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SHORT COMMUNICATION

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An *N*-myristoylated protein kinase C-α pseudosubstrate peptide that functions as a multidrug resistance reversal agent in human breast cancer cells is not a P-glycoprotein substrate

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Abstract Protein kinase C-α (PKC-α) activation is an important contributing factor in human breast cancer MCF-7 MDR cell drug resistance. We recently reported the use of *N*-myristoylated PKC-α pseudosubstrate peptides with potent PKC-α inhibitory activity as reversal agents of drug resistance in MCF-7 MDR cells. The peptides potently inhibit phosphorylation of the PKC-α substrates P-glycoprotein (P-gp), raf kinase and PKC-α itself in MCF-7 MDR cells in association with a severalfold induction of intracellular uptake of P-gp substrate chemotherapeutics and a statistically significant twofold increase in cellular chemosensitivity. We now report that the *N*-myristoylated PKC-α pseudosubstrate peptide *N*-myristoyl-RFARKGALRQKNV (P3) is not a P-gp substrate in MCF-7 MDR cells based on a comparison of the cellular uptake of [^{125}I]-radiolabeled P3 in MCF-7 MDR vs MCF-7 WT cells. The extent of cellular uptake of the radiolabeled peptide in the drug-resistant cell line MCF-7 MDR was either greater than or equivalent to the uptake in the parental drug-sensitive MCF-7 WT cell line over a time course of 30 min to 6 h, and across a peptide concentration range of 25–100 μM. Additionally, treatment of the MCF-7 MDR cells with verapamil (VPL), a known P-gp efflux inhibitor, had no effect on the cellular accumulation of radiolabeled P3. Our results provide direct evidence that the N-myristoylated pseudosubstrate peptide is taken up equivalently by drug-sensitive and MDR cancer cells and therefore has potential value as an MDR reversal agent that operates by a novel mechanism.

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Introduction

The failure of aggressive chemotherapy to eradicate malignant disease because of innate or acquired drug resistance mechanisms is a major obstacle to successful cancer treatment [11]. A broad category of multidrug resistant (MDR) cancer cells is characterized by reduced intracellular accumulation of chemotherapeutic drugs as a result of overexpression of the drug efflux pump P-glycoprotein (P-gp) [6, 11]. The abundant expression of P-gp and its message *mdr*-1 observed in specimens of intrinsically resistant cancers and malignant human and canine tumors that have relapsed during or after chemotherapy indicates the potential relevance of P-gp-associated MDR in clinical drug resistance [1, 9, 11, 21].

Protein kinase C (PKC) is an isozyme family that includes at least 11 mammalian members [15]. There are several lines of evidence suggesting that PKC activation is integrally related to P-gp-associated MDR. First, selective PKC activators such as phorbol esters induce resistance to cytotoxic P-gp substrates in cancer cells [7, 12]. Second, the isozyme PKC-α is overexpressed in several drug-selected MDR cancer cell lines, including MCF-7 MDR [2, 7]. Third, both selective activation of PKC-α in human colon cancer cells [12] and PKC-α overexpression in an *mdr*-1-transfected human breast cancer MCF-7 subline [22] induce MDR. Finally, PKC phosphorylates the linker region of P-gp in MDR human KB-VI cancer cells, and the phosphorylation is tightly coupled to the regulation of intracellular drug accumulation [3, 4]. However, because recent studies [8, 10] have shown that specific mutations of P-gp at the phosphorylation sites do not affect the function or expression of the transporter, the effects of PKC activation on P-gp are most likely indirect and probably result from

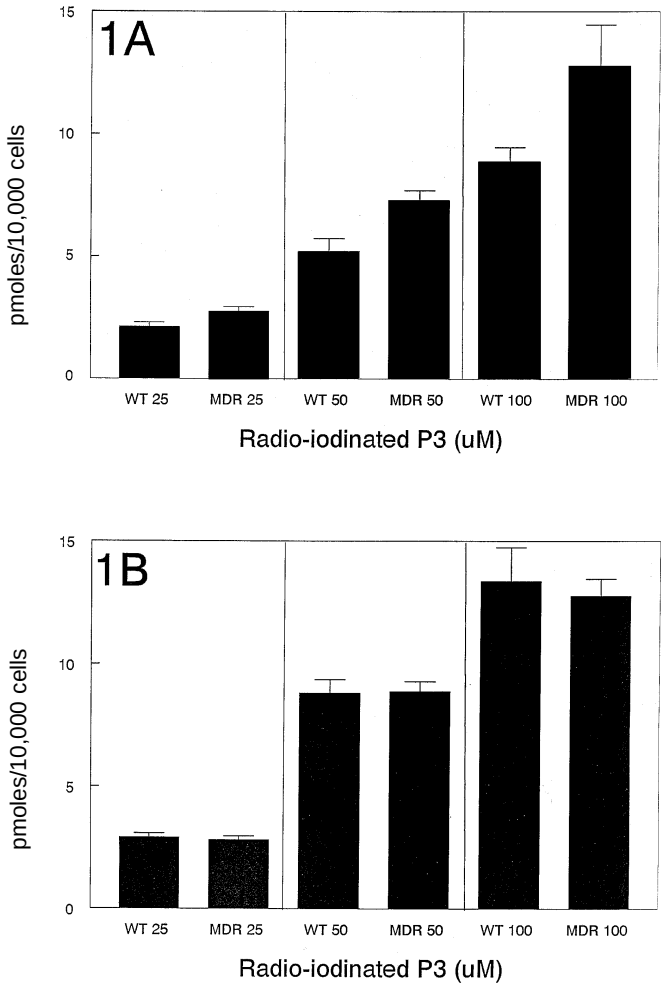

Fig. 1A, B A comparison of ^{125}I-P3 uptake by MCF-7 WT (*WT*) and MCF-7 MDR (*MDR*) cells. Experimental values shown are the means ($\pm$SEM) of four experiments done in quadruplicate (**A** 30-min incubation period, **B** 2-h incubation period)

phosphorylation of other PKC substrates that influence P-gp function and expression.

We have previously characterized the inhibition of PKC by *N*-myristoylated peptides corresponding to the autoinhibitory pseudosubstrate sequence of PKC-α and other PKC substrate recognition sequences [20]. We subsequently reported that the *N*-myristoylated PKC-α pseudosubstrate peptides *N*-myristoyl-FARKGALRQ (P1) and *N*-myristoyl-RFARKGALRQKNV (P3) function as MDR reversal agents in human breast cancer MCF-7 MDR cells operating by a novel mechanism involving a sharp increase in intracellular drug accumulation concomitant with potent inhibition of the phosphorylation of P-gp and two other PKC-α substrates, Raf-1 kinase and PKC-α itself, whereas the non-myristoylated counterparts (P2 and P4, respectively) neither increase drug accumulation nor inhibit phosphorylation of substrates [13]. Classical MDR reversal agents such as verapamil (VPL) and cyclosporin A cannot be used to reverse clinical drug resistance because of their severe toxicity at therapeutic concentrations [18]. Importantly, the *N*-myristoylated PKC-α pseudosubstrate peptides can be distinguished from VPL and other classical MDR reversal agents [5, 19, 23] in that they did not compete with [^{3}H]azidopine for drug binding sites on P-gp [13]. Other compounds have also been recently described with partial inhibition of MDR without competing for drug binding sites on P-gp [16]. Because P-gp has multiple drug binding sites [19], the possibility remained that the peptides could serve as P-gp substrates.

P-gp substrates are typically hydrophobic and positively charged [6, 11] and in some cases peptidic in nature [17, 19], features shared by the *N*-myristoylated PKC-α pseudosubstrate peptides [20]. P-gp-mediated efflux of the pseudosubstrate peptides would compromise their value as MDR reversal agents by limiting their availability to drug-resistant cancer cells. To ascertain whether the *N*-myristoylated pseudosubstrate peptide P3 was a P-gp substrate in MCF-7 MDR cells, we compared the uptake of a radiolabeled P3 analog in MCF-7 MDR vs MCF-7 WT cells.

Materials and methods

The effects of P3 on the intracellular accumulation of P-gp substrate chemotherapeutics and on the phosphorylation of PKC substrates in MCF-7 MDR cells were observed during a period of 30 min to 6 h with a P3 concentration range of 25–100 μ*M* [13]. [Y-^{125}I] *N*-myristoyl-RYARKGALRQKNV (^{125}I-P3) was synthesized at The M. D. Anderson Cancer Center Synthetic Antigen Facility and radiolabeled and HPLC purified at New England Nuclear Laboratories (Wilmington, Del.). A stock solution of *N*-myristoyl-RYARKGALRQKNV was spiked with ^{125}I-P3, resulting in a specific activity of 100–200 cpm/pmol. MCF-7 MDR and the parental WT cell lines were plated at 40 000 cells per well in 48-well plates (CoStar, Cambridge, Mass.) with Eagle's modified essential medium (Gibco-BRL, Grand Island, N.Y.) supplemented with 5% heat-inactivated fetal bovine serum (FBS), nonessential amino acids, vitamins, sodium pyruvate, L-glutamine, and penicillin/streptomycin [13]. After incubation overnight at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, the medium was removed and replaced with medium containing 0 μ*M*, 25 μ*M*, 50 μ*M*, or 100 μ*M* ^{125}I-P3. After the indicated incubation period, the medium was removed and the wells were washed with ice-cold phosphate-buffered saline three times [13]. The cells were then trypsinized for 45 min and the radioactivity counted using a Packard Auto Gamma Counter [13]. For each peptide concentration and incubation period employed, the counts retained by control wells lacking cells provided background values; these values were 23$\pm$3% of the total counts per minute. Cellular uptake of ^{125}I-P3 was defined as total minus background counts per minute and converted to picomoles ^{125}I-P3/10 000 cells. Cell viability of $>$95% was demonstrated prior to the assay by Trypan Blue exclusion and before trypsinization by morphology with light microscopy.

Results

The results shown in Fig. 1A indicate that the extent of ^{125}I-P3 (25–100 μM) uptake was similar in the drug-sensitive and MDR MCF-7 cell lines after a 30-min incubation period, with somewhat higher ^{125}I-P3 uptake values in the MDR line, arguing against P-gp-mediated ^{125}I-P3 efflux

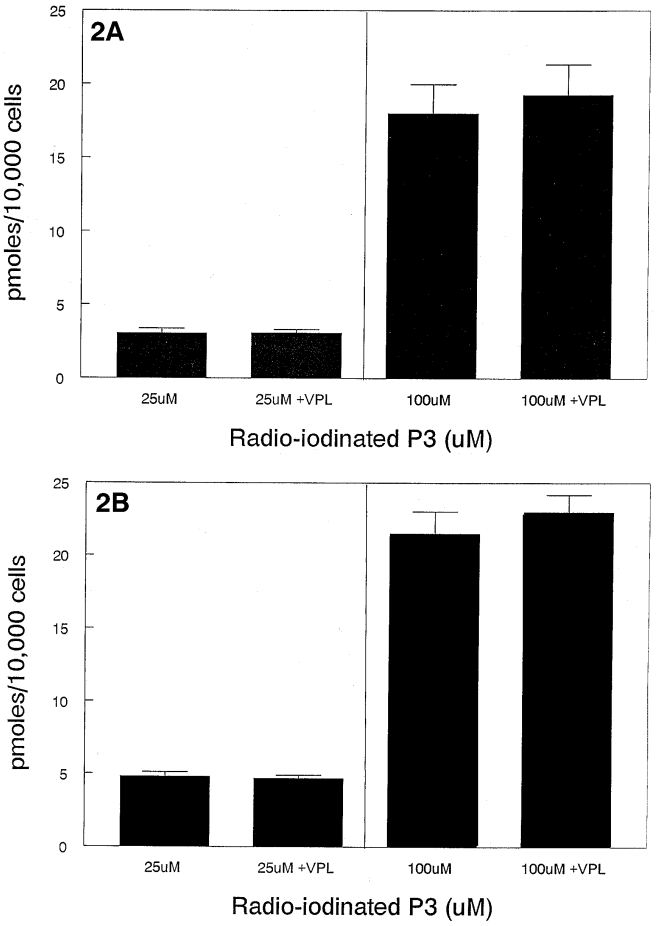

Fig. 2A, B Effect of 10 μM VPL on ^{125}I-P3 uptake by MCF-7 MDR cells. Experimental values shown are the means (±SEM) of four experiments done in quadruplicate. (**A** 30-min incubation period, **B** 2-h incubation period)

from the drug-resistant cells. Likewise, there was no difference in ^{125}I-P3 uptake between the two cell lines after incubation periods of 2 h (Fig. 1B) and 6 h (data not shown). The concentration-dependent rise in intracellular ^{125}I-P3 uptake (Fig. 1) correlated with the concentration dependence observed in the inhibition of P-gp phosphorylation by the *N*-myristoylated pseudosubstrate peptide [13].

VPL is a potent inhibitor of MCF7-MDR efflux of P-gp substrates [7]. VPL at a concentration of 10 μM induces a severalfold increase in the accumulation of doxorubicin and other P-gp substrates in MCF-7 MDR cells after incubation for 30 min to 2 h [13]. As an independent test of whether ^{125}I-P3 was a P-gp substrate in MCF-7 MDR cells, we determined the effect of VPL on ^{125}I-P3 accumulation in the cells. Figure 2A shows that 10 μM VPL had no effect on ^{125}I-P3 uptake by MCF-7 MDR cells after a 30-min incubation period. Figure 2B shows that 10 μM VPL was also without effect on ^{125}I-P3 uptake at the 2 h time-point. In separate experiments, the addition of 10 μM VPL to MCF7-MDR cells significantly increased the accumulation of the P-gp substrates [^{14}C]doxorubicin and [3H]vincristine severalfold, whereas accumulation of [^{3}H]5-fluorouracil (which is not a P-gp substrate) was unaffected (data not shown).

Discussion

Our results provide direct evidence that the *N*-myristoylated pseudosubstrate peptide ^{125}I-P3 is not a P-gp substrate in MCF7-MDR cells. We found that MCF-7 MDR accumulated as much or slightly more ^{125}I-P3 than the drug-sensitive parental cell line under conditions where P3 was a potent modulator of PKC substrate phosphorylation and chemotherapeutic drug uptake in the cells. We also found that VPL had no effect on ^{125}I-P3 uptake in the MCF-7 MDR cells. It is well-established that PKC substrate motifs contain multiple basic residues which are of critical importance to the affinity of the substrate at the active site [14]. Thus, the PKC substrate recognition motif in P3, which contains five basic residues, is typical in this respect. Therefore, our results demonstrate that a peptide containing a typical PKC substrate motif is not recognized as a substrate by P-glycoprotein. The *N*-myristoylated PKC-α pseudosubstrate peptides represent a distinct and novel modality for MDR reversal and PKC-α blockade which may escape the commonly encountered toxicities presently recognized with P-gp binding reversal agents, such as cyclosporin and VPL, and which is not compromised by P-gp-mediated efflux of the peptidic reversal agent.

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References

1. Bergman PJ, Ogilvie GK, Powers BE (1996) Monoclonal antibody C219 immunohistochemistry in canine lymphoma: predictive ability and sequential analysis. J Vet Intern Med 10:354
2. Blobe GC, Sachs CW, Khan WA, Fabbro D, Stabel S, Wetsel WC, Obeid LM, Fine RL, Hannun YA (1993) Selective regulation of expression of protein kinase C (PKC) isoenzymes in multidrug resistance MCF7 cells. J Biol Chem 268:658
3. Chambers TC, Zheng B, Kuo JF (1992) Regulation by phorbol ester and protein kinase C inhibitors and by a protein phosphatase inhibitor (okadaic acid) of P-glycoprotein phosphorylation and relationship to drug accumulation in multidrug resistant human KB cells. Mol Pharmacol 41: 1008
4. Chambers TC, Pohl J, Raynor RL, Kuo JF (1993) Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. J Biol Chem 268:4592
5. Chen G, Ramachandran C, Krishan A (1993) Thaliblastine, a plant alkaloid, circumvents multidrug resistance by direct binding to P-glycoprotein. Cancer Res 53:2544
6. Endicott JA, Ling V (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Review Biochem 58:137
7. Fine RL, Patel J, Chabner BA (1988) Phorbol esters induce multidrug resistance in human breast cancer cells. Proc Natl Acad Sci USA 85:582
8. Germann UA, Chambers TC, Ambudkar SV, Licht T, Cardarelli CO, Pastan I, Gottesman MM (1996) Characterization of phosphorylation-defective mutants of P-glycoprotein expressed in mammalian cells. J Biol Chem 271:1708

9. Goldstein LJ, Galski H, Fojo A, Willingham M, Lai S-L, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM, Pastan I (1989) Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst 81:116
10. Goodfellow HR, Sardini A, Ruetz S, Callaghan R, Gros P, McNaughton PA, Higgins CF (1996) Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. J Biol Chem 271:13668
11. Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62:385
12. Gravitt KR, Ward NE, Fan D, Skibber JM, Levin B, O′Brian CA (1994) Evidence that protein kinase C-α activation is a critical event in phorbol ester-induced multiple drug resistance in human colon cancer cells. Biochem Pharmacol 48:375
13. Gupta KP, Ward NE, Gravitt KR, Bergman PJ, O′Brian CA (1996) Partial reversal of multidrug resistance in human breast cancer cells by an *N*-myristoylated protein kinase C-α pseudosubstrate peptide. J Biol Chem 271:2102
14. House C, Wetterhall RE, Kemp BE (1987) The influence of basic residues on the substrate specificity of protein kinase C. J Biol Chem 262:772
15. Newton AC (1995) Protein kinase C: structure, function and regulation (minireview). J Biol Chem 270:28495
16. Sachs CW, Safa AR, Harrison SD, Fine RL (1995) Partial inhibition of multidrug resistance by safingol is independent of modulation of P-glycoprotein substrate activities and correlated with inhibition of protein kinase C. J Biol Chem 270:26639
17. Sharma RC, Inoue S, Roitelman J, Schimke RT, Simon RD (1992) Peptide transport by the multidrug resistance pump. J Biol Chem 267:5731
18. Shustik C, Dalton W, Gros P (1995) P-glycoprotein-mediated multidrug resistance in tumor cells: biochemistry, clinical relevance and modulation. Mol Aspects Med 16:1
19. Tamai I, Safa AR (1991) Azidopine noncompetitively interacts with vinblastine and cyclosporin A binding to P-glycoprotein in multidrug resistant cells. J Biol Chem 266:16796
20. Ward NE, O′Brian CA (1993) Inhibition of protein kinase C by *N*-myristoylated peptide substrate analogs. Biochemistry 32:11903
21. Weinstein RS, Jakate SM, Dominguez JM, Lebovitz MD, Koukoulis GK, Kuszak JR, Klusens LF, Grogan TM, Saclarides TJ, Roninson IB, Coon JS (1991) Relationship of the expression of the multidrug resistance gene product (P-glycoprotein) in human colon carcinoma to local tumor aggressiveness and lymph node metastasis. Cancer Res 51: 2720
22. Yu G, Ahmad S, Aquino A, Fairchild CR, Trepel JB, Ohno S, Suzuki K, Tsuruo T, Cowan KH, Glazer RI (1991) Transfection with protein kinase C-α confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein. Cancer Commun 3:181
23. Zhang L, Sachs CW, Fu HW, Fine RL, Casey PJ (1995) Characterization of prenylcysteines that interact with P-glycoprotein and inhibit drug transport in tumor cells. J Biol Chem 270:22859