Enhancement of Drug Accumulation by Andrastin A Produced by Penicillium sp. FO-3929 in Vincristine-resistant KB Cells

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In the course of our screening for compounds that reverse multidrug resistance, we found that the cytotoxicity of vincristine was enhanced 1.5 ~ 20-fold depending on the concentration of andrastin A in vincristine-resistant KB cells (VJ-300). Andrastin A alone had no effect on the growth of drug sensitive KB cells and VJ-300 cells. On the other hand, andrastin A (25 and 50 µg/ml) significantly enhanced accumulation of [3H]vincristine in VJ-300 cells. Andrastin A (50 µg/ml) completely inhibited the binding of [3H]azidopine to the P-glycoprotein in VJ-300 cells. The result suggests that andrastin A directly interacts with P-glycoprotein and inhibits the efflux of antitumor agents in drug resistant cells.

Tumor cells having acquired resistance to an anticancer agent generally show cross-resistance to a wide variety of structurally and mechanistically unrelated antitumor agents. A major mechanism of this type of resistance is often associated with the overexpression of plasma membrane phosphoglycoprotein (P-glycoprotein). P-glycoprotein, a product of the multidrug resistance 1 (MDR 1) gene, is a membrane-associated active transport protein that utilizes ATP hydrolysis to pump cytotoxic drugs out of cells, reducing the intracellular concentration of the drug and, as a result, toxicity. Therefore, P-glycoprotein-mediated MDR appears to be an important clinical component of tumor resistance in chemotherapy.

Recently, many classes of chemicals, including verapamil and some other calcium channel blockers, cyclosporins, steroid hormones, and other compounds, were found to enhance the intracellular accumulation and cytotoxic action of P-glycoprotein transported drugs. 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 compounds from microorganisms, we discovered that andrastin A enhances the cytotoxicity of vincristine in vincristine-resistant VJ-300 cells. Andrastins were recently isolated from Penicillium sp. FO-3929 as protein farnesyltransferase inhibitors by our group. Here we report the enhancement of sensitivity of vincristine-resistant KB cells (VJ-300) to vincristine by andrastin A and the mechanism of action of andrastin A on VJ-300 cells.

Materials and Methods

Drugs
Andrastin A was prepared as described previously. Vincristine was purchased from Shionogi Co., Ltd. (Osaka, Japan). [G-3H]Vincristine sulphate (7.8 Ci/mmol) and [3H]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
The human KB epidermoid carcinoma cells (KB-3-1-4) and VJ-300 cells were obtained from Prof. M. KUWANO (Department of Biochemistry, Kyushu University School of Medicine).
of Medicine).

KB-3-1-4 and VJ-300 cells were maintained in culture flasks in MEM medium supplemented with 10% fetal bovine serum and kanamycin (100 µg/ml). For the in vitro drug treatment experiments, tumor cells (2 × 10⁴ cells) were seeded in 0.2 ml of culture medium/well in 96-well plates (Corning Glass Works). The cells were treated in triplicate with graded concentrations of antitumor agents in the absence or presence of andrastin A and were then incubated in a CO₂ 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 (15).

**Cellular Accumulation of [³H]Vincristine**

Suspensions of KB-3-1-4 and VJ-300 cells (1 × 10⁶ cells/ml) in growth medium containing 10 mM HEPES buffer were incubated at 37°C with 30 nM [³H]vincristine (7.8 Ci/mmoll) in the presence or absence of either andrastin A (25 or 50 µg/ml) or verapamil (5 µg/ml). At various intervals, the amount of intracellular [³H]vincristine was determined as described by Sugimoto et al. (16). 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.

**Preparation and Photoaffinity Labeling of Plasma Membranes**

Preparation of the plasma membranes was performed as described by Yang et al. (17), 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 x g for 10 minutes at 4°C. The supernatant fraction was overlaid on 35% sucrose and centrifuged at 18,000 x g for 60 minutes. The membrane fraction at the interface was then centrifuged at 100,000 x 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 [³H]azidopine (47.0 Ci/mmoll). 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 (18). A total 50 µg of protein was loaded onto each lane. The gel was fixed in a mixture of 25% isopropl 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 Kodak XAR-5 film.

**Results and Discussion**

Reversal of Vincristine Resistance by Andrastin A

We examined the effect of andrastin A on the sensitivity of KB-3-1-4 and VJ-300 cells to vincristine. KB-3-1-4 and VJ-300 cells were incubated with increasing concentrations of vincristine in the presence of andrastin A for 72 hours and the drug toxicity was determined by the MTT cytotoxicity assay. The IC₅₀ values of vincristine for KB-3-1-4 and VJ-300 cells were 1.3 and 600 ng/ml, respectively, in the absence of andrastin A (Fig. 2). Andrastin A alone had no significant effect on the growth of KB-3-1-4 and VJ-300 cells at 50 µg/ml. In the presence of increasing concentration of andrastin A from 6.25 µg/ml to 50 µg/ml, the IC₅₀ values of vincristine decreased 1.5~20-fold depending on the concentration of andrastin A in the VJ-300 cells, but they decreased only 2-fold by the presence of 50 µg/ml of andrastin A in the KB-3-1-4 cells (Fig. 2).

Effect of Andrastin A on the Cellular Accumulation of [³H]Vincristine

The intracellular accumulation of [³H]vincristine in KB-3-1-4 and VJ-300 cells was examined with or without addition of andrastin A to the culture medium. [³H]-Vincristine efficiently accumulated in KB-3-1-4 cells, and 50 µg/ml of andrastin A slightly enhanced cytotoxicity and accumulation of [³H]vincristine (Fig. 2A and 3A), suggesting that the possibility of undetectable expression of P-glycoprotein or the existence of unknown MDR mechanisms in KB-3-1-4 cells because of verapamil also slightly enhanced accumulation of [³H]vincristine (Fig. 2B). Although, [³H]vincristine accumulation was extremely reduced in VJ-300 cells as compared with that of KB-3-1-4 cells (Fig. 3B), a significant accumulation of [³H]vincristine was observed by the addition of andrastin A (25 and 50 µg/ml) and verapamil (5 µg/ml) in VJ-300 cells (Fig. 3B). The result suggests that the potentiating effect of cytotoxicity in VJ-300 cells (Fig. 2B) was due to enhancement of intracellular accumulation of vincristine by andrastin A.
Fig. 2. The effect of andrastin A on drug toxicity in KB-3-1-4 and VJ-300 cells.

The KB-3-1-4 (A) and VJ-300 (B) cells were treated in triplicate with graded concentrations of antitumor agents in the absence (control) or presence of andrastin A.

Fig. 3. Effect of andrastin A on the uptake of [3H]vincristine in KB-3-1-4 and VJ-300 cells.

Suspension of KB-3-1-4 (A) and VJ-300 cells (B) in growth medium containing 10 mM HEPES buffer were incubated at 37°C with 30 nM [3H]vincristine (7.8 Ci/mmol) in the presence or absence (control) of either andrastin A or verapamil.

Inhibitory Effect of Andrastin A on [3H]Azidopine Photolabeling of P-Glycoprotein

The arylazide 1,4-dihydropyridine, azidopine, photolabels P-glycoprotein in plasma membranes of MDR cells and this labeling is inhibited by vinblastine and some calcium channel blockers. Since overexpression of the MDR 1 gene was observed in VJ-300 cells compared with the KB-3-1-4 cells, we investigated whether andrastin A inhibited the [3H]azidopine photolabeling of P-glycoprotein. [3H]Azidopine specifically labeled a 170 KDa P-glycoprotein in VJ-300 cells but not KB-3-1-4 cells, and andrastin A (50 μg/ml) completely inhibited the binding of [3H]azidopine to P-glycoprotein (Fig. 4). Although overexpression of the 170 KDa P-glycoprotein in many MDR cell lines is correlated with multidrug
resistance\(^{20-22}\), the molecular mechanism by which P-glycoprotein mediates the efflux of drugs is presently not understood. However, by using photoactive drug analogue, the direct binding between P-glycoprotein and drugs has been demonstrated extensively\(^{23-25}\). In addition andrastin A was firstly isolated as a protein farnesyltransferase inhibitor but it has no effect on ras processing in cellular model (data not shown). Therefore it was regarded that protein farnesyltransferase and P-glycoprotein function may not be related. These findings, atoning with photolabeling of P-glycoprotein was completely inhibited by andrastin A (50 µg/ml), suggests that andrastin A directly interacts with P-glycoprotein and inhibits the efflux of antitumor agents.

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References


25) Greenberger, L. M.: Major photoaffinity drug labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. J. Biol. Chem. 268: 11471-11475, 1993