Abstract. Multidrug resistance protein 4 (MRP4) is capable of transporting acyclic nucleotide phosphonates, but little is known about its role in lamivudine (LAM) and entecavir (ETV) transport. In the present study, the involvement of MRP4 in the transport of LAM and ETV was investigated through in vitro experiments. The cytotoxicity of three anti-viral drugs and their activities against HBV as characterized in HepG2.4D14 [wild-type hepatitis B virus (HBV)] and HepG2.A64 (ETV-resistant HBV) cells. LAM, ETV and tenofovir (TFV) demonstrated a 50% effective concentration against HBV of 4.14±0.03, 0.13±0.02 and 3.24±0.01 µM in HepG2.4D14 cells and of 5.94±0.20, 6.28±0.07 and 11.43±0.09 µM in HepG2.A64 cells, respectively. After administering 3-[(3-(2-[7-chloro-2-quinolinyl]ethyl)phenyl)-[(3-dimethylamino-3-oxoporphyl)-thio]-methyl]-thio)propanoic acid (MK571), the intracellular concentrations of all three drugs were much lower than the extracellular drug concentrations in these two cell types, whereas the intracellular levels of LAM, ETV and TFV were enhanced and the extracellular concentrations were reduced by addition of MK571. Thus, MRP4 is mainly responsible for the efflux of LAM and ETV in hepatocyte cultures. These results may contribute to enhancing antiviral efficacy.

Introduction

Multidrug resistance protein 4 (MRP4) transports nucleoside monophosphates (1), and an increasing number of studies have indicated that MRP4 transports an array of diverse substrates [eicosanoids, prostaglandins, bile acids, cyclic adenosine monophosphate, cyclic guanosine monophosphate, dehydroepiandrosterone 3-sulfate (DHEAS), conjugated steroids and folate] (2,3), anticancer agents (methotrexate and etoposide), and antiviral drugs [nelfinavir, adefovir and tenofovir (TFV)] (4,5).

Nucleos(t)ide analogues (NAs) are very effective anti-viral agents that function by inhibiting the replication of the hepatitis B virus (HBV). Currently available NAs include the nucleoside analogues lamivudine (LAM), telbivudine, and entecavir (ETV) and the nucleotide analogues adefovir and TFV. LAM, ETV, adefovir and TFV all inhibit HBV polymerase. The structure of TFV is similar to adefovir, differing only by the addition of a methyl group in the sugar-like aliphatic linker (4). Furthermore, adefovir and TFV are both transported by MRP4 (3,6,7). To the best of our knowledge, whether the transport of LAM and ETV involves MRP4 has not been reported in the literature to date. Thus, the present study aimed to evaluate the possibility that the transport of LAM and ETV may involve MRP4 in vitro.
NAs are broadly used in antiviral and anti-tumor therapy, and their intracellular concentrations affect the clinical response. Based on knowledge of the intracellular concentrations of NAs, the most simple and cost-effective technique for the determination of NA content is high performance liquid chromatography (HPLC), but in many cases this technique is not sufficiently sensitive (8). Compared with other routine techniques, the recent improvements in column technology and mass spectrometers associated with liquid chromatography-mass spectrometry (LCMS) methods have resulted in more sensitive, specific and efficient results, making them commonly used methods (9). Furthermore, they reduce the relative sample preparation and analysis time. Moreover, the intracellular concentrations of NAs (like small molecule ETV) are extremely low at pg/ml levels; thus, the LCMS method is needed for detection because the concentrations of these molecules are below the detection limits and sensitivity of typical reversed-phase HPLC methods (10). Therefore, LCMS technology is more suitable for evaluating the pharmacokinetics of NAs in vitro.

The aim of the present study was to evaluate whether MRP4 is involved in the hepatocyte efflux of LAM and ETV by i) detecting the expression of MRP4 in HepG2.4D14 cells containing wild-type HBV and HepG2.A64 cells containing ETV-resistant HBV; ii) evaluating the cytotoxic effects of LAM, ETV, TFV, 3-((3-(2-[7-chloro-2-quinoliny]ethyl)phenyl)-[3-dimethylamino-3-oxoporphyl]-thio)-methyl]-thio) propanoic acid (MK571) and the activities of these three NAs against HBV in vitro; and iii) investigating the intracellular concentrations of LAM, ETV and TFV (a positive control) in the presence or absence of MK571 in the two cell lines.

Materials and methods

Reagents. HPLC-grade LAM, ETV and MK571 were provided by Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and TFV was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The water was purified by a Purelab Classic UF purification system. Formic acid (LCMS grade) was obtained from Sigma-Aldrich; Merck KGaA and HPLC-grade methanol, ammonium acetate were supplied by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). TFV was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The water was purified by a Purelab Classic UF purification system. Formic acid (LCMS grade) was obtained from Sigma-Aldrich; Merck KGaA and HPLC-grade methanol, ammonium acetate were supplied by Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. The HepG2.4D14 (wild-type HBV) and HepG2.A64 (ETV-resistant HBV) cell lines were gifts from Dong-Ping Xu (Beijing 302 Hospital, Beijing, China) (11,12). They were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in 5% CO2 and 300 µg/ml genetin was added to the medium (Sigma-Aldrich; Merck KGaA).

Cell proliferation assay. Cells were seeded at 3x10³ cells/well into 96-well plates for five replicates in three independent experiments. After incubation for 24 h at 37°C in 5% CO2, dilution series of drugs in 100 µl conditioned medium were added and changed every other day for 4 days. The medium was removed and 10 µl Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) was added to each well and agitated for 10 min. The cells were further incubated for 1–4 h, then the absorbance was detected by an EnSpire® Multimode Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) at 450 nm wavelength. The concentrations of 50% inhibition of growth (IC50) were calculated individually by nonlinear regression using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Quantitative polymerase chain reaction (qPCR) analysis. Total RNA was extracted using TRizol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer’s protocol. The primer sequences were as follows: MRP4 sense, 5’-TGG TGCAGAAGGGAGCTTAC-3′ and antisense, 5’-GCTC TC CAGAGCCACCATCTT-3′; β-actin sense, 5’-GCCAACAACA GTGCTGTCTGG-3′ and antisense, 5’-GCTCAGGAGGAG CAATGATCTTG-3′. qPCR was performed in a thermocycler for 40 cycles according to the following procedure: 95°C for 30 sec, 95°C for 5 sec, 57°C for 30 sec, 65°C for 15 sec and then stored at 4°C. It was carried out on an Applied Biosystems Prism 7900HT Sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a one-step SYBR® PrimeScript™ Real-Time PCR kit (Takara, Dalian, China). β-actin served as an endogenous control. The relative mRNA expression levels were calculated using the 2-ΔΔct method (13).

Western blot analysis. The cells and tissues were lysed on ice using RIPA buffer and PMSF protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. The lysate was centrifuged at 12,000 x g for 20 min at 4°C, and the clear supernatant was mixed with 5X Loading sample buffer. The protein concentrations were quantified using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). Total protein (30 µg) was separated by 10% SDS-PAGE gel at room temperature for 2 h and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA) for 2 h. The membranes were incubated with anti-MRP4 (cat. no. ab180712; 1:20 dilution; Abcam, Cambridge, MA, USA) and anti-β-actin (cat. no. 12620; 1:500 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) primary monoclonal antibodies overnight at 4°C. Following this, they were incubated with the horse-radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. sc-2004; 1:4,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. The protein bands were visualized by enhanced chemiluminescence (Beyotime Institute of Biotechnology) and analyzed using Image Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

DNA extraction and HBV DNA qPCR. HBV DNA was extracted using a Tissue DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. Quantitative analysis was performed by qPCR using the One Step Hepatitis B viral DNA quantitative fluorescence diagnostic kit (Hunan Sansure Biotech, Hunan, China). Briefly, after addition of 5 µl nucleic acid lysis buffer, HBV DNA was subsequently released from the 5-µl samples. After 10 min, the above PCR reaction mixtures were added to each well. The
qPCR was performed according to the manufacturer's instructions using the absolute quantitative PCR fluorescence probing method and quantified using the 2^ΔΔCq method (13,14). Forward and reverse primer sequences for HBV were, 5'-GTGTCGCG CCCTTTTATCAT-3' and 5'-ACACAGCAGGCAACATACC TTG-3', respectively, and the specific fluorescent probe was 5'FAM-CATCTGCTGCTATGCTCATTTCTTT-Dabcyl3'. A dilution series of the WHO international reference standard for HBV DNA [NIBSC 97/746, genotype B (accession nos. D00329, AF100309, AB033554)], 0, 4x10^4, 4x10^5, 4x10^6, 4x10^7 IU/ml was PCR amplified. The thermocycling conditions were as follows: 50°C for 2 min for Taq enzyme reaction, 94°C for 5 min for Taq enzyme activation; 45 cycles of 94°C for 15 sec and 57°C for 30 sec, and 25°C for 10 sec.

Standard solutions and quality control (QC) samples. Stock solutions of LAM and ETV (2 µg/ml) and TFV (10 mM) were prepared by dissolving the reagents in 50% methanol in water, storing them at -20°C and serially diluting them in 50% methanol to generate the sample concentrations. Calibration curves (LAM and ETV for cells and culture supernatants: 0.5, 2, 10, 25, 50 and 100 ng/ml and 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/ml, respectively; TFV for cells: 0.45, 0.9, 4.5, 9.0, 45.0 and 90.0 ng/ml; TFV for culture supernatants: 9.0, 18.0, 45.0, 90.0, 180.0 and 450.0 ng/ml) and two QC samples with low and high concentrations (LAM and ETV for cells and culture supernatants: 0.5, 80 and 0.5, 8.0 ng/ml; TFV for cells: 0.9 and 72.0 ng/ml; TFV for culture supernatants: 18.0 and 360 ng/ml) were prepared by spiking stock solutions into blank cell samples or blank culture supernatants (controls).

Sample preparation. Cells (3x10^5/ml) were transferred to 6-well culture plates and the following day were treated with LAM, ETV or TFV for 4 days in the presence or absence of 5 µM MK571 (a widely used MRP4 inhibitor) for 48 h (HepG2.4D14) or 4 h (HepG2.A64). At the end of each time-point, 5x10^6 cells were centrifuged at 4°C for 5 min at 12,000 x g and washed twice with ice cold phosphate-buffered saline (PBS). The cells were fully lysed on ice by ultrasonic treatment in 200 µl 70% methanol. Culture medium was extracted with an equivalent volume of ice-cold 100% methanol. For calibration, 10 µl working standard solutions were spiked into 90 µl blank cell samples or blank supernatant tubes. After vortexing for 5 min and centrifuging at 4°C for 10 min at 12,000 x g, 100 µl samples were drawn for LCMS analysis.

LCMS conditions. The LCMS system consisted of two LC-20AD pumps, a SIL-20AHT autosampler, a CTO-20AC column oven and a DGU-20A3 degasser (Shimadzu Corporation, Kyoto, Japan). A Leapsil C18 column (150x2.1 mm, 2.7-µm particle size; Dikma, Richmond Hill, NY, USA) with a pre-column (4.0x3.0 mm I.D., 5 µm; Phenomenex, Torrance, CA, USA) was used for the sample separation. The mobile phase for ETV consisted of 5 mM ammonium acetate in water (solvent A) and methanol (solvent B). The mobile phase for LAM and TFV was 10 mM ammonium acetate, 0.5% formic acid in water (solvent A) and 0.5% formic acid in 100% methanol (solvent B). The analytical column was operated at a flow rate of 0.25 ml/min at 40°C, and a volume of 10 µl was injected at 4°C. The linear gradient profile consisted of the following proportions of solvent A and B applied at time t (min); (t, %): 1, 95%; 1.5, 5%; 3.0, 5%; 3.5, 95%.

The samples were detected using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc.) equipped with electrospray ionization (ESI). The ESI ion source temperature was set at 650°C, and the capillary voltage was 5.5 kV. Multiple reaction monitoring transitions were applied for quantification in comparison with standards: m/z 230.1→112.1 for LAM, m/z 278.3→152.1 for ETV, and m/z 288.2→176.2 for TFV. Integration of the peak area and data analysis was performed using Analyst 1.6.2 software (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Method validation. Validation of the specificity, linearity, lower limit of quantification (LLOQ), precision, accuracy, recovery (RE), matrix effect (ME), dilution and stability of the method was evaluated according to the guidance of the Food and Drug Administration (FDA) Guidance for industry bioanalytical method validation (15).

LLOQ was defined as the lowest concentration of the calibration curve at which precision and accuracy was within 20% with a signal-to-noise ratio >10.0. The selectivity of each ingredient over interference from endogenous substances was assessed. The presence of components for which the response was <20% LLOQ was accepted. A calibration curve was established based on external standards using a weighted least-squares linear regression with 1/x² weighting. Accuracy and precision were assessed by analysing five replicates of the two QC samples on three subsequent days, and accuracy and precision bias within ±15% was accepted. The RE and intra- and inter-day relative standard deviation should be <15%. ME was determined by comparing the percentage of the peak area for each ingredient spiked into the drug-free blank sample (control) with those in the two QC levels. The stability of each ingredient demonstrated that the accuracy and precision bias introduced by storage at room temperature for 4 h and 30 days at -80°C and 8 h in the autosampler were all within 15%. The dilution integrity was assessed at 2 -fold, 4-fold or 20-fold the upper limit of the quantification concentration for all samples.

Statistical analysis. All data are presented as the mean ± standard error of at least three independent experiments. Data were compared using an unpaired two-tailed Student's t-test, two-way analysis of variance followed by Bonferroni's post hoc test or nonlinear regression. The statistical analyses were performed using SPSS software version 12.0.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of MRP4 in HepG2.4D14 and HepG2.A64 cell lines. MRP4 transcriptional levels were detected in HepG2.4D14 cells containing wild-type HBV and HepG2.A64 cells containing ETV-resistant HBV. MRP4 mRNA expression was higher in HepG2.A64 cells than that in HepG2.4D14 cells (Fig. 1A; P<0.001). Furthermore, the protein expression levels of MRP4 were consistent with the MRP4 transcriptional levels (Fig. 1B and C; P<0.01).

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Cytotoxicity of compounds in HepG2.4D14 and HepG2.A64 cells. To investigate the cytotoxicity of antiviral drugs (LAM, ETV and TFV) and the MRP inhibitor MK571 in hepatic cells, HepG2.4D14 and HepG2.A64 cells were incubated with various concentrations of these drugs for 96 h (Fig. 2). In both cell types, apparent cytotoxicity was observed for LAM, ETV, TFV and MK571, at doses above 10 µM (Fig. 2). The calculated IC\textsubscript{50} was 180.80, 0.14, 256.27 and 44.57 µM in HepG2.4D14 cells for LAM, ETV, TFV and MK571, respectively, and similar IC\textsubscript{50} values were obtained in HepG2.A64 cells (Table I). Therefore, noncytotoxic doses were used for subsequent experiments.

NAs against HBV in HepG2.4D14 and HepG2.A64 cells. HepG2.4D14 and HepG2.A64 cells expressing HBV were used to analyze the anti-HBV activities of LAM, ETV and TFV. The levels of supernatant HBV DNA in HepG2.4D14 and HepG2.A64 cells treated with antiviral drugs at various non-cytotoxic concentrations for 4 days were quantified by qPCR. LAM, ETV and TFV demonstrated anti-HBV activities in HepG2.4D14 cells [concentrations for 50% of the maximal effect (EC\textsubscript{50}) values were 4.14±0.03, 0.13±0.02 and 3.24±0.01 µM, respectively] and in HepG2.A64 cells (EC\textsubscript{50} values were 5.94±0.20, 6.28±0.07 and 11.43±0.09 µM, respectively; Table II). Compared with wild-type HepG2.4D14 cells, the potency of ETV was decreased by at least 48-fold in ETV-resistant HepG2.A64 cells (Table II). The latter conferred a 3- to 4-fold decreased susceptibility to TFV in cell culture (Table II). Taken together, these data demonstrated that HepG2.4D14 and HepG2.A64 cells could be used to investigate drug resistance, suggesting that they may be helpful to screen for inhibitors of HBV replication.

Optimization of chromatographic conditions. To optimize chromatographic behavior, several types of aqueous and organic phases were evaluated: Different concentrations of ammonium acetate buffer, ammonium formate or formic acid were used instead of water; methanol was used to supplant...
acetonitrile; and different volumes of formic acid were added to the organic phase to adjust the pH value. The mobile phase selected for each ingredient was revealed to be suitable based on method validation. The ESI source conditions for MS were also optimized to obtain a good signal with a high sensitivity. The drugs concentrations were varied between the cell and supernatant samples, which led to a wide linear range of measurements. For LAM and ETV, the linear range can cover both cell and supernatant samples, and thus the same calibration curves and QCs were used. However, for TFV, the linear range did not encompass them both, and therefore two different calibration curves were used to ensure accuracy. The results for the inter-run (n=5) precision, accuracy, RE, ME and LLOQs for LAM, ETV and TFV in cell and culture supernatant samples at two QC levels are presented in Table III.

For the selectivity study, no significant peaks at the retention time of each ingredient were identified in the blank samples (control and MK571; Fig. 3A and B). Cell samples of LAM, ETV and TFV, as well as the supernatant samples of LAM and ETV displayed good chromatographic behavior using the same precipitation method with acetonitrile mixed with methanol (Fig. 4A and B). However, for the supernatant samples of TFV, the above method was not suitable for analysis of the peak shape and response values. Only when the pH was adjusted to the samples using formic acid did we obtain a suitable chromatographic behavior for TFV (Fig. 5). The retention time of LAM, ETV and TFV was 1.64, 4.25 and 4.51 min, respectively.

Effect of a transport inhibitor on LAM and ETV concentrations in HepG2.4D14 and HepG2.A64 cells. To study the pharmacokinetics of LAM and ETV in different cell types, incubations with LAM, ETV and TFV were conducted over 4 days in the presence or absence of MK571. It is well known that TFV is transported by MRP4 (3,6,7); thus, it was used as a positive control. After dosing with MK571, LAM, ETV and TFV demonstrated lower extracellular drug concentrations than those in the culture medium both in HepG2.4D14 and HepG2.A64 cells (Fig. 6), whereas their intracellular concentrations in HepG2.4D14 cells exceeded those in HepG2.A64 cells (Fig. 7). In contrast to the control group (absence of MK571), the extracellular concentrations of all three drugs were slightly reduced by the presence of MK571 (Fig. 6A-C), and the intracellular concentrations of all three drugs were increased by the presence of MK571 (Fig. 7A-C), as expected.

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while their intracellular accumulation was markedly increased by the presence of MK571 in the two cell lines, especially for ETV (P<0.05) and TFV (0.5 µM, P<0.05; 2 µM, P<0.01) in HepG2.4D14 cells (Fig. 7A-C).

Discussion

MRP4 is capable of transporting acyclic nucleotide phosphonates, but little is known about its role in LAM and ETV transport. LCMS method for estimating NA concentration in the cells is more sensitive, specific and efficient. The present study assessed the ability of MRP4 to interact with LAM and ETV using LCMS analysis, and demonstrated that MRP4 is capable of transporting LAM and ETV.

Analyses of NAs have been performed in plasma, urine, cultured cells and tissues (3,8,16-18). Plasma drug...
concentrations are usually thought to predict clinical efficacy in pharmacokinetic studies. However, the plasma or medium concentrations of drugs with low membrane permeability or those that are substrates for drug transporters, such as adefovir and TFV, may not reflect their intracellular concentrations (19). The results of the present study supported this perspective and indicated that medium concentrations of LAM, ETV and TFV were different from their intracellular concentrations. Therefore, it is important to not only monitor plasma or medium concentration but also variations in the intracellular concentrations of NAs, according to the pharmacological mechanism of drug action (8).

Figure 6. Extracellular concentrations of LAM, ETV and TFV in HepG2.4D14 and HepG2.A64 cells. HepG2.4D14 and HepG2.A64 cells were treated with (A) LAM, (B) ETV and (C) TFV for 4 days in the presence or absence of MK571. At the indicated time-points, concentrations were quantified by liquid chromatography-mass spectrometry. Data are expressed as the mean ± standard deviation. LAM, lamivudine; ETV, entecavir; TFV, tenofovir; MK571, 3-[(3-(2-[7-chloro-2-quinolinyl]ethyl)phenyl)-(3-dimethylamino-3-oxoporphyl)-thio]-methyl]-thio) propanoic acid.

Figure 7. Intracellular concentrations of LAM, ETV and TFV in HepG2.4D14 and HepG2.A64 cells. HepG2.4D14 and HepG2.A64 cells were treated with (A) LAM, (B) ETV and (C) TFV for 4 days in the presence or absence of MK571. At the end of each time-point, cells were lysed and centrifuged, and the amounts of intracellular LAM, ETV and TFV were measured by liquid chromatography-mass spectrometry. Data are expressed as the mean ± standard error. *P<0.05, **P<0.01 vs. control group. LAM, lamivudine; ETV, entecavir; TFV, tenofovir; MK571, 3-[(3-(2-[7-chloro-2-quinolinyl]ethyl)phenyl)-(3-dimethylamino-3-oxoporphyl)-thio]-methyl]-thio) propanoic acid; LCMS, liquid chromatography-mass spectrometry.
To better evaluate the intracellular concentration of NAs, the LCMS conditions were optimized. Drug detection may be seriously affected by the matrix effects caused by the use of bio-analytical methods. Many approaches are capable of reducing matrix effects, and the most important approaches include sample preparation methods, efficient chromatographic separation and use of quantitative stable isotopically labeled internal standards (20,21). The latter are often overlooked, probably owing to their high cost and limited availability in recently emerging LCMS assays (22,23). In most analytical methods, sample pretreatment is very important because the main purpose of this step is to reduce matrix effects (24). The extraction of unstable NAs is performed on ice and in the dark. Common sample preparation methods are generally divided into protein precipitation (PP) and solid phase extraction (SPE). PP is the simplest method to remove proteins using PP reagents such as inorganic acid (perchloric acid) (25), organic acid (trichloroacetic acid) (26) or an organic solvent (acetoni trile, methanol) (27,28) in biological matrices. In the present study, after lysing the cells completely using appropriate conditions such as ultrasonic vibration, methanol was used to sufficiently reduce the matrix effects. Different LC columns, mobile phase compositions and flow rates were evaluated separately for each ingredient in this study to optimize the peak shapes. A Leapsil C18 column could provide satisfactory results for all ingredients. The parameters for ESI positive ion mode were optimized separately for the detection specificity. These improved LCMS methods for the determination of the intracellular concentration of NAs were simpler, faster and more sensitive.

Because NAs inhibit serum HBV DNA and only induce low rates of HBsAg seroconversion, most patients require long-term treatment to prevent the progression of liver disease. Long-term therapy requires the ability to manage NA treatment failure. It has been demonstrated that NA treatment failure is associated with increased expression of MRP4 in antiretroviral therapy (29). Furthermore, the absence of MRP4 increases the concentrations of adeovir and TFV in MRP4 knockout mice (3). This finding provides a possible link between the levels of NA and the expression of MRP4. The results of the present study demonstrated that inhibition of MRP4 increased the intracellular concentrations of LAM, ETV and TFV in HepG2.A64 cells, thus, MRP4 may regulate LAM and ETV concentrations in human hepatocyte cell lines.

MRP4 mediates ATP-dependent unidirectional transport. It is widely present in human epithelial cells and is mostly localized to the basolateral membrane (30), except for the luminal side of the brain capillary endothelium and the renal proximal tubular cells (2). MRP4 is localized to the sinusoidal membrane of human hepatocytes, and MRP4-mediated export is potently suppressed by the quinoline derivative MK571 (31). The amounts of expressed MRP4 determine the intracellular concentration of MRP4-mediated transported drugs (4). The results of the present study demonstrated that the intracellular concentrations of the three antiviral agents were enhanced by inhibiting MRP4 export. Consequently, compared with HepG2.4D14 cells, HepG2.A64 cells expressing high levels of MRP4 displayed increased drug export and a reduced intracellular accumulation of NAs. Thereby, the intracellular concentrations of LAM, ETV and TFV in HepG2.A64 cells were lower than those in HepG2.4D14 cells.

Prostaglandin E2 may stimulate MRP4 ATPase activity; however, concentration-dependent biphasic kinetics may have an influence (32). Similarly, the MRP4-mediated transport of DHEAS and estradiol 17-β-D-glucuronide is suppressed by low concentrations of steroid analogues and sulfated bile acids in a competitive manner, whereas no inhibition is observed at a high concentration (33). The results of the present study further confirmed this finding and indicated that low doses of NAs in the presence of an MRP4 inhibitor accumulated to a greater extent than high doses of LAM (data not shown), and even high concentrations of NAs demonstrated no significant change, compared with the control group.

In conclusion, the present study demonstrated the involvement of MRP4 in LAM and ETV efflux, and elucidated the mechanism of its distribution in two hepatocyte cell lines. These results may contribute to enhancing antiviral efficacy and will be applied in the case of NA treatment failure.

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