Partial Reversal of Multidrug Resistance in Human Breast Cancer Cells by an N-Myristoylated Protein Kinase C-α Pseudosubstrate Peptide*

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The predominant characteristics of multidrug resistant (MDR) cancer cells are broad spectrum resistance to chemotherapeutic agents and a pronounced defect in intracellular accumulation of the drugs, in association with overexpression of the drug efflux pump P-glycoprotein. Protein kinase C (PKC) phosphorylates the linker region of P-glycoprotein. Evidence has been presented that the isozyme PKC-α may contribute to the drug resistance phenotype of human breast cancer MCF7-MDR cells. PKC-α is markedly overexpressed in MCF7-MDR cells, and artificial overexpression of PKC-α in MCF7 constructs that overexpress P-glycoprotein significantly enhances the MDR phenotype of the cells in association with increased P-glycoprotein phosphorylation. Verapamil, cyclosporin A, and a number of other agents that compete with cytotoxic drugs for binding sites on P-glycoprotein can potently reverse MDR, but this is accompanied by severe toxicity in vivo. In this report, we demonstrate that an N-myristoylated peptide that contains a sequence corresponding to the pseudosubstrate region of PKC-α (P1) partially reverses multidrug resistance in MCF7-MDR cells by a novel mechanism that involves inhibition of PKC-α. P1 and two related PKC inhibitory N-myristoylated peptides restored intracellular accumulation of chemotherapeutic drugs in association with inhibition of the phosphorylation of three PKC-α substrates in MCF7-MDR cells: PKC-α, Raf-1 kinase, and P-glycoprotein. A fourth N-myristoylated peptide substrate analog of PKC, P7, did not affect drug accumulation in the MCF7-MDR cells and failed to inhibit the phosphorylation of the PKC-α substrates. The effects of P1 and verapamil on drug accumulation in MCF7-MDR cells were additive. P1 did not affect P-glycoprotein expression. MCF7-MDR cells were not cross-resistant to P1, which suggests that the peptide was not transported by P-glycoprotein. Furthermore, P1 was distinguished from MDR reversal agents such as verapamil and cyclosporin A by its inability to inhibit [3H]jodizopine photoaffinity labeling of P-glycoprotein. P1 actually increased [3H]jodizopine photoaffinity labeling of P-glycoprotein in MCF7-MDR cells, providing evidence that the effects of P1 on P-glycoprotein in MCF7-MDR cells are not restricted to inhibition of the phosphorylation of the pump. P1 may provide a basis for developing a new generation of MDR reversal agents that function by a novel mechanism that involves inhibition of PKC-α-catalyzed P-glycoprotein phosphorylation.

Resistance to chemotherapy is a major obstacle to successful cancer treatment, and it often accounts for the failure of aggressive chemotherapy to eradicate malignant disease (1). Most metastatic cancers are either innately resistant to chemotherapy or acquire drug resistance during the course of chemotherapy (1). Multidrug resistant (MDR) cancer cells are characterized by broad spectrum resistance to chemotherapeutic drugs, markedly reduced intracellular accumulation of the drugs, and overexpression of the drug efflux pump P-glycoprotein (1, 2). The relevance of MDR to clinical drug resistance in cancer therapy is indicated by the abundant expression of P-glycoprotein and its message mdr1 in specimens of human cancer that are intrinsically resistant to chemotherapy and in malignant tumors from patients who have relapsed during or after chemotherapy (1, 3, 4).

Protein kinase C (PKC) is an isozyme family with at least ten mammalian members (5). Highly selective phorbol-ester PKC activators induce resistance in cancer cells to multiple cytotoxic drugs that are P-glycoprotein substrates in association with a sharp reduction in the intracellular accumulation of the drugs, providing evidence that PKC activation contributes to MDR. The phorbol-ester effects on chemosensitivity and drug accumulation have been observed in several drug-sensitive and MDR cancer cell lines including human breast cancer MCF7-WT and MCF7-MDR, and the magnitude of the effect is generally a 2–6-fold increase in the IC50 values of cytotoxic drugs (6–12). PKC phosphorylates the linker region of P-glycoprotein in human KB-V1 cancer cells (9, 13, 14), and this is tightly coupled to the effects of PKC on intracellular drug accumulation and MDR, providing evidence that PKC regulates the function of P-glycoprotein in this system (11). The isozyme PKC-α has been shown to be overexpressed in several MDR cancer cell lines including MCF7-MDR (8, 15–18). Evidence that PKC-α activation contributes to MDR has been provided by observations that MDR is induced by the selective activation of PKC-α in human colon cancer cells (12) and by transfection of an mdr1-transfected human breast cancer MCF7 subline with PKC-α (19).

Verapamil, cyclosporin A, and related MDR reversal agents

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1 The abbreviations used are: MDR, multidrug-resistant or multidrug resistance; ADR, Adriamycin; 5FU, 5-fluouracil; PKC, protein kinase C; VLB, vinblastine; VP, verapamil; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAb, monoclonal antibody; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
that compete with chemotherapeutic drugs for binding sites on P-glycoprotein (20–23) generally cause severe toxicity in vivo at therapeutic concentrations, and this precludes their use in the treatment of drug-resistant cancer (24, 25). In a previous report, we characterized the mechanism of PKC inhibition by an N-myristoylated peptide corresponding to the autoinhibitory pseudosubstrate sequence of PKC-α (P1) that had been shown to selectively inhibit PKC in human fibroblasts (26, 27).

In this report, we show that the peptide P1 reverses MDR by a novel mechanism that is associated with a sharp increase in intracellular drug accumulation and inhibition of the phosphorylation of P-glycoprotein and two other PKC-α substrates, Raf-1 kinase and PKC-α itself. P1 was distinguished from verapamil and related MDR reversal agents (20–23) in that it did not inhibit photoaffinity labeling of drug-binding sites on P-glycoprotein by [3H]lazidopine. P1 did not affect P-glycoprotein expression in MCF7-MDR cells. MCF7-MDR cells were not cross-resistant to P1, providing indirect evidence that the peptide was not transported by P-glycoprotein. The N-myristoylated PKC-α pseudosubstrate peptide P1 may be a valuable starting point for developing a new generation of MDR reversal agents that function by a novel mechanism that involves inhibition of PKC-α-catalyzed P-glycoprotein phosphorylation.

### MATERIALS AND METHODS

#### Cell Lines

The drug-sensitive wild-type human breast cancer cell line MCF7-WT and the MDR subline MCF7-MDR were supplied by Dr. Kenneth H. Cowan (National Cancer Institute). The MCF7-MDR cells were derived from the parental line MCF7-WT by serial passage with increasing ADR concentrations and are maintained in the presence of 1 μg/ml ADR. The MCF7-MDR subline is >100-fold resistant to ADR, vincristine, and VLB (18). MCF7-MDR cells were grown in the absence of ADR for at least two passages prior to their use in experiments. Cells were maintained in Eagle’s minimum essential medium containing 5% heat-inactivated fetal calf serum, nonessential amino acids, vitamins, sodium pyruvate, L-glutamine, and penicillin-streptomycin.

#### Synthetic Peptides

The peptides were synthesized using the VEGAS peptide synthesizer at the M.D. Anderson Cancer Center Synthetic Antigen Facility.

#### Drug Accumulation and Efflux Assays

Drug accumulation was measured by an established method (10, 12). Direct growth-inhibitory effects of the peptides against MCF7-WT and MCF7-MDR were measured by a modification of this procedure, in which the cells were exposed to the peptides for the entire 96-h growth inhibition assay.

#### P-glycoprotein Immunoprecipitation

P-glycoprotein immunoprecipitation was performed using a modification of previous methods (18). MCF7-MDR cells were determined using established methods (18, 29). Nearly confluent MCF7 cells grown in 75-cm² flasks were labeled with [32P]Pi and scraped from the plates with lysis buffer (50 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 5 mM EGTA, and 5 mM β-mercaptoethanol) (1 ml/plate) and incubated at 37°C for 15 min, lysates were spun at 13,800 × g for 15 min, and supernatants were collected.

#### Western Blot Analysis

Western blot analyses were performed using 10 μg of protein as described previously. 

#### Drug and Reagents—[3H]ATP (30 Ci/mmol), [3H]2-DG (200 Ci/mmol), [3H]Adriniamycin (55 Ci/mmol), [3H]Lazidopine (49 Ci/mmol), and [3H]vinblastine sulfate (11 Ci/mmol) were purchased from Amer sham Corp. [3H]5-Fluorouracil (14 Ci/mmol) was obtained from Dupont NEN. The P-glycoprotein monoclonal antibody C219 was obtained from Signet Laboratories (Dedham MA), adriamycin and vinblastine sulfate were from Cetus Laboratories (Emeryville CA), S-fluorouracil was from SdoPak (Franklin Park, IL), and protein assay solution and SDS-PAGE reagents were from Bio-Rad Laboratories. Tissue culture reagents were purchased from Life Technologies, Inc. Proteinase inhibitors, tetratcinol, myristic acid, N,N-dimethylmyristamide, verapamil, protein A-Sepharose, and all other reagents were obtained from Sigma. Purified rat brain PKC was prepared from rat brains, and its Ca²⁺- and phosphatidylserine-stimulated histone kinase activity was assayed as described previously (28).

### Drug Accumulation and Efflux Assays

The intracellular accumulation of cytotoxic drugs was measured by an established method (10, 12). Stock solutions of 50 μM [3H]ADR, 23 μM [3H]2-DG, and 70 μM [3H]5FU were diluted before use in tissue culture medium containing 10 mM HEPES (pH 7.3). Drug stocks were made up to 10 mM. MCF7-MDR cells were plated at a density of 5 × 10⁵ cells/well. Following a 20–24-h attachment period at 37°C, cells were preincubated with the peptides under investigation at indicated concentrations for 30 min at 37°C. Treatment with buffer alone served as a negative control, and treatment with 10 μM verapamil was used as a positive control. Next, the treated cells were incubated at 37°C with radiolabeled cytotoxic drug ([3H]ADR, [3H]2-DG, or [3H]5FU) in the presence of the peptides (or controls) for the duration of the drug accumulation period (5 min to 6 h). The drug accumulation assay was terminated by rapidly washing the cells three times with ice-cold PBS, detaching the cells by a 30-min exposure to trypsin-EDTA at 37°C, harvesting the cells, and counting them in vials containing 15 ml of scintillation fluid. To measure net drug efflux rates, MCF7-MDR cells were preloaded with 0.2 μM [3H]ADR for 2 h, washed rapidly with 15 ml PBS four times, and reincubated at 37°C in the presence of N-myristoylated peptides, verapamil, or medium alone. At the indicated time intervals, the incubation was stopped and the radioactivity released into the medium was counted, as described previously (10).

### Growth Inhibition Assay

The reduction in viable cell number affected by a 24-h exposure to cytotoxic drugs (ADR, VLB, and 5FU) was measured as described previously using a tetrazolium dye (12). Cells were harvested in their exponential growth phase, and single cell suspensions with a viability of >95% were seeded into 96-well microculture plates at a density of 3,000 MCF7-WT or 4,500 MCF7-MDR cells/well. Following a 24–24-h attachment period, cells were preincubated for 1 h at 37°C with either the synthetic peptide under investigation or buffer (which served as a vehicle control). Next, the peptide-containing medium was removed, and the cells were incubated with either medium alone or medium containing cytotoxic drugs (ADR, VLB, and 5FU) for 24 h at 37°C. Drug-containing medium was then removed from the cells, fresh medium was applied, and the cells were further incubated for 72 h. At the end of the incubation period, 40 μl of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (2.5 mg/ml VLB) was pipetted into each well and allowed to react for 12 h. Unreacted dye and medium were removed from the wells, 100 μl of dimethyl sulfoxide was added to each well, and the conversion of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan by viable cells was quantitated by measuring the absorbance at 570 nm with a microplate-scanning spectrophotometer (10, 12). 

### Reporter Gene Assay

The reporter gene assay was performed as described previously (20), with the following modifications. 

African green monkey kidney (293) cells were transfected with plasmid DNA using FuGENE 6 reagent (Boehringer Mannheim). Cells were incubated for 20 h at 37°C prior to the addition of protein A-Sepharose to the cell lysate, and the cell lysate was incubated for an additional 2 h at 37°C prior to centrifugation. The supernatant was then removed and subjected to SDS-PAGE. Calf intestinal alkaline phosphatase activity was determined as described previously (20).

### Immunoprecipitation

PKC-α was immunoprecipitated from MCF7 cell lysates as described previously (26, 27).
that corresponds to a PKC phosphorylation site in P-glycoprotein does not inhibit PKC-catalyzed histone phosphorylation. Analogs of P2, P4, and P6, respectively. Each of these sequence of the pseudosubstrate region of PKC and growth-inhibitory activity of cytotoxic anticancer drugs in mammalian cells (26, 31, 32) provides indirect evidence that the peptides enter mammalian cells, which is consistent with their membrane-active nature (27). P5 partially reverses adriamycin resistance in murine fibrosarcoma cells (33). Specific activation of PKC is correlated with enhancement of MDR in tumor cells, and inhibition of the enzyme is often associated with partial reversal of MDR (10, 11). We hypothesized that P1 and related PKC-inhibitory N-myristoylated peptides might antagonize MDR in human breast cancer MCF7-MDR cells.

Table I shows the structures of the peptides under investigation for modulatory effects on the intracellular accumulation and growth-inhibitory activity of cytotoxic anticancer drugs in human breast cancer MCF7-MDR cells. P2 contains the core sequence of the pseudosubstrate region of PKC-α (PKC(20-28)), P4 contains a more extensive PKC-α pseudosubstrate sequence (PKC(19-31)) (26, 27), and P6 contains the sequence of the PKC phosphorylation site of the epidermal growth factor receptor at Thr654 (27). P1, P3, and P5 are N-myristoylated analogs of P2, P4, and P6, respectively. Each of these N-myristoylated peptides inhibits the histone kinase reaction of purified PKC (Table I); the corresponding nonmyristoylated peptides do not inhibit PKC-catalyzed histone phosphorylation (Table I). P7 is an N-myristoylated peptide with a sequence that corresponds to a PKC phosphorylation site in P-glycoprotein (residues 656–666) (13). Although P7 does not inhibit the histone kinase activity of PKC, it is weaker than P1, P3, and P5 in this regard, and, at concentrations ≤10 μM, P7 actually enhanced the accumulation of [14C]ADR (Fig. 1A) and [3H]VLB accumulation in MCF7-MDR cells were enhanced by 5- and 10-fold higher than those in MCF7-MDR. The enhancement of drug uptake was most pronounced when the N-myristoylated peptide concentration was 100 μM. However, at a concentration of 50 μM, P1 and P3 each significantly enhanced [14C]ADR uptake, and P1 and P5 significantly enhanced [3H]VLB uptake in the MCF7-MDR cells (Fig. 1, A and B). In parallel experiments, we found that like VP, P1–P6 did not significantly affect [3H]SFU accumulation in MCF7-MDR cells (Fig. 1C). This demonstrates a degree of specificity in the effects of the N-myristoylated peptides on drug accumulation in MCF7-MDR cells. [14C]ADR and [3H]VLB accumulation in MCF7-MDR cells were enhanced by less than 5% by myristic acid (25–100 μM) and by N,N-dimethylmyristamide (25–100 μM), which is a nonpeptidic amide of myristic acid. Thus, specificity is also indicated by the lack of effect of the acyl head group of the peptides on [14C]ADR and [3H]VLB accumulation. In addition, P7 (25–100 μM) failed to enhance [14C]ADR and [3H]VLB accumulation in the cells, indicating that not all N-myristoylated cationic peptides can enhance the retention of these cytotoxic drugs in MCF7-MDR cells.

Table II shows that P1–P6 increased [14C]ADR and [3H]VLB accumulation in the drug-sensitive cell line MCF7-WT only marginally (≤1.8-fold) (P2, P3, P4) or not at all (P1, P5, P6), and they had no detectable effect on [3H]SFU accumulation in the cells. Of the N-myristoylated peptides, only P3 detectably increased [14C]ADR and [3H]VLB accumulation in the MCF7-WT cells (Table II). Thus, the marked enhancement of [14C]ADR and [3H]VLB uptake achieved by the N-myristoy-
Effects of synthetic peptide substrate analogs of PKC on cytotoxic drug accumulation in MCF7-MDR cells. Effects of the N-myristoylated peptides P1, P3, and P5 and the corresponding nonmyristoylated peptides P2, P4, and P6 at concentrations of 25, 50, and 100 μM on drug accumulation in MCF7-MDR cells are shown. Drug accumulation was assayed as described under "Materials and Methods." Under each experimental condition examined in A–C, >95% cell viability was observed at the end of the drug accumulation period by trypan blue exclusion. Each experimental value shown is the average value from three experiments that were done in triplicate. Values that differ significantly from the untreated control are identified by asterisks. **, p < 0.015; *, p < 0.05. A, [14C]ADR accumulation in MCF7-MDR cells is shown. % of Control, the amount of [14C]ADR accumulation observed in the presence of the indicated peptide expressed as a percentage of [14C]ADR accumulation observed in untreated control (CTRL) MCF7-MDR cells. Treatment with 10 μM VP served as a positive control. The [14C]ADR concentration was 0.1 μM, and accumulation was measured after a 2-h incubation period. Untreated cells retained 2.2 ± 0.2 pmol [14C]ADR/10^6 cells. B, [3H]VLB accumulation in MCF7-MDR cells is shown. Assays were done with 10 nM [3H]VLB, and accumulation was measured after incubating the cells with drugs for 30 min. Untreated MCF7-MDR cells retained 0.066 ± 0.005 pmol [3H]VLB/10^6 cells. For other details, see the description of A, C, [3H]5FU accumulation in MCF7-MDR cells is shown. Where indicated, peptides (P1–P6) were present at 100 μM; [3H]5FU was at 10 nM. Drug accumulation was measured after 30 min and 2 h, as shown. [3H]5FU accumulation in control (CTRL) cells was 0.051 ± 0.002 (30 min) and 0.058 ± 0.004 pmol [3H]5FU/10^6 cells (2 h). For other details, see the description of A.

| TABLE II | Effects of synthetic peptide substrate analogs of PKC on cytotoxic drug accumulation in MCF7-WT cells |
|-----------------|--------------------------------------------------|
| Drug uptake*    | [14C]ADR | [3H]VLB | [3H]5FU |
| VP              | 93 ± 13  | 124 ± 13 | 97 ± 12  |
| P1              | 99 ± 14  | 115 ± 13 | 92 ± 5   |
| P2              | 95 ± 5   | 160 ± 9  | 97 ± 7   |
| P3              | 173 ± 13 | 153 ± 6  | 102 ± 1  |
| P4              | 136 ± 20 | 137 ± 24 | 103 ± 6  |
| P5              | 104 ± 6  | 107 ± 10 | 97 ± 2   |
| P6              | 98 ± 3   | 121 ± 10 | 93 ± 2   |

* Drug uptake is expressed as a percentage of the uptake observed in untreated MCF7-WT cells. Drug uptake was measured as described under "Materials and Methods.""
which was comparable with the decline of 19 ± 2% observed with 10 μM verapamil.

At a concentration of 50 μM, the N-myristoylated pseudosubstrate peptide P1 significantly enhanced [14C]ADR and [3H]VLB accumulation in the MCF7-MDR cells, but in each case the degree of enhancement was very modest when compared with 10 μM VP (Fig. 1). Likewise, at concentrations of 2.5 μM, VP had little effect on [14C]ADR accumulation (Table III). In an attempt to demonstrate efficacy of P1 at concentrations of 50 μM, we measured the enhancement of [14C]ADR uptake in MCF7-MDR cells by combinations of P1 (50 μM) and VP (2.5 μM). We found that 2.5 μM VP in combination with 25 μM P1 enhanced [14C]ADR accumulation in MCF7-MDR cells approximately as effectively as 10 μM VP, and the degree of enhancement was significantly greater than that achieved by either 25 μM P1 or 2.5 μM VP alone (Table III). We observed a similar but more marked statistically significant effect by 50 μM P1 in combination with 2.5 μM VP (Table III). These results demonstrate that the effects of P1 and VP on drug accumulation in MCF7-MDR cells are additive, and they show that P1 can modulate drug accumulation in the MDR breast cancer cells at concentrations as low as 25 μM. (5 μM P1 was ineffective in combination with VP in enhancing [14C]ADR accumulation) (data not shown).

The restoration of [14C]ADR and [3H]VLB accumulation by the N-myristoylated pseudosubstrate peptide P1 in MCF7-MDR cells suggested that the peptide might also sensitize MCF7-MDR cells to the cytotoxic drugs. To test this, we measured the effect of a 1-h exposure to P1 on MCF7-MDR cell growth using a 96-h assay system and found that under these conditions, P1 was not growth-inhibitory (Fig. 3). Thus, P1 could be tested for MDR reversal activity in this system under conditions where it potently stimulated drug accumulation in MCF7-MDR cells (Fig. 2, A and C). Under these conditions, P1 significantly reduced the ADR concentration required for 50% MCF7-MDR cell growth inhibition (IC50) approximately 2-fold, from a value of 26.33 ± 0.40 μg/ml to 14.11 ± 0.41 μg/ml (p < 0.001, n = 6). Representative results obtained in one experiment are shown in Fig. 3A. Similarly, the IC50 of VLB was reduced by P1 approximately 2-fold in MCF7-MDR cells, from

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Effects of synthetic peptide substrate analogs of PKC on the kinetics of net drug uptake in MCF7-MDR cells. Intracellular accumulation of [14C]ADR (A and B) and [3H]VLB (C and D) was measured in MCF7-MDR cells at the indicated time intervals. Where specified, assays were done in the presence of 100 μM peptide (A and C, P1 and P2; B and D, P3, P4, P5, and P6) or 10 μM VP (A and C). In these experiments, cell viability was >95% according to trypan blue exclusion measurements. For definition of % Control, assay conditions, and other experimental details, see the legend to Fig. 1 and "Materials and Methods." Each experimental value represents an average of triplicate determinations, and the results shown were determined to be reproducible in a duplicate experiment.

| [P1] | [VP] | Statistical significance |
|------|------|--------------------------|
| μM   | μM   | [14C]ADR uptake*         |
| 25   | 0.5  | 145 ± 21                 |
| 25   | 2.5  | 212 ± 12                 |
| 50   | 0.5  | 189 ± 31                 |
| 25   | 2.5  | 317 ± 15                 |
| 25   | 0    | 131 ± 15                 |
| 50   | 0    | 153 ± 4                  |
| 0    | 0.5  | 107 ± 10                 |
| 0    | 2.5  | 136 ± 15                 |
| 0    | 10   | 236 ± 16                 |
| 0    | 0    | 100 ± 8                  |

* [14C]ADR uptake is expressed as the percentage of Control, i.e., as the percentage of [14C]ADR uptake observed in untreated MCF7-MDR cells. For experimental details, see "Materials and Methods" and the legend to Fig. 1.

* P values are indicated wherever the [14C]ADR uptake observed with a combination of P1 and VP was significantly different from the uptake observed both with P1 alone and with VP alone at equivalent concentrations.

* n.s., not significant.

* n.a., not applicable. The results shown are an average of four experiments done in triplicate.
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2.56 ± 0.32 μg/ml to 1.18 ± 0.08 μg/ml VLB (p < 0.02, n = 5); representative data are shown in Fig. 3B. In contrast, P1 was without effect on 5FU cytotoxicity in MCF7-MDR cells. Fig. 3C shows that the percentage of MCF7-MDR cell growth inhibition achieved by 5FU was approximately the same in the presence (open circles) and in the absence of P1 (closed circles); the results shown are an average of three experiments. Taken together with the effects of P1 on drug accumulation in MCF7-MDR cells (Figs. 1 and 2), these results provide evidence that P1-mediated chemosensitization of MCF7-MDR cells involves inhibition of P-glycoprotein-mediated drug transport.

We next tested whether restoration of drug accumulation in MCF7-MDR cells by P1 and related N-myristoylated PKC-inhibitory peptides was associated with inhibition of P-glycoprotein phosphorylation. Fig. 4A shows that exposure of MCF7-MDR cells to P1 (25–100 μM) during a 3-h 32P-labeling period was associated with a concentration-dependent inhibition of P-glycoprotein phosphorylation; maximal inhibition was achieved with 100 μM P1. Similarly, P3 and P5 inhibited P-glycoprotein phosphorylation in MCF7-MDR cells, although P5 was considerably less effective than either P1 or P3 in this respect (Table IV). In contrast, the effects of the nonmyristoylated peptides P2, P4, P6, and the N-myristoylated peptide P7 on P-glycoprotein phosphorylation in MCF7-MDR cells were negligible (Table IV). In control experiments, we determined by immunoblot analysis of P-glycoprotein with C219 mAb that P1, P3, and P5 had only minor or negligible effects on P-glycoprotein expression under the conditions of the P-glycoprotein phosphorylation experiments (Fig. 4B; Table IV).

To determine whether the PKC-inhibitory effects of the N-myristoylated peptides in MCF7-MDR cells were restricted to integral membrane protein PKC substrates such as P-glycoprotein, we examined the phosphorylation state of the PKC substrate Raf-1 kinase (34), which shuttles between the cytoplasmic compartment and the plasma membrane of mammalian cells (35). Raf-1 kinase has been implicated in MDR (36), and it plays a pivotal role in PKC-α-mediated signal transduction (34). Activation of cellular PKC has been linked to Raf-1 kinase activation (37). Raf-1 kinase phosphorylation was analyzed by immunoprecipitation of the protein from lysates of 32P-labeled cells with a Raf-1 kinase-specific mAb. We found that the phosphorylation of Raf-1 kinase was at least 10 times greater in the MCF7-MDR line compared with the drug-sensitive line MCF7-WT (Fig. 5A, lanes F and G), when the phosphorylation data in Fig. 5A were subjected to densitometric analysis and then normalized for the approximately 2-fold increase in Raf-1 kinase expression that we observed in...
the MDR line by Western analysis (Fig. 5B). This increase in the phosphorylation of the PKC-\(\alpha\) substrate Raf-1 kinase in MCF7-MDR is consistent with the reported 30-fold overexpression of PKC-\(\alpha\) in the cells (18). As in the case of P-glycoprotein phosphorylation, 100 \(\mu\)M P1, P3, and P5 each potently inhibited Raf-1 kinase phosphorylation in the MCF7-MDR cells, whereas 100 \(\mu\)M P7 was noninhibitory (Fig. 5A, lanes B–E). In contrast, the peptides had little or no effect on Raf-1 kinase expression in MCF7-MDR cells, according to immunoblot analysis of cell lysates (Fig. 5B). Densitometric analysis revealed that 100 \(\mu\)M P3 and P5 each inhibited Raf-1 kinase phosphorylation in MCF7-MDR cells by more than 95\% (Fig. 5A, lanes C and D), and 200 \(\mu\)M P1 achieved a similar degree of inhibition (Fig. 5A, lane A). The inhibition of Raf-1 kinase phosphorylation achieved by 100 \(\mu\)M P1 was approximately 80\% (Fig. 5A, lane B).

**TABLE IV**

Inhibition of P-glycoprotein phosphorylation by synthetic peptide substrate analogs of PKC.

| Peptide (a) | % P-glycoprotein phosphorylation (b) | % P-glycoprotein expression (c) |
|------------|--------------------------------------|---------------------------------|
| None       | 100                                  | 100                             |
| P1         | 15 ± 3                               | 100 ± 3                         |
| P2         | 79 ± 4                               | n.d.                            |
| P3         | 17 ± 4                               | 79 ± 5                          |
| P4         | 114 ± 14                             | n.d.                            |
| P5         | 59 ± 2                               | 86 ± 6                          |
| P7         | 115 ± 1                              | n.d.                            |
| MCF7-WT cells | <5                                  | <5                              |

(a) All peptides were present at 100 \(\mu\)M.

(b) Unless otherwise indicated, the cell line employed was MCF7-MDR.

(c) Conditions for exposure of MCF7-MDR cells to peptides were the same as for measurements of P-glycoprotein phosphorylation and P-glycoprotein expression. Immunoblot analysis of P-glycoprotein expression was done with 500 ng/ml C219 mAb, and the resultant band at 160 kDa was quantitated by computerized densitometric analysis. For further details, see "Materials and Methods." Where indicated, MCF7-MDR cells were treated with synthetic peptides (P1, P3, P5, and P7) for the duration of the labeling period. \(\text{[}\text{P}\text{]}\text{32P}\) labeled cells were lysed with detergent, and PKC-\(\alpha\) was immunoprecipitated from the cell lysates, as described under "Materials and Methods." Immunoprecipitated PKC-\(\alpha\) was detected by SDS-PAGE and autoradiography of the gel; the autoradiogram was subjected to densitometric analysis. Results are shown for MCF7-WT (lane A), MCF7-MDR treated with 100 \(\mu\)M P1 (lane B), 100 \(\mu\)M P3 (lane C), 100 \(\mu\)M P5 (lane D), 100 \(\mu\)M P7 (lane E), and untreated MCF7-MDR (lane F). PKC-\(\alpha\) is the radiolabeled band migrating at 82 kDa. B, immunoblot analysis of PKC-\(\alpha\) expression in MCF7 cell lysates (50 \(\mu\)g of protein/lane) using 1 \(\mu\)g/ml PKC-\(\alpha\) monodonal antibody as the primary antibody (12) is shown in the upper panel; a control analysis done in the absence of the primary antibody is shown in the lower panel. The arrows indicate the positions of PKC-\(\alpha\) (82 kDa). Cells were treated with peptides as described for A. Lane 1, MCF7-WT; lane 2, MCF7-MDR; lane 3, P1-treated MCF7-MDR; lane 4, P3-treated MCF7-MDR; lane 5, P5-treated MCF7-MDR; lane 6, P7-treated MCF7-MDR.

PKC-\(\alpha\)-catalyzed Raf-1 kinase phosphorylation is preceded by PKC-\(\alpha\) activation and autophosphorylation (38, 39). To test whether the alterations in Raf-1 kinase phosphorylation shown in Fig. 5A were reflective of changes in PKC-\(\alpha\) activity, we analyzed the MCF7-WT and MDR cells for comparable changes in PKC-\(\alpha\) phosphorylation. Like Raf-1 kinase, PKC-\(\alpha\) is localized in the plasma membrane and the cytoplasmic compartment of mammalian cells (17, 40). Following a 3-h labeling period, a major 82-kDa radiolabeled band corresponding to phosphorylated PKC-\(\alpha\) was detected in \(\text{[}\text{P}\text{]}\text{32P}\)-labeled MCF7-MDR but not in MCF7-WT by immunoprecipitation of the protein from the cell lysates with a PKC-\(\alpha\)-specific mAb (Fig. 6A, lanes A and F). Similarly, PKC-\(\alpha\) was readily detected in an MCF7-MDR cell lysate but not in an MCF7-WT cell lysate by immunoblot analysis (Fig. 6B, lanes 1 and 2). Densitometric analysis of Fig. 6A showed that as in the case of Raf-1 kinase phosphorylation, PKC-\(\alpha\) phosphorylation was inhibited in MCF7-MDR cells >95\% by 100 \(\mu\)M P3 (lane C) and 100 \(\mu\)M P5 (lane D) and approximately 80\% by 100 \(\mu\)M P1 (lane B); 100 \(\mu\)M P7 affected <25\% inhibition of PKC-\(\alpha\) phosphorylation (lane E). The peptides had little or no effect on the expression of PKC-\(\alpha\) in MCF7-MDR cells under these experimental conditions according to immunoblot analysis of cell lysates (Fig. 6B). At 200 \(\mu\)M, P1 inhibited PKC-\(\alpha\) phosphorylation in MCF7-MDR cells >95\%, but P7 still achieved <25\% inhibition (data not shown). Thus, the N-myristoylated peptides P1, P3, and P5 potently inhibited the phosphorylation of three PKC-\(\alpha\) substrates (P-glycoprotein, Raf-1 kinase, and PKC-\(\alpha\)) in MCF7-MDR cells under conditions where the peptides restored intracellular drug accumulation, whereas the N-myristoylated peptide P7 inhibited the phosphorylation of these PKC-\(\alpha\) substrates very weakly or not at all and was without effect on the uptake of cytotoxic drugs in the MDR breast cancer cells. In most cases, the peptide concentration required for potent induction of drug uptake by P1, P3, and P5 (100 \(\mu\)M) (Fig. 1) caused >80\% inhibition of the phosphorylation of P-glycoprotein, Raf-1-kinase, and PKC-\(\alpha\) in the MCF7-MDR cells (Figs. 4–6; Table IV), suggesting that nearly complete inhibition of PKC-\(\alpha\) catalysis may be required for substantial reversal of MDR by the peptides.
Prenylcyotene methyl esters and several cyclic peptides have been shown to compete for drug-binding sites on P-glycoprotein in studies of photoaffinity labeling of the transporter with \(^{[3H]}\)azidopine (41, 42), and several hydrophobic linear peptides have been reported to serve as P-glycoprotein substrates (42-45). In contrast, the amphiphilic linear peptides melittin and alamethicin do not interact with P-glycoprotein (42). To test whether the mechanism of MDR reversal by the amphiphilic peptide P1 could involve direct interactions between P1 and drug-binding sites of P-glycoprotein in addition to inhibition of P-glycoprotein phosphorylation, we analyzed the effects of P1 and related peptides on the photoaffinity labeling of P-glycoprotein with \(^{[3H]}\)azidopine in MCF7-MDR cells. Results of photoaffinity labeling experiments done in whole MCF7 cells are shown in Fig. 7. A single prominent \(^{[3H]}\)azidopine-labeled band that corresponded to P-glycoprotein (160 kDa) was observed in the lane corresponding to untreated MCF7-MDR cells (lane B); the absence of this band in the MCF7-WT sample (lane A) confirmed its identity as P-glycoprotein. Exposure of the MCF7-MDR cells to vinblastine achieved about 50% inhibition of the labeling of P-glycoprotein according to densitometric analysis (lane C). Exposure to 100 \(\mu M\) P1, P3, and P5 (lanes D-F) had no inhibitory effect on photoaffinity labeling of P-glycoprotein in MCF7-MDR cells. In fact, the peptides actually enhanced the labeling of P-glycoprotein by \(^{[3H]}\)azidopine.

Comparisons of the chemosensitivities of MCF7-MDR and MCF7-WT cells to cytotoxic drugs have shown that the relative resistances of MCF7-MDR cells to the P-glycoprotein substrates ADR and VLB are, respectively, 610- and 360-fold (18). As a test of whether P1 could serve as a P-glycoprotein substrate in MCF7-MDR cells, we analyzed the MCF7-MDR cells for cross-resistance to P1 by comparing the growth-inhibitory activity of P1 and related peptides against MCF7-WT and MCF7-MDR cells. In these experiments, cells were exposed to the N-myristoylated peptides during the entire growth inhibition assay period (96 h; the 1-h exposure period employed in Fig. 3 was not used because it would have required very high peptide concentrations to achieve >50% cell growth inhibition). Although statistically significant cross-resistance was observed in MCF7-MDR cells with each peptide, the very modest degree of cross-resistance to the pseudosubstrate peptides P1 and P3 (<1.5-fold) (Table V) provided evidence that P1 and P3 did not serve effectively as P-glycoprotein substrates in the cells. In contrast, the cross-resistance of the cells to P5, which contains a sequence that corresponds to a PKC phosphorylation site in the EGF receptor, was pronounced (10-fold) (Table V), providing evidence that P5 may be transported by P-glycoprotein. Ideally, an MDR reversal agent should be equipotent against P-glycoprotein.

**Table V**

| IC\(_{50}\) | Fold resistance | P value |
|---------|----------------|---------|
| MCF7-WT | MCF7-MDR       |         |
| P1      | 29 ± 1         | 42 ± 3  | 1.45  | <0.05 |
| P3      | 48 ± 1         | 62 ± 3  | 1.29  | <0.05 |
| P5      | 5.9 ± 0.3      | 63 ± 3  | 10.68 | >0.01 |

**DISCUSSION**

Previous reports have shown that the isozyme PKC-\(\alpha\) is selectively overexpressed in human breast cancer MCF7-MDR cells (18), and artificial overexpression of PKC-\(\alpha\) in MCF7 constructs that overexpress P-glycoprotein increases the drug resistance of the cells in association with increased P-glycoprotein phosphorylation (19), providing evidence that PKC-\(\alpha\)-catalyzed P-glycoprotein phosphorylation may contribute to MDR in MCF7 cells. In this report, we demonstrate that an N-myristoylated PKC-\(\alpha\) pseudosubstrate peptide, N-myristoyl-FARKGLRQ(P1), partially reverses drug resistance in MCF7-MDR by a novel mechanism that involves PKC-\(\alpha\) inhibition. P1 induced cytotoxic drug accumulation in MCF7-MDR cells as effectively as the potent MDR reversal agent verapamil (Fig. 1) in association with potent inhibition of P-glycoprotein phosphorylation (Fig. 4). P1 also inhibited the phosphorylation of two other PKC-\(\alpha\) substrates in MCF7-MDR cells, Raf-1 kinase (Fig. 5), and PKC-\(\alpha\) (Fig. 6) under these conditions. Thus, induction of drug accumulation by P1 was associated with PKC-\(\alpha\) inhibition in MCF7-MDR cells. Based on the evidence described above (18, 19), that PKC-\(\alpha\)-catalyzed P-glycoprotein phosphorylation may be a contributing factor in the MDR phenotype of MCF7-MDR cells, it is evident that the mechanism of MDR reversal by P1 most likely involves inhibition of P-glycoprotein phosphorylation.

The mechanism of P1-mediated MDR reversal clearly does not involve competitive binding at \(^{[3H]}\)azidopine binding sites on P-glycoprotein (Fig. 7). This distinguishes P1 from MDR reversal agents such as PKC-inhibitory staurosporines (47, 48), verapamil, and cyclosporin A, which are highly effective MDR reversal agents in vitro but cannot be used to reverse MDR in vivo due to severe toxic effects at therapeutic concentrations (24, 47). It is also evident that MDR reversal by P1 does not involve altered P-glycoprotein expression and that it is not compromised by cross-resistance in the MDR cells (Fig. 4B, Table V). It should be noted that modulation of the ATPase...
activity of isolated P-glycoprotein and the [3H]VLB binding activity of P-glycoprotein-containing membrane vesicles by phospholipid-interacting peptides, such as melittin (42) and the N-myristoylated peptides described here (27), cannot be used to characterize interactions between the peptides and P-glycoprotein because of the pronounced nonspecific effects of phospholipid-interacting peptides in these assay systems (42). It is also worthwhile to note that because treatment of breast cancer patients with tamoxifen, which is PKC-inhibitory at therapeutic concentrations (49), is associated with little toxicity, it appears that PKC-inhibitory MDR reversal agents such as P1 could potentially give rise to a new generation of MDR reversal agents that are associated with acceptably low toxicity.

The inability of P1 to antagonize [3H]azidopine labeling of P-glycoprotein in MCF7-MDR cells and the lack of cross-resistance of MCF7-MDR cells to P1 suggest that P1 is not a P-glycoprotein substrate. However, these results do not exclude the possibility that linear myristoylated peptides such as P1 may interact with a P-glycoprotein site that is nonoverlapping and distinct from the azidopine binding site. In fact, the enhancement of [3H]azidopine labeling of P-glycoprotein by P1 is suggestive of such interactions. The enhanced labeling affected by P1 cannot be explained simply by the inhibitory activity of P1 against P-glycoprotein phosphorylation, because PKC-α-catalyzed P-glycoprotein phosphorylation increases azidopine-labeling of the pump (46). Nor can it be explained by the amphiphilicity of P1, because other linear amphiphilic peptides do not enhance azidopine labeling of P-glycoprotein (42).

In this study, we compared the effects of P1 and other N-myristoylated peptide-substrate analogs of PKC in MCF7-MDR cells. In general, the ability of the N-myristoylated peptide-substrate analogs to inhibit the phosphorylation of endogenous PKC-α substrates (P-glycoprotein, Raf-1 kinase, and PKC-α) in MCF7-MDR cells correlated with restoration of drug uptake in the cells by the peptides, i.e., P1, P3, and P5 were active, whereas P7 was inactive. Discrepancies within this general trend may be due to the pronounced cross-resistance of the MCF7-MDR cells to P5, effects of the N-myristoylated peptides on P-glycoprotein in addition to inhibition of the phosphorylation of the pump (these putative effects are inferred from the enhancement of photoaffinity labeling of P-glycoprotein by the peptides as discussed above), differences among the amphiphilic peptides in their interactions with cell membranes, etc. Furthermore, because P1 and P3 contain PKC-α pseudophosphorylation sequences (26, 27), they are likely to be recognized not only by PKC-α but also by other proteins that interact with naturally occurring PKC-α phosphoylation sites, e.g., protein phosphatases. It is also important to note that because of the overlapping substrate specificities of PKC isoforms (50), P1 and P3 may also antagonize the function of several other PKC isoforms in MCF7-MDR cells, and these inhibitory effects may contribute to their MDR reversal activity. Finally, complexity is also introduced by the existence of multiple mechanisms of drug resistance in drug-selected MDR cancer cells that overexpress P-glycoprotein, such as MCF7-MDR. Contributing factors to MDR can include multidrug resistance protein (MRP), glutathione S-transferase, topoisomerases, etc. (25), and some of these drug resistance mechanisms might also be affected by the peptides.

Studies of the inhibition of purified PKC by N-myristoylated peptide substrate analogs demonstrated that the N-myristoylated peptides interacted with the phospholipid cofactor, whereas their nonmyristoylated counterparts did not (27). The membrane-active nature of the N-myristoylated peptides most likely accounts for their ability to access cellular PKC (26, 31, 33). As the phospholipid cofactor concentration is increased in the PKC assay system, the inhibitory potency of the N-myristoylated peptides declines (27), providing evidence that their inhibitory effects are subject to surface dilution. This may account for the sharp increase in the potencies of P1, P3, and P5 as inducers of drug uptake in MCF7-MDR cells when the bulk peptide concentration was increased from 50 to 100 μM.

Bioactive peptides such as P1 are subject to proteolytic degradation, which often limits their potency. This may explain the superior potency of P1 in inducing drug uptake in short-term assays (6 h) compared with its potency in reversing MDR over a 96-h time course. In some cases, this problem can be overcome by designing retro-inverso analogs of the peptides. In a retro-inverso analog, the sequence of the parent peptide is reversed, and the residues are replaced by the corresponding d-enantiomers (51). The side chain surfaces of parent and retro-inverso peptides are similar or identical, but the topologies of their backbones differ (51). Thus, superior bioactivity can be achieved by retro-inverso analogs, when the side chain surface prevails in the interaction of the bioactive peptide with its target (52). A retro-inverso analog of a PKC-α pseudosubstrate peptide has been shown to potently inhibit phosphorylation of a synthetic peptide-substrate by PKC (53). Efforts are now underway to design retro-inverso analogs of P1 and related N-myristoylated peptides that are superior to the parent peptides in the reversal of MDR in human breast cancer cells. In a recent report, computer-based algorithms were successfully used to predict MDR reversal activity based on structural features of compounds entered into the data base (54); this type of strategy may also be useful for designing peptide or peptidomimetic P1 analogs with optimized MDR reversal activity.

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