Biochemical and Biophysical Research Communications **266,** 76–80 (1999)
Article ID bbrc.1999.1768, available online at http://www.idealibrary.com on IDEAL®

Modulation of cIAP-1 by Novel Antitubulin Agents When Combined with Bryostatin 1 Results in Increased Apoptosis in the Human Early Pre-B Acute Lymphoblastic Leukemia Cell Line Reh

Nathan R. Wall,* Ramzi M. Mohammad,* Sanaa M. Nabha,*
George R. Pettit,† and Ayad M. Al-Katib*

**Division of Hematology and Oncology, Department of Internal Medicine, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan 48201; and †Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287*

Received October 29, 1999

Previous studies have shown that bryostatin 1 induces a decrease in the expression of the antiapoptotic protooncogene Bcl-2 in the human acute lymphoblastic leukemia (ALL) cell line Reh. This down-regulation has been shown to reduce drug resistance of the Reh cells to anti-tubulin polymerization agents. In the present study we investigated the effect of bryostatin 1 alone and in combination with novel anti-tubulin agents (dolastatin 10 and auristatin PE) and the chemotherapeutic vincristine on the inhibitor of apoptosis protein cIAP-1. Cells were cultured with bryostatin 1 (1 nM), dolastatin 10 (0.1 ng/ml), auristatin PE (0.1 ng/ml), or vincristine (0.5 ng/ml) alone or the combination of these anti-tubulins with bryostatin 1. Western blots were conducted to assess the effects of the above agents on cIAP-1 protein level. Flow-cytometric analysis [7-amino-actinomycin D (7AAD)] was conducted to assess apoptosis as well as staining for morphology using tetrachrome stain. Our results show that cIAP-1 is induced in a time-dependent fashion after bryostatin 1 exposure up to 72 h. However, upon treatment of cells with a combination of bryostatin 1 and dolastatin 10 or auristatin PE, the induction of cIAP-1 was abolished, leading to a significant increase in apoptosis. The initial 24- and 48-h reduction in cIAP-1 protein level recorded in the bryostatin 1 and vincristine combination recovered to control levels by 72 h. We believe that this phenomenon is responsible for the reduced apoptosis recorded in this combination. Results of this study should prove useful in guiding the clinical application of these novel agents in the treatment of ALL. **© 1999 Academic Press**

Key Words: **bryostatin 1, dolastatin 10, auristatin PE, cIAP-1, Bcl-2, acute lymphoblastic leukemia (ALL).**

Acute lymphoblastic leukemia (ALL) is the most common malignancy found in children and accounts for one fourth of all childhood cancers and approximately 75% of all cases of childhood leukemia. There are approximately 2000 new cases of ALL diagnosed in the United States annually. Genetic factors are presumed to play a significant role in the cause of ALL with evidence based upon chromosomal abnormalities such as translocations and deletions occurring in every case (1).

Bcl-2 was initially discovered as an overexpressed protein in human B-cell lymphomas arising as a result of a t(14;18) chromosomal translocation (2, 3). Overexpression of Bcl-2 protects many cell types against apoptosis in response to such diverse stimuli as viral infection, hypoxia, ionizing radiation or chemotherapeutic agents (4–8). In contrast, reduction of Bcl-2 expression by cytokine- or antisense-mediated approaches, increase the sensitivity of certain types of tumor cell lines *in vitro* to several cytotoxic drugs (9–11). However, in contrast to the aforementioned studies, it has been shown that cellular levels of Bcl-2 in ALL cells is not correlated with response to cytotoxic drugs or ionizing radiation (IR) either *in vivo* or *in vitro* (11–13). Furthermore, Bcl-2 protein in the Reh ALL cell line underwent down-regulation after IR without induction of the proapoptotic protein Bax or apoptosis (11). Recently we have shown that the macrocyclic lactone bryostatin 1 induced low levels of Bcl-2 are also insufficient in inducing apoptosis in the Reh cell line. Apoptosis in Reh can be induced by co-treating cells with bryostatin 1 and the anti-tubulin agents dolastatin 10, auristatin PE or vincristine, which induce Bax protein (14). Thus, higher levels of Bax relative to Bcl-2 are necessary to increase sensitivity to apoptosis in the Reh cell line.

0006-291X/99 $30.00

The induction of apoptosis or the progression through the process of apoptosis has recently been shown to be inhibited by a family of proteins called Inhibitors of Apoptosis (IAPs) (15–20). Here we show that cIAP-1 is induced after bryostatin 1 exposure in the Reh ALL cell line. However, upon treating cells with a combination of bryostatin 1 and the novel antitubulin agents dolastatin 10 or auristatin PE, the induction of cIAP-1 was abolished leading to increased apoptosis. The combination of bryostatin 1 and vincristine failed to down regulate cIAP-1. We believe that this phenomenon is responsible for the reduced apoptosis recorded in this combination.

METHODS AND MATERIALS

Cell culture. The human pre-B ALL cell line Reh was obtained from the American Type Culture Collection (Rockville, MD). The Reh cell line was established from a 15-year-old girl with ALL and was characterized as being at the early pre-B stage (21). Reh cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Biological agents. Bryostatin 1, a macrocyclic lactone, is extracted and purified from the marine bryozoan *Bugula neritina* (22). It is a protein kinase C activator (23, 24) and a biological response modifier (25, 26). Bryostatin 1 has been synthesized and made available for clinical trials and experimental research through the United States National Cancer Institute. Dolastatin 10 is an agent that was isolated from the sea hare *Dolabella auricularia* and has been found to be antineoplastic. It inhibits cell proliferation and arrests cells in mitosis by binding to microtubule components, mainly tubulin (27). Auristatin PE is a structural analog of dolastatin 10 with the dolaphenine unit substituted with phenethylamide (28). The vinca alkaloid vincristine is widely used clinically in the treatment of a variety of human cancers and is an integral part of induction chemotherapy regimens for ALL (29, 30). Vincristine has been shown to disrupt the mitotic spindle apparatus by binding to the microtubular protein tubulin during metaphase (30, 31), arresting cells in mitosis.

Culture of Reh with bryostatin 1, auristatin PE, dolastatin 10, and vincristine. Reh cells were seeded at 2×10^5/ml in T-75 tissue culture flasks (Falcon Labware, Oxnard, CA). Bryostatin 1 (1 nM), auristatin PE (0.1 ng/ml), dolastatin 10 (0.1 ng/ml), vincristine (0.5 ng/ml), or vehicle was added to flasks containing Reh cells. Bryostatin 1 was dissolved in 0.09% dimethyl sulfoxide (DMSO) and PBS at a concentration of 10^{-5} M and then further diluted to the final concentrations in culture medium. Dolastatin 10 and auristatin PE (George R. Pettit, Arizona State University) were dissolved in 0.09% DMSO and PBS and then diluted to the final concentrations in culture medium. Vincristine was diluted to the final concentration in culture medium. Cultures were incubated for up to 120 h at 37°C and 5% CO_2. Cell viability and growth inhibition were determined daily using trypan blue (0.4%) exclusion (Gibco, Grand Island, NY).

7AAD staining and flow cytometry. 7-Amino-actinomycin D (Calbiochem-Novabiochem, La Jolla, CA) was dissolved in acetone, diluted in PBS to a concentration of 200 μg/ml, and was kept at −20°C, protected from light as described previously (32). Briefly, 100 μl of 7AAD solution was added to 1×10^6 cells, suspended in 1 ml of PBS and mixed well. Reh cells were stained for 20 min on ice while protected from light. Cells were pelleted, the supernatant removed, and the pellet washed twice with PBS. Samples from untreated and 24, 48 and 72 h bryostatin 1-, auristatin PE-, dolastatin 10- and vincristine-treated cells and combinations of bryostatin 1 and individual antitubulin agents were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Data on 20,000 cells was acquired and processed using Lysys II software (Becton Dickinson, Mountain View, CA). Scattergrams were generated by combining forward light scatter with 7AAD fluorescence (Molecular and Cellular Imaging and Analytical Cytometry Core Facility of the Barbara Ann Karmanos Cancer Institute and Wayne State University School of Medicine, Grant P30 CA22453-20).

Western blot analysis. Whole-cell extracts (25–50 μg) were resolved by 12% SDS-PAGE, transferred to Hybond C-extra membranes (Amersham Life Science, Arlington Heights, IL), and detected with antiserum specific for Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or cIAP-1 (Pharmingen, San Diego, CA) with the use of an ECL assay (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Briefly, cells were washed twice with cold PBS and lysed at 4°C for 30 min in lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris-Cl, 1 mM phenylmethylsulfonyl fluoride) with occasional vortexing. Protein concentrations were determined using the Micro BCA protein assay (Pierce Chemical Company, Rockford, IL) The Hybond C-extra membranes were blocked (5% milk, 0.05% Tween 20, PBS) for 1 h at room temperature. The membranes were then incubated with the primary antibody (1:1000 dilution in PBS, 0.05% Tween 20) overnight at 4°C. The membranes were washed well in PBS with 0.05% Tween 20 and then incubated with the horseradish peroxidase-conjugated antimouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000 dilution in PBS, 0.05% Tween 20). Protein levels were visualized by peroxidase reaction using the ECL kit (Amersham Life Science, Arlington Heights, IL). Equal sample loading was confirmed by reprobing the same blots with a rabbit polyclonal antiserum against GAPDH (1:5000; Trevigen Inc., Gaithersburg, MD). Blots were stripped by submerging the membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5-mM Tris-HCl, pH 6.7) and incubating at 60° C for 25 min with occasional agitation.

Statistical analysis. The significance of differences between experimental conditions was determined using a one-way analysis of variance (ANOVA). Comparisons were made between the control, all single agent treatments and all combination treatments using the Tukey-Kramer multiple comparisons test. All numbers are means ± the standard error of the mean (SEM).

RESULTS AND DISCUSSION

Apoptosis has been shown to play multiple roles both in normal cellular and tissue physiology and over the past decade it has become the focus of intense inquiry into what role apoptosis plays in the disease process. The realization that defective apoptosis plays a role in the pathogenesis of a number of diseases offers new opportunities for the identification of novel targets for therapeutic intervention (33). Bcl-2 was the first protooncogene discovered which was found to contribute to tumor growth by decreasing the rate of programmed cell death rather than by accelerating cell division (34). It was later shown that reduction of Bcl-2 expression by cytokine- or antisense-mediated approaches, increase the sensitivity of certain types of tumor cell lines *in vitro* to several cytotoxic drugs (9–11).

We therefore determined to ascertain why when the human early pre-B ALL cell line Reh is treated with the experimental therapeutic and protein kinase C activator bryostatin 1, the recorded decrease in Bcl-2

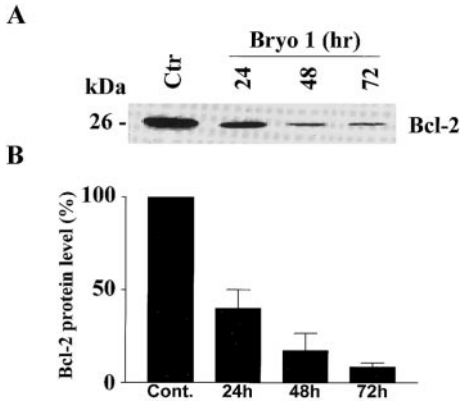

FIG. 1. Bryostatin 1-induced down-regulation of Bcl-2. (A) Western blot analysis of Bcl-2 protein after treatment of Reh cells (2×10^5/ml) with bryostatin 1 (1 nM). (B) Bcl-2 protein levels relative to those of G3PDH were measured in bryostatin-treated cells and expressed as a percentage of Bcl-2 level in control cells at the same time. Results represent the mean ± SD of three different experiments.

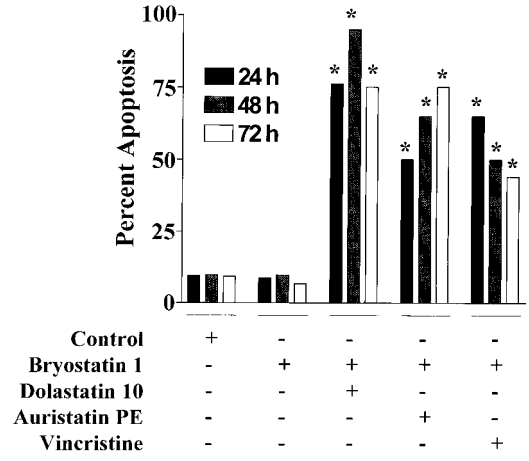

FIG. 2. 7AAD flow cytometric analysis of apoptosis. Reh cells (2×10^5/ml) were exposed to bryostatin 1 (1 nM) alone or in combination with dolastatin 10 (0.1 ng/ml), auristatin PE (0.1 ng/ml), or vincristine (0.5 ng/ml). Combination therapy included a 24-h pretreatment in 1 nM bryostatin 1 followed by 24-, 48-, or 72-h treatments with the antitubulin agent. Percentage of apoptotic cells was determined on a FACS-scan. *Significant difference from control, bryostatin 1 alone and between agents given in combination ($P < 0.001$).

protein (Fig. 1A) does not result in the induction of apoptosis. Densitometric measuring of Bcl-2 protein levels in three separate experiments indicated that bryostatin 1 treatment of Reh ALL cells resulted in a ten-fold reduction in Bcl-2 protein levels over 72 h (Fig. 1B). Bcl-2 protein levels were held at this reduced level for 120 h without recovery (data not shown). 7AAD flow cytometric analysis for apoptosis did not record an increase in apoptosis in the Reh cells treated with bryostatin 1 alone (Fig. 2). We therefore contemplated the existence of other putative factors which may be maintaining apoptosis resistance in lieu of significant reduced levels of Bcl-2.

cIAP-1 is a member of an evolutionarily conserved family of homologous proteins that suppress apoptosis induced by multiple stimuli. Some IAP family proteins, including XIAP, cIAP-1, and cIAP-2, can bind and directly inhibit selected caspases, a group of intracellular cell death proteases (35). Here we show that cIAP-1 is induced after bryostatin 1 exposure in the Reh ALL cell line (Fig. 3A). Densitometric measurement of cIAP-1 protein level in two separate experiments indicated that treatment with bryostatin 1 for 72 h resulted in a two-fold increase in cIAP-1 protein level (Fig. 3B).

To further study the association between apoptosis induction and the modulation of Bcl-2 and cIAP-1 proteins in the Reh cell line we used three antitubulin polymerization agents: dolastatin 10, auristatin PE and vincristine. Indeed, in the presence of bryostatin 1 as a 24 h pretreatment, Reh cells subsequently treated with dolastatin 10 (0.1 ng/ml) or auristatin PE (0.1 ng/ml) showed an increase in apoptosis by 7AAD flow cytometric analysis. Bryostatin 1 combined with vincristine (0.5 ng/ml) failed to elicit this same cell-killing phenomenon (Fig. 2). When Reh cells are treated with bryostatin 1 for 24 h followed by 24, 48 and 72 h of dolastatin 10, auristatin PE or vincristine, significant ($P < 0.001$) apoptosis is induced (Fig. 3). However, when vincristine was given after bryostatin the level of apoptosis in Reh cells increased significantly compared with bryostatin or vincristine alone treated cells but over time there was a reduction in percent apoptosis. We speculate that this recovery is the result of the Reh cells becoming resistant to the combination of bryostatin 1 and vincristine. We therefore undertook comparing levels of apoptosis with levels of cIAP-1 in bryostatin 1 and antitubulin-agent combinations.

In Reh cells treated with dolastatin 10 or auristatin PE alone, no change in the level of cIAP-1 was recorded

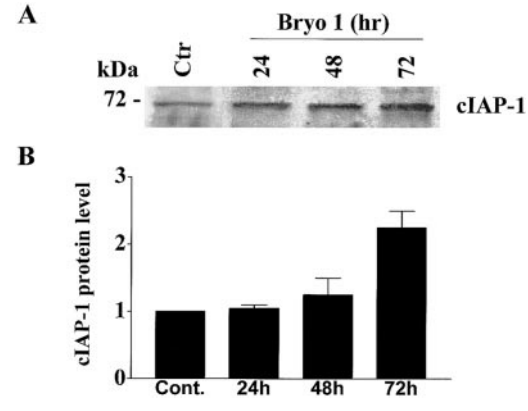

FIG. 3. Bryostatin 1-induced induction of cIAP-1. (A) Western blot analysis of cIAP-1 protein after treatment of Reh cells (2×10^5/ml) with bryostatin 1 (1 nM). (B) cIAP-1 protein levels relative to those of G3PDH were measured in bryostatin-treated cells and expressed in comparison to control cells. Results represent the mean ± SD of two different experiments.

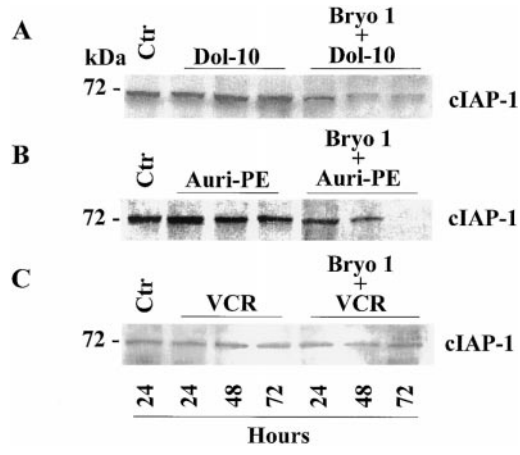

FIG. 4. Bryostatin 1 and antitubulin agents modulate the levels of cIAP-1. Reh cells (2×10^5/ml) were exposed to (A) dolastatin 10 (0.1 ng/ml), (B) auristatin PE (0.1 ng/ml), or (C) vincristine (0.5 ng/ml) alone or in combination with bryostatin 1 (1 nM). Combination therapy included a 24-h pretreatment in bryostatin 1 followed by 24-, 48-, or 72-h treatments with the antitubulin agent. Protein levels were checked against those of G3PDH as a control for loading accuracy. Results represent findings from two different experiments.

(Figs. 4A and 4B). However in the combinations of bryostatin 1 and dolastatin 10 (Fig. 4A) or bryostatin 1 and auristatin PE (Fig. 4B), the induction of cIAP-1 was down regulated in a time dependent manner. The treatment of Reh cells with vincristine alone also failed to induce a change in the level of cIAP-1 but unlike the results recorded for the previous combination of bryostatin 1 and dolastatin 10 or auristatin PE, those recorded of bryostatin 1 and vincristine failed to induce significant down regulation of cIAP-1 (Fig. 4C). A slight reduction in the level of cIAP-1 is recorded after 24 and 48 h as compared to the control but by 72 h the level of cIAP-1 strengthens. Maximum percent apoptosis was recorded at 72 h post bryostatin followed by dolastatin 10 or auristatin PE treatment when cIAP-1 was completely down regulated. However, high levels of apoptosis were recorded 24 and 48 h after combination treatment occurred and yet cIAP-1 was expressed. This phenomenon may be the result of an intermediate state where cIAP-1 protein is expressed but may be nonfunctional. The initial decrease in cIAP-1 together with the increase in the proapoptotic protein Bax (14) at these time points may be enough to elicit apoptosis. However, we believe that cIAP-1's recovery at 72 h may in part be responsible for the reduced apoptosis recorded in this combination. This phenomenon is currently under investigation in our laboratory.

In conclusion, we have found that bryostatin 1's down regulation of the proapoptotic protooncogene Bcl-2 does not induce apoptosis as would be expected because of its ability to simultaneously stimulate the induction of the inhibitor of apoptosis protein cIAP-1. cIAP-1 protein, which was unaffected by the antitubulin agents alone, was abolished when combining dolastatin 10 or auristatin PE with bryostatin 1 resulting in increased apoptosis. The finding that dolastatin 10 and auristatin PE together with bryostatin 1 work more efficiently to induce apoptosis in the Reh ALL cell line then when the clinically tested vinca alkaloid vincristine is combined with bryostatin 1 further strengthens the argument for the development of newer antitubulin agents to be used against acute lymphoblastic leukemia.

ACKNOWLEDGMENTS

This work was supported by Grant 6323-99 from the Leukemia Society of America and by Grant CA79837 from the National Institutes of Health. For help with the flow cytometry the authors thank Eric Van Buren, Evano Piasentin, and Dr. Stephen Lerman from the Molecular and Cellular Imaging and Analytical Cytometry Core Facility of the Barbara Ann Karmanos Cancer Institute and Wayne State University School of Medicine. This facility is supported in part by National Institutes of Health Grant P30 CA22453-20.

REFERENCES

1. Rubnitz, J. E., and Look, A. T. (1998) *J. Ped. Hem. Oncol.* **20,** 1–11.
2. Pegoraro, L., Palumbo, A., Erikson, J., Falda, M., Giovanazzo, B., Emanuel, B. S., Rovero, G., Nowell, P. C., and Croce, C. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7166–7170.
3. Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C. M. (1985) *Science* **228,** 1440–1443.
4. Gejewski, T. F., and Thompson, C. B. (1996) *Cell* **87,** 589–593.
5. Ibrado, A. M., Huang, Y., Fang, G., Liu, L., and Bhalla, K. (1996) *Cancer Res.* **56,** 4743–4748.
6. Ibrado, A. M., Liu, L., and Bhalla, K. (1997) *Cancer Res.* **57,** 1109–1115.
7. Reed, J. C. (1996) *Behring Inst. Mitt* **97,** 72–100.
8. Shimizu, S., Eguchi, Y., Kosaka, H., Kamuke, W., Matsuda, H., and Tsuijimoto, Y. (1995) *Nature* **374,** 811–813.
9. Lotem, J., and Sachs, L. (1994) *Cell Growth Differ.* **5,** 321–327.
10. Reed, J. C., Kitada, S., Takayama, S., and Miyashita, T. (1994) *Ann Oncol.* **5,** 61–65.
11. Findley, H. W., Lubing, G., Yeager, A. M., and Zhou, M. (1997) *Blood* **89,** 2986–2993.
12. Gala, J. L., Vermylen, C., Cornu, G., Ferrant, A., Michaux, J. L., Philippe, M., and Martiat, P. (1994) *Ann. Hematol.* **69,** 17–24.
13. Coustan-Smith, E., Kitanaka, A., Pui, C. H., McNinch, L., Evans, W. E., Raimondi, S. C., Behm, F. G., Arico, M., and Campana, D. (1996) *Blood* **87,** 1140–1146.
14. Wall, N. R., Mohammad, R. M., and Al-Katib, A. M. (1999) *Leukemia Res.* **23,** 881–888.
15. Hay, B. A., Wassarman, D. A., and Rubin, G. M. (1995) *Cell* **83,** 1253–1262.
16. Rothe, M., Pan, M-G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995) *Cell* **83,** 1243–1252.
17. Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dongen, J. L., Gilfillan, M. C., Shiels, H., Hardwick, J. M., and Thompson, C. B. (1996) *EMBO J.* **15,** 2685–2689.
18. Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A., and Korneluk, R. G. (1996) *Nature* **379,** 349–353.

19. Uren, A. G., Pakusch, M., Hawkins, C. J., Puls, K. L., and Vaux, D. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4974–4978.
20. Ambrosini, G., Adida, C., and Altieri, D. C. (1997) *Nat. Med.* **3,** 917–921.
21. Koziner, B., Stavnezer, J., and Al-Katib, A. (1985) *Clin. Immunopathol.* **37,** 135–141.
22. Pettit, G. R., Herald, S. L., Doubek, D. L., Arnold, E., and Clardy, J. (1982) *J. Am. Chem. Soc.* **104,** 6846–6848.
23. Stone, R. M., Sariban, E., Pettit, G. R., and Kufe, D. W. (1988) *Blood* **72,** 208–213.
24. Kiss, Z., Deli, E., Girard, P. R., Pettit, G. R., and Kuo, J. F. (1987) *Biochem. Biophys. Res. Commun.* **146,** 208–215.
25. Jones, R. J., Sharkis, S. J., Miller, C. B., Towinsky, E. K., Burke, P. J., and May, W. S. (1990) *Blood* **75,** 1319–1323.
26. Tuttle, T. M., Inge, T. H., Bethke, K. P., McCrady, C. W., Pettit, G. R., and Bear, H. D. (1992) *Cancer Res.* **52,** 548–553.
27. Bai, R., Roach, M. C., Jayaram, S. K., Barkoczy, J., Pettit, G. R., Luduena, R. F., Hamel, E. (1993) *Biochem. Pharmacol.* **45,** 1503–1515.
28. Pettit, G. R., Srirangam, J. K., Barkoczy, J., Williams, M. D., Durkin, K. P., Boyd, M. R., Bai, R., Hamel, E., Schmidt, J. M., and Chapuis, J. C. (1995) *Anti-Cancer Drug Design* **10,** 529–544.
29. Hill, B. T., and Whelan, R. D. H. (1981) *J. Natl. Cancer Inst.* **67,** 437–443.
30. Jackson, D. V., and Bender, R. A. (1979) *Cancer Res.* **39,** 4346–4349.
31. Jordan, M. A., Thrower, D., and Wilson, L. (1991) *Cancer Res.* **51,** 2212–2222.
32. Philpott, N. J., Turner, A. J. C., Scopes, J., Westby, M., Marsh, J. C. W., Gordon-Smith, E. C., Dalgleish, A. G., and Gibson, F. M. (1996) *Blood* **87,** 2244–2251.
33. Granville, D. J., Carthy, C. M., Hunt, D. W. C., and McManus, B. M. (1998) *Lab. Invest.* **78,** 893–913.
34. Vaux, D. L., Cory, S., and Adams, J. M. (1988) *Nature* **335,** 440–442.
35. Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G. S., and Reed, J. C. (1998) *J. Biol. Chem.* **273,** 7787–7790.