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MODULATORS OF THE MULTIDRUG-TRANSPORTER, P-GLYCOPROTEIN, EXIST IN THE HUMAN PLASMA

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SUMMARY: P-glycoprotein (P-gp) is thought to mediate the transport of anti-cancer drugs and to be responsible for the multidrug-resistant (MDR) phenotype. P-gp is also expressed in normal human tissues, such as the adrenal gland, kidney, liver, colon and capillary endothelium of the brain. However, the function and transporting substrates of P-gp in normal tissues are still not understood. This paper explains that some compounds in the human plasma can modulate the transporting activity of P-gp. A partially purified fraction from the human plasma enhanced the accumulation of anti-cancer agents in MDR cells. This fraction inhibited the efflux of vinblastine from MDR cells, and also inhibited the photoaffinity labeling of P-gp with azidopine as effectively as vinblastine, quinidine and cepharanthine. The compounds in this purified fraction may be physiological substrates of P-gp and can probably overcome MDR.

One possible cause of multidrug resistance (MDR) in some human tumor cell lines is the decreased accumulation of a variety of chemically and functionally unrelated drugs. Overexpression of a membrane glycoprotein termed P-glycoprotein (P-gp) has been widely observed in various MDR cell lines. Although definitive proof has not yet been presented, P-gp is thought to be a pump molecule that transports anti-cancer drugs outside the cells (1,2,3).

The physiological function of P-gp is not known. However, its distribution in the liver, kidney, jejunum and colon suggests that it plays a role in the secretion of anti-cancer drugs, alkaloids, toxic compounds and

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Abbreviations used: MDR, multidrug resistance; VCR, vincristine; VBL, vinblastine; DAU, daunomycin; AZP, azidopine; ODS, octadecyl silanol; HPLC, high performance liquid chromatography.

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certain metabolites into the bile, urine and directly into the gastrointestinal tract (4). Recently, P-gp has been shown to be expressed in the capillary endothelium of the brain and testes, which also suggests that P-gp regulates the entry of certain molecules into specific anatomic compartments (5). P-gp is also expressed in the adrenal grand and in the trophoblast of the placenta in man (6), and in the gravid uterus of mice (7). However, the physiological role and transporting substrates of P-gp in these organs are still unknown. Yang et al. reported that progesterone and some steroid hormones can interact with P-gp and modulate its pumping activity (8). However, the apparent inhibitory constant (Ki) values of most steroids for ATP-dependent binding of [^{3}H]VCR to P-gp were more than 10^{-5}M (9), which is much higher than the physiological concentration of steroid hormones. There is also no evidence that steroids are physiological substrates for P-gp.

We find that there are certain kinds of compounds in the human plasma that modulate the transporting activity of P-gp. This is the first report that provides physiological evidence for the presence of candidates for transporting substrates. Large scale preparation and further purification of the compounds in the human plasma will make the chemical structure and physiological function of these substrates clear.

MATERIALS AND METHODS

Cells: A human KB epidermal carcinoma cell line and its multidrug-resistant subline, KB-8-5 (10) were maintained in a minimal essential medium (MEM) containing 10 % newborn calf serum (NCS).

Assay for Accumulation of Anti-Cancer Drugs: Monolayer cultures of KB and KB-8-5 cells grown on 24-well dishes were incubated with 200 μl of 0.1 μCi/ml [^{3}H]vinblastine (VBL) or [^{3}H]daunomycin (DAU) for 60 min at 37°C. After being washed with ice cold phosphate buffered saline (PBS) three times, the cells were solubilized with 0.5% Triton X-100 and the cell-associated radioactivity was measured.

Determination of Resistance to Drugs: Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (11). Briefly, the cells were plated in 96-well dishes at 5,000 cells per well in the presence of various concentrations of anti-cancer drugs and partially purified fraction from human plasma. After 5 days incubation, the cells were incubated with 0.2 mg/ml MTT in MEM for 4 h. The formazan formed was dissolved in dimethyl sulfoxide and its absorbance at 570 nm was measured.

Photoaffinity Labeling of P-gp with Azidopine (AZP): Preparation of membrane vesicles from KB-C2 cells (a multidrug-resistant subline of KB cells maintained in 2 μg/ml of colchicine), and photoaffinity labeling with [^{3}H]AZP were carried out as described previously (12,13).

Purification of Modulators from The Human Plasma: Organic compounds from 1 liter of human plasma obtained from blood drawn from normal subjects were extracted into an equal volume of ethylacetate. The materials were dried under reduced pressure and dissolved in 50 ml of acetone, and then kept at 4°C. After 1 h, insoluble material was removed by centrifugation. The soluble material was dried and then dissolved in 30 ml of absolute ethanol. The

ethanol-soluble compounds were dried and then suspended in 20 ml of 50% ethanol. After centrifugation, the soluble material was concentrated under reduced pressure, and then applied to an ODS-HPLC column (4.5 mm x 25 cm). Bound matter was eluted with a linear gradient (30-100%) of organic solvent mixture (isopropanol: acetonitril = 1:1). Five μl of each fraction was used for [^{3}H]VBL accumulation assay in KB-8-5 cells. The active fractions were collected and concentrated.

Chemicals: [^{3}H]VBL and [^{3}H]AZP were obtained from Amersham; [^{3}H]DAU from Dupont NEN; Vincristine, Adriamycin and MTT from Sigma; ODS column (TSK-120A) from Tosoh Co. LTD.

RESULTS AND DISCUSSION

Anti-cancer drugs and agents that overcome MDR have been known to competitively inhibit drug-efflux from MDR cells that overexpress P-gp. We applied this phenomenon to the purification of the modulators of P-gp in the human plasma. Fractions that enhanced the accumulation of [^{3}H]VBL in monolayers of an MDR cell line, KB-8-5 were collected. We used human plasma to purify the modulators, because [1] the human plasma is easy to collect, [2] P-gps in the human liver and kidney are thought to transport unknown compounds from the blood to the bile or urine, and [3] P-gp in the adrenal gland may be involved in the excretion of unknown compounds, such as metabolites or hormones, into the blood.

Organic compounds were extracted with ethylacetate from the human plasma. The active compounds that enhanced [^{3}H]VBL accumulation in KB-8-5 cells were completely soluble in acetone and ethanol, and about 80% of the active compounds were soluble in 50% ethanol. The 50% ethanol soluble fraction was further purified with reversed phase HPLC. Active compounds were eluted from an ODS-HPLC column as several peaks at a high concentration (more than 75%) of the organic solvent (Fig.1). We obtained about 1.0 mg of partially purified material consisting of several peaks from 1 liter of the human plasma (see Fig.1). This material dissolved in 500 μl ethanol was called HPLC purified fraction and used for experiments as described below.

Figure 2 shows the effect of the HPLC purified fraction on the accumulation of [^{3}H]VBL and [^{3}H]DAU in KB and KB-8-5 cells. The HPLC purified fraction enhanced the accumulation of [^{3}H]VBL in KB-8-5 cells 6-7 times, but only 1.5 times in KB cells (Fig.2, left). The activity of the fraction was not susceptible to heat and proteases. The HPLC purified fraction also enhanced the accumulation of [^{3}H]DAU in KB-8-5 cells, but not in KB cells (Fig.2, right). Verapamil which is a well characterized agent that overcomes MDR and has been shown to interact with P-gp (14) also enhanced the accumulation of [^{3}H]VBL and [^{3}H]DAU in KB-8-5 cells. The HPLC purified fraction inhibited the rapid efflux of [^{3}H]VBL from KB-8-5 cells, but not affected the efflux from KB cells (Fig.3). Verapamil also had similar effect on the efflux (data not shown). These data suggest that inhibition of the active efflux of VBL is the cause of

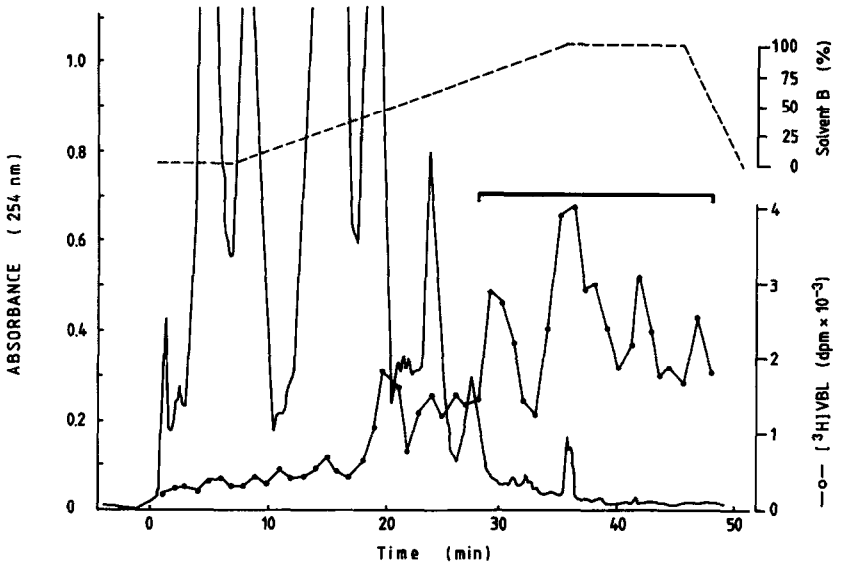

Fig.1 Analysis of the compounds from the human plasma by reversed phase HPLC. 50% ethanol soluble fraction from 1 liter of human plasma was applied to an ODS-HPLC column, and bound compounds were eluted using a given gradient of organic solvent with flow rate of 1.0 ml/min. Solvent A contained 15% acetonitril and 15% isopropanol in water; solvent B contained 50% acetonitril and 50% isopropanol. The gradient from A to B is shown by a dotted line. Fractions (1 ml) were collected and 5 μl was used for the accumulation of [^{3}H]VBL in KB-8-5 cells. The fractions indicated by a bold line were combined (HPLC purified fraction) and used for further experiments.

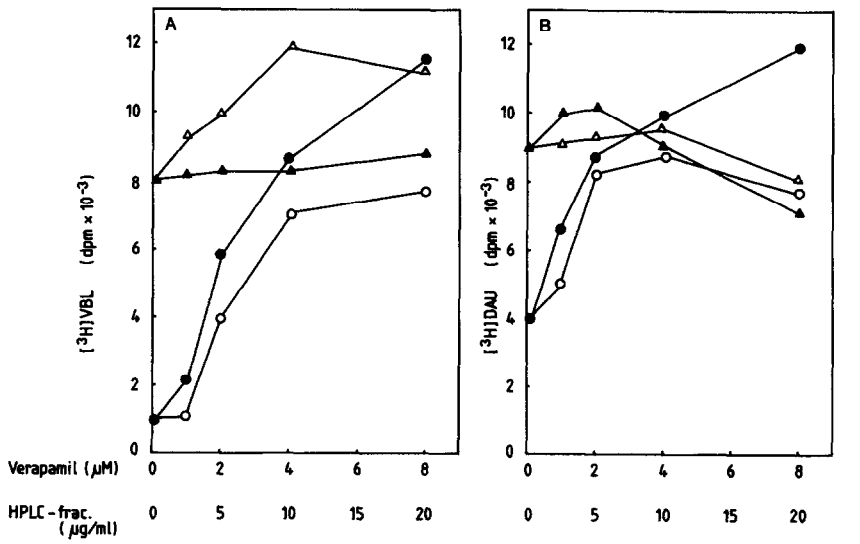

Fig.2 The effect of HPLC purified fraction on the accumulation of [^{3}H]VBL (A) and [^{3}H]DAU (B) in KB cells (▲ , △) and KB-8-5 cells (● , ○). Cells were incubated with [^{3}H]VBL and [^{3}H]DAU in the presence of various concentrations of verapamil (● , ▲) and HPLC purified fraction (HPLC-frac.) from human plasma (○ , △). After 60 min, the cell-associated radioactivity in each well was measured. Each point is the average of duplicate trials.

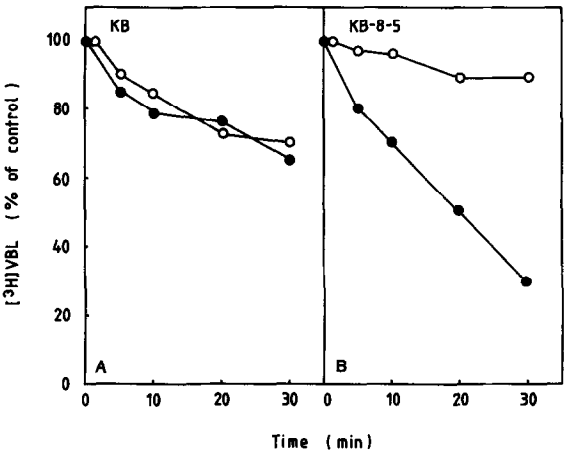

Fig.3 The effect of HPLC purified fraction from human plasma on the efflux of [3H]VBL from KB cells (A) and KB-8-5 cells (B). The cells were incubated with 0.1 μCi/ml (A) or 0.5 μCi/ml (B) of [^{3}H]VBL for 60 min at 37°C. After being washed with cold PBS, the cells were further incubated with prewarmed MEM without (●) and with (○) 20 μg/ml of the HPLC purified fraction at indicated periods. After the cells were washed with PBS, cell-associated radioactivity was measured.

the enhanced accumulation of VBL by the HPLC purified fraction in KB-8-5 cells.

P-gp has been shown to be specifically labeled by the photoactive analog of transporting substrates, such as VBL, verapamil and azidopine (AZP). Most transporting substrates (anti-cancer drugs and agents that overcome MDR) inhibit the photolabeling of P-gp with [^{3}H]AZP (12,15,16). The HPLC purified fraction from the plasma also inhibited the photoaffinity labeling of P-gp with [^{3}H]AZP. As shown in Fig.4, 5 μg/ml of this fraction inhibited photolabeling to a similar degree to 10 μM of VBL, quinidine and cepharanthine. Since the molecular weight of the compounds in the HPLC purified fraction is not known, the affinity of the compounds to P-gp cannot be estimated. However, the molecular weight range of the known transporting substrates of P-gp is 300-2000. If the molecular weight of the compounds in the HPLC purified fraction is within this range, the affinity of these compounds to P-gp may be similar to or stronger than vinblastine. Verapamil did not affect photolabeling of P-gp with AZP at the same concentration. The binding site of verapamil in P-gp may be different from that of AZP, or the binding affinity of verapamil to P-gp may be weaker than that of VBL, quinidine, and cepharanthine.

The effect of the HPLC purified fraction on the sensitivity of KB-8-5 cells to vincristine and Adriamycin was examined (Fig.5). Ten μg/ml of this fraction increased the sensitivity of KB-8-5 cells to these anti-cancer drugs as effectively as 5 μM verapamil. This suggests that the compounds in this fraction have an ability to overcome MDR.

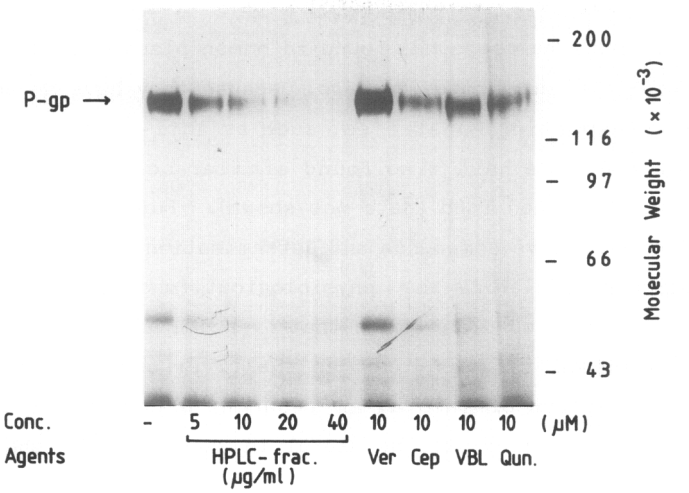

Fig.4 The effect of HPLC purified fraction and agents on the photoaffinity labeling of P-gp with [^{3}H]AZP. Membrane vesicles from KB and KB-C2 cells were photolabeled with 0.5 μM [^{3}H]AZP in the absence of any drugs (-) and in the presence of 10 μM VBL, verapamil (Ver), cepharanthine (Cep), quinidine (Qun) and indicated concentrations of HPLC purified fraction from human plasma. Photolabeled samples were subjected to 7.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis and fluorography. The arrow indicates 140-kDa P-gp.

Recently, McGrath and Varshavsky have suggested that P-gp in yeast is involved in the transport of a-factor pheromone (17). P-gp in the adrenal gland and other normal tissues might also be involved in excretion of unknown

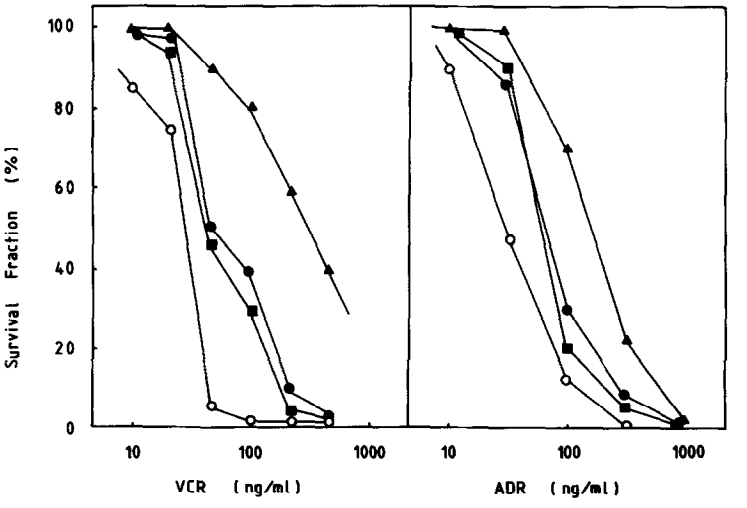

Fig.5 The effect of partially purified material from the human plasma on the sensitivity of KB-8-5 cells to vincristine (VCR) and Adriamycin (ADR). KB cells (open circle) and KB-8-5 cells (closed symbols) were plated at 5,000 per well in 96-well dishes in the absence (▲) and presence (●) of 10 μg/ml HPLC purified fraction and in the presence of 5 μM verapamil (■). They were incubated for 5 days, and cell viability was assayed by the MTT method.

hormones or factors. The compounds found in human plasma are likely candidates for the physiological substrates of P-gp, because they have an ability similar to the other transporting substrates, such as anti-cancer drugs and agents that overcome MDR. We have also found similar compounds in the plasma of rabbits bred with difined food (data not shown). Further purification of the molecules of these active compounds and determination of their chemical structures is necessary to clarify the physiological function of these molecules. These compounds may be used as agents that overcome MDR. They may be transported by P-gp in normal tissues, such as the liver and kidney, and they may modulate the retention of several anti-cancer drugs in the blood. Further analysis of these compounds will be useful in understanding the physiological role of P-gp.

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