Bryostatin 1 Affects P-Glycoprotein Phosphorylation but not Function in Multidrug-resistant Human Breast Cancer Cells

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ABSTRACT

The function of P-glycoprotein (Pgp), which confers multidrug resistance by active efflux of drug, is thought to be dependent on phosphorylation. Previous studies have suggested that protein kinase C (PKC) plays an important role in Pgp phosphorylation. We report here the effects of bryostatin 1, a unique PKC activator and inhibitor, on Pgp function in a multidrug-resistant MCF-7 human breast cancer subtype in which overexpresses PKC-α. Bryostatin 1 (100 nM) decreased Pgp phosphorylation after 24 h of treatment. In contrast, it did not affect Pgp function as demonstrated by the accumulation of [3H]vinblastine and rhodamine 123.

We compared the effect of bryostatin 1 treatment on PKC-α with that of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (200 nM). 12-O-tetradecanoylphorbol-13-acetate caused translocation of PKC-α from the cytosol to the cell membrane after a 10-min treatment and its down-regulation after 24 h of treatment. Likewise, bryostatin 1 (100 nM) caused translocation, but only after longer treatment (1 h), and it caused down-regulation of PKC-α at 24 h of treatment. Thus, while the MCF-7TH cells overexpress the PKC-α isoform, and its down-regulation by bryostatin 1 is associated with decreased Pgp phosphorylation, these alterations do not modulate drug transport. We conclude that, while bryostatin 1 may be useful clinically because of its ability to inhibit PKC, it is not able to reverse Pgp-mediated multidrug resistance.

INTRODUCTION

The identification of clinically suitable agents able to overcome drug resistance would have great impact for cancer treatment. In tumor cell lines, the best characterized mechanism of drug resistance is "multidrug resistance" mediated by overexpression of an energy-dependent membrane-bound drug efflux pump known as Pgp. Pgp undergoes covalent modification by rapid cycles of phosphorylation and dephosphorylation in which several different protein kinases have been implicated (1–6). The earliest reports suggested that phosphorylation played a role in the activity of the efflux pump (1, 7–9). Increased transport results from increased Pgp phosphorylation, whereas impaired function occurs with inhibition of phosphorylation (2, 3).

PKC (2, 10–13), PKA (4, 13), and other potentially more specific kinases (3, 5, 6) have been linked to Pgp phosphorylation. Chambers et al. (12) reported that PKC phosphorylates Pgp at multiple sites in the linker region between the two halves of the molecule. Characterization of specific residues for PKC and PKA phosphorylation has been reported in both murine and human Pgp (11, 13). Several studies have confirmed that activation of PKC by the phorbol ester TPA results in an increase in Pgp phosphorylation associated with increased drug efflux, decreased drug accumulation, and a more resistant phenotype (1–3, 7, 14, 15). Conversely, PKC down-regulation by long-term TPA treatment or inhibition by staurosporine or calphostin C impairs Pgp function, resulting in decreased drug efflux and increased accumulation (7, 14, 16). Increased expression or activity of the PKC isoforms has been reported in multidrug-resistant cell lines (2, 7, 17), with a differential increase in the expression of the PKC-α isoform being the most frequent observation (17–19). This finding has been reported for a multidrug-resistant subtype of MCF-7 human breast cancer cells (19).

Bryostatin 1, which is structurally distinct from the phorbol esters, can activate PKC in vitro but in vivo it can paradoxically antagonize other activators of PKC such as the tumor-promoting phorbol esters (20, 21). Bryostatin 1 is the best studied member of a family of more than 13 compounds that have a macrocyclic lactone structure with varying side chains (22, 23). As the most abundant of the bryostatins isolated from the marine bryozoan Bugula neritina, bryostatin 1 is the first member of this class of compounds to be tested in humans. These macrocyclic lactones possess potent antineoplastic properties in human and murine tumor cell lines (23, 24–26) and in vivo activity in murine B16 melanoma. The range of in vitro activities includes cytotoxicity and terminal differentiation of malignant cells (27, 28). Moreover, in contrast to the phorbol esters, bryostatin 1 lacks tumor-promoting activity. These properties suggested that bryostatin 1 could be useful as an antineoplastic agent. The first Phase I clinical trial with bryostatin 1 was recently completed and demonstrated that concentrations of drug as high as 25 μg/ml could be safely achieved in humans (29).

Since PKC modulation has been shown to impair Pgp function, and bryostatin 1 has been shown to be well tolerated clinically, we studied the effect of bryostatin 1 on a multidrug-resistant MCF-7 human breast cancer subtype which overexpresses PKC-α. We sought an agent suitable for clinical studies which could decrease Pgp phosphorylation and thereby impair its function. The results of these studies are described here.
Fig. 1 Expression of PKC isoforms in MCF-7 (Parent) and MCF-7TH (TH) cells. Cells were harvested and fractionated into a cytosol (S) and a membrane (P) fraction. Following electrophoresis and transfer to nitrocellulose, immunoblotting was performed with antibodies specific for the isoenzymes detected. Identical results were obtained in a second series of independent experiments.

MATERIALS AND METHODS

Materials. Bryostatin 1 was provided by the Developmental Therapeutics Program (Division of Cancer Treatment, National Cancer Institute); TPA was obtained from Sigma; monoclonal anti-PKC-α antibody was obtained from United Biomedical, Inc.; and polyclonal anti-PKC-β, -ε, -δ, -γ, and -ζ antibodies were obtained from GIBCO Life Technologies-BRL.

Cell Lines. MCF-7TH multidrug-resistant cells were selected from the parental MCF-7 human breast cancer cells by intermittent exposure to doxorubicin and were maintained in 200 ng/ml drug. The cells were cultured in DMEM, 10% FCS, penicillin, streptomycin, and glucose at 37°C in 5% CO₂. MCF-7TH cells overexpress Pgp and are cross-resistant to doxorubicin (250-fold), vinblastine (480-fold), paclitaxel (73-fold), and actinomycin D (220-fold), relative to MCF-7 cells. A multidrug-resistant human colon carcinoma subline, SW620 Ad300, was utilized as a control in certain experiments. These cells overexpress mdr-1 and are maintained in 300 ng/ml doxorubicin (30). The multidrug-resistant sublines were cultured in media free of drug for 1 week prior to each experiment.

Drug Accumulation Assay. The accumulation of vinblastine, daunorubicin, and colchicine were assayed as previously described (31). Briefly, cells were plated in 6-well dishes (350,000/well), and after 24 h were treated with TPA and bryostatin 1 for the indicated periods of time. Cells were then rinsed once and incubated for 10 min with assay medium before starting the incubation. Cells were incubated for 45 min with 2 × 10⁶ dpm/well, which is equivalent to 14 nM [³H]vinblastine. Rhodamine 123 accumulation was also assayed as previously described (16). Briefly, after treatment for the indicated time period, cells were trypsinized and resuspended at 1 × 10⁶/ml in phenol red-free improved MEM + 10% FBS containing 0.5 μg/ml rhodamine. Following a 30-min incubation at 37°C in 5% CO₂, cells were washed in ice-cold improved MEM and analyzed on a FACSsort flow cytometer (Becton Dickinson) equipped with a 488-nm laser. The green fluorescence of rhodamine 123 was detected with a 530-nm band-pass filter.

PKC Isoform Immunoblotting. Pretreated and untreated cells were harvested in PKC buffer [2 mM EDTA, 0.5 mM EGTA, 5 mM DTT, 25 mM Tris-Cl (pH 7.4), and 50 μg/ml aprotinin (32)]. After sonication, the disrupted cells were centrifuged at 1,000 × g to remove nuclei; the samples were then centrifuged at 100,000 × g for 30 min. The supernatant, containing the cytosol, was removed, and the membrane pellet was resuspended in PKC buffer. After protein quantitiation (Bio-Rad), equal amounts (25 μg) of cytosolic and membrane protein were subjected to SDS-PAGE on a 7.5% SDS-polyacrylamide gel.
RESULTS

We first studied the pattern of expression of PKC isoforms in MCF-7/TH multidrug-resistant breast cancer cells. As shown in Fig. 1, MCF-7/TH cells overexpress PKC-α when compared with parental MCF-7 cells. This blot, which compares equal amounts of protein in the cytosolic, or supernatant, fraction, and in the membrane, or particulate, fraction, demonstrates that PKC-α is overexpressed in both fractions. The isoforms ε and ζ are expressed at comparable levels in both cell lines, while β, γ, and η are not reliably detected in either cell line. The overexpression of PKC-α in the multidrug-resistant cells suggested that inhibition could affect Pgp phosphorylation and function.

To exclude the possibility that bryostatin 1 is a substrate for Pgp, cytotoxicity assays were performed in parental and resistant cells, with and without verapamil. Doses of bryostatin 1 of less than or equal to 100 nM did not result in growth inhibition over the 4-day incubation in the cytotoxicity assay. The 50% inhibitory concentration for MCF-7/TH was 20 μM, and for MCF-7 was 2 μM, suggesting that MCF-7/TH is 10-fold resistant to bryostatin 1 as shown in Fig. 2. However, the addition of verapamil (10 μg/ml) did not significantly increase the sensitivity to bryostatin 1 (Fig. 2). The effect of verapamil on bryostatin 1 cytotoxicity was also tested in multidrug-resistant SW620 Ad300 human colon carcinoma cells, again demonstrating no sensitization by the Pgp antagonist (data not shown).
Fig. 5  Effect of bryostatin 1 on Pgp phosphorylation in MCF-7TH cells. Cells were treated for 24 h with 10 pm or 100 nm of bryostatin 1 or 200 nm TPA prior to metabolic labeling with 250 μCi [32P]P, for 90 min. Next, immunoprecipitation was carried out with a polyclonal antibody against recombinant protein fragments of Pgp. Similar results were obtained in three independent experiments.

To study the effect of bryostatin 1 on Pgp function, drug accumulation studies were performed in MCF-7TH cells, as shown in Fig. 3. Previous studies described an increase in vinblastine accumulation to 300% of control in MCF-7TH cells after treatment with calphostin C, a PKC inhibitor (16). As shown in Fig. 3, treatment of MCF-7TH cells with either 200 nm TPA or 100 nm bryostatin 1 decreased [3H]vinblastine accumulation 10–30% after 15 min. Treatment with TPA for 72 h increased vinblastine accumulation to 160% of control, which is suggestive of PKC inhibition. However, 100 nm bryostatin 1 did not significantly affect [3H]vinblastine accumulation after a 24- or 72-h treatment. Likewise, no effect of 10 pm bryostatin 1 on vinblastine accumulation could be observed (data not shown). For comparison, vinblastine accumulation in the presence of verapamil and after staurosporine treatment is shown (Fig. 3, inset). Verapamil increased vinblastine accumulation to 700% of control, while staurosporine, a nonspecific PKC inhibitor, increased vinblastine accumulation to 350% of control. Thus, neither TPA nor bryostatin 1 increased vinblastine accumulation as expected. Accumulation experiments with [3H]daunomycin, [3H]colchicine, [3H]vinblastine, and rhodamine 123 also showed no appreciable effect of treatment with either bryostatin 1 or TPA (Fig. 4). No significant effect of bryostatin 1 was observed in vinblastine accumulation in MCF-7 parental cells (data not shown).

We asked whether Pgp dephosphorylation and PKC-α down-regulation were occurring under the conditions used in the vinblastine accumulation assay. Cells were treated with 10 pm and 100 nm bryostatin 1 for 24 h prior to labeling with [32P]P, at 37°C for 90 min in the presence of bryostatin 1. Immunoprecipitation of the labeled Pgp is shown in Fig. 5, with densitometric quantitation in the lower panel. There is a significant reduction in Pgp phosphorylation in cells treated with 100 nm bryostatin 1 for 24 h, while a slight reduction is also detected after a 10 pm treatment. Pgp phosphorylation is also reduced by 24-h treatment with 200 nm TPA. Since both Pgp dephosphorylation and PKC-α down-regulation result from bryostatin 1 treatment, we sought an explanation for the failure to see an effect on drug accumulation and examined the possibility that increased mdr-1 expression results from bryostatin I treatment. Northern blot analysis was performed following bryostatin 1 treatment of MCF-7TH cells; mdr-1 expression was not regulated (data not shown). Thus, up-regulation of Pgp expression cannot explain the failure of bryostatin 1 to modulate Pgp activity.

The effect of bryostatin 1 on the PKC isoforms in MCF-7TH was compared to the previously reported results with TPA in human multidrug-resistant breast cancer cells (7, 19). As shown in Fig. 6, in the MCF-7TH cells PKC-α was translocated by TPA after a 10-min treatment and was still in the active form after a 1-h treatment as demonstrated by a shift in signal from the supernatant to the particulate fraction, representing translocation from the cytosol to the cell membrane. After 24 h, the level of PKC-α was reduced in both cytosolic and membrane fractions, consistent with down-regulation of this isoform. Bryostatin I treatment with 100 nm showed a weaker effect compared to TPA in PKC-α translocation. A partial shift was detected after a 10-min treatment, and the shift from the cytosol to the membrane was still not complete at 1 h of treatment. Subsequently, PKC-α was completely down-regulated, as shown at
the 24-h time point. Modulation of the other two detectable isoforms of PKC, ε and ζ, was also evaluated in MCF-7TH cells. As shown in Fig. 6, PKC-ε appeared to be partially down-regulated by both agents at 24 h, whereas no significant effect on PKC-ζ was observed (the slight differences observed in Fig. 6 are experimental vari-ations). These results demonstrate that PKC-α in MCF-7TH responded to both TPA and bryostatin 1, but a more pronounced effect was detected in TPA-treated cells compared to bryostatin 1-treated cells. Since differential results have been observed with lower doses of bryostatin 1 in other model systems (20, 37, 38), we next examined the effect of 10 μM bryostatin 1 in both MCF-7 and MCF-7TH cells. Bryostatin 1 at 10 μM failed to down-regulate PKC-α levels after 24-h treatment in contrast to 100 nM bryostatin 1 (Fig. 7). Vinblastine accumulation performed with 10 μM bryostatin 1 also demonstrated no significant difference in accumulation after 15 min, 1 h, or 24 h of treatment (data not shown).

**DISCUSSION**

The present study reports the effect of bryostatin 1, a PKC activator and inhibitor, on drug transport and Pgp phosphorylation in multidrug-resistant human breast cancer cells. Bryostatin 1 was unable to increase rhodamine or vinblastine accumulation in MCF-7TH cells, although it did reduce Pgp phosphorylation. PKC-α, which is overexpressed in MCF-7TH cells relative to the parental MCF-7 cells, was effectively down-regulated by bryostatin 1. Partial down-regulation of PKC-ε was also observed, with no major effect on PKC-ζ. Thus, although the PKC isoforms responded to bryostatin 1 as expected, no effect on Pgp function could be measured.

The lack of an effect on Pgp function was unexpected, since a role for PKC-α in stimulating Pgp activity has been suggested in previous reports. Increased PKC-α expression has been described in other MCF-7 doxorubicin-resistant cells, a KB cell line, and multidrug-resistant murine cell lines including P388, murine fibrosarcoma cells, and UV-2237 (19, 39–42). Blöbe et al. (19) reported a 10-fold increase in total PKC activity in multidrug-resistant MCF-7 cells with increased phosphorylation following TPA treatment. Ahmad and Glazer (43) reported that transfection of MCF-7Adr cells with an expression vector containing the cDNA for PKC-α in the antisense orientation inhibited PKC-α, which then sensitized the cells to chemotherapeutic agents. These studies documented an overexpression of PKC-α in multidrug-resistant cells and raised the possibility that chemosensitivity could be modified through PKC-α inhibition.

The lack of an effect on drug accumulation in the MCF-7TH subline was also unexpected in view of observations showing decreased Pgp function and increased drug accumulation following treatment of MCF-7TH cells with both calphostin C and staurosporine. Similarly, bryostatin 1 had no effect on drug accumulation in a multidrug-resistant subline of SW620 human colon cancer cells (data not shown).

Bryostatin 1 and TPA treatment effects on the various PKC isoforms were comparable and similar to findings in previously published reports (21, 44–46). Both induced an early translo-
translocation of PKC-\(\alpha\) from the cytosol to the membrane in MCF-7TH cells, although bryostatin 1 activation of PKC-\(\alpha\) was less pronounced than that of TPA and both resulted in down-regulation by 24 h. Identical results were observed in a multidrug-resistant human colon carcinoma subline (data not shown) which does not overexpress PKC-\(\alpha\) compared to parental cells as do the multidrug-resistant MCF-7TH cells reported here.

TPA and bryostatin 1 induced a partial down-regulation of PKC-\(\epsilon\), and had no obvious effect on PKC-\(\zeta\). This was also consistent with previous reports showing partial down-regulation of PKC-\(\epsilon\) in response to TPA or bryostatin 1 treatment of NIH3T3 cells, mouse keratinocytes, and SH-SY5Y neuroblastoma cells (21, 46),2 while neither translocation nor down-regulation of PKC-\(\zeta\) was observed in NIH3T3 mouse fibroblast cells, platelets, and murine epidermis (21, 47–49).

The present results contrast with previous observations showing that decreased Pgp phosphorylation is associated with decreased drug transport (2, 3, 16, 33). Decreased Pgp phosphorylation and impaired vinblastine transport were seen after sodium butyrate treatment of SW620 human colon cancer cells, although colchicine transport was preserved (33). Similar findings were observed in SW620Ad300 cells treated with calphostin C, a PKC inhibitor which impaired vinblastine while increasing verapamil transport (16). We now report a decrease in Pgp phosphorylation following bryostatin 1 treatment accompanied by unaffected vinblastine, rhodamine, and daunomycin transport. These discrepancies may be explained by a differential effect of these agents on distinct sites of phosphorylation by several kinases which may include but are not limited to the PKC family. These data suggest that kinases other than PKC may be more important in regulating Pgp. Taken together, these data suggest that Pgp phosphorylation modulates drug binding and affinity and resulting transport, rather than having a direct effect on Pgp function.

Our results show that bryostatin 1 down-regulates PKC-\(\alpha\) and reduces Pgp phosphorylation in MCF-7TH multidrug-resistant cells overexpressing PKC-\(\alpha\), without an effect on drug transport. Although bryostatin 1 appears to be clinically well tolerated and may have a role as an antineoplastic agent, based on these observations, its use clinically to modulate Pgp-mediated multidrug resistance cannot be recommended.

**REFERENCES**


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