Antitumor Activity of Bruceantin: An Old Drug with New Promise[§,⊥]

Muriel Cuendet[†] and John M. Pezzuto*[,‡]

Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612, and Schools of Pharmacy, Nursing, and Health Sciences, Purdue University, West Lafayette, Indiana 47907

Received July 3, 2003

Bruceantin was first isolated from *Brucea antidysenterica*, a tree used in Ethiopia for the treatment of cancer, and activity was observed against B16 melanoma, colon 38, and L1210 and P388 leukemia in mice. Phase I and II clinical trials were then initiated, but no objective tumor regressions were observed and clinical development was terminated. Recently, the activity of bruceantin has been studied with a number of leukemia, lymphoma, and myeloma cell lines. Cell differentiation was induced and c-MYC was down-regulated, suggesting a mechanistic correlation between c-MYC down-regulation and induction of cell differentiation or cell death. Treatment of HL-60 and RPMI 8226 cell lines induced apoptosis, and this involved the caspase and mitochondrial pathways. Moreover, an in vivo study using RPMI 8226 human-SCID xenografts demonstrated that bruceantin induced regression in early as well as advanced tumors, and these significant antitumor responses were facilitated in the absence of overt toxicity. Apoptosis was significantly elevated in tumors derived from animals treated with bruceantin. In sum, bruceantin interferes with the growth of leukemia, lymphoma, and myeloma cells in culture and xenograft models. Responses of this type suggest bruceantin should be reinvestigated for clinical efficacy against hematological malignancies.

Introduction

Of the scores of natural products that have played an integral role in drug therapies benefiting countless human beings, it is especially intriguing to recall the historical development of camptothecin, isolated from *Camptotheca acuminata* Decne (Nyssaceae), and taxol, found in *Taxus brevifolia* Nutt. (Taxaceae). Camptothecin was first isolated in 1966 by Wall et al. and was found to demonstrate remarkable life prolongation with mice infected with L1210 and P388 leukemia cells.[1] However, in the early 1970s, clinical trials did not yield promising results, and interest in camptothecin and analogues remained quelled until 1985, when it was discovered that the compound, by a unique mechanism, inhibited the enzyme topoisomerase I.[2] Finally, new clinical trials were performed with camptothecin and analogues, significant objective responses were found on treatment of many resistant solid tumors,[3] and useful antitumor drugs were developed. Taxol was isolated during the same time period as camptothecin, and activity was found with a number of in vivo rodent models, such as P388 leukemia, B16 melanoma, and L1210 leukemia. A particularly strong response was observed in the B16 melanoma assay.[4] Nonetheless, it was only years later that this was one criterion for placing taxol in clinical trial.[5] The other major development occurred when it was established that the mechanism of taxol was unique in that it stabilized microtubules and inhibited their depolymerization.[6] Then, although taxol showed great efficacy against many solid tumors,[5] supply of the compound was limited. Ultimately, more than 25 years after the initial discovery of a novel natural product with excellent activity in a number of animal models, taxol became available in adequate quantities for therapeutic use.[4]

In this review, we discuss another drug discovered in the era of camptothecin and taxol. Bruceantin (Figure 1) is a quassinoid obtained from *Brucea* species (Simaroubaceae). Bruceantin and analogues are capable of inducing an array of biological responses including antiinflammatory and antileukemic effects with murine models.[7] The major mechanism responsible for antineoplastic activity at the molecular level has been attributed to inhibition of protein synthesis.[8] Such inhibition has been shown to occur via interference at the peptidyltransferase site, thus preventing peptide bond formation.[9] To assess toxicity, bruceantin was evaluated in three separate phase I clinical trials in patients with various types of solid tumors. Hypotension, nausea, and vomiting were common side effects at higher doses, but hematologic toxicity was moderate to insignificant and manifested mainly as thrombocytopenia.[10,11] Bruceantin was then tested in two separate phase II trials including adult patients with metastatic breast cancer[12] and malignant melanoma.[13] No objective tumor regressions were observed, and clinical trials were terminated.

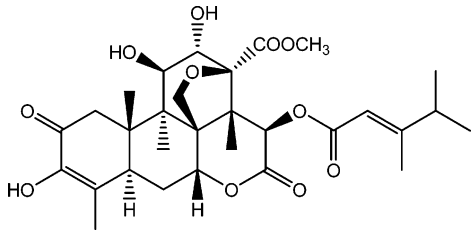

Figure 1. Chemical structure of bruceantin.

In our program for the procurement of novel plant-derived chemotherapeutic/chemopreventive agents, HL-60 cell differentiation activity has been used as one marker of activity.[14] This led to the identification of brusatol (a structural analogue of bruceantin) as a potent inducer of HL-60 cell differentiation,[15] and bruceantin was found to demonstrate even greater potency. The effect of bruceantin was evaluated with a panel of leukemia, lymphoma, and myeloma cell lines, with representative chromosomal translocations and other gene mutations. A significant finding was potent down-regulation of c-MYC oncoproteins. Cell lines expressing high levels of c-MYC oncoprotein were most sensitive to bruceantin-mediated effects.[16] In HL-60 and RPMI 8226 cell lines, treated cells underwent apoptosis. Moreover, bruceantin induced regression in early

[§] Some of the information given in this review was presented at the 43rd meeting of the American Society of Pharmacognosy, New Brunswick, NJ, July 27−31, 2002.

[⊥] Dedicated to the late Dr. Monroe E. Wall and to Dr. Mansukh C. Wani of Research Triangle Institute for their pioneering work on bioactive natural products.

* Corresponding author. Tel: (765) 494-1368. Fax: (765) 494-7880. E-mail: jpezzuto@purdue.edu.

[†] University of Illinois at Chicago.

[‡] Purdue University.

10.1021/np030304+ CCC: $27.50
Published on Web 12/24/2003

tumors as well as advanced tumors in RPMI 8226 human-SCID xenografts.[17] In sum, these data suggest bruceantin is a strong candidate for the chemotherapy of hematological malignancies.

Differentiation, Antiproliferative, and Cytotoxic Effects of Bruceantin on Leukemic Cells. The HL-60 cell system has been utilized as a tool to study the molecular and cellular events that lead to maturation. Various chemical entities have shown remarkable activities as inducers of HL-60 cell differentiation. These compounds act through gene expression modulation of important signals that regulate differentiation, proliferation, and cell death processes. For instance, all-*trans*-retinoic acid was discovered as a differentiating agent using this system[18] and, together with its natural and synthetic analogues, constitutes one of the most important categories of chemopreventive and chemotherapeutic agents.[19,20] Bruceantin-induction of differentiation was determined by the ability of treated cells to produce superoxide anions [nitroblue tetrazolium (NBT)-reduction], a functional marker of mature macrophages or granulocytes. An EC_{50} of approximately 20 nM was observed.[21] The potential of bruceantin to induce antiproliferative and differential cytotoxic effects in a panel of 11 leukemic cell lines showing various chromosomal aberrations was evaluated.[16] Assessment of viability using the Trypan blue exclusion method demonstrated that bruceantin was preferentially cytotoxic to the NB4, U937, BV173, SUPB13, RS4;11, Daudi, and DHL-6 cell lines, showing IC_{50} values of less than 15 ng/mL. On the other hand, HL-60, Kasumi-1, and Reh cell lines showed increased resistance to cytotoxic effects, with IC_{50} values in the range 20−45 ng/mL. K562 and normal lymphocytic cells (stimulated with concanavalin A) were the least sensitive of all cells tested, demonstrating approximately 90% viability after 4 days of treatment with 50 ng/mL of bruceantin. There was no obvious correlation between cytotoxic activity and a particular chromosomal aberration. The effects of bruceantin on proliferation of normal human lymphocytes or leukemic cells by incorporation of [^{3}H]-thymidine into DNA over an 18 h incubation period, after exposure to various concentrations of bruceantin for 4 days, were also analyzed. Bruceantin inhibited the proliferation of normal human lymphocytes, HL-60, K562, Kasumi-1, SUPB13, RS4;11, and Reh cells in a dose-dependent manner. Interestingly, these cell lines represent those that were most resistant to bruceantin-mediated cytotoxicity, while the compound actually increased the amount of radioactive precursor incorporation in some cytotoxic-sensitive cell lines, NB4, U937, BV173, and Daudi.

Although the reason for the difference in the response of the various cell lines is unknown, it was observed that bruceantin exerted strong cytotoxicity in those cell lines reported to express wild-type p53, including NB4, U937, BV173, and Daudi,[22−24] while some of the less sensitive cell lines have been reported to be p53-null or mutant p53-expressing cell lines, e.g., HL-60, K562, Kasumi-1, and Reh.[22−26] Bruceantin was also tested for potential to inhibit 7,12-dimethylbenz(*a*)anthracene-induced preneoplastic lesion formation in the mouse mammary organ culture (MMOC) model. This model correlates with in vivo chemopreventive activity in models such as the DMBA-induced rat mammary adenocarcinoma and the DMBA/12-*O*-tetradecanoylphorbol 13-acetate (TPA) two-stage mouse skin papilloma models.[27] Bruceantin showed 70% inhibition when tested at a concentration of 2 μM.[21]

Bruceantin Down-Regulates c-*myc*. The mechanism of action of various differentiation and apoptosis inducers remains largely unknown, but the participation of certain key genes has been demonstrated for some active compounds, such as all-*trans*-retinoic acid and CGP 57148.[28,29] c-*myc* deregulation is involved in blockage of differentiation, increased apoptosis, and proliferation. Previous studies examined c-*myc* status in leukemia, lymphoma, and myeloma cell lines after a short exposure to bruceantin (10 ng/mL). Bruceantin induced marked decreases of c-*myc* mRNA and protein expression in all cell lines. In nearly all cell lines, down-regulation of c-*myc* mRNA was less intense than the decrease observed with c-Myc protein levels (Figure 2), suggesting translational and/or post-translational regulation of this oncogene.[16,17]

The biological consequences of down-regulating c-*myc* are numerous. In the hematopoietic system, this gene inhibits differentiation[30−32] and functions as a leukemogenic protein in various lymphomas and leukemias.[33,34] Moreover, it is known that deregulation of c-*myc*, in conjunction with *p53* and *bcl-2* mutations, is associated with malignant phenotype, leading to the hypothesis that *myc* deregulation decreases the probability of maturation, while p53 and bcl-2 mutations enhance cell survival, therefore favoring leukemic cell renewal.[35] Thus, bruceantin-induced c-*myc* down-regulation might trigger cell death mechanisms preferentially in those cell lines with wild-type p53 protein expression, while triggering terminal differentiation in other cell lines with genetic defects in their apoptotic pathways.

Mechanism of Bruceantin-Induced Apoptosis in HL-60 and RPMI 8226 Cells. Treatment of HL-60 and RPMI 8226 cells with bruceantin resulted in the formation of apoptotic bodies, as observed by DAPI staining. After a 24 h treatment, the IC_{50} for apoptosis was 6.7 ng/mL (12.2 nM) and 7.0 ng/mL (12.8 nM), respectively, for these two cell lines. Pretreatment with a caspase inhibitor (z-VAD) reduced apoptosis to the level of the nontreated cells, indicating that activation of caspases was necessary for this response. These cysteine proteases form a proteolytic cascade which can be initiated by ligation of the cell surface Fas death receptor.[36,37] It was found that bruceantin led to the proteolytic processing of procaspases-3, -8, and -9 and induced caspase activity, as determined by the DEVD-R110 cleavage assay in RPMI 8226 cells.[17] Moreover, the proteolytic degradation of the caspase-3 substrate PARP was observed in HL-60 and RPMI 8226 cells (Figure 3). Further, based on data obtained with two additional myeloma cell lines, U266 and H929, it can be suggested that c-MYC down-regulation induced by bruceantin is a critical event leading to cell death.[17] The level of c-MYC was low in U266 cells and did not change after exposure to bruceantin. In H929 cells, bruceantin induced a mild up-regulation at 4 h and a stronger up-regulation at 24 h. The corresponding IC_{50} values for apoptosis were 26.8 ng/mL (49 nM) in U266 cells, and 63.3 ng/mL (115 nM) in H929 cells, demonstrating a casual relationship between c-MYC down-regulation and induction of apoptosis.

Recently, it has been found that the mitochondrial release of cytochrome *c* plays an important role in amplifying the caspase cascade.[38,39] Released cytochrome *c* forms a complex with Apaf-1, resulting in activation of caspase-9 and consequent activation of downstream caspases. Cytochrome *c* release from mitochondria is a consequence of the proteolytic processing of BID (a pro-apoptotic member of the Bcl-2 family),[40] secondary to the activation of caspase-8. Proteolytic generation of the cleaved product of BID results in translocation of BID to the mitochondria and insertion into the mitochondrial membrane, where it inhibits the anti-apoptotic action of Bcl-2 and results in

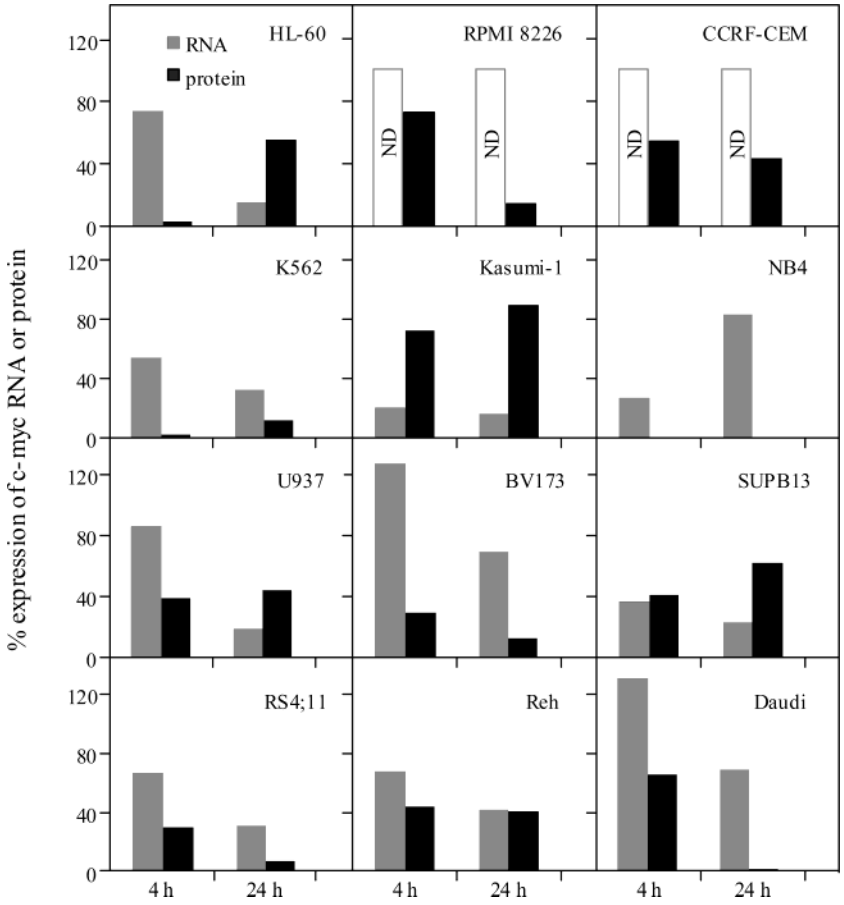

Figure 2. Brusatol down-regulates c-*myc* expression. Cells were treated with solvent (0.1% v/v DMSO, control) or bruceantin (10 ng/mL) for 4 or 24 h and then analyzed for c-*myc* mRNA using real time RT-PCR and protein expression by western blotting.[10] Results are shown as a percentage of c-*myc* mRNA or protein expression, relative to levels observed in cells treated with solvent (0.1%, v/v, DMSO) only, after normalization relative to β-actin. ND: not measured.

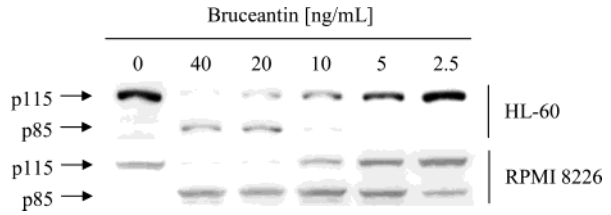

Figure 3. Dose-dependent cleavage of PARP. HL-60 and RPMI 8226 cells were treated with various concentrations of bruceantin (0−40 ng/mL) for 24 h and then analyzed by western blotting as described previously.[17]

the release of cytochrome *c*. In RPMI 8226 cells, bruceantin induced BID cleavage.[17] Mitochondrial dysfunction, in particular the induction of the mitochondrial membrane permeability transition (MPT), has been implicated in the cascade of events involved in the induction of apoptosis. Inhibition of the mitochondrial electron-transport chain is an early event and reduces the mitochondrial transmembrane potential ($\Delta\Psi_m$), which may induce the formation of the mitochondrial permeability transition pore and the subsequent MPT.[41,42] $\Delta\Psi_m$ of HL-60 and RPMI 8226 cells treated with bruceantin was analyzed using the $DiOC_6$ fluorescent probe. In both cell lines, a dose- and time-dependent decreased incorporation of $DiOC_6$, indicating the disruption of $\Delta\Psi_m$, was observed (Figure 4). This drop in $\Delta\Psi_m$ correlated well with other parameters of apoptosis and indicates that bruceantin activates the capsase and mitochondrial pathways of apoptosis.

In Vivo Study Using a Multiple Myeloma Model. An in vivo study, using 6-week-old female, 13-week-old female, and 6-week-old male SCID mice, showed that bruceantin was effective in treating RPMI 8226 human-SCID xenografts with doses as low as 1.25 mg/kg.[17] Doses of 2.5 and 5.0 mg/kg significantly induced regression in early tumors as well as advanced tumors (Figure 5), without mediating overt toxicity. The percentage of apoptotic cells evaluated by the TUNEL assay in the peripheral proliferating areas was 14.0% in control tumors, and this significantly increased to 36.6% in the tumors of animals treated with bruceantin, confirming the relevance of in vitro data obtained with RPMI 8226 cells treated with bruceantin.

Conclusions

In leukemic and myeloic cells, bruceantin down-regulated c-*myc* in all cell lines, but to a larger extent in those most sensitive to cell death or terminal differentiation. This indicates a possible role for c-*myc* in the mechanism of cell differentiation and apoptosis induced by bruceantin. Moreover, bruceantin induced regression in early tumors as well as advanced tumors in RPMI 8226 human-SCID xenografts by inhibiting cell proliferation and inducing apoptotic cell death, without mediating overt toxicity. These data suggest bruceantin should be reinvestigated in a clinical setting for effectiveness against hematological malignancies.

Acknowledgment. This work was supported by program project P01 CA48112 funded by the National Cancer Institute, NIH, Bethesda, MD. We thank the National Cancer Institute

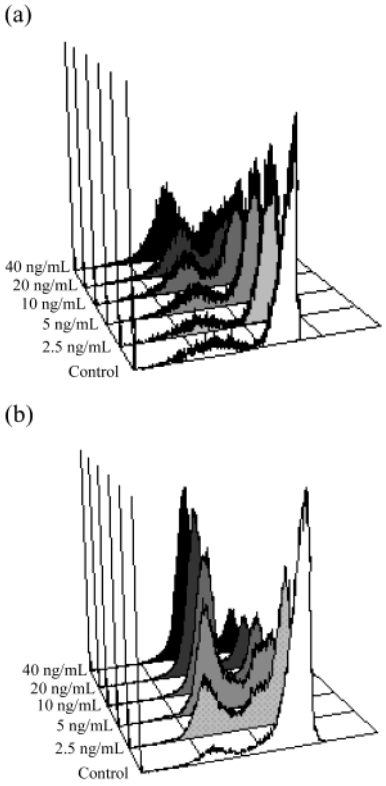

Figure 4. $DiOC_6$ labeling of mitochondria. HL-60 (a) and RPMI 8226 (b) cells were treated with the indicated concentrations of bruceantin (0−40 ng/mL) for 24 h, then mitochondrial membrane potential was determined as described previously.[17]

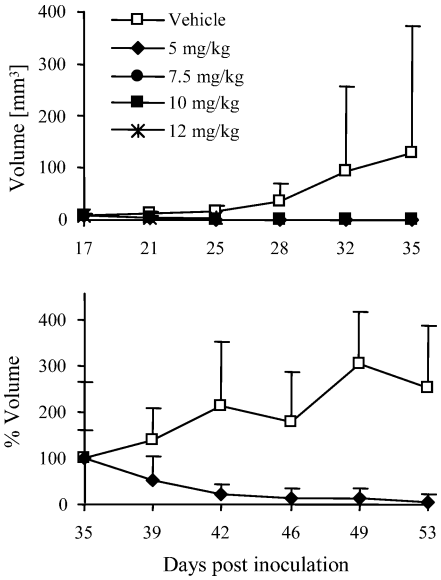

Figure 5. Effect of bruceantin on xenograft tumors derived from RPMI 8226 human multiple myeloma cells. Approximately 1×10^7 RPMI 8226 cells were inoculated subcutaneously in the right rear flank of 6-week-old female SCID mice. Mice bearing tumors from the injection of RPMI 8226 cells were treated with vehicle control (5% ethanol solution in saline) or bruceantin (5−12 mg/kg, every 3 days, i.p.) from day 17 to day 35 post-inoculation. Tumor diameters were measured and tumor volumes in mm^3 were calculated as described previously.[17] After day 35, mice from the control group were treated with bruceantin (5 mg/kg, 5 mice) or vehicle (2 mice) until day 53. Tumor volumes were measured at the indicated time points. Differences in the growth rate of tumors during the treatment period between the control group and the treated groups were statistically significant. Data are the mean ± 95% CI.

for supplying bruceantin and Dr. K. Hagen of the Research Resources Center (UIC) for analysis of samples by flow cytometry.

References and Notes

(1) Wall, M.; Wani, M.; Cook, C.; Palmer, K.; McPhail, H.; Sim, G. *J. Am. Chem. Soc.* **1966**, *88*, 3888−3890.
(2) Hsiang, Y. H.; Hertzberg, R.; Hecht, S.; Liu, L. F. *J. Biol. Chem.* **1985**, *260*, 14873−14878.
(3) Slichenmyer, W. J.; Rowinsky, E. K.; Donehower, R. C.; Kaufmann, S. H. *J. Natl. Cancer Inst.* **1993**, *85*, 271−291.
(4) Wall, M. E.; Wani, M. C. *J. Ethnopharmacol.* **1996**, *51*, 239−253; discussion 253−254.
(5) Schiff, P. B.; Fant, J.; Horwitz, S. B. *Nature* **1979**, *277*, 665−667.
(6) Suffness, M. In *Annual Reports in Medicinal Chemistry*; Bristol, J., Ed.; Academic Press: San Diego, CA, 1993; pp 305−314.
(7) Tang, W.; Eisenbrand, G. In *Chinese Drugs of Plant Origin: Chemistry, Pharmacology, and Use in Traditional and Modern Medicine*; Tang, W., Eisenbrand, G., Eds.; Springer-Verlag: Berlin, 1992; pp 207−222.
(8) Liao, L. L.; Kupchan, S. M.; Horwitz, S. B. *Mol. Pharmacol.* **1976**, *12*, 167−176.
(9) Fresno, M.; Gonzales, A.; Vazquez, D.; Jimenez, A. *Biochim. Biophys. Acta* **1978**, *518*, 104−112.
(10) Liesmann, J.; Belt, R. J.; Haas, C. D.; Hoogstraten, B. *Cancer Treat. Rep.* **1981**, *65*, 883−885.
(11) Bedikian, A. Y.; Valdivieso, M.; Bodey, G. P.; Murphy, W. K.; Freireich, E. J. *Cancer Treat. Rep.* **1979**, *63*, 1843−1847.
(12) Wiseman, C. L.; Yap, H. Y.; Bedikian, A. Y.; Bodey, G. P.; Blumenschein, G. R. *Am. J. Clin. Oncol.* **1982**, *5*, 389−391.
(13) Arsenau, J. C.; Wolter, J. M.; Kuperminc, M.; Ruckdeschel, J. C. *Invest. New Drugs* **1983**, *1*, 239−242.
(14) Suh, N.; Luyengi, L.; Fong, H. H. S.; Kinghorn, A. D.; Pezzuto, J. M. *Anticancer Res.* **1995**, *15*, 233−240.
(15) Luyengi, L.; Suh, N.; Fong, H. H. S.; Kinghorn, A. D.; Pezzuto, J. M. *Phytochemistry* **1996**, *43*, 409−412.
(16) Mata-Greenwood, E.; Cuendet, M.; Sher, D.; Gustin, D.; Stock, W.; Pezzuto, J. M. *Leukemia* **2002**, *16*, 2275−2284.
(17) Cuendet, M.; Christov, K.; Lantvit, D.; Deng, Y.; Hedayat, S.; Helson, L.; McChesney, J.; Pezzuto, J. M. *Clin. Cancer Res.*, in press.
(18) Breitman, T. R.; Selonick, S. E.; Collins, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2936−2940.
(19) Huang, M. E.; Ye, Y. C.; Chen, S. R.; Chai, J. R.; Lu, J. X.; Zhoa, L.; Gu, L. J.; Wang, Z. Y. *Blood* **1988**, *72*, 567−572.
(20) Moon, R. C.; McCormick, D. L.; Mehta, R. G. *Cancer Res.* **1983**, *43*, 2469s−2475s.
(21) Mata-Greenwood, E.; Daeuble, J. F.; Grieco, P. A.; Dou, J.; McChesney, J. D.; Mehta, R. G.; Kinghorn, A. D.; Pezzuto, J. M. *J. Nat. Prod.* **2001**, *64*, 1509−1513.
(22) Bocchia, M.; Xu, Q.; Wesley, U.; Xu, Y.; Korontsvit, T.; Loganzo, F.; Albino, A. P.; Scheinberg, D. A. *Leuk. Res.* **1997**, *21*, 439−447.
(23) Lubbert, M.; Miller, C. W.; Crawford, L.; Koeffler, H. P. *J. Exp. Med.* **1988**, *167*, 873−886.
(24) Hendrikse, A. S.; Hunter, A. J.; Keraan, M.; Blekkenhorst, G. H. *Int. J. Radiat. Biol.* **2000**, *76*, 11−21.
(25) Furuwatari, C.; Yagi, A.; Yamagami, O.; Ishikawa, M.; Hidaka, E.; Ueno, I.; Furihata, K.; Ogiso, Y.; Katsuyama, T. *Am. J. Clin. Pathol.* **1998**, *110*, 368−373.
(26) Aldridge, D. R.; Radford, I. R. *Cancer Res.* **1998**, *58*, 2817−2824.
(27) Steele, V. E.; Sharma, S.; Mehta, R.; Elmore, E.; Redpath, L.; Rudd, C.; Bagheri, D.; Sigman, C. C.; Kelloff, G. J. *J. Cell Biochem.* (Suppl.) **1996**, *26*, 29−53.
(28) Dyck, J. A.; Maul, G. G.; Miller, W. H., Jr.; Chen, J. D.; Kakizuka, A.; Evans, R. M. *Cell* **1994**, *76*, 333−343.
(29) Dan, S.; Naito, M.; Tsuruo, T. *Cell Death Differ.* **1998**, *5*, 710−715.
(30) Collins, J. F.; Herman, P.; Schuch, C.; Bagby, G. C., Jr. *J. Clin. Invest.* **1992**, *89*, 1523−1527.
(31) Negroni, A.; Scarpa, S.; Romeo, A.; Ferrari, S.; Modesti, A.; Raschella, G. *Cell Growth Differ.* **1991**, *2*, 511−518.
(32) Nguyen, H. Q.; Selvakumaran, M.; Liebermann, D. A.; Hoffman, B. *Oncogene* **1995**, *11*, 2439−2444.
(33) Dalla-Favera, R.; Bregni, M.; Erikson, J.; Patterson, D.; Gallo, R. C.; Croce, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7824−7827.
(34) Boxer, L. M.; Dang, C. V. *Oncogene* **2001**, *20*, 5595−5610.
(35) Adams, J. M.; Cory, S. *Science* **1991**, *254*, 1161−1167.
(36) Nagata, S. *Cell* **1997**, *88*, 355−365.
(37) Budihardjo, I.; Oliver, H.; Lutter, M.; Luo, X.; Wang, X. *Annu. Rev. Cell. Dev. Biol.* **1999**, *15*, 269−290.
(38) Yabuki, M.; Tsutsui, K.; Horton, A.; Yoshioka, T.; Utsumi, K. *Free Radical Res.* **2000**, *32*, 507−514.
(39) Gree, D.; Reed, J. *Science* **1998**, *281*, 1309−1312.
(40) Kaufmann, S.; Hengartner, M. *Trends Cell. Biol.* **2001**, *11*, 526−534.
(41) Zamzami, N.; Marchetti, P.; Castedo, M.; Decaudin, D.; Macho, A.; Hirsh, T.; Susin, S.; Petit, P.; Mignotte, B.; Kroemer, G. *J. Exp. Med.* **1995**, *182*, 367−377.
(42) Kroemer, G.; Petit, P.; Zamzami, N.; Vayssiere, J.-L.; Mignotte, B. *FASEB J.* **1995**, *9*, 1277−1287.

NP030304+