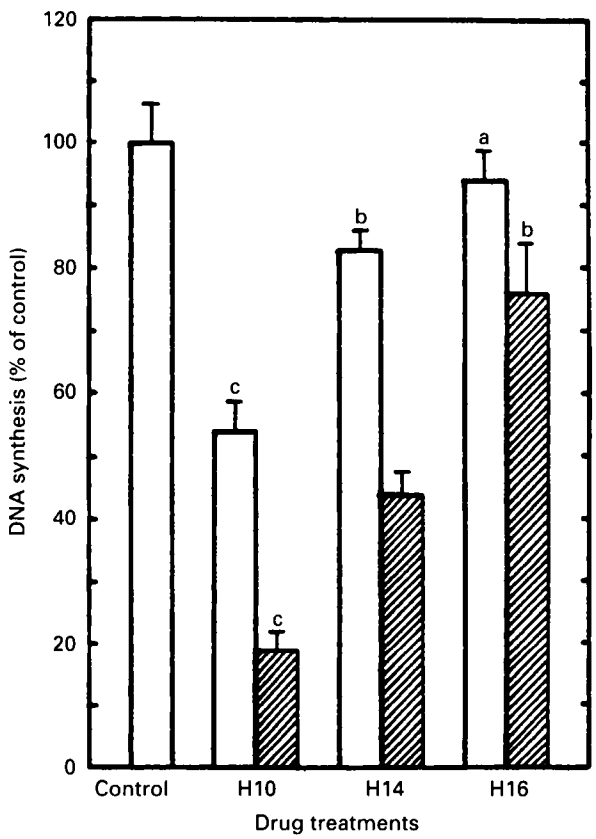

Figure 2. Comparison of the inhibitory effects of TP analogs on DNA synthesis in EMT-6 tumor cells *in vitro*. EMT-6 cells (1×10^5 cells/0.5 ml/well) were plated for 24 h at 37°C, incubated for 2 h in the presence or absence (control) of 10 µM (open) and 25 µM (striped) concentrations of the indicated drugs, and then pulse labeled for an additional 60 min to determine the rate of [^{3}H]thymidine incorporation into DNA. DNA synthesis in vehicle-treated control cells was 100 622±6339 c.p.m. (100±6%). The blank value (230±12 c.p.m.) for cells incubated and pulse labeled at 0°C with 1 µCi [^{3}H]thymidine has been subtracted from the results. Bars: means±SD (n=3). [a]Not significantly different from control; [b]$p<0.025$, significantly smaller than control; [c]$p<0.005$, smaller than respective H14 concentrations.

divided by the percentage of mitotic cells in non-treated controls.[17,18] Data of all *in vitro* experiments were analyzed using Student's *t*-test with the level of significance set at $p < 0.05$.

Results

Drugs

The chemical structures of the TP analogs tested for their antitumor activity against EMT-6 mammary sarcoma cells are shown in Figure 1. Because these compounds were synthesized in Dr DH Hua's chemistry laboratory,[11,13,14] the code names used in the biological studies include the letter H followed by serial numbers indicating the order of synthesis.[12,15] From top to bottom, the three rings of the TPs are labeled A, B and C. With a pyridyl ring at C-3 of the A-ring, a pyran B-ring and no alkylation at C-7 of the C-ring, the correct nomenclature for H10 is 3-(3-pyridyl)-1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano [4,3-*b*][1] benzopyran.[11] Addition of isopropenyl group at C-7 of the 3-pyridyl benzopyran H10 yields H14, which is a (5a*S*,7*S*)-7-isopropenyl-3-(3-pyridyl)-1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran.[11] In contrast to H10, the TP H16 has a non-linear skeleton, a methyl group at C-3 of its A-ring and its central B-ring is a pyridine. The correct nomenclature for H16 is 1*H*-3-methyl-7,8,9,10-tetrahydro-pyrano[4,3-c]quinolin-1-one.

Inhibition of DNA synthesis by TPs

H16 has no or very little effect, but H10 and H14 inhibit DNA synthesis in a concentration-dependent manner (Figure 2). On an equal 10 or 25 μM concentration basis, H10 is significantly more effective than H14 in the EMT-6 tumor cell system. For instance, concentrations of 10 and 25 μM H10, respectively, inhibit DNA synthesis by 46 and 81%, whereas, under similar conditions, H14 inhibits this response by 17 and 56% (Figure 2). Concentrations of 1.6 μM or higher must be used to demonstrate the inhibitory effect of H10 on DNA synthesis (Figure 3). The concentration of H10 that inhibits DNA synthesis by 50% (IC_{50}) in EMT-6 cells is about 10 μM (Figure 3). Since H10 might disrupt MT dynamics, its ability to inhibit DNA synthesis was compared to that of a spectrum of representative antimitotic agents (Figure 4). At 10 μM, H10 inhibits DNA synthesis by 48%, and is significantly more potent than nocodazole and podophyllotoxin, whereas colchicine, paclitaxel and

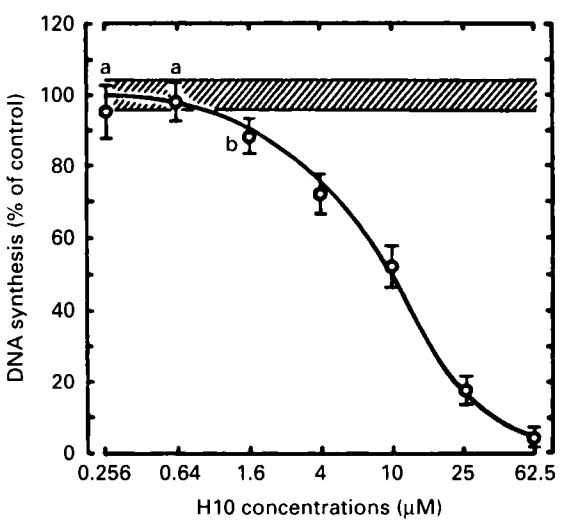

Figure 3. Concentration-dependent inhibition of DNA synthesis by the TP analog H10 (○) in EMT-6 cells *in vitro*. The protocol of the experiment was identical to that of Figure 2. DNA synthesis in vehicle-treated control cells was 114 476 ± 4 922 c.p.m. (100 ± 4%; striped area). The blank value (355 ± 19 c.p.m.) has been subtracted from the results. The concentrations of drugs are plotted on a logarithmic scale. Bars: means ± SD (n=3). [a]Not different from control; [b]$p < 0.025$, smaller than control.

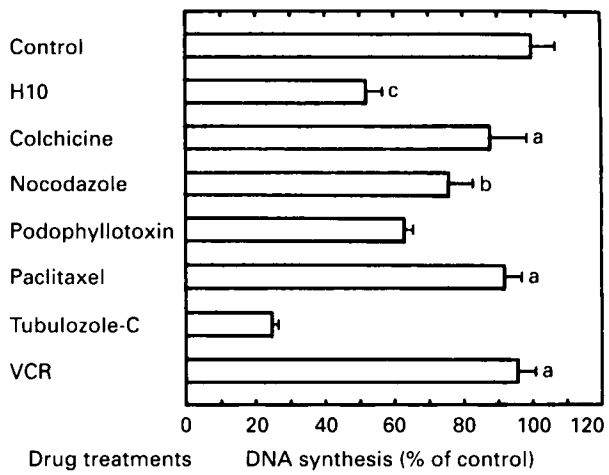

Figure 4. Comparison of the inhibitory effects of the TP analog H10 and a spectrum of known MT-disrupting agents on DNA synthesis in EMT-6 cells *in vitro*. The protocol of the experiment was identical to that of Figure 2 and the drugs were all tested at 10 μM. DNA synthesis in vehicle-treated control cells was 98 406 ± 7282 c.p.m. (100 ± 7%). The blank value (182 ± 14 c.p.m.) has been subtracted from the results. Bars: means ± SD (n=3). [a]Not different from control; [b]$p < 0.025$, smaller than control; [c]$p < 0.025$, smaller than nocodazole and podophyllotoxin but $p < 0.005$, greater than tubulozole-C.

VCR have no inhibitory effects. Tubulozole-C, which reduces this DNA response by 75%, is the only antimitotic drug more potent than H10 against DNA synthesis (Figure 4).

Antiproliferative activity of TPs

Over a 4 day period, there is a 17-fold increase in the number of control EMT-6 cells grown in the absence of drug *in vitro* (Figure 5). The ability of H10 to inhibit this rate of tumor cell growth is clearly concentration dependent at all time points tested. At 0.31 μM, H10 has no significant effect but concentrations of 1.25, 5 and 20 μM H10 increasingly reduce the number of untreated EMT-6 cells observed in control wells after 4 days by 50, 88 and 94%, respectively (Figure 5). At 10 μM, H10 is an antiproliferative agent more potent than H14, whereas H16 has no significant effect (Figure 6). When these antiproliferative effects are expressed as percentage of the increasing numbers of untreated tumor cells present each day in control culture wells, the effectiveness of each TP tested clearly increases over time. For instance, 10 μM H14 inhibits tumor cell growth by 24, 51, 64 and 74% at days 1, 2, 3 and 4, respectively. At 10 μM, the more

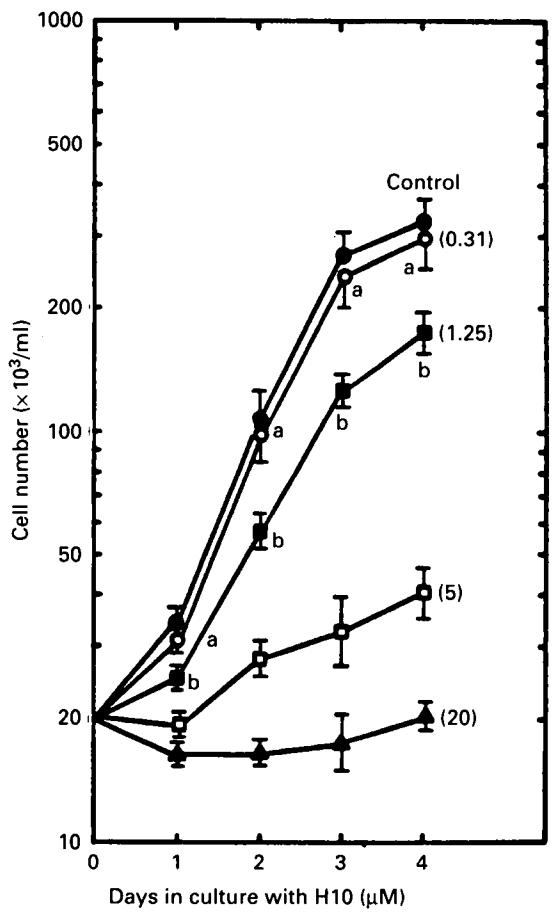

Figure 5. Concentration-dependent inhibition of the growth of EMT-6 cells *in vitro* by the TP analog H10. EMT-6 cells were plated at an initial density of 1×10^4 cells/0.5 ml/well, incubated at 37°C for 4 days in the presence or absence (●, control) of 0.3125 (○), 1.25 (■), 5 (□) and 20 μM H10 (▲) and their density was monitored in triplicate every 24 h using a Coulter counter. Cell numbers are plotted on a logarithmic scale. Bars: means ± SD (n=3). [a]Not different from control; [b]$p < 0.005$, smaller than control.

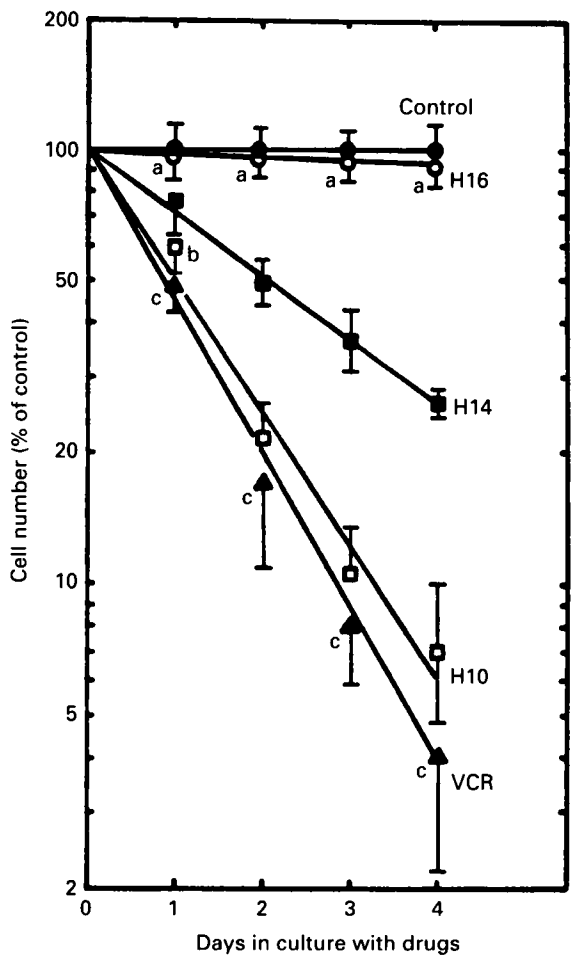

Figure 6. Comparison of the abilities of 10 μM concentrations of the TP analogs H10 (□), H14 (■) and H16 (○), and the known MT de-stabilizing agent VCR (▲) to inhibit the growth of EMT-6 cells over a 4 day period *in vitro*. The protocol of the experiment was identical to that of Figure 5. The results, expressed as percentage of the increasing numbers of vehicle-treated control cells (●) after 1 (32 157 ± 4 759 cells/ml), 2 (94 533 ± 12 006 cells/ml), 3 (227 495 ± 18 655 cells/ml) and 4 (308 821 ± 41 999 cells/ml) days in culture, are plotted on a logarithmic scale. Bars: means ± SD (n=3). [a]Not different from control; [b]$p < 0.025$, smaller than control; [c]not different from H10.

effective H10 reduces the same increasing numbers of untreated EMT-6 cells observed at 1, 2, 3 and 4 days in control wells by 40, 79, 90 and 93%, respectively. Interestingly, the magnitude of this antiproliferative effect of H10 matches that of 10 μM VCR (Figure 6). When full concentration-response curves are compared, however, it appears that the maximal antiproliferative activity of 10 μM H10 at 4 days can be mimicked by concentrations of VCR as low as 0.04–0.1 μM (Figure 7). Indeed, the smallest concentrations of VCR and H10 inducing significant antiproliferative effects are 1 nM and 0.78 μM, respectively. As a result, VCR (IC_{50}: 5 nM) is an inhibitor of EMT-6 tumor cell growth about 300 times more potent than H10 (IC_{50}: 1.5 μM) (Figure 7). Moreover, H10 cannot match the antiproliferative effects of the other antimitotic drugs tested (Figure 8). Although 2 μM H10 can inhibit tumor cell growth at 4 days by 62%, the six other known antimitotic agents tested at this concentration all inhibit this response by 85–95%, tubulozole-C being again the most effective (Figure 8).

Antitubulin activity of TPs

Glycerol and paclitaxel stabilize tubulin and lower the critical concentration (CC) of protein required to initiate its polymerization.[16] In the absence of 10% glycerol, therefore, purified tubulin cannot polymerize at protein concentrations below 10 mg/ml (Figure 9). However, the MT-stabilizing drug paclitaxel can easily induce the polymerization of such low concentration (2.2 mg/ml) of purified tubulin in our assay. At 25 μM, H10 cannot promote tubulin polymerization in the

Table 1. Comparison of the abilities of the TP analog H10 and the known MT de-stabilizing anticancer drug VCR to increase the mitotic index of EMT-6 mammary tumor cells *in vitro*

Drug treatment[a]	Mitotic cells (%)[b]	Mitotic index[c]
Control	1.93 ± 0.86	
H10 (0.25 μM)	2.85 ± 0.21[d]	1.48
VCR (0.25 μM)	23.20 ± 4.38[e]	12.02
H10 (2.5 μM)	13.72 ± 2.27[f]	7.11
VCR (2.5 μM)	33.84 ± 1.35	17.53

[a]EMT-6 cells (0.5×10^6 cells/0.5 ml/well) were incubated in triplicate for 24 h at 37 C in the presence or absence (control) of the indicated concentrations of drugs.
[b]Results are expressed as percent of a total of 500 cells scored for mitotic figures (means ± SD; n=3).
[c]Percent of mitotic cells in drug-treated cultures divided by the percent of mitotic cells in vehicle-treated controls.
[d]Not different from control.
[e]$p<0.05$; greater than 2.5 μM H10 and $p<0.025$, smaller than 2.5 μM VCR.
[f]$p<0.005$, greater than control.

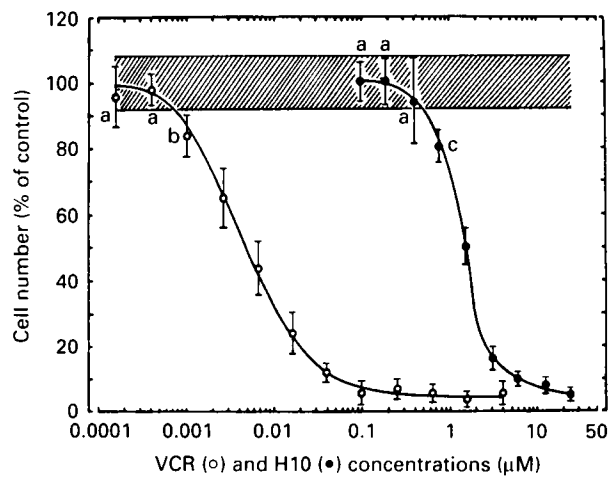

Figure 7. Comparison of the concentration-dependent inhibitions of the growth of EMT-6 cells *in vitro* by the TP analog H10 (●) and the known MT de-stabilizing agent VCR (○). The protocol of the experiment was identical to that of Figure 5. The results are expressed as percentage of the number of vehicle-treated control cells after 4 days in culture (336 173 ± 28 239 cells/ml; 100 ± 8%; striped area). Drug concentrations are plotted on a logarithmic scale. Bars: means ± SD (n=3). [a]Not different from control; [b]$p<0.05$ and [c]$p<0.025$, smaller than control.

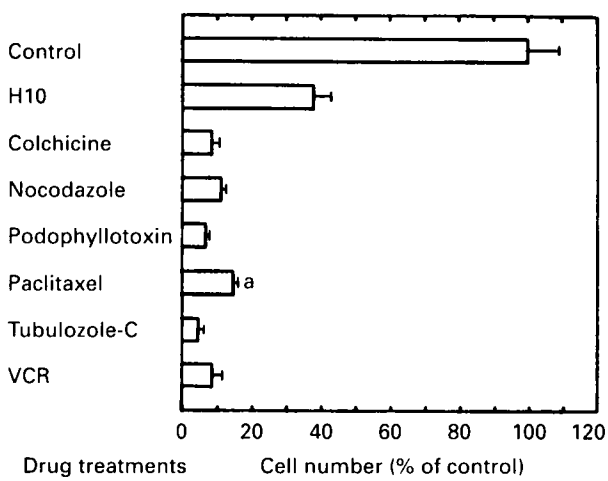

Figure 8. Comparison of the inhibitory effects of the TP analog H10 and a spectrum of known MT-disrupting agents on the growth of EMT-6 cells *in vitro*. The protocol of the experiment was identical to that of Figure 5 and all drugs were tested at 2 μM. The results are expressed as percentage of the number of vehicle-treated control cells after 4 days in culture (294 840 ± 25 887 cells/ml; 100 ± 9%). Bars: means ± SD (n=3). [a]$p<0.005$, smaller than H10.

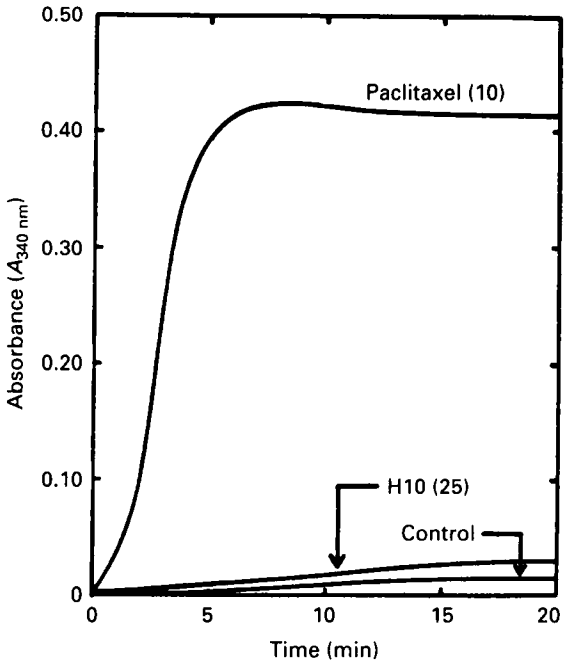

Figure 9. Comparison of the abilities of the TP analog H10 and a known MT-stabilizing anticancer drug to induce tubulin polymerization in the absence of glycerol. Purified tubulin was diluted to a final concentration of 2.2 mg/ml in 80 mM PIPES buffer, pH 6.8, containing 1 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP. The polymerization reactions were placed in quartz microcells and incubated at 35 C in the presence or absence (control) of 25 μM H10 or 10 μM paclitaxel. The rate of MT assembly was continuously monitored by scanning over 20 min the increase in turbidity at $A_{340\ nm}$. Assays were performed in duplicate.

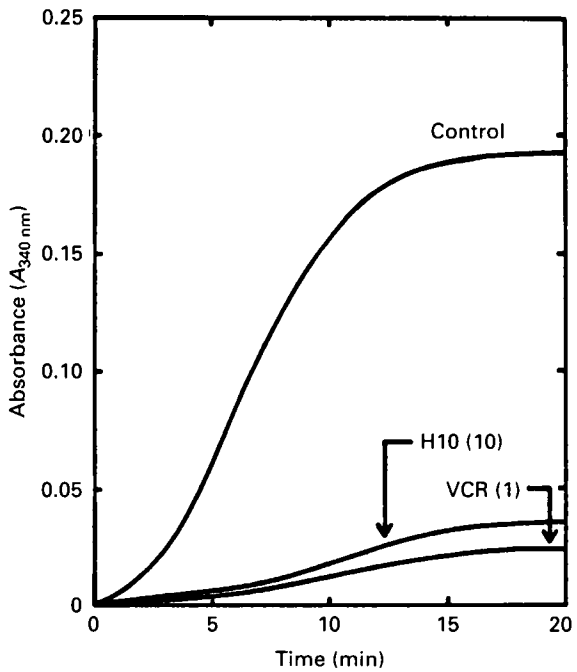

Figure 10. Comparison of the abilities of the TP analog H10 and a known MT de-stabilizing anticancer drug to inhibit tubulin polymerization in the presence of glycerol. The assay mixtures were identical to those of Figure 9 but contained 10% glycerol. The polymerization reactions were incubated in the presence or absence (control) of 10 μM H10 or 1 μM VCR and the rate of MT assembly was monitored as described in Figure 9. Assays were performed in duplicate.

absence of glycerol and, thus, is not a MT-stabilizing agent that blocks MT disassembly like paclitaxel (Figure 9). Figure 10 shows the three typical phases of MT polymerization occuring when purified tubulin (2.2 mg/ml) undergoes polymerization in the presence of 10% glycerol: a short lag phase, an exponential growth phase almost linear between 200 and 600 s, and a steady phase reaching a plateau after 15 min.[16] At 10 μM, H10 inhibits the rate and plateau of glycerol-induced tubulin polymerization by more than 80% and mimics the inhibitory effect of 1 μM VCR, indicating that this TP is a novel MT de-stabilizing agent that prevents MT assembly (Figure 10).

H10 inhibits the control rate of glycerol-induced tubulin polymerization between 200 and 600 s in a concentration-dependent manner and with an $IC_{50} = 1.5$ μM (Figure 11), which are identical to those observed for the concentration-dependent inhibition of EMT-6 tumor cell growth by this TP at day 4 (Figure 7). Even though VCR appears to be an antiproliferative agent about 300 times more potent than H10 in the EMT-6 tumor cell system (Figure 7), it inhibits the control rate of glycerol-induced tubulin polymerization between 200 and 600 s with an $IC_{50} = 0.15$ μM, which is only 10 times smaller than that of H10 (Figure 11).

In relation with their antiproliferative activities, 2.5 μM H10 inhibits the rate of tubulin polymerization more effectively than a similar concentration of H14, whereas H16 has no effect (Figure 12). Tubulozole-C is the most effective inhibitor of tubulin polymerization among a spectrum of five known MT de-stabilizing agents tested at 0.25 μM. Since 0.25 μM H10 cannot inhibit the rate of tubulin polymerization (Figures 11 and 12), a greater concentration of H10 (2.5 μM) must be used to match or surpass the inhibitions of tubulin polymerization caused by 0.25 μM colchicine, nocodazole, podophyllotoxin and VCR (Figure 12).

Antimitotic activity of TPs

In relation with its ability to inhibit tubulin polymerization and tumor cell growth, a 24 h treatment with 2.5 μM H10 produces a 7-fold increase in the mitotic

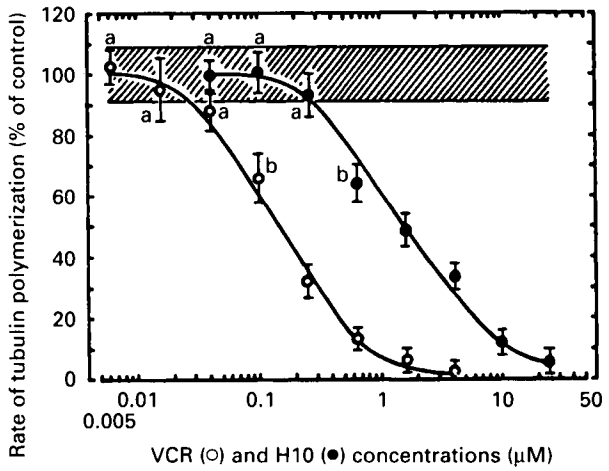

Figure 11. Comparison of the concentration-dependent inhibitions of tubulin polymerization in the presence of glycerol by the TP analog H10 (●) and the known MT destabilizing anticancer drug VCR (○). The conditions of the assays were identical to those of Figure 10. The polymerization reactions were incubated in the presence or absence (control) of the indicated concentrations of drugs, which are plotted on a logarithmic scale. Results are expressed as percentage of the rate of tubulin polymerization between 200 and 600 s in vehicle-treated control reactions ($\Delta A_{340\ nm} = 0.115 \pm 0.010$; $100 \pm 9\%$; striped area). Bars: means ± SD ($n=2$). [a]Not different from control; [b]$p < 0.05$, smaller than control.

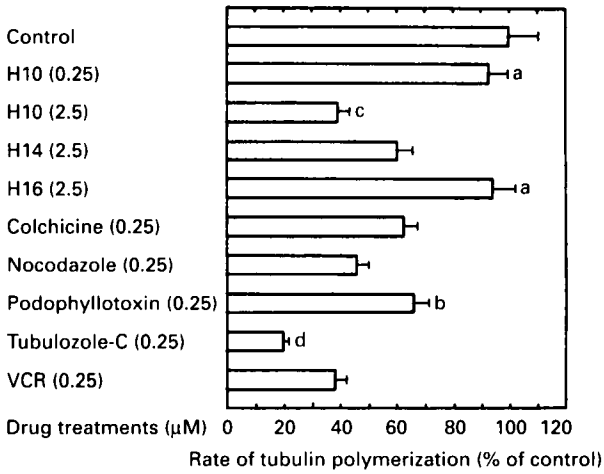

Figure 12. Comparison of the abilities of the TP analogs H10, H14 and H16, and a spectrum of known MT destabilizing agents to inhibit tubulin polymerization in the presence of glycerol. The conditions of the assays were identical to those of Figure 10. The polymerization reactions were incubated in the presence or absence (control) of the indicated concentrations of drugs. Results are expressed as percentage of the rate of tubulin polymerization between 200 and 600 s in vehicle-treated control reactions ($\Delta A_{340\ nm} = 0.126 \pm 0.013$; $100 \pm 10\%$). Bars: means ± SD ($n=2$). [a]Not different from control; [b]$p < 0.05$, smaller than control but not different from colchicine or H14; [c]$p < 0.05$, smaller than H14 and colchicine but not different from nocodazole or VCR; [d]$p < 0.05$, smaller than VCR.

index of EMT-6 cells but is ineffective at 0.25 μM (Table 1). In contrast, both of these concentrations of VCR increase to a greater degree than 2.5 μM H10 the percent of mitotic cells. The novel synthetic antitumor agent H10, therefore, may arrest cell cycle progression in M-phase but its antimitotic activity is weaker than that of VCR (Table 1).

Discussion

The KHJJ tumor line, derived from a primary mammary tumor arising in a BALB/c mouse after implantation of a hyperplastic alveolar nodule, originally contained mixed subpopulations of acidophilic spindle and basophilic epithelial neoplastic cells which, upon transplantation, formed dimorphic tumors with both sarcoma-like and carcinoma-like regions.[19] After the 30th transplant generation, however, only carcinoma-like patterns were observed. The EMT-6 mammary tumor cell line, derived from the 25th transplant generation of the KHJJ tumor, is composed only of elongated, acidophilic, spindle cells that produce morphologically uniform tumors resembling the original sarcoma-like pattern of the early passages of the KHJJ tumor.[19] Since EMT-6 mammary sarcoma cells have identical properties and respond similarly to therapeutic treatments *in vivo* and *in vitro*,[19,20] the present antitumor effects of H10 *in vitro* suggest that this TP might also inhibit the growth of EMT-6 mammary tumors *in vivo*. H10, which prevents murine L1210 lymphoblastic leukemia cells from synthesizing macromoledules and proliferating,[12,15] is also effective against human MCF-7 breast cancer cells *in vitro* (data not shown). Moreover, the concentration-dependent antitumor effects of H10 in the L1210[12,15] and EMT-6 tumor cell systems have comparable IC_{50} values for DNA synthesis (8.5 and 10 μM, respectively) and tumor cell growth (1.1 and 1.5 μM, respectively). The fact that H10 is equally effective against different tumor cell lines known to form ascites or solid tumors *in vivo* suggests that TPs might be valuable to develop a new synthetic class of anticancer drugs.

The discrepancies previously observed between the inhibitory effects of various TPs on nucleic acid synthesis and tumor cell growth in the L1210 system suggested that other molecular targets were involved in their mechanism of action and that these com-

pounds might inhibit tumor cell proliferation in relation with their ability to disrupt MT dynamics rather than macromolecule synthesis.[15] This hypothesis is substantiated by the finding that the inhibitions of tubulin polymerization and EMT-6 tumor cell growth by H10 both share similar IC_{50} values of 1.5 μM, whereas higher concentrations of H10 are required to inhibit DNA synthesis (IC_{50} = 10 μM) in the same EMT-6 system. Nevertheless, H10 is an inhibitor of DNA synthesis more potent than all other MT-disrupting agents tested, except tubulozole-C. In fact, only half of the known antimitotic drugs tested at 10 μM in our study can inhibit DNA synthesis in EMT-6 cells. The ability of colchicine to inhibit the incorporation of [^{3}H]thymidine into the DNA of mitogen-stimulated cells was not observed in this study.[21] Even though it is a weaker MT-disrupting agent than other antimitotic drugs, H10 might be a more versatile anticancer drug able to target a wider range of molecular events and affect several phases of the cell cycle because of its ability to inhibit nucleic acid and protein syntheses. Moreover, the antiproliferative activities of H10 and H14 in EMT-6 cells are related to the abilities of these TPs to inhibit both DNA synthesis and tubulin polymerization, whereas H16 fails to alter all three biomarkers. Because H10 increasingly blocks mitotic cells and slows down the rate of EMT-6 cell proliferation, the difference between the number of exponentially growing untreated control cells and the reduced number of H10-treated cells keeps increasing with the numbers of days in culture. These results suggest that the effectiveness of H10 as an inhibitor of mammary tumor cell growth increases over time *in vitro* and is a combination of drug concentration and duration of drug exposure.

The chemical structure of the 3-pyridyl benzopyran H10 has the most antitumor activity among 16 various TP analogs synthesized so far.[12,15] In spite of its effectiveness against DNA synthesis, a 3,4-dimethoxyphenyl benzopyran loses its ability to inhibit tubulin polymerization and L1210 tumor cell growth at concentrations below 25 μM, suggesting that a 3-pyridyl is a more desirable aryl substituent than a 3,4-dimethoxyphenyl.[15] Since substitution of the pyridyl ring by a methyl group at C-3 of the A-ring abolishes the effectiveness of H10 in the L1210 system, the presence of a nitrogen atom in the aromatic ring attached at C-3 appears essential for the antitumor activity of this TP.[15] Introduction of methyl group at C-5a and hydroxy or formyloxy groups at C-6 has previously been shown to considerably reduce the ability of H10 to inhibit DNA synthesis and proliferation in L1210 cells.[12] Even though it possesses the more desirable 3-pyridyl group at C-3, H14 is less effective than H10 against DNA synthesis, tubulin polymerization and EMT-6 cell growth, demonstrating that introduction of isopropenyl group at C-7 of the C-ring is clearly detrimental to the antitumor activity of the H10 structure. Since the structure of H16 differs in a number of ways from that of H10, it is difficult to precisely explain why H16 totally lacks antitumor activity without synthesizing and testing its 3-pyridyl analog. The major reason for its inactivity may be that H16 is a C-3 methyl substituent lacking the 3-pyridyl group so critical for the antitumor effects of H10. However, the L-shaped skeleton and central pyridine B-ring of H16 could also contribute to its inactivity. The linear skeleton of H10 should be tested for its ability to interact with tubulin at the vinblastine or colchicine binding sites.[22] Adding a substituent onto the cyclohexane C-ring or other chemical modifications that increase the molecular size or disrupt the linear skeleton of H10 might decrease the binding of TPs to tubulin and their antitumor activity. As compared to other MT-disrupting anticancer drugs currently used clinically,[22] the structure of H10 is very simple, easy to synthesize in few steps and in high yields, and its antitumor activity might be ameliorated by modification of the nitrogen-containing aromatic ring attached at C-3 of the A-ring and replacement of one carbon at positions C-7 or C-8 of the C-ring by a heteroatom, such as nitrogen.[11,13,14] Of course, the anticancer potential of TPs should be confirmed *in vivo* by testing the water-soluble carboxylic acid salt of H10 in mice inoculated with ascites or solid tumors.

The *Vinca* alkaloid VCR is a spindle poison which binds to tubulin, prevents MT assembly, causes metaphase arrest and kills cells attempting mitosis.[22-24] The IC_{50} of 5 nM characterizing the antiproliferative activity of VCR in the present EMT-6 cell system is within the 1.1-14 nM range of IC_{50} values reported for the cytostatic/cytotoxic effects of VCR and vinblastine in different cell lines.[22,25,26] At 10 μM, H10 can match the maximal inhibition of EMT-6 tumor cell growth caused by VCR but is not as potent as VCR since the IC_{50} for its antiproliferative activity is about 300 times greater than that of VCR. Although the polymerization of purified tubulin is assayed in a cell-free system, VCR inhibits EMT-6 cell proliferation with an IC_{50} value which is 30 times smaller than that required for this antimitotic drug to inhibit tubulin polymerization. In contrast, H10 inhibits tubulin polymerization with an IC_{50} value which is identical to that of its antiproliferative activity and only 10 times greater than that characterizing the inhibition of tubulin polymerization by VCR. This apparent discrepancy between the abilities of VCR to inhibit tubulin polymerization and EMT-6 cell growth suggests that, even though H10

inhibits DNA synthesis more than most antimitotic agents and blocks tubulin polymerization about 10 times less effectively than VCR, something is preventing H10 from inhibiting tumor cell growth also 10 times less than VCR. Either VCR disrupts other molecular events besides MTs in order to inhibit tumor cell growth so well, actions which H10 fails to mimic, or something is preventing H10 from fully expressing its MT de-stabilizing and antiproliferative potential in intact cells *in vitro*. The cellular uptake, distribution, metabolism and retention of H10 remain to be investigated. It is not known whether protein components of the serum-containing medium interact with H10 and limit its uptake, binding to tubulin and antiproliferative activity in cell culture. The fact that the concentrations of antimitotic agents effective in the tubulin polymerization assay are consistently higher than those with cytostatic activity has been noticed before.[27-32] Antimitotic drugs interacting with a few essential sites in the MTs might disrupt the mitotic spindle and be cytostatic over a 4-day period at concentrations much lower than those required to directly block tubulin polymerization.[32]

Tubulin is a labile protein, which is unstable below 80 mM PIPES, should not be exposed to pH values below 6.8 or above 7.0 and will not polymerize in the presence of Ca^{2+}.[16] The propensity of tubulin subunits to assemble into MTs is dependent upon their affinity for MT ends. In order to achieve MT polymerization, the value of this affinity (called CC) has to be less than the total concentration of free tubulin subunits.[16] GTP and Mg^{2+} are necessary for tubulin nativity, and glycerol stabilizes tubulin and lowers the CC required to initiate polymerization.[16] Paclitaxel, which also lowers the CC and eliminates the requirement for GTP, promotes tubulin polymerization in the absence of glycerol and stabilizes MTs by inhibiting their depolymerization.[22,33,34] The effect of paclitaxel on tubulin minus glycerol and the effect of glycerol on tubulin, therefore, can be used to screen for MT-stabilizing and de-stabilizing drugs.[22] A short lag phase is necessary to create nucleation sites, which are small tubulin oligomers from which larger MT polymers can form. Because MT polymerization is readily reversible, a given population of MTs is continually growing and shortening, a phenomenon called dynamic instability.[16] Thus, the growth phase observed between 200 and 600 s in Figure 10 reflects the rapid increase in the ratio of MT assembly:disassembly. Finally, a steady phase is established when the residual concentration of free tubulin heterodimer becomes equal to the CC required to initiate polymerization.[16] The kinetics of paclitaxel- or glycerol-induced MT polymerization shown in Figures 9 and 10 appear consistent with the initial concentration of 2.2 mg tubulin/ml used in our reactions.

In contrast to the MT-stabilizing drug paclitaxel,[22,33,34] the most effective antiproliferative concentration of H10 cannot induce the polymerization of tubulin minus glycerol, suggesting that this antimitotic TP neither promotes MT assembly nor blocks tubulin depolymerization and MT disassembly. However, H10 inhibits in a concentration-dependent manner the rate and plateau of glycerol-induced tubulin polymerization, demonstrating that this TP blocks MT assembly like the known MT de-stabilizing drugs that bind to tubulin on the colchicine or vinblastine sites.[22] At 1.5 μM, the IC_{50} value for the inhibition of glycerol/Mg^{2+}-induced polymerization (rate) of purified tubulin by H10 is within, or very close to, the following ranges of IC_{50} values reported for known MT de-stabilizing drugs using the turbidity assay: colchicine, 0.2–20 μM; nocodazole, 1–5 μM; podophyllotoxin, 0.3–3 μM; tubulozole-C, 0.3–0.5 μM; and VCR, 0.1–2 μM.[22,27,28,32,35-37] The superior inhibitions of tubulin polymerization, DNA synthesis and EMT-6 cell growth caused by tubulozole-C in our study confirm the reports that tubulozole-C is a more potent antitubulin agent than nocodazole and colchicine, and is more effective against solid tumors than VCR.[22,35,37-39] H10 might inhibit tubulin polymerization as a consequence of its binding interaction with the α/β tubulin dimer, though other mechanisms such as interactions with Mg^{2+} ions or GTP are conceivable.[22]

Based on its ability to disrupt MT dynamics, H10 would be expected to arrest cells in G_2/M phase. The mitotic index can differentiate between the antimitotic drugs that cause G_2 or M phase arrest. Agents that arrest cells in M phase, such as VCR, increase the mitotic index but agents that cause G_2 arrest, such as etoposide (VP-16), decrease it.[18] Since H10 increases the percentage of mitotic figures and the mitotic index within 24 h, it is clearly capable of causing metaphase arrest and blocking the progression of EMT-6 tumor cells in the M phase of their cycle like VCR. TPs, which inhibit nucleic acid and protein syntheses, might also decrease tubulin production and alter other phases of the cell cycle.

Conclusion

TPs represent a novel synthetic class of MT de-stabilizing drugs, which block tubulin polymerization and prevent mammary tumor cells from synthesizing DNA and proliferating *in vitro*. The anticancer potential and molecular mechanism of action of these antimitotic drugs warrants further investigation.

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