Biochemical Pharmacology, Vol. 41, No. 5, pp. 829–832, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

SHORT COMMUNICATIONS

Effect of bryostatin 1 on drug-induced, topoisomerase II-mediated DNA cleavage and topoisomerase II gene expression in human leukemia cells

(*Received* 9 *April* 1990; *accepted* 6 *September* 1990)

Treatment of HL-60 human leukemia cells with phorbol 12-myristate 13-acetate (PMA*) for 24 hr produces a 10-fold decrease in etoposide-induced, topoisomerase II-mediated DNA cleavage [1]. PMA-induced cellular adherence, an early sign of terminal differentiation, occurs prior to the decline in drug-induced cleavage. This suggests that the decreased cleavage may be associated with terminal monocytoid differentiation rather than result from a proximal phorbol-initiated biochemical event such as phosphorylation of topoisomerase II by the phorbol ester receptor protein kinase C [2–7]. To study this theory more completely, we employed bryostatin 1 [8], a protein kinase C stimulator that *does not* induce monocytoid differentiation [9, 10], to address whether pharmacologic activation of protein kinase C alone is sufficient to down-regulate topoisomerase II-mediated events.

Materials and Methods

The HL-60 cell line used in these experiments was a gift from Dr. Robert Hall, Guthrie Research Institute, Sayre, PA [11, 12]. The cells were propagated in our laboratory in Iscove's modified Dulbecco's medium (Hazleton, Lenexa, KS) and 10% fetal bovine serum at 37° in 5% CO_2. All cells were free of mycoplasma (American Type Culture Collection, Rockville, MD). Mouse leukemia L1210 cells served as internal standard cells in alkaline elution experiments. HL-60 cells were radiolabeled with 0.05 to 0.1 μCi/mL of [2-^{14}C]thymidine (DuPont-New England Nuclear, Boston, MA) for 24–72 hr to label their cellular DNA [13] for alkaline elution experiments (see below). L1210 cells were labeled with 0.1 μCi/mL of [*methyl*-^{3}H]thymidine; these served as an internal standard in alkaline elution experiments. Cells were incubated for at least 1 hr in label-free medium prior to treatment with any drug.

Phorbol 12-myristate 13-acetate (PMA) from the LC Services Corp. (Woburn, MA) was constituted as 10^{-2} M stock solutions in 100% dimethyl sulfoxide (DMSO). Bryostatin 1 was kept as a 10^{-3} M stock solution in 100% DMSO. The *m*-AMSA (amsacrine, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide) (NSC 249992) was from the National Cancer Institute, and the etoposide was a gift from Drs. Byron Long and James H. Keller of the Bristol-Myers Co. Both drugs were constituted as 10^{-2} M solutions in 100% DMSO.

Colony formation was quantified by the previously described [13] soft agar method of Chu and Fisher [14]. DNA alkaline elution was performed as previously described [1, 13, 15] to quantify drug-induced DNA cleavage frequencies. Northern blotting was performed using standard techniques [13]. The human topoisomerase II cDNA probe (ZII69) used was a gift from Dr. Leroy Liu of the Johns Hopkins School of Medicine [16]. The 1.3-kilobase (kb) *Cla*I/*Eco*RI fragment from the 3′ end of the c-*myc* gene [17] was a gift from Dr. Mark Blick, M.D. Anderson Cancer Center. These probes were radiolabeled using the Amersham Multiprime labeling system (Amersham Corp., Arlington Heights, IL).

Results and Discussion

Rapidly proliferating cells contain more topoisomerase II [18, 19] than quiescent cells do. Furthermore, the DNA of proliferating cells exhibits a greater sensitivity to drug-induced, topoisomerase II-mediated DNA cleavage [18, 20–22] than does the DNA of quiescent cells. We therefore evaluated the effects of bryostatin 1 on the growth of HL-60 cells to be employed in assays of drug-induced DNA cleavage. Bryostatin 1 concentrations of 1000 and 100 nM had almost no effect on cell growth. Lower concentrations of bryostatin 1 did slow cell growth to some degree, but this effect was relatively small during the first 2 days of treatment, the duration of the longest bryostatin 1 treatment used in the experiments reported here.

PMA down-regulates topoisomerase II-mediated DNA cleavage in HL-60 cells [1]. Furthermore, this down-regulation was drug-specific, being greater for etoposide than for *m*-AMSA (see Table 1) [1]. In contrast, bryostatin 1 (1000 nm for 24 hr) had virtually no effect on drug-induced DNA cleavage (Table 1), although prolonged treatment (48 hr) did decrease drug-induced DNA cleavage slightly (data not shown). The only pronounced effect of bryostatin 1 on drug-induced DNA cleavage was seen in the small subpopulation of adherent cells (8–12%) treated with low concentrations of bryostatin 1 (1 and 10 nM, data not shown). Thus, bryostatin 1 can down-regulate drug-induced, topoisomerase II-mediated DNA cleavage; however, it took longer to achieve this effect than with PMA, the extent of the decrease was less than with PMA (Table 1), and a much smaller segment of the treated cell population was affected.

Others have demonstrated that bryostatin 1 can actually block (rather than mimic) some of the effects of PMA, presumably by competing for the phorbol receptor (protein kinase C) [9, 23–27]. The data in Table 1 demonstrate the same to be true in our system. When added just prior to PMA, 1000 nM bryostatin 1 prevented PMA-induced down-regulation of topoisomerase II-mediated DNA cleavage. Figure 1 demonstrates this more clearly. As the bryostatin 1 concentration that preceded the addition of 10 nM PMA decreased, so did the ability of bryostatin 1 to overcome the down-regulation of drug-induced cleavage. Bryostatin 1 can also overcome the ability of PMA to produce cellular adherence; this too depends on the concentration of bryostatin 1 that precedes the PMA. Furthermore, the ability of a 24-hr bryostatin 1 treatment to block cleavage paralleled the ability of this treatment to block adherence (Fig. 1, inset). These results suggest that the signal to adhere and the down-regulation of topoisomerase II-mediated DNA cleavage may be mechanistically related.

To examine this possibility more closely, we studied the effect of separating the combined PMA–bryostatin 1 treatments in time to see whether the effects of one drug could escape the influence of the other (data not shown). When bryostatin 1 was added before PMA, very few cells adhered and no decrease in drug-induced DNA cleavage occurred (also see Table 1). In contrast, when PMA was

* Abbreviations: PMA, phorbol 12-myristate 13-acetate; *m*-AMSA, amsacrine, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; and etoposide, 4-(4,6-*O*-ethylidene-β-D-glucopyranoside.

Table 1. Effect of pretreatment of HL-60 human leukemia cells with 1000 nM bryostatin just prior to addition of 10 nM PMA for 24 hr on subsequent topoisomerase II-mediated DNA cleavage produced by 5 μM etoposide or 0.5 μM *m*-AMSA*

	DNA cleavage frequency ratio		
	Bryostatin 1†	PMA alone†	Bryostatin 1 then PMA†
Etoposide	0.98 ± 0.22	0.14 ± 0.03	0.91 ± 0.11
m-AMSA	0.71 ± 0.17	0.24 ± 0.05	0.82 ± 0.27

* Values are means ± 1 SD (N = 4) for etoposide- or means ± range (N = 2) for *m*-AMSA-induced DNA cleavage frequency in cells exposed to bryostatin 1 or PMA divided by the drug-induced DNA cleavage frequency in cells receiving neither bryostatin 1 nor PMA.

† Only adherent cells were used following PMA. All cells were used following bryostatin 1 alone or in combination with PMA.

added first, a delay in the addition of bryostatin 1 of 2–6 hr reduced the ability of the latter to restore drug-induced DNA cleavage to control levels. Surprisingly, bryostatin 1 was able to block the majority of the PMA-induced adherence even when added 6 hr after PMA. Thus, bryostatin 1 can dissociate the effects of PMA on drug-induced DNA cleavage and cell adherence. The PMA-induced down-regulation of DNA cleavage was locked in more rapidly than was PMA-induced cell adherence. This seems to contrast with findings from our previous work using PMA alone [1], wherein PMA's maximum reduction of drug-induced DNA cleavage lagged behind the full development of PMA-induced cellular adherence [28]. Taken together, these results suggest that the PMA-induced *signal* resulting in reduced DNA cleavage occurs *before* the PMA-induced *signal* to adhere even though the adherence can be developed more fully prior to the maximum decrease in etoposide-induced DNA cleavage. Thus, the PMA-induced decrease of drug-induced, topoisomerase II-mediated DNA cleavage is not a consequence of PMA-induced adherence alone. Instead, it may be related to as yet undescribed biochemical correlates of monocytoid differentiation that occur after protein kinase C activation but *before* terminal differentiation.

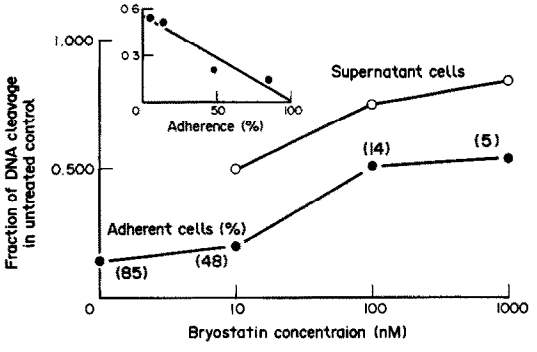

Fig. 1. Effect of pretreatment with various concentrations of bryostatin 1 immediately prior to addition of 10 nM PMA on etoposide-induced DNA cleavage in HL-60 human leukemia cells. Cells were treated with bryostatin 1 and PMA for 24 hr prior to the removal of both agents, the addition of 5 μM etoposide for 1 hr, and the quantification of etoposide-induced DNA cleavage using alkaline elution with proteinase. Drug-induced cleavage was quantified in nonadherent (supernatant) (○) and adherent (●) cell populations separately. Data are expressed as the ratio of the etoposide-induced DNA cleavage frequency in the cells exposed to bryostatin 1 and PMA divided by the cleavage frequency in cells exposed to neither agent. Numbers in parentheses represent the percent of cells adherent just prior to the addition of etoposide. The inset shows the plot of the DNA cleavage frequency ratio in the adherent cells versus the amount of adherence.

PMA (10 nM for 24 hr) was able to block the cytotoxicity of etoposide (86% survival compared with 6% for etoposide alone). In contrast, bryostatin 1 (1000 nM for 24 hr) had only a small effect on etoposide-induced cytotoxicity (16% survival). Bryostatin 1 added before PMA protected few cells from the cytotoxicity of etoposide (20%), whereas PMA added first offered greater protection (60%). Finally, bryostatin 1 was able to blunt the cytotoxic effects of etoposide only in the small population of cells that adhered to plastic (4%) after a 48-hr treatment with 10 nM bryostatin 1 (etoposide alone = 4% survival; 10 nM brostatin 1 for 48 hr plus etoposide = 46%). These results are consistent with those obtained when quantifying the other effects of PMA, bryostatin 1, and the various combinations of the two on other etoposide-induced effects.

Bryostatin 1 had little effect on the amount of topoisomerase II message in HL-60 cells, whereas PMA produced a pronounced down-regulation of both the topoisomerase II and c-*myc* [29] messages (Fig. 2). A small decrease in topoisomerase II mRNA was seen in cells exposed to 10 nM bryostatin for 48 hr; however, this was not accompanied by a marked decline in c-*myc* message nor was this change of the magnitude produced by PMA in half the time (Fig. 2). Surprisingly, bryostatin 1, whether added before or after PMA, was able to overcome the PMA-induced down-regulation of these genes. However, this may simply indicate that the 2- to 3-fold differences detectable in other assays may not be detectable using Northern blotting.

In summary, unlike PMA, bryostatin 1 has been found to have a minimal effect on drug-induced topoisomerase II-mediated DNA cleavage and no effect on topoisomerase II mRNA levels. Furthermore, bryostatin 1 overcame the down-regulatory effects of PMA treatment on (1) drug-induced, topoisomerase II-mediated DNA cleavage, (2) drug-induced cytotoxicity, and (3) topoisomerase II gene expression. Thus, it is unlikely that the effects of phorbol ester treatment on topoisomerase II-mediated events are a direct consequence of protein kinase C activation *per se*.

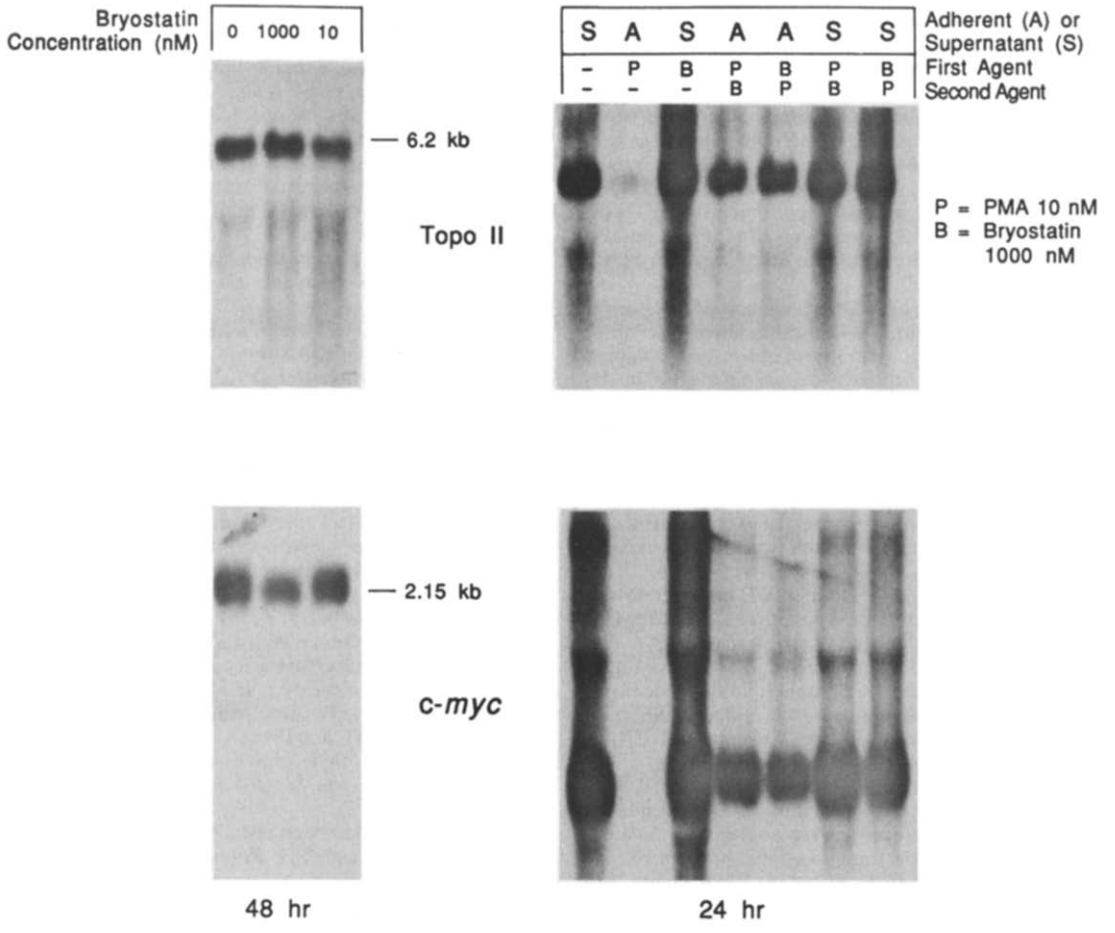

Fig. 2. Effect of bryostatin 1 and PMA on expression of topoisomerase II mRNA in HL-60 human leukemia cells. (Left) Forty-eight hour treatments with bryostatin 1 alone. All cells were used in this experiment. (Right) Twenty-four hour treatments with (from left to right) vehicle control (supernatant cells) 10 nM PMA alone (adherent cells, 48% adherence), 1000 nM bryostatin 1 alone (supernatant cells), PMA and then bryostatin 1 (adherent cells, 4% adherence), bryostatin 1 and then PMA (adherent cells, 5% adherence), PMA and then bryostatin 1 (supernatant cells), and bryostatin 1 and then PMA (supernatant cells).

Rather, the results with bryostatin 1 suggest that the phorbol ester effects are related to more distal effects of phorbol ester treatment that may be related to monocytoid differentiation.

Acknowledgements—This work was supported by PHS Research Grant CA40090, Grant CH-324C from the American Cancer Society, and a contribution from the Nucleopore Corporation of Pleasanton, CA, to L.A.Z., and by the Fannie E. Rippel Foundation, the Arizona Disease Control Research Commission, the Robert B. Dalton Endowment Fund, and an Outstanding Investigator Grant CA-44344-01A1 (NCI, DHHS) to G.R.P. We thank Dr. Robert Hall of the Guthrie Research Institute, Sayre, PA, for providing the HL-60 cells; Dr. Leroy Liu of the Johns Hopkins School of Medicine for his gift of the human cDNA topoisomerase II gene probe; Dr. Mark Blick of the M.D. Anderson Cancer Center for supplying the c-*myc* probe; Dr. Cherry L. Herald for other assistance; and Diane Rivera for editing the manuscript.

* To whom correspondence should be addressed at: Box 52, 1515 Holcombe Blvd., Houston, TX 77030.

Department of Medical Oncology
Division of Medicine
The University of Texas M.D. Anderson Cancer Center
Houston, TX; and

LEONARD A. ZWELLING*
DIANA CHAN
ELIZABETH ALTSCHULER
JANICE MAYES
MICHAEL HINDS

The Cancer Research Institute
Arizona State University
Tempe, AZ, U.S.A.

GEORGE R. PETTIT

REFERENCES

1. Zwelling LA, Chan D, Hinds M, Mayes J, Silberman LE and Blick M, Effect of phorbol ester treatment on drug-induced, topoisomerase II-mediated DNA cleavage in human leukemia cells. *Cancer Res* **48**: 6625–6633, 1988.
2. Niedel JE, Kuhn LJ and Vendenbark GR, Phorbol diester receptor copurifies with protein kinase C. *Proc Natl Acad Sci USA* **80**: 36–40, 1983.

3. Nishizuka; Y, The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**: 693–698, 1984.
4. Blumberg PM, Protein kinase C as the receptor for the phorbol ester tumor promoters: Sixth Rhoads Memorial Award Lecture. *Cancer Res* **48**: 1–8, 1988.
5. Sahyoun N, Wolf M, Besterman J, Hsieh T-s, Sander M, LeVine H III, Chang K-J and Cuatrecasas P, Protein kinase C phosphorylates topoisomerase II: Topoisomerase II activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc Natl Acad Sci USA* **83**: 1603–1607, 1986.
6. Rottmann M, Schroder HC, Gramzow M, Renneisen K, Kurelec B, Dorn A, Friese U and Muller WEG, Specific phosphorylation of proteins in pore complex-laminae from the sponge *Geodia cydonium* by the homologous aggregation factor and phorbol ester. Role of protein kinase C in the phosphorylation of topoisomerase II. *EMBO J* **6**: 3939–3944, 1987.
7. Constantinou A, Henning-Chubb C and Huberman E, Novobiocon- and phorbol-12-myristate-13-acetate-induced differentiation of human leukemia cells associated with a reduction in topoisomerase II activity. *Cancer Res* **49**: 1110–1117, 1989.
8. Pettit GR, Herald CL, Doubek DL and Herald DL, Isolation and structure of bryostatin 1. *J Am Chem Soc* **104**: 6846–6848, 1982.
9. Kraft AS, Smith JB and Berkow RL, Bryostatin, an activator of the calcium phospholipid-dependent protein kinase, blocks phorbol ester-induced differentiation of human promyelocytic leukemia cells HL-60. *Proc Natl Acad Sci USA* **83**: 1334–1338, 1986.
10. Blumberg PM, Petti GR, Warren BS, Szallasi A, Schuman LD, Sharkey NA, Nakauma H, Dell-Aquila ML and deVries DJ, The protein kinase C pathway in tumor promotion. In: *Skin Carcinogenesis: Mechanisms and Human Relevance* (Eds. Slaga TJ, Klein-Szanto AJP, Boutwell RK, Stevenson DE, Spitzer HL and D'Motto B), pp. 201–212. Alan R. Liss, New York, 1989.
11. Leftwich JA, Carlson P, Adelman B and Hall RE, HL-60-1E3, a novel phorbol diester-resistant HL-60 cell line. *Cancer Res* **47**: 1319–1324, 1987.
12. Ely CM, Leftwich JA, Chenevix-Trench G, Hall RE and Westin EH, Altered regulation of c-*myc* in an HL-60 differentiation resistant subclone, HL-60-1E3. *Cancer Res* **47**: 4594–4600, 1987.
13. Zwelling LA, Hinds M, Chan D, Mayes J, Sie KL, Parker E, Silberman L, Radcliffe A, Beran M and Blick M, Characterization of an amsacrine-resistant line of human leukemia cells. *J Biol Chem* **264**: 16411–16420, 1989.
14. Chu M-Y and Fischer GA, The incorporation of ^{3}H-cytosine-arabinoside and its effect on murine leukemic cells (L5178Y). *Biochem Pharmacol* **17**: 753–767, 1968.
15. Zwelling LA, Michaels S, Erickson LC, Ungerleider RS, Nichols M and Kohn KW, Protein-associated DNA strand breaks in L1210 cells treated with the DNA intercalating agents 4′(9-acridinylamino)methanesulfon-*m*-anisidide and Adriamycin. *Biochemistry* **20**: 6553–6563, 1981.
16. Tsai-Pflugfelder M, Liu LF, Liu AA, Tewey KM, Whang-Peng J, Knutsen T, Huebner K, Croce CM and Wang JC, Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21–22. *Proc Natl Acad Sci USA* **85**: 7177–7181, 1988.
17. Dalla-Favera R, Martinotti S, Gallo RC, Erickson J and Croce CM, Translocation and rearrangements of the c-*myc* oncogene locus in human undifferentiated B-cell lymphomas. *Science* **219**: 963–967, 1983.
18. Sullivan DM, Latham MD and Ross WE, Proliferation-dependent topoisomerase II content as a determinant of antineoplastic drug action in human, mouse, and Chinese hamster ovary cells. *Cancer Res* **47**: 3973–3979, 1987.
19. Hsiang Y-H, Wu H-Y and Liu LF, Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* **48**: 3230–3235, 1988.
20. Sullivan DM, Glisson BS, Hodges PK, Smallwood-Kentro S and Ross WE, Proliferation dependence of topoisomerase II mediated drug action. *Biochemistry* **25**: 2248–2256, 1986.
21. Zwelling LA, Estey E, Silberman L, Doyle S and Hittelman W, Effect of cell proliferation and chromatin conformation on intercalator-induced, protein-associated DNA cleavage in human brain tumor cells and human fibroblasts. *Cancer Res* **47**: 251–257, 1987.
22. Markovits J, Pommier Y, Kerrigan D, Covey JM, Tilchen EJ and Kohn KW, Topoisomerase II-mediated DNA breaks and cytotoxicity in relation to cell proliferation and the cell cycle in NIH 3T3 fibroblasts and L1210 leukemia cells. *Cancer Res* **47**: 2050–2055, 1987.
23. Smith JB, Smith L and Pettit GR, Bryostatins: Potent, new mitogens that mimic phorbol ester tumor promoters. *Biochem Biophys Res Commun* **132**: 939–945, 1985.
24. Hennings H, Blumberg PM, Pettit GR, Herald CL, Shores R and Yuspa SH, Bryostatin 1, an activator of protein kinase C, inhibits tumor promotion by phorbol esters in SENCAR mouse skin. *Carcinogenesis* **8**: 1343–1346, 1987.
25. Stone RM, Sariban E, Pettit GR and Kufe DW, Bryostatin 1 activates protein kinase C and induced monocytic differentiation of HL-60 cells. *Blood* **72**: 208–213, 1988.
26. de Vries DJ, Herald CL, Petti GR and Blumberg PM, Demonstration of sub-nanomolar affinity of bryostatin 1 for the phorbol ester receptor in rat brain. *Biochem Pharmacol* **37**: 4069–4073, 1988.
27. Gschwendt M, Furstenberger G, Rose-John S, Rogers M, Kittstain W, Pettit GR, Herald CL and Marks F, Bryostatin 1, an activator of protein kinase C, mimics as well as inhibits biological effects of the phorbol ester TPA *in vivo* and *in vitro*. *Carcinogenesis* **9**: 555–562, 1988.
28. Zwelling LA, Hinds M, Chan D, Altschuler E, Mayes J and Zipf TF, Phorbol ester effects on topoisomerase II activity and gene expression in HL-60 human leukemia cells with different proclivities toward monocytoid differentiation. *Cancer Res* **50**: 7116–7122, 1990.
29. Kraft AS, Baker VV and May WS, Bryostatin induces changes in protein kinase C location and activity without altering c-*myc* expression in human promyelocytic leukemia cells (HL60). *Oncogene* **1**: 111–118, 1987.