医薬品相互作用研究
一 般 論 文
24(4) 9 -19 (2001)

Preliminary Observation of Enhancement of Vinblastine Activity in Multidrug Resistance Cells by Haloperidol and Dihydrohaloperidol

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(Received February 13, 2001
Accepted March 15, 2001)

Abstract

In order to identify clinically effective drugs for reversing multidrug resistance (MDR) mediated by P-glycoprotein, haloperidol, an antipsychotic, was investigated for its efficacy to reverse MDR in cells overexpressing P-glycoprotein. A nontoxic concentration of haloperidol (1-30 μM) enhanced the cytotoxic effects of vinblastine (VBL), concentration-dependently, in VBL-resistant human leukemia (K562/VBL) cells, but had no effect in the parent cells. Haloperidol also enhanced the cytotoxicities of epirubicin, doxorubicin and actinomycin D in the K562/VBL cells, but not those of idarubicin and cisplatin; this enhancement was less than that of the VBL toxicity in the VBL-resistant tumor line. Haloperidol increased the intracellular accumulation of VBL in the K562/VBL cells, and the binding of [^{3}H]-azidopine to the cell-surface protein, P-glycoprotein, was inhibited by haloperidol in a concentration-dependent manner. Haloperidol was less potent than verapamil. Thus, haloperidol appeared to potentiate anticancer agents through the reversal of MDR by competitively inhibiting drug-binding to P-glycoprotein. In contrast, the main metabolite of haloperidol, dihydrohaloperidol, without antipsychotic activity, had less of an effect. Therefore, haloperidol might be useful for reversing drug-resistance.

Key words —— K562 cell, haloperidol, dihydrohaloperidol, vinblastine, multidrug resistance

Introduction

A major problem in the clinical application of vinca alkaloids as antitumor agents is the development of multidrug resistance (MDR) in tumors. It is well known that myelomas and lymphomas frequently develop drug resistance, despite an initial response to chemotherapy. The typical MDR phenotype includes cross-resistance to anthracyclines, vinca alkaloids, podophyllotoxins, taxans, and other cytotoxic compounds, with increased expression of the membrane protein, P-glycoprotein. P-Glycoprotein

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functions as an efflux pump on the surface membranes of resistant cells, and transports antitumor agents out of the cancer cell. The reversal or modulation of multidrug resistance has been achieved using a wide variety of pharmacological agents including calcium channel blockers, cyclosporins, cardiovasucular drugs, steroid analogs, antibiotics, calmodulin inhibitors, and antimalarials. These agents reverse the MDR by competitively inhibiting the binding of the chemotherapeutic agents to P-glycoprotein, a mechanism unrelated to their primary pharmacological roles[1,2].

We have been investigating the effects of several drugs used in combination with an antitumor drug, to promote antitumor activity. There has been interest in the ability of calcium channel blocking agents and calmodulin inhibitors to enhance the responses of drug-resistant tumor cells to vinca alkaloids and anthracycline antibiotics[3,4]. The antipsychotic drug, haloperidol is one of the most potent calmodulin inhibitors among the tranquilizers currently approved for clinical use[5-7]. A well-known dopaminergic antagonist has long been used clinically for purposes other than the reversal of MDR. The main metabolite of haloperidol in humans and rats is dihydrohaloperidol, which has no antipsychotic activity. Since the function of P-glycoprotein was inhibited by some calmodulin inhibitors in multidrug resistant cells, we examined whether or not haloperidol could potentiate the effect of vinblastine and overcome the MDR in cultured drug-sensitive human cancer cell lines. Furthermore, we compared the activity of dihydrohaloperidol to overcome multidrug resistance.

Materials and Methods

Cell lines and culture A human leukemic cell line (K562) and a multidrug-resistant clone derived from it (K562/VBL) were used. The properties of K562/VBL and the parental K562 have been described previously[7,8]. The cells were routinely kept in RPMI 1640 medium, supplemented with 10% fetal calf serum and penicillin G (100U/mL)/streptomycin (100 μg/mL) at 37℃ in a humidified 5% CO_2-95% air incubator under standard conditions.

Chemicals Vinblastin (Exal, Shionogi Pharmaceutical Co., Osaka, Japan) and doxorubicin (Adriamycin, Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan) were obtained commercially. Haloperidol and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrohaloperidol was synthesized in our laboratories. The drugs were dissolved in dimethylsulfoxide (at 10 nmol) and stored at -20 ℃. Light exposure was kept to a minimum for all drugs used. [^{3}H]-Vinblastine and [^{3}H]-azidopine were purchased from Amersham LIFE SCIENCE (Tokyo, Japan).

Cytotoxicity assay Cells were inoculated into each well of 96-well plates with 50 μL of the culture medium at $4x10^4$ cells. After an overnight incubation, 50 μL of antitumor drug solution or 25 μL of vinblastine solution and 25 μL of multidrug-resistant blocker solution, at final concentration of indicated dose were added in triplicate and the plates incubated for 48 hr. The cytotoxicity was determined by the trypan

blue exclusion[9,10]. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue and the cells were counted with a haemocytometer. The results are expressed as the percentage of dead cells (ratio of stained cells vs. the control number of cells).

Accumulation and efflux of vinblastine In the accumulation experiment, the cells ($1x10^6$) were suspended in 1 ml 20 mM HEPES-buffered G-medium (pH 7.4) and incubated in the presence of 37 kBq [^{3}H]-vinblastine at 37℃. After the incubation, they were chilled on ice and collected by centrifugation (2000 rpm x 5 min) at 4℃. The cells were then washed twice with chilled PBS (pH 7.4). The vinblastine accumulated in the cells was measured for radioactivity after solubilization with NaOH and neutralization with acetic acid[11]. In the efflux experiment, the cells were loaded with 20 nM [^{3}H]-vinblastine (74 kBq) by incubation in glucose-free Hanks' solution (pH 7.4) containing 10 mM NaN_3. The cells were washed once with chilled PBS and incubated, with or without the test drugs, in 20 mM HEPES-buffered G-medium (pH 7.4). After incubation of the cells for the designated periods at 30 ℃, the radioactivity remaining in the cells was measured as described above. The results were expressed as the percentage of vinblastine in the cells, relative to the amounts initially loaded.

Binding of [^{3}H]-azidopine to the plasma membrane of K563/VBL cells Membrane vesicles from the K562/VBL cells were prepared as described[12] from cells grown in 24-24 mm dishes under standard growth conditions. The protein concentrations were determined by the method of Bradford[13]. The membrane vesicles (50 μg of protein) were photolabeled in 40 mM Tris-HC1 buffer (pH 7.2) containing 4% dimethylsulfoxide and 100 nM [^{3}H]-azidopine in a final volume of 25 μL in the presence or absence of the test drugs. The photolabeled membranes were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using gradient gels (4-20 %). A total of 20 μg of protein was loaded onto each lane. The gel was fixed, treated with the fluorographic reagent Amplify (Amersham Japan), dried, and then exposed to Kodak XAR-5 film at -80 ℃ for 12-14 days.

Statistical analysis Statistical significance was assessed using a Student's t-test for unpaired two tailed comparisons. A P-value of less than 0.05 was considered significant.

Results

Chemosensitization by haloperidol and dihydrohaloperidol The drug-resistant cell line K562/VBL was established by growing the parental cell line K562 in a medium in which the concentration of vinblastine was increased stepwise for several months, up to the indicated value. The cells thus selected were able to grow in the presence of the highest possible concentrations of vinblastine (0.3 μM). The cells were cultured for at least 3 days under the same conditions prior to use in the sensitization assays[7,8]. A typical set of data from an experiment to determine the vinblastine sensitivity of the parent K562 cells and the vinblastine-resistant subline K562/VBL is shown in Fig. 1. Each value represents the mean value of 3 separate

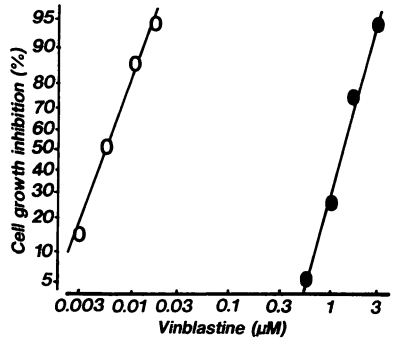

Fig. 1 Effect of Vinblastine (VBL) on the Survival of K562 and K562/VBL Cells

A human chronic myelogenous leukemia cell line, K562, was obtained from Cancer Cell Repository (Tohoku University). Cells were cultured in RPMI 1640 supplement with 10% fetal bovine serum, and 100 U/mL penicillin and 100 μg/mL streptomycin in a CO_2 incubator at 37℃. The K562 cell line resistant to vinblastine (K562/VBL) used in our study was selected in the presence of increasing doses of VBL without prior mutagenization. The K562/VBL cell line was maintained in medium containing 0.3 μM VBL. The K562/VBL was washed out at least 2 day before the experiments. For the evaluation of cell survival, cells were inoculated in culture plates and incubated for 24 h at 37℃, then they were treated with VBL at the indicated concentration in the K562 (open circles) or K562/VBL (closed circles) cells, and incubated for 2 day before cytotoxicity assay. The level of cell death was expressed as the percent ratio of stained vs. unstained cells by the trypan blue exclusion test. Cells unable to exclude trypan blue (stained) are referred to as dead cells. The median concentration of tumor cells by 50% (IC_{50}) was determined by plotting the logarithm of the drug concentration versus the growth rate (percentage of control) of the treated cells. SD was usually within 10% of the mean value. Fold resistance was determined by dividing the IC_{50} for the resistance line K562/VBL by the IC_{50} for the sensitive K562 cells.

wells and the lines were fitted to the points by eye. In this example, the IC_{50} (dose to reduce trypan blue exclusion to 50% of control) is 0.006 μM for the parent cells and 1.2 μM for K562/VBL. The ratio of these values is defined as the resistance factor, RF=IC_{50}(resistant subline)/IC_{50}(parent line) and is therefore 200 for the K562/VBL

In order to estimate the chemosensitized effect of haloperidol in the K562/VBL cells, the haloperidol concentration was kept constant while the anticancer drug concentration was varied. The potentiating effects of haloperidol, dihydrohaloperidol and verapmil on vinblastine cytotoxicity are illustrated in Table 1. The IC_{50} values of vinblastine for the K562 and K562/VBL cells were 0.032 and 4.0 μM, respectively, indicating an approximate 125-fold resistance.

In the positive control, verapamil at 1, 6 or 10 μM enhanced the cytotoxicity of vinblastine for the K562/VBL cells, the IC_{50} of vinblastine shifting from 4.0 to 2.1, 1.3 and 0.41 μM, respectively. When haloperidol was added at a final concentration of 1, 6, 10 or 30 μM to the K562/VBL cell cultures, the cytotoxicity of vinblastine was enhanced dose-dependently, and the IC_{50} of vinblastine shifted from 4.0 to 3.0, 2.2, 1.0 and 0.19 μM, respectively, although the degree of enhancement was less than that obtained with verapamil (Table 1).

In contrast, the major metabolite of haloperidol, dihydrohaloperidol, at 1, 6, 10 or 30 μM minimally enhanced the cytoxtoxicity of vinblastine on the K562/VBL cells, the IC_{50} of vinblastine shifting form 4.0 to 4.2, 3.1, 2.1 and 1.2 μM, respectively, although the degree of enhancement was less than that

Table 1 Effect of Haloperidol and Dihydrohaloperidol on Sensitivity of Vinblastine in K562/VBL Cells

Drugs	Dose (μM)	Cell lines	Vinblastine IC_{50} (μM)
Verapamil	0	K562	0.032
	0	K562/VBL	4.0 (125)
	1	K562/VBL	2.1 (64)
	6	K562/VBL	1.3 (39)
	10	K562/VBL	0.41 (11)
	10	K562	0.071 (1)
Haloperidol	1	K562/VBL	3.0 (93)
	6	K562/VBL	2.2 (68)
	10	K562/VBL	1.0 (31)
	30	K562/VBL	0.19 (6)
	30	K562	0.059 (2)
Dihydro-haloperidol	1	K562/VBL	4.2 (131)
	6	K562/VBL	3.1 (96)
	10	K562/VBL	2.1 (65)
	30	K562/VBL	1.2 (37)
	30	K562	0.035 (1)

Cells were grown in suspension culture in the continuous presence of the drug for 48 hr. The experimental conditions and analysis are same as Fig. 1. Each value in parentheses represents the relative resistance to vinblastine.

obtained by haloperidol (Table 1).

Furthermore, as dihydrohaloperidol is a racemic mixture, we investigated the activities of its enantiomers, (R)-dihydrohaloperidol and (S)-dihydrohaloperidol, to potentiate the cytotoxicity of vinblastine. Against K562/VBL, (R)-dihydrohaloperidol was more effective than (S)-dihydrohaloperidol at doses of 10 and 30 μ M. Since (R)-dihydrohaloperidol is more active than its stereoisomer (S)-dihydrohaloperidol, the configuration of the asymmetric C-3 carbon seems to play a role in the anti-MDR activity of dihydrohaloperidol. Such differences in activity on MDR and a decrease of in *in vitro* toxicity have also been reported for the L-and D-2 steroisomers of verapamil[14].

The mechanisms by which haloperidol exerts its action on the drug efflux function of tumor cells have recently been investigated. It has been demonstrated that haloperidol competes with antitumor drugs at a drug-binding site on P-glycoprotein[15]. The difference in the *in vitro* activity of dihydrohaloperidol could result from a different affinity for this site. However, a lipophilic agent such as haloperidol can interact with the membrane lipids of tumor cells and this led to an indirect inhibition of the drug efflux by modification of the membrane fluidity[16].

Furthermore, the effects of haloperidol on

the sensitivities of the K562/VBL cells to doxorubicin, epirubicin, vincristine, actinomycin D, daunorubicin, idarubicin and cisplatin were assayed in the same manner (data not shown). The K562/VBL cells were resistant to much higher concentrations of doxorubicin, epirubicin, vincristine, actinomycin D and daunorubicin than the parental K562 cells. Haloperidol at 1, 6, 10 and 30 μM also enhanced the cytotoxicity of doxorubicin, epirubicin, vincristine, actinomycin D and daunorubicin, although the degree of enhancement was less than that obtained with verapamil, but not idarubicin or cisplatin. Again haloperidol had little or no effect on the sensitivity to various antitumor agents in the sensistive parental clone.

Drug accumulation P-glycoprotein is believed to function as an ATP-dependent drug efflux pump for which drug transport is coupled to ATP hydrolysis. To examine the impairment of drug accumulation by haloperidol, we measured the amount of [^{3}H]-vinblastine accumulated by P-glcoprotein in the K562/VBL cells. The cellular accumulation and retention of [^{3}H]-vinblastine by K562 and K562/VBL is shown in Fig.2. [^{3}H]-Vinblastine accumulated in the cells and reached a plateau within 20 min; the amount of vinblastine in K562/VBL was then about 11% of that in the K562 cells. It can be seen that the K562/VBL cells show a considerably reduced ability to accumulate and retain the labeled drug.

Haloperidol potently increased the accumulation of vinblastine in a dose-dependent manner and dihydrohaloperidol was less effective than haloperidol (Fig. 2a). The effect of verapamil, however, was the most potent.

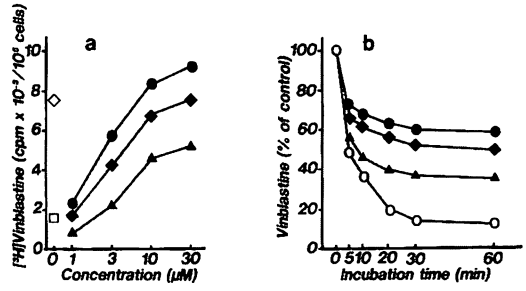

Fig.2 Effect of Haloperidol on the Accumulation and Retention of Vinblastine in K562/VBL Cells

To evaluate the cellular accumulation (a) and retention (b) of vinblastine, cells were incubated with [^{3}H]-vinblastine, and then cellular vinblastine content was determined as outlined in "Materials and Methods".

Left panel; Cells were incubated with [^{3}H]- vinblastine for 20 min in the absence (◇, K562; □,K562/VBL) or presence of verapamil (●), haloperidol (◆) and dihydrohaloperidol (▲). Each point represents the mean of two experiments done in triplicate.

Right panel; K562/VBL cells were incubated with [^{3}H]-vinblastine for 20 min in the absence (○, Control) or presence of verapamil (●, 30 μM), haloperidol (◆ ,30 μM) and dihydrohaloperidol (▲, 30 μM), washed, and resuspended in drug-free medium with (●, ◆ and ▲) and without (○) 30 μM verapamil, haloperidol and dihydrohaloperidol. The percentage of vinblastine retention was obtained from the ratio of the amount of drug retained to that originally accumulated in 20 min. Each point represents the mean of two experiments done in triplicate.

Furthermore, as shown in Fig. 2b, the efflux of vinblastine from K562/VBL was inhibited by haloperidol and dihydrohaloperidol at 30 μM, but the inhibitory effect of haloperidol was the more potent. In

the K562 cells, haloperidol and dihydrohaloperidol did not affect vinblastine accumulation and retention (data not shown). These results indicate that the vinblastine accumulation induced by haloperidol and its metabolites is dependent on the inhibition of efflux. Consequently, the effect of overcoming drug resistance (Table 1) seems to be closely related to the potency of these compounds to induce drug accumulation in cells.

Inhibition of [^{3}H]-azidopine photolabeling of P-glycoprotein by haloperidol and dihydrohaloperidol Azidopine, a photoactive analogue of dihydropyridine, photolabels P-glycoprotein in the plasma membranes of multidrug-resistant cells and the labeling is inhibited by MDR modulators or chemosensitizers[17,18]. By using this photolabeling system, we investigated whether haloperidol inhibited the [^{3}H]-azidopine photolabeling of p-glycoprotein. As shown in Fig. 3, [^{3}H]-azidopine specifically labeled a 170,000-180,000 dalton protein in the K562/VBL cells, but not in the drug-sensitive K562 cells. In the presence of 100 μM verapamil, the radiolabeling of P-glycoprotein was almost completely inhibited, but in the presence of 10 or 100 μM haloperidol and dihydrohaloperidol, it was only partially inhibited, the inhibitory effect of haloperidol being the most potent. This result shows that the inhibitory effect of haloperidol and dihydrohaloperidol are weaker than verapamil.

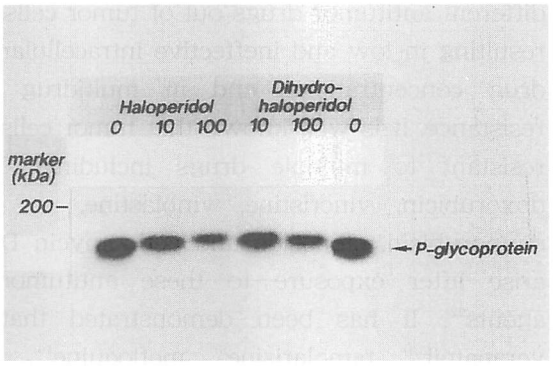

Fig.3 Effect of Haloperidol on [^{3}H]-Azidopine Photolabeling of P-Glycoprotein of Membrane Vesicles from K562/VBL Cells

Typical photolabeling patterns are presented. K562/VBL membrane vesicles (50 μg of protein) were incubated with 100 nM [^{3}H]-azidopine in the absence or presence of either haloperidol or dihydrohaloperidol at the indicated concentrations. After solubilization, photolabeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in "Materials and Methods".

Discussion

Drug resistance is a crucial problem in the treatment of cancer. A part from intrinsically resistant tumors, some sensitive tumors gradually develop drug resistance during multiple cycles of chemotherapy. A variety of mechanisms of drug resistance are considered, including reduced cellular drug accumulation, increased detoxification, intracellular vesicularization of drugs, altered enzymatic activities, down-regulation of target and enhanced DNA repair[19]. The expression of P-glycoprotein in the membranes of tumor cells leads to cross-resistance to chemically unrelated antitumor drugs, because P-glycoprotein has been demonstrated to have more than 2 distinct drug acceptor sites that allosterically couple[20,21]. P-glycoprotein is an ATP-dependent drug efflux pump that transports

different antitumor drugs out of tumor cells, resulting in low and ineffective intracellular drug concentrations, and in multidrug resistance. It is well known that tumor cells resistant to multiple drugs including doxorubicin, vincristine, vinblastine, daunorubicin, etoposide and actinomycin D arise after exposure to these antitumor agents[22]. It has been demonstrated that verapamil[3,4], tamolarizine[9], mefloquine[10], reserpine[23], quinidine[24], phenothiazine derivatives[25], cepharanthine[26], dipyridamole[27], pyridine derivatives[28] and other drugs reverse the multidrug resistance. These drugs are supposed to inhibit the activity of P-glycoprotein to pump out chemotherapeutic agents from the cells through binding with P-glycoproten[29-31]. Verapamil has attracted much attention as a chemosensitizer. Several combination therapies using verapamil plus anthracyclines or vinca alkaloids have been tried[32,33], but verapamil causes serious cardiovascular side effects due to its calcium antagonistic activity[34]. Therefore, a multi-drug resistance blocker with fewer side effects is needed. We examined the reversing effects of haloperidol on multidrug resistance because vinca alkaloids, such as vinblastine, are known to bind P-glycoprotein, and agents capable of binding P-glycoprotein often have chemotherapeutic effects on tumors or reverse multidrug resistance.

It was found that haloperidol exerts reversing effects on multidrug resistance *in vitro*, but, the effect was weaker than that of verapamil *in vitro*. The binding of [^{3}H]-azidopine to P-glycoprotein is a valuable technique for the evaluation of drug binding sites and the elucidation of the mechanism of multidrug resistance. [^{3}H]-Azidopine is often used as a binding agent for P-glycoproten[17,18]. Haloperidol inhibited the azidopine binding less potently than verapamil. Furthermore, haloperidol augmented the intracellular accumulation of vinblastine in tumor cells. Thus, it is considered that haloperidol increases the accumulation of antitumor drugs in tumor cells by inhibiting the pump activity of P-glycoprotein.

Haloperidol, however, had little effect on the vinblastine activity in drug-sensitive parent K562 cells. Haloperidol causes movement disorders such as neuroleptic malignant syndrome, dystonias, and tardive dyskinesia, but it is a drug widely used for the treatment of schizophrenia and other affective disorders. These properties make haloperidol a candidate as a multidrug resistance modifier. Furthermore, the fact that the haloperidol and dihydrohaloperidol show resistance-reversing activity by a mechanism similar to that of verapamil and dihydrohaloperidol is weaker than haloperidol regarding pharmacological activity and side effect suggest that this compound could serve as a mechanistic prove of multidrug resistance modifier.

In conclusion, the antipsychotic drug, haloperidol shows resistance- reversing activity by a mechanism similar to that of verapamil; haloperidol increases the intra-cellular concentrations of chemotherapeutic agents by inhibiting the function of P-glycoprotein in K562/ VBL, but has no effect on the vinblastine activity in drug-sensitive K562 cells.

Acknowledgment

This research was supported in part by a Grant-in-Aid for Science Research Promotion Fund (Japan Private School Promotion Foundation).

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