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Sensitivity to Antitumor Drugs and Vinblastine Binding to Membrane in Rat Ascites Hepatoma AH66 Cells

Shinya WAKUSAWA, Shigeo NAKAMURA, Kohsei INOKO, and Ken-ichi MIYAMOTO*

Research Laboratory for Development of Medicine, School of Pharmacy, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–11, Japan.
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Rat ascites hepatoma AH66 cells have lower sensitivity to *Vinca* alkaloids and anthracycline antibiotics than AH66F cells, a subline of AH66 cells. AH66 cells expressed P-glycoprotein, while the protein was not detectable in AH66F cells. There are two affinity sites for [^{3}H]vinblastine binding in the AH66 cell membrane, while AH66F cells have only one affinity site. The high affinity [^{3}H]vinblastine binding in AH66 cells was inhibited by Adriamycin, verapamil, nicardipine, and reserpine. The high affinity site of the binding may be the multidrug transporter, P-glycoprotein. [^{3}H]Vinblastine binding was not influenced by adenosine 3′,5′-monophosphate (AMP), adenosine triphosphate (ATP), or guanosine triphosphate (GTP). The multidrug resistance in AH66 cells may depend on P-glycoprotein which is not modulated by nucleotide.

Keywords [^{3}H]vinblastine binding assay; rat ascites hepatoma; AH66 cell; P-glycoprotein; nucleotide

Introduction

Multidrug resistance is an important problem in cancer chemotherapy. Multidrug-resistant tumor cells show reduced accumulation of a variety of chemically unrelated drugs, and the cells have been indicated to overexpress P-glycoprotein in the plasma membrane.[1] Recent studies have shown that the protein is photolabeled with a photoactive derivative of vinblastine[2] or the calcium channel blocker azidopine,[3] and the labeling is blocked by verapamil, reserpine, or several other compounds,[3-6] which reverse multidrug resistance *in vitro*. Binding assays have been shown to be useful for characterization of the vinblastine-binding protein in the membrane fraction of resistant cells and the evaluation of drugs that reverse multidrug resistance.[7,8] Rat ascites hepatoma AH66 cells show resistance to vinblastine, actinomycin D, and Adriamycin.[9] We investigated the characteristics of drug binding in AH66 cell membranes compared with a sensitive cell line, AH66F.

Materials and Methods

Materials The radioactive, photoactive vinblastine analogue, *N*-(*p*-azido-3-^{125}I-salicyl)-*N*′-*β*-aminoethylvindesine (1.85 TBq/mmol), [^{125}I]-NASV was generously provided by Dr. H. Hidaka (Nagoya University School of Medicine, Japan). [^{3}H]Vinblastine (592 GBq/mmol) was obtained from Amersham Co., Japan, Ltd. The antitumor drugs used were vinblastine, vincristine (Shionogi & Co., Osaka, Japan), Adriamycin, 5-fluorouracil, mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan), daunorubicin (Meiji Seika Co., Tokyo, Japan), cisplatin (Bristol-Myers Research Institute, Tokyo, Japan), and methotrexate (Sigma Chemical Co., St. Louis, MO, U.S.A.). The following drugs were also obtained commercially: verapamil, nicardipine, reserpine, adenosine triphosphate (ATP), ATP-*γ*S, guanosine triphosphate (GTP), and adenosine 3′,5′-monophosphate (AMP) (Wako Pure Chemical, Industries, Osaka, Japan). Dimethylaminoethylreserpilinate hydrochloride (DMAR) was kindly donated from Hokuriku Seiyaku Co., Ltd., Katsuyama, Japan. H-87 was a kind gift from Dr. H. Hidaka (Nagoya University School of Medicine, Nagoya, Japan).

Cells and Culture Rat ascites hepatoma AH66 and AH66F cells, which were obtained from the Sasaki Institute, Tokyo, Japan, were maintained by weekly passage in the abdominal cavities of female Donryu rats weighing 110—150 g (Nippon SLC, Hamamatsu, Japan). Cells were suspended in a minimum essential medium supplemented with 10% fetal calf serum, and 100 μM kanamycin, and 10^5 cells were seeded in 24-well plastic dishes. The effects of drugs on cell growth were evaluated after consecutive culture for 48 h.

Preparation of Plasma Membrane Cells were harvested from the tumor-bearing rats 7 d after transplantation and were used for experiments after the removal of red cells by repeated washing with phosphate-buffered saline (PBS; pH 7.4) using gentle centrifugation. Membrane was prepared by the method described before.[10]

Protein was measured by the method of Lowry *et al.*[11] using bovine serum albumin as the standard.

Immunoblotting The plasma membrane preparation (100 μg protein) was solubilized in a Laemmli sample buffer,[12] electrophoresed on sodium dodecyl sulfate (SDS)–polyacrylamide 7.5% gel, and transferred onto nitrocellulose membrane filters (Schleicher & Schuell, Dassel, Germany). After it was blocked with 3% gelatin, the membrane was incubated overnight with 10 μg/ml C219 monoclonal antibody against P-glycoprotein[13] (Centocor, Inc., Malvern, PA, U.S.A.), and with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (Organon Teknika Co., West Chester, PA, U.S.A.) for 1 h. Following each incubation, the membrane was washed extensively with PBS containing 0.05% Tween-20. The immunopositive band was made visible in a PBS solution containing 0.5 mg/ml diaminobenzidine and 0.03% H_2O_2.

Photoaffinity Labeling with [^{125}I]NASV The plasma membrane (100 μg protein) was incubated in 100 μl of 40 mM phosphate buffer (pH 7.4), 4% dimethyl sulfoxide, and 7.4 kBq [^{125}I]NASV. Then the photolabeled protein was detected as described previously.[14]

[^{3}H]Vinblastine Binding Assay Cell membrane preparations (30—50 μg protein) were incubated in a buffer containing 5 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.4) with various concentrations of [^{3}H]vinblastine in the presence of 1 mM vinblastine or, in its absence, in a total volume of 0.25 ml for 20 min at 30 °C unless otherwise mentioned. Incubation was then stopped by the addition of 3 ml of the buffer and rapid filtration through glass filters (Whatmann GF/B). Each filter was rapidly washed three times with 3 ml of the buffer, added to 6 ml of toluene–Triton based scintillation fluid, and the radioactivity on the filter was measured. Specific binding was defined as the difference between the amount of radioligand bound in the absence (total binding) and in the presence (nonspecific binding) of 1 mM unlabeled vinblastine. The vinblastine binding depended only slightly on temperature.

Results

The AH66 cell line has been shown to have lower sensitivity to vinblastine than several other rat ascites hepatoma cell lines.[9,15] We investigated the sensitivity of AH66 cells to several other antitumor drugs, and compared it with AH66F cells, which are a subline of AH66 cells. Table I shows that AH66 cells are less sensitive to vinblastine, vincristine, Adriamycin, and daunorubicin than AH66F cells, while there is only a slight difference in their sensitivities to 5-fluorouracil, methotrexate, cisplatin, and mitomycin C. Immunoblot analysis with anti-P-glycoprotein monoclonal antibody C219 showed that AH66 cells express 150—160 kDa P-glycoprotein. [^{125}I]NASV also

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Table I. *In Vitro* Sensitivities to Antitumor Drugs of AH66 and AH66F Cells

Antitumor agent	IC_{50} (nM)		
	AH66F	AH66	
Adriamycin	42	295	(7)
Daunorubicin	28	230	(8)
Vinblastine	3.4	63	(19)
Vincristine	3.3	313	(95)
Mitomycin C	24	70	(3)
5-Fluorouracil	638	801	(1)
Methotrexate	6.9	8.6	(1)
Cisplatin	222	447	(2)

Cells were incubated with each drug at 37 °C for 48 h. Each IC_{50} value represents the mean of duplicate experiments. Numbers in parentheses represent the relative resistance to AH66F cells.

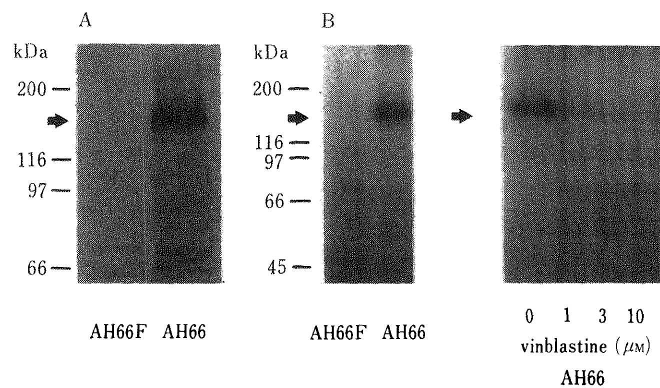

Fig. 1. Immunoblotting with C219 Antibody (A) and Photoaffinity Labeling with [^{125}I]NASV (B) of P-Glycoprotein in AH66F and AH66 Cell Membrane Preparations

Position of P-glycoprotein is shown by the arrow.

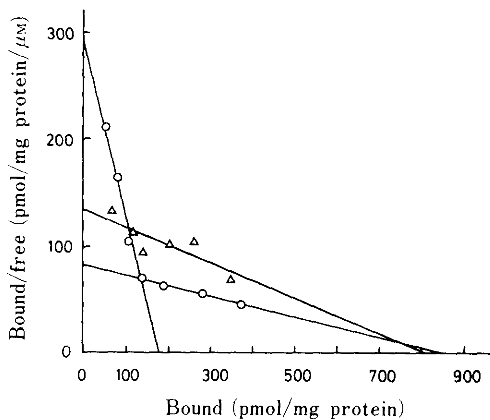

Fig. 2. Scatchard Plot of the Specific Binding of [^{3}H]Vinblastine to the Membrane Preparations from AH66F and AH66 Cells

The plasma membrane preparations (○, AH66, △, AH66F) were incubated with various concentrations of [^{3}H]vinblastine at 30 °C for 20 min.

photolabeled this protein band, and this photolabeling was selectively inhibited by unlabeled vinblastine. There was no detectable amount of this protein in the AH66F cell membrane (Fig. 1).

Then we investigated the characteristics of vinblastine binding in the plasma membrane fraction. When the membrane preparation was incubated with 0.5 μM [^{3}H]-vinblastine at 30 °C, the specific binding of [^{3}H]vinblastine depended on time. The fraction of specific binding was about 75% of the total binding after incubation for 10—20 min, when the specific binding reached maximum (data not shown). Results of Scatchard analysis of the saturable specific [^{3}H]vinblastine binding are indicated in Fig. 2 and Table II. AH66 cells have high and low affinity binding sites, but AH66F cells lack the high affinity binding site and have only a low affinity site.

Table II. Binding Parameters of Vinblastine in Plasma Membrane Preparations from AH66F and AH66 Cells

	K_d (μM)	B_{max} (pmol/mg protein)
AH66F	5.9	803
AH66		
High affinity site	0.6	177
Low affinity site	10	845

The plasma membrane preparations were incubated with [^{3}H]vinblastine at 30 °C for 20 min.

Table III. IC_{50} Values of Various Compounds for Specific Binding in AH66 Membrane Preparation

Compound	IC_{50} (μM)
Vinblastine	0.5
Adriamycin	26
H-87	9.2
Verapamil	5.0
Nicardipine	2.0
Reserpine	5.8
DMAR	30

The plasma membrane preparation was incubated with 0.5 μM [^{3}H]vinblastine in the absence or presence of each drug at 30 °C for 20 min. Each value represents the mean of duplicate experiments.

Table IV. Effects of Nucleotides on Specific Binding of Vinblastine in AH66 Plasma Membrane Preparation

Compound (3 mM)	Specific binding (pmol/mg protein)	% of control
None	75	100
AMP	75	100
ATP	70	93
ATP-γS	68	90
GTP	69	93

The plasma membrane preparation was incubated with 0.5 μM [^{3}H]vinblastine in the absence or presence of each nucleotide at 30 °C for 20 min. Each value represents the mean of duplicate experiments.

Next, we investigated the competition by some drugs with the vinblastine binding at the high affinity site in AH66 cell membranes. Specific [^{3}H]vinblastine binding competed with vinblastine itself and with Adriamycin in a dose-dependent manner, and the IC_{50} values were 0.5 and 26 μM, respectively (Table III). Other drugs such as H-87, verapamil, nicardipine, and reserpine, which have been shown to reverse multidrug resistance,[16-19] also effectively inhibited the binding of vinblastine (Table III). However, DMAR, which is less effective in overcoming vinblastine resistance,[20] showed only weak inhibition.

It has been reported that the binding of *Vinca* alkaloids was modulated by ATP in human myelogenous leukemia K562/ADR cells.[8] However, in AH66 cells, nucleotides

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such as AMP, ATP, ATP-γS, and GTP did not influence the binding of vinblastine (Table IV).

Discussion

Generally, multidrug-resistant cells show resistance to *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and several other drugs.[1] These cells extrude the antitumor drugs in an energy-dependent manner. Rat ascites hepatoma AH66 cells, which are a differentiated cholangioepitherial cell type,[21] also show an energy-dependent efflux of vinblastine from the cells and inherent resistance to vinblastine.[9,15]

In this study, we ascertained that AH66 cells were more resistant to *Vinca* alkaloids and anthracyclines than the sensitive subline AH66F cells, and the difference in sensitivities of AH66 and AH66F cells to mitomycin C, 5-fluorouracil, cisplatin, and methotrexate was small. The relative resistance to antitumor drugs in AH66 cells is like that of multidrug-resistant cells.[22] Therefore, while the AH66F cell line is a sensitive counterpart, the AH66 cell line is an inherent multidrug-resistant phenotype dependent on a 150—160 kDa P-glycoprotein in the plasma membrane (Fig. 1).

Our investigation using the radioligand binding assay technique found that the membrane fraction expressed the specific binding sites of vinblastine. Scatchard analysis gave a profile of two affinity classes of [^{3}H]vinblastine binding site in the AH66 membrane and only one low affinity binding site in the AH66F membrane. The vinblastine binding to the high affinity site in the AH66 cell membrane was inhibited by vinblastine itself, Adriamycin, and other drugs known to reverse the multidrug resistance, which were also reported to bind to P-glycoprotein.[16-19] [^{125}I]NASV-photolabeled 150—160 kDa protein was observed in the AH66 cell membrane, and such protein was not detectable in the AH66F cell membrane, which lacks the high affinity binding site. This evidence strongly suggests that the high affinity binding site is a fraction of P-glycoprotein. The low affinity vinblastine binding sites could not be identified by photoaffinity labeling, and the binding proteins remain unclear. P-Glycoprotein has been reported to contain two ATP binding domains in a molecule,[23,24] and Naito *et al.*[8] have reported that the vinblastine binding to the membrane from Adriamycin-resistant human myelogenous K562/ADR cells was dependent on ATP concentration. In this study, ATP and other nucleotides did not affect the binding of vinblastine. These facts indicate that the binding of resistant drugs to P-glycoprotein in AH66 cells is essentially independent of the nucleotides.

In conclusion, we indicate that AH66 cells are of a multidrug-resistant phenotype dependent on P-glycoprotein which is not modulated by nucleotides.

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