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## Preclinical report

# Inhibition of macromolecular synthesis by cryptophycin-52

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**Cryptophycin (CP)-52, a synthetic analog of CP-1, possesses potent and selective antiproliferative activity against human solid tumors both *in vitro* and *in vivo*. Based on an algorithm developed in this laboratory using HCT-116 human colon adenocarcinoma cells, CP-52 exhibited a time- and concentration-dependent antiproliferative effect in the *in vitro* clonogenic assay. Inhibition of both DNA and RNA synthesis was observed in the absence of any effect on protein synthesis following a 24-h exposure to CP-52, at a time when proliferating cells were arrested in the $G_2$/M phase of the cell cycle. In summary, we interpret these data to indicate that the selective inhibition of DNA synthesis may be a major causative factor responsible for the antiproliferative activity of CP-52 and subsequent $G_2$/M arrest. [© 2002 Lippincott Williams & Wilkins.]**

*Key words:* Cryptophycin-52, drug action profile, $G_2$/M arrest, macromolecular synthesis.

## Introduction

Cryptophycin (CP) is a dioxadiazacyclo-hexadecene-tetrone isolated from cyanobacteria of the genus *Nostoc*[1,2] with potent and selective antiproliferative activity against a broad spectrum of human solid tumors, including mammary, colon and pancreatic adenocarcinoma in mouse xenografts, as well as multidrug-resistant tumors.[3–6] Following extensive analog synthesis and preclinical evaluation, CP-52, a dimethyl synthetic derivative of the parent compound CP-1 (Figure 1), was selected for clinical development based on its potency, broad spectrum of human antitumor activity and chemical stability.[7] As an antiproliferative agent, CP-52 had antiproliferative activity several-fold more potent than either paclitaxel or vinblastine.[8] CP-52 possesses antimitotic activity by depolymerization of spindle microtubules and also has been reported to be a potent suppressor of microtubule dynamics.[9] Previous studies comparing CP-52 to known microtubulin inhibitors have led us to conclude that CP-52 has important targets other than microtubules.[10] In this communication, we present new data supporting the selective inhibition of DNA synthesis as an important target for manifesting the antiproliferative activity of CP-52.

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## Materials and methods

### Chemicals

RPMI 1640 with L-glutamine and sodium bicarbonate, 2.5% trypsin and L-glutamine were obtained from Celox (St Paul, MN), bovine calf serum (BCS) from Hyclone (Logan, UT), Dulbecco's phosphate-buffered saline with calcium and magnesium, Hank's balanced salt solution (HBSS) without calcium and magnesium, and penicillin–streptomycin from Gibco/BRL (Rockville, MD). Nobel agar, collagenase type XI-S, DNase I type II, EDTA disodium salt, dimethylsulfoxide (DMSO), 2-mercaptoethanol, RNase, propidium iodide (PI) and Trypan blue were purchased from Sigma (St Louis, MO). T-25 culture flasks were purchased from Falcon Products (Becton Dickinson, Franklin Lakes, NJ) and all other culture ware from Corning (Corning, NY).

### CP-52

Dr Chuan Shih at Lilly Research Laboratories (Eli Lilly, Indianapolis, IN) provided CP-52 in fine powder form. A stock solution of 10 mg/ml was prepared by dissolving the powder in absolute

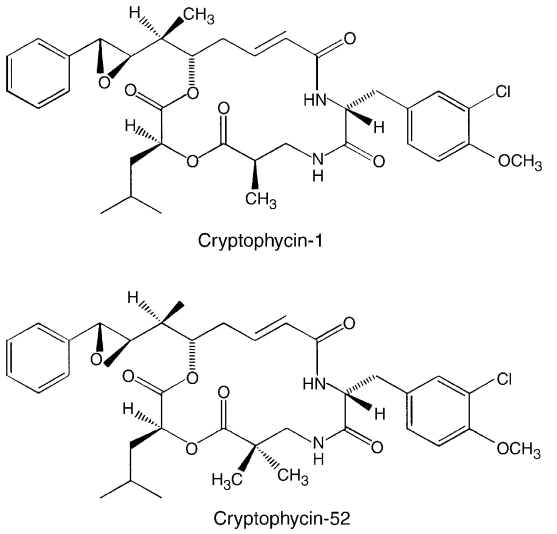


**Figure 1.** Structure of CP-52 and its parent compound CP-1.

ethanol as well as additional dilutions. Stock solutions stored at –20°C were stable under the described conditions.

## Cell culture

HCT-116 human colon adenocarcinoma cells were obtained from the National Cancer Institute (Bethesda, MD) and maintained as a monolayer culture in culture medium (RPMI 1640 supplemented with 15% heat-inactivated bovine calf serum, 1% L-glutamine and 1% penicillin–streptomycin). Cells were passaged twice a week by harvesting with an enzyme cocktail containing 0.25% trypsin, 0.53 mM disodium EDTA, 4 μg/ml DNase and 100 μg/ml collagenase. Cell cultures in exponential growth (day 3) and plateau phase (day 14) were used for drug exposure, and triplicate cultures were pooled for each drug concentration tested. Growth characteristics of HCT-116 cells *in vitro* have been described.[11]

## S phase analysis by flow cytometry

An aliquot of cell suspension was centrifuged at 200*g* for 5 min at 18°C, washed twice with ice-cold PBS, mixed thoroughly and re-centrifuged for 5 min. The cell pellet was fixed in 1 ml of ice-cold 70% ethanol with constant shaking and stored at 4°C. Ethanol-fixed cells were re-centrifuged for 5 min at 18°C, and the supernatant decanted by inverting the tubes and blotting the residual supernatant. To the cell pellet, 0.3 ml of PI/RNase mixture (50 μg/ml and 5 U/ml, respectively) was added, mixed thoroughly by vortexing and analyzed using a BD FACScan flow cytometer (Becton Dickinson Biosciences, San Jose, CA). DNA analysis was performed using Modfit software (Verity Software House, Topsham, ME).

## Determination of cell survival

HCT-116 cells were grown in 5 ml RPMI 1640 with 15% BCS supplemented with 1% antibiotic antimycotic at 37°C and 5% $CO_2$ at a starting concentration of $10^5$ cells/T-25 flask. On day 3, cells were exposed to different concentrations of CP-52. Flasks were incubated for 2 and 24 h in a 5% $CO_2$ incubator at 37°C, and the cells harvested with enzyme cocktail, washed once with HBSS and resuspended in HBSS with viability obtained by Trypan blue staining. Cells were prepared in RPMI 1640 containing 15% BCS, 1% antibiotic–antimycotic, $5 \times 10^{-5}$ M $\beta$-mercaptoethanol and agar at a final concentration of 0.3%. After thorough mixing, aliquots were pipetted over a hardened layer of 0.6% agar so as to yield 2000 cells per plate. The plates were incubated in a 5% $CO_2$ incubator maintained at 37°C for 14 days. For continuous exposure, appropriate amounts of CP-52 were added directly to the top layer mixture. Colonies (50 cells or more) were scored under a stereozoom microscope (Bausch & Lamb, model BVB-125).

## Influx studies

Exponentially growing HCT-116 cells in six-well plates were incubated with 3 and 30 μg/ml of CP-52 for 0, 1, 2, 3, 4, 5, 10, 15, 30, 45 and 60 min at 37°C in a 5% $CO_2$ incubator. At the end of each incubation, medium was aspirated and cells centrifuged at 200*g* for 5 min. CP-52 was extracted from the supernatant with 2 volumes of acetonitrile and washed once with 5 volumes of HBSS for analysis by HPLC (see below). Adherent cells in each well were sonicated in HBSS using a Branson Sonifier 450 with a micro tip in a constant pulsed mode for 2 min. Sonicate was centrifuged at 300*g* for 5 min. To 500 μl of sonicate, 1 ml of methanol was added, extracted by solid-phase extraction (using Waters SepPak $C_{18}$ 1 ml column and 2 ml of acetonitrile to elute the sample) and evaporated to dryness at 50°C under nitrogen.

Aliquots of the extract in mobile phase were analyzed by HPLC.

### Efflux studies

Exponentially growing HCT-116 cells in six-well plates were incubated with different concentrations of CP-52. After 30 min, medium was aspirated and wells were washed with 5 ml of HBSS (washing media added and removed in 30 s). To each well, 5 ml of fresh RPMI 1640 with 15% BCS was added, and incubation continued for 0, 1, 2, 3, 4, 5, 10, 15, 30, 45 and 60 min. At the end of incubation, the medium and cells from each well were processed and analyzed by HPLC as mentioned above.

### HPLC analysis

CP-52 analysis was carried out with a Waters Maxima Workstation HPLC and model AS100 Bio-Rad autosampler (Hercules, CA) set at 4°C, two model 510B Waters solvent pumps, and a model 490E detector set at 220 nm. Two analytical columns of $3.9 \times 150$ mm Novapak $C_{18}$ (Waters) were joined in tandem and preceded by a $\mu$Bondapak $C_{18}$ precolumn cartridge (Millipore, Milford, MA). The mobile phase was 60% acetonitrile and 40% deionized water with a flow rate of 1 ml/min and a run time of 120 min. The analytical columns were maintained at 28°C using a Waters temperature control module. The lower limit of quantitation was 25 ng/ml and the assay was linear over the range tested (up to 30 $\mu$g/ml). Under these conditions, the CP-52 peak eluted near 45 min. Absolute values of CP-52 were obtained using a standard curve (five concentrations) of a stock CP-52 solution added to growth media. Validation of each set of results was accomplished using run standards (stock drug solution dissolved in mobile phase at 2 mg/ml).

### Inhibition of macromolecular synthesis

Exponentially growing HCT-116 cells were incubated with a range of concentrations of CP-52 for 2 and 24 h. Cells were pulse-labeled for an additional 30 min with [$^3$H]thymidine (5 $\mu$Ci/ml), [$^3$H]uridine (10 $\mu$Ci/ml) or [$^3$H]leucine (50 $\mu$Ci/ml) in a 5% $CO_2$ incubator maintained at 37°C. At the end of incubation, cells were harvested by incubating with the enzyme cocktail, and the cell pellet was washed once with HBSS and resuspended in HBSS at a density of $1 \times 10^6$ cells/ml. Cell count and viability were determined using Trypan blue. Then 1 ml of the cell suspension was filtered through Whatman GF/C glass microfiber disks, and cells deposited on the filter disks were lysed with ice-cold 10% trichloroacetic acid (4 ml $\times$ 3), washed with ice-cold absolute ethanol (4 ml $\times$ 3) and the filters were air-dried. Radioactivity in each filter was quantitated in biodegradable scintillation cocktail using Beckman Liquid Scintillation Counter. Appropriate ethanol-treated HCT-116 cells were maintained as controls and results expressed as percent control.

### Apoptosis

Apoptosis was measured in HCT-116 cells treated with CP-52 using the APO-DIRECT kit (PharMingen, San Diego, CA). Cells exposed to CP-52 were fixed in 5 ml of 1% (v/v) paraformaldehyde in PBS, then in 5 ml of ice-cold 70% (v/v) ethanol and then stained in a solution of TdT reaction buffer, TdT enzyme, FITC-dUTP for 60 min at 37°C. At the end of incubation, cells were rinsed with buffer and resuspended in 1 ml of PI/RNase solution. After 30 min incubation, cells were analyzed by dual-parameter flow cytometry (as described above) for determination of cell cycle-specific apoptosis.

## Results

### Inhibition of colony formation

As shown in Figure 2, concentration– and time–response data for CP-52 on clonogenic HCT-116 cells in log phase (day 3, solid symbols) and plateau phase (day 14, open symbols) were obtained over a wide range of drug concentrations following 2, 24 and 336 h of exposure. The surviving fraction of these cells, plotted as a function of drug concentration (Figure 2A), demonstrated a concentration- and time-dependent cell kill, characterized by an exponential decline in survival with increasing concentration and time. Cell killing was minimal following a 2-h exposure to concentrations of CP-52 up to 10 $\mu$g/ml. However, increasing exposure time to 24 h resulted in 50% loss of clonogenic cells at a concentration of 25 pg/ml and surviving fractions of 0.1 following an exposure to 1 ng/ml, and 0.01 at $4 \times 10^{-1}$ $\mu$g/ml following an exposure to 400 ng/ml. Continuous exposure to various concentrations of

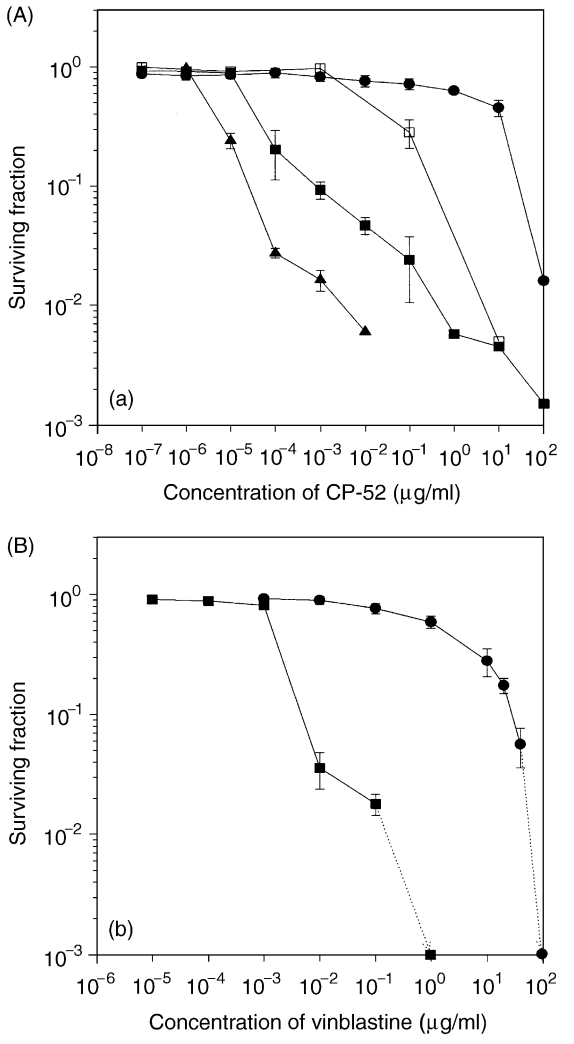


**Figure 2.** Concentration- and time-dependent cytotoxic effect of (A) CP-52 and (B) vinblastine on clonogenic HCT-116 cells *in vitro*. Log-phase (closed symbols) and plateau-phase (open symbols) HCT-116 cells were exposed to various concentrations of: (A) CP-52 as indicated for 2 (circles), 24 (solid squares) and 336 (triangles) h, and plateau-phase cells for 24 h (open squares) at 37°C or (B) concentrations of vinblastine as indicated for 2 (circles) and 24 (squares) h at 37°C. Clonogenic assays were then carried out in triplicate with data expressed as a fraction of control (untreated) HCT-116 clonogenic cells. The data are from three separate experiments done in triplicate and the results are shown as the means ± SE.

CP-52 resulted in a surviving fraction of 0.01 at an exposure level of 20 ng/ml. When compared to the inhibition of colony formation by the known tubulin inhibitor vinblastine following a 24-h exposure, the concentration–survival curves were similar to those found for CP-52 (surviving fractions of 0.01 at concentrations of 0.2 and 0.7 μg/ml, respectively, Figure 2B).

## Cell cycle progression delay and apoptosis

The effect of CP-52 on cell cycle progression of proliferating (day 3) HCT-116 cells is shown in Figure 3. At a CP-52 concentration of $10^{-6}$ μg/ml for 24 h that resulted in no clonogenic cell killing (Figure 2), $G_2$/M arrest was observed, with 62% of single cells detected in $G_2$/M. This degree of $G_2$/M arrest was identical to that observed following a 24-h exposure to 10 μg/ml that resulted in a cell surviving fraction of 0.01. Also, as shown in Figure 4, 24-h exposures to concentrations of CP-52 and vinblastine responsible for comparable clonogenic cell kill (surviving fractions of 0.01–0.001, Figure 2) resulted in the same degree of $G_2$/M arrest (71 versus 72% of cycling cells following 1 μg/ml, respectively). The APO-DIRECT assay demonstrated that the corresponding degree of apoptosis also was similar in terms of the fraction of FITC-dUTP-positive cells (as well as those cells with $< 2C$ DNA content, Figure 4).

## Cellular uptake and efflux of CP-52

Exponentially growing HCT-116 cells were plated in six-well plates ($10^5$ cells/well), incubated with 30 μg/ml of CP-52 and the amount of drug accumulated inside the cells measured as a function of exposure time (Figure 5A). The initial (5 min) cellular uptake of CP-52 at 30 μg/ml was rapid, followed by a plateau from 10 to 60 min at 1–1.2 ng/$10^6$cells with similar uptake kinetics (Figure 5A). Efflux measurements were obtained after cells had been exposed to 30 μg/ml of CP-52 for 60 min, then washed and placed in medium without drug at time 0. As can be seen in Figure 5(B), only 24% (i.e. 287 pg/$10^6$ cells) of maximum intracellular CP-52 (following the initial 60 min exposure to 30 μg/ml of CP-52) was retained within the cell after the subsequent 60 min incubation in drug-free medium.

## Macromolecular synthesis inhibition

The effect of CP-52 on macromolecular synthesis inhibition following exposure of HCT-116 cells for 2 and 24 h to various concentrations of CP-52 is shown in Figure 6. After a 2-h exposure, CP-52 inhibited DNA synthesis to 49% of control and RNA synthesis to 30% of control at the highest concentration of 100 μg/ml while having little or no effect on inhibition of protein synthesis at this concentration (Figure 6A). However, by lengthening the exposure time of

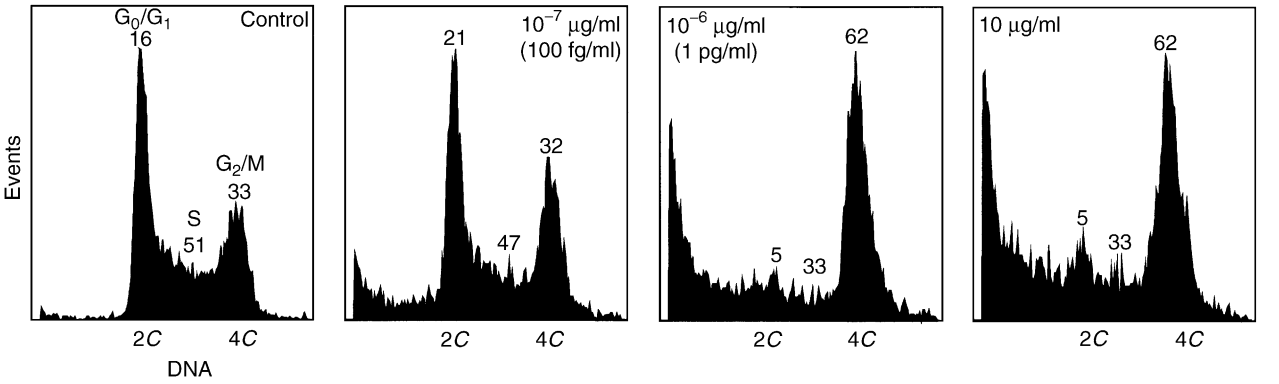


**Figure 3.** Effect of a 24-h exposure to various concentrations of CP-52 on the cell cycle progression of exponentially growing (day 3) HCT-116 cells using multivariate flow cytometry. Numbers represents the percent of gated single cells in different phases of the cell cycle.

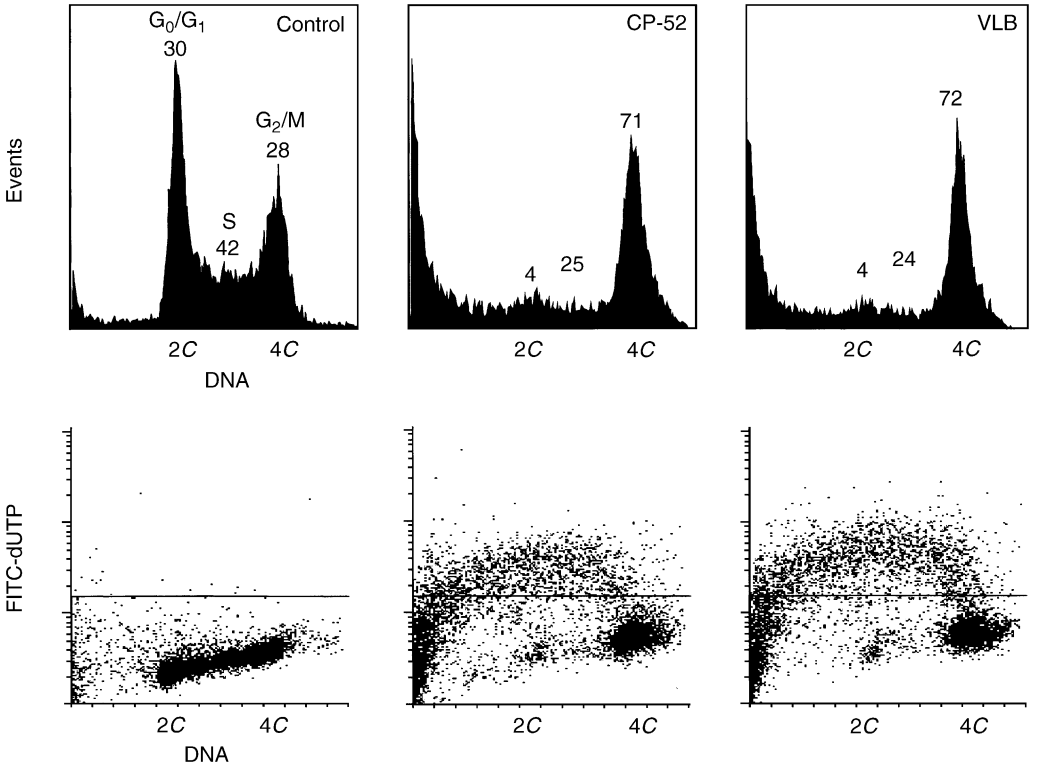


**Figure 4.** Phase-specific apoptosis induced by exposure of exponentially growing HCT-116 cells to CP-52 and vinblastine (VLB) for 24 h at 1 μg/ml.

proliferating HCT-116 cells to CP-52 from 2 to 24 h, both DNA and RNA synthesis was inhibited at much lower doses (down to 7 and 11% of control, respectively, at 1 ng/ml of CP-52), with protein synthesis being inhibited to 42% of control at this CP-52 concentration. At 10 ng/ml, DNA synthesis was inhibited significantly to 27% of control while RNA synthesis was inhibited to 73% of control with minimal protein synthesis inhibition (97% of control).

## Discussion

CP-52 is a potent antitumor drug whose mechanism of action linking its selective cytotoxicity to its

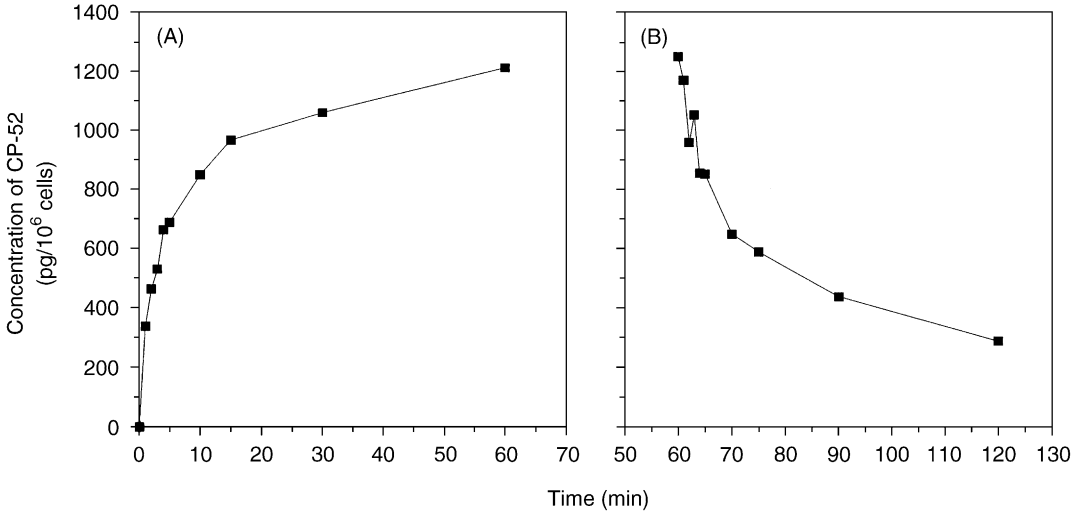


**Figure 5.** Concentration- and time-dependent cellular influx (A) and efflux (B) of CP-52 in exponentially growing HCT-116 cells exposed to 30 $\mu$g/ml of CP-52 (see Materials and methods for details). The amount of intracellular CP-52 was quantitated by HPLC following sonication of the cells and extraction of CP-52 with acetonitrile.

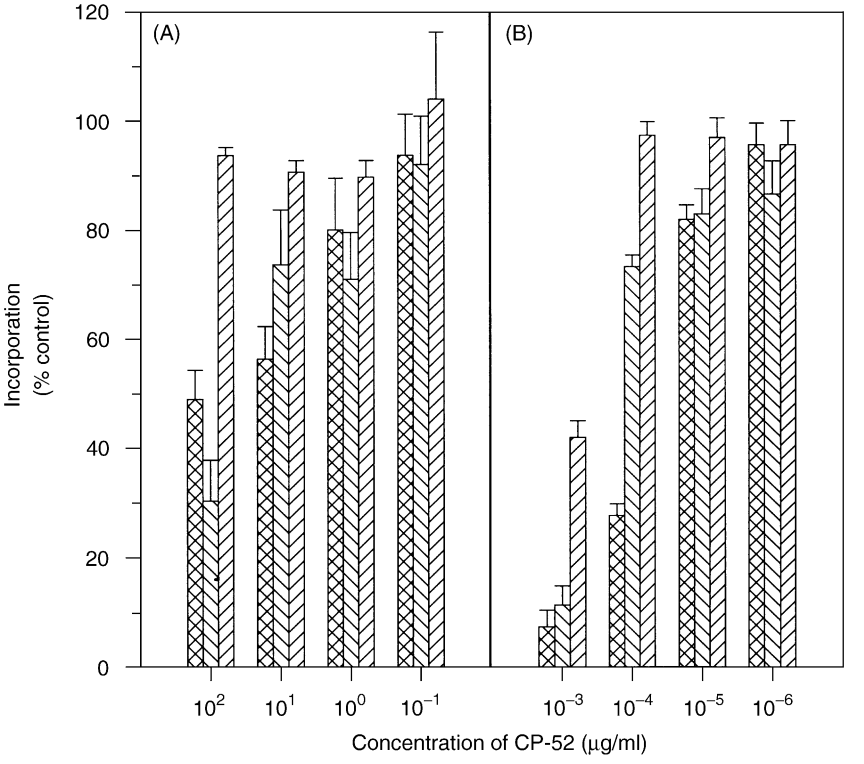


**Figure 6.** Effect of CP-52 on macromolecular synthesis inhibition. Following exposure of HCT-116 cells to different concentrations of CP-52 for (A) 2 and (B) 24 h, cells were pulse-labeled with [$^3$H]thymidine for DNA (left-hand columns), [$^3$H]uridine for RNA (center columns) or [$^3$H]leucine for protein (right-hand columns) synthesis for 30 min. Cells were lysed with ice-cold 10% TCA on glass fiber filters, washed with ice-cold ethanol, air-dried and the radioactivity measured by liquid scintillation counting. The data are from three separate experiments done in duplicate and the results are shown as the means $\pm$ SE.

antiproliferative activity is thought due to its antitubulin activity,[9] but is likely more complex[12,13] and important to its therapeutic success.[14] The application of anticancer drugs, possibly sharing the same target but exerting different effects, is valuable in combination chemotherapy for the treatment of drug

resistant tumors.[15] Martello *et al.* have attributed this type of interaction to the synergistic effects of taxol and discodermolide that, although sharing a similar mechanism of action, can potentiate apoptosis at doses that do not induce mitotic arrest.[16] Similarly, comparison of the scope of activity of CP-52 to the classic microtubulin inhibitor vinblastine leads to the conclusion that the cytotoxicity of CP-52 may not relate solely to microtubules.

Cellular mechanism of action studies were conducted in HCT-116 human colon adenocarcinoma cells since they constituted one of the human solid tumor cell lines in the *in vitro* discovery panel used to discover the antiproliferative activity of CP-52. On the basis of a comparative *in vitro* clonogenic cell assay, we have shown that CP-52 exhibits similar antiproliferative activity to HCT-116 cells following a 24-h exposure when expressed on a molar basis to Vinblastine and confirms data in earlier studies with the parent compound, CP-1.[3] The $IC_{50}$ values for antiproliferative activity in various other solid-tumor and leukemia cell lines also have been reported to be in the low picomolar range.[12,13] In addition, there was approximately a 10-fold concentration difference following a 24-h exposure to CP-52, in cell killing between log and plateau phase cells (Figure 2A) that probably reflected the phase-specific cytotoxicity of CP-52. Lastly, the present study indicates that a 24-h exposure of HCT-116 cells to CP-52, like Vinblastine, leads both to their arrest in $G_2$/M and apoptosis.

Our data suggest that the dose-dependent and selective effect of CP-52 on DNA synthesis inhibition contributes to loss of clonogenic cell growth. This is supported by the observation that clonogenic cell kill was observed following 2-h exposures to CP-52 only when the selective inhibition of DNA synthesis was more than 50% of control. Following longer CP-52 exposure times of 24 h, inhibition of DNA synthesis was inhibited at much lower drug concentrations (e.g. 1 ng/ml resulted in a surviving fraction of 0.1 while DNA, RNA and protein synthesis were inhibited to 7, 12 and 42% of control).

No clonogenic cell killing was observed following a 24-h exposure to 1 pg/ml, a dose where there was no inhibition of macromolecular synthesis but clear flow cytometric evidence of $G_2$/M arrest. Since intracellular drug concentration are known to mediate cytotoxicity,[17] pharmacokinetic studies were carried out with CP-52 that indicated initial concentration-dependent kinetics, with rapid accumulation of CP-52 by HCT-116 cells within 10 min of exposure at 30 $\mu$g/ml that reached a plateau by 60 min. When these cells were placed in drug-free medium, a rapid efflux of CP-52 was observed, with 50% of the drug remaining within the cell 15 min later that decreased steadily to 24% by 1 h.

Recent insights gained from target-oriented drug discovery have led to a re-evaluation of previous 'standard mechanisms',[18] with the addition of novel targets like DNA topoisomerase[19,20] and cyclins.[21,22] For example, although the effectiveness of adriamycin as an anticancer agent was first related to DNA binding,[23] new evidence has shown that topoisomerase II inhibition is also involved.[24] Similarly, although the binding of CP-52 to tubulin with altered microtubule dynamics may lead to $G_2$/M arrest, we have presented evidence that suggests that the antiproliferative effect of CP-52 may initially be the result of DNA synthesis inhibition followed by RNA and protein synthesis inhibition and apoptosis of drug-treated HCT-116 cells arrested in $G_2$/M.

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