The Disposition of Reduced Haloperidol and HPP,⁺ Metabolites of Haloperidol, is Not Influenced by P-Glycoprotein

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=국문초록=

Haloperidol 대사물 Reduced Haloperidol과 HPP⁺의 체내 분포에
P-glycoprotein 수송체의 관련성 평가
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연구배경: HPP⁺와 reduced haloperidol(RHAL) 및 haloperidol의 분포 및 소실(disposition)에 대한 P-glycoprotein(P-gp)의 관련성을 평가하였다.

방 법: P-gp(-)-MDA435/LCC6 세포주와 P-gp-과발현-MDA435/LCC6^{MDR1} 세포주에서, HPP⁺와 RHAL 및 haloperidol의 세포내 축적을 비교하였고 잘 알려진 P-gp 기질인 doxorubicin의 세포내 축적에 대한 다약제내성 환원 효과(MDR1-reversing effect)를 평가하였다.

결 과: Haloperidol과 RHAL은 두 세포주에 용량 의존적으로 축적되었고 경미한 다약제내성 환원 효과를 보였으나, HPP⁺는 매우 적게 축적되었으며 다약제내성 환원효과가 없었다. Haloperidol과 RHAL 및 HPP⁺의 축적 정도는 두 세포주 간에 차이가 없었다.

결 론: P-gp가 HPP⁺와 RHAL 및 haloperidol의 분포 및 소실에 영향을 주지 않으므로, 이 화합물들이 뇌로 분포하는 것에 P-gp의 기질들과 유의하게 상호작용하지는 않을 것이다.

주제어: Haloperidol · Reduced haloperidol · HPP⁺ · P-Glycoprotein · 다약제내성(multidrug resistance, MDR)

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INTRODUCTION

P-glycoprotein (P-gp), a product of the human *MDR1* gene, is a membrane transport protein and is localized in normal tissues including intestine, kidney, liver, testis, and brain as well as cancer cells.^{1),2)} The tissue distribution of P-gp suggests that this protein may contribute to the protection of organisms against xenobiotics by excreting these compounds into urine or bile and by minimizing their accumulation in critical organs such as brain. For example, P-gp located in the blood-brain barrier pumps ivermectin out of the brain in normal animals, but 100-fold higher concentrations of ivermectin can be accumulated in the brains of *mdr1a* (-/-) knock-out mice.³⁾

In humans, the antipsychotic haloperidol is biotransformed to many metabolites including reduced haloperidol and a number of pyridinium metabolites, including 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxybutyl]pyridinium ion (HPP⁺) and reduced form of HPP⁺ (RHPP⁺).^{4),5)}

Haloperidol pyridinium metabolites are structurally similar to neurotoxic 1-methyl-4-phenylpyridinium (MPP⁺), an agent known to cause neurodegeneration, and they have been reported to have similar or more potent toxic effects including inhibition of mitochondrial respiration, irreversible inhibition of tyrosine hydroxylase and depletion of dopamine and serotonin.^{6),8)} The formation of toxic pyridinium metabolites of haloperidol has been reported to be mediated by hepatic cytochrome P450 3A (CYP3A) *in vitro*,⁵⁾ but it has not been clearly established that HPP⁺ formed in peripheral tissue is distributed into the

brain.⁹⁾

Most P-gp substrates contain electron donor groups in their chemical structures (groups with an unshared electron pair on an electronegative atom, e.g. O, N, S, F and Cl, or groups with a π -electron orbital of an unsaturated system).¹⁰⁾ Haloperidol is not a known substrate of P-gp, although it does contain some of these electron donor groups.¹¹⁾ However, metabolites are in some cases better substrates of P-gp than their parent drugs, and it remains to be established whether HPP⁺ is a substrate.¹²⁾ Indeed, when comparing a number of steroid analogues as substrates and inhibitors of P-gp, Barnes *et al.*¹²⁾ suggested that some hydrophilicity is required for an efficient transport, and metabolites are generally more hydrophilic than their parent drugs. Idarubicinol and daunorubicinol, metabolites of idarubicin and daunorubicin, have been reported to be more susceptible to P-gp than their parent compounds.¹³⁾ Therefore, it is possible that haloperidol metabolites may be P-gp substrates, whereas the parent drug is not.¹¹⁾ This question seems particularly important for two reasons. First, P-gp is a component of the blood-brain barrier. If HPP⁺ is a substrate of P-gp, HPP⁺ may be expected to accumulate more in the brain of a subject with limited P-gp in the blood brain barrier, which may cause greater haloperidol-induced neurotoxicity. Second, although there is no evidence for metabolism of haloperidol to HPP⁺ in gastrointestinal epithelial cells, the disposition of HPP⁺ may be influenced by P-gp there because formation of HPP⁺ is mediated by CYP3A4 that is present together with and frequently shares substrates with P-gp

in these epithelial cells.^{5,14)} If this is true, the systemic uptake of HPP⁺ formed in gastrointestinal epithelial cells may vary among subjects whose P-gp activities are different.¹⁵⁾

To test the hypothesis that P-gp is involved in the disposition of haloperidol metabolites, we compared the intracellular accumulation of haloperidol, reduced haloperidol, and HPP⁺ in a P-gp over-expressing multidrug resistant (MDR) cell line (MDA435/LCC6^{MDR1}) and a P-gp-negative control cell line (MDA435/LCC6). We also evaluated the MDR reversing effect of haloperidol metabolites by measuring their effect on doxorubicin accumulation in these cell lines.

MATERIALS AND METHODS

Materials

Haloperidol, doxepin, doxorubicin hydrochloride and verapamil hydrochloride were purchased from Sigma Chemical Company (Saint Louis, MO, USA) and reduced haloperidol was obtained from Janssen Pharmaceutica Naamloze Vennootschap (Beerse, Belgium). 4-(4-Chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium ion (HPP⁺) was a generous gift from Doctor Neal Castagnoli, Junior (Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA). Levallorphan was purchased from United States Pharmacopeial Convention (USPC, Rockville, MD, USA). All other chemicals and reagents used were of highest commercially available quality.

Cell Culture

P-gp-negative MDA435/LCC6 cells and P-gp over-expressed MDA435/LCC6^{MDR1} cells were provided by Doctor Robert. Clarke (Vincent T. Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA). MDA435/LCC6^{MDR1} cells have been obtained by transduction of MDA435/LCC6 cells with a retroviral vector directing the constitutive expression of the human *MDR1* gene. In these cells, *MDR1* expression was demonstrated by Western blotting and its function confirmed in terms of increased resistance to doxorubicin, taxol, and vinblastine, and decreased intracellular accumulation of vinblastine.¹⁶⁾ These cells were maintained in improved minimum essential medium (IMEM, Biofluids, Rockville, MD, USA) containing phenol red and supplemented with 5% fetal calf serum, in a humidified 5% CO₂/95% air atmosphere at 37°C, as described previously.¹⁶⁾ Forty-eight hours before the accumulation study, both cell lines were seeded into 24-well culture dishes (Costar, Cambridge, MA, USA) at a seeding density of 2.5×10^5 cells/well.

Accumulation of Haloperidol and Its Metabolites

On the study day, cells in 24-well plates were treated by exchanging 250 μ L of spent IMEM containing 0.31 to 20 μ M of haloperidol, reduced haloperidol, or HPP⁺ or doxorubicin (a well-known P-gp substrate) as a positive control. Haloperidol

and reduced haloperidol were initially dissolved in methanol and then serially diluted with fresh IMEM, while iodine salt of HPP⁺ was dissolved in the distilled water. The methanol concentrations were kept constant (0.2%) in all samples as those in the IMEM containing the highest concentration. All treatments were carried out in triplicate. Cell cultures were reincubated at 37°C for 5 hours. Treatments were stopped by carefully washing wells once with 0.5 mL/well ice-cold NaCl (0.15 M). Cells from two reference wells in each plate were counted in order to allow expression of the accumulation of compounds based on the same number of cells (10^6 cells), and average cell counts of MDA435/LCC6 and MDA435/LCC6^{MDR1} were 7.6×10^5 and 6.3×10^5 cells/well, respectively.

Intracellular concentrations of haloperidol and reduced haloperidol were measured by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection by the method of Kim *et al.*¹⁷⁾ with minor modifications, and intracellular HPP⁺ concentration was measured by the fluorescence HPLC method of Igarashi and Castagnoli, Junior.¹⁸⁾ with modification. In brief, haloperidol, reduced haloperidol, and HPP⁺ were extracted from cell monolayers in the remaining wells by adding 1 mL of acetonitrile, 20 μ L of internal standard, (i.e. 10 μ g/mL of doxepin into haloperidol- and reduced haloperidol-treated wells), and 100 μ g/mL of levallorphan into HPP⁺ treated wells. Then the whole content of each well including scraped cell debris was transferred into 1.5 mL microfuge tubes, shaken vigorously on

the vortex for 3 minutes, and centrifuged at $16,000 \times g$ for 15 minutes. The supernatants were dried by SpeedVac (Savant Instrument, Incorporated, Farmingdale, NY, USA) in clean microfuge tubes and reconstituted with 100 μ L of HPLC mobile phase consisting of 0.01 M phosphate buffer containing 10 mM triethylamine and 5mM pentanesulfonic acid acetonitrile (pH 4.0) and acetonitrile in a ratio of 65.5:34.5 (volume/volume). Haloperidol, reduced haloperidol, and the internal standard (doxepin) were separated on a Spherisorb[®] octadecylsilane (ODS, particle size 5 μ m, internal diameter $\times$ length: 4.6 $\times$ 250 mm, Alltech Associates, Incorporated, Deerfield, IL, USA) column with a NovaPak[®] C18 guard column (Waters Corporation, Milford, MA, USA) at a flow rate of 1.0 mL/min and detected by a Waters 490 UV detector set at 254 nm wavelength. The separation of HPP⁺ and levallorphan was performed using the same column and detection was carried out with a Spectrovision FD-300 Dual Monochromator Fluorescence detector (Groton Technology Incorporated, Concord, MA, USA) set at an excitation wavelength of 304 nm and emission wavelength of 374 nm. With this method, the calibration curves for the analytes with an internal standard were linear over a concentration range of 10~1,000 nM in 1 mL acetonitrile added wells containing MDA435/ LCC6 cells or MDA435/ LCC6^{MDR1} cells. The minimum detectable concentration of the assay was 5 nM for haloperidol and reduced haloperidol, and 2 nM for HPP⁺. Results were presented as pmol/ 10^6 cells.

MDR1-Reversing Effect of Haloperidol, Reduced Haloperidol, and HPP⁺ on Doxorubicin Accumulation

The MDR1-reversing activities of haloperidol, reduced haloperidol, and HPP⁺ were determined by measuring their effect on doxorubicin accumulation in MDA435/LCC6^{MDR1} cells. The effects of these compounds were compared to the MDR1 reversing effect of verapamil, a well-known P-gp inhibitor. P-gp-negative MDA435/LCC6 cells were used as a negative control to evaluate non-specific effects. Cells in 24-well plates were treated by exchanging media containing 4 μ M doxorubicin and 4 different concentrations (0.31, 1.25, 5, or 20 μ M) of haloperidol, reduced haloperidol, HPP⁺, or verapamil as a positive control. All treatments were carried out in triplicate, and cell cultures were reincubated at 37°C for 3 hours. Treatments were stopped by carefully washing wells once with 0.5 mL/well ice-cold 0.15 M NaCl. Doxorubicin was extracted from the cell monolayer in each well by adding 0.5 mL of 20% trichloroacetic acid. Plates were placed overnight at 4°C in the dark to precipitate. Samples of 400 μ L supernatant were transferred to measure doxorubicin concentration in a Hitachi F4500 spectrofluorometer (Hitachi, Limited, Tokyo, Japan) set at an excitation wavelength of 500 nm and emission wavelength of 580 nm. Results were plotted as amount of doxorubicin per 10⁶ cells.

RESULTS

After a 5-hour exposure to haloperidol, redu-

Fig. 1. Accumulation of haloperidol, reduced haloperidol and HPP⁺ in P-gp-negative (MDA435/LCC6) and P-gp-positive (MDA435/LCC6^{MDR1}) cell lines. The amount of intracellular haloperidol, reduced haloperidol or HPP⁺ was measured after 5-hour exposure of 0.31 to 20 μ M (0.31, 1.25, 5, or 20 μ M) of these compounds in improved minimum essential medium (IMEM). The symbols are as follows: haloperidol in MDA435/LCC6 cells (○), haloperidol in MDA435/LCC6^{MDR1} cells (●), reduced haloperidol in MDA435/LCC6 cells (▽), reduced haloperidol in MDA435/LCC6^{MDR1} cells (▼), HPP⁺ in MDA435/LCC6 cells (□), HPP⁺ in MDA435/LCC6^{MDR1} cells (■), doxorubicin in MDA435/LCC6 cells (◇), and doxorubicin in MDA435/LCC6^{MDR1} cells (◆). Each data indicate mean $\pm$ standard deviation of triplicate.

ced haloperidol, and HPP⁺, these compounds were accumulated into both MDA435/LCC6 cells and MDA435/LCC6^{MDR1} cell lines in a dose dependent manner (Fig. 1). However, there was no difference in the intracellular accumulation of any of the compounds tested between P-gp-positive and P-gp-negative cells. The accumulation of HPP⁺ was minimal compared to that of reduced haloperidol and haloperidol in both cell lines, and reduced haloperidol showed the

highest cellular accumulation. In contrast to haloperidol and its metabolites, MDA435/LCC6 cells and MDA435/LCC6^{MDR1} cells showed a huge difference in accumulation of doxorubicin. Doxorubicin, a well-known P-gp substrate, accumulated in MDA435/LCC6 cells in a dose

Fig. 2. MDR1-reversing effects of haloperidol, reduced haloperidol and HPP⁺ on doxorubicin accumulation in P-gp-negative (MDA435/LCC6) and P-gp-positive (MDA435/LCC6^{MDR1}) cell lines. The amount of intracellular doxorubicin was measured after 3-hour exposure of 4 μ M doxorubicin and 0.31–20 μ M (0.31, 1.25, 5, or 20 μ M) of haloperidol, reduced haloperidol or HPP⁺. Upper and lower dashed lines indicate baseline doxorubicin accumulation in MDA435/LCC6 and MDA435/LCC6^{MDR1} cells in the absence of any inhibitors (haloperidol, reduced haloperidol or HPP⁺) tested, respectively. The symbols are as follows: haloperidol in MDA435/LCC6 cells (○), haloperidol in MDA435/LCC6^{MDR1} cells (●), reduced haloperidol in MDA435/LCC6 cells (▽), reduced haloperidol in MDA435/LCC6^{MDR1} cells (▼), HPP⁺ in MDA435/LCC6 cells (□), HPP⁺ in MDA435/LCC6^{MDR1} cells (■), verapamil in MDA435/LCC6 cells (◇), and verapamil in MDA435/LCC6^{MDR1} cells (◆). Each data indicate mean $\pm$ standard deviation of triplicate.

dependent manner, but no significant accumulation of doxorubicin was found in MDA435/LCC6^{MDR1} cells (Fig. 1).

Doxorubicin accumulated in P-gp negative MDA435/LCC6 cells (1212.0 pmol/10⁶ cells), while MDA435/LCC6^{MDR1} cells were able to maintain lower intracellular levels of doxorubicin (207.7 pmol/10⁶ cells) (Fig. 2). This difference between the two cell lines was partly reversed by adding haloperidol or reduced haloperidol at a high concentration (20 μ M). However, the effect was small. At 20 μ M, haloperidol and reduced haloperidol decreased the MDR1 effect on doxorubicin accumulation by only 45.3% and 23.4%, respectively. No reversing effect of MDR1 by 20 μ M HPP⁺ was observed. In contrast, the known MDR1 reversing agent verapamil, at 20 μ M, almost completely restored doxorubicin accumulation in MDA435/LCC6^{MDR1} cells to the levels of the parental P-gp-negative MDA435/LCC6 cells (Fig. 2).

DISCUSSION

In the present study, haloperidol, reduced haloperidol, and HPP⁺ were accumulated into both MDA435/LCC6 cells and MDA435/LCC6^{MDR1} cell lines in a dose dependent manner. In contrast to the known P-gp substrate doxorubicin, there was no difference in the accumulation of any compound between P-gp-positive and P-gp-negative cells. These data suggest that P-gp has little influence on the disposition of haloperidol, a known poor substrate of P-gp,¹¹⁾ but also on the disposition of its metabolites, reduced haloperidol and HPP⁺. It follows that P-gp in the blood

brain barrier is unlikely to influence the penetration of HPP⁺ formed in the peripheral tissues, into brain. For the same reasons, HPP⁺ is not expected to be exported by P-gp located in gastrointestinal epithelial cells.

HPP⁺ was accumulated into both cell lines tested in a dose dependent manner although the amount of its accumulation was very small compared to that of haloperidol or reduced haloperidol. This indicates that HPP⁺ can cross lipid cell membranes despite its ionic structure. Igarashi⁹⁾ reviewed data related to this question and concluded that it was likely that HPP⁺ formed in peripheral tissue does distribute into brain. Our data seems to support this hypothesis because HPP⁺ did distribute into the cells that we studied, but it would appear unlikely that P-gp in the blood brain barrier has a role in the protection of the brain from the transport of this neurotoxic haloperidol pyridinium metabolite across the barrier. All these findings imply that a patient who has higher plasma concentration of HPP⁺ would be expected to have greater accumulation into the brain and higher risk of neurological toxicity due to this toxic metabolite.⁶⁻⁸⁾

In the present study, haloperidol and reduced haloperidol showed very weak MDR1-reversing effects. Twenty μ M of haloperidol and reduced haloperidol increased the intracellular accumulation of doxorubicin in P-gp-negative MDA 435/LCC6^{MDR1} cells by 45% and 23%, respectively. In comparison, the reference MDR1 reversing agent verapamil, at the same concentration, almost completely eliminated the doxorubicin accumulation differential. No reversing

effect of MDR1 by HPP⁺ was observed at all. These data are consistent with those of Ibrahim *et al.*¹⁹⁾ who reported that several antipsychotics including haloperidol weakly block P-gp function in MDR1 expressed Caco-2 cells. From these results, the MDR1-reversing effect of all these compounds *in vivo* would not be expected to alter the disposition of other P-gp substrates. Normal therapeutic plasma concentrations of haloperidol are in the range of 10 to 53 nM and concentrations of reduced haloperidol and HPP⁺ are in a similar range,⁹⁾ but inhibition of P-gp occurs at concentrations above 1 μ M (Fig. 2).

In conclusion, P-gp does not influence the disposition of HPP⁺, reduced haloperidol, and metabolites of haloperidol. In addition, these haloperidol metabolites are unlikely to have any effect on the disposition of P-gp substrates *in vivo*. Although the metabolites of haloperidol are more polar than the parent drug, it does not inevitably follow that they are better substrates of P-gp.

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