Effect of Therapeutic Moderate Hypothermia on Multi-drug Resistance Protein 1-Mediated Transepithelial Transport of Drugs

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Abstract

To clarify the effect of therapeutic moderate hypothermia on drug distribution, transepithelial transport via multi-drug resistance protein 1 (MDR1) (also called P-glycoprotein or ABCB1) was evaluated at various temperatures in vitro using LLC-GA5-COL150 cells, which were established by transfecting human MDR1 complementary deoxyribonucleic acid into kidney epithelial LLC-PK, cells and express MDR1 on the apical membrane. MDR1 is expressed in the blood-brain barrier to limit drug distribution to the brain by exporting exogenous substances including calcium blockers and antiarrhythmic drugs. Digoxin was used as a typical substrate, as well as the non-substrate tetracycline and paracellular marker inulin. MDR1-mediated transport of digoxin decreased at lower temperatures. Transport of tetracycline also decreased at lower temperatures, probably due to changes in membrane fluidity. However, no change was found at over 32°C, suggesting that passive diffusion does not change during moderate hypothermia. The distribution of MDR1 substrates should be considered during hypothermic conditions, as the clinical outcome could be affected.

Key words: therapeutic moderate hypothermia, pharmacokinetics, multi-drug resistance protein 1, digoxin, quinidine, tetracycline

Introduction

Moderate hypothermia with a target temperature of 32–34°C to treat severe traumatic brain injury improved outcomes in preclinical15,32) and preliminary clinical investigations,9,28,29) which led to widespread use in neurological emergencies such as spinal cord injury,45) ischemic stroke,23,25) subarachnoid hemorrhage,14,18,24) cardiopulmonary arrest,7,52) and hypoxic-anoxic encephalopathy,1,3,4,16) A recent large prospective and randomized clinical trial of therapeutic moderate hypothermia for severe traumatic brain injury found no difference in the number of poor outcomes including severe disability, vegetative state, or death between hypothermia and normothermia in patients with good recovery 6 months after injury in a cohort of 392 patients.10) In contrast, clinical trials have shown significant benefit for patients with cardiac arrest based on survival after hospital discharge with sufficiently good neurological function.6,20) The resulting controversy11,31,33,34) necessitates additional preclinical and clinical studies.

Proof of the efficacy of moderate hypothermia may be obscured by the large interindividual differences in pathophysiological conditions.37) Adverse events such as arrhythmia, polyuria,34–36) wound infection,26) thrombocytopenia,22) and abnormal clotting23) may also obstruct evaluation of therapeutic mild hypothermia. Arrhythmia and polyuria occur in the initial phase of cooling, and severe disruptions in fluid balance, blood pressure, and electrolyte levels are likely to occur.34–36) Active treatment of these adverse events by administration of electrolytes and antiarrhythmic agents is the key to use of therapeutic moderate hypothermia, but little information is available on the pharmacokinetics and pharmacodynamics involved.

Drug transporters, e.g., multi-drug resistance protein 1 (MDR1) (also called P-glycoprotein or ABCB1), and drug metabolizing enzymes, e.g.,
cytochrome P450s, are thought to be the two major biological factors determining the pharmacokinetics of drugs,27,40,41 in particular the limitation of distribution to the brain through the blood-brain barrier (BBB).13,43) MDR1 is expressed in normal tissues including the liver, kidneys, small and large intestines, brain, testes, muscle, placenta, and adrenals, and confers intrinsic resistance to normal tissues by transporting exogenous substances out of the cell.27,40,41) MDR1 expressed in the BBB limits the brain distribution of various types of substances. Therefore, changes in the MDR1 function might affect the pharmacological effect of drugs.12,43) Many clinically important drugs are substrates of MDR1 including immunosuppressants, protease inhibitors, anticancer drugs, and calcium blockers.

In the present study, the effect of therapeutic moderate hypothermia on transepithelial transport via MDR1 was evaluated in vitro using the MDR1-overexpressing cell line LLC-GA5-COL150 and typical substrates, [3H]digoxin and [3H]quinidine.

Materials and Methods

I. MDR1 transporter assay

LLC-GA5-COL150 cells were established by transfection of human MDR1 complementary deoxyribonucleic acid into porcine kidney epithelial LLC-PK1 cells,48,49) and are useful for the assessment of MDR1-mediated drug transport and drug interactions.42,47) LLC-PK1 and LLC-GA5-COL150 cells are seeded onto microporous membrane filters to form monolayers. The apical membranes face up and the basal membranes are attached to the microporous polycarbonate membrane. MDR1 is highly expressed on the apical membrane in LLC-GA5-COL150 cells and acts as an efflux transporter, so transport from the basal side to the apical side (B to A transport) is increased and transport from the apical side to the basal side (A to B transport) is decreased for MDR1 substrates in LLC-GA5-COL150 cells compared to LLC-PK1 cells (Fig. 1). Consequently, MDR1-mediated transport can be assessed by measuring B to A transport and A to B transport in LLC-GA5-COL150 cells and LLC-PK1 cells.48,49)

II. Cell culture

LLC-PK1 (220–230 passages) and LLC-GA5-COL150 cells (9–12 passages) were maintained in a culture medium consisting of Medium199 (Dainippon Pharmaceutical Co., Ltd., Osaka) supplemented with 10% fetal bovine serum (Lot No. AMJ17247; HyClone, Logan, Utah, U.S.A.). No antibiotics were added. For LLC-GA5-COL150 cells, 150 ng/ml of colchicine was added to the culture medium for the stable expression of MDR1. LLC-PK1 (1.0 × 10^6 cells; 1.82 × 10^6 cells/cm²) and LLC-GA5-COL150 (1.5 × 10^6 cells; 2.73 × 10^4 cells/cm²) cells were seeded on plastic culture dishes (100 mm diameter) in 10 ml of culture medium. Monolayer cultures were grown in a humidified atmosphere of 5% CO₂-95% air at 37°C, and subcultured every 4 and 7 days for LLC-PK1 and LLC-GA5-COL150 cells, respectively, with 0.02% ethylenediaminetetra-acetic acid and 0.05% trypsin (Invitrogen Corp., Carlsbad, Calif., U.S.A.).

III. Assay of substrate transport

The transepithelial transport of [3H]digoxin (595.7 GBq/mmol; PerkinElmer Inc., Boston, Mass., U.S.A.), [3H]tetracycline (45.1 GBq/mmol; PerkinElmer Inc.), [3H]quinidine (740.0 GBq/mmol; Amersham Biosciences, Piscataway, N.J., U.S.A.), and [methoxy-14C]inulin (308.0 GBq/mmol; Amersham Biosciences) across LLC-PK1 and LLC-GA5-COL150 cell monolayers was examined as described previously.42,47) B to A and A to B transports were assayed independently. Cells were seeded on microporous filters (Transwell™, Cat. No. 3414; Corning Costar Corp., Cambridge, Mass., U.S.A.) at a density of 2.0 × 10^6 cells/well (4.26 × 10^5 cells/cm²) and 2.4 × 10^6 cells/well (5.11 × 10^5 cells/cm²) for LLC-PK1 and LLC-GA5-COL150 cells, respectively, and cultured in a humidified atmosphere of 5% CO₂-95% air at 37°C for 3 days. The culture medium was replaced with fresh culture medium 3 hours before the start of transport experiments. Colchicine was not included even for LLC-GA5-COL150 cells during the transport experiment.

The transport experiment was initiated by replacing the culture medium on the basal side for B to A transport or the apical side for A to B transport (donor side) with 2 ml of fresh culture medium containing [3H]digoxin (100 nM, 18.5 kBq/ml), [3H]quinidine (100 nM, 18.5 kBq/ml), or [3H]tetracycline (1000 nM, 45.1 kBq/ml) and the culture medium on the opposite side (receiver side) with 2 ml of fresh culture medium. [Methoxy-14C]inulin (6.0 μM, 1.85
Fig. 2 Time course of transepithelial transport of [3H]digoxin in LLC-PK1 and LLC-GA5-COL150 cells at 37°C (A) and 32°C (B). Open (circle, triangle) and closed (circle, triangle) symbols show transepithelial transport across LLC-PK1 and LLC-GA5-COL150 cell monolayers, respectively. Circles show B to A transport, and triangles indicate A to B transport. Each point represents the mean ± standard deviation of results from at least three independent experiments.

kBq/ml) was also included on the donor side. The monolayers were incubated at the required temperature, and aliquots (25 μl) of the culture medium on the receiver side were taken at 1, 2, and 3 hours.

The level of radioactivity in the medium was measured in 3 ml of ACS II (Amersham Biosciences) with a liquid scintillation counter (LSC-5100; Aloka Co., Ltd., Tokyo). The clearance was calculated by dividing the rate of transport by the concentration applied. The net B to A transport ratio, which correlates with brain distribution, was calculated by dividing B to A transport by A to B transport for assessment of MDR1-mediated transport.

IV. Data analysis

Results are expressed as the mean ± standard deviation. Statistical analysis of the data was performed with one-way analysis of variance followed by Scheffe’s post-hoc test (two-tailed), with p < 0.05 considered significant.

Results

Transepithelial transport of [3H]digoxin, [3H]quinidine, [3H]tetracycline, and [methoxy-14C]inulin across LLC-PK1 and LLC-GA5-COL150 cell monolayers was time-dependent at 37°C (data only shown for [3H]digoxin in Fig. 2). Table 1 shows the concentration-normalized transport rate, or the clearance, of [3H]digoxin, [3H]quinidine, [3H]tetracycline, and [methoxy-14C]inulin. The B to A and A to B transport of [methoxy-14C]inulin across LLC-PK1 and LLC-GA5-COL150 cell monolayers was about 4.67–10.8 μl/hr without marked differences in the direction, cell type, or temperature except at 4°C, suggesting that paracellular transport was not affected by temperatures of 25°C or over. The B to A transport of [3H]digoxin across LLC-GA5-COL150 cell monolayers was markedly higher than the A to B transport (Fig. 2, Table 1). The B to A transport was higher and the A to B transport was lower in LLC-GA5-COL150 cell monolayers compared with LLC-PK1 cells, showing that the net B to A transport was faster in LLC-GA5-COL150 cells than LLC-PK1 cells (Fig. 2, Table 1). This finding indicates that this method is appropriate for the evaluation of MDR1-mediated transport. Similar results were obtained for [3H]quinidine, another substrate for MDR1 (Table 1). In contrast, the B to A and A to B transport of [3H]tetracycline were similar in both LLC-GA5-COL150 and LLC-PK1 cell monolayers (Table 1), confirming the transport of tetracycline to be independent of MDR1.

The B to A transport and A to B transport of [3H]digoxin, [3H]quinidine, and [3H]tetracycline across LLC-PK1 and LLC-GA5-COL150 cell monolayers decreased with lower temperature (Table 1). Figure 3 shows the effects of lower temperature on the net B to A transport ratio for [3H]digoxin, [3H]quinidine, and [3H]tetracycline in LLC-GA5-COL150 cells. The net B to A transport ratio of [3H]digoxin decreased with lower temperature, suggesting that MDR1 transport activity was reduced at 32°C and lower. The net B to A transport ratio of [3H]quinidine was only reduced at 4°C. Although the net B to A transport ratio of [3H]quinidine was only reduced at 4°C. Although the net B to A transport ratio of [3H]tetracycline, not a substrate for MDR1, was not changed at lower temperatures (Fig. 3), both the B to A and A to B transport of [3H]tetracycline were decreased at lower temperatures, suggesting that transcellular transport mediated by passive diffusion was also affected by lower temperatures, although less than MDR1 transport activity.
Table 1  Effect of temperature on the transepithelial transport of drugs in LLC-PK₁ and LLC-GA5-COL150 cells

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Temperature (°C)</th>
<th>LLC-PK₁ (µl/hr/well)</th>
<th>LLC-GA5-COL150 (µl/hr/well)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>B to A</td>
<td>A to B</td>
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<tr>
<td>[³H]digoxin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>78.4 ± 8.9</td>
<td>39.0 ± 6.6</td>
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<tr>
<td>32</td>
<td></td>
<td>47.2 ± 4.5*</td>
<td>25.1 ± 4.1*</td>
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<td>30</td>
<td></td>
<td>21.4 ± 2.2*</td>
<td>37.9 ± 5.1*</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>19.0 ± 0.3*</td>
<td>12.0 ± 0.2*</td>
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<td>4</td>
<td></td>
<td>1.84 ± 0.18*</td>
<td>2.67 ± 0.50*</td>
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<tr>
<td>[³H]quinidine</td>
<td></td>
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<td>37</td>
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<td>224 ± 19</td>
<td>237 ± 21</td>
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<td>32</td>
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<td>178 ± 10*</td>
<td>207 ± 26</td>
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<td>25</td>
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<td>155 ± 17*</td>
<td>182 ± 7*</td>
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<td></td>
<td>24.6 ± 5.4*</td>
<td>36.5 ± 4.4*</td>
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<td>[³H]tetracycline</td>
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<td>37</td>
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<td>8.84 ± 0.84</td>
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<td>32</td>
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<td>7.02 ± 0.27</td>
<td>7.40 ± 0.92</td>
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<td>5.67 ± 0.67</td>
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<td>6.83 ± 1.82</td>
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<td>1.64 ± 0.17*</td>
<td>3.13 ± 2.26*</td>
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<tr>
<td>[Methoxy-14C]inulin</td>
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<td>37</td>
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<td>6.04 ± 0.37</td>
<td>6.73 ± 0.46</td>
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<td>32</td>
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<td>6.13 ± 1.36</td>
<td>7.21 ± 0.51</td>
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<td>4.67 ± 1.04</td>
<td>6.31 ± 1.43</td>
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<td>5.88 ± 0.74</td>
<td>7.91 ± 1.46</td>
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<td>1.80 ± 0.47*</td>
<td>3.04 ± 1.89*</td>
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</table>

Values are the mean ± standard deviation of results from at least three independent experiments. Comparison with the data at 37°C was performed by one-way analysis of variance followed by Scheffe’s post-hoc test (two-tailed), with p < 0.05 considered significant (*).

Fig. 3  Temperature-dependent net B to A transport ratio of [³H]digoxin (A), [³H]quinidine (B), and [³H]tetracycline (C). Each bar represents the mean ± standard deviation of results from at least three independent experiments. Comparison with the data at 37°C was performed by one-way analysis of variance followed by Scheffe’s post-hoc test (two-tailed), with p < 0.05 considered significant (*).
Discussion

This study clearly showed that the net B to A transport ratio of digoxin, a MDR1 substrate, was decreased at 32°C, which is the lowest target temperature for therapeutic mild hypothermia. However, the decrease in the net transport ratio of quinidine, another MDR1 substrate, was only observed at 4°C. The reason for the discrepancy between MDR1 substrates digoxin and quinidine is not clear. Quinidine is also a substrate for the organic cation transporter, which is expressed by LLC-PK1 cells. Therefore, the organic cation transporter might have affected the findings of temperature dependency. Tetracycline was used as a non-substrate for MDR1. Although tetracycline is a substrate for human organic anion transporter, LLC-PK1 cells are deficient in organic anion transporters, suggesting that the transport of tetracycline involved passive diffusion in the present study. No significant difference in the passive diffusion process was found at 32°C. In addition, the transport of inulin, as a marker of the paracellular pathway, showed no effect of temperature at 25°C, suggesting that the tight junction is not affected during therapeutic hypothermia. Therefore, this study indicated that therapeutic moderate hypothermia causes the suppression of drug transport via MDR1 rather than via passive diffusion.

Hypothermia is reported to change the pharmacokinetics of various drugs, including phenytoin, vecuronium, and neostigmine in humans, and flesinoxan and fentanyl in experimental animals. Such changes are thought to be due to decrease in blood flow, hepatic metabolism, and biliary excretion. The present study showed suppression of MDR1 transport during hypothermia. MDR1 transport may be the main mechanism for MDR1 substrates such as vecuronium, especially for brain distribution, because MDR1 in the BBB is critical for limiting the distribution and controlling the efflux of xenobiotic compounds from the brain.

In conclusion, therapeutic moderate hypothermia may suppress hepatobiliary excretion and renal secretion, and enhance the distribution in the brain and testis of drugs, especially those which are substrates for MDR1 such as calcium blockers and antiarrhythmic drugs.

Acknowledgment

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MDR1-Mediated Transport in Hypothermia

Commentary

The authors have reported the results of an elegant experiment in which they determined the effect of moderate hypothermia on transepithelial transport via multi-drug resistance protein-1 (MDR1). The in vitro evaluation utilized an MDR1-over-expressing cell line established by transfection of human MDR1, complementary deoxyribonucleic acid into porcine kidney epithelial cells. Digoxin, quinidine, tetracycline and inulin were used to evaluate typical substrate, non-substrate and passive diffusion.

The results indicate that MDR1 transport activity is reduced at 32 degrees centigrade and lower, yet transcellular transport, mediated by passive diffusion, was affected less than MDR1 transport activity. The transport of inulin, a marker of the paracellular pathway, showed no effect. These findings support the hypothesis that moderate hypothermia suppresses drug transport via MDR1 rather than passive diffusion.

These findings may explain some of the contradictory results obtained in the laboratory and in clinical trials utilizing hypothermia for a wide variety of conditions whose treatment utilizes various pharmacological agents. It is through careful experimentation such as this that the precise role of hypothermia in the clinical situation can be best determined.

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This paper describes the pharmacokinetics and pharmacodynamics of drugs under conditions of therapeutic moderate hypothermia. The authors focused on MDR1 because MDR1 expressed in the BBB limits the brain distribution of various types of substances. By using a unique method of transfecting human MDR1 into LLC-GA5-COL150 cells, this study showed that therapeutic moderate hypothermia caused the suppression of drug transport via MDR1 rather than via passive diffusion.

Although this study evaluates the mechanism of change in pharmacokinetics and pharmacodynamics in hypothermia in vitro, the authors are encouraged to advance this study by using primary neuron culture and to extend it into in vivo study in order to apply this unique study for the direction of therapy.

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