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Short communication

Involvement of phosphatidylinositol-3-kinase in membrane ruffling induced by P-glycoprotein substrates in multidrug-resistant carcinoma cells

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Abstract

P-glycoprotein (P-gp) is a transmembrane protein that transports a variety of structurally and functionally diverse drugs. We recently found that the interaction of drugs with P-gp promoted invasion and metastasis. In this study, we sought to determine the mechanism by which the interaction of P-gp with its substrates leads to the earliest membrane changes associated with cellular invasion, i.e., membrane ruffling. We focused on the activation of phosphatidylinositol-3-kinase (PI-3-kinase), a lipid kinase that regulates actin cytoskeletal organization and cell movement. Sensitive or multidrug-resistant (MDR) MCF-7 (human breast cancer) or KB (human oral carcinoma) cells were treated with drugs or vehicle, and then were stained with phalloidin-tetramethyl-rhodamine isothiocyanate. Membrane ruffles were visualized using a fluorescence microscope. PI-3-kinase activity was determined by an *in vitro* immune-complex kinase assay and thin-layer chromatography. Drugs transported by P-gp, vinblastine and *trans*-flupenthixol, increased membrane ruffling and PI-3-kinase activity in the MDR cell lines, MCF-7/AdrR and KBV-1, which overexpress P-gp. This effect was not seen with mechlorethamine, a drug that is not transported by P-gp, and was not detected in sensitive parental cell lines that do not express P-gp. A similar effect was also observed in the *MDR1* transfectant, MCF-7/BC-19. Wortmannin, an inhibitor of PI-3-kinase, blocked the effect of VBL and *t*FPT on membrane ruffling and the activity of PI-3-kinase in MDR cells. These results indicate that drugs transported by P-gp induce membrane ruffling, an early indicator of cellular motility and metastatic potential, in cancer cells overexpressing P-gp and that this effect may be mediated through activation of PI-3-kinase.

Keywords: P-glycoprotein; Membrane ruffling; Phosphatidylinositol-3-kinase; P-glycoprotein substrate; Multidrug resistance; Signal transduction

1. Introduction

MDR mediated by the expression of P-gp has been carefully characterized over the last two decades. P-gp, the *MDR1* gene product, is a 150–180-kDa plasma membrane phosphoglycoprotein belonging to the ATP-binding cassette family, which includes multidrug-resistance protein (MRP), cystic fibrosis transmembrane conductance regulator (CFTR), sulfonylurea receptor (SUR), and lung-resistance protein (LRP). P-gp functions as an energy-dependent drug transporter with broad substrate specificity [1–3]. It shares homology with bacterial transport proteins [4], has ATP binding [5] and hydrolysis [6] activities, drug binding [7] and efflux [8] properties, and possesses the ability to bind compounds that reverse MDR, such as verapamil and cyclosporin A [9,10]. Expression of P-gp in human cancers is known to be associated with a poor clinical outcome [11].

Overexpression of P-gp results in decreased intracellular drug concentrations [2]. Based on this mechanism it was generally assumed that the poor prognosis associated with P-gp expression was due to the inability of cytotoxic drugs to reach effective concentrations at intracellular targets. Yet, a variety of compounds that restore intracellular drug concentrations by interfering with the function of P-gp (MDR modulators) have thus far failed to change the overall prognosis of treated patients [12,13]. To investigate this problem, we developed an MDR cell line that readily grows in culture and in a simple animal model [14]. During

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Abbreviations: MDR, multidrug-resistant or multidrug resistance; P-gp, P-glycoprotein; PI-3-kinase, phosphatidylinositol-3-kinase; VBL, vinblastine; *t*FPT, *trans*-flupenthixol; bFGF, basic fibroblast growth factor.

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our studies, we were surprised to find that treatment of animals implanted with tumor cells overexpressing P-gp with vincristine, paclitaxel, or *trans*-flupenthixol (*t*FPT), compounds that are transported by P-gp, increased invasiveness and metastases [15]. In contrast, this effect was not observed in parental, sensitive cells or when MDR cells were treated with drugs whose transport was not affected by P-gp [15]. Siegfried *et al*. previously demonstrated that membrane fluidity is decreased in drug-resistant cells and that the degree of changes correlates with the degree of resistance to doxorubicin [16], but whether this alteration in membrane fluidity is associated with tumor cell invasion and metastasis is not known. Upon closer analysis, we found that treated MDR cells had greater membrane ruffling, an early indicator of cellular motility and invasiveness [17]. Membrane ruffles are specialized plasma membrane ultrastructures that contain fine actin filaments. Since the formation of membrane ruffles depends upon actin polymerization, a process that is mediated by PI-3-kinase [18], in the current study we investigated whether drugs transported by P-gp activated PI-3-kinase and whether this was exclusive to MDR cell lines. We found that P-gp substrate drugs induced membrane ruffling in cancer cells that overexpressed P-gp through activation of PI-3-kinase.

2. Materials and methods

2.1. Cell culture

The MDR human breast carcinoma cell lines MCF-7/AdrR and MCF-7/BC-19 (*MDR1* transfectant), and the sensitive parental line MCF-7, were supplied by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer and were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37° in a humidified atmosphere containing 5% CO_2/95% air. The MDR human oral carcinoma line KBV-1, developed by Dr. Michael Gottesman's laboratory, and the sensitive parental line KB3-1 were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under identical conditions as described above. KBV-1 cells were maintained in 0.5 μg/mL of VBL for the maintenance of the MDR phenotype. Both of the MDR cell lines used in this study, KBV-1 and MCF-7/AdrR, overexpress P-gp [19]. Cells were checked routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after 3 months, and new lines were obtained from frozen stocks.

2.2. Membrane ruffling

Membrane ruffling was determined using fluorescently labeled phalloidin as previously described [20]. Briefly, cells grown in drug-free medium for 3 days were plated at an identical density (1×10^5 cells/mL) on glass coverslips in 35-mm tissue culture dishes, incubated for 24 hr, and then treated with different concentrations of drugs or vehicle. The drug concentrations were chosen based on concentration–response studies. Next, the glass coverslips were rinsed twice with pre-warmed PBS, and the cells were fixed in 3.7% formaldehyde in PBS for 15 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then were treated with 50 mM ammonium chloride in PBS for 10 min. Membrane ruffling was visualized by staining with 5 μM phalloidin-tetramethyl-rhodamine isothiocyanate (TRITC) (Sigma) for 1 hr at room temperature. The slides were viewed using a fluorescence microscope with the PC Image-Pro Plus system (Media Cybernetics).

2.3. PI-3-kinase

Cells grown in drug-free medium were treated with vehicles or drugs in 100-mm tissue culture dishes. At the end of the treatment period, the cells were quickly washed with ice-cold, freshly prepared Buffer A (137 mM NaCl, 20 mM Tris–HCl (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM sodium orthovanadate), and then lysed with ice-cold lysis buffer (Buffer A containing 1% Nonidet P-40 (NP-40) and 1 mM phenylmethylsulfonyl fluoride). Cell lysates (1 mg) were immunoprecipitated with 5 μL of polyclonal anti-PI-3-kinase (p85) antibody (Upstate Biotechnology). Antibody–enzyme immunocomplexes were precipitated by adding 60 μL of a 50% slurry of Protein-A Sepharose CL-4B in lysis buffer. Beads were washed three times with lysis buffer, three times with 0.1 M Tris–HCl (pH 7.4), 5 mM LiCl, and 0.1 mM sodium orthovanadate, and twice with TNE buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.1 mM sodium orthovanadate). Beads were then suspended in 50 μL of TNE buffer, and 10 μL of 100 mM $MgCl_2$ and 10 μL (20 μg) of sonicated phosphatidylinositol were added. The reaction was initiated by the addition of 0.88 mM (final concentration) ATP, 10 μCi of [γ-^{32}P]ATP, incubated for 15 min at 37°, and stopped by adding 10 μL of 6 N HCl. Lipids were then extracted with chloroform:methanol (1:1). The lower organic phase was spotted on silica gel TLC plates, which were developed in chloroform:methanol:water:ammonium hydroxide (60:47:11.3:2). Radioactive spots were visualized by autoradiography and quantified with a phosphorimager.

2.4. Western blot analysis

Cell lysates (50 μg protein) prepared from treated samples were subjected to 8% SDS–PAGE, and separated proteins were transferred to nitrocellulose. The nitrocellulose was immunoblotted with the polyclonal anti-PI-3-kinase (p85) antibody after incubation in PBS containing

3% nonfat milk for 1 hr. Detection of proteins was carried out using an enzyme-linked chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Inc.), as specified by the manufacturer.

3. Results and discussion

In an earlier report, we found that treatment of P-gp(+) cell lines with drugs transported by P-gp increased invasion

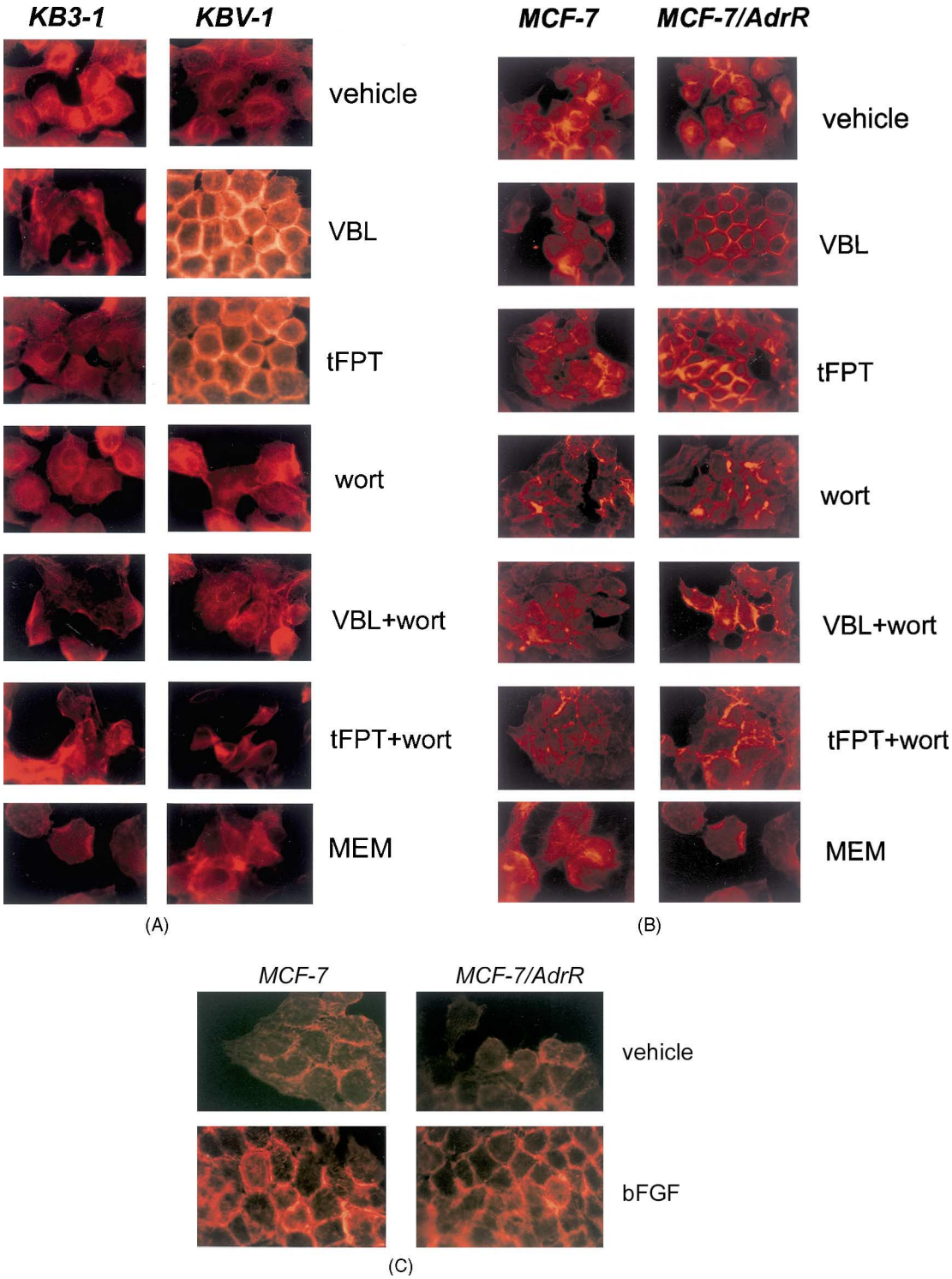

Fig. 1. Effects of drugs on membrane ruffling in sensitive and drug-resistant KB (A) and MCF-7 (B, C) cells. Cells plated at an identical density (1×10^5 cells/mL) on glass coverslips in 35-mm cell culture dishes were treated with VBL (1 nM for KB3-1 and MCF-7; 500 nM for KBV-1 and MCF-7/AdrR), *t*FPT (1 μM for KB3-1 and MCF-7; 5 μM for KBV-1 and MCF-7/AdrR), wortmannin (wort, 1 μM), mechlorethamine (MEM, 0.5 μM), bFGF (50 ng/mL), or vehicle for 24 hr, then fixed in 3.7% formaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS. Drug concentrations were chosen that produced $\leq$10% cell killing during the 24-hr incubation. bFGF was used as a control agent to induce membrane ruffling. Membrane ruffling was visualized by staining with 5 μM phalloidin-tetramethyl-rhodamine isothiocyanate (TRITC) as described in Section 2. Results are representative of three similar experiments.

and metastasis [15]. To begin to define the mechanism(s) underlying these observations, we determined the effects of drugs on the activity of PI-3-kinase, an enzyme shown to regulate the structure and function of the actin network [21]. Treatment of KBV-1 and MCF-7/AdrR cells for 24 hr with P-gp-transportable drugs, VBL and *t*FPT, at concentrations that produced $\leq$10% cell killing, increased membrane ruffling as compared with that seen with vehicle alone (Fig. 1A and B). In contrast, mechlorethamine, a drug that is not transported by P-gp, did not induce membrane ruffling in MDR cells (Fig. 1A and B). In parental, sensitive cell lines (KB3-1 and MCF-7), which do not express P-gp, neither P-gp-transportable nor non-P-gp-transportable drugs increased membrane ruffling (Fig. 1A and B). In these experiments, concentrations of drugs were chosen based on concentration–response curves generated for both sensitive and resistant cell lines. Because of the 500-fold difference in sensitivity to VBL between the MDR cell lines and the parental lines, and the 5-fold difference in sensitivity to *t*FPT, we used 1 nM VBL and 1 μM *t*FPT in sensitive lines and 500 nM VBL and 5 μM *t*FPT in resistant lines. We found no induction of membrane ruffling in sensitive cells at any of the concentrations tested. In contrast, we observed membrane ruffling in MDR cells exposed to a wide range of drug concentrations. bFGF was used as a control agent to induce membrane ruffling in both sensitive and MDR MCF-7 cells (Fig. 1C). bFGF is not a P-gp substrate, and has been shown previously to induce membrane ruffling in breast cancer cells [20]. These results indicate that both P-gp and drugs transported by P-gp are required to induce membrane ruffling in MDR cells.

We next investigated the effects of drugs on PI-3-kinase, an enzyme that links phosphatidylinositol signaling

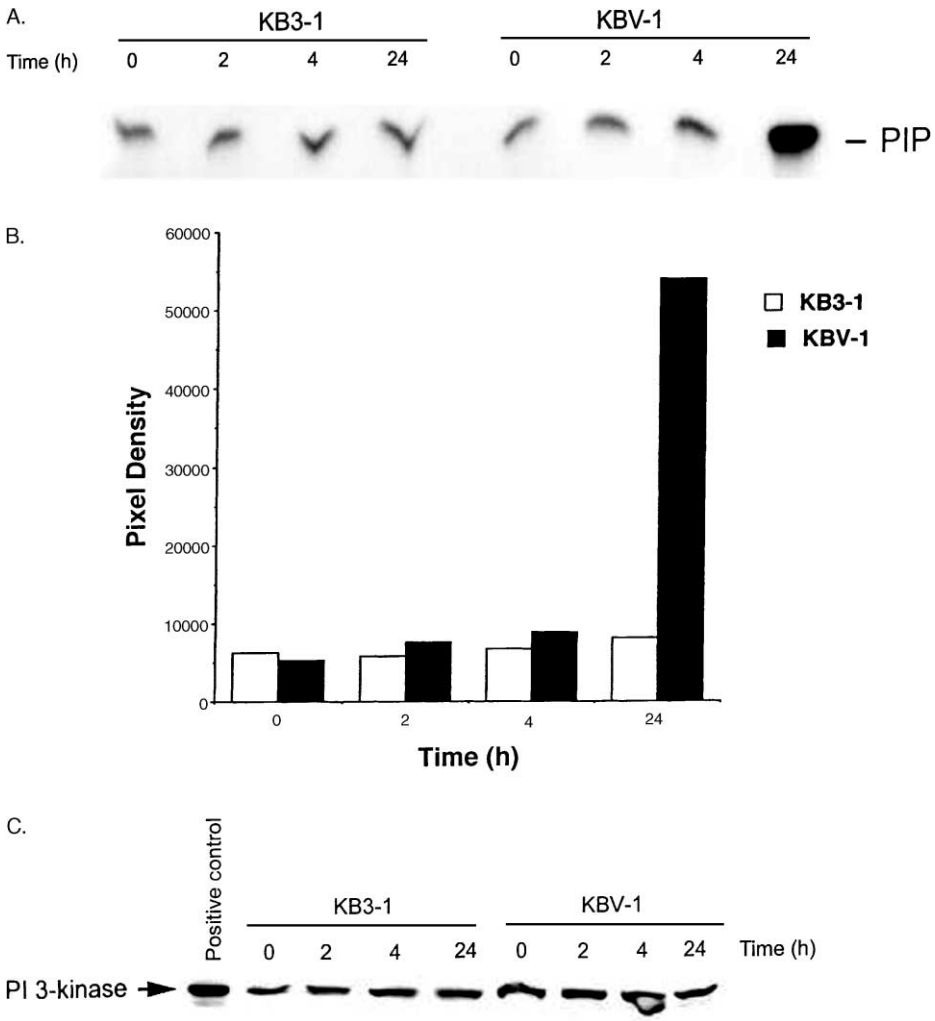

Fig. 2. Time course of the effect of VBL on PI-3-kinase activity and contents in sensitive and drug-resistant KB cells. (A, B) Cells were grown in drug-free medium in 100-mm tissue culture dishes for 3 days, then treated with VBL (1 nM, KB3-1 and 500 nM, KBV-1), or vehicle for various periods of time. Cell lysates were prepared from the treated samples and immunoprecipitated with anti-PI-3-kinase p85 antibody. PI-3-kinase activity in anti-p85 immnoprecipitates was measured as described in Section 2. Results are representative of three similar experiments. PIP: phosphatidylinositol-3-phosphate. (C) Equal amounts (50 μg) of proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with an anti-PI-3-kinase p85 antibody as described in Section 2. Human Jurkat cell lysate was used as a positive control. Results are representative of two similar experiments.

pathways to a number of cellular processes including membrane ruffling [21,22]. PI-3-kinase catalyzes the reaction that phosphorylates phosphatidylinositol on the D-3 position of the inositol ring. The 3-phosphoinositide products have been shown to increase membrane ruffling, vesicle movement, as well as cellular differentiation and invasion [18,23–25]. Panels A and B in Fig. 2 demonstrate that VBL increased PI-3-kinase activity in KBV-1 cells in a time-dependent manner at concentrations shown to induce membrane ruffling. Increased activity of the enzyme could be seen by 4 hr of incubation with 500 nM VBL, and a 10-fold increase occurred by 24 hr (Fig. 2A and B). Increased activity of the enzyme was sustained for 36 hr (data not shown). In contrast, VBL did not increase PI-3-kinase activity in KB3-1 cells (Fig. 2A and B). The content of PI-3-kinase, as determined by Western blotting, was not affected by treatment with VBL for the same period of time (Fig. 2C), suggesting that the increases in PI-3-kinase activity caused by VBL did not require increased synthesis of the protein.

*t*FPT is the most potent of a series of compounds of the thioxanthene class used to reverse MDR mediated by P-gp [3]. This compound has been shown to require phenylalanine at position 983 in the putative transmembrane domain region 12 of P-gp for its activity [26]. *t*FPT increased PI-3-kinase activity (Fig. 3) and membrane ruffling (Fig. 1) in KBV-1 and MCF-7/AdrR cells. In contrast, mechlorethamine did not stimulate activation of PI-3-kinase in these cell lines. None of these drugs increased PI-3-kinase activity in the parental control lines.

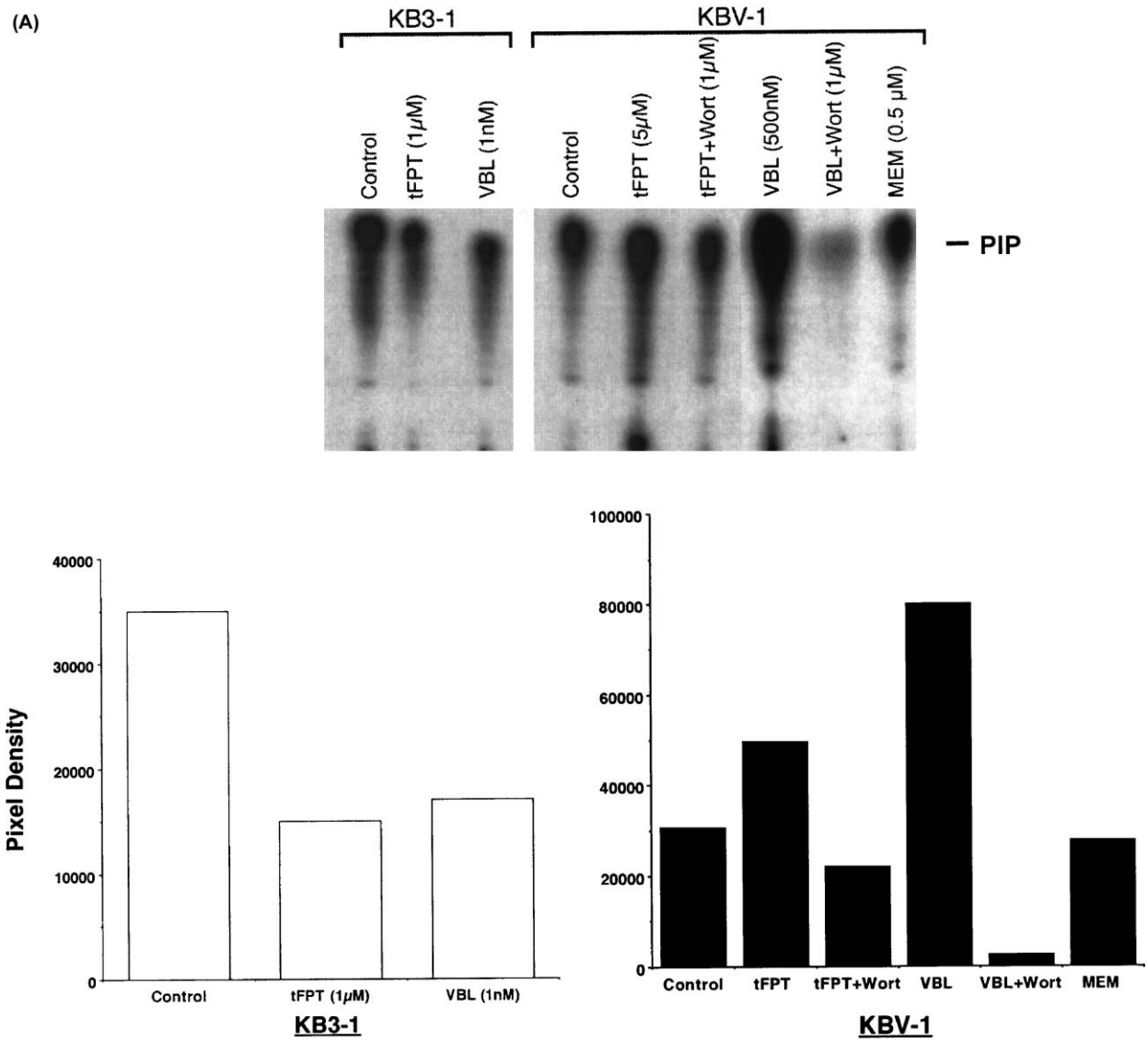

Fig. 3. Effects of drugs on PI-3-kinase activity in sensitive and drug-resistant KB (A) and MCF-7 (B) cells. Cells grown in drug-free medium were treated with VBL, *t*FPT, mechlorethamine (MEM), wortmannin (wort), or vehicle for 24 hr. The concentrations were chosen based on concentration–response studies. PI-3-kinase activity was measured as described in Fig. 2. Results are representative of two similar experiments. PIP: phosphatidylinositol-3-phosphate.

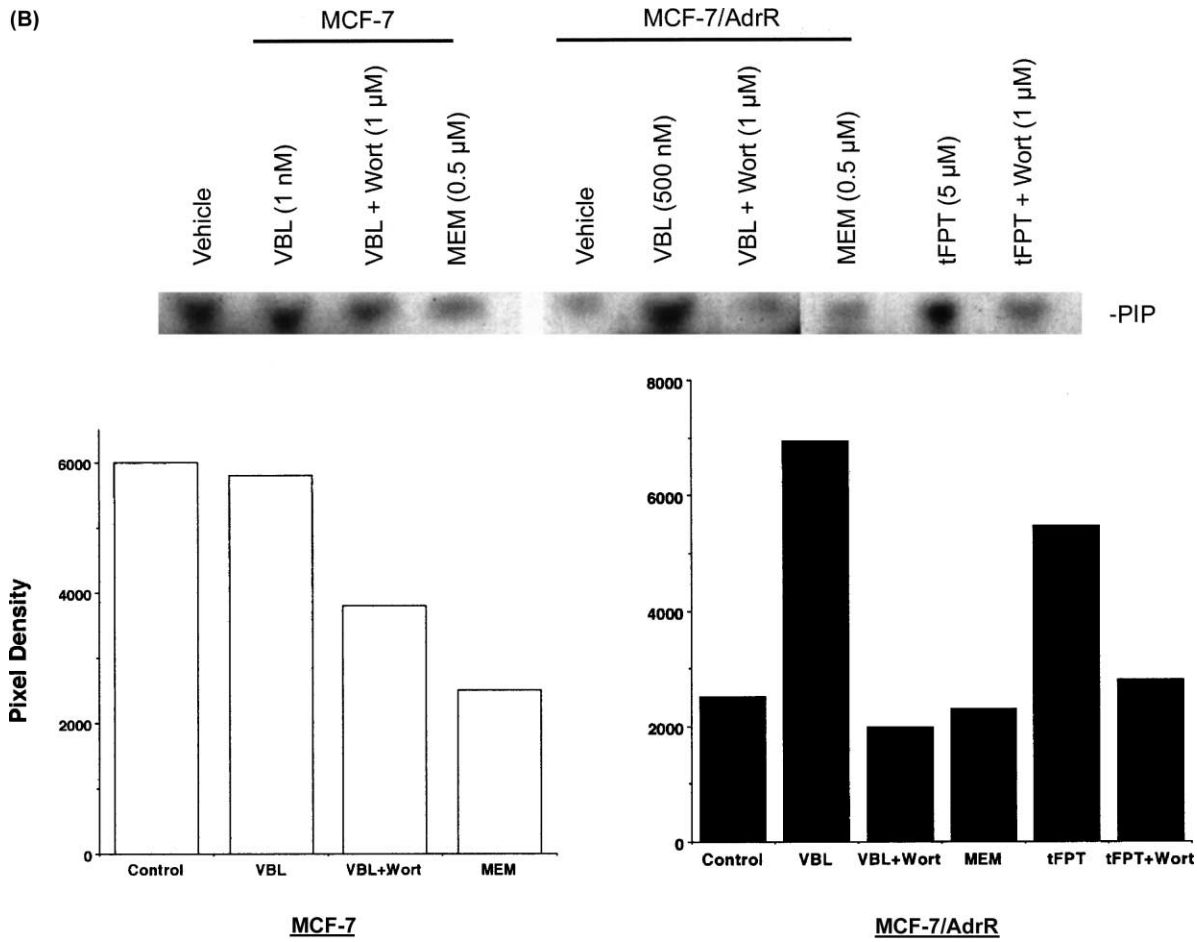

Fig. 3. (*Continued*).

To further test the role of PI-3-kinase in drug-induced membrane ruffling, we studied the effect of wortmannin, a fungal metabolite that selectively and irreversibly inhibits PI-3-kinase [27]. Figs. 1 and 3 demonstrate that wortmannin inhibited both membrane ruffling and PI-3-kinase activation stimulated by VBL or *t*FPT. The greater inhibition of PI-3-kinase activity by wortmannin seen in KBV-1 cells treated with VBL, as compared with the cells treated with *t*FPT (Fig. 3A), was probably due to the lower concentration of VBL (500 nM) than of *t*FPT (5 μM) used to treat the cells, while the wortmannin concentration was the same (1 μM). Probably this concentration of the inhibitor could almost abolish the effect of 500 nM VBL, but not 5 μM *t*FPT. However, in MCF-7/AdrR cells, a similar degree of inhibition by wortmannin was observed in the cells treated with either VBL or *t*FPT (Fig. 3B). This may reflect the biochemical difference between the cell lines. Higher concentrations of wortmannin were tested in our studies, but cytotoxicity was observed (data not shown). These experiments strengthen the conclusion that the enhanced membrane ruffling resulting from treatment with P-gp-transportable drugs is mediated through the activation of PI-3-kinase.

Since MDR cell lines such as KBV-1 and MCF-7/AdrR selected with chemotherapeutic agents display multiple mechanisms of drug resistance, we carried out similar experiments in the *MDR1* transfectant, the MCF-7/BC-19 cell line. As shown in Fig. 4, although MCF-7/BC-19 cells had a different morphology than the MCF-7/AdrR

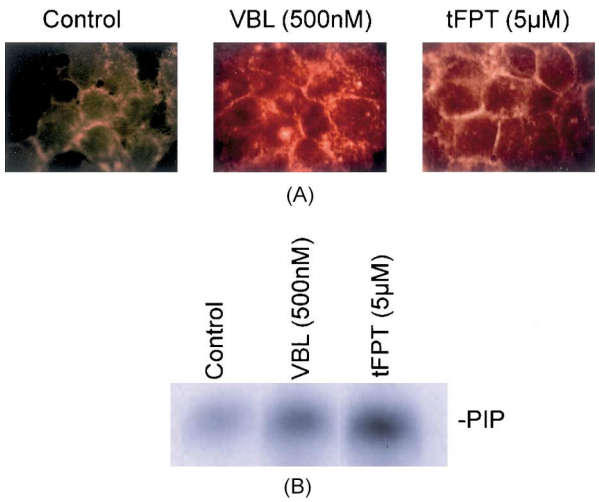

Fig. 4. Effects of drugs on membrane ruffling and PI-3-kinase activity in MCF-7/BC-19 cells. Cells grown in drug-free medium were treated with VBL, *t*FPT, or vehicle for 24 hr. Membrane ruffling (A) and PI-3-kinase activity (B) were assayed as described in Figs. 1 and 2. Results are representative of two similar experiments. PIP: phosphatidylinositol-3-phosphate.

and parental lines due to transfection of the *MDR1* gene, VBL and *t*FPT also increased membrane ruffling and PI-3-kinase activity in the transfected cell line. These data provide clearer evidence linking the expression of P-gp to drug-induced membrane ruffling and PI-3-kinase activation. The differences in induction of PI-3-kinase activity in MCF-7/BC19 cells (Fig. 4B) compared with that seen in KBV-1 cells (Fig. 2A) were likely due to the fact that these results were obtained in different cell lines.

The mechanism by which interaction of P-gp with drugs triggers PI-3-kinase activity is unknown. We therefore determined if this effect required a physical interaction between the transporter and the enzyme using a co-immunoprecipitation technique. We did not detect a physical association in treated or untreated MDR cells (data not shown). The lack of a direct interaction between P-gp and PI-3-kinase indicates that other mediators are involved. We have previously shown a direct interaction between P-gp and isoforms of protein kinase C [19], and a link between PI-3-kinase and protein kinase C signaling has been reported [28,29]. Whether protein kinase C is involved in drug-induced activation of PI-3-kinase and membrane ruffling remains to be determined. The interaction of P-gp with protein kinase C was stimulated by epidermal growth factor, and epidermal growth factor-mediated signaling may provide a clue to PI-3-kinase activation [30].

In summary, our studies reveal that drugs known to be transported by P-gp can induce membrane ruffling through PI-3-kinase. These findings confirm and extend our previous work on drug-induced metastasis and implicate a role for PI-3-kinase in this process.

Acknowledgment

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