Regular Article

Efflux Transport of N-monodesethylamiodarone by the Human Intestinal Cell-Line Caco-2 Cells

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Summary: Amiodarone (AMD) is a benzofurane derivative with class III antiarrhythmic activity that is effective in controlling intractable cardiac arrhythmias. One of the most common and serious drug interactions in clinical practice is the interaction between digoxin and an antiarrhythmic agent. It has been reported that AMD and N-monodesethylamiodarone (DEA), the active metabolite of AMD, inhibit the P-glycoprotein (P-gp/MDR1)-mediated digoxin transport. However, the intestinal transport processes of AMD and DEA have not been fully revealed. In this study, we focused on the intestinal transport mechanism of DEA and characterized the intestinal transport of DEA using Caco-2 cells. Basal-to-apical transport of DEA by Caco-2 cells was greater than apical-to-basal transport. The relationship between concentration and basal-to-apical flux rate appeared to approach saturation. The uptake of DEA by Caco-2 cells was increased in the presence of typical ATP-depletion compounds and thyroid hormones. On the other hand, substrates for P-gp, multidrug resistance-associated proteins (MRPs/ABCCs) and breast cancer resistance protein (BCRP/ABCG2) had no effect on the efflux of DEA. These results suggest that an ATP-binding cassette (ABC) transporter, which is different from P-gp, MRPs and BCRP, mediates the efflux of DEA across the apical membrane in Caco-2 cells and that thyroid hormone inhibits this transporter.

Key words: N-monodesethylamiodarone; amiodarone; thyroid hormones; transporter

Introduction

Recent studies have shown that multiple transporters are expressed in the gastrointestinal tract and play important roles in the secretion and absorption of many compounds.¹ It is well known that drug-drug interactions involving transporters can often directly affect the therapeutic safety and efficacy of many important drugs.

Amiodarone (AMD) is a benzofurane derivative with class III antiarrhythmic activity that is effective in controlling intractable cardiac arrhythmias.²,³ Clinical evidence suggests that this drug plays a role in reducing the relative risk for arrhythmia or sudden death and the overall mortality in survivors of myocardial infarction and in heart failure patients.⁴–⁷ AMD is an arrhythmic agent that is widely used and coadministered with many kinds of drugs.

One of the most common and serious drug interactions in clinical practice is the interaction between digoxin and an antiarrhythmic agent. Digoxin is recognized to be transported by an ATP-dependent efflux pump, P-glycoprotein (P-gp/MDR1).⁸ It has been reported that AMD and N-monodesethylamiodarone (DEA), the active metabolite of AMD, inhibited the basal-to-apical transport of [³H]digoxin by human MDR1 cDNA-transfected LLC-PK1 cells. Therefore, MDR1 is thought to be responsible for the previously reported drug interaction between digoxin and AMD.⁹ It is not known, however, P-gp recognizes AMD and DEA as a substrate or not. Since AMD is widely used in clinical practice despite its frequent side effects after chronic administration, it is important to elucidate the transport mechanisms of AMD and DEA.

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Fig. 1. Chemical structures of amiodarone (AMD) and N-monodesethylamiodarone (DEA).

The human colon adenocarcinoma cell line Caco-2 has been used as a model in which to study intestinal absorption or secretion of various drugs.10 This cell line spontaneously differentiates in culture into polarized cell monolayers with many enterocyte-like properties of transporting epithelia. Caco-2 cells retain various transporters expressed in the intestine. Using this model, a number of studies have been performed to characterize intestinal transport mechanisms. We have found that a specific transport system is responsible for the uptake of AMD across the apical membrane in Caco-2 cells (submitted to journal). We therefore focused on the intestinal transport mechanism of DEA.

DEA is more cytotoxic than AMD suggesting that DEA plays an important role in the development of AMD-induced toxicity.11–13 The purpose of this study was to investigate the intestinal efflux transport mechanism of DEA.

Materials and Methods

**Chemicals:** DEA was kindly supplied by Taisho Pharmaceutical (Tokyo, Japan). All other reagents were of the highest grade available and used without further purification. DEA was dissolved in methanol (1% w/v final concentration) due to their hydrophobic properties and poor solubility in water.

**Cell culture:** Caco-2 cells obtained from American Type Culture Collection (Rockville, MD) were maintained in plastic culture flasks (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) as described previously.14 These stock cells were subcultivated before reaching confluence. The medium consisted of Dulbecco’s Modified Eagle’s Medium (Sigma) supplemented with 10% fetal bovine serum (ICN Biomedicals, Inc, Aurora, OH), 1% nonessential amino acid (Gibco), 2 µM L-glutamine (Gibco) and 100 IU/mL penicillin-100 µg/mL streptomycin (Sigma). The monolayer cultures were grown in an atmosphere of 5% CO₂-95% O₂ at 37°C. The cells were given fresh growth medium every 2 days. When the Caco-2 cells had reached confluence, they were harvested with 0.25 mM trypsin and 0.2% EDTA (0.5-1 min at 37°C), resuspended, and seeded into a new flask.

In the present study, Caco-2 cells were used between passages 45 and 60. For the transport study, Caco-2 cells were seeded at a cell density of 2 × 10⁶ cells/cm² on 6-well (3-µm pores, 4.71-cm² growth area) Transwell™ (Corning Costar Corp., Cambridge, MA). The cell monolayers were fed a fresh growth medium every 2 days and were used at 16 to 21 days for the transport experiments. TEER was used to monitor the integrity of the monolayers. Monolayers with TEER above 350 Ωcm² (after subtracting the back group value of the transwell) were used in the efflux study. For the uptake studies, Caco-2 cells were seeded at a cell density of 1 × 10⁷ cells/cm² on 12-well plastic plates (Corning Costar Corp., Cambridge, MA). The cell monolayers were fed a fresh growth medium every 2 days and were used at 4 to 6 days for the uptake experiments.

**Transcellular transport across Caco-2 cell monolayers:** Transcellular transport of DEA was measured using monolayer cultures grown in 6-well Transwell™. The incubation medium used for the transcellular transport study was HBSS-MES (pH 5.0) buffer. After removal of the growth medium from both sides of monolayers, the cells were preincubated at 37°C for 10 min with HBSS-MES (pH 5.0) buffer (6-well; 2.6 mL of outside and 1.5 mL of inside). After removal of the medium, incubation medium containing DEA was added outside. The monolayers were incubated for 60 min at 37°C. For transport measurements, aliquots of incubation medium on the other side were taken at specified times and samples were collected for immediate analysis.

**Uptake study in Caco-2 cell monolayers:** The uptake experiment was performed as described previously.15 The uptake of DEA was measured using monolayer cultures grown in 12-well plastic plates. The incubation medium used for the uptake study was HBSS-MES (pH 5.0) buffer (25 mM D-glucose, 137 mM NaCl, 5.37 mM KCl, 0.3 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.26 mM CaCl₂, 0.8 mM MgSO₄ and 10 mM MES). After removal of the growth medium, cells were preincubated at 37°C for 10 min with 1.0 mL of HBSS-MES buffer (pH 5.0). After removal of the medium, 1.0 mL of incubation medium containing a substrate was added. The
monolayers were incubated for the indicated time at 37 °C. Each cell monolayer was washed rapidly twice with a 2.0 mL ice-cold incubation medium at the end of the incubation period. The cells were solubilized with 0.5 mL of 1N NaOH and neutralized with 0.5 mL HCl. After vortexing briefly, a part of the mixture (100 μL) was transferred to a fresh tube and 400 μL MeOH was added. After centrifugation of the mixture (15,000 x g, for 10 min), the concentration of DEA in the supernatant was measured.

**Analytical procedures:** DEA was determined using an HPLC system equipped with a Shimadzu LC liquid chromatograph pump and UV detector. Data collection, integration and calibration were accomplished using. The column was a Mightysil RP-8GP column (4.6 x 250 mm (5 μm), Kanto Chemical). A mobile phase containing 9.5 mM H3PO4: acetonitrile (1:1, v/v) was used. Column temperature and flow rate were 40°C and 1.0 mL/min, respectively. The wavelength for detection of DEA was 242 nm. Protein was measured by the method of Lowry et al. with bovine serum albumin as a standard.16)

The apparent permeability coefficient (P_app) was calculated using the following equation:

\[ P_{app} = \frac{dQ/dt}{A \cdot C_0} \]

where dQ/dt is the linear appearance rate of mass in the receiver solution, A is the filter/cell surface area (4.71 cm²), and C₀ is the initial concentration of substrate (100 μM).

Statistical significance was evaluated using ANOVA followed by Student’s t-test, and a value of p < 0.05 was considered significant.

**Results**

**Transcellular transport of DEA:** Figure 2A shows the transcellular transport of DEA by Caco-2 cells. Basal-to-apical transport of DEA was greater than apical-to-basal transport. To characterize the basal-to-apical transport of DEA, the concentration-dependence of DEA transcellular transport by Caco-2 cell monolayers was examined. The relationship between concentration and basal-to-apical flux rate appeared to approach saturation (Fig. 2B).

**Effects of various compounds on the efflux of DEA by Caco-2 cells:** Next, we focused on the efflux mechanisms of DEA involving transporters in the apical membrane. Since the Caco-2 cells were grown in wells, basolateral transporters make a minor contribution to the transport of DEA.17) In order to characterize the efflux transport of DEA from Caco-2 cells, an uptake study was carried out. Since the uptake of DEA was reached an almost steady state at 30 min after the start of incubation (data not shown), the efflux of DEA was characterized by the amount of DEA remaining in Caco-2 cells for 60 min in the presence of inhibitors (Table 1). The efflux of DEA was inhibited by typical ATP-depletion compounds, carbonyl cyanide m-chlorophenylhydrazone (CCCP), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP). ATP-binding cassette (ABC) transporters, such as P-gp, multidrug resistance-associ-
### Table 1. Effects of various compounds on the uptake of DEA by Caco-2 cells

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (mM)</th>
<th>DEA uptake (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>100.0 ± 3.2</td>
</tr>
<tr>
<td>CCCP</td>
<td>0.05</td>
<td>196.5 ± 20.1*</td>
</tr>
<tr>
<td>FCCP</td>
<td>0.05</td>
<td>192.7 ± 14.8*</td>
</tr>
<tr>
<td>DNP</td>
<td>0.05</td>
<td>121.7 ± 8.4*</td>
</tr>
<tr>
<td>Verapamil</td>
<td>1.0</td>
<td>99.3 ± 8.3</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>1.0</td>
<td>96.4 ± 8.1</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>1.0</td>
<td>108.8 ± 13.1</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>1.0</td>
<td>96.6 ± 7.8</td>
</tr>
<tr>
<td>T₃</td>
<td>0.05</td>
<td>195.8 ± 5.4*</td>
</tr>
<tr>
<td>T₄</td>
<td>0.05</td>
<td>207.8 ± 3.5*</td>
</tr>
</tbody>
</table>

The uptake of DEA was incubated with DEA (50 µM) over a period of 60 min at 37°C in the presence or absence of inhibitors. The control value is 59.7 nmol/mg protein/60 min. Each value is the mean ± S.D. of three measurements. *; significantly different from the control (p<0.05)

### Table 2. Effects of CCCP and thyroid hormones on transcellular transport of DEA by Caco-2 cell monolayers

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (mM)</th>
<th>DEA transcellular transport (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>100.0 ± 4.9</td>
</tr>
<tr>
<td>CCCP</td>
<td>0.05</td>
<td>68.7 ± 0.6*</td>
</tr>
<tr>
<td>T₃</td>
<td>0.1</td>
<td>78.9 ± 0.5*</td>
</tr>
<tr>
<td>T₄</td>
<td>0.05</td>
<td>83.0 ± 4.7*</td>
</tr>
</tbody>
</table>

The monolayers were incubated at 37°C for 60 min with DEA (100 µM) added to the basolateral side in the absence or presence of CCCP, T₃ and T₄. After incubation, DEA efflux to the apical side was measured. The control value is 0.36 nmol/cm²/60 min. Each value is the mean ± S.D. of three measurements. *; significantly different from the control (p<0.05)

The uptake of DEA was increased in the presence of thyroid hormones (T₃ and T₄), which have an iodophenolic ring and molecular conformation similar to that of AMD. We then examined the effects of CCCP, T₃ and T₄ on the transcellular transport of DEA. All of these compounds significantly decreased DEA transport (Table 2).

### Discussion

In clinical practice, changes in pharmacokinetics due to drug-drug interactions can often directly affect the therapeutic safety and efficacy of drugs. It has been reported that various transporters are expressed in the intestine. There are many clinical examples of drug-drug interactions due to inhibition of transport proteins in the intestine.

Among the various antiarrhythmic agents, AMD has electrophysiological effects that most closely approximate those of the ideal antiarrhythmic agent. AMD has a number of side effects, including hepatic, pulmonary and thyroid toxicity, and multiple drug interactions that limit its clinical use. Moreover, it has been reported that AMD and DEA inhibit the P-gp-mediated digoxin transport. However, the intestinal transport processes of AMD and DEA have not been fully revealed. Since we have found that a specific transport system plays a role in the uptake of AMD across the apical membrane in Caco-2 cells, we characterized the intestinal transport of DEA using Caco-2 cells. We found that basal-to-apical transport of DEA was greater than the apical-to-basal transport and basal-to-apical transport of DEA was saturable (Fig. 2), suggesting that DEA was secreted by carrier-mediated system. In order to characterize the efflux transport of DEA from Caco-2 cells, an inhibition study was carried out. The results suggest that an ABC transporter, which is different from P-gp, BCRP nor MRPs, plays a role in the efflux of DEA across the apical membrane in Caco-2 cells (Table 1). Thyroid dysfunction is one of the most common adverse effects of AMD because of its high iodine content (37% of the molecular weight) and structural similarity to thyroid hormones. We focused on thyroid hormones and examined the effects of thyroid hormones on the transport of DEA by Caco-2 cells. Significantly enhanced uptake of DEA and reduced basal-to-apical transport of DEA were found in the presence of thyroid hormones (Tables 1, 2). Ribeiro et al. reported that the efflux of thyroid hormones is saturable and that this transport is inhibited by verapamil. Recently, Mitchell et al. reported that P-gp contributes to the export of thyroid hormone from cells. However, we found that verapamil had no effect on the efflux of DEA (Table 1). Taking all of these findings into consideration, it is possible that a specific transport system, which is different from P-gp, BCRP nor MRPs, mediates the efflux of DEA across the apical membrane in Caco-2 cells and that thyroid hormone inhibits this transporter.

There have been not so much studies on the transport of thyroid hormone. It has been reported that the rat thyroid cell line FRTL-5 cells, fibroblast NIH-3T3 cells show T₄-efflux. However, neither FRTL-5 cells nor NIH-3T3 cells synthesize P-gp. It is possible that intestinal thyroid hormone-sensitive transporter, which contributes to the intestinal secretion of DEA, plays a role in the efflux of T₃ in these cell line. However, this transport system has not yet been elucidated at the molecular level.
level. Further studies are needed to clarify the transport mechanisms of DEA and thyroid hormones.

In summary, our results suggest that an ABC transporter, which is different from P-gp, BCRP nor MRPs, mediates the eﬄux of DEA across the apical membrane in Caco-2 cells and that thyroid hormone inhibits this transporter. It is now recognized that eﬄux transporters function as an absorptive barrier and limit the oral bioavailability of various drugs. The inhibition of this transporter may lead to increase plasma concentration of oral administrated drug. Further studies are needed to clarify the characteristics of this transporter.

References
