Anti-Cancer Drugs 1999, **10**, pp. 489-504

Report

Tricyclic pyrone analogs: a new synthetic class of bifunctional anticancer drugs that inhibit nucleoside transport, microtubule assembly, the viability of leukemic cells *in vitro* and the growth of solid tumors *in vivo*

Elisabeth M Perchellet, James B Ladesich, Molly J Magill, Yi Chen,[1] Duy H Hua[1] and Jean-Pierre Perchellet

Anti-Cancer Drug Laboratory, Division of Biology, Ackert Hall, and [1]Department of Chemistry, Kansas State University, Manhattan, KS 66506-4901, USA.

Tricyclic pyrones (TPs) may represent a novel synthetic class of microtubule (MT) de-stabilizing anticancer drugs previously shown by us to inhibit macromolecule synthesis, tubulin polymerization, and the proliferation of leukemic and mammary tumor cells *in vitro*. A linear skeleton with a N-containing aromatic ring attached at C3 of the top A-ring, a central pyran B-ring and a six-membered bottom C-ring with no alkylation at C7 are required for the antitumor activities of the lead compounds, a 3-pyridyl benzopyran (code name H10) and its somewhat weaker 2-pyridyl regioisomer (code name H19). Increasing concentrations of H10 do not alter the binding of [^{3}H]vinblastine and [^{3}H]GTP to tubulin but mimic the ability of unlabeled colchicine (CLC) to reduce the amount of [^{3}H]CLC bound to tubulin, suggesting that TPs may interact with the CLC binding site to inhibit tubulin polymerization. Exogenous Mg^{2+} cations absolutely required for the binding of GTP to tubulin and MT assembly cannot overcome the antitubulin action of H10. H10 reduces the viability of L1210 cells *in vitro* (IC_{50}: 0.5 μM) but its antitumor activity may be related to its ability to inhibit tubulin polymerization and rapidly increase the mitotic index rather than to induce DNA cleavage and apoptosis. The anticancer potential of TPs *in vivo* is demonstrated by the fact that i.p. injections of the water-soluble H10–HCl decrease the growth of solid tumors in mice inoculated s.c. with Lewis lung carcinoma. A critical finding is that the antimitotic H10 is a bifunctional anticancer drug, which also blocks the cellular transport of nucleosides (IC_{50}: 6 μM) to inhibit DNA synthesis. Since few CLC site-binding antimitotic agents are active in solid tumor models *in vivo*, the ability of these new MT de-stabilizing TPs to totally block nucleoside transport might be valuable in polychemotherapy to arrest tumor cells at several phases of their cycle, potentiate the action of antimetabolites and sensitize multidrug-resistant tumor cells. [© 1999 Lippincott Williams & Wilkins.]

Key words: Apoptosis, DNA cleavage, DNA synthesis, L1210 cell viability, Lewis lung carcinoma, mitotic index, nucleoside transport, tubulin binding site and polymerization.

Introduction

Novel tricyclic pyrone (TP) analogs, derived from the tetracyclic structures of pyripyropene and arisugacin, have been synthesized in Hua's laboratory, using a simple one-pot coupling reaction of pyrones and enals.[1-4] When screened for their antitumor activity in Perchellet's laboratory, these TPs are designated by a code name, including the letter H followed by a serial number indicating the order of synthesis.[5-7] From top to bottom, the three rings of the TPs are labeled A, B and C. When tested at 50 μM, a tetracyclic pyripyropene analog and a monocyclic pyrone are totally inactive but several 3,5a,6-substituted 1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyrans decrease the ability of L1210 leukemic cells to synthesize DNA and grow *in vitro* by 79-100%.[5]

The aryl groups of these antitumor agents are either 3,4-dimethoxyphenyl or 3-pyridyl and TPs lacking such

---

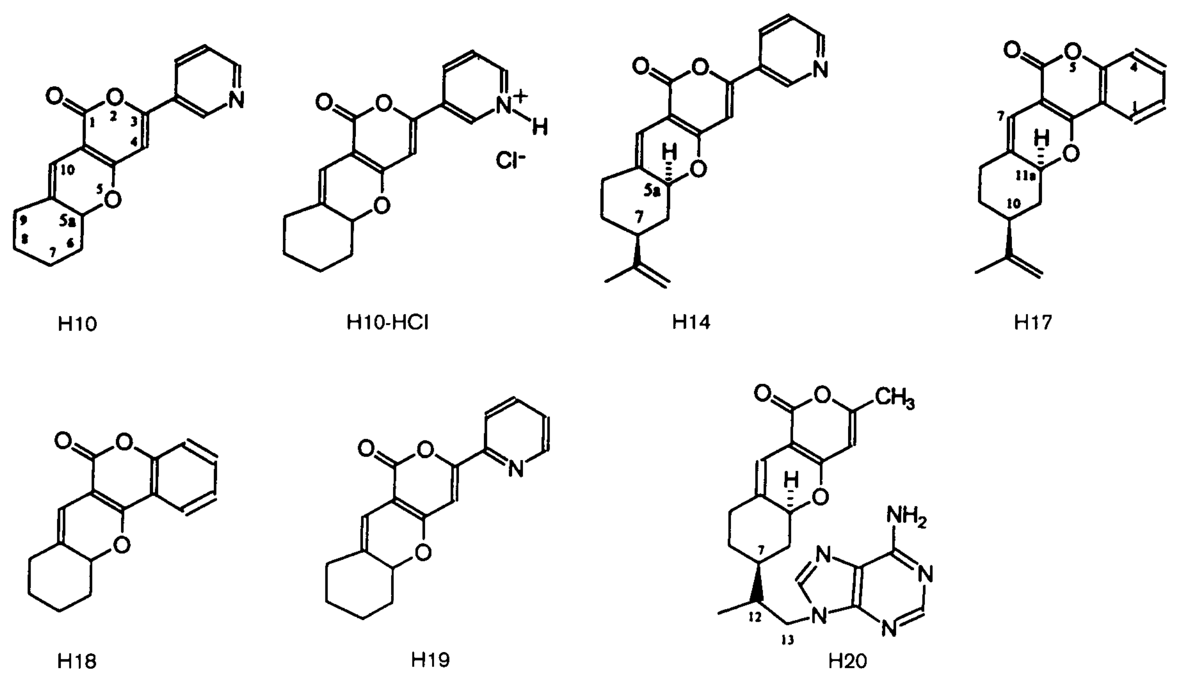
This study was supported by a Special Group Incentive Research Award from Kansas State University, BioServe Space Technologies (NASA Grant NAGW-1197), the American Heart Association, Kansas Affiliate (grant KS-96-GS-69), Great Plains Diabetes Inc. (Wichita, KS) and the Center for Basic Cancer Research at Kansas State University.

Correspondence to J-P Perchellet, Anti-Cancer Drug Laboratory, Division of Biology, Ackert Hall, Kansas State University, Manhattan, KS 66506-4901, USA.
Tel: (+1) 785 532-7727; Fax: (+1) 785 532-6653;
E-mail: jpperch@ksu.edu

aryl groups at C3 of their A-ring have no or very little antitumor activity.[5] However, at concentrations of 25 μM or below, which are required to assess structure-activity relationships (SARs), the most effective antitumor TP synthesized so far is the 3-pyridyl benzopyran H10,[6] which is our lead compound (Figure 1). The hypothesis that the N atom in the aromatic ring attached at C3 is crucial for the antitumor activity is substantiated by the fact that the 3-pyridyl benzopyran H10 is much more potent against tubulin polymerization and L1210 cell growth/viability than the 3,4-dimethoxyphenyl benzopyran H5, and deletion or substitution of the pyridyl ring by a methyl group at C3 decreases the effectiveness of H10.[5,6] Introductions of an isopropenyl group at C7, methyl group at C5a and hydroxy or formyloxy groups at C6 also reduce considerably the antiproliferative activity of H10.[5,6] Moreover, a non-linear TP with a central pyridine B-ring and missing the essential 3-pyridyl ring at C3 has no bioactivity.[7] Our state of the knowledge, therefore, is that a linear skeleton with a pyridyl ring at C3 of the top A-ring, a central pyran B-ring and a six-membered bottom C-ring with no alkylation at C7 are the preferred structures for the antitumor activity of these TPs.[5-7]

H10 has already been shown to inhibit the synthesis of DNA, RNA and protein, and the proliferation of murine L1210 leukemic and EMT-6 mammary tumor cells *in vitro* to the same maximal degree than a spectrum of known anticancer drugs used clinically but is an antitumor agent several fold less potent on an equal molecular basis.[5-7] The short-term inhibition of nucleic acid synthesis by H10 is reversible following drug removal but its long-term inhibition of tumor cell growth is not reversible after 24 h of drug exposure.[6] In L1210 cells, some TPs are more potent than H10 against DNA synthesis but their antiproliferative activities are much weaker than that of H10, a discrepancy suggesting that other molecular targets may be involved in their mechanism of action.[6] Indeed, the antiproliferative activity of TPs may be linked to their ability to disrupt microtubule (MT) dynamics and work as novel spindle poisons.[6]

Since H10 mimics the inhibition of tubulin polymerization caused by vincristine (VCR) in the presence of 10% glycerol but not the induction of tubulin polymerization caused by paclitaxel in the absence of 10% glycerol, TPs may represent a novel synthetic class of MT de-stabilizing drugs that prevent MT assembly.[6,7] Interestingly, H10 is less potent than VCR against glycerol-induced tubulin polymerization in a 20 min turbidity assay (IC_{50}: 1.5 versus 0.15 μM) and the proliferation of EMT-6 cells after 4 days in culture (IC_{50}: 1.5 μM versus 5 nM) but inhibits DNA synthesis after 2 h in EMT-6 cells (IC_{50}: 10 μM) more effectively than all other known MT-disrupting agents tested, except tubulozole-C.[7] Although H10 inhibits DNA synthesis and might affect several phases of the cell cycle, its ability to block MT assembly and increase



H10 H10-HCl H14 H17

H18 H19 H20

Figure 1. Chemical structures and code names of the TP analogs tested for their antitumor activity.

the mitotic index of tumor cells indicates that these novel TPs may be cell cycle-specific anticancer drugs useful for arresting mammalian cells in M phase.[6,7]

Since the MT de-stabilizing H10 might have a wider spectrum of antitumor effects than VCR, the present study was undertaken to (i) demonstrate the antitumor potential of water-soluble H10 against solid tumor *in vivo*, (ii) elucidate further the molecular mechanism of action of H10 by characterizing its interaction with tubulin and DNA, and (iii) screen novel H10 analogs to clarify SARs *in vitro*.

Materials and methods

Cell culture and drug treatments

All solutions of synthetic TPs and VCR (a gift from Lilly Research Laboratories, Indianapolis, IN), vinblastine (VBL), colchicine (CLC) and camptothecin (CPT) (all from Sigma, St Louis, MO) were dissolved and diluted in dimethyl sulfoxide (DMSO), whereas daunomycin (DAU; from Sigma) solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl. Suspension cultures of murine L1210 lymphocytic leukemia cells (ATCC, Rockville, MD) were maintained in continuous exponential growth in RPMI 1640 medium supplemented with 7.5% fortified bovine calf serum (FCS; HyClone, Logan, UT) and penicillin (100 IU/ml)-streptomycin (100 μg/ml), and incubated in the presence or absence of drugs at 37°C in a humidified atmosphere containing 5% CO_2 essentially as described previously.[5-7]

Nucleoside transport and DNA synthesis

L1210 cells (10^6/0.5 ml) were preincubated for 15 min at 37°C in the presence or absence of TPs and then exposed to 1 μCi of [methyl-^{3}H]thymidine (50 Ci/mmol; Amersham, Arlington Heights, IL) for only 15-30 s to assess the cellular uptake of nucleoside over such a very short period of time.[8] Reactions were diluted with 2 ml of ice-cold Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) and the unincorporated radiolabel was removed by centrifugation at 200 *g* for 10 min. After washing thrice with 2 ml of ice-cold PBS, intact cell pellets were harvested by centrifugation and incubated for 30 min in 1 ml of hypotonic lysis buffer (HLB) containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.2% Triton X-100. Cell lysates were mixed with 9 ml of Bio-Safe II (Research Products International, Mount Prospect, IL) and counted to estimate the cellular uptake of [^{3}H]thymidine. Drug inhibition was expressed as percent of [^{3}H]thymidine transported into vehicle-treated control cells over similar 15-30 s periods.[8] For DNA synthesis, L1210 cells (10^6/0.5 ml of FCS-free RPMI 1640 medium) were preincubated for 90 min at 37°C in the presence or absence of TPs and then pulse-labeled with 1 μCi of [^{3}H]thymidine for another 30 min to estimate the rate of incorporation of [^{3}H]thymidine into DNA over such a longer period of time.[5,6] Incubations were terminated by the addition of 0.5 ml of 10% trichloroacetic acid (TCA) and the radioactivity bound to the TCA-precipitable material was recovered over Whatman GF/A glass microfiber filters, washed thrice with 2 ml of 5% TCA and twice with 2 ml of 100% EtOH, and determined by liquid scintillation counting (LSC) in 10 ml of Bio-Safe NA (Research Products International) as reported previously.[5,6]

Cell viability and mitotic index

L1210 cells suspended in FCS-containing RPMI 1640 medium were grown in 48-well Costar cell culture plates (Costar, Cambridge, MA) for 4 days in the presence or absence of TPs to evaluate drug cytotoxicity and calculate the mitotic index. Decreasing concentrations of cells were initially plated at time 0 in order to collect control samples with approximately equal cell densities after 1, 2, 3 and 4 days in culture. The viability of TP-treated cells was assessed from their ability to bioreduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product which absorbs at 490 nm.[9] At the appropriate time after drug treatment, cell samples (about 10^6/0.5 ml/well) were further incubated at 37°C for 3 h in the dark in the presence of 0.1 ml of MTS:PMS (2:0.1) reagent and their relative cell viability was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL). Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were substracted from the results. At the appropriate time after drug treatment, cell populations were collected by centrifugation at 200 *g* for 10 min to determine their mitotic index.[10,11] For hypotonic treatment, cells were resuspended in 1 ml of 75 mM KCl for 20 min at 4°C. After fixation in 1 ml of MeOH:acetic acid (3:1), the final cell pellets were collected by centrifugation, resuspended in 75 μl of MeOH:acetic acid (3:1), dispensed onto glass slides, air dried and stained by spreading 40 μl of 0.1% crystal violet under a coverslip as described before.[7] The percent of cells in mitosis was determined microscopically by counting 500 cells/slide. The mitotic index was calculated as the percent of mitotic cells in drug-treated cultures divided by the percent of mitotic cells in non-treated controls.[7,10,11]

Tubulin polymerization and binding assays

The polymerization of purified tubulin protein from bovine brain in the presence of glycerol was analyzed using the Tubulin/Microtubule Biochem kit purchased from Cytoskeleton (Denver, CO).[12] The polymerization reactions contained, in a final volume of 0.2 ml, tubulin (2.2 mg/ml) in 80 mM PIPES buffer, pH 6.8, supplemented with 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP and 10% glycerol.[6,7] MT de-stabilizing drugs were added to the assay mixture in 1 μl of DMSO:tubulin buffer (40:60) as reported previously.[6,7] Samples were immediately incubated at 35°C in quartz microcells, and the rate and plateau of tubulin polymerization were followed over 20-30 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.[6,7]

Tubulin binding assays were performed using the DEAE-cellulose filter method.[13,14] Increasing concentrations of unlabeled TP, VBL, CLC and GTP were compared for their ability to alter the bindings of [^{3}H(G)]VBL sulfate (5.3 Ci/mmol; Moravek Biochemicals, Brea, CA), [ring C, methoxy-^{3}H]CLC (61.4 Ci/mmol; NEN Life Science Products, Boston, MA) and [8-^{3}H]GTP tetrasodium salt (10 Ci/mmol; ICN Pharmaceuticals, Irvine, CA) to purified tubulin. Tubulin was diluted to a final concentration of 0.5 mg/ml in 80 mM PIPES buffer, pH 6.8, containing 1 mM EGTA and 1 mM $MgCl_2$ (PEM buffer). Glycerol must be omitted since CLC binds to tubulin dimers but not to polymerized MTs.[15] The assay mixtures (0.1 ml) were supplemented with increasing concentrations of unlabeled drugs and then incubated with [^{3}H]VBL (0.5 μCi, 10 μM) or [^{3}H]CLC (0.5 μCi, 1 μM) for 90 min at 37°C, or with [^{3}H]GTP (1 μCi, 5 μM) for only 10 min at 4°C.[16] After dilution with 5 ml of ice-cold 0.1 × PEM buffer, the CLC and VBL reaction mixtures were filtered through stacks of three Whatman DE81 ion exchange paper disks and the drug-protein complexes retained on the filters were washed with 3 × 10 ml of 0.1 × PEM buffer to eliminate residual levels of free radiolabeled drugs. However, the GTP binding reactions were diluted with 2.5 ml of ice-cold 0.1 × PEM buffer and the filters were washed only once with 2.5 ml of this buffer to avoid gradual dissociation of the GTP-tubulin complex on the filters during chronic washing.[16] After drying the filters, the radioactivity bound to tubulin was estimated by LSC in 20 ml of Bio-Safe NA. Control VBL, CLC and GTP binding assays were incubated in the absence of unlabeled drugs and blank values for free [^{3}H]VBL, [^{3}H]CLC or [^{3}H]GTP absorbed on filters in the absence of tubulin were substracted from the results.

DNA cleavage and apoptosis

Drug-induced DNA cleavage was determined by intact chromatin precipitation, using 10^6 L1210 cells which were prelabeled with 1 μCi of [^{3}H]thymidine for 2 h at 37°C, washed with 3 × 1 ml of ice-cold PBS, collected by centrifugation and resuspended in fresh medium containing FCS.[17,18] Such cells containing prelabeled DNA were then exposed for 24 h to TPs and drugs known to induce DNA fragmentation. After centrifugation at 200 *g* for 10 min to discard the drugs and wash the cells, the intact cell pellets were lysed for 30 min in 0.5 ml of HLB, centrifuged at 12 000 *g* for 30 min to collect the supernatants and resuspended in 0.5 ml of HLB. After another similar centrifugation, the radioactivities in the pooled supernatants (detergent-soluble low molecular weight DNA fragments) and the pellet (intact chromatin DNA) were determined by LSC: % DNA fragmentation = [c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100.[17,18]

For apoptosis, L1210 cells were incubated for various periods of time in the presence or absence of drugs and DNA was extracted from samples with equal cell densities (10^7/ml), using a salting out procedure.[19,20] After centrifugation at 200 *g* for 10 min, the cell pellets were washed twice with PBS and lysed overnight at 37°C in 0.34 ml of 10 mM Tris-HCl, pH 8.0, containing 2 mM EDTA, 400 mM NaCl, 1% SDS and proteinase K (0.5 mg/ml). Cell lysates were vortexed for 15 s with 0.1 ml of 6 M NaCl and centrifuged (3000 r.p.m. × 30 min). The supernatants (0.44 ml) were mixed with 0.88 ml of ice-cold 100% EtOH and kept at 4°C for 15 min for DNA precipitation. After centrifugation (13 000 r.p.m. × 15 min) at 4°C, the air-dried DNA pellets were dissolved in 0.34 ml of 10 mM Tris-HCl, pH 8.0, with 1 mM EDTA (TE buffer) and incubated for 2 h at 37°C in the presence of RNase (0.1 mg/ml). After another round of EtOH precipitation and centrifugation, the final air-dried pellets were dissolved in 50 μl of TE buffer and their DNA concentrations determined spectrophotometrically at 260 nm. Equal amounts of DNA samples (@ 3 μg/7.5 μl of TE buffer) were mixed with 1.5 μl of

10 mM Tris-HCl, pH 7.5, containing 50 mM EDTA, 10% Ficoll 400 and 0.4% orange G, and loaded on each lane. About 0.5 μg of 100 bp DNA ladder and 0.75 μg of lambda DNA/*Eco*RI+*Hind*III (both from Promega) were similarly applied to each gel to provide size markers in the range 100-1500 and 125-21 226 bp, respectively. Horizontal electrophoresis of DNA samples was performed for 3.7 h at 60 V in 1.5% agarose gels containing ethidium bromide (1 μg/ml) with 90 mM Tris-HCl, pH 8.0, containing 90 mM boric acid and 2 mM EDTA as a running buffer. DNA fragments were visualized and photographed with Polaroid 667 film under UV transillumination.[20]

Solid tumor model *in vivo*

Lewis lung carcinoma (LL/2) cells (ATCC) were maintained in continuous exponential growth by twice a week passage in RPMI 1640 medium supplemented with 7.5% FCS and penicillin (100 IU/ml)-streptomycin (100 μg/ml). For injection, mixed monolayer and suspension cultures were collected by aspirating several times with medium, washed and centrifuged (200 *g* for 10 min) twice in PBS, and resuspended in sterile 0.9% NaCl at a density of 10^6 LL/2 cells/0.2 ml. Female C57BL/6 mice (20 g), from Harlan Sprague Dawley (Indianapolis, IN), were inoculated s.c. in the right abdominal flank with 10^6 LL/2 cells/0.2 ml of sterile 0.9% NaCl on day 0 and randomized (six or seven animals per dose). Doses of water-soluble H10-HCl (Figure 1) ranging from 8 to 30 μg/g body weight were injected i.p. in 0.2 ml of 0.9% NaCl, pH 2.0, either once a day from days 1 to 6 (experiment 1) or every other day from days 1 to 11 (experiment 2). Starting on day 1, LL/2-inoculated control mice received similar i.p. injections of 0.2 ml of vehicle at the above frequencies. Mice were sacrificed on days 14 (experiment 1) or 20 (experiment 2), and their primary s.c. solid tumors were excised and weighed. Antitumor activity was determined by expressing the mean tumor weights of the TP-treated mice as percent of the mean tumor weights of the untreated control mice.

Data of all biochemical and tumor experiments were analyzed using Student's *t*-test with the level of significance set at $p < 0.05$.

Results

Drugs

Since their inhibition of DNA synthesis and L1210 and EMT-6 tumor cell growth have already been established *in vitro*,[6,7] H10 and H14 can be used as references to assess and compare the antitumor effects of the newly synthesized TP analogs illustrated in Figure 1. The most potent antitumor TP discovered so far is H10, a racemic compound with a 3-pyridyl moiety and a stereocenter at C5a.[1,6,7] 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 and addition of an isopropenyl group at C7 of 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. H14 is an optically pure material containing two stereocenters at C5a and C7. H10-HCl, a water-soluble hydrochloric salt of H10, exists most likely as the depicted ammonium chloride salt (ionic species). H19, a 2-pyridyl analog of H10, is also a racemic compound with a stereocenter at C5a. The correct nomenclature for this regioisomer of H10 is 3-(2-pyridyl)-1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran. H18 possesses a benzene ring fused with its 2-pyrone A-ring and misses the important 3-pyridyl ring attached at C3 of the pyrone ring of H10. H18, a tetracyclic pyrone with a chromen-2-one ring system, is a racemic compound with a stereocenter at C11a. Its nomenclature is 6-oxo-6*H*,8*H*-9,10,11,11a-tetrahydro-[1]benzopyrano[4,3-*b*][1]benzopyran. H17 is an optically pure analog of H18 containing two stereocenters at C10 and C11a. H17, which contains an isopropenyl group at C10 of its TP skeleton, is a (10*S*,11a*S*)-10-isopropenyl-6-oxo-6*H*,8*H*-9,10,11,11a-tetrahydro-[1]benzopyrano[4,3-*b*][1]benzopyran. H20 is an optically active compound containing an adenine moiety attached to the isopropyl substituent at C7 of its TP nucleus. This 1-methylethyl group at C7 represents a linker between the TP and adenine. The nomenclature of H20 is (5a*S*,7*S*)-7-[2-(*N*9-adeninyl)-1-methylethyl]-3-methyl-1*H*,7*H*,5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran (Figure 1).

Cytotoxicity of TPs

Newer TP analogs were screened for their cytotoxicity *in vitro* in an attempt to improve on the potency of H10 and select the most promising agent for preclinical study (Figure 2). Indeed, decreased tumor cell viability after several days of drug treatment may be a better predictor of anticancer activity than antiproliferation since growth delay may allow survivors to resume dividing and expand clonally once the drug is metabolized and its effect is waning.[21] However, on an equal molecular basis, none of the new TP analogs proved to be more effective than H10 at decreasing the viability of L1210 cells over a 4-day period *in vitro* (Figure 2). All TPs inhibit tumor cell viability when tested at 25 μM, H10 and H19 producing nearly total inhibition (Figure 2). Besides the known H10, however, very few TPs remain effective at lower concentrations. H14 has a marginal effect at 5 μM and only the new H19, which closely resembles H10, can inhibit tumor cell viability by 61% at 5 μM and 22% at 1 μM but these effects are still weaker than those of H10, which reduces cell viability by 90% at 5 μM and 68% at 1 μM (Figure 2).

The relative potencies of H10 and H19 are more evident when full concentration-response curves are compared in Figure 3, where the striped area at 100% represents the control levels of viable L1210 cells growing in culture at days 2 and 4. The magnitudes of the cytotoxic effects of both TPs are clearly related to the combination of their increasing concentration and duration of action (Figure 3). For instance, 0.256 μM H10 and 0.64 μM H19 are ineffective at day 2 but their cytotoxicities become apparent at day 4. Moreover, 0.64 μM H10 and 1.6 μM H19 are marginally cytotoxic at day 2 (16 and 11% inhibition, respectively) but decrease cell viability by 57 and 49%, respectively, at day 4. As a result, the cytotoxic activities of H10 and H19 are, respectively, characterized by IC_{50} values of 2.5 and 7.5 μM at day 2 but 0.5 and 1.5 μM at day 4, suggesting that H10 is about 3 times more potent than its regioisomer H19 (Figure 3). Under similar conditions, however, the cytotoxicity of the known MT destabilizing drug VCR is characterized by an $IC_{50} \leqslant$ 5 nM (data not shown). Thus, the most bioactive H10 was the only TP worth selecting for *in vivo* testing.

Anticancer potential *in vivo*

H10-HCl (Figure 1) is fully water soluble at acid pH, whereas H10 needs to be sonicated in potentially toxic solvents for solubilization and i.p. injection in 0.2 ml of a mixture of EtOH:Tween 80:PBS (15:10:75) *in vivo*. Therefore, to demonstrate the anticancer potential of TPs *in vivo*, the water-soluble HCl form of H10 was

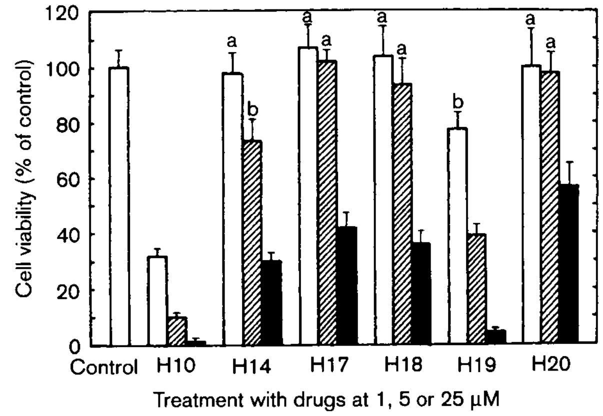


Figure 2. Comparison of the cytotoxic effects of 1 (open), 5 (striped) and 25 (closed) μM concentrations of novel TP analogs on L1210 cells at day 4. Cells were seeded in triplicate at an initial density of 5000 cells/0.5 ml/well in RPMI 1640 medium, containing 7.5% FCS and penicillin (100 IU/ml)–streptomycin (100 μg/ml), and grown for 4 days in the presence or absence (control) of TPs in a humidified incubator at 37°C with 5% CO_2 in air. The ability of viable cells/0.5 ml/well to bioreduce 0.1 ml of MTS:PMS (20:1) reagent over a 3 h incubation period at 37°C was assessed by measuring the absorbance of the water-soluble formazan products at $A_{490\ nm}$. Cell viability results are expressed as percent of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells ($A_{490\ nm}$= 1.588±0.092; 100±6%) after 4 days in culture. The blank value ($A_{490\ nm}$=0.194) for cell-free medium supplemented with MTS:PMS reagent has been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.01$, smaller than control.

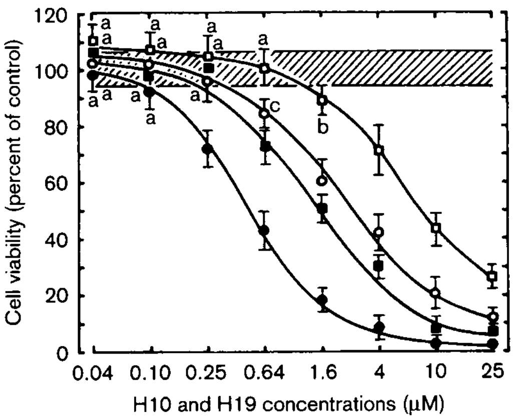


Figure 3. Concentration–response curves for the cytotoxic effects of H10 (circles) and H19 (squares) on L1210 cells at days 2 (open symbols) and 4 (closed symbols). Cells were seeded in triplicate at initial densities of 75 000 or 5000 cells/0.5 ml/well and, respectively, incubated for 2 or 4 days in the presence or absence (control) of the indicated concentrations of TPs, which are plotted on a logarithmic scale. The ability of viable cells/0.5 ml/well to bioreduce 0.1 ml of MTS:PMS (20:1) reagent over a 3 h incubation period was assessed as described in Figure 2. Cell viability results are expressed as percent of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells (100±6%; striped area) at days 2 ($A_{490\ nm}$=1.051±0.063) and 4 ($A_{490\ nm}$=1.379±0.088). Blank values ($A_{490\ nm}$=0.203 and 0.172 at days 2 and 4) for culture medium supplemented with MTS:PMS reagent have been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.05$ and [c]$p<0.025$, smaller than control.

tested in mice inoculated s.c. with LL/2 cells on day 0. Starting on day 1, a total of six i.p. injections of H10-HCl were administered either at 24 h intervals in experiment 1 or at 48 h intervals in experiment 2 and the tumor weights were compared to those of drug-untreated control mice after sacrifice at days 14 or 20 (Table 1). In these two protocols, doses of H10-HCl ranging from 8 to 30 μg/g body weight all decrease the growth of solid LL/2 tumors by 32 to 67% (Table 1).

Antitubulin activity of TPs

H10 has been shown to be a MT de-stabilizing agent, which inhibits in a concentration-dependent manner (IC$_{50}$: 1.5 μM) the rate of tubulin polymerization induced by 10% glycerol and 1 mM MgCl$_2$.[7] When compared at 5 μM, H10, H19 and H14, respectively, inhibit the control rate of glycerol-induced tubulin polymerization in the presence of 1 mM MgCl$_2$ by 68, 50 and 25% (Figure 4), suggesting that, as compared to the inhibitory effects of H10, the weaker cytotoxicities of H19 and H14 in the L1210 cell system *in vitro* (Figures 2 and 3) may be related to their weaker antitubulin activities in the turbidity assay (Figure 4). MTs require hydrolysis of GTP for their assembly and the tubulin dimer is a GTPase protein containing 2 mol of GTP. Only one of them is loosely bound, complexed to Mg^{2+}, and can be exchanged within 5 s to 5 min with the free GTP or GDP present in the medium.[15,16] However, GTP has a higher affinity for the exchangeable site than GDP. Since exogenous Mg^{2+} is required

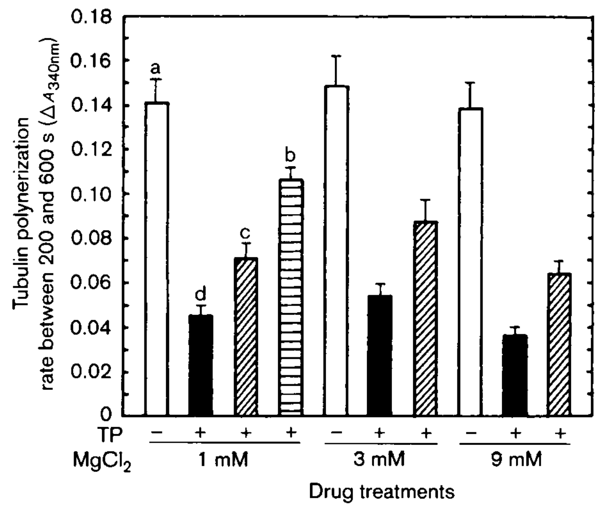


Figure 4. Effect of increasing concentrations of MgCl$_2$ on the inhibition of glycerol-induced tubulin polymerization by TPs. Purified tubulin was diluted to a final concentration of 2.2 mg/ml in 80 mM PIPES buffer, pH 6.8, containing 10% glycerol, 1 mM EGTA, 1 mM GTP and either 1, 3 or 9 mM MgCl$_2$. The polymerization reactions were placed in quartz microcells and incubated at 35°C in the presence (+) or absence (−) of 5 μM TPs. The rate of MT assembly was continuously monitored by scanning over 30 min the increase in turbidity at $A_{340\ \mathrm{nm}}$. Columns represent the linear increases in turbidity ($\Delta A_{340\ \mathrm{nm}}$) observed between 200 and 600 s: control (open), H10 (closed), H19 (oblique stripes) and H14 (horizontal stripes). Bars: means ± SD (n=3). [a]Not different from controls at 3 and 9 mM MgCl$_2$; [b]$p<0.05$, smaller than control; [c]$p<0.05$, smaller than H14 but not different from H19 at 3 and 9 mM MgCl$_2$; [d]$p<0.05$, smaller than H19 but not different from H10 at 3 and 9 mM MgCl$_2$.

Table 1. Antitumor activity of H10–HCl against Lewis lung carcinoma in mice[a]

Treatment[b] (dose/injection)	Results at day 14 (means ± SD, n=7)		
	Body weight (g)	Tumor weight/mouse (g)	Percent of control
Experiment 1			
control[c]	19.18 ± 1.86	0.685 ± 0.263	100
H10 (15 μg/g body weight)	19.85 ± 2.03[d]	0.377 ± 0.198[e]	55
H10 (30 μg/g body weight)	17.88 ± 1.34[d]	0.226 ± 0.167[f]	33
	Results at day 20 (means ± SD, n=6)		
	Body weight (g)	Tumor weight/mouse (g)	Percent of control
Experiment 2			
control	21.58 ± 2.02	2.233 ± 0.460	100
H10 (8 μg/g body weight)	20.76 ± 1.92[d]	1.518 ± 0.353[e]	68
H10 (20 μg/g body weight)	20.13 ± 2.14[d]	1.246 ± 0.567[g]	56

[a] In each group, 10^6 LL/2 cells were inoculated s.c. in the right flank of female C57BL/6 mice on day 0.
[b] The indicated doses of H10–HCl were injected i.p. in 0.2 ml of 0.9% NaCl, pH 2.0, every day from day 1 to 6 (experiment 1) or every other day from day 1 to 11 (experiment 2).
[c] Starting on day 1, LL/2-inoculated control mice received similar i.p. injections of 0.2 ml of vehicle daily or every other day.
[d] Not different from control; [e]$p<0.025$, [f]$p<0.005$ and [g]$p<0.01$, smaller than control.

for the binding of GTP to the exchangeable site on tubulin and for MT assembly,[12,15,22] it is of interest to determine if the antitubulin action of TPs involves Mg^{2+} and may be overcome by increasing concentrations of exogenous Mg^{2+}. However, the respective abilities of 5 μM H10 and H19 to inhibit the control rate of glycerol-induced tubulin polymerization persist in the presence of up to 9 mM $MgCl_2$ (Figure 4). Such failure of increasing concentrations of $MgCl_2$ to prevent or reverse the inhibitions of tubulin polymerization caused by H10 and H19 suggests that Mg^{2+} is unlikely to play a role in the antitubulin action of TPs.

MT de-stabilizing agents interact with tubulin either on the CLC or vinca alkaloid binding sites.[15] The striped areas at 100% in Figure 5 represent the control bindings of radiolabeled VBL, CLC or GTP to purified tubulin (0.5 mg/ml). As expected, the control bindings of [^{3}H]VBL (0.5 μCi, 10 μM) or [^{3}H]GTP (1 μCi, 5 μM) to tubulin are respectively inhibited by increasing concentrations (1.28–125 μM) of unlabeled VBL and GTP but not by similar concentrations of H10 (Figures 5A and C). In contrast, increasing concentrations (1.28–125 μM) of H10 mimic the ability of increasing concentrations (0.51–125 μM) of cold CLC to reduce the control amount of [^{3}H]CLC (0.5 μCi, 1 μM) bound to tubulin (Figure 5B), suggesting that TPs may interact with tubulin at the CLC binding site to inhibit MT assembly without disrupting the binding sites for GTP or vinca drugs. However, the TP and CLC binding sites might not fully overlap or these agents might have different affinities for tubulin since 50 μM cold CLC and H10, respectively, inhibit the control level of [^{3}H]CLC bound to tubulin by 89 and 53% (Figure 5B).

Antimitotic activity of TPs

The MT de-stabilizing drugs H10 and VCR have been compared for their ability to increase the mitotic index of L1210 cells in a time (Figure 6)- and concentration

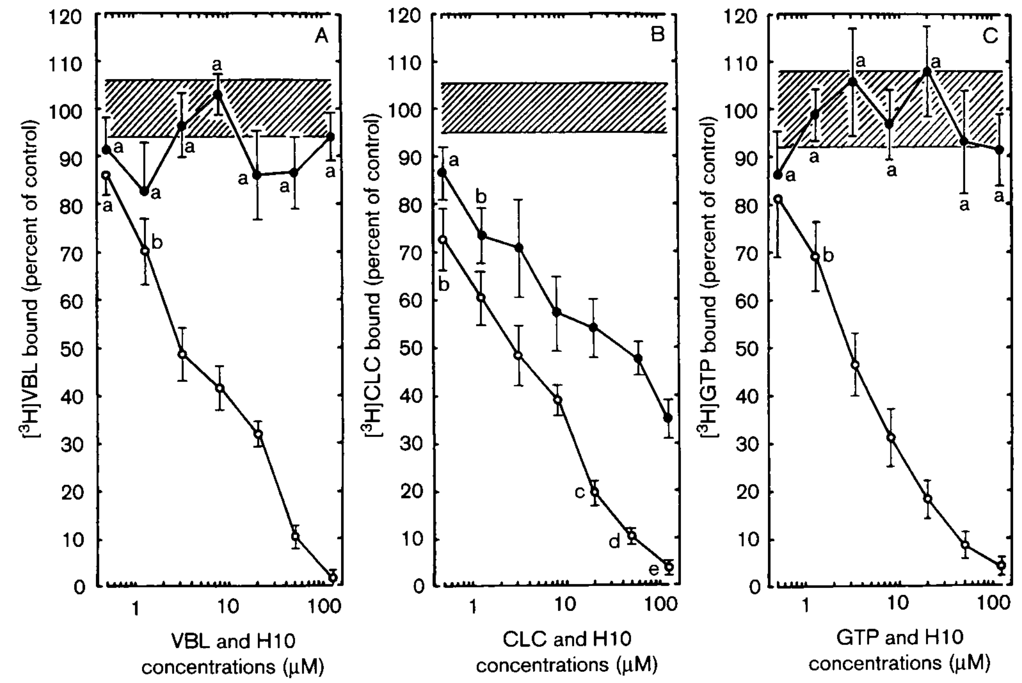


Figure 5. Comparison of the effects of H10, VBL, CLC and GTP on the binding of [^{3}H]VBL (A), [^{3}H]CLC (B) and [^{3}H]GTP (C) to purified tubulin. Tubulin was diluted to a final concentration of 0.5 mg/ml in 80 mM PIPES buffer, pH 6.8, containing 1 mM $MgCl_2$ and 1 mM EGTA. The assay mixtures (0.1 ml) were supplemented with the indicated concentrations of unlabeled H10 (●) or VBL, CLC and GTP (○), which are plotted on a logarithmic scale, and were incubated with [^{3}H]VBL (0.5 μCi, 10 μM) or [^{3}H]CLC (0.5 μCi, 1 μM) for 90 min at 37°C (A and B) or with [^{3}H]GTP (1 μCi, 5 μM) for 10 min at 4°C (C). Unlabeled drugs were added to the assay mixtures about 5 min before the radiolabeled compounds and the radioactivity bound to tubulin was determined by the DEAE-cellulose filter method. Results are expressed as percent of [^{3}H]VBL (31 675±1979 c.p.m.; 100±6%; striped area in A), [^{3}H]CLC (92 844±4921 c.p.m.; 100±5%; striped area in B) or [^{3}H]GTP (28 116±2165 c.p.m.; 100±8%; striped area in C) bound to tubulin in control assays incubated in the absence of unlabeled drugs. Blank values for free [^{3}H]VBL (15 459±726 c.p.m.), [^{3}H]CLC (382±19 c.p.m.) and [^{3}H]GTP (16 745±682 c.p.m.) retained on filter stacks in the absence of tubulin have been respectively substracted from the results in (A), (B) and (C). Bars: means±SD (n=2). [a]Not different from control; [b]$p<0.05$, smaller than control; [c]$p<0.025$, [d]$p<0.005$ and [e]$p<0.01$, smaller than H10.

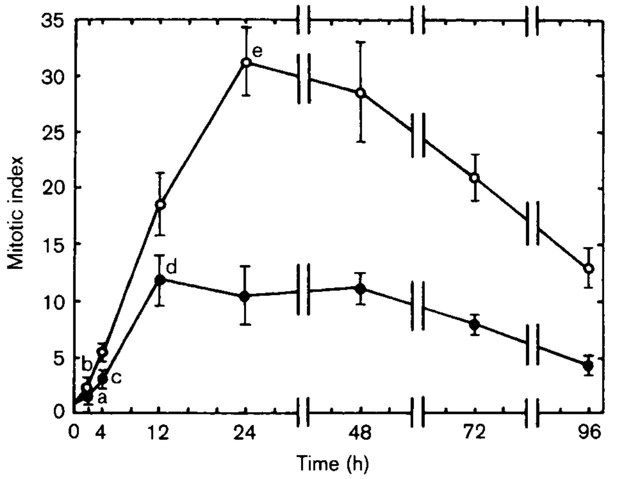


Figure 6. Time–response curves for the abilities of H10 and VCR to increase the mitotic index of L1210 cells *in vitro*. L1210 cells were seeded in triplicate at initial densities of 1 000 000, 250 000, 62 500 or 15 625 cells/0.5 ml/well and, respectively, incubated for periods of 2–24, 48, 72 or 96 h at 37°C in the presence or absence (control) of 1 μM H10 (●) or VCR (○). After fixation with MeOH:acetic acid (3:1) and staining with 0.1% crystal violet, about 500 cells/slide were scored for mitotic figures and the mitotic index was expressed as the percent of mitotic cells in drug-treated cultures divided by the percent of mitotic cells in non-treated controls. The mean percent of mitotic cells in controls at each time point over the 4-day period was 1.54±0.38%. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.025$ and [c]$p<0.005$, greater than control; [d]Not different from H10 at 24 and 48 h but $p<0.05$, greater than H10 at 72 h; [e]not different from VCR at 48 h but $p<0.01$, greater than VCR at 72 h.

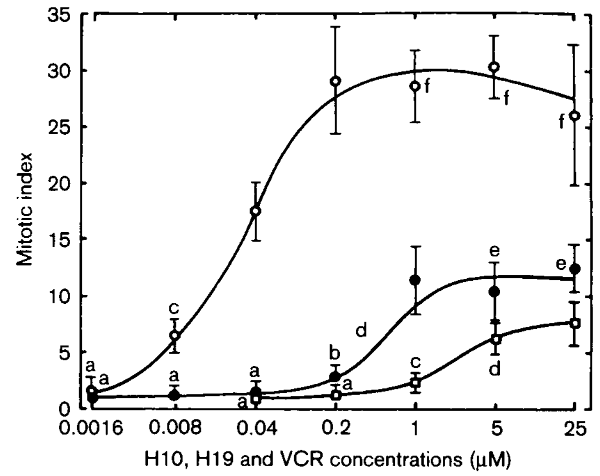


Figure 7. Concentration–response curves for the abilities of H10, H19 and VCR to increase the mitotic index of L1210 cells *in vitro*. L1210 cells (10^6/0.5 ml/well) were incubated in triplicate for 24 h at 37°C in the presence or absence (control) of the indicated concentrations of H10 (●), H19 (□) or VCR (○), which are plotted on a logarithmic scale. Results are expressed as indicated in Figure 6. The mean percent of mitotic cells in controls at 24 h was 1.46±0.19%. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.05$ and [c]$p<0.005$, greater than control; [d]not different from 25 μM H19; [e]not different from 1 μM H10 but $p<0.05$, greater than H19; [f]not different from 0.2 μM VCR.

(Figure 7)-dependent manner. At 1 μM, VCR produces a rapid and prolonged 30-fold increase in the mitotic index of L1210 cells, which starts as early as 2 h and peaks at 24 h before slowly declining between 2 and 4 days (Figure 6). In relation with its weaker antitubulin activity,[7] 1 μM H10 causes an 11-fold increase in the mitotic index of L1210 cells, which starts at 4 h and reaches a plateau of maximal stimulation between 12 and 48 h before declining thereafter (Figure 6). Therefore, the abilities of various concentrations of TPs and VCR to raise the mitotic index of L1210 cells were compared at 24 h (Figure 7), the first time at which both compounds maximally increase the percent of mitotic cells in Figure 6. Concentrations of H10 and H19, respectively, 25 and 125 times greater than that of VCR must be used to significantly increase the percent of mitotic cells at 24 h (Figure 7). Moreover, all concentrations of VCR of 0.2 μM or greater produce maximal 28-fold increases in the mitotic index of L1210 cells, whereas smaller 12- and 7-fold elevations in the percent of mitotic cells can only be achieved by concentrations of H10 and H19, respectively, 1 and 5 μM or greater (Figure 7). Nevertheless, the antimitotic effects of 24 h pretreatments with either 1 μM H10 or 0.2 μM VCR are similarly irreversible following drug removal (data not shown).

Effects of TPs on DNA cleavage and apoptosis

L1210 cells containing [^{3}H]thymidine-prelabeled DNA were used to quantitatively determine whether TPs could induce DNA fragmentation (Figure 8). Treatments for 24 h with 1 μM CPT or DAU, anticancer drugs known to induce DNA-strand breaks, result in 57 and 47% DNA cleavage, respectively (Figure 8). The extent of DNA cleavage is only 24% in cells treated for 24 h with 1 μM of the MT de-stabilizing drug VCR (Figure 8). In contrast, 1 μM H10 and H19 do not break DNA and much higher concentrations of these TPs (5–25 μM) must be used to induce significant levels of DNA cleavage (11–20%) over a 24 h period (Figure 8). Since 1 μM H10 maximally increases the mitotic index at 12–24 h (Figures 6 and 7) and is cytotoxic at 2–4 days (Figures 2 and 3) but fails to

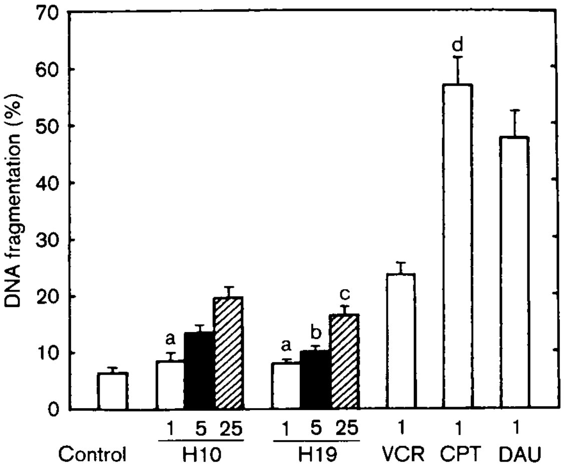


Figure 8. Comparison of the effects of novel TPs, MT destabilizing agent and known DNA-damaging drugs on DNA cleavage in L1210 cells *in vitro*. Cells (10^6/0.5 ml/well) were prelabeled with 1 μCi of [^{3}H]thymidine for 2 h, washed and resuspended in fresh medium containing 10% FCS, and incubated at 37°C for 24 h in the presence or absence (control) of 1 (open), 5 (closed) or 25 (striped) μM concentrations of the indicated drugs. After lysing the cells in a HLB containing 0.2% Triton X-100, the detergent-soluble DNA fragments present in the supernatants and the intact chromatin DNA remaining in the pellets were separated by centrifugation and their radioactivity estimated by LSC. Results are expressed as [c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100. For untreated controls, the supernatant is 871 ± 104 c.p.m. and the pellet is 12739 ± 1554 c.p.m. Bars: means ± SD (n=3). ^{a}Not different from control; $^b p < 0.005$, greater than control but not different from 5 μM H10; ^{c}not different from 25 μM H10; $^d p < 0.05$, greater than DAU.

cause any DNA cleavage at 24 h (Figure 8), the antitumor activities of short-term treatments with low concentrations of TPs are probably linked to their antitubulin rather than DNA-damaging effects.

Analysis of drug-induced internucleosomal DNA cleavage further indicates that the time- and concentration-dependent effects of H10 on DNA fragmentation are substantially weaker than those of VCR (Figure 9). No or little DNA cleavage is visualized in controls, whereas DNA cleavage bands with a characteristic pattern of the internucleosomal ladder suggestive of apoptosis become increasingly visible over a 4-day period in cells treated with 10 μM H10 or VCR (Figure 9A). At each time point, the intensities of the DNA ladders caused by H10 are less pronounced than those resulting from VCR treatment (Figure 9A). When compared at 72 h, no or only faint DNA fragmentation bands are detected in controls and cells treated with 0.256 or 0.64 μM H10 but bands of lower molecular weight DNA become increasingly apparent at or above 1.6 μM H10 (Figure 9B). In contrast, all concentrations of VCR tested in this range (0.256-25 μM) can induce the formation of DNA ladders indicative of apoptosis at 72 h (Figure 9B).

Inhibition of nucleoside transport by TPs

A critical finding is that a 15 min treatment with H10 is sufficient to block in a concentration-dependent manner the cellular transport of [^{3}H]thymidine occurring over only 15-30 s *in vitro* (Figures 10 and 11). Interestingly, the concentration-dependent inhibitions of nucleoside transport (IC_{50}: 6 μM in Figure 10) and DNA synthesis (IC_{50}: 8.5 μM)[5] by H10 are nearly identical, suggesting that the reversible inhibition of [^{3}H]thymidine incorporation into DNA caused by H10 at 2 h[6] may largely be due to the ability of this TP to immediately block the uptake of [^{3}H]thymidine by the cell. Moreover, the effectiveness of various TPs as inhibitors of DNA synthesis correlates with the magnitudes at which they inhibit the cellular transport of nucleosides (Figure 11). Indeed, H14, which inhibits DNA synthesis to a greater degree than H10[6] and H19 in the L1210 tumor cell system, is also more effective than H10 and H19 against the cellular transport of nucleosides (Figure 11). Finally, the inhibitions of nucleoside transport and DNA synthesis, which persist at the same level for 3 days in TP-treated cells, are both fully reversible upon drug removal (data not shown), suggesting that TPs must remain present in the medium to continually block the cellular transport of [^{3}H]thymidine and thereby prevent its incorporation into DNA.

Discussion

The only TPs cytotoxic at 5 μM are H10, H14 and H19, confirming the finding that a N-containing aromatic ring attached at C3 of the top pyrone A-ring is required for their antitumor activity. Alkylation at C7 of the bottom C-ring is undesirable since H14, which is a H10 analog with an isopropenyl substituent at C7, is less potent than H10 and H19 at 5 μM, and cannot mimic the cytotoxicity of these TPs at 1 μM. Whether or not an isopropenyl group or a 1-methylethyl group linked to an adenine moiety are present at C7, the inability of H17, H18 and H20 to decrease tumor cell viability at 5 μM is likely due to the fact that their top pyrone A-ring is either fused with a benzene ring or posseses a methyl substituent at C3 and, therefore, lacks the essential pyridyl substituent at C3. Since the 3-pyridyl

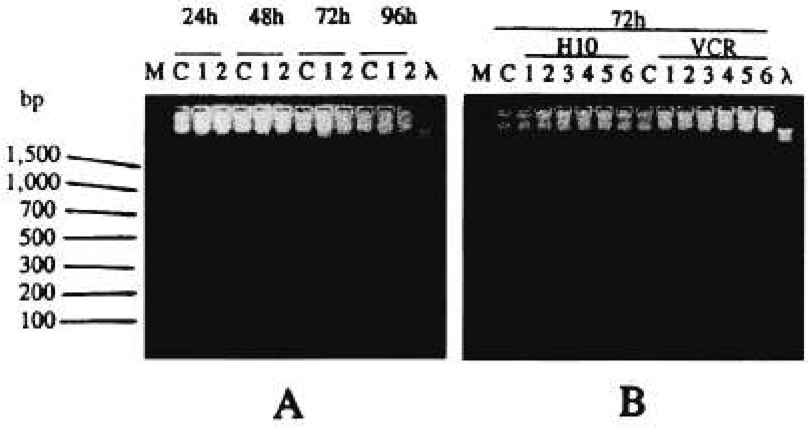


Figure 9. Agarose gel analysis of drug-induced internucleosomal DNA cleavage in L1210 cells *in vitro*. (A) Time courses of DNA fragmentation induced by H10 and VCR in L1210 cells. Cellular DNA was extracted from 10^7 cells incubated at 37°C for 1, 2, 3 and 4 days in the presence or absence (control: lanes C) of 10 μM H10 (lanes 1) or VCR (lanes 2). (B) Concentration-dependent level of DNA laddering in 10^7 cells incubated at 37°C for 72 h in the presence or absence (control: lanes C) of 0.256, 0.64, 1.6, 4, 10 and 25 μM H10 (left lanes 1–6) or VCR (right lanes 1–6). Equal amounts of DNA (3 μg/well) were loaded onto a 1.5% agarose gel containing ethidium bromide (1 μg/ml), separated by electrophoresis for 3.7 h at 60 V and photographed under UV light. A typical ladder pattern indicating the presence of DNA equivalent to the size of single and oligo nucleosomes is characteristic of apoptosis. Size markers are shown in lanes M (0.5 μg of 100 bp standard DNA ladder) and λ (0.75 μg of lambda DNA/*Eco*RI+*Hin*dIII markers).

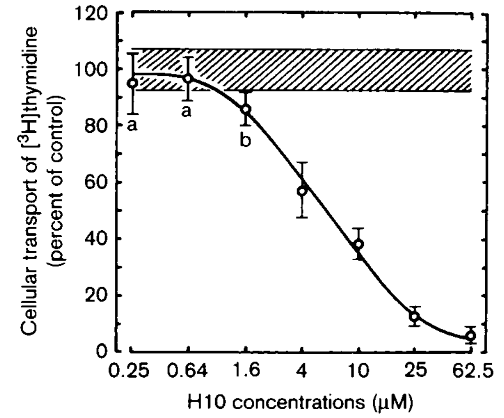


Figure 10. Concentration-dependent inhibition of nucleoside transport by the TP analog H10 in L1210 cells *in vitro*. Cells (2.5×10^6/0.5 ml of RPMI 1640 medium) were preincubated for 15 min at 37°C in the presence or absence (control) of the indicated concentrations of H10 (○), which are plotted on a logarithmic scale, before being exposed to 1 μCi of [^{3}H]thymidine for 30 s at 37°C. After washing thrice with PBS, intact cell pellets were harvested by centrifugation, incubated for 30 min in 1 ml of HLB containing 0.2% Triton X-100, and these lysates were mixed with scintillation cocktail and counted to estimate the cellular uptake of [^{3}H]thymidine. Results are expressed as percent of [^{3}H]thymidine transported into vehicle-treated control cells over 30 s (51 720$\pm$3724 c.p.m.; 100$\pm$7%; striped area). Bars: means$\pm$SD (n=3). ^{a}Not different from control; ^{b}$p<0.05$, smaller than control.

benzopyran H10 blocks tubulin polymerization, increases the mitotic index and reduces tumor cell viability to a greater degree than its 2-pyridyl regioisomer H19, the position of the N atom in the pyridyl moiety may play a role in the mechanism by which TPs interact with the CLC binding site on tubulin to block MT assembly, and inhibit tumor cell growth and viability. Interestingly, H14 inhibits nucleoside transport and DNA synthesis to a greater degree than H10 and H19 in the L1210 tumor cell system, suggesting that its structure may be better suited for interacting with nucleoside transporters at the plasma membrane than with MT proteins. A critical finding is that TPs may represent a new synthetic class of bifunctional anticancer drugs, which block the cellular transport of nucleosides and MT assembly to prevent leukemic cells from synthesizing DNA and dividing *in vitro*. Extending earlier findings,[6,7] the present study with H10, H14 and H19 suggests that the potency of these TPs against tumor cell proliferation and viability is better correlated to the magnitude of their antitubulin and antimitotic activities than to their ability to block the cellular uptake of thymidine and its incorporation into DNA. In spite of its superior inhibition of nucleoside transport and DNA synthesis, therefore, H14 may be a weaker antitumor agent than H10 and H19 because it is less

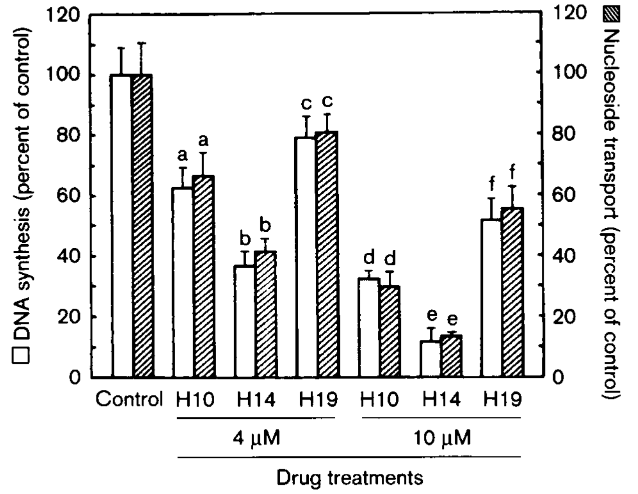


Figure 11. Comparison of the inhibitory effects of three new TP analogs on DNA synthesis (open) and nucleoside transport (striped) in L1210 cells *in vitro*. The cellular uptake of [^{3}H]thymidine (striped) and the rate of [^{3}H]thymidine incorporation into DNA (open) were determined in cells (2×10^6/0.5 ml of RPMI 1640 medium) respectively preincubated at 37°C for 15 or 90 min in the presence or absence (control) of 4 and 10 μM concentrations of the indicated TP analogs. For the cellular transport of nucleosides, the protocol of the experiment was identical to that of Figure 10, except that preincubated cells were then exposed to 1 μCi of thymidine for 15 s at 37°C. Results are expressed as percent of [^{3}H]thymidine transported into vehicle-treated control cells over 15 s (22530 ± 2456 c.p.m.; 100 ± 11%; striped control). For DNA synthesis, preincubated cells were then pulse-labeled with 1 μCi of [^{3}H]thymidine for an additional 30 min at 37°C. Results are expressed as percent of [^{3}H]thymidine incorporation into DNA in vehicle-treated control cells over 30 min (26003 ± 2548 c.p.m.; 100 ± 10%; open control). The blank value (1187 ± 132 c.p.m.) for cells incubated and pulse-labeled at 2°C with 1 μCi of [^{3}H]thymidine has been substracted from the results. Bars: means ± SD (n=3). Values with similar superscripts are not significantly different from each other. $^a p<0.05$ and $^d p<0.01$, smaller than respective H19 concentrations; $^b p<0.01$ and $^e p<0.005$, smaller than respective H10 concentrations; $^c p<0.005$, smaller than respective controls; ^{f}not different from 4 μM H10.

effective than these TPs against tubulin polymerization.

The present data substantiate the hypothesis that TPs might inhibit tumor cell proliferation and viability in relation with their ability to block tubulin polymerization rather than macromolecule synthesis.[6,7] However, the apparent discrepancies between the concentrations of H10 required to inhibit nucleoside transport and DNA synthesis (IC_{50}: 6-10 μM), block tubulin polymerization and increase the mitotic index, or reduce tumor cell proliferation and viability (IC_{50}: 0.5-1.5 μM) may be due in part to the different experimental conditions: the polymerization of purified tubulin is monitored over 20-30 min in a cell-free turbidity assay; the rates of nucleoside transport over 15-30 s and DNA synthesis over 30 min are determined in cells treated with H10 for 15 min to 2 h; and the ability of TP-treated cells to undergo mitosis, grow and remain viable is followed for 1-4 days.[5-7] Moreover, H10 must remain in the medium to maintain nucleoside transport and DNA synthesis inhibition, whereas the antimitotic, cytostatic and cytotoxic effects caused by H10 pretreatments are irreversible following drug removal. Although the cellular uptake, retention, metabolism and half-life of H10 in cell culture remain to be determined, greater concentrations of H10 may simply be required over time to maintain reversible effects inhibited than to sustain irreversible inhibitory effects. Nevertheless, because both responses are reversible and share similar IC_{50} values (6-10 μM),[5-7] the rapid inhibition of thymidine incorporation into DNA caused by H10 is likely to be due to its ability to immediately block the cellular uptake of this nucleoside. That different tumor cell lines often possess different types or ratios of nucleoside transporters might explain why H14 inhibits DNA synthesis more effectively than H10 in L1210 cells but not in EMT-6 cells.[6,7]

The cytostatic and cytotoxic effects of each concentration of H10 increase with the time in culture, suggesting that the effectiveness of TPs as inhibitors of tumor cell proliferation[5-7] and viability *in vitro* is a combination of drug concentration and duration of drug exposure. As a result, H10 inhibits L1210 and EMT-6 tumor cell growth at 2 and 4 days with IC_{50} values of 2 and 1-1.5 μM, respectively,[5,7] in relation with its ability to reduce L1210 cell viability with IC_{50} values of 2.5 μM at day 2 and 0.5 μM at day 4 (Figure 3). Cytotoxicity may predict anticancer potential more accurately than cytostatic activity[21] and the IC_{50} required to reduce cell viability at day 4 (0.5 μM) may be lower than that observed for tumor cell growth (1.5 μM) because the Coulter counter data[5-7] include all viable and non-viable tumor cells that have previously accumulated and remain in the medium at day 4, irrespective of their metabolic status. It should be noted that 0.5 μM H10 also increases the mitotic index to about half of its maximum level (Figure 7), suggesting that such concentration may be sufficient to induce 50% of the antimitotic, cytostatic and cytotoxic effects of H10.

Although tumor response in mice *in vivo* does not assure clinical activity, it is the closest surrogate assay we have. The fact that H10 is equally effective against L1210 and EMT-6 cell lines known to form ascites and solid tumors *in vivo* suggests that TPs might be valuable to develop a new class of anticancer drugs.[5-7] Since H10 abolishes L1210 cell viability *in vitro*, it should also increase the survival of mice inoculated with fast-growing L1210 leukemia *in vivo*. Despite its overall sensitivity, the L1210 leukemia model has some limitations for screening agents active against relatively slow-growing solid tumors, is not expected to recognize all drugs potentially effective in the tumor panel, and is inappropriate to evaluate the factor of distribution of the drug to the tumor tissue since both tumor cells and test compounds are injected i.p.[23,24] To overcome these limitations, it is important to demonstrate the effectiveness of water-soluble H10-HCl against LL/2 cells *in vivo*, a solid tumor model which permits the evaluation of the factor of distribution of the drug administered i.p. to the tumor tissue transplanted s.c. Tubulozole-C is the only MT-disrupting agent that can inhibit DNA synthesis like H10.[7] Compared to other MT de-stabilizing and CLC site-binding agents, tubulozole-C is a more potent antitubulin compound than nocodazole, CLC and VCR *in vitro*.[7,25] *In vivo*, tubulozole-C is more effective than VCR on solid tumors while VCR is superior to tubulozole-C in the L1210 model.[25] Doses of tubulozole-C (10-320 μg/g body weight) and VCR (0.1-2 μg/g body weight) have been successfully tested i.p. 1-13 days following tumor inoculation *in vivo*.[25-27] Thus, it is significant that six i.p. injections of H10-HCl (8-30 μg/g body weight) are sufficient to inhibit the growth of LL/2 tumors *in vivo* by 32-67%. These preliminary results demonstrate that H10 injected at a site remote from the site of tumor transplantation can effectively inhibit the development of solid malignant neoplasms with metastatic potential *in vivo*.

In contrast to paclitaxel, H10 cannot promote the polymerization of a low concentration of tubulin (2.2 mg/ml) in the absence of glycerol and, thus, is not a MT-stabilizing agent that blocks MT disassembly like paclitaxel.[7] When purified tubulin (2.2 mg/ml) undergoes polymerization in the presence of 10% glycerol, MT assembly is characterized by 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.[7] In this turbidity assay, H10 inhibits the control rate and plateau of glycerol-induced tubulin polymerization (IC_{50}: 1.5 μM) and mimics the antitubulin activity of VCR (IC_{50}: 0.15 μM), indicating that H10 is a MT de-stabilizing agent that prevents MT assembly but is less potent than VCR.[7] Such antitubulin action of TPs is likely to play a major role in their molecular mechanism of anticancer activity since H10, H14 and H19 inhibit the rate of glycerol-induced tubulin polymerization between 200 and 600 s in relation to their ability to reduce tumor cell viability. 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.[11] Since H10 increases the percent of mitotic figures as rapidly as VCR, it is likely to cause metaphase arrest and block the progression of L1210 cells in the M phase of their cycle, although to a lesser degree than VCR.

Interestingly, H10 inhibits DNA synthesis, whereas few of the known antimitotic drugs can do so.[7] Even though it might disrupt MTs to a lesser degree than other antimitotic drugs, H10 might be a more versatile anticancer agent able to target a wider range of molecular events and affect several phases of the cell cycle because of its ability to inhibit the cellular transport of nucleosides and DNA synthesis. As a nucleoside transport inhibitor which inhibits [^{3}H]thymidine incorporation into DNA, the antimitotic H10 might also arrest cell cycle progression in S phase. Thus, H10 may be a bifunctional anticancer drug with a self-limiting mechanism of action: by arresting in S phase the tumor cells of an unsynchronized population, H10 might limit the fraction of tumor cells entering M phase and susceptible to MT disruption. This might explain why the increase of mitotic index caused by H10 reaches an earlier plateau at a much lower level than that caused by VCR.

H10 inhibits tubulin polymerization with an IC_{50} value of 1.5 μM, which is 3 times greater than the concentration of 0.5 μM required for H10 to induce 50% of its increase of mitotic index and decrease of cell viability. The fact that the concentrations of antimitotic agents effective in the tubulin polymerization assay are consistently higher than those with cytotoxic activity has been noticed before.[7,14,15] Antimitotic drugs interacting with a few essential sites in the MTs might disrupt the mitotic spindle and be cytotoxic over a 4-day period at concentrations much lower than those required to directly block the rate of glycerol/Mg^{2+}-induced tubulin polymerization in a cell-free turbidity assay.[14] Indeed, mitotic arrest occurs when less than 5% of the cellular tubulin is complexed by CLC.[15] In addition to MT disruption, the ability of H10 to impair nucleoside transporters should also contribute to the overall cytostatic/cytotoxic actions of this antimitotic drug.

GTP and Mg^{2+} are necessary for tubulin nativity, and glycerol stabilizes tubulin and lowers the critical concentration of free subunits required to initiate polymerization.[12] Cytotoxic concentrations of H10 prevent the binding of [^{3}H]CLC to tubulin, and inhibit the rate and plateau of glycerol/Mg^{2+}-induced tubulin polymerization, demonstrating that this TP blocks MT assembly like the known MT de-stabilizing drugs that interact with the CLC binding site of tubulin.[15] H10 is unlikely to displace, or interfere with, Mg^{2+} in order to inhibit glycerol/Mg^{2+}-induced tubulin polymerization since the antitubulin effect of H10 persists in the presence of 1800 times more $MgCl_2$, suggesting that the exogenous Mg^{2+} cations absolutely required for the binding of GTP to tubulin and MT assembly cannot overcome the antitubulin action of TPs. H10 may inhibit tubulin polymerization as a consequence of its interaction with the CLC binding site but is unlikely to disrupt, or bind to other sites of, the α/β tubulin dimer since 125 μM H10 fails to alter the binding of 5 μM GTP and 10 μM VBL. On an equal molecular basis, however, H10 is less effective than unlabeled CLC at reducing the control binding of [^{3}H]CLC to tubulin, suggesting that the binding affinity of H10 for tubulin is lower than that of CLC or that the binding sites of those two drugs on tubulin do not fully overlap. Although they do not affect the binding of GTP to tubulin, most CLC site-binding agents enhance tubulin-dependent GTP hydrolysis.[15] CLC binds to a site on the free tubulin heterodimer which is not exposed when this unit is assembled into MTs *in vitro*.[12,15] CLC site-binding agents induce conformational changes which promote the fluorescence of tubulin.[28] Free sulfhydryl (SH) groups are essential for MT assembly and may be used as potential probes to characterize drug binding sites, based on their reactivity with the alkylating agent iodo[^{14}C]acetamide.[29,30] CLC-induced conformational changes of tubulin block SH groups and prevent cysteine cross-links.[29] The hypothesis that conformational changes caused by the interaction of H10 with tubulin might alter the fluorescence of this protein, the alkylation of SH groups by iodo[^{14}C]acetamide and the hydrolysis of exchangeable GTP[31] remains to be investigated. When MT assembly is prevented in CLC- or nocodazole-treated cells, the level of unpolymerized tubulin is increased and this, in turn, inhibits the formation of new tubulin mRNA while the pre-existing message decays rapidly.[32] Since H10 inhibits DNA, RNA and protein syntheses,[5-7] its ability to affect the level of translatable tubulin mRNA and tubulin synthesis should be studied.

As compared to known DNA-damaging agents, tumor cells exposed for long periods of time to highly cytotoxic concentrations of H10 exhibit only low levels of DNA cleavage and apoptosis, suggesting that the antitumor action of TPs does not primarily involve the formation of DNA strand breaks. In contrast to its ability to rapidly block nucleoside transport and DNA synthesis, prevent MT assembly, and increase the mitotic index, which are probably the causes of its antitumor activity, the delayed and weak stimulation of DNA fragmentation and apoptosis observed 1-4 days after H10 treatment may be merely the consequence of its cytostatic/cytotoxic activity. Most MT-stabilizing and de-stabilizing anticancer drugs which arrest cell cycle progression in G_2/M phases also initiate internucleosomal DNA fragmentation indicative of apoptosis.[20,33,34] G_2/M arrest may not be sufficient to induce apoptosis and additional phosphoregulatory pathways may be required. Activation of c-*jun* N-terminal kinase/stress-activated protein kinase may be a common cellular response to MT-disrupting agents.[33] Hence, apoptosis does not necessarily result from mitotic block and these two phenomena can occur independently of each other.[34]

Because anticancer drugs with different mechanisms of action may have complementary or synergistic effects, the use of multiple drug combinations is an important strategy in cancer chemotherapy. A TP inhibiting both nucleoside transport and MT assembly might differ from standard CLC site-binding agents and alter a larger number of molecular targets in unsynchronized cells than another drug inhibiting a single one of these events. Nucleoside fluxes in mammalian cells are mediated by a family of plasma membrane transporters that function by equilibrative (facilitated diffusion) or Na^+-dependent (concentrative) mechanisms, and can be identified by their permeant selectivities and sensitivities to various inhibitors. All nucleosides are substrates for the Na^+-independent transporters but Na^+-dependent transporters generally accept purines rather than pyrimidine nucleosides as substrates.[35] Dipyridamole (DPR), 6-(4-nitrobenzylmercapto)purine ribonucleoside (NBMPR) and dilazep are standard potent inhibitors of equilibrative nucleoside transport, whereas phloridzin specifically inhibits Na^+-dependent nucleoside transport.[36,37] Like H10, NBMPR and DPR also reduce [^{3}H]thymidine incorporation into DNA.[38] Nucleoside transport inhibitors block equally well the influx and efflux of nucleosides.[39] L1210 cells possess three distinct nucleoside transporters: two equilibrative transporters sensitive (es) or insensitive (ei) to NBMPR and one Na^+-dependent transporter (cif) of low sensitivity to NBMPR and DPR.[35,37,40,41] An important objective, therefore, would be to determine if H10 affects preferentially the bidirectional fluxes of purines and/or pyrimidine nucleosides, mimics or modulates the cellular action of the above nucleoside transporter probes, binds to specific plasma membrane nucleoside transporters, and uses such pathway for its cellular uptake.

For nucleoside synthesis, cells use purine and pyrimidine nucleosides generated either through *de novo* synthesis or through the utilization of salvage pathways. Multidrug resistance (MDR) is sometimes associated with increases in the number of nucleoside transporters and their rate of transport, resulting in the increased uptake of adenosine.[42] By blocking the rescue effect of exogenous nucleosides, NBMPR, DPR and dilazep may potentiate or prolong the antitumor activity of antimetabolites which inhibit the *de novo* pathway for nucleoside synthesis.[43-46] Acquisition or resistance to antimitotic agents may proceed via both P-glycoprotein- and non-P-glycoprotein-mediated processes.[47] The DPR analog, BIBW 22, is a bifunctional modulator which reverses the MDR phenotype by interfering with both P-glycoprotein and nucleoside transport in MDR cells.[8] As a bifunctional inhibitor of nucleoside transport and MT assembly, H10 might be valuable in polychemotherapy to enhance the cytotoxicity of VCR, potentiate the antitumor activity of methotrexate and 5-fluorouracil, and sensitize MDR tumor cells.

Conclusion

The TP H10 inhibits the growth of solid tumors *in vivo* and may represent a novel synthetic class of bifunctional anticancer drugs, which block nucleoside transport and MT assembly to prevent leukemic cells from synthesizing DNA and dividing *in vitro*.

Acknowledgments

We thank Dr Thomas L Jeatran, Lilly Research Laboratories, Indianapolis, IN, for the generous gift of VCR sulfate.

References

1. Hua DH, Chen Y, Sin H-S, *et al.* A one-pot condensation of pyrones and enals. Synthesis of 1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran. *J Org Chem* 1997; **62**: 6888-96.
2. Robinson PD, Hua DH, Chen Y, Meyers CY. Structure of *cis*-3,5a-dimethyl-6-formyloxy-1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran. *Acta Cryst* 1997; **C53**: 1642-4.
3. Hua DH, Chen Y, Robinson PD, Meyers CY. Structure of (5a*S*,7*S*)-7-isopropenyl-3-methyl-1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran. *Acta Cryst* 1997; **C53**: 1995-7.
4. Robinson PD, Beatty A, Hua DH, *et al.* Racemic 3-(3,4-dimethoxyphenyl)-1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran, an antitumor agent formed from the condensation of 1-cyclohexenecarboxaldehyde with 4-hydroxy-6-(3,4-dimethoxyphenyl)-2-pyrone. *Acta Cryst* 1999; in press.
5. Perchellet JP, Newell SW, Ladesich JB, *et al.* Antitumor activity of novel tricyclic pyrone analogs in murine leukemia cells *in vitro*. *Anticancer Res* 1997; **17**: 2427-34.
6. Newell SW, Perchellet EM, Ladesich JB, *et al.* Tricyclic pyrone analogs: a new class of microtubule-disrupting anticancer drugs effective against murine leukemia cells *in vitro*. *Int J Oncol* 1998; **12**: 433-42.
7. Perchellet EM, Ladesich JB, Chen Y, *et al.* 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*. *Anti-Cancer Drugs* 1998; **9**: 565-76.
8. Chen H-X, Bamberger U, Heckel A, Guo X, Cheng Y-C. BIBW 22, a dipyridamole analogue, acts as a bifunctional modulator on tumor cells by influencing both P-glycoprotein and nucleoside transport. *Cancer Res* 1993; **53**: 1974-7.
9. Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 1991; **3**: 207-12.
10. Ishida R, Miki T, Narita T, *et al.* Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. *Cancer Res* 1991; **51**: 4909-16.
11. Yoshimatsu K, Yamaguchi A, Yoshino H, Koyanagi N, Kitoh K. Mechanism of action of E7010, an orally active sulfonamide antitumor agent: inhibition of mitosis by binding to the colchicine site of tubulin. *Cancer Res* 1997; **57**: 3208-13.
12. Engelborghs Y. Dynamic aspects of microtubule assembly. In: Avila J, ed. *Microtubule proteins*. Boca Raton, FL: CRC Press 1990: 1-35.
13. Borisy GG. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. *Anal Biochem* 1972; **50**: 373-85.
14. Goldbrunner M, Loidl G, Polossek T, Mannschreck A, von Angerer E. Inhibition of tubulin polymerization by 5,6-dihydroindolo[2,1-*a*]isoquinoline derivatives. *J Med Chem* 1997; **40**: 3524-33.
15. Hamel E. Interaction of tubulin with small ligands. In: Avila J, ed. *Microtubule proteins*. Boca Raton, FL: CRC Press 1990: 89-191.
16. Arai T, Ihara Y, Arai K-I, Kaziro Y. Purification of tubulin from bovine brain and its interaction with guanine nucleotides. *J Biochem (Tokyo)* 1975; **77**: 647-58.
17. Ling Y-H, Priebe W, Perez-Soler R. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. *Cancer Res* 1993; **53**: 1845-52.
18. Kolber MA, Broschat KO, Landa-Gonzalez B. Cytochalasin B induces cellular DNA fragmentation. *FASEB J* 1990; **4**: 3021-7.
19. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**: 1215.
20. Lai Y-L, Chen Y-J, Wu T-Y, *et al.* Induction of apoptosis in human leukemic U937 cells by tetandrine. *Anti-Cancer Drugs* 1998; **9**: 77-81.
21. Brown JM. NCI's anticancer drug screening program may not be selecting for clinically active compounds. *Oncol Res* 1997; **9**: 213-5.

22. Correia JJ, Baty LT, Williams RCJr. Mg^{2+} dependence of guanine nucleotide binding to tubulin. *J Biol Chem* 1987; **262**: 17278-84.
23. Johnson RK. Screening methods in antineoplastic drug discovery. *J Natl Cancer Inst* 1990; **82**: 1082-3.
24. Grindley GB. Current status of drug development: failure or limited success? *Cancer Cells (Cold Spring Harbor)* 1990; **2**: 163-71.
25. De Brabander M, Geuens G, Nuydens R, *et al.* Tubulozole: a new stereoselective microtubule inhibitor. *Ann NY Acad Sci* 1986; **466**: 757-66.
26. Koyanagi N, Nagasu T, Fujita F, *et al. In vivo* tumor growth inhibition produced by a novel sulfonamide, E7010, against rodent and human tumors. *Cancer Res* 1994; **54**: 1702-6.
27. Tsuruo T, Iida H, Naganuma K, Tsukagoshi S, Sakurai Y. Promotion by verapamil of vincristine responsiveness in tumor cell lines inherently resistant to the drug. *Cancer Res* 1983; **43**: 808-13.
28. Bhattacharyya B, Wolff J. Promotion of fluorescence upon binding of colchicine to tubulin. *Proc Natl Acad Sci USA* 1974; **71**: 2627-31.
29. Wallin M, Hartley-Asp B. Effects of potential aneuploidy inducing agents on microtubule assembly *in vitro*. *Mutat Res* 1993; **287**: 17-22.
30. Luduena RF, Roach MC. Interaction of tubulin with drugs and alkylating agents. 2. Effects of colchicine, podophyllotoxin, and vinblastine on the alkylation of tubulin. *Biochemistry* 1981; **20**: 4444-50.
31. Kobayashi T. Dephosphorylation of tubulin-bound guanosine triphosphate during microtubule assembly. *J Biochem (Tokyo)* 1975; **77**: 1193-7.
32. Ben-Ze'ev A, Farmer SR, Penman S. Mechanisms of regulating tubulin synthesis in cultured mammalian cells. *Cell* 1979; **17**: 319-25.
33. Wang T-H, Wang H-S, Ichijo H, *et al.* Microtubule-interfering agents activate c-*jun* N-terminal kinase/stress-activated protein kinase through both *ras* and apoptosis signal-regulating kinase pathways. *J Biol Chem* 1998; **273**: 4928-36.
34. Lieu C-H, Chang Y-N, Lai Y-K. Dual cytotoxic mechanisms of submicromolar taxol on human leukemia HL-60 cells. *Biochem Pharmacol* 1997; **53**: 1587-96.
35. Crawford CR, Ng CYC, Noel LD, Belt JA. Nucleoside transport in L1210 murine leukemia cells: evidence for three transporters. *J Biol Chem* 1990; **265**: 9732-6.
36. Hammond JR. Comparative pharmacology of the nitrobenzylthioguanosine-sensitive and -resistant nucleoside transport mechanisms of Ehrlich ascites tumor cells. *J Pharmacol Exp Ther* 1991; **259**: 799-807.
37. Dagnino L, Bennett LL Jr, Paterson ARP. Sodium-dependent nucleoside transport in mouse leukemia L1210 cells. *J Biol Chem* 1991; **266**: 6308-11.
38. Moorghen M, Ince P, Finney KJ, Watson AJ, Harris AL. Organ culture as a model for investigating the effects of antimetabolites and nucleoside transport inhibitors on rodent colonic mucosa. *In Vitro Cell Dev Biol* 1991; **27A**: 873-7.
39. Gu JG, Foga IO, Parkinson FE, Geiger JD. Involvement of bidirectional adenosine transporters in the release of L-[^{3}H]adenosine from rat brain synaptosomal preparations. *J Neurochem* 1995; **64**: 2105-10.
40. Cory JG, Downes DL, Ng CYC, Belt JA. 5-Hexyl-2'-deoxyuridine inhibition of nucleoside transport in L1210 cells. *Oncol Res* 1992; **4**: 175-9.
41. Vijayalakshmi D, Dagnino L, Belt JA, Gati WP, Cass CE, Paterson ARP. L1210/B23.1 cells express equilibrative, inhibitor-sensitive nucleoside transport activity and lack two parental nucleoside transport activities. *J Biol Chem* 1992; **267**: 16951-6.
42. Morgan PF, Fine RL, Montgomery P, Marangos PJ, Multidrug resistance in MCF-7 human breast cancer cells is associated with increased expression of nucleoside transporters and altered uptake of adenosine. *Cancer Chemother Pharmacol* 1991; **29**: 127-32.
43. Marina NM, Belt JA. Effect of nucleoside transport inhibitors on thymidine salvage and the toxicity of nucleoside analogs in mouse bone marrow granulocyte-macrophage progenitor cells. *Cancer Commun* 1991; **3**: 367-72.
44. Cao SS, Zhen YS. Potentiation of antimetabolite antitumor activity *in vivo* by dipyridamole and amphotericin B. *Cancer Chemother Pharmacol* 1989; **24**: 181-6.
45. Hughes JM, Tattersall MHN. Potentiation of methotrexate lymphocytotoxicity *in vitro* by inhibitors of nucleoside transport. *Brit J Cancer* 1989; **59**: 381-4.
46. Muggia FM, Slowiaczek P, Tattersall MHN. Characterization of conditions in which dipyridamole enhances methotrexate toxicity in L1210 cells. *Anticancer Res* 1987; **7**: 161-6.
47. Parekh H, Wiesen K, Simpkins H. Acquisition of taxol resistance via P-glycoprotein- and non-P-glycoprotein-mediated mechanisms in human ovarian carcinoma cells. *Biochem Pharmacol* 1997; **53**: 461-70.

(Received 11 March 1999; accepted 25 March 1999)