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ORIGINAL ARTICLE

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In vitro pharmacology of cryptophycin 52 (LY355703) in human tumor cell lines

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Abstract *Purpose*: Cryptophycin 52 (LY355703) is a new member of the cryptophycin family of antitumor agents that is currently undergoing clinical evaluation for cancer chemotherapy. The mechanism of action of the cryptophycin class of compounds is associated with an action on microtubules. This report details the pharmacological profile of this new clinical compound in a panel of human tumor cell lines. *Methods*: Antiproliferative effects of cryptophycin 52 were measured indirectly by detection of the metabolic reduction of alamarBlue®. Cytoxicity was assessed by enzymatic dye activation (calcein AM) combined with dye exclusion (ethidium homodimer) and by clonogenicity assay. Cell cycle effects were evaluated using flow cytometry and fluorescence microscopy. *Results*: Both antiproliferative and cytotoxic effects of cryptophycin 52 were concentration- and time-dependent. IC_{50} values for antiproliferative activity in both solid and hematologic tumor cell lines were in the low picomolar range, and without exception, were significantly below values for the antimitotic agents paclitaxel and vinblastine. Flow cytometry and microscopic examination of tumor cells treated with cryptophycin 52 indicated that they accumulated in the mitotic phase of the cell cycle. Cryptophycin 52 was tested for its sensitivity to multidrug-resistance in several paired cell lines in which a sensitive parental line was matched with a multidrug-resistant derivative line. The resistant lines have been shown to over express Pgp and/or MRP multidrug-resistance transport factors. Compared to other antimitotic agents (paclitaxel, vinblastine, vincristine), the potency of cryptophycin 52 was shown to be minimally affected in multidrug-resistant cells compared to their sensitive parental lines. *Conclusion*: Cryptophycin 52 has potent antimitotic, antiproliferative and cytotoxic activity in in vitro human tumor cell models. It is significantly more potent and less sensitive to multidrug resistance mechanisms than other antimitotic antitumor agents currently used in cancer therapy. These characteristics may translate into therapeutic advantages for the clinical use of cryptophycin 52 in cancer chemotherapy.

Key words LY355703 · Cryptophycin · Antimitotic · Oncolytic · Cell cycle

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Introduction

Cryptophycins are a class of experimental depsipeptide antitumor agents. The parent compound of the series, cryptophycin 1 (cryptophycin, cryptophycin A), was isolated from the cyanobacterium *Nostoc* sp. [36, 41], and subsequently produced by chemical synthesis [2]. Cryptophycin 1 was initially described as an antifungal agent [36], and was later characterized as a cytotoxic agent with activity against tumors in vivo and cultured tumor cell lines, including drug-resistant cells [39, 41]. The mechanism of action of the cryptophycin class of compounds has been associated with an action on microtubules [1, 18, 23, 27, 28, 38, 39].

Cryptophycin 52 (LY355703; Fig. 1) is a new member of the cryptophycin family of antitumor agents that is currently undergoing clinical evaluation for cancer chemotherapy. It has been produced by total chemical synthesis. As noted by Golakoti et al. [6], the ester functionality connecting units C and D of cryptophycins (Fig. 1, box) is very susceptible to base hydrolysis. The dimethyl substitution in cryptophycin 52 was designed to reduce this susceptibility. Cryptophycin 52 is an extremely potent antiproliferative agent against a broad spectrum of cultured human tumor cells including several cell lines with a multidrug-resistant phenotype. It

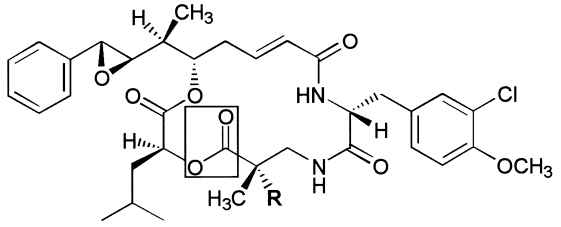


Fig. 1 Cryptophycin structure. Cryptophycin 52 (LY355703) differs from cryptophycin 1 by substitution of a methyl group at the R position. The *box* indicates the ester functionality connecting the C and D portions of the cryptophycin molecule

has activity in the low picomolar range that is comparable to that of cryptophycin 1. Like other microtubule agents, such as paclitaxel (Taxol®) and the vinca alkaloids [e.g. 16], the antiproliferative effect of cryptophycin 52 appears to be associated with its ability to block mitotic progression in cells. Its most sensitive action appears to be suppression of microtubule dynamics [25, 28]. Cryptophycin 52 is the most potent agent thus far studied with respect to suppression of microtubule dynamics [28]. At low concentrations (at or near the IC_{50}), its antimitotic effects on cells occur in the absence of significant reduction in microtubule polymer mass [44].

We demonstrated that cryptophycin 52 has antimitotic, antiproliferative and cytotoxic activity in the picomolar concentration range against a variety of human tumor cells. It was 40- to 400-fold more potent than either paclitaxel or the vinca alkaloids in all cell lines tested. Both paclitaxel and the vinca alkaloids are very sensitive to multidrug-resistance mechanisms mediated by P-glycoprotein (Pgp) and/or multidrug-resistance associated protein (MRP) transporters [22, 24]. However, in this study we showed that cryptophycin 52 was significantly less sensitive to these multidrug-resistance mechanisms based on its antiproliferative effects in cell lines containing the Pgp and MRP multidrug-resistance phenotypes.

Materials and methods

Materials

Vinblastine, vincristine and paclitaxel were purchased from Sigma Chemical Company (St.Louis, Mo.). Cryptophycin 52 (LY355703) was produced by total chemical synthesis at Lilly Research Laboratories (Indianapolis, Ind.). The compounds were dissolved in dimethyl-d_6 sulfoxide (DMSO) as 2–10 m*M* stocks, and stored at −70 °C. For experimental use, aliquots were thawed at room temperature and dilutions prepared in DMSO at 1000 or 10 000× concentrates prior to dilution into cell culture medium and addition to the cells.

Cell culture

The tumor cells lines used in the evaluation of cryptophycin 52 are listed in Table 1. Human tumor cell lines were grown at 37 °C in an atmosphere containing in 5% carbon dioxide in Corning (Corning Costar, Cambridge, Mass.) 75-cm^2 vented flasks, in cell culture medium purchased either from BioWhittaker (Walkersville, Md.) or Gibco BRL (Grand Island, N.Y.), containing 10% fetal bovine serum (HyClone, Logan, Utah), in the absence of antibiotics and supplemented with 2 m*M* L-glutamine (BioWhittaker). Exceptions are noted as footnotes to Table 1.

Cell proliferation assay

Subcultures of cells were plated to Corning 24-well tissue culture plates in densities of $1.5–2.0 \times 10^4$ cells per well. Attached cells were incubated for 48 h and suspension cultures were incubated for 24 h prior to compound addition. Except where noted in the text, half-log serial compound dilutions were prepared in DMSO and diluted into cell culture medium. The final DMSO concentration in culture was ≤0.1%. Compound dilutions were added as a total medium change to the attached cells and as a concentrated supplement to the suspension cells; the cultures were then incubated for an additional 72 or 96 h (three to four cell generations in the control cultures). Cell proliferation was measured indirectly by determining the ability of a cell population to metabolically reduce the indicator dye alamarBlue® (Sensititer/Alamar, Westlake, Ohio). The alamarBlue dye reduction assay was carried out according to the manufacturer's protocol with fluorescence measured at 560 nm excitation and 590 nm emission using a Cytofluor™ 2350 fluorescence plate reader (PerSeptive Biosystems, Framinghan, Mass.). The IC_{50} was calculated from concentration response curves following 72 or 96 h of incubation after a single compound exposure.

Drug resistance assay

Assays for multidrug resistance were carried out in several paired cell lines in which a sensitive parental line was matched with a multidrug-resistant derivative line (see Table 2). The resistant lines have been shown to overexpress Pgp and/or MRP multidrug-resistance transport factors. The IC_{50} for antiproliferative activity of test compounds in both sensitive and resistant cell lines was determined using metabolic reduction of alamarBlue as a surrogate measure for cell number. The ratio of the IC_{50} for the resistant line to that of the sensitive parental line was used to calculate a resistance factor (fold difference).

Flow cytometry

Cells were seeded to Corning 25-cm^2 flasks at 5×10^4 cells/ml and incubated 24 (suspension cells) or 48 (attached cells) h prior to compound addition. Compound dilutions for each cell line were prepared in DMSO, then diluted into cell culture medium and added as a concentrated supplement to the flasks of cells. The final compound concentrations used were the IC_{50} and three times the IC_{50}, as determined in the antiproliferation assays. Following compound exposures ranging from 6 to 48 h, the cells were removed from the flasks, centrifuged, resuspended in 300 µl PBS (calcium- and magnesium-free), fixed by addition of 700 µl reagent grade methanol added dropwise, and stored at −20 °C until analysis. Fixed cells were collected by centrifugation (Beckman GS-6KR, 2000 rpm × 10 min) and treated with modified Vindelov's propidium iodide solution, containing 5 µg/ml RNase [40]. Samples were analyzed using a Coulter EPICS XL-MCL flow cytometer with an argon ion laser and 625 nm filter to determine the cell cycle phase based upon cellular DNA content. Flow cytometry data were integrated using MultiCycle AV software (Phoenix Flow Systems, San Diego, Calif.).

Microscopy

Fluorescence microscopy of cells was carried out on an MRC 1024-UV confocal microscope system (BioRad Laboratories, Hercules, Calif.), using a Diaphot 200 inverted microscope (Nikon, Melville,

Table 1 Cell lines used

Cell	Tumor type	Growth medium	Doubling time (h)	Source
U937	Human histocytic lymphoma	RPMI-1640 with 25 m*M* HEPES	17–18	American Type Tissue Culture, Rockville, Md.
U-A10/p50	Human histocytic lymphoma MRP^+, PGP^+	RPMI-1640 with 25 m*M* HEPES	20–24	Dr. C.A. Slapak at Dana Farber Cancer Institute, Boston, Mass.
CCRF-CEM	Human acute lymphoblastic leukemia	RPMI-1640 with 25 m*M* HEPES	17–18	St Judes Children's Research Hospital, Memphis, Tenn.
HL60	Human acute myeloblastic leukemia	RPMI-1640 with 25 m*M* HEPES[a]	22	Dr. Melvin Center, Kansas State University, Manhattan, Kan.
HL60/ADR	Human acute myeloblastic leukemia, MRP^+, PGP^-	RPMI-1640 with 25 m*M* HEPES[a]	20	Dr. Melvin Center, Kansas State University, Manhattan, Kan.
HL60/Vinc	Human acute myeloblastic leukemia, MRP^-, PGP^+	RPMI-1640 with 25 m*M* HEPES[a]	20	Dr. Melvin Center, Kansas State University, Manhattan, Kan.
Caco-2	Human colon adenocarcinoma	DMEM with high glucose[b]	29	Dr. J. Fogh, Sloan Kettering Hospital, New York, N.Y.
HT-29	Human colon adenocarcinoma	McCoy's 5 A	20	Purdue University, Lafayette, Ind.
GC-3	Human colon carcinoma	RPMI-1640 with 25 m*M* HEPES	23–25	Peter Houghton, St Judes Children's Research Hospital, Memphis, Tenn.[f]
HeLa-C1	Human cervical epithelioid carcinoma transfected with pRc/CMV vector	RPMI-1640 with 25 m*M* HEPES[c]	20–21	Drs. S. Cole & R. Deeley, Queens University, Ontario, Canada
HeLa-T5	Human cervical epithelioid carcinoma transfected with pRc/CMV-MRP1	RPMI-1640 with 25 m*M* HEPES[c]	19	Drs. S. Cole & R. Deeley, Queens University, Ontario, Canada
HeLa-S3	Human cervical epithelioid carcinoma	DMEM with low glucose[d]	21	American Type Tissue Culture, Rockville, Md.
MCF-7	Human breast adenocarcinoma	MEM (phenol-red free)[e]	35	Dr. K. Hirsch, Lilly Research Laboratories, Indianapolis, Ind.
MDA-MB-231	Human breast adenocarcinoma	EMEM	20–22	American Type Tissue Culture, Rockville, Md.

[a] Medium contains 50 μg/ml gentamicin (Gibco BRL, Grand Island, N.Y.)
[b] DMEM with high glucose from GibcoBRL; 0.1 m*M* non-essential amino acids (GibcoBRL) also added
[c] 10% FBS replaced with 5% enriched calf serum (HyClone); 400 μg/ml Geneticin® (G-418 Sulfate, GibcoBRL) added for selection
[d] DMEM with low glucose from GibcoBRL; 0.1 m*M* non-essential amino acids (GibcoBRL) also added
[e] Phenol red-free MEM with Earls Salts from GibcoBRL; 10 ng/ml insulin and 0.1 m*M* non-essential amino acids (both from GibcoBRL) added
[f] Cells isolated from human tumor xenograft at Lilly Research Laboratories by R. Shultz

N.Y.). Cells were cultured in a Lab-Tek chambered coverglass, and exposed to cryptophycin 52 as described in the cell proliferation assay. After 24 h incubation, the medium was removed and the cells were fixed in 4% EM grade formaldehyde (Polysciences, Warrington, Pa.) diluted in Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride (Ca^{++}/Mg^{++}-free PBS; Gibco BRL, Grand Island, N.Y.). The cells were then rinsed in cold 70% ethanol and stored in 70% ethanol at −20 °C until ready for staining. The cells were stained using a primary monoclonal anti-β-tubulin antibody (N357, Amersham Life Science, Arlington Heights, Ill.) followed by an antimouse Ig fluorescein-linked whole antibody (N1031, Amersham Life Science). The nuclei of the cells were counterstained using Vindelov's propidium iodide solution diluted 1:10 with Vindelov's buffer. Color, digitized optical section images of the red (propidium iodide) and green (fluorescein) fluorescent signals were collected. The red and green image stacks were merged and projected onto a single plane (maximum intensity projection). For publication, the color image was converted to gray scale using Confocal Assistant software, version 4.02 (developed by T.C. Brelje).

Table 2 Resistance factors[a] in paired multidrug-resistant cell lines

Resistant cell line / Sensitive cell line (Resistance mechanism)	Cryptophycin 52	Paclitaxel	Vinblastine	Vincristine
HL-60/ Vinc / HL-60 (Pgp)	6	> 450	70	> 1000
HL-60 /ADR / HL-60 (MRP)	0.5	1.0	1.1	7
HeLa-T5 / HeLa-C1 (MRP)	1.0	1.3	2.1	32
U-A10/p50 / U937 (Pgp, MRP)	4	60	10	191

[a] Resistance factor $= \frac{IC_{50} \text{ resistant cell line}}{IC_{50} \text{ sensitive cell line}}$

Cell death determination

The Live/Dead® Viability/Cytotoxicity Kit for animal cells from Molecular Probes (Eugene, Ore.) was used with fluorescence microscopy according to the manufacturer's protocol. In this fluorescence-activation/dye-exclusion assay, cells in culture were exposed to calcein AM and ethidium homodimer. Calcein AM, a nonfluorescent dye precursor, is readily taken up by live cells. Once inside the live cell, calcein AM is metabolically converted to the fluorescent derivative calcein by esterase activity (i.e. calcein AM will only "stain" live cells with active esterases). The plasma membrane permeability barrier of live cells, however, prohibits the cellular uptake of ethidium homodimer, a fluorescent DNA nucleic acid stain, so that ethidium homodimer will only stain dead cells in which the plasma membrane permeability barrier no longer functions.

Attached cells were seeded to four-well, chambered coverglasses (Lab-Tek Nalge Nunc International, Naperville, Ill.); the coverglasses for HeLa-S3 cells were freshly coated with poly-lysine [17]. All attached cells were seeded to chambered coverglasses, 2.5–5×10^3 cells per chamber, 48 h prior to compound addition as a medium change. The suspension cells were grown and treated in Corning 75-cm^2 flasks with vented caps. Aliquots of 1 ml were removed from these flasks and placed in the chambered coverglasses for staining and counting on a Nikon Diaphot inverted fluorescence microscope. In each of the duplicate chambered coverglasses, a minimum of 200 cells per well were counted for each cell line at each time-point. The results are expressed as the percent of dead cells per total cells counted.

Clonogenicity

In the clonogenic assay, cells are exposed to test compounds for a defined period of time, washed free of the compound, diluted and replated in fresh medium, and cultured for 10 to 14 days. Cells that are viable after the exposure to test agents usually retain the ability to proliferate and form colonies when replated, whereas nonviable cells undergo no more than one or two rounds of division when replated and fail to form colonies. The attached cells were initially seeded to Corning 25-cm^2 vented flasks, 5×10^4 cells/ml, and incubated 48 h prior to compound addition. Compound dilutions for each cell line were prepared in DMSO, then diluted into cell culture medium and added as a medium change. The final concentrations used were one, three and ten times the IC_{50}, as determined in the cell proliferation assay. Following the compound exposure times, the cells were removed from the flasks and concentrated by centrifugation (Beckman GS-6KR, 1000 rpm × 10 min). The cells were then resuspended in fresh medium in log dilutions from 1×10^5 to 100 cells per ml and plated in Corning six-well plates. The plates were incubated at 37 °C in an atmosphere containing 5% carbon dioxide until colonies formed, 10 to 14 days later. Colonies were rinsed with PBS, fixed in 100% methanol and stained with 0.5% crystal violet. Colonies of greater than 32 cells [5] were then counted manually, using a stereomicroscope.

Statistics

Statistical significance was evaluated using a Dunnett's test, based upon a 95% confidence interval ($P \leq 0.05$).

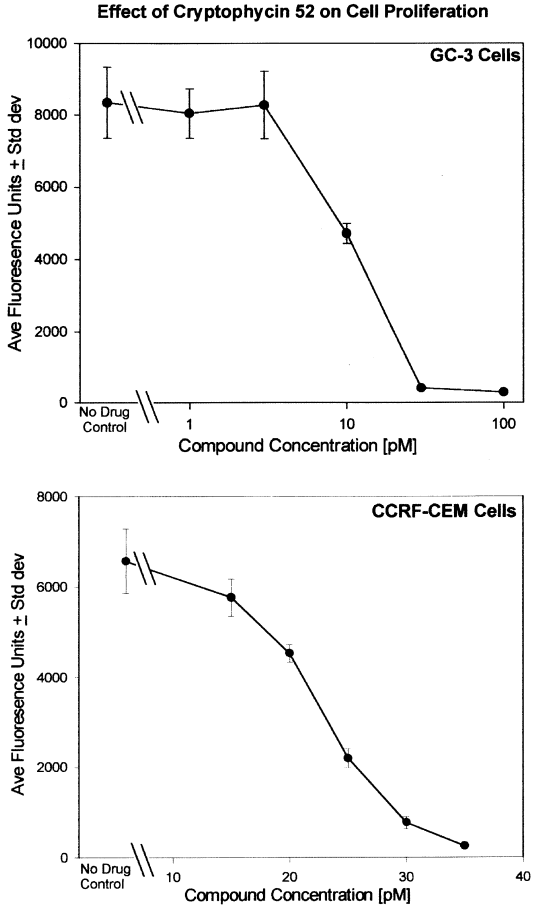
Results

Cryptophycin 52 had potent antiproliferative activity in a variety of human tumor cell lines. Table 3 lists the IC_{50} values in multiple cell lines, and a comparison with values for the other antimitotic oncolytics, paclitaxel and vinblastine. IC_{50} values for cryptophycin 52 in the cell lines we have tested were consistently in the picomolar range and without exception were significantly below comparative values in the same cell lines for the other antimicrotubule (antimitotic) agents. Half-log concentration intervals were typically used to determine IC_{50} values for cell lines; however, for the CCRF-CEM leukemia cells, 5 p*M* concentration steps were found to be necessary for accurate IC_{50} determination owing to the relatively steep concentration response (e.g. Fig. 2). The IC_{50} for the majority of cells was determined after exposure to cryptophycin 52 for three to four generations (doublings) of the nontreated control population. For most cells a 72-h exposure time was used; however, a 96-h exposure was used for Caco-2 and MCF-7 cells which had relatively long doubling times (29 and 35 h, respectively). The MDA-MB-231 cells were an exception. The 96-h incubation time for these cells resulted in approximately 4.5 generations and was used because the MDA-MB-231 cells had a very shallow concentration response curve and showed only a partial response to the antimitotic compounds (maximum of about 60% inhibition). With the exception of MDA-MB-231, the IC_{50} values for cryptophycin 52 were less than 40 p*M* in all of

Table 3 IC_{50} values (n*M*; ± SE) for antiproliferative effects of cryptophycin 52, paclitaxel and vinblastine in cultured human tumor cells following compound exposure for three to four cell generations in the control cell population (72 or 96 h incubation depending on the cell doubling time, see Table 1). MDA-MB-231 cells were exposed to compound for 4.5 generations (see Results)

Cell line	Cryptophycin 52	Paclitaxel	Vinblastine
U937, leukemia	0.015 ± 0.001[a]	1.2 ± 0.172[a]	1.11 ± 0.002[a]
CCRF-CEM, leukemia	0.022 ± 0.0003[a]	2.1 ± 0.21[a]	2.7 ± 0.29[a]
HL60, leukemia	0.018 ± 0.003[a]	2.3 ± 0.14[a]	2.9 ± 0.06[a]
HT-29, Colon carcinoma	0.036 ± 0.004[a]	3.8 ± 0.6[a]	4.6 ± 0.7[a]
GC3, colon carcinoma	0.013 ± 0.002[a]	5.6 ± 1.77[a]	2.8 ± 0.17[a]
Caco-2, colon carcinoma	0.027 ± 0.002[b]	5.3 ± 0.16[b]	4.1 ± 0.16[b]
MCF-7, mammary carcinoma	0.037 ± 0.002[b]	2.0 ± 0.9[b]	4.0 ± 0.13[b]
MDA-MB-231 mammary carcinoma	0.232 ± 0.09[b]	9.70 ± 0.07[b]	30 ± 0.26[b]
HeLa, cervical carcinoma	0.020 ± 0.002[a]	1.7 ± 0.04[a]	1.9 ± 0.29[a]

[a] 72-h incubation following compound exposure
[b] 96-h incubation following compound exposure



the nonmultidrug-resistant cell lines tested. Cryptophycin 52 was 40- to over 400-fold more potent than paclitaxel in the cell lines (average 135-fold more potent), and 70- to over 200-fold more potent than vinblastine (average 131-fold more potent).

Cryptophycin 52 was tested for its sensitivity to multidrug-resistance mechanisms in paired cell lines in which a sensitive parental cell line was matched with a multidrug-resistant derived line which overexpressed one or both of the characterized ATP-binding cassette (ABC) transporters associated with multidrug resistance (Pgp and MRP). The resistance factors calculated for cryptophycin 52 were consistently less than those for other antimitotic oncolytics evaluated in the same cell systems (Table 2). The potency of cryptophycin 52, vinblastine or paclitaxel were not markedly different from that of the sensitive parental line in the two resistant lines expressing only MRP. The MRP-expressing cell lines were however 7- and 32-fold more resistant to vincristine than the corresponding sensitive parental cells. In the cell lines expressing Pgp (HL60/Vinc and U-A10/50), cryptophycin 52 was only 6-fold and 4-fold

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Fig. 2 Examples of inhibition curves used to calculate IC_{50} values in the antiproliferative assay based on metabolic reduction of alamarBlue fluorometric dye. GC-3 human colon carcinoma and CCRF-CEM human lymphoblastic leukemia cells 72 h after a single exposure to cryptophycin 52

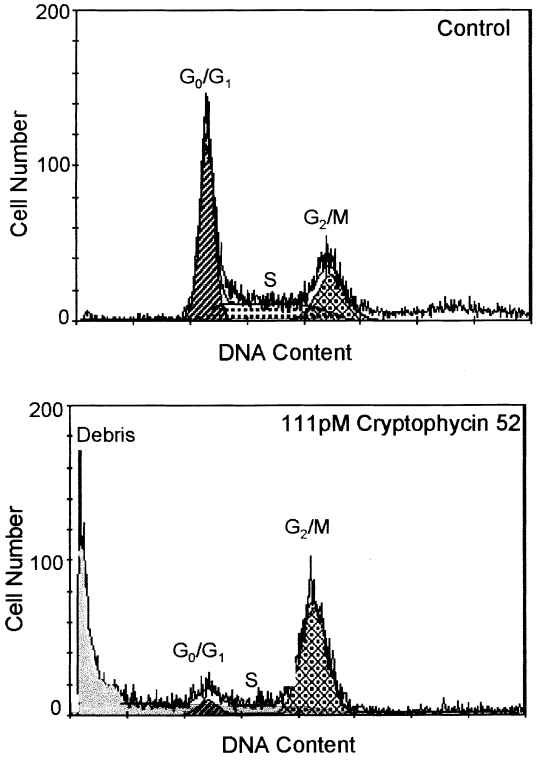

Fig. 3 Histograms of flow cytometry data showing G_2/M accumulation of MCF-7 human breast adenocarcinoma cells 24 h after exposure to 111 p*M* cryptophycin 52 (three times the IC_{50}). After compound treatment, 89% of the cells were in G_2/M and less than 10% of the cells were estimated to be in G_0/G_1. Untreated control cells contained 47% G_0/G_1 and 24% G_2/M. Significant cell debris can be detected with a low DNA content after exposure to cryptophycin 52 . The G_2/M accumulation shown for the MCF-7 cells was typical for all cells evaluated in this study. Peak G_2/M accumulation following cryptophycin 52 treatment could exceed 90% in some analyses. Cell debris appeared at varying times following treatment, depending on the cells being evaluated

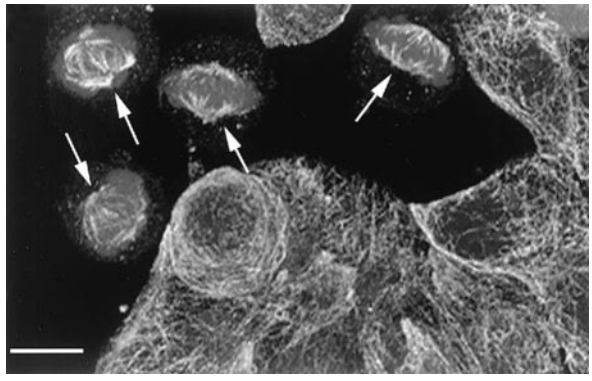

Fig. 4 HT-29 cells stained with anti-β-tubulin primary antibody and a fluorescein-linked secondary antibody to stain microtubules, then counterstained with propidium iodide to stain nuclei. The cells were fixed 24 h after exposure to 45 p*M* cryptophycin 52. The majority of cells in the culture were in mitosis (60 to 80% after the 24-h exposure period). This micrograph was selected to show both mitotic and interphase cells. Several of the mitotic cells can be seen to be in a metaphase-like configuration (*arrows*), and interphase cells contain abundant microtubules (*bar* 10 μm)

less potent in the resistant lines, compared to the sensitive parental line. By comparison, however, vinblastine was 70-fold less potent in the HL60/Vinc and 10-fold less potent in the U-A10/50 cells than in the parental lines. Vincristine was more than 1000-fold less potent in the HL60/Vinc and 191-fold less potent in the U-A10/50 cells; and paclitaxel was more than 450-fold less potent in the HL60/Vinc and 60-fold less potent in the U-A10/50 cells than in their sensitive counterparts.

Cells incubated with cryptophycin 52 at one to three times the IC_{50} concentration, accumulated in the G_2/M phase of the cell cycle, as determined by flow cytometry (e.g. Fig. 3). Microscopic examination indicated that the majority of cells appeared to have a metaphase-like morphology when examined 6 to 24 h after exposure to cryptophycin 52 at one to three times the IC_{50} (e.g. Fig. 4).

The cytotoxic effects of cryptophycin 52 in cultured tumor cells were evaluated by both a fluorescence-activation/dye-exclusion (live/dead) assay and by a clonogenicity assay. Figure 5 illustrates data from five of the cell lines tested. Each cell line was evaluated at 0, 1, 3 and 10 times the IC_{50} determined in the alamarBlue dye reduction assay. For Caco-2 and MCF-7 cells the concentrations used were based on the 72-h IC_{50} rather than the 96-h IC_{50} shown in Table 3. In all cell lines examined, there was a time- and concentration-dependent increase in the occurrence of ethidium homodimer-positive (dead) cells in the population. In the leukemia cell line, CCRF-CEM, the response was variable at 20 p*M* ($0.9 \times IC_{50}$) which reflects the steep dose–response curve in these cells. In the assay shown, no significant cell death was noted even after prolonged incubation (96 h). At higher cryptophycin 52 concentrations (60 and 200 p*M*), however, there was significant ($P \leq 0.05$) cell death at 48 and 96 h in CCRF-CEM cells. In the Hela-S3 cervical carcinoma cell line and the Caco-2 colon carcinoma cells there were no differences between treated and control groups at 24 h, but a concentration-dependent, progressive cell death was evident with longer incubation periods. The data for the HT-29 colon carcinoma cells and the MCF-7 mammary carcinoma cells were more variable because of the difficulty in distinguishing individual cells due in part to the fragmentation of dead cells at the later time-points. Nevertheless, the data indicate that significant cell death had occurred in MCF-7 cells at 700 p*M* by 24 h, at 210 and 700 p*M* by 72 h, and at all concentrations of cryptophycin 52 by 96 h. In HT-29 cells the data for cytotoxicity were statistically significant only at 35 and 350 p*M* after 24 h exposure to compound.

The percentage of dead cells (ethidium homodimer-positive) in the CCRF-CEM, Caco-2 and HeLa-S3 cell populations reached about 80% by 96 h. In contrast, MCF-7 cells were less sensitive to cryptophycin 52. In the MCF-7 cell line, the percentage of dead cells did not exceed 50 to 60% even after prolonged incubation. A similar result was noted in the proliferation assay, where the percent inhibition in the MCF-7 cell population did

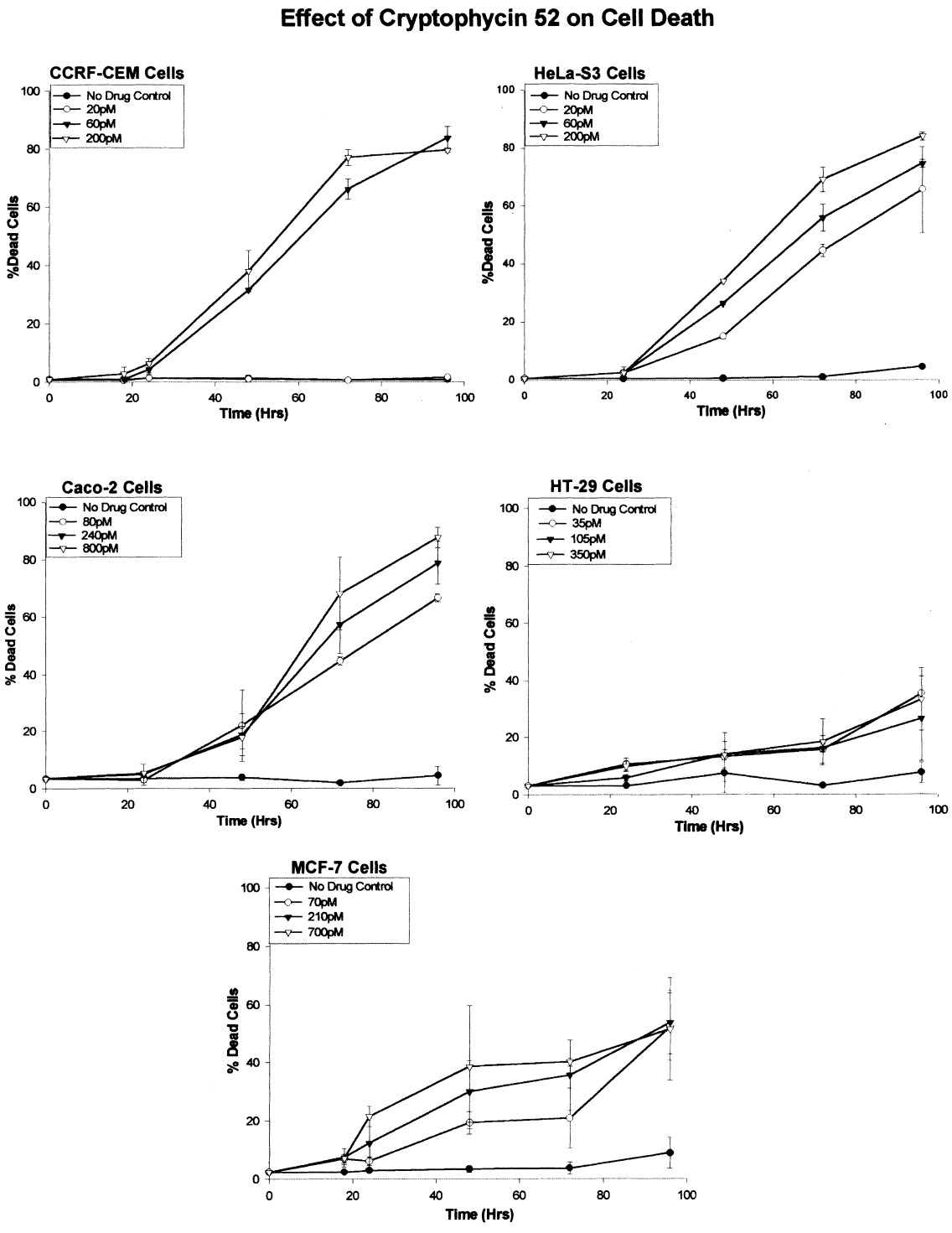

Fig. 5 Concentration-dependent cytotoxic effects of cryptophycin 52 on different cell lines as assessed by the calcein AM/ethidium homodimer fluorescence-activation/dye-exclusion (live/dead) assay. The data represent microscopic counts of live cells (calcein stained) and dead cells (ethidium homodimer stained) and are expressed as the percentage of dead cells in the population. At least 200 cells were counted in each of two duplicate cultures for each graph point

not exceed 60 to 70% in the presence of high concentrations ($>100 \times IC_{50}$) of cryptophycin 52, paclitaxel or vinblastine. Inhibition of proliferation after 72 h incubation with cryptophycin 52 at 100 to 300 p*M* in the HT-29 cells was 85 to 90%. By comparison, only about 30% of the HT-29 cells appeared ethidium homodimer-positive following 96 h of exposure to cryptophycin 52. We note, however, that quantitation of the effect of cryptophycin 52 on HT-29 cells by microscopic counting was very difficult. HT-29 cells grew as compact clusters,

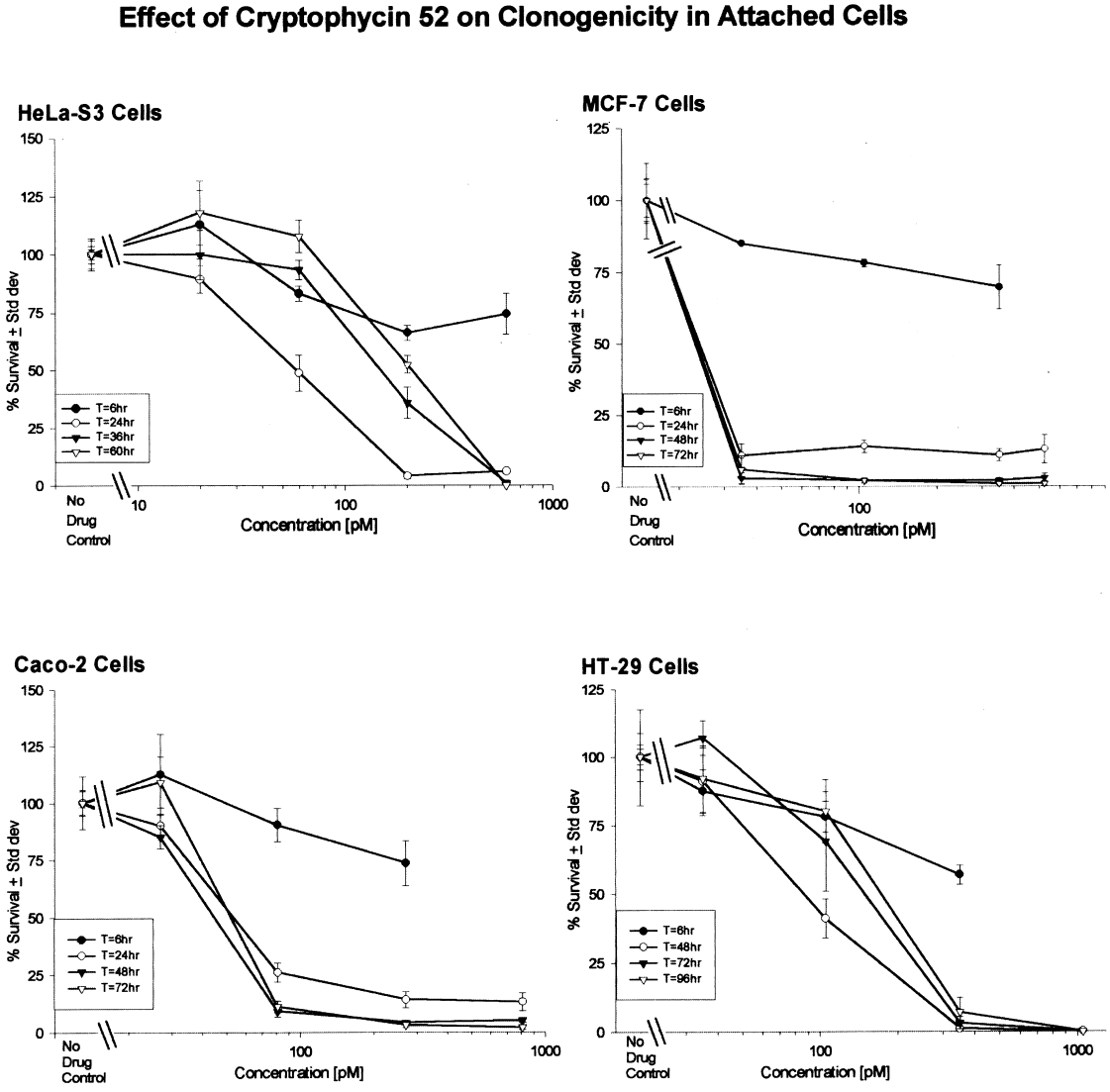

Fig. 6 Concentration-dependent cytotoxic effects of cryptophycin 52 on different cell lines as assessed by the clonogenic cell survival assay. The data represent the number of colonies that formed when cells treated with cryptophycin 52 for 6 to 96 h were diluted and replated in fresh medium. The data are expressed as the percentage of the plated cells that survived and formed colonies after 10 to 14 days in culture. Data represent the mean and standard deviation from duplicate cultures

and cells exposed to the higher cryptophycin 52 concentrations fragmented, making them difficult to count.

We examined the cytotoxic effect of cryptophycin 52 in a clonogenic assay using four attached cell lines, HeLa-S3 cervical carcinoma, MCF-7 mammary cancer, and Caco-2 and HT-29 colon carcinoma cells. Cryptophycin 52 concentrations of 1, 3, 10 and 30 times the antiproliferative IC_{50} were used in a single exposure for a 6- to 96-h incubations. We observed a concentration-dependent decrease in cell viability in each of the cell lines (Fig. 6). In HeLa-S3 cells, there was a statistically significant ($P \leq 0.05$) reduction in viability at the IC_{50} only after 24 h exposure, and at three times IC_{50} after 6 or 24 h exposure to the compound. At higher concentrations of cryptophycin 52, there was a significant reduction in cell viability with all exposure times. In MCF-7 cells, cryptophycin 52 significantly reduced viability with all exposure times and concentrations. In Caco-2 cells, statistically significant cytotoxicity occurred after 24 and 48 h exposure to cryptophycin 52 at the $IC_{50,}$ and after 24, 48 or 72 h exposure to higher concentrations of the compound. There was a slight concentration-dependent reduction in viability after 6 h exposure to cryptophycin 52. The data, however, were not statistically significant owing to sample variability. In HT-29 cells, only the 48-h data showed significant cytotoxicity at the IC_{50} level, but at higher concentrations, cell viability was significantly reduced with all exposure times.

Discussion

A number of natural products have been described which are antimitotic in cells and bind to and disrupt microtubule function [8, 15]. Several of these natural

products or derived analogs have clinical utility in the treatment of neoplasms. The vinca alkaloids, vinblastine (Velban®, Velbe®) and vincristine (Oncovin®), were isolated from the plant *Catharanthus rosea* and characterized as antitumor agents over 40 years ago [13]. Vinblastine, vincristine, vindesine (Eldisone®) and more recently vinorelbine (Navelbine®) are used both alone and in combination therapy for treatment of leukemia and lymphomas as well as other tumors [9, 12, 14]. The antimicrotubule agent paclitaxel has been used effectively in both advanced cancers of diverse types and in initial therapy for early stage cancers [33]. Antimitotic agents may differ significantly in the spectrum of tumors that they affect therapeutically. Recently, paclitaxel or vinblastine in combination with a third antimicrotubule agent, estramustine (estramustine phosphate, Emcyt®), has been found to be useful in the treatment of prostate cancer [9–11]. Thus, antitumor agents with the same cellular target (microtubules), but with somewhat different mechanisms of action and binding sites [26] may be useful in combination as cancer chemotherapy. Cryptophycin 52 binds to the vinca domain of microtubules and is the most potent suppressor of microtubule dynamics so far studied [28]. At low to moderate concentrations (near the antiproliferative IC_{50}), cryptophycin 52 blocks mitosis at the metaphase-anaphase transition without demonstrating significant effects on microtubule polymer mass. The cytotoxic effects of cryptophycin 52 appear to be mediated at least in part through an apoptotic mechanism of action [44].

Preliminary reports (abstracts) have noted the antiproliferative activity of cryptophycin 52 against cultured human tumor cells [35, 43], its antitumor activity in animal models [4, 30, 45], and its potent activity in suppressing microtubule dynamics in vitro [25]. The data we have presented in this report detail the broad spectrum of antiproliferative and cytotoxic activity of cryptophycin 52 against a diverse panel of human tumor cell lines. We used IC_{50} values based on a dye reduction assay to evaluate compound effects. The assay is both time- and concentration-dependent. Reduced incubation time after compound exposure resulted in increased IC_{50} values. For example, the 72-h (2.5 generations) Caco-2 IC_{50} was 80 pM, whereas the 96-h (3.3 generations) IC_{50} was 27 pM. We standardized assay time at three to four cell generations using exposure times of 72 or 96 h. With the exception of the mammary cell line MDA-MB-231, IC_{50} values for cryptophycin 52 were less than 40 pM in all of the nonmultidrug-resistant cell lines tested. Even in the HL60/Vinc multidrug-resistant cell line, cryptophycin 52 had an IC_{50} of 126 pM. The antiproliferative potency for cryptophycin 52 in this panel of human tumor lines is significantly greater than both paclitaxel and vinblastine. The average IC_{50} for cryptophycin 52 was about 130-fold less than the IC_{50} of either vinblastine or paclitaxel in the same cell lines. In addition, cryptophycin 52 was several 100- to over a 1000-fold more potent than vinblastine or paclitaxel in the Pgp multidrug-resistant cell lines.

Cryptophycin 52 induced cytotoxicity measured by either the calcein AM/ethidium homodimer assay or clonogenicity was concentration-dependent in the cell lines tested. The response of cells to cryptophycin 52 in the clonogenic assay suggests that a relatively short exposure (6 h) to cryptophycin 52 was sufficient to cause significant decreases in viability, especially at three to ten times the antiproliferative IC_{50}. At longer exposure times the cytotoxic effects of cryptophycin 52 in all of the cell lines were enhanced. An essential difference between the two cytotoxicity assays was that in the calcein AM/ethidium homodimer assay, cells were incubated with cryptophycin 52 for the full time period of the assay, whereas, in the clonogenic assay, cells were exposed to the compound for a period of time, then washed free of the compound, and replated in fresh medium. Several issues complicate interpretation of these results. Because of the extreme potency of cryptophycin 52 in cell culture (i.e. effective concentrations in the low picomolar range), we were unable to make direct measurements of the half-life of the molecule in cell culture medium. At compound concentrations in the micromolar range, however, the half-life was estimated to be relatively short (less than a few hours). In addition, cryptophycin 52 has been reported to adsorb readily to glass, plastic and serum proteins [35]. Thus the effective concentration at the cell level may differ significantly from the levels initially placed into the culture medium. This is further complicated by the fact that cells have the ability to concentrate cryptophycin 52 several 100-fold [28]. While these issues make it difficult to assess just how potent cryptophycin 52 actually is, it remains true that the addition of picomolar quantities of this agent to cultures of human tumor cells is sufficient to inhibit growth and kill the cells.

Multidrug resistance limits the therapeutic utility of a variety of anticancer natural products, including the taxanes and vinca alkaloid antimicrotubule agents. The best-characterized factors associated with the multidrug-resistant phenotype are the members of the ATP-binding cassette (ABC) superfamily of transporter molecules, Pgp and MRP [22]. The exact mechanism by which these molecules mediate drug efflux remains somewhat controversial [32], but there is little doubt that they significantly effect a cell's susceptibility to a diverse family of cytotoxic agents. The sensitivity of a potential therapeutic antitumor agent to drug resistance mechanisms is viewed as an important criterion for determining whether it should be carried forward through preclinical and clinical evaluation. A number of cell lines have been developed which overexpress either one or both of these molecules [7, 20, 37]. These cell lines make useful tools to evaluate, in the preclinical setting, how a new experimental agent may be affected by these important mediators of clinical drug resistance. Some caution is warranted in the interpretation of data from cell lines selected for multidrug resistance with respect to the mechanism of resistance. Of the resistant cell lines we used, three were obtained by exposing cells to a toxic concentration of a chemotherapeutic agent and then

selecting surviving cells for propagation. HL60/Vinc was selected using vincristine, and HL60/ADR and U-A10/p50 were selected using doxorubicin (Adriamycin). The multidrug resistance associated with these cells correlated with the expression of the ABC transporters Pgp (HL60/Vinc) and MRP (HL60/ADR), or a combination of the two (U-A10/p50), though additional resistance mechanisms may also be present in these selected lines [19–21, 37]. These other resistance mechanisms may be specific to the selective agent rather than associated with broader resistance to multiple classes of toxic agents. HeLa-T5 cells were obtained by transfection of the MRP gene into this cell, and multidrug resistance is presumably directly attributable to MRP expression [7].

Cryptophycin 1, the parent compound in the cryptophycin class of antimitotic agents, has been shown to have reduced susceptibility to Pgp-mediated multidrug resistance [39]. Our data indicate that cryptophycin 52 also has reduced susceptibility to drug resistance mechanisms in Pgp-expressing cell lines as compared to paclitaxel, vinblastine and vincristine. In addition, we examined drug resistance in cell lines expressing MRP, and cryptophycin 52 showed no evidence of susceptibility to MRP-mediated drug resistance. As expected, paclitaxel and vinblastine also showed relatively little drug resistance due to MRP, whereas vincristine was sensitive to MRP-mediated resistance [3, 29, 46]. Cryptophycin 52 displayed very modest effects in the Pgp-expressing cell lines (four- to sixfold) as compared to the other antimicrotubule agents. In the well-characterized HL60/Vinc cell line, the cells were 70-fold resistant to vinblastine, greater than 450-fold resistant to paclitaxel and greater than 1000-fold resistant to vincristine when compared to the HL60 parental cells. Resistance to the effects of antimicrotubule agents may be caused by mechanisms other than those associated with drug efflux by multidrug-resistance transporters. Modification of target molecules [31, 34] or intracellular drug sequestration (as observed in the U-A10/p50 cell line) [42] are examples of non-efflux-related resistance mechanisms. Nevertheless, efflux-related mechanisms are implicated in limiting the clinical utility of a variety of anticancer agents. The data indicate that cryptophycin 52 has decreased sensitivity to efflux-related drug resistance mechanisms, and this, coupled with picomolar potency, support cryptophycin 52 as a strong clinical candidate for cancer chemotherapy.

Cryptophycin 52 was selected from an extensive group of over 450 experimental cryptophycin compounds for clinical evaluation. It has a number of features which make it an attractive candidate for clinical use. We have shown that cryptophycin 52 is an exceptionally potent antiproliferative and cytotoxic agent against human tumor cell lines. Mechanistically, cryptophycin 52 inhibits microtubule polymerization and powerfully suppresses microtubule dynamics and function [28], blocks cells in mitosis and induces apoptosis [44]. Compounds with a similar antimitotic mechanism of action have proven to be very useful in the clinical setting as antitumor agents. Preliminary reports have shown that cryptophycin 52 reduces tumor mass and/or delays tumor progression in mouse tumors and human tumor xenograft models [4, 30, 45], and, as demonstrated in this study, unlike paclitaxel and the vinca alkaloids, it is relatively insensitive to the multidrug-resistance factors Pgp and MRP.

References

1. Bai R, Schwartz RE, Kepler JA, Pettit GR, Hamel E (1996) Characterization of the interaction of cryptophycin 1 with tubulin: binding in the vinca domain, competitive inhibition of dolastatin 10 binding, and an unusual aggregation reaction. Cancer Res 56: 4398–4406
2. Barrow RA, Hemscheidt T, Liang J, Paik S, Moore RE, Tius MA (1995) Total synthesis of cryptophycins: revision to the structures of cryptophycins A and C. Am Chem Soc 117: 2479–2490
3. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 54: 5902–5910
4. Corbett T, Valeriote F, Simpson C, Moore R, Tius M, Barrow R, Hemscheidt T, Liang J, Paik S, Polin L, Pugh S, Kushner J, Harrison S, Shih C, Martinelli M (1997) Preclinical antitumor activity of cryptophycin-52/55 (C-52;C-55) against mouse tumors. Proc Am Assoc Cancer Res 38: 225 (abstract 1515)
5. Freshney RI (1987) Measurement of cytotoxicity and viability. In: Culture of animal cells: a manual of basic technique, 2nd edn. Wiley-Liss, New York Chichester Brisbane Toronto Singapore, pp 246–248
6. Golakoti T, Ogino J, Heltzel CE, Husebo TL, Jensen CM, Larsen LK, Patterson GML, Moore RE, Mooberry SL, Corbett TH, Valeriote FA (1995) Structure determination, conformational analysis, chemical stability studies, and antitumor evaluation of the cryptophycins. Isolation of 18 new analogs from *Nostoc* sp. Strains GSV 224. J Am Chem Soc 117(49): 12030–12049
7. Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, Deeley RG (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Res 54: 357–361
8. Hamel E (1996) Antimitotic natural products and their interactions with tubulin. Med Res Rev 16: 207–231
9. Hudes GR, Greenberg R, Krigel RL, Fox S, Scher R, Litwin S, Watts P, Speicher L, Tew K, Comis R (1992) Phase II study of estramustine and vinblastine, two microtubule inhibitors in hormone-refractory prostate cancer. J Clin Oncol 10: 1754–1761
10. Hudes GR, Obasaju C, Chapman A, Gallo J, McAleer C, Greenberg R (1995) Phase I study of paclitaxel and estramustine: preliminary activity in hormone-refractory prostate cancer. Semin Oncol 22(3) [Suppl 6]: 6–11
11. Hudes GR, Nathan FE, Khater C, Greenberg R, Gomella L, Stern C, McAleer C (1995) Paclitaxel plus estramustine in metastatic hormone refractory prostate cancer. Semin Oncol 22(5) [Suppl 12]: 41–45
12. Jacobs AD, Gale RP (1984) Recent advances in the biology and treatment of acute lymphoblastic leukemia in adults. N Engl J Med 311: 1219–1231
13. Johnson IS, Wright HF, Svoboda GH, Vlantis J (1960) Antitumor principles derived from vinca rosea Linn: I. Vinca leucoblastine and leurosine. Cancer Res 20: 1016–1022
14. Johnson SA, Harper P, Hortobagyi GN, Pouillart P (1996) Vinorelbine: an overview. Cancer Treat Rev 22: 127–144
15. Jordan MA, Wilson L (1998) Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr Opin Cell Biol 10: 123–130

16. Jordan MA, Toso RJ, Thrower D, Wilson L (1993) Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc Natl Acad Sci USA 90: 9552–9556
17. Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L (1996) Mitotic block induced in HeLa cells by low concentrations of paclitaxel (taxol) results in abnormal mitotic exit and apoptotic cell death. Cancer Res 56: 816–825
18. Kerksiek K, Mejillano MR, Schwartz RE, Georg GI, Himes RH (1995) Interaction of cryptophycin 1 with tubulin and microtubules. FEBS Lett 377: 59–61
19. Marsh W, Center MS (1987) Adriamycin resistance in HL60 cells and accompanying modification of a surface membrane protein contained in drug-sensitive cells. Cancer Res 47: 5080–8086
20. McGrath T, Center MS (1987) Adriamycin resistance in HL60 cells in the absence of detectable P-glycoprotein. Biochem Biophys Res Commun 145: 1171–1176
21. McGrath T, Latoud C, Arnold ST, Safa AR, Felsted RL, Center MS (1989) Mechanisms of multidrug resistance in HL60 cells: analysis of resistance associated membrane proteins and levels of *mdr* gene expression. Biochem Pharmacol 38(20): 3611–3619
22. McKenna SL, Padua RA (1997) Multidrug resistance in leukemia. Br J Haematol 96: 659–674
23. Mooberry SL, Taoka CR, Busquets L (1996) Cryptophycin 1 binds to tubulin at a site distinct from the colchicine binding site and at a site that may overlap the vinca binding site. Cancer Lett 107: 53–57
24. Nooter K, Stoter G (1996) Molecular mechanisms of multidrug resistance in cancer chemotherapy. Pathol Res Pract 192: 768–780
25. Panda D, Williams DC, Wagner MM, Paul DC, Habeck LL, Mendelsohn LG, Shih C, Moore RE, Wilson L (1997) Inhibition of microtubule polymerization and dynamics by two novel cryptophycins, cryptophycins 52 and 55. Proc Am Assoc Cancer Res 38: 225 (abstract 1517)
26. Panda D, Miller HP, Islam K, Wilson L (1997) Stabilization of microtubule dynamics by estramustine by binding to a novel site in tubulin: a possible mechanistic basis for its antitumor action. Proc Natl Acad Sci U S A 94: 10560–10564
27. Panda D, Himes RH, Moore RS, Wilson L, Jordan MA (1997) Mechanism of action of the unusually potent microtubule inhibitor, cryptophycin 1. Biochemistry 36: 12948–12953
28. Panda D, DeLuca K, Williams D, Jordan MA, Wilson L (1998) Antiproliferative mechanism of action of cryptophycin 52: kinetic stabilization of microtubule dynamics by high affinity binding to microtubule ends. Proc Natl Acad Sci USA 95: 9313–9318
29. Paul S, Breuninger LM, Kruh GD (1996) ATP-dependent transport of lipophilic cytotoxic drugs by membrane vesicles prepared from MRP-overexpressing HL60/ADR cells. Biochemistry 35(44): 14003–14011
30. Polin L, Valeriote F, Moore R, Tius M, Barrow R, Hemscheidt T, Liang J, Paik S, White K, Harrison S, Shih C, Martinelli M, Corbett T (1997) Preclinical antitumor activity of cryptophycin-52/55 (C-52;C-55) against human tumors in SCID mice. Proc Am Assoc Cancer Res 38: 225 (abstract 1514)
31. Ranganathan S, Dexter DW, Benetatos CA, Chapman AE, Tew KD, Hudes GR (1996) Increase of βb_{III}- and βb_{IVa}-tubulin isotypes in human prostate carcinoma cells as a result of estramustine resistance. Cancer Res 56: 2584–2589
32. Roepe PD, Wei L, Hoffman MM, Friederike F (1996) Altered drug translocation mediated by the MDR protein: direct, indirect, or both? J Bioenerg Biomembr 28: 541–555
33. Rowinsky EK (1997) The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 48: 353–374
34. Schibler MJ, Cabral F (1986) Taxol-dependent mutants of chinese hamster ovary cells with alterations in α- and β-tubulin. J Cell Biol 102: 1522–1531
35. Schultz RM, Shih C, Harrison S, Martinelli MJ, Toth JE, Andis SL (1997) In vitro antiproliferative activity of the epoxide cryptophycin analogue, LY355703. Proc Am Assoc Cancer Res 38: 225 (abstract 1518)
36. Schwartz RE, Hirsch CF, Sesubm DF, Flor JE, Chartrain M, Fromtling RE, Harris GH, Salvatore MJ, Liwaxh JM, Yudin K (1990) Pharmaceuticals from cultured algae. J Indian Microbiol 5: 113–124
37. Slapak CA, Mizunuma N, Kufe DW (1994) Expression of the multidrug resistance associated protein and P-glycoprotein in doxorubicin-selected human myeloid leukemia cells. Blood 84: 3113–3121
38. Smith CD, Zhang X (1996) Mechanism of action of cryptophycin. Interaction with the vinca alkaloid domain of tubulin. J Biol Chem 271: 6192–6198
39. Smith CD, Zhang X, Mooberry SL, Patterson GML, Moore RE (1994) Cryptophycin: a new antimicrotubule agent active against drug-resistant cells. Cancer Res 54: 3779–3784
40. Tonkinson JL, Marder P, Andis SL, Schultz RM, Gossett LS, Shih C, Mendelsohn LG (1997) Cell cycle effects of antifolate antimetabolites: implications for cytotoxicity and cytostasis. Cancer Chemother Pharmacol 39: 521–531
41. Trimurtulu G, Ohtani I, Patterson GML, Moore RE, Corbett TH, Valeriote FA, Demchik L (1994) Total structures of cryptophycins, potent antitumor depsipeptides from the blue-green alga *Nostoc* sp. strain GSV. J Am Chem Soc 116: 4729–4737
42. Williams DC, Paul DC, Wagner MM, Slapak CA (1997) Daunorubicin, paclitaxel Bodipy® FL, rhodamine 123 and the fluorescent lysosomal probes LysoSensor™ yellow/blue and acridine orange accumulate in a common vesicular compartment in multidrug resistant U-A10 myeloid leukemia cells. Proc Am Assoc Cancer Res 38: 386 (abstract 2589)
43. Williams DC, Wagner MM, Law KL, Shepard RL, Paul DC, Starling JJ, Dantzig AH, Moore RE, Shih C (1997) In vitro pharmacology of cryptophycin 52 (LY355703) and cryptophycin 55 (LY355702) in human tumor cell lines. Proc Am Assoc Cancer Res 38: 226 (abstract 1519)
44. Williams DC, Wagner MM, Paul DC, Jordan MA, Wilson L, Shih C (1998) The mechanism of action of cryptophycin 52 (LY355703): cellular effects on human colon carcinoma cell lines HT-29 and Caco-2 (in press)
45. Worzalla JF, Cao J, Ehlhardt WJ, Harrison SD, Law KL, Martinelli MJ, Self TD, Starling JJ, Shih C, Theobald KS, Toth JE, Zimmermann JL, Corbett TH (1997) LY355702 and LY355703, new cryptophycin analogues with antitumor activity against human tumor xenografts. Proc Am Assoc Cancer Res 38: 225 (abstract 1516)
46. Zaman GJR, Flens MJ, vanLeusden MR, deHaas M, Mulder H, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ, Borst P (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA 91: 8822–882