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Long-lasting Accumulation of Vinblastine in Inostamycin-treated Multidrug-resistant KB Cells

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Inostamycin, a novel polyether compound, reverses multidrug resistance in KB cells. The mechanism of its action was studied by use of radioactively labeled vinblastine. Inostamycin dose-dependently increased the accumulation of [^{3}H]vinblastine in multidrug-resistant KB-C4 cells at 0.5–2 μg/ml, while it did not enhance accumulation in the drug-sensitive KB-3-1 cells. At a concentration of 1 μg/ml inostamycin inhibited active [^{3}H]vinblastine efflux from KB-C4 cells, but not from KB-3-1 cells, and inhibited [^{3}H]vinblastine binding to KB-C4 membranes with an IC_{50} of 0.94 μg/ml (1.3 μM). Furthermore, [^{3}H]vinblastine accumulated by treatment with 1 μg/ml of inostamycin was resistant to efflux from KB-C4 cells, even after the removal of inostamycin.

Key words: KB cells — Inostamycin — Multidrug resistance

Multidrug-resistant cells show cross-resistance to many structurally unrelated drugs.[1] On the surface of multidrug-resistant cells P-glycoproteins encoded by *mdr*1 are often overexpressed, and are thought to be a cause of drug resistance.[2–5] It has been proposed that P-glycoprotein expels anticancer drugs energy-dependently from inside the drug-resistant cells.[3, 6, 7] To circumvent drug resistance in cancer chemotherapy and to elucidate its mechanism, many chemosensitizers such as verapamil,[6, 8] quinidine,[9] quinacrine,[10] and cyclosporin A[11] have been investigated. Among them, verapamil is the best studied chemosensitizer. It increases drug accumulation by inhibiting the drug efflux and competitively inhibits vincristine binding to the P-glycoprotein in multidrug-resistant K562/ADM cells.[8, 12] But the precise mechanism of P-glycoprotein action and the reason for its broad substrate specificity are still unclear.

In a previous paper, we reported that inostamycin (Fig. 1), a novel polyether compound, reversed colchicine, adriamycin, and vinblastine resistance in KB-C4 cells, which were isolated by stepwise selections with colchicine,[13, 14] and we showed that it inhibited adriamycin efflux and also enhanced the influx of this drug.[15] In this study, to understand the mechanism of inostamycin action more precisely, we have examined its action on vinblastine resistance and compared the results with those for verapamil. We found that inostamycin prevents vinblastine binding to the membranes of multidrug-resistant cells and that its action on vinblastine accumulation lasts longer than that of verapamil.

MATERIALS AND METHODS

Drugs Inostamycin was isolated from *Streptomyces* as reported previously.[16] Colchicine and vinblastine (VBL) were purchased from Sigma; and verapamil came from Wako Chemicals. [G-^{3}H]VBL sulfate (11.7 Ci/mmol) was obtained from Amersham.

Cell culture Human carcinoma multidrug-resistant KB-C4 and sensitive KB-3-1 cells were generous gifts from Dr. T. Tsuruo, Institute of Applied Microbiology, University of Tokyo, with the permission of Dr. I. Pastan, National Cancer Institute. The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum (Cell Culture Laboratories), 100 units/ml penicillin G (Sigma), and 100 μg/ml kanamycin (Sigma) at 37°C in 5% CO_2. KB-C4 cells were cultured in medium containing 4 μg/ml colchicine until a few days before use to maintain their drug resistance.

Growth inhibition assay KB-C4 or KB-3-1 cells were plated at 3×10^4 cells/well in 12-well plates (Costar) in 1 ml of medium. After incubation for 24 h, chemicals were added and the cells were further incubated for 48 h. Then, the medium was removed and the cells were washed twice with Ca^{2+},Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS[−]) and treated with trypsin. Cell numbers were counted with a hemocytometer.

Accumulation of [^{3}H]VBL KB-C4 or KB-3-1 cells were plated at 3×10^5 cells/well in 12-well plates, and incubated at 37°C for 24 h. Cells were washed with PBS[−] and incubated in 0.5 ml of serum-free RPMI 1640 medium (50 mM HEPES: pH 7.4) containing 0.2 μCi/ml (17 nM) [^{3}H]VBL with or without test chemicals for 2 h, according to the method of Fojo *et al.*[6] After incubation at 37°C, the assay medium was removed on ice and the cells were washed twice with ice-cold PBS[−]. The cells were trypsinized, and dissolved in 3 ml of Atomlight (DuPont). Then, the cell-associated radioactivity was counted.

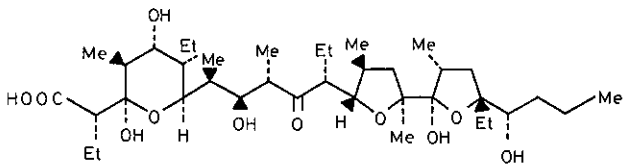

Fig. 1. Structure of inostamycin.

To examine the effect of removal of test chemicals on [^{3}H]VBL accumulation, cells were incubated in the assay medium containing [^{3}H]VBL for 1 h at 37°C, and rapidly washed with ice-cold PBS[−]. Then, the cells were further incubated in [^{3}H]VBL-free fresh medium with or without test chemical for 1 h at 37°C.

Efflux of [^{3}H]VBL Efflux of [^{3}H]VBL was measured as described previously.[15] KB-C4 or KB-3-1 cells (3×10^5 cells/well) incubated for 24 h at 37°C were washed twice with PBS[−]. Then, the cells were incubated in 0.5 ml of glucose-free Hanks' balanced salt solution containing 0.2 μCi/ml (17 n*M*) [^{3}H]VBL and 10 m*M* sodium azide. After incubation at 37°C for 30 min the plate was kept at 4°C for 10 min, and then the medium was removed. The cells were quickly washed twice with ice-cold PBS[−], and 0.5 ml of pre-warmed (37°C) serum-free RPMI 1640 medium (50 m*M* HEPES: pH 7.4) with or without test chemicals was added. After incubation at 37°C for the indicated periods, the cells were washed twice with ice-cold PBS[−], and the radioactivity was counted.

Preparation of plasma membranes KB-C4 plasma membranes were prepared by the method of Cornwell *et al.*[17] with minor modifications. Cells were grown in 145-cm^2 dishes (Greiner), washed with phosphate-buffered saline (PBS), and scraped off in PBS with a rubber policeman. The cells were washed by centrifugation (1000*g*, 10 min) in PBS and then in buffer A (10 m*M* Tris-HCl [pH 7.5], 25 m*M* sucrose, 0.2 m*M* $CaCl_2$, and 0.02 m*M* phenylmethylsulfonyl fluoride [PMSF]). The cells were resuspended in buffer A, kept at 4°C for 15 min, and then homogenized with 40 strokes using a Dounce homogenizer. EDTA was added to the cell homogenate to give a final concentration of 1 m*M*. The cell homogenate was diluted 4 times with buffer B (10 m*M* Tris-HCl [pH 7.5], 25 m*M* sucrose) and then centrifuged at 1000*g* for 10 min. The supernatant was layered onto 35% sucrose solution (10 m*M* Tris-HCl [pH 7.5], 35% sucrose, 1 m*M* EDTA) and centrifuged at 18,600*g* for 30 min. The membrane fraction at the interface was collected, resuspended in buffer B, and then centrifuged at 62,800*g* for 60 min. Pellets were resuspended in 10 m*M* Tris-HCl (pH 7.5) containing 250 m*M* sucrose and 0.1 μ*M* PMSF and stored at −80°C prior to use. Protein concentration was determined by using the Bio-Rad protein assay kit with bovine plasma γ-globulin as the standard.

[^{3}H]VBL binding assay Binding of [^{3}H]VBL to KB-C4 membranes was measured by the method of Naito *et al.*[7] KB-C4 membranes containing 80 μg of protein were incubated at 25°C in 50 μl of assay buffer (10 m*M* Tris-HCl [pH 7.5], 250 m*M* sucrose, 5 m*M* $MgCl_2$, 3 m*M* ATP) containing 0.17 μ*M* [^{3}H]VBL with or without test chemicals. After 20 min, the reaction was stopped by addition of 4 ml ice-cold washing buffer (10 m*M* Tris-HCl [pH 7.5], 250 m*M* sucrose, 5 m*M* $MgCl_2$). Membranes were collected by filtration on a glass microfiber filter (Whatman GF/C) pretreated with 1% bovine serum albumin solution. Then, the membrane was washed with another 4 ml of ice-cold washing buffer. The filters were dried, and the radioactivity was counted.

RESULTS

Effect of inostamycin on vinblastine resistance in KB cells Inostamycin alone inhibited the growth of KB-C4 and KB-3-1 cells with IC_{50}s of 2.5 and 1.9 μg/ml, respectively. As reported before,[15] inostamycin dose-dependently increased the activity of vinblastine toward KB-C4 cells, as shown in Table I.

Effect of inostamycin on [^{3}H]VBL accumulation Figure 2 shows that in the absence of inostamycin the amount of [^{3}H]VBL accumulated in KB-C4 cells was about 6 times lower than that in KB-3-1 cells. When inostamycin was added to the assay medium, the amount of [^{3}H]VBL in KB-C4 cells was increased to the control level of KB-3-1 cells. At a concentration of 2 μg/ml inostamycin increased [^{3}H]VBL accumulation in KB-C4 cells 6-fold. However, it did not affect that of KB-3-1 cells at 0.5–2 μg/ml. Verapamil also increased [^{3}H]VBL accumulation in KB-C4 cells, but the dose of verapamil required to obtain the same effect was about 10 times higher (data not shown).

Table I. Effect of Inostamycin on Vinblastine Resistance in KB Cells

	Dose (μg/ml)	Relative resistance[a]	
		KB-3-1	KB-C4
Inostamycin	0	1.0	366.7
	0.5	0.9	56.7
	1	0.9	15.0
	2	0.7	1.3

a) Relative resistance was calculated by dividing the IC_{50} of vinblastine in KB-C4 or KB-3-1 cells in the presence or absence of inostamycin by the IC_{50} of vinblastine in KB-3-1 cells in the absence of inostamycin. IC_{50} values of vinblastine in KB-3-1 and KB-C4 cells were 0.0012 and 0.44 μg/ml, respectively. Values are means of duplicate determinations.

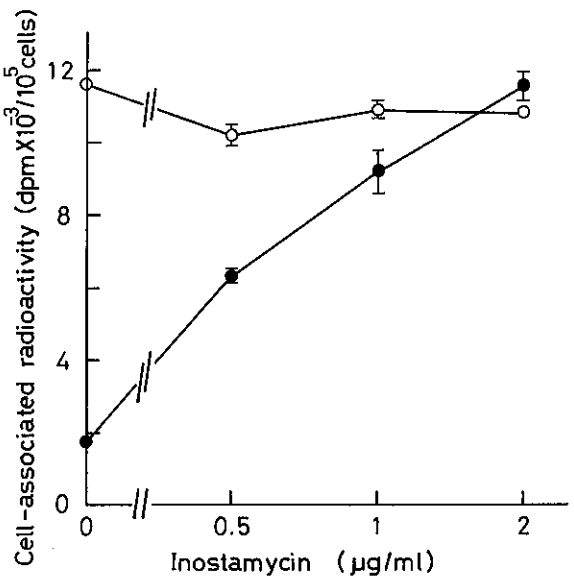

Fig. 2. Effect of inostamycin on accumulation of [^{3}H]VBL in KB cells. KB-C4 (●) or KB-3-1 (○) cells were incubated with 17 n*M* [^{3}H]VBL with or without various concentrations of inostamycin for 2 h. Values are means±SD of triplicate determinations.

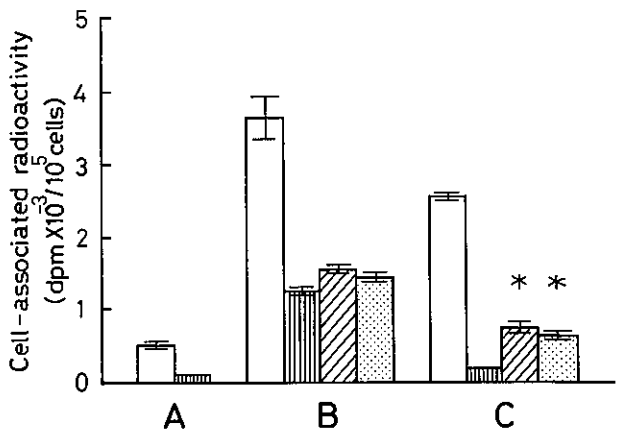

Fig. 3. Effect of removal of inostamycin or verapamil on [^{3}H]-VBL previously accumulated in KB-C4 cells. Once [^{3}H]VBL had accumulated for 1 h (white bars) in the absence of drug (A) or in the presence of 1 μg/ml inostamycin (B) or 10 μg/ml verapamil (C), the cells were washed with PBS[−] and then further incubated without drug (▥) or with 1 μg/ml inostamycin (▨) or 10 μg/ml verapamil (▩) for 1 h. Values are means±SD of triplicate determinations. * Statistically significant difference ($P<0.001$) from (▥) in (C).

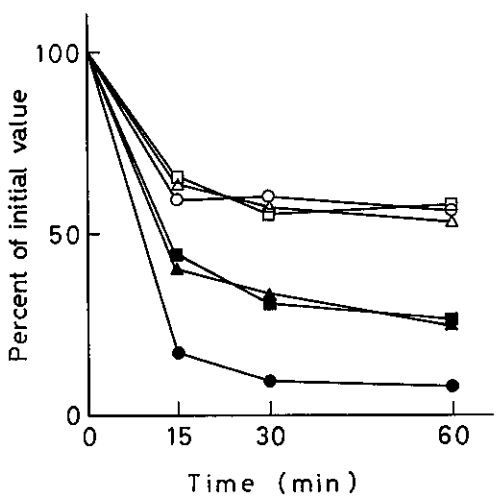

Fig. 4. Inhibition of [^{3}H]VBL efflux from KB cells by inostamycin. KB-C4 (closed plots) or KB-3-1 (open plots) cells were preincubated with 17 n*M* [^{3}H]VBL and sodium azide for 30 min. Then, the cells were incubated without drug (●, ○) or with 1 μg/ml inostamycin (▲, △) or 10 μg/ml verapamil (■, □). Values are means of triplicate determinations.

Next we examined the effect of the removal of inostamycin on [^{3}H]VBL accumulation in KB-C4 cells. First [^{3}H]VBL was accumulated in the presence of 1 μg/ml inostamycin or 10 μg/ml verapamil for 1 h, and then the medium was replaced with [^{3}H]VBL-free medium with or without inostamycin or verapamil, and the cells were further incubated for 1 h. As shown in Fig. 3, when accumulation of [^{3}H]VBL was increased by inostamycin, about 40% of the accumulated [^{3}H]VBL remained even 1 h after the removal of inostamycin (Fig. 3B). The continuous presence of inostamycin or the addition of verapamil after the removal of inostamycin did not markedly increase the amount of [^{3}H]VBL that remained (Fig. 3B). On the other hand, when [^{3}H]VBL accumulation was increased by verapamil, only about 10% of the [^{3}H]VBL remained 1 h after the removal of verapamil (Fig. 3C). The [^{3}H]VBL content was made significantly higher by the continuous presence of verapamil or by the addition of inostamycin (Fig. 3C). Inostamycin had a similar effect on the maintenance of accumulated [^{14}C]adriamycin (data not shown).

Effect of inostamycin and verapamil on [^{3}H]VBL efflux

To obtain sufficient drug accumulation for the assay, we added 10 m*M* sodium azide to the preincubation medium and allowed the drug to accumulate for 30 min. The initial value (0 time) of KB-3-1 cells was 2 times higher than that of KB-C4 cells. The drug efflux was started by replacement of the radioactive medium with fresh medium lacking or containing inostamycin or verapamil. As shown in Fig. 4, in the absence of test chemicals, the amount of [^{3}H]VBL in KB-C4 cells quickly decreased, and by 60 min had become 10% of the initial value; but that of KB-3-1 cells decreased to a lesser extent, and at 15 min it reached the steady state, at 60% of the initial

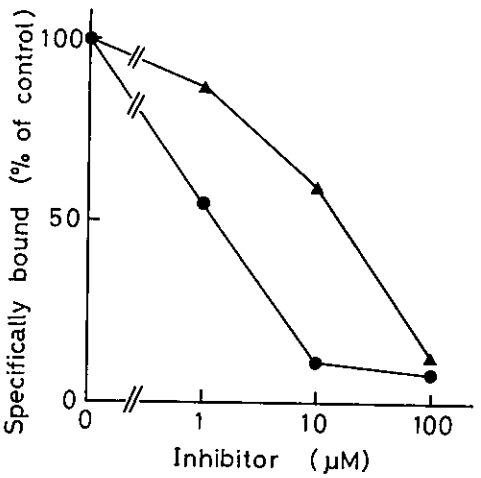

Fig. 5. Inhibition of [^{3}H]VBL binding to plasma membranes of KB-C4 cells. KB-C4 membranes were incubated with 0.17 μM [^{3}H]VBL and inostamycin (●) or verapamil (▲) at 25°C for 20 min and then processed as described in "Materials and Methods." Values are means of triplicate determinations.

value. When we added inostamycin at 1 μg/ml into the efflux medium, the active efflux of [^{3}H]VBL from KB-C4 cells was inhibited and at 60 min 25% remained. Verapamil at 10 μg/ml also inhibited the efflux from KB-C4 cells like inostamycin. On the other hand, the drug efflux from KB-3-1 cells was not influenced by either chemical.

Inhibition of [^{3}H]VBL binding to KB-C4 plasma membranes by inostamycin The [^{3}H]VBL specifically bound to the membranes was determined by subtraction of non-specifically bound material, which was assayed in the presence of excess (100 μM) cold VBL, from the total bound. The dose-response relationships of inostamycin and verapamil are shown in Fig. 5. Inostamycin decreased the specific binding of [^{3}H]VBL to KB-C4 membranes with an IC_{50} of 0.94 μg/ml (1.3 μM). Verapamil also inhibited the binding, with an IC_{50} of 7.4 μg/ml (15 μM).

DISCUSSION

It has been reported that common features shared by anticancer drugs and chemosensitizers acting on P-glycoprotein include a basic nitrogen atom and two planar aromatic rings.[18, 19] Inostamycin, being a polyether compound, does not have these features. Therefore, mechanistic studies with polyether compounds such as inostamycin may give some new insights into the nature of drug resistance.

In our assay system, the active efflux of [^{3}H]VBL from KB-C4 cells was inhibited about 50% by inostamycin, although it restored the accumulation rather completely. When the concentrations of inostamycin and verapamil were increased to 2 μg/ml and 10 μg/ml, respectively, the inhibitory effects on the drug efflux were not enhanced. Our preliminary results indicated that inostamycin increase passive transport of vinblastine in KB-C4 cells, as we had observed for adriamycin.

Although inostamycin enhanced the accumulation of adriamycin even in KB-3-1 cells in our previous report,[15] it showed no effect on the vinblastine accumulation. This discrepancy may be due to the difference of membrane affinity between the two drugs. Adriamycin is considered to bind to the membrane in a rather non-specific manner. In fact, unlike with vinblastine, inostamycin and verapamil could not inhibit adriamycin binding to the KB-C4 membranes (data not shown).

[^{3}H]VBL that had accumulated for 1 h in the presence of verapamil was mostly lost after removal of the verapamil, but not in its continuous presence. Verapamil would be effective only in the co-presence of anticancer drugs. It is reported that the accumulation of [^{3}H]-verapamil in multidrug-resistant K562/ADM cells was 3 times lower than that in drug-sensitive K562 cells.[20] Verapamil is presumably a substrate for P-glycoprotein and thus would be removed from the cells in the place of anticancer drugs. On the other hand, [^{3}H]VBL accumulation once increased by inostamycin remained at about 40%, even after the inostamycin had been removed. [^{3}H]VBL accumulated by inostamycin appeared to be resistant to efflux. At least two possible explanations for this phenomenon might be suggested. First, inostamycin might inhibit P-glycoprotein action irreversibly. Second, inostamycin, once it has entered the cell, may not be easily expelled. Our preliminay experiments showed no difference in the accumulations of inostamycin in KB-C4 and KB-3-1 cells, which were extracted and analyzed for the drug by thin-layer chromatography. Therefore, unlike verapamil, inostamycin may not be a substrate for P-glycoprotein and may not be removed from the drug-resistant cells.

We are studying the effect of inostamycin on drug binding to P-glycoprotein by using azidopine. Since long-lasting accumulation of the drug should be advantageous for clinical use, we are also planning to examine the effect of inostamycin on drug resistance *in vivo*.

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