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ORIGINAL ARTICLE

Ku Lu · Jack Dempsey · Richard M. Schultz
Chuan Shih · Beverly A. Teicher

Cryptophycin-induced hyperphosphorylation of Bcl-2, cell cycle arrest and growth inhibition in human H460 NSCLC cells

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Abstract Bcl-2 has been described as a factor that can protect from apoptosis. The protective effect of Bcl-2 may be lost if the protein is phosphorylated. Bcl-2 phosphorylation can be induced by agents that affect microtubule depolymerization or prevent microtubule assembly. In 13 human tumor cell lines there was a high degree of heterogeneity in Bcl-2 protein expression. Human H460 non-small-cell lung carcinoma (NSCLC) cells expressed high levels of Bcl-2 and were selected for study. Western blot analysis for Bcl-2 phosphorylation was carried out after 4 h and 24 h of exposure to cryptophycin 52, cryptophycin 55, paclitaxel or vinblastine. Cryptophycin 52 and cryptophycin 55 were very potent inducers of Bcl-2 phosphorylation. After 4 h of exposure, Bcl-2 phosphorylation was evident with 0.05 n*M* cryptophycin 52, 0.25 n*M* cryptophycin 55, 5 n*M* vinblastine and 50 n*M* paclitaxel. The hyperphosphorylated form of Bcl-2 was evident after 24 h exposure of H460 cells to 0.25 n*M* cryptophycin 52 or cryptophycin 55 and 50 n*M* vinblastine or paclitaxel. The effects of the compounds on the cell cycle paralleled those on Bcl-2 phosphorylation. In H460 cells 90% cell killing was obtained with 0.13 n*M* cryptophycin 52, 0.2 n*M* cryptophycin 55, 20 n*M* paclitaxel and > 100 n*M* vinblastine after 24 h of exposure as determined by colony formation. In Bcl-2-negative Calu-6 NSCLC cells, 90% cell killing was obtained with 0.03 n*M* cryptophycin 52, 0.1 n*M* cryptophycin 55, 11 n*M* paclitaxel and 0.5 n*M* vinblastine using the same experimental design. Thus, cryptophycins are potent inducers of Bcl-2 phosphorylation. The cryptophycins were more potent cytotoxic agents in Bcl-2-negative Calu-6 cells than in Bcl-2-positive H460 cells indicating that pathways triggered by Bcl-2 phosphorylation are involved in cryptophycin-induced lethality.

Key words Bcl-2 · Cryptophycins · Cell cycle arrest · Microtubule inhibitors

K. Lu · J. Dempsey · R. M. Schultz · C. Shih · B. A. Teicher (✉)
Lilly Research Laboratories, Lilly Corporate Center,
Indianapolis, IN 46285, USA
e-mail: teicher_beverly_a@lilly.com
Tel.: +1-317-2762739; Fax: +1-317-2776285

Introduction

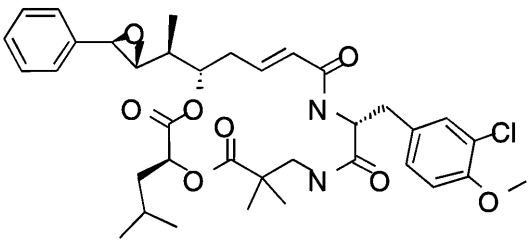
The Bcl-2 protein family is a family of apoptosis regulators protecting cells from stresses. Expression of Bcl-2 is an intracellular suppressor of apoptosis or programmed cell death [1, 2, 3, 4, 5, 6]. Bcl-2 expression has been shown to protect cells from a variety of external stresses including chemotherapeutic agents and radiation. Bcl-2 expression correlates with poor outcome in several cancers [7, 8]. Microtubule-stabilizing agents such as paclitaxel and docetaxel, and microtubule-disrupting drugs such as vincristine and vinblastine, have antimitotic and apoptosis-inducing activity. These drugs induce apoptosis by disorganization of microtubule structure. Several studies have demonstrated that bcl-2 phosphorylation can be specifically induced by drugs that affect microtubule depolymerization or prevent microtubule assembly [9, 10, 11, 12, 13, 14, 15, 16, 17]. Human glioma cells, breast cancer cells, ovarian cancer cells, multiple myeloma cells and prostate cancer cells exposed to paclitaxel express a phosphorylated form of Bcl-2 and undergo apoptosis [9, 10, 11, 12, 13, 14, 15]. These and other data suggest that phosphorylation of Bcl-2 may inhibit bcl-2 function [16]. Furthermore, Bcl-2 phosphorylation occurs relatively early after paclitaxel exposure, much earlier than other observable features of cell death. Bcl-2 phosphorylation may, thus, be an early signal of apoptosis.

Cryptophycin-1, a desipeptide isolated from cyanobacteria of the genus *Nostoc*, was initially described as an antifungal agent [18]. It later was shown to have antimitotic activity and cytotoxicity toward tumor cells in culture and anticancer activity against murine solid tumor models and human tumor xenografts [19, 20, 21,

22]. Subsequently, many cryptophycins have been isolated and prepared by chemical synthesis [23]. The mechanism of anticancer action of the cryptophycins has been associated with their action on intracellular microtubules [24, 25, 26, 27, 28]. In cell culture, the cryptophycins maintain activity against ovarian and breast carcinoma cells that overexpress the multidrug resistance efflux pump P-glycoprotein (MDR-1) as well as the MRP multidrug resistance protein, and in in vivo tumor models, the cryptophycins have been shown to lack cross resistance with paclitaxel and doxorubicin [19, 23, 25, 26].

Microtubules are dynamic assemblies involved in the maintenance of cell structure, regulation of membrane transport processes, cell motility and proliferation. Agents such as paclitaxel promote tubulin assembly and hyperstabilize microtubules, while agents such as colchicine and vinca alkaloids inhibit tubulin polymerization and destabilize microtubules [29]. The actions of the cryptophycins resemble those of the vinca alkaloids, so that it is likely that the cryptophycins interact with the vinca alkaloid binding domain or at a site that overlaps with the vinca alkaloid binding domain of tubulin [25, 26, 30, 31, 32, 33].

Cryptophycin 52 (LY355703), a synthetic cryptophycin, is currently undergoing phase II clinical trial [34]. Cryptophycin 52 has demonstrated very potent activity against human tumor cell lines in culture [35, 36] and a broad range of antitumor activity against both murine and human tumors. Cryptophycin 52 has demonstrated marked antitumor activity against both early and well-established tumors [19, 21, 22]. The aim of the current study was to explore the effects of cryptophycins on Bcl-2 phosphorylation and the cell cycle, and their cytotoxicity, in Bcl-2-positive and negative cells.



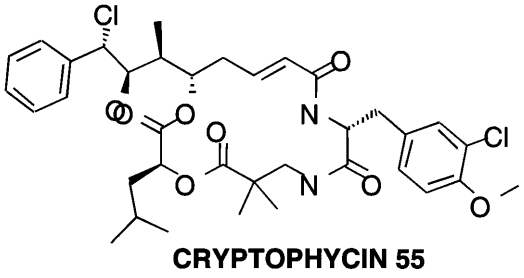


Fig. 1 Chemical structures of cryptophycin 52 and cryptophycin 55

Materials and methods

Drugs

Cryptophycin 52 (LY355703) and cryptophycin 55 were produced by total chemical synthesis at Lilly Research Laboratories (Indianapolis, Ind.) (Fig. 1). Paclitaxel and vinblastine were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cryptophycin 52, cryptophycin 55, paclitaxel and vinblastine were prepared in DMSO. The final concentration of DMSO in the cell culture medium was 0.5%. The mouse monoclonal IgG_1 Bcl-2 primary antibody was purchased from Santa Cruz (sc-509; Santa Cruz, Calif.). The secondary antibody (goat anti-mouse IgG_1, HRP conjugate) was purchased from Upstate (12-349; Lake Placid, N.Y.).

Tumor cell lines

The following 13 human tumor cell lines were studied: NCI-H460 non-small-cell lung carcinoma (NSCLC), Calu-6 NSCLC, SW2 small-cell lung carcinoma, H82 small-cell lung carcinoma, HCT116 colon carcinoma, HT29 colon carcinoma, LNCaP prostate carcinoma, DU-145 prostate carcinoma, PC-3 prostate carcinoma, MCF-7 breast carcinoma, MDA-MB-468 breast carcinoma, SaOS-2 osteosarcoma and one murine tumor cell line, EMT-6 mouse mammary carcinoma. All of the cell lines were purchased from the American Type Culture Collection (Manasses, Md.) except SW2 cells and EMT-6 cells [37, 38, 39]. The H460, HCT116, HT29, DU-145, PC-3, MCF-7, H82 and MDA-MB-468 cells were grown in RPMI-1640 medium supplemented 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, N.Y.). The SW2 cells were grown in RPMI-1640 medium supplemented 2.0 g/l sodium bicarbonate, 10% FBS and 1% penicillin-streptomycin (GIBCO BRL). The Calu-6 cells were grown in Earle's MEM supplemented with 1×MEM nonessential amino acids, 1×MEM sodium pyruvate, 10% FBS and 1% penicillin-streptomycin (GIBCO BRL). The LNCaP cells were grown in RPMI-1640 medium (without phenol red) supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO BRL). The SaOS-2 cells were grown in McCoy's 5A medium supplemented 10% FBS and 1% penicillin-streptomycin (Bio-Whittaker, Walkersville, Md.). The EMT-6 cells were grown in Waymouth's MB 752/1 medium supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO BRL).

Western blotting analysis

The 13 cell lines (MCF-7, MB-468, H460, Calu-6, H82, SW2, HCT116, HT29, LNCaP, DU-145, PC3, EMT-6 and SaOs) were grown until 85% confluent and collected for protein determination. Adherent cells were suspended by exposure to 0.25% trypsin/1 m*M* EDTA (GIBCO BRL), then medium containing serum was added and the cultures were centrifuged at 1000 rpm for 5 min and the pellets collected. The cells washed twice with phosphate-buffered saline (PBS) and cell pellets collected. The suspended cells (H82 and SW2) were collected by centrifugation at 1000 rpm for 5 min, then washed twice with PBS and the cell pellets collected. The cells were lysed on ice in 50 m*M* Tris, (pH 7.4), 150 m*M* NaCl, 0.1% Triton X-100, 1% of Nonidet P-40, 4 m*M* EDTA, 1 m*M* sodium orthovanadate, 20 m*M* sodium fluoride, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 m*M* PMSF) and the samples were shaken at 4 °C for 10 min. After sonication for 12 s, the samples were centrifuged at 14,000 rpm for 20 min in the cold and supernatants were collected. The protein concentrations of the supernatants were determined using a Bio-Rad DC Protein Assay Kit (500-0112).

The human H460 NSCLC cell line was selected for the Bcl-2 phosphorylation study. H460 cells were grown in 75 cm^2 flasks until about 70% confluent, then the cells were exposed to various concentrations of cryptophycin 52, cryptophycin 55, paclitaxel and vinblastine (0, 0.05, 0.25, 0.5, 5.0, 50, 100 n*M*) for 4 h or 24 h. Both dead cells and live cells were collected. The cells were lysed on ice in 50 m*M* Tris (pH 7.4), 150 m*M* NaCl, 0.1% Triton X-100, 1% of Nonidet P-40, 4 m*M* EDTA, 1 m*M* sodium orthovanadate, 20 m*M* sodium fluoride, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 m*M* PMSF). The supernatants were collected after centrifugation at 14,000 rpm for 20 min. The protein concentrations of supernatants were determined using a Bio-Rad DC Protein Assay Kit (500-0112). The samples were combined with 4×NuPAGE LDS sample buffer (NP0007; Novex, San Diego, Calif.) and 10×LC5625 reducing agent (Novex), and then heated at 70 °C for 10 min. The protein samples (22 μg/per lane) were loaded onto 12% Tris-glycine SDS gels (1.5 mm 15 wells, Pro-Cast SDS-page; Novex).

After electrophoresis, the proteins were transferred from the gel to a PVDF membrane (Novex). The membrane was blocked with 1×PBS containing 5% non-fat milk and 0.2% Tween-20 overnight at 4 °C. The membrane was washed twice for 10 min each time in washing buffer (0.1% Tween-20 in 1×PBS) and then incubated at room temperature with the primary antibody for bcl-2 (mouse monoclonal IgG_1, sc-509) in 1×PBS containing 5% non-fat dried milk for 1 h. After washing three times with washing buffer for 10 min each time, the membrane was incubated at room temperature with the secondary antibody (goat anti-mouse IgG_1, HRP conjugate, 12-349) in 1×PBS buffer containing 5% non-fat dried milk for 1 h. After the membrane had been washed with washing buffer three times for 10 min each time, it was exposed to the ECL Western blotting kit (RPN2106; Amersham, Arlington Heights, Ill.) and the resulting chemiluminescence was detected using Hyperfilm ECL (Amersham).

Flow cytometry analysis

Exponentially growing H460 cells were seeded at 1×10^6 cells/flask in a 75 cm^2 flask containing 25 ml RPMI-1640 medium (Bio-Whittaker) supplemented with 10% FBS. The cells were incubated for 24 h at 37 °C in humidified air/5% carbon dioxide. The cells were then exposed to paclitaxel, cryptophycin 52, cryptophycin 55 or vinblastine prepared in DMSO. After 24 h incubation, the cells were harvested by treatment with 0.25% trypsin/1 m*M* EDTA and washed once with 1×PBS. After centrifugation, 0.5 ml PBS was added to the pellet and the cells were resuspended. To the cell suspension was added ice-cold 70% ethanol (5 ml) with mixing. The cells were then stored at −20 °C for 2–24 h. The cells were removed from storage at −20 °C and washed once with 1×PBS. Buffer comprising 0.1% (v/v) Triton X-100 (Sigma) in PBS and 2 mg DNAse-free RNAse and 200 μl of 1 mg/ml propidium iodide (Molecular Probes) was added to each ethanol-fixed tube of cells. The cells were then analyzed on a Coulter Epics flow cytometer, exciting at 488 n*M*. WinMDI 2.8 software was used to illustrate the DNA histograms.

Cell survival analysis

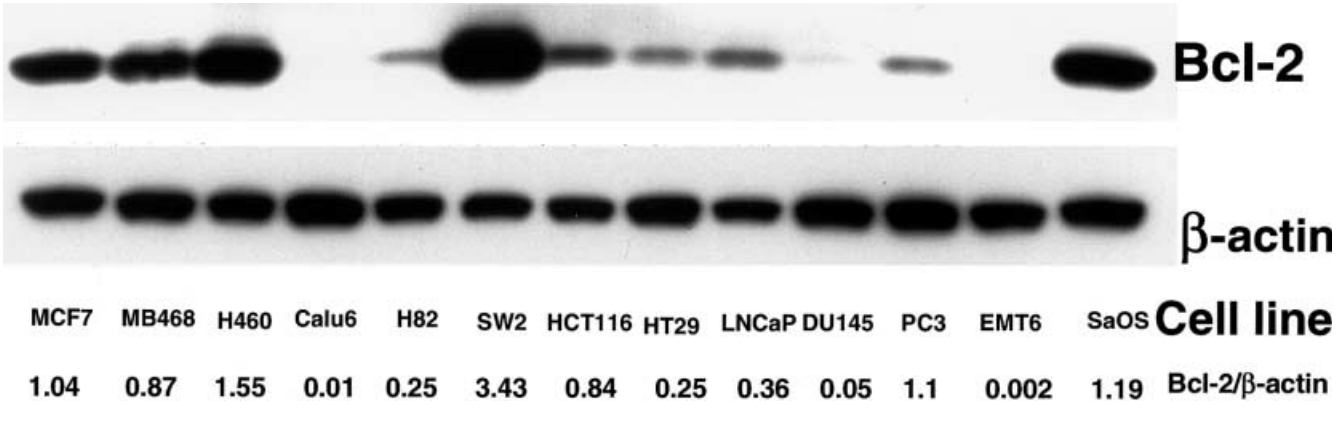
Human H460 and Calu6 NSCLC cells were grown in 25 cm^2 flasks until about 70% confluent, then various concentrations of paclitaxel, cryptophycin 52, cryptophycin 55 and vinblastine (0, 0.05, 0.25, 0.5, 5.0, 50, 100 n*M*) were added to the cultures for 24 h. After the 24-h exposure to the agent, the cells were washed with 0.9% PBS and suspended by exposure to 0.25% trypsin/0.1% EDTA. The cells were plated in duplicate at three or more dilutions for colony formation. After 7 to 10 days, the colonies were visualized by staining with crystal violet in methanol. Colonies of 50 cells or more were counted. The results are expressed as the surviving fraction of treated cells compared with control cultures.

Results

The structures of cryptophycin 52 and cryptophycin 55 differ in that cryptophycin 52 is an epoxide and cryptophycin 55 is the corresponding chlorohydrin (Fig. 1).

Western blot methodology was used to analyze 13 tumor cell lines in exponential growth in monolayer culture for expression of Bcl-2 using β-actin as a standard for comparison. The human tumor cell lines included two breast cancer lines (MCF-7 and MDA-MB-468), two NSCLC lines (NCI-H460 and Calu-6), two small-cell lung cancer lines (NCI-H82 and SW2), two colon cancer lines (HCT116 and HT29), three prostate cancer lines (LNCaP, DU-145 and PC-3) and one osteosarcoma (SaOS-2). There was a high degree of heterogeneity in the expression of Bcl-2 over the 13 lines analyzed (Fig. 2). The two breast cancer lines expressed moderate levels of Bcl-2. The H460 NSCLC line expressed high levels of Bcl-2, while the Calu-6 NSCLC was Bcl-2-negative. The SW2 small-cell cancer line markedly overexpressed Bcl-2, while the H82 small-cell cancer line expressed very low levels of Bcl-2. The HCT116 colon cancer line expressed moderate levels of

Fig. 2 Western blot determination of the expression of Bcl-2 using β-actin as a standard for comparison in 13 human tumor cell lines in exponential growth in monolayer culture. The data were analyzed by densitometric comparison of Bcl-2 and β-actin



Bcl-2 and the HT29 colon cancer line expressed low levels of Bcl-2. Of the three prostate cancer lines, LNCaP expressed moderate levels of Bcl-2, PC-3 expressed low levels of Bcl-2 and DU-145 was negative for Bcl-2. The human osteosarcoma line SaOS-2 expressed high levels of Bcl-2.

The human NCI-H460 NSCLC cell line was selected for the study of the effects of anticancer agents on the phosphorylation state of Bcl-2. Western blot analysis for Bcl-2 phosphorylation was carried out after 4 h of exposure of the cells to paclitaxel or cryptophycin 52 (Fig. 3). Under these conditions, phosphorylated Bcl-2 was evident but the hyperphosphorylated form was not seen. The phosphorylated form of Bcl-2 was evident at a cryptophycin 52 concentration as low as 0.05 n*M* and through 100 n*M*. The phosphorylated form of Bcl-2 was clearly present at both 50 n*M* and 100 n*M* of paclitaxel. Further Western blot analysis for Bcl-2 phosphorylation was carried out after 24 h of exposure of the cells to paclitaxel or cryptophycin 52. Under these conditions, both phosphorylated and hyperphosphorylated forms of Bcl-2 were evident (Fig. 4). The phosphorylated form of Bcl-2 was evident at a cryptophycin 52 concentration as low as 0.05 n*M* and through 100 n*M*. The hyperphosphorylated form of Bcl-2 was clearly detectable at cryptophycin 52 concentrations of 0.25 n*M* through 100 n*M*. Both the phosphorylated and hyperphosphorylated forms of Bcl-2 were clearly present at paclitaxel concentrations of both 50 n*M* and 100 n*M*.

Western blot analysis for Bcl-2 phosphorylation was carried out after 4 h of exposure of the H460 cells to paclitaxel, cryptophycin 55 or vinblastine (Fig. 5). Under these conditions, phosphorylated Bcl-2 was evident but the hyperphosphorylated form was not seen. The phosphorylated form of Bcl-2 was evident at cryptophycin 55 concentrations of 0.25 n*M* through 100 n*M*. The phosphorylated form of Bcl-2 was evident at vinblastine concentrations of 5 n*M* through 100 n*M*. The phosphorylated form of Bcl-2 was clearly present at paclitaxel concentrations of both 50 n*M* and 100 n*M*. Further Western blot analysis for Bcl-2 phosphorylation was carried out after 24 h of exposure of the cells to paclitaxel or cryptophycin 55. Under these conditions, both phosphorylated and hyperphosphorylated forms of Bcl-2 were evident (Fig. 6). The phosphorylated form of Bcl-2 was evident at cryptophycin 55 concentrations of 0.25 n*M* through 100 n*M*. The hyperphosphorylated form of Bcl-2 was also clearly detectable at cryptophycin

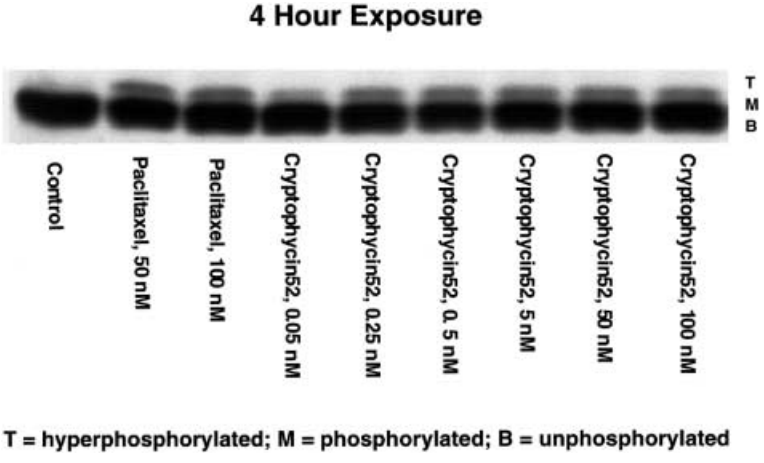


Fig. 3 Western blot determination in human H460 NSCLC cells of Bcl-2 phosphorylation after 4 h of exposure of the cells to paclitaxel (50 or 100 n*M*) or cryptophycin 52 (0.05 to 100 n*M*)

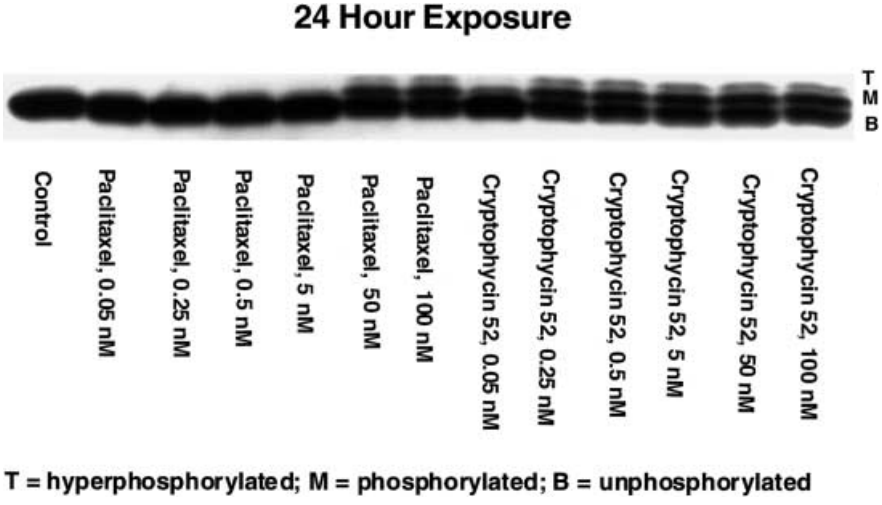


Fig. 4 Western blot determination in human H460 NSCLC cells of Bcl-2 phosphorylation after 24 h of exposure of the cells to paclitaxel (0.05 to 100 n*M*) or cryptophycin 52 (0.05 to 100 n*M*)

55 concentrations of 0.25 n*M* through 100 n*M*. Both the phosphorylated and hyperphosphorylated forms of Bcl-2 were clearly present at both paclitaxel concentrations of 50 n*M* and 100 n*M*. Finally, Western blot analysis for Bcl-2 phosphorylation was carried out after 24 h of exposure of the cells to paclitaxel or vinblastine. Under these conditions, both phosphorylated and hyperphosphorylated forms of Bcl-2 were evident (Fig. 6). The phosphorylated form of Bcl-2 was evident at vinblastine concentrations of 5 n*M* through 100 n*M*. The hyperphosphorylated form of Bcl-2 was clearly detectable at vinblastine concentrations of 50 n*M* and 100 n*M*. Both the phosphorylated and hyperphosphorylated forms of Bcl-2 were clearly present at paclitaxel concentrations of both 50 n*M* and 100 n*M*.

The human NCI-H460 NSCLC cell line was used for the study of the effects of anticancer agents on the distribution of the cell population through the cell cycle (Fig. 7). Staining with propidium iodide and flow cytometry was used to analyze cell cycle distribution after exposure of H460 cells to various concentrations of paclitaxel for 24 h. The distribution of the cells through

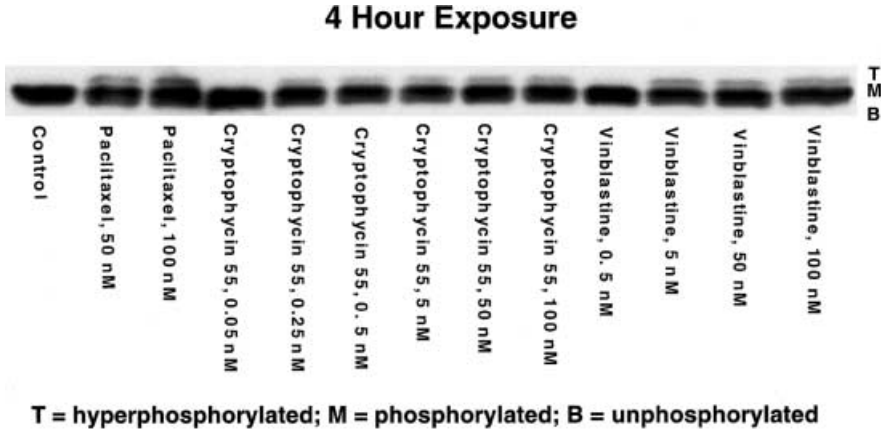


Fig. 5 Western blot determination in human H460 NSCLC cells of Bcl-2 phosphorylation after 4 h of exposure of the cells to paclitaxel (50 or 100 n*M*), cryptophycin 55 (0.05 to 100 n*M*) or vinblastine (0.5 to 100 n*M*)

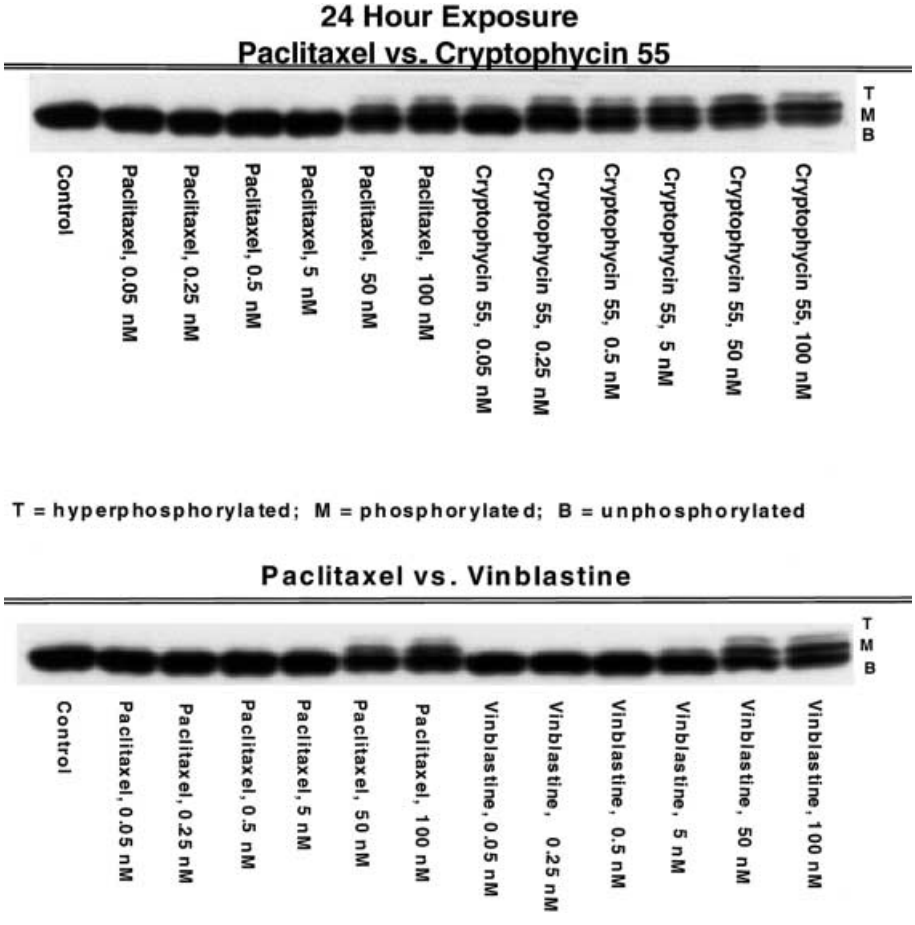


Fig. 6 Western blot determination in human H460 NSCLC cells of Bcl-2 phosphorylation after 24 h of exposure of the cells to paclitaxel (0.05 to 100 n*M*), cryptophycin 55 (0.05 to 100 n*M*) or vinblastine (0.05 n*M* to 100 n*M*)

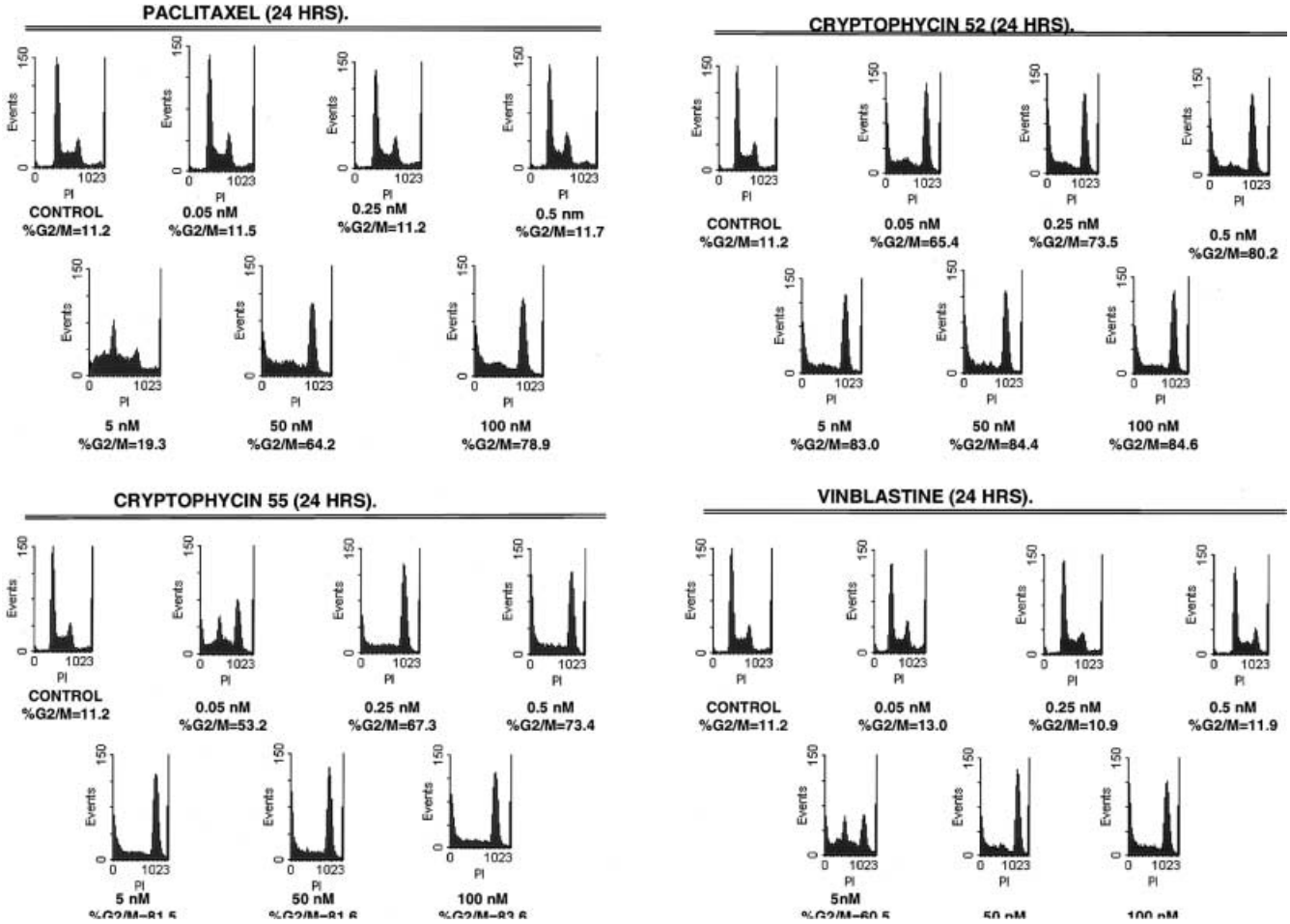


Fig. 7 Cell cycle distribution of H460 cells after exposure to various concentrations (0.05 n*M* to 100 n*M*) of paclitaxel, cryptophycin 52, cryptophycin 55 or vinblastine for 24 h. The distribution of the cells was determined by staining with propidium iodide and flow cytometry

the cell cycle was not altered after exposure to paclitaxel at concentrations of 0.05 n*M* through 5 n*M*. However, after exposure to 50 n*M* or 100 n*M* of paclitaxel there was a marked accumulation of the cells in the G2/M phase of the cell cycle. The distribution of the cells through the cell cycle was altered after 24 h exposure to cryptophycin 52 at concentrations of 0.05 n*M* through 100 n*M* with a marked accumulation of the cells in the G2/M phase of the cell cycle. The distribution of the cells through the cell cycle was also altered after 24 h exposure to cryptophycin 55 at concentrations of 0.05 n*M* through 100 n*M* with a marked accumulation of the cells in the G2/M phase of the cell cycle at concentrations of 0.25 n*M* and greater. Finally, the distribution of the cells through the cell cycle was altered after 24 h exposure to vinblastine at concentrations of 5 n*M* through 100 n*M* with a marked accumulation of the cells in the G2/M phase of the cell cycle at concentrations of 50 n*M* and 100 n*M*. Over the concentration range of the compounds tested, cryptophycins 52 and 55 were the most potent in blocking cells in the G2/M phase of the cell cycle (Fig. 8), paclitaxel was the least potent, being at least 3.5 logs less potent than the cryptophycins, and vinblastine was of intermediate potency.

Survival of exponentially growing human H460 NSCLC cells in monolayer culture after exposure to various concentrations of cryptophycin 52, cryptophycin 55, vinblastine or paclitaxel for 24 h was determined by colony formation (Fig. 9). One log of cell killing (90%) was obtained with 0.13 n*M* cryptophycin 52, 0.2 n*M* cryptophycin 55, 20 n*M* paclitaxel and >100 n*M* vinblastine. Survival of exponentially growing human Calu-6 NSCLC cells in monolayer culture after exposure to various concentrations of cryptophycin 52, cryptophycin 55 or paclitaxel for 24 h was also determined by colony formation. One log of cell killing (90%) was obtained with 0.03 n*M* cryptophycin 52, 0.1 n*M* cryptophycin 55, 11 n*M* paclitaxel and 0.5 n*M* vinblastine.

Discussion

Bcl-2 has been described as a factor that can protect cells from apoptosis [2, 3, 5]. The protective effect of Bcl-2 may be lost if the protein is phosphorylated [3, 4, 6].

Bcl-2 was very heterogeneously expressed in the 13 human tumor cell lines examined in the current study. The human H460 NSCLC cell line had a high expression of Bcl-2 and therefore was selected for further study.

Cryptophycin 52 and cryptophycin 55 were very potent inducers of Bcl-2 phosphorylation in H460 cells in culture. After 4 h of exposure to 0.05 n*M* cryptophycin 52, bcl-2 phosphorylation was evident while 50 n*M* of paclitaxel was required to produce the same effect. A cryptophycin 55 concentration of 0.25 n*M* and a vinblastine concentration of 5 n*M* produced similar Bcl-2 phosphorylation after exposure of H460 cells to the compounds for 4 h. The hyperphosphorylated form of bcl-2 was evident after 24 h of exposure of H460 cells to the compounds. The cryptophycins were very potent inducers of the hyperphosphorylated form of Bcl-2. In H460 cells after 24 h exposure, hyperphosphorylated Bcl-2 was clearly evident at cryptophycin 52 and cryptophycin 55 concentrations of 0.25 n*M* or greater, and after 24 h exposure to 50 n*M* or 100 n*M* paclitaxel or vinblastine.

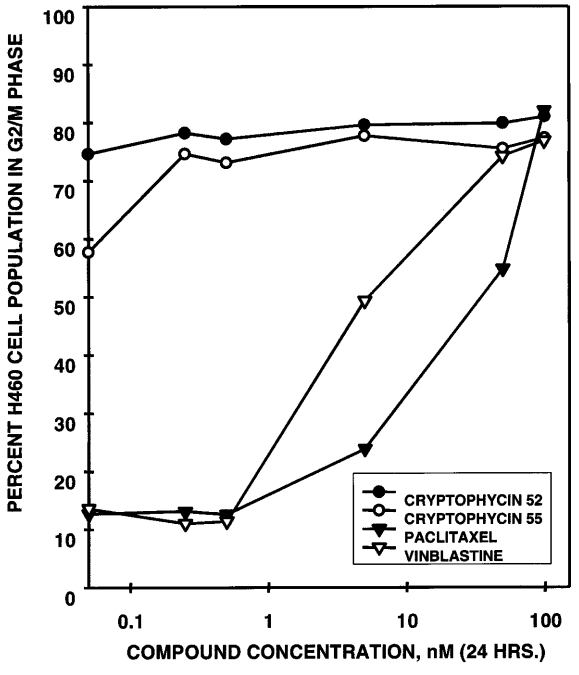


Fig. 8 Percent of cells in the G2/M phase of the cell cycle after exposure to a concentration range (0.05 n*M* to 100 n*M*) of paclitaxel, cryptophycin 52, cryptophycin 55 or vinblastine for 24 h as determined from the cycle distribution data

The effects of the compounds on the cell cycle paralleled those on Bcl-2 phosphorylation. Exposure for 24 h to cryptophycin 52 or cryptophycin 55 markedly increased the proportion of the population of exponentially growing H460 cells in the G2/M phase of the cell cycle even at the lowest concentration (0.05 n*M*) of the compounds tested. The percent of cells in the G2/M phase seemed to parallel the expression of both the phosphorylated and the hyperphosphorylated forms of Bcl-2. G2/M phase arrest was evident upon exposure to vinblastine at 5 n*M* while paclitaxel concentrations of 50 n*M* or 100 n*M* were required to achieve the same effect.

The cytotoxicity of the compounds was determined using H460 cells that expressed high levels of Bcl-2 and Calu-6 cells that were negative for Bcl-2 expression. The pattern of cell killing by the compounds in the two cell lines was very similar. In fact, the concentrations of the cryptophycins and paclitaxel required to kill one log (90%) of the cells were lower in the Bcl-2-negative Calu-6 cells than in the Bcl-2-positive H460 cells. Working in human HeLa cervical carcinoma cells and human SKOV-3 ovarian carcinoma cells, Ling et al. [40] have found that upon exposure of the cells to paclitaxel, Bcl-2 phosphorylation is closely associated with M phase arrest, accumulation of cyclin B1 and activation of cdc2/cyclin B1 kinase but not with apoptosis of the cells. These investigators have shown that Bcl-2 phosphorylation is tightly associated with mitotic arrest but did not

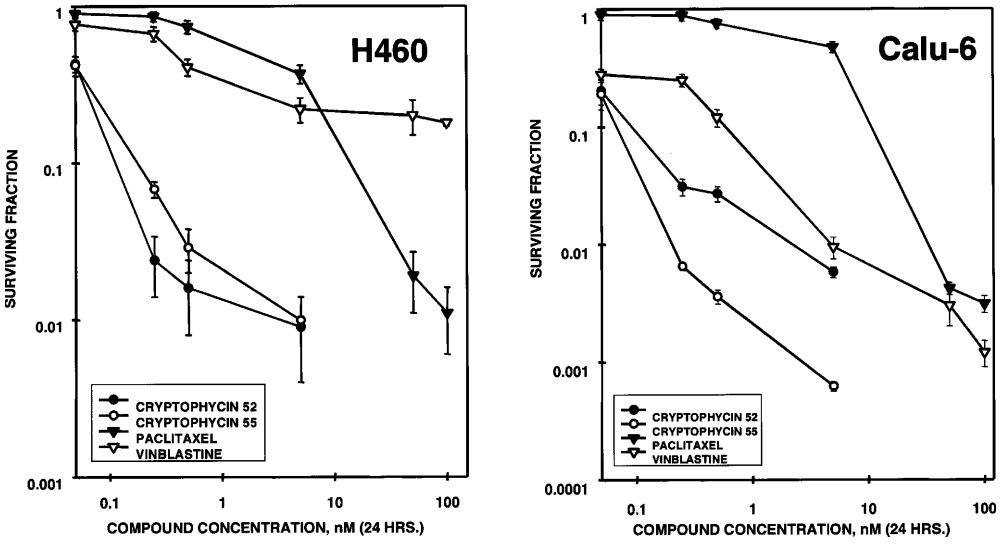


Fig. 9 Survival for human H460 or Calu-6 NSCLC cells after exposure to various concentrations (0.05 n*M* to 100 n*M*) of paclitaxel, cryptophycin 52, cryptophycin 55 or vinblastine for 24 h. Points are the means of two determinations (*bars* SEM)

show that Bcl-2 phosphorylation is a determinant of progression into apoptosis after mitotic arrest induced by antitubulin agents [40].

Elliott et al. [41] studied HeLa cells stably transfected to overexpress Bcl-2. They exposed the cells to etoposide and examined several determinants of apoptosis and other indicators of cell death. They found that the inhibition of etoposide-induced apoptosis by Bcl-2 results in the accumulation of giant multinucleated cells that lose viability without apoptotic morphology or DNA degradation by laddering [41]. In an in vivo study comparing the Bcl-2-expressing murine lymphoma cell line LY-ar and the Bcl-2-negative line LY-as, Story et al. [42] found that after treatment of tumor-bearing animals with six chemotherapeutic agents, apoptotic indices are higher in the bcl-2-negative tumors but that tumor response (tumor growth delay) cannot be predicted from apoptotic indices. In the current study, the antitubulin agents were as lethal to tumor cells that were Bcl-2-negative as to tumor cells that were Bcl-2-positive.

Cryptophycin 52 and cryptophycin 55 were very potent inducers of Bcl-2 phosphorylation with a potency three orders of magnitude greater than that of paclitaxel. After 4 h of exposure to the compounds the phosphorylated form of Bcl-2 was evident and after 24 h of exposure the hyperphosphorylated form was seen. The blocking of cells in the G2/M phase of the cell cycle by these compounds occurred at the same concentrations as the phosphorylation and hyperphosphorylation of Bcl-2 in the cells. The cytotoxicity of the cryptophycins and paclitaxel was altered by the presence or absence of expression of Bcl-2 in the human NSCLC cell lines H460 and Calu-6, thus indicating that pathways triggered by Bcl-2 phosphorylation are involved in cryptophycin-induced lethality.

References

1. Wang LG, Liu XM, Kreis W, Budman DR (1999) The effect of microtubule agents on signal transduction pathways of apoptosis: a review. Cancer Chemother Pharmacol 44: 355–361
2. Reed JC (1995) Prevention of apoptosis as a mechanism of drug resistance. Hematol Oncol Clin North Am 9: 451–473
3. Basu A, You S-A, Haldar S (2000) Regulation of Bcl2 phosphorylation by stress response kinase pathway. Int J Oncol 16: 497–500
4. You S-A, Basu A, Haldar S (1999) Potent antitumor proteasome inhibitors: a novel trigger for Bcl2 phosphorylation to induce apoptosis. Int J Oncol 15: 625–628
5. Reed JC, Miyashita T, Takayama S, Wang HG, Sato T (1996) Bcl-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 60: 23–32
6. Hu Z-B, Minden MD, McCulloch EA (1998) Phosphorylation of Bcl-2 after exposure of human leukemic cells to retinoic acid. Blood 92: 1768–1775
7. Berardo MD, Elledge RM, Moor C, Clark GM, Osborne CK (1998) Bcl-2 and apoptosis in lymph node positive breast carcinoma. Cancer 82: 1296–1302
8. Webb A, Cunningham D, Cotter F, Clarke PA, diStefano F, Ross P, Corbo M, Dziewanowska Z (1997) Bcl-2 antisense therapy in patients with non-Hodgkin lymphoma. Lancet 349: 1137–1141
9. Blagosklonny MV, Schulte T, Nguyen P, Trepel J, Neckers LM (1996) Taxol-induced apoptosis and phosphorylation of Bcl-2 protein involves c-Raf-1 and represents a novel c-Raf-1 signal transduction pathway. Cancer Res 56: 1851–1854
10. Haldar S, Chintapalli J, Croce CM (1996) Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res 56: 1253–1255
11. Blagosklonny MV, Giannakakou P, El-Deiry WS, Kingston DGI, Higgs PI, Neckers L, Fojo T (1997) Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. Cancer Res 57: 130–135
12. Kroning R, Lichtenstein A (1998) Taxol can induce phosphorylation of bcl-2 in multiple myeloma cells and potentiate dexamethasone-induced apoptosis. Leuk Res 22: 275–286
13. Scatena CD, Stewart ZA, Mays D, Tang LJ, Keefer CJ, Leach SD, Pietenpol JA (1998) Mitotic phosphorylation of bcl-2 during normal cell cycle progression and Taxol-induced growth arrest. J Biol Chem 273: 30777–30784
14. Roth W, Wagenknecht B, Grimmel C, Dichgans J, Weller M (1998) Taxol-mediated augmentation of CD95 ligand-induced apoptosis of human malignant glioma cells: association with bcl-2 phosphorylation but neither activation of p53 nor G2/M cell cycle arrest. Br J Cancer 77: 404–411
15. Makhija S, Taylor DD, Gibb RK, Gercel-Taylor C (1999) Taxol-induced bcl-2 phosphorylation in ovarian cancer cell monolayer and spheroids. Int J Oncol 14: 515–521
16. Fan W (1999) Possible mechanisms of paclitaxel-induced apoptosis. Biochem Pharmacol 57: 1215–1221
17. Srivastava RK, Mi Q-S, Hardwick JM, Longo DL (1999) Deletion of the loop of Bcl-2 completely blocks paclitaxel-induced apoptosis. Proc Natl Acad U S A 90: 3775–3780
18. Schwartz RE, Hirsch CF, Sesin DF, Flor JE, Chartrain M, Fromtling RE, Harris GH, Salvatore MJ, Liesch JM, Yudin K (1990) Pharmaceuticals from cultured algae. J Ind Microbiol 5: 113–124
19. Corbett TH, Valeriote FA, Demchik L, Polin L, Panchapor C, Pugh S, White K, Knight J, Jones J, Jones L, LoRusso P, Foster B, Wiegand RA, Lisow L, Golakoti T, Heltzel CE, Ogino J, Patterson GML, Moore RE (1996) Preclinical anticancer activity of cryptophycin-8. J Exp Ther Oncol 1: 95–108
20. Panda D, Himes RH, Moore RE, Wilson L, Jordan MA (1997) Mechanism of action of the unusually potent microtubule inhibitor, cryptophycin 1. Biochemistry 36: 12948–12953
21. 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 (abstract 1514). Proc Am Assoc Cancer Res 38: 225
22. Worzalla JF, Cao J, Ehlhardt WJ, Harrison SD, Law KL, Martinelli MJ, Self TD, Starling JJ, Shih C, Thoebald KS, Toth JE, Zimmermann JL, Corbett TH (1997) LY355702 and LY355703, new cryptophycin analogues with antitumor activity against human tumor xenografts (abstract 1516). Proc Am Assoc Cancer Res 38: 225
23. Trimurtulu G, Ohtani I, Patterson GML, Moore RE, Corbett TH, Valeriote FA, Demchik L (1994) Total structures of cryptophycins, potent antitumor desipeptides from the blue-green algae *Nostoc* sp. Strain GSV. J Am Chem Soc 116: 4729–4737
24. Hyams JS, Lloyd CW (1994) Microtubules. Wiley-Liss, New York
25. Smith CD, Zhang X (1996) Mechanism of action of cryptophycin. J Biol Chem 271: 6192–6198
26. Smith CD, Zhang X, Mooberry SL, Patterson GML, Moore RE (1994) Cryptophycin: a new microtubule agent active against drug-resistant cells. Cancer Res 54: 3779–3784
27. Wilson L, Jordan MA (1994) Pharmacological probes of microtubule function. In: Hyams J, Lloyd C (eds) Microtubules. Wiley-Liss, New York, pp 59–84

28. Wilson L, Jordan MA (1995) Microtubule dynamics: taking aim at a moving target. Chem Biol 2: 569–573
29. Hamel E (1996) Antimitotic natural products and their interactions with tubulin. Med Res Rev 16: 207–231
30. 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
31. Kerksiek K, Meijillano MR, Schwartz RE, Georg GI, Himes RH (1995) Interaction of cryptophycin 1 with tubulin and microtubules. FEBS Lett 377: 59–61
32. Mooberry SL, Busquets L, Tien G (1997) Induction of apoptosis by cryptophycin 1, a new antimicrotubule agent. Int J Cancer 73: 440–448
33. 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
34. Stevenson JP, Gallagher M, Vaughn D, Schucter L, Haller D, Fox K, Algazy K, Aviles V, Hahn S, O'Dwyer PJ (1999) Phase I trial of the cryptophycin analogue LY355703 administered as an IV infusion, D1 and D8, repeated every 21 days. Proc Am Assoc Cancer Res 40: 92
35. Schultz RM, Shih C, Wood PG, Harrison SD, Ehlhardt WJ (1998) Binding of the epoxide cryptophycin analog, LY355703 to albumin and its effects on in vitro antiproliferative activity. Oncol Rep 5: 1089–1094
36. Wagner MM, Paul DC, Shih C, Jordan MA, Wilson L, Williams DC (1999) In vitro pharmacology of LY355703 (cryptophycin 52) in human tumor cell lines. Cancer Chemother Pharmacol 43: 115–125
37. Francis J, Bernal SD, Gazdar AF, Thompson R, Baylin S (1980) L-Dopa decarboxylase activity (DDC): a distinguishing biomarker for the growth of small cell lung cancer (SCCL) in tissue culture. Proc Am Assoc Cancer Res 21: 52
38. Francis K, Thompson R, Bernal SD, Luk G, Baylin S (1983) Effects of dibutyryl cyclic adenosine 3′, 5′-monophosphate on the growth of cultured human small cell lung carcinoma and the specific cellular activity of L-dopa decarboxylase. Cancer Res 43: 639–345
39. Teicher BA, Herman TS, Holden SA, Wang YY, Pfeffer MR, Crawford JW, Frei E (1990) Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo, Science 247*:* 1457–1461
40. Ling Y-H, Tornos C, Perez-Soler R (1998) Phosphorylation of Bcl-2 is a marker of M phase events and not a determinant of apoptosis. J Biol Chem 273: 18984–18991
41. Elliott MJ, Murphy KM, Stribinskiene L, Ranganathan V, Sturges E, Farnsworth ML, Lock RB (1999) Bcl-2 inhibits early apoptotic events and reveals post-mitotic multinucleation without affecting cell cycle arrest in human epithelial tumor cells exposed to etoposide. Cancer Chemother Pharmacol 44: 1–11
42. Story MD, Mirkovic N, Hunter N, Meyn RE (1999) Bcl-2 expression correlates with apoptosis induction but not tumor growth delay in transplantable murine lymphomas treated with different chemotherapy drugs. Cancer Chemother Pharmacol 44: 367–371