VOL. 53 NO. 10, OCT. 2000 THE JOURNAL OF ANTIBIOTICS pp. 1201 — 1206

Reversal of Multidrug Resistance by 7-*O*-Benzoylpyripyropene A in Multidrug-Resistant Tumor Cells

MUN-CHUAL RHO[†], MASAHIKO HAYASHI, AKIKO FUKAMI, RIKA OBATA, TOSHIAKI SUNAZUKA, HIROSHI TOMODA, KANKI KOMIYAMA and SATOSHI ŌMURA*

The Kitasato Institute and School of Pharmaceutical Sciences, The Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

(Received for publication March 15, 2000)

7-*O*-Benzoylpyripyropene A (7-*O*-BzP), a semi-synthetic analog of pyripyropene, was investigated for its reversing effect on multidrug-resistant (MDR) tumor cells. 7-*O*-BzP (6.25 μg/ml) completely reversed resistance against vincristine and adriamycin in vincristine-resistant KB cells (VJ-300) and adriamycin-resistant P388 cells (P388/ADR), respectively. 7-*O*-BzP alone had no effect on the growth of drug sensitive and drug-resistant cells. 7-*O*-BzP (6.25 μg/ml) significantly enhanced accumulation of [^{3}H]vincristine in VJ-300 cells and completely inhibited the binding of [^{3}H]azidopine to the P-glycoprotein in VJ-300 cells and P388/ADR cells. The result suggests that 7-*O*-BzP effectively reverses P-glycoprotein-related MDR by interacting directly with P-glycoprotein in drug resistant VJ-300 and P388/ADR cells.

Intrinsic or acquired resistance of tumor cells to anticancer drugs remains a serious impediment in the chemotherapy of cancer. Once tumor cells acquire resistance to an antitumor agent, they generally show cross-resistance to a wide variety of structurally and mechanistically unrelated antitumor agents[1~3]. This multidrug resistance of tumor cells often involves the expression of P-glycoprotein, which is an ATP-dependent drug pump that can exclude a broad range of commonly used chemotheraputic drugs[4~6]. Recently, both P-glycoprotein and the resulting multidrug resistance (MDR) phenotype were found to be inhibited by a variety of pharmacological products[7~11]. However, the mechanisms by which these agents reverse MDR is not fully understood, and these agents have not been widely applied as chemosensitizing agents for the treatment of cancer because of their toxicity and/or low efficacy.

As a part of our search for new modulators of MDR, we discovered that 7-*O*-benzoylpyripyropene A (7-*O*-BzP) enhances the cytotoxicity of vincristine and adriamycin in vincristine-resistant KB cells (VJ-300) and adriamycin-resistant P388 cells (P388/ADR). Fungal metabolites pyripyropenes were originally isolated as potent inhibitors of acyl-coA: cholesterol acyltransferase (ACAT) by our group[12~14], and 7-*O*-BzP were recently synthesized as one of the derivatives[15,16]. Here we report the enhancement of sensitivity of VJ-300 cells and P388/ADR cells to vincristine or adriamycin by 7-*O*-BzP.

Materials and Methods

Drugs

7-*O*-Benzoylpyripyropene A (7-*O*-BzP) was prepared as described previously[15,16]. Vincristine was purchased from Shionogi Co., Ltd. (Osaka, Japan). Adriamycin was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). [G-^{3}H]Vincristine sulphate (7.8 Ci/mmol) and [^{3}H]azidopine (47.0 Ci/mmol) were purchased from Amersham Japan Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

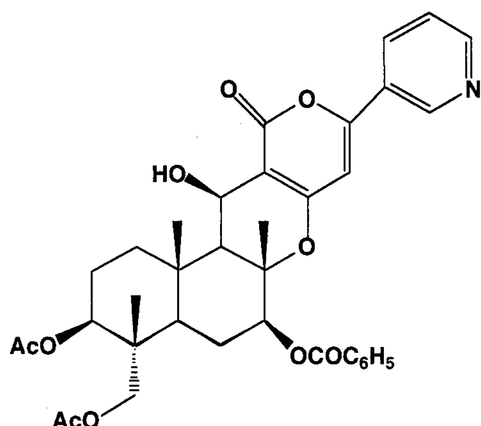
Cell Culture and Drug Treatment

Vincristine sensitive human epidermoid carcinoma cells

Dedicated to the memory of Sir Edward Abraham.

† Present address: Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cardiovascular Research Lab., 52 Oun-dong, Yusong-gu, Taejon, 305-333 Korea.

Fig. 1. Structure of 7-*O*-benzoylpyripyropene A (7-*O*-BzP).



(KB-3-1-4) and vincristine resistant human epidermoid carcinoma cells (VJ-300)[17] were obtained from Prof. M. KUWANO (Department of Biochemistry, Kyushu University School of Medicine). Adriamycin sensitive mouse leukemia cells (P388/S) and adriamycin resistant mouse leukemia cells (P388/ADR)[18] were kindly provided by Dr. M. INABA (Cancer Research Institute).

KB cells and P388 cells were maintained in culture flasks in MEM medium supplemented with 10% fetal bovine serum or RPMI 1640 medium supplemented with 10% FCS, respectively. For the *in vitro* drug treatment experiments, tumor cells (2×10^4 cells/ml for KB cells and 1×10^4 cells/ml for P388 cells) were seeded in 0.2 ml of culture medium/well in 96-well plates (Corning Glass Works). To determine the cytotoxicity of 7-*O*-BzP, cells suspended in 200 μl of the medium ($1\sim2\times10^4$ cells/ml) were plated in a 96-well culture plate (Corning) and incubated for 24 hours at 37°C in a 5% CO_2-95% air atmosphere. The cells were treated in triplicate with graded concentrations of antitumor agents in the absence or presence of 7-*O*-BzP and were then incubated in a CO_2 incubator at 37°C for 72 hours. The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was used to measure the cytotoxic effect[19].

Assay for [^{3}H]Vincristine Uptake in Cancer Cells

Suspensions of KB-3-1-4 and VJ-300 cells (1×10^6 cells/ml) suspended in growth medium containing 10 mM HEPES buffer were incubated at 37°C with 30 nM [^{3}H]vincristine (7.8 Ci/mmol) in the presence or absence of either 7-*O*-BzP (6.25 or 0.4 μg/ml) or verapamil (5 μg/ml). At various intervals, the amount of intracellular [^{3}H]vincristine was determined as described by SUGIMOTO *et al.*[20]. In brief, 0.3 ml aliquots were transferred onto an oil layer (0.5 ml) consisting of Toray Silicon SH550 and oil paraffin (4 : 1) in a 1.5 ml microtube. After centrifugation, the supernatant fluid was removed. The cells comprising the pellet were then lysed with 0.3 ml of 0.5 N KOH and the radioactivity was counted by liquid scintillation system.

Assay for Photoaffinity Labeling of Plasma Membrane with [^{3}H]Azidopine

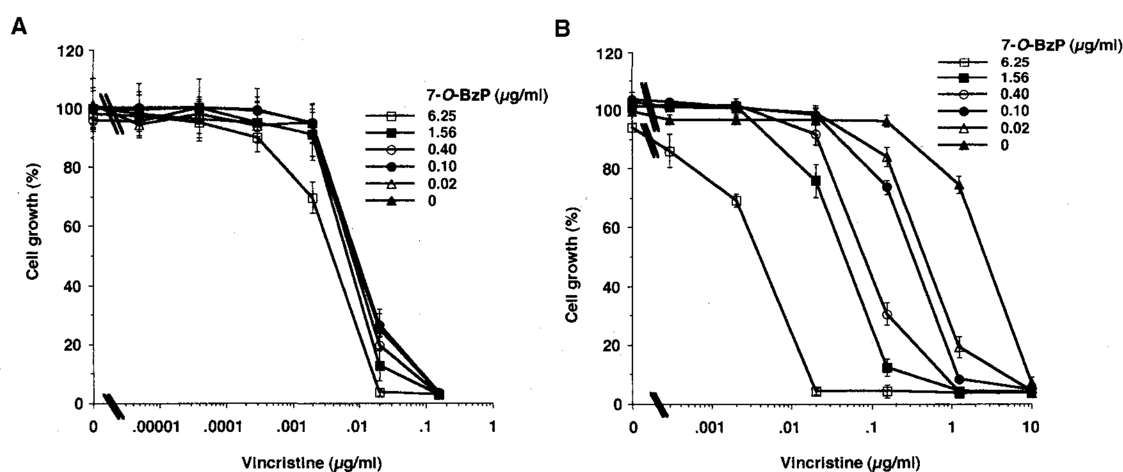
Preparation of the plasma membranes was performed as described by YANG *et al.*[21], with slight modifications. In brief, cells were washed with phosphate-buffered saline (0.15 M NaCl and 20 mM sodium phosphate, pH 7.4) and were homogenized in 10 mM Tris-HCl, pH 7.4, 250 mM sucrose and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at $3000\times g$ for 10 minutes at 4°C. The supernatant fraction was overlaid on 35% sucrose and centrifuged at $18,000\times g$ for 60 minutes. The membrane fraction at the interface was then centrifuged at $100,000\times g$ for 70 minutes. The pellet was resuspended (2 mg/ml) and photolabeled in 50 mM Tris-HCl, pH 7.4, containing protease inhibitors (0.1 mM PMSF, 1% aprotinin and 10 μg/ml leupeptin) and 200 nM [^{3}H]azidopine (47.0 Ci/mmol). The mixture was preincubated for 1 hour at 25°C and then irradiated for 15 minutes at 4°C with UV lamp (254 nm) at distance of 8 cm. Photolabeled membranes were analyzed by SDS-PAGE on 5~20% gradient gels by a modification of the method of LAEMMLI[22]. A total 50 μg of protein was loaded onto each lane. The gel was fixed in a mixture of 25% isopropyl alcohol and 10% acetic acid, treated with the fluorographic reagent amplify (Amersham Japan, Ltd.) for 30 minutes, dried, and then exposed for 14 days at −70°C using Kodac XAR-5 film.

Results and Discussion

Enhancement of Sensitivity of MDR Tumor Cells to Drugs by 7-*O*-BzP

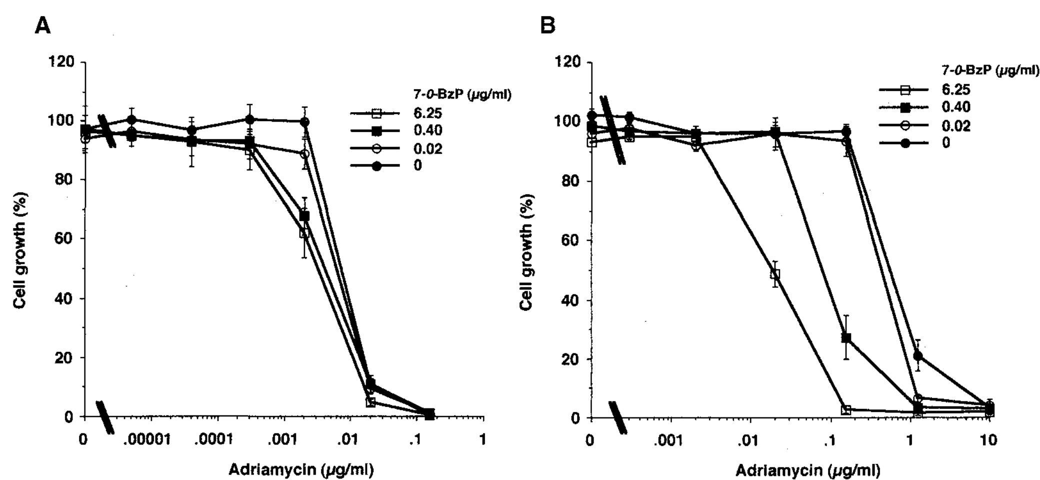
We examined the effect of 7-*O*-BzP on the sensitivity of KB-3-1-4 and VJ-300 cells to vincristine and of P388/S and P388/ADR cells to adriamycin. The IC_{50} values of vincristine for KB-3-1-4 and VJ-300 cells were 8.5 ng/ml and 2.8 μg/ml, respectively, in the absence of 7-*O*-BzP (Fig. 2). The sensitivity to vincristine was enhanced 700-fold by 6.25 μg/ml of 7-*O*-BzP in VJ-300 cells with the IC_{50} value of vincristine being 4.0 ng/ml (Fig. 2B) in the presence of 7-*O*-BzP. On the other hand, the IC_{50} values of adriamycin

Fig. 2. Effect of 7-*O*-BzP on sensitivity of KB-3-1-4 and VJ-300 cells to vincristine.



Vincristine-sensitive KB-3-1-4 cells (A) and -resistant VJ-300 cells (B) were treated in triplicate with graded concentrations of vincristine in the absence (control) or presence of 7-*O*-BzP.

Fig. 3. Effect of 7-*O*-BzP on sensitivity of P388/S and P388/ADR cells to adriamycin.

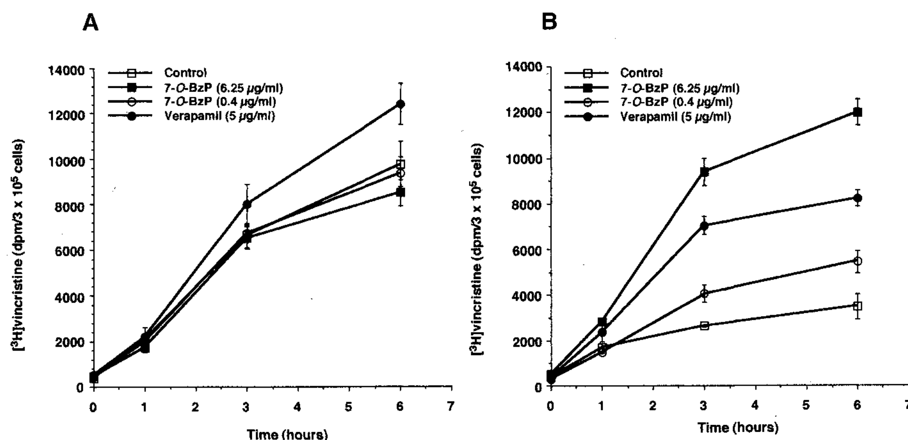


Adriamycin-sensitive P388/S cells (A) and -resistant P388/ADR cells (B) were treated in triplicate with graded concentrations of adriamycin in the absence (control) or presence of 7-*O*-BzP.

for P388/S and P388/ADR cells were 7.0 ng/ml and 0.6 μg/ml, respectively, in the absence of 7-*O*-BzP (Fig. 3). The sensitivity to adriamycin was enhanced 30-fold by 6.25 μg/ml of 7-*O*-BzP in P388/ADR cells and the IC_{50} value of adriamycin being 18.0 ng/ml (Fig. 3B) in the presence of 7-*O*-BzP. 7-*O*-BzP alone had no significant effect on the growth of KB cells and P388 cells at 25 μg/ml (data not shown). The results suggest that 7-*O*-BzP (6.25 μg/ml) completely reversed resistance against vincristine and adriamycin in VJ-300 cells and P388/ADR cells, respectively. On the other hand, the IC_{50} values of vincristine and adriamycin decreased only 2.3-fold by 6.25 μg/ml of 7-*O*-BzP in KB-3-1-4 cells and P388/S cells (Fig. 2A and Fig. 3A).

Previously, we reported the circumvention of MDR of two new compounds, andrastin A[9] and kopsiflorine[11].

Fig. 4. Effect of 7-*O*-BzP on the uptake of [^{3}H]vincristine in KB-3-1-4 and VJ-300 cells.



KB-3-1-4 (A) and VJ-300 cells (B) suspended in growth medium containing 10 mM HEPES buffer were incubated at 37°C with 30 nM [^{3}H]vincristine (7.8 Ci/mmol) in the presence or absence (control) of either 7-*O*-BzP or verapamil. Cells associated [^{3}H]vincristine was determined as described in Materials and Methods.

Although these compounds had no cytotoxicity in VJ-300 cells, kopsiflorine and andrastin A caused a 5-fold and a 20-fold increase in the activity of vincristine in the cells, respectively. However, the recovery from vincristine resistance was not complete. In contrast to this, despite of no cytotoxicity, 7-*O*-BzP completely recovered the activity of vincristine in VJ-300 cells. MDR antagonist such as calcium channel blocker, cyclospsorine A have cytotoxicity *in vitro*; nevertheless it is considered that they are a suitable drug for clinical trials of malignancies[23~25]. Therefore, these results strongly suggest that 7-*O*-BzP may be a potential candidate for circumvention of MDR *in vivo*.

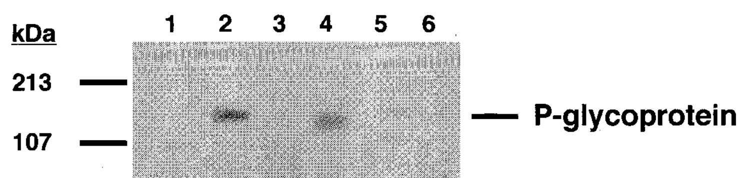
Accumulation of [^{3}H]Vincristine in MDR Tumor Cells by 7-*O*-BzP

Since cellular accumulation of anti-cancer drugs is decreased in MDR tumor cells, the intracellular amount of [^{3}H]vincristine in KB-3-1-4 and VJ-300 cells was measured with or without addition of 7-*O*-BzP. [^{3}H]Vincristine was accumulated time-dependently in KB-3-1-4 cells, and 7-*O*-BzP or verapamil had no significant effect on [^{3}H]vincristine accumulation (Fig. 4A). On the other hand, the amount of [^{3}H]vincristine in VJ-300 cells was about one-third that of KB-3-1-4 cells (Fig. 4), a significant accumulation of [^{3}H]vincristine was observed following the addition of 7-*O*-BzP (6.25 and 0.4 μg/ml) and verapamil (5 μg/ml) in VJ-300 cells (Fig. 4B). This result suggests that the potentiating effect of cytotoxicity in VJ-300 cells (Fig. 2B) was due to enhancement of intracellular accumulation of vincristine by 7-*O*-BzP.

Inhibition of [^{3}H]Azidopine Photolabeling of P-Glycoprotein by 7-*O*-BzP

It was reported that dihydropyridine azidopine photolabels P-glycoprotein in plasma membranes of MDR cells and that this labeling is inhibited by various P-glycoprotein substrates including vinblastine, andrastin A[9], kopsiflorine[11] and some calcium channel blockers[23~25]. Since overexpression of 170 kDa P-glycoprotein in many MDR cell lines is correlated with MDR[26~28], we investigated whether 7-*O*-BzP inhibited the [^{3}H]azidopine photolabeling of P-glycoprotein. [^{3}H]Azidopine specifically labeled a 170 kDa P-glycoprotein in VJ-300 cells and P388/ADR cells but not KB-3-1-4 cells and P388/S cells. 7-*O*-BzP (6.25 μg/ml) completely inhibited the binding of [^{3}H]azidopine to P-glycoprotein in VJ-300 cells and P388/ADR cells (Fig. 5). On the other hand, 7-*O*-BzP was firstly synthesized as an inhibitor of ACAT (IC_{50}: 85 ng/ml) *in vitro* but it has no effect on this cellular model (data not shown). Furthermore, the structure-activity relationships of pyripyropene analogs between inhibition effect of ACAT and reversal effect of MDR was not corresponded[29]. Therefore it was regarded that ACAT and P-glycoprotein function may not be related.

Fig. 5. Effect of 7-*O*-BzP on [^{3}H]azidopine photolabeling of P-glycoprotein.



KB-3-1-4 (lane 1), P388/S (lane 3), VJ-300 (lane 2 and 5) and P388/ADR (lane 4 and 6) membrane vesicles (50 μg of protein) were incubated with 200 nM [^{3}H]azidopine in the absence (lane 1~4) or presence of 7-*O*-BzP at 6.25 μg/ml (lane 5 and 6).

These results suggest that the reversal effect of 7-*O*-BzP on MDR tumor cells is due to at least its direct interaction with P-glycoprotein. The resulting increase in cellular accumulation of anti-cancer drugs lead to a high cytotoxic potency.

Acknowledgments

We thank Dr. M. KUWANO (Department of Biochemistry, Kyushu University School of Medicine) and Dr. M. INABA (Cancer Research Institute) for providing the human KB epidermoid carcinoma cells and P388 mouse leukemia cells, respectively. This study was supported in part by a Grant-in Aid from the Ministry of Education, Science Sports and Culture, Japan and funds from the Japan Keirin Association.

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