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Human arylacetamide deacetylase is responsible for deacetylation of rifamycins: Rifampicin, rifabutin, and rifapentine

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ABSTRACT

Rifamycins such as rifampicin, rifabutin, and rifapentine are used for the treatment of tuberculosis and induce various drug-metabolizing enzymes. Rifamycins have been reported to be mainly deacetylated by esterase(s) expressed in human liver microsomes (HLM) to 25-deacetylrifamycins, but the responsible enzyme remained to be determined. In this study, we found that recombinant human arylacetamide deacetylase (AADAC) could efficiently deacetylate rifamycins, whereas human carboxylesterases, which are enzymes responsible for the hydrolysis of many prodrugs, showed no activity. The involvement of AADAC in the deacetylation of rifamycins in HLM was verified by the similarities of the K_m and K_i values and the inhibitory characteristics between recombinant AADAC and HLM. Rifamycins exhibited potent cytotoxicity to HepG2 cells, but their 25-deacetylated metabolites did not. Luciferase assay using a reporter plasmid containing CYP3A4 direct repeat 3 and everted repeat 6 motifs revealed that 25-deacetylrifamycins have lesser potency to transactivate CYP3A4 compared with the parent drugs. Supporting these results, HepG2 cells infected with a recombinant adenovirus expressing human AADAC showed low cytotoxicity and induction potency of CYP3A4 by rifamycins. In addition, CYP3A4 induction in human hepatocytes by rifamycins was increased by transfecting siRNA for human AADAC. Thus, we found that human AADAC was the enzyme responsible for the deacetylation of rifamycins and would affect the induction rate of drug-metabolizing enzymes by rifamycins and their induced hepatotoxicity.

1. Introduction

Tuberculosis is one of the major health problems in developing countries and its incidence remains higher in Japan among developed countries [1]. Rifamycins are a group of structurally similar, complex, macrocyclic antibiotics produced by *Streptomyces mediterranei* [2] and are the most important first-line antituberculosis drugs. The representative rifamycins are rifampicin, rifabutin, and rifapentine (Fig. 1). Among them, rifampicin and rifabutin have been in clinical use for a long time. Rifampicin is used for the treatment of tuberculosis, leprosy, and some types of osteomyelitis and endocarditis [3]. Rifabutin is a broad-spectrum antibiotic widely prescribed for the treatment and prophylaxis of *Mycobacterium avium*-intracellular complex in patients with acquired immunodeficiency syndrome (AIDS) [4]. Rifapentine is a newly developed agent structurally similar to rifampicin, and the minimum inhibitory concentration of this drug was 2–4-fold lower than that of rifampicin [3]. It is generally accepted that rifamycins can induce many drug-metabolizing enzymes including cytochrome P450 (CYP) 3A4 [5]. Thus, drug–drug interactions sometimes occur with the combination of rifamycins and drugs metabolized by the same enzymes. For example, patients taking rifampicin had low area under the curve (AUC) ratio of fluconazole [6], which are both metabolized by CYP3A4. In addition, rifampicin has been reported to cause toxic injury to hepatocytes [7]. However, the involvement of rifamycins in hepatic injury remains unclear because rifamycins are frequently used in combination with other antitubercular drugs such as isoniazid and pyrazinamide [8].

It has been reported in a study using human liver microsomes (HLM) that a main metabolic pathway of rifamycins is 25-deacetylation [4]. The 25-deacetylation of rifampicin and rifabutin did not require nicotinamide adenine dinucleotide phosphate and was completely inhibited by paraoxon and diisopropyl fluorophosphate (DFP), which are inhibitors of esterases [4]. However, it remains to be clarified which esterase in HLM, such as arylacetamide deacetylase (AADAC), carboxylesterase (CES), cholinesterase (ChE), and paraoxonase (PON), is responsible for the reaction. Previously, ChE was considered to be a candidate based

Abbreviations: AADAC, arylacetamide deacetylase; AdAADAC, a recombinant adenovirus expressing human AADAC; AdGFP, a recombinant adenovirus expressing a green fluorescence protein; BNPP, bis-*p*-nitrophenylphosphate; CES, carboxylesterase; ChE, cholinesterase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate; HLM, human liver microsomes; HPLC, high performance-liquid chromatography; NaF, sodium fluoride; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PON, paraoxonase.

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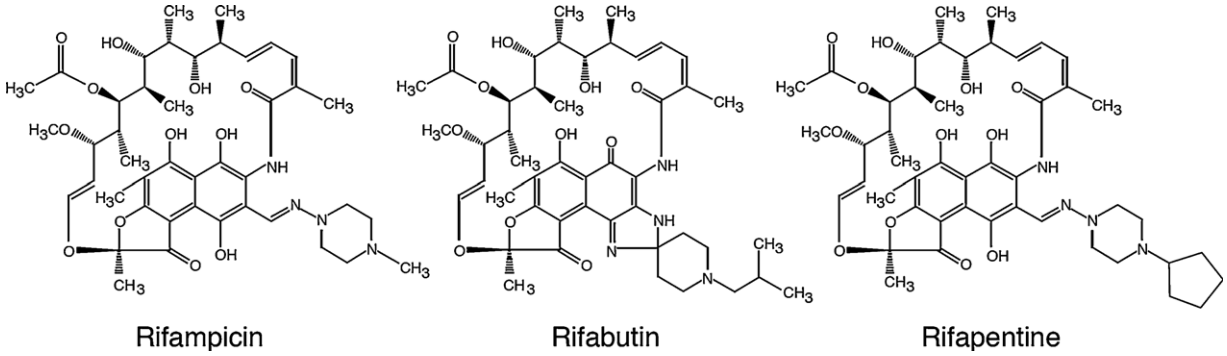

Fig. 1. Chemical structures of rifampicin, rifabutin and rifapentine.

on the fact that the reaction in HLM was inhibited by eserine, a potent inhibitor of ChE [9]. However, human serum or whole blood including ChE could not catalyze the rifabutin 25-deacetylation [9]. Therefore, it was speculated that an esterase other than ChE would be responsible for the deacetylation of rifamycins.

Human AADAC is a major serine esterase expressed in HLM [10]. AADAC was first identified as the enzyme that catalyzes the deacetylation of 2-acetylaminofluorene [11]. Until recently, AADAC has been classified as a lipase because of the high homology of the active site domain of AADAC with that of hormone-sensitive lipase [12,13]. However, we demonstrated that human AADAC is involved in the hydrolysis of the clinical drugs, flutamide and phenacetin [14,15]. Because the AADAC enzyme activity was inhibited by DFP and eserine [14], it was conceivable that AADAC is responsible for the deacetylation of rifamycins. In this study, we identified that human AADAC is the enzyme responsible for the deacetylation of rifamycins. The effect of this deacetylation on the induced cytotoxicity and enzyme induction potency was also investigated.

2. Materials and methods

2.1. Materials and reagents

Flutamide, 5-amino-2-nitrobenzotrifluoride (FLU-1), rifampicin, DFP, physostigmine sulfate (eserine), phenylmethylsulfonyl fluoride (PMSF) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 25-Desacetylrifampicin (25-deacetylrifampicin), rifabutin, 25-desacetylrifabutin (25-deacetylrifabutin), rifapentine, and 25-desacetylrifapentine (25-deacetylrifapentine) were purchased from Toronto Research Chemicals (Toronto, Canada). Cell Counting Kit-8 (CCK-8) was purchased from Dojin Chemical Laboratories (Kumamoto, Japan). Bis-*p*-nitrophenylphosphate (BNPP) and sodium fluoride (NaF) were purchased from Sigma–Aldrich (St. Louis, MO). Pooled HLM (prepared from 50 individuals) were purchased from BD Gentest (Woburn, MA). The Adenovirus Expression Vector Kit (Dual version) and Adenovirus genome DNA-TPC were purchased from Takara (Shiga, Japan). The QuickTiter Adenovirus Titer Immunoassay kit was from Cell Biolabs (Tokyo, Japan). Stealth Select RNAi for human AADAC (Accession NM_HSS100016) (siAADAC) and Stealth RNAi Negative Control Medium GC Duplex #2 (siScramble) were obtained from Invitrogen (Carlsbad, CA). Restriction enzyme *Swa*I was from New England Biolabs (Beverly, MA). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Human cryopreserved hepatocytes (lot 82 (Hispanic, female, 23 years) and lot 100 (Caucasian, female, 74 years)) were purchased from In Vitro Technologies (Baltimore, MD). Other chemicals and solvents were of analytical or the highest grade commercially available.

2.2. Rifamycin deacetylase activity

The rifamycins (rifampicin, rifabutin, and rifapentine) deacetylase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4) and various enzyme sources (HLM and Sf21 cell homogenates expressing esterases, 0.5 mg/ml). Sf21 cell homogenates expressing AADAC, CES1A1, or CES2 were prepared previously [15,16]. In a preliminary study, we confirmed that the formation rate of 25-deacetylrifamycins was linear with respect to the protein concentration (<1.5 mg/ml) and incubation time (<90 min). The rifamycins were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 5–1000 μM rifampicin, 2–500 μM rifabutin, or 5–500 μM rifapentine after 2-min preincubation at 37 °C. After 60-min incubation, the reaction was terminated by the addition of 0.1 ml of ice-cold acetonitrile. After removal of the protein by centrifugation at 9500 × *g* for 5 min, a 60-μl portion of the supernatant was subjected to high-performance liquid chromatography (HPLC). The HPLC analysis was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), an L-7405 UV detector (Hitachi), and a D-2500 Chromato-Integrator (Hitachi) equipped with an Inertsil ODS-3 column (5-μm particle size, 4.6 mm i.d. × 250 mm; Kanto Chemical, Tokyo, Japan). The eluent was monitored at 339 nm for the rifampicin and rifapentine deacetylase activities and 278 nm for the rifabutin deacetylase activity with a noise-base clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increase the signal 3-fold by differentiating the output, and 5-fold by further amplification with an internal amplifier, resulting in a maximum 15-fold amplification of the signal. The mobile phases were 34% acetonitrile containing 0.1% triethylamine and 10 mM ammonium acetate (pH 4.0) for rifampicin deacetylase activity and 40% acetonitrile containing 0.1% triethylamine and 10 mM ammonium acetate (pH 4.0) for rifabutin and rifapentine deacetylase activities. The flow rate was 1.0 ml/min. The column temperature was 35 °C. The quantification of 25-deacetylrifamycins was performed by comparing the HPLC peak height with that of an authentic standard. Because few 25-deacetylrifamycin contaminants exist in the commercially available rifamycins, the content of 25-deacetylrifamycins in the mixture incubated without the enzyme was subtracted from that with the enzyme to correct the activity. The activity in each concentration was determined as the mean value in triplicate. For kinetic analyses of the rifamycins deacetylase activity, the parameters were estimated from the fitted curves using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis.

2.3. Inhibition analyses

To clarify whether AADAC is the main esterase responsible for the deacetylation of rifamycins in HLM, the inhibition analysis was performed using representative esterase inhibitors. Organophosphates BNPP and DFP are general CES inhibitors [17,18]. Eserine and NaF are ChE inhibitors [19,20]. PMSF is a serine esterase inhibitor [21]. DTNB is an arylesterase inhibitor [22]. Among above inhibitors, DFP and eserine were strong inhibitors against AADAC [14]. The concentrations of inhibitors were 10 μM for BNPP, DFP, eserine, and DTNB, and 1 mM for PMSF and NaF. PMSF and DTNB were dissolved in DMSO such that the final concentration of DMSO in the incubation mixture was 1.5%. Other inhibitors were dissolved in distilled water. The experimental procedures and conditions were the same as described above except that 50 μM rifampicin, 10 μM rifabutin, or 20 μM rifapentine were added. These rifamycin concentrations are similar to K_m values of each deacetylase activity in HLM. It was confirmed that 1.5% DMSO did not inhibit the deacetylase activity of the rifamycins, and the control activity was determined in the presence of 1.5% DMSO.

2.4. Cell culture

Human embryonic kidney 293 cells and human hepatoma cell line HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). The 293 and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 3% glutamine, 16% sodium bicarbonate, and 0.1 mM non-essential amino acids (Invitrogen) in a 5% CO_2 atmosphere at 37 °C. Cells were infected with the adenovirus in medium containing 5% FBS.

2.5. Construction of recombinant adenovirus

A recombinant adenovirus expressing human AADAC (AdAADAC) was constructed using the cosmid-terminal protein complex (COS-TPC) method according the manufacturer's instructions. AADAC cDNA was amplified by PCR from pTARGET vector including AADAC cDNA constructed in the previous study [14] using PrimeSTAR HS DNA Polymerase (Takara) and the following primer sets: forward primer, 5′-TAGAGACCAAGAAGCGGGAC-3′ and reverse primer, 5′-GCTACATGTTTTACTATAGATTTTCC-3′. The PCR product was inserted into the *Swa*I site of the pAxcwtit vector. This vector and the parental adenovirus DNA terminal protein complex were co-transfected into 293 cells by Lipofectamine 2000 (Invitrogen). The recombinant adenovirus was isolated and propagated into the 293 cells. The recombinant adenovirus expressing a green fluorescence protein (AdGFP) for negative control was generated in a previous study [23]. Viral titers were determined by a QuickTiter Adenovirus Titer Immunoassay kit. The titers of AdAADAC and AdGFP were 9.2×10^8 plaque forming units (PFU)/ml and 2.1×10^8 PFU/ml, respectively.

2.6. Immunoblot analysis and enzyme activity of human AADAC in HepG2 cells infected with AdAADAC

HepG2 cells (3.5×10^5 cells/well) were seeded in 12-well plates. After 24 h incubation, the cells were infected with AdAADAC at MOI 0, 5, 10, 20, 50, or 100 for 24, 48, or 72 h. Immunoblot analysis and flutamide hydrolase activity were analyzed for the AADAC protein expression and enzyme activity, respectively. Immunoblot analysis was performed using total cell homogenates of adenovirus-infected HepG2 cells (30 μg) as described previously [14]. For the flutamide hydrolase activity, after the cells were incubated with 500 μM flutamide for 1 h, the FLU-1 concentration in the supernatant was measured using HPLC analysis as described previously [14].

The activity in HepG2 cells infected with AdAADAC was compared with that in human hepatocytes. Human hepatocytes were maintained in hepatocyte culture medium (Cambrex, East Rutherford, NJ) at 37 °C under an atmosphere of 5% CO_2. Human hepatocytes (3.0×10^5 cells/well) were seeded in 12-well plates. After 1-h incubation, cells were incubated with 500 μM flutamide for 1 h and the FLU-1 concentration in the supernatant was measured.

2.7. Cytotoxicity assay

To evaluate the effects of rifamycins and their 25-deacetylated metabolites on the cell viability, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt) assay, which is a modified MTT assay, was performed as follows: HepG2 cells (1.5×10^5 cells/well) were seeded in 24-well plates. After 48 h incubation, the cells were treated with 0–200 μM rifampicin, rifabutin, or their 25-deacetylated metabolites, and 0–100 μM rifapentine or deacetylrifapentine for 24 h or 48 h. CCK-8 reagent was added and the absorbance of WST-8 formazan was measured at 450 nm. The cell viability was calculated by comparison with the absorbance of cells incubated without chemicals.

To evaluate the effects of the deacetylation of rifamycins by AADAC on the rifamycins-induced cytotoxicity, WST-8 assay was performed as follows: HepG2 cells (1.5×10^5 cells/well) were seeded in 24-well plates. After 24 h incubation, cells were infected with AdAADAC or AdGFP at MOI 20. Twenty-four hours after infection, the cells were treated with 0–200 μM rifampicin and rifabutin, or 0–100 μM rifapentine for 24 h or 48 h. CCK-8 reagent was added and absorbance of WST-8 formazan was measured at 450 nm. The cell viability was calculated by comparison with the absorbance of cells incubated without chemicals.

To determine the concentration of rifamycins and their deacetylated metabolites in the medium, each medium was collected and a 60-μl portion of the supernatant after removal of the protein by centrifugation at $9500 \times g$ for 5 min was subjected to HPLC. The conditions for analyses were described above.

2.8. Luciferase assay

To evaluate whether or not 25-deacetylrifamycins have the potency to induce pregnane X receptor (PXR)-target genes, luciferase assay was performed. HepG2 cells were seeded into 24-well plates at 1.5×10^5 cells/well. After 24 h incubation, transfection was performed using Tfx-20 reagent (Promega, Madison, MI) as follows: The transfection mixtures consisted of 290 ng of pCYP3A4-362-7.7K and 10 ng of phRL-TK plasmid (Promega). The reporter construct pCYP3A4-362-7.7K contained the promoter region (−362 to −11) including the ER6 (everted repeat separated by six nucleotides) motif and the distal enhancer region (−7836 to −7200) including the DR3 (direct repeat separated by three nucleotides) motif of the *CYP3A4* gene, to which PXR binds [24]. After 24 h incubation, the cells were treated with 0–20 μM rifamycins or their 25-deacetylmetabolites for 48 h, and the cells were harvested and lysed to measure the luciferase activity using a Dual Luciferase Reporter Assay System (Promega). The relative luciferase activities were normalized with the *Renilla* luciferase activities.

2.9. Isolation of total RNA from cryopreserved human hepatocytes treated with rifamycins and real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

The effects of the deacetylation of rifamycins on the induction rates of PXR-target genes were evaluated using human cryopreserved

hepatocytes (lots 82 and 100) (In Vitro Technologies) as follows: Human hepatocytes (4×10^5 cells/well) were reverse transfected with 25 nmol siRNA by Lipofectamine RNAiMAX (Invitrogen). The cells were seeded into 12-well plates. After 24 h incubation, the medium was changed to hepatocyte culture medium (epidermal growth factor-, antibiotics-, and fatty acid–free bovine serum albumin (FAF-BSA)-free) containing 10 μM rifamycins. After 48 h incubation, the human hepatocytes were harvested and total RNA was extracted using RNAiso (Takara). Reverse transcription was performed using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Human CYP3A4 mRNA was quantified by real-time RT-PCR using MX3000P real-time PCR system (Stratagene, La Jolla, CA) with MxPro QPCR software as follows: The forward and reverse primers used for CYP3A4 were 5′-CCAAGCTATGCTCTTCACCG-3′ and 5′-TCAGGCTCCACTTACGGTGC-3′, respectively. A 1-μL portion of the reverse-transcribed mixture was added to a PCR mixture containing 10 pmol of each primer and 12.5 μl of SYBR Premix Ex Taq solution in a final volume of 25 μl. After an initial denaturation at 95 °C for 30 s, the amplification was performed by denaturation at 94 °C for 10 s, annealing and extension at 60 °C for 20 s for 45 cycles. Human glyceraldehyde 3-phosphate dehydrogenase mRNA was also quantified according to a method described previously [25].

To confirm the suppression of AADAC mRNA expression by siAADAC, real-time RT-PCR was performed using a primer pair as described previously [14]. In addition, to confirm the effect of siAADAC on the AADAC enzyme activity, the flutamide hydrolase activity at 500 μM was measured as described above.

2.10. Statistical analysis

Comparison of two groups was made with unpaired, two-tailed Student's t test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Kinetic analyses of the deacetylase activities of rifamycins in HLM

Because rifamycins have been reported to be deacetylated by human liver esterase-like enzymes [4], it was first investigated whether HLM could hydrolyze the three rifamycins, rifampicin, rifabutin, and rifapentine (Fig. 2A–C, Table 1). The rifampicin and rifapentine deacetylase activities in HLM followed the substrate inhibition kinetics (Fig. 2A and C), whereas the rifabutin deacetylase activity followed the Michaelis–Menten kinetics (Fig. 2B). The K_m value of rifampicin deacetylation (195.1 ± 7.5 μM) was higher than those of rifabutin and rifapentine deacetylations (20.5 ± 1.8 μM and 37.1 ± 5.9 μM, respectively), resulting in a lower CL_{int} value for rifampicin deacetylation (0.8 ± 0.1 μL/min/mg) compared with those of rifabutin and rifapentine deacetylations (5.0 ± 0.2 μL/min/mg and 6.9 ± 0.3 μL/min/mg, respectively) (Table 1). These deacetylase activities were not detected in human liver cytosol (HLC) (data not shown). Thus, these results suggested that rifamycins could be efficiently deacetylated in HLM, but not in HLC.

3.2. Deacetylase activities of rifamycins by recombinant human AADAC

To investigate whether human AADAC can catalyze the deacetylation of rifamycins, the deacetylase activities were measured using recombinant human AADAC (Fig. 2D–F, Table 1). Human AADAC could efficiently catalyze the deacetylation of rifamycins, and the deacetylase activities by AADAC followed similar kinetics to those in HLM (Fig. 2A–C). The K_m and K_i values of recombinant AADAC were quite similar to those in HLM (Table 1). These results suggested that AADAC could be the major enzyme responsible for the deacetylation of rifamycins in human liver.

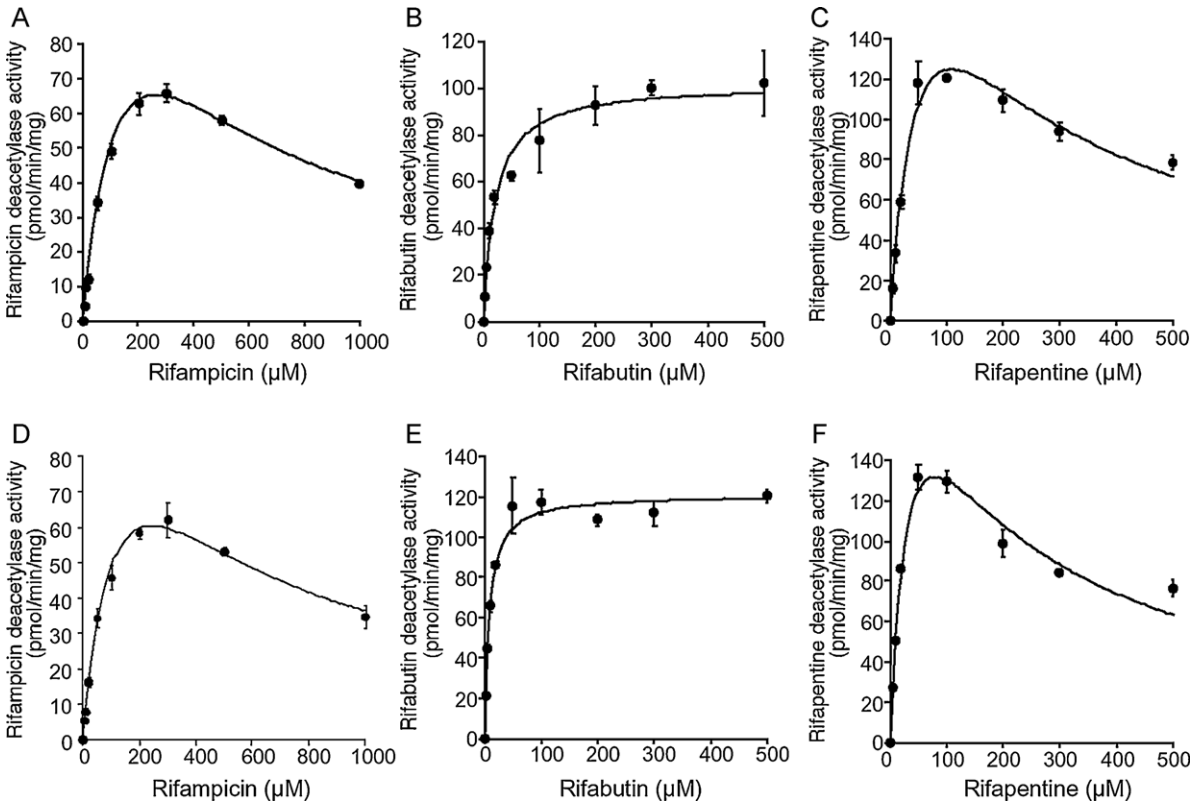

Fig. 2. Kinetic analyses of rifampicin (A, D), rifabutin (B, E), and rifapentine (C, F) 25-deacetylase activities in HLM (A, B, and C) and recombinant human AADAC (D, E, and F). Each point represents the mean ± S.D. of triplicate determinations.

Table 1
Kinetic parameters of the deacetylase activities of rifamycins.

	Enzyme	K_m (μM)	V_{max} (pmol/min/mg)	K_i (μM)	CL_{int} (μL/min/mg)
Rifampicin	HLM	195.1 ± 7.5	162.6 ± 10.2	355.6 ± 31.1	0.8 ± 0.1
	AADAC	162.9 ± 19.2	140.7 ± 0.8	370.3 ± 39.2	0.9 ± 0.1
Rifabutin	HLM	20.5 ± 1.8	102.0 ± 5.7		5.0 ± 0.2
	AADAC	8.0 ± 0.4	121.1 ± 3.1		15.1 ± 0.7
Rifapentine	HLM	37.1 ± 5.9	255.2 ± 32.5	174.3 ± 43.8	6.9 ± 0.3
	AADAC	63.1 ± 12.0	269.7 ± 33.6	194.6 ± 45.3	4.3 ± 0.5

Data are the mean ± S.D. of triplicate determinations.

3.3. Effects of chemical inhibitors on deacetylase activities of rifamycins in HLM and recombinant AADAC

To further investigate whether AADAC is the principal enzyme for the deacetylation of rifamycins in human liver, the effects of several inhibitors of the deacetylase activities in HLM and the recombinant AADAC were analyzed (Fig. 3). The three rifamycin deacetylase activities in HLM were potently inhibited by 10 μM eserine and DFP. In addition, the activities in HLM were moderately inhibited by 10 μM BNPP, 1 mM NaF, and 1 mM PMSF. No inhibition occurred by 10 μM DTNB. A similar inhibition pattern was obtained by the recombinant AADAC. These results could also support that AADAC is the esterase responsible for the deacetylation of rifamycins in human liver.

3.4. MOI- and time-dependent changes of AADAC expression and enzyme activity in HepG2 cells

To investigate the significance of the deacetylation of rifamycins by AADAC, AADAC-overexpressing HepG2 cells were constructed by infection with AdAADAC. First, to evaluate the optimum MOI for the expression of AADAC, the AADAC expression level and flutamide hydrolase activity, which is specifically catalyzed by AADAC [14], at 500 μM were measured with HepG2 cells infected with MOI 0–100 AdAADAC for 48 h (Fig. 4A). The AADAC protein level and flutamide hydrolase activity were increased MOI-dependently. High AADAC expression and enzyme activity were observed in cells infected with AdAADAC at MOI 50 and MOI 100 (flutamide hydrolase activity: 3.18 nmol/min/mg and 3.48 nmol/min/mg, respectively), but cells appeared to be slightly damaged. The flutamide hydrolase activity at MOI 20 (1.35 nmol/min/mg) was higher than that in human hepatocytes (lot 82 and lot 100: 0.68 nmol/min/mg and 0.41 nmol/min/mg, respectively). With HepG2 cells infected with AdAADAC at MOI 20 for 24 h, 48 h, and 72 h, the highest flutamide hydrolase activity was observed after 48 h infection (1.58 nmol/min/mg), although the protein level appeared to be highest after 72 h infection (Fig. 4B). Because the drugs were incubated for 24 or 48 h in the subsequent experiments, AdAADAC infection to HepG2 cells was performed at MOI 20 for 24 h. It was also confirmed that the AADAC expression and flutamide hydrolase activity were not observed in AdGFP-infected HepG2 cells (data not shown).

3.5. Effect of rifamycin deacetylation by AADAC on the induced cytotoxicity

Rifampicin has been reported to have a cytotoxic effect on hepatocytes [7]. To investigate whether the 25-deacetylated metabolites of rifamycins cause cytotoxicity, the viabilities of HepG2 cells treated with rifamycins and 25-deacetylrifamycins were measured by WST-8 assay. After 24 h treatment, rifabutin and rifapeintine showed potent toxicity against HepG2 cells in a dose-dependent manner with 20% and 50% cytotoxic concentrations (CC_{20} and CC_{50}) of 86 μM and 119 μM, and 14 μM and 54 μM, respectively (Fig. 5B and C). On the other hand, rifampicin showed less toxicity (CC_{20}: 194 μM) compared with rifabutin and rifapentine (Fig. 5A). After 48 h treatment, rifampicin showed potent toxicity in a dose-dependent manner with CC_{20} and CC_{50} of

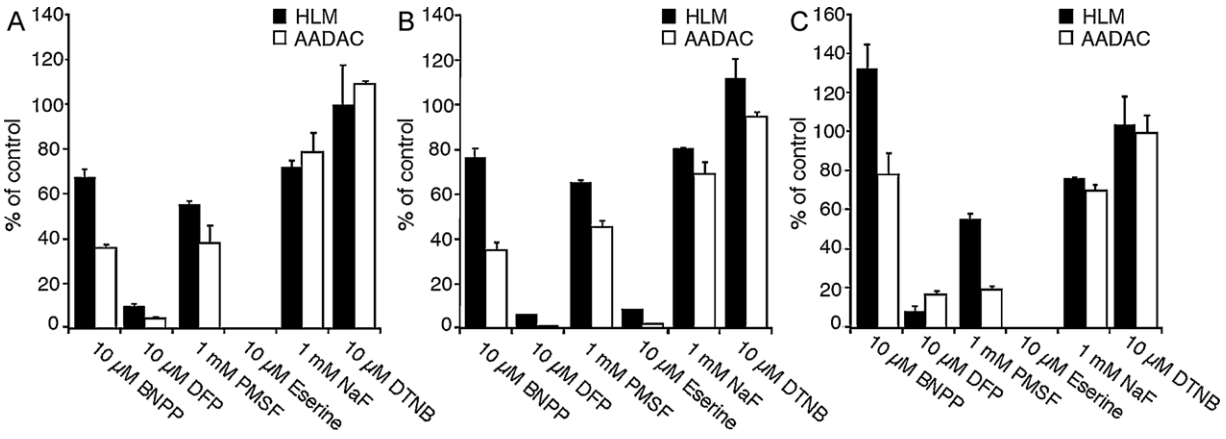

Fig. 3. Effects of chemical inhibitors on rifamycin deacetylase activities in HLM and recombinant human AADAC. Rifampicin (A), rifabutin (B), and rifapentine (C) deacetylase activities in HLM (black bar) and recombinant AADAC (white bar) were determined at 50 μM, 10 μM and 20 μM, respectively. Each column represents the mean ± S.D. of triplicate determinations. The control activities of rifampicin deacetylation in HLM and recombinant human AADAC were 34.3 ± 2.0 pmol/min/mg and 34.4 ± 2.6 pmol/min/mg, respectively. The control activities of rifabutin deacetylation in HLM and recombinant human AADAC were 38.9 ± 3.4 pmol/min/mg and 65.7 ± 2.7 pmol/min/mg, respectively. The control activities of rifapentine deacetylation in HLM and recombinant human AADAC were 59.1 ± 3.4 pmol/min/mg and 86.4 ± 1.1 pmol/min/mg, respectively.

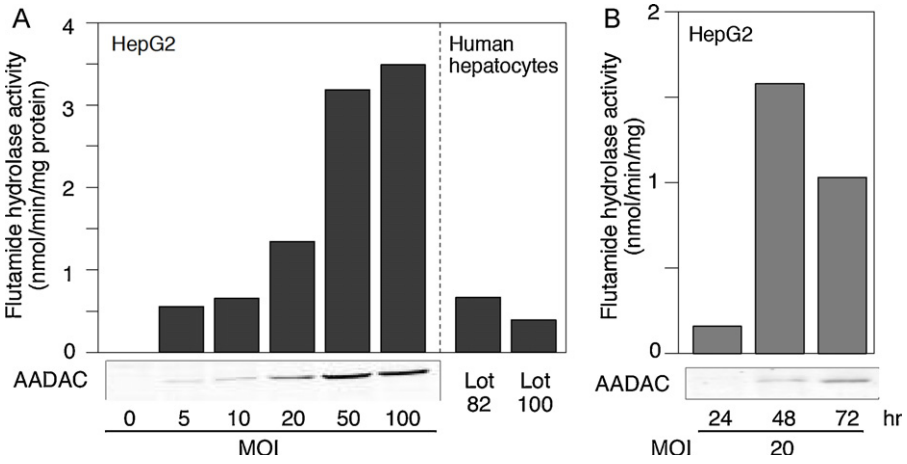

Fig. 4. MOI- (A) and time-dependent changes (B) of flutamide hydrolase activity (500 μM) and AADAC protein expression in AdAADAC-infected HepG2 cells. HepG2 cells were infected with AdAADAC for 48 h (A) or at MOI 20 (B). Total cell homogenates (30 μg) from AdAADAC-infected HepG2 cells were separated by electrophoresis using 10% SDS-polyacrylamide gel. Data are mean of duplicate determinations.

35 μM and 185 μM, respectively (Fig. 5D). 25-Deacetylated metabolites of rifampicin, rifabutin, and rifapentine showed no or less cytotoxicity compared with the parent rifamycins (Fig. 5).

To investigate the effect of rifamycin deacetylation by AADAC on the induced cytotoxicity, the cytotoxicity assay was performed using HepG2 cells infected with AdAADAC (Fig. 6). After 48 h treatment with rifampicin, the viabilities in HepG2 cells infected with AdAADAC (CC_{20}: 136 μM) were significantly higher than those infected with AdGFP as the negative control (CC_{20} and CC_{50}: 64 μM and 142 μM) (Fig. 6A). Similar results were obtained by 24 h treatment with rifabutin or rifapentine (Fig. 6B and C). It was confirmed that rifamycins were deacetylated in this assay system by measuring rifamycins/their deacetylated metabolites in the medium (their concentrations after incubation with 100 μM rifampicin, rifabutin, and rifapentine: 5.7 μM/82.1 μM, 29.2 μM/56.2 μM, and 33.9 μM/52.7 μM, respectively). These results suggested that AADAC would protect against rifamycins-induced cytotoxicity.

3.6. Effect of rifamycin deacetylation by AADAC on CYP3A4 promoter activity

Rifamycins are known as activators of PXR, which affects the expression of many genes. Particularly, CYP3A4 is well-known to

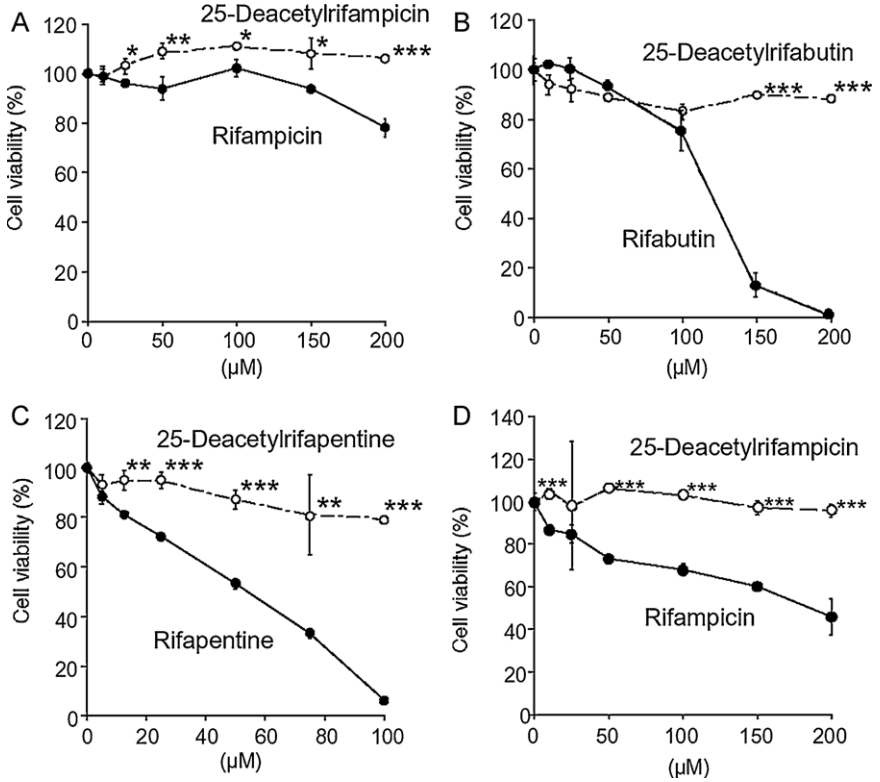

Fig. 5. The viability of HepG2 cells treated with rifampicin/25-deacetylrifampicin (A, D), rifabutin/25-deacetylrifabutin (B), and rifapentine/25-deacetylryfapentine (C). HepG2 cells were treated with rifamycins (closed circle) or 25-deacetylrifamycins (open circle) for 24 h (A, B, and C) or 48 h (D). Data are mean ± S.D. of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with rifamycins.

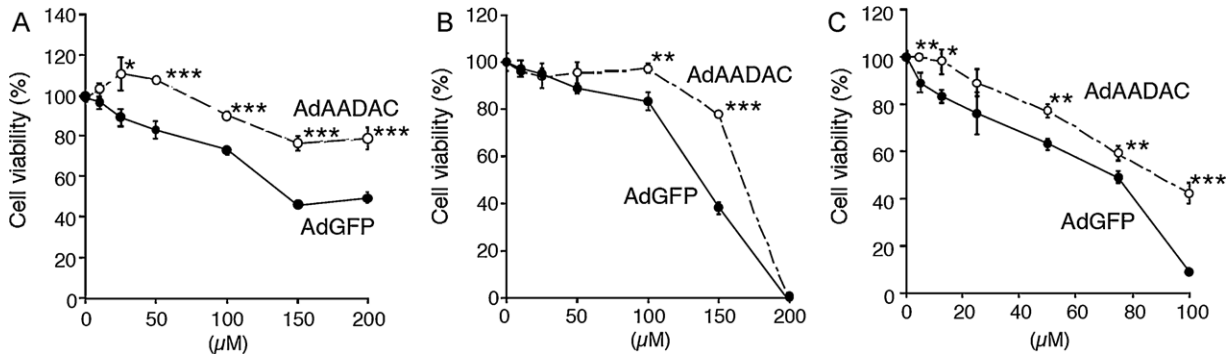

Fig. 6. The effect of rifamycin 25-deacetylation by human AADAC on the induced cytotoxicity. After infection of AdAADAC (open circle) or AdGFP (closed circle) at MOI 20 for 24 h, adenovirus-infected HepG2 cells were treated with rifampicin (A) for 48 h or rifabutin (B) and rifapentine (C) for 24 h. Data are mean ± S.D. of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control cells infected with AdGFP.

be highly induced by rifamycins [5]. However, it remains unknown whether 25-deacetylrifamycins have enzyme induction potency. To investigate whether 25-deacetylrifamycins have the potency to induce the CYP3A4 promoter activity, luciferase assays were performed with a pCYP3A4-362-7.7K plasmid in HepG2 cells. Rifampicin, rifabutin, and rifapentine could induce the transcriptional activity of CYP3A4 in a dose-dependent manner at 10 μM with inductions of 3.7-fold, 2.1-fold, and 3.8-fold, respectively (Fig. 7). On the other hand, their 25-deacetylated metabolites induced no or a little activity compared with the corresponding parent rifamycins.

To further investigate the effect of rifamycin deacetylation by AADAC on the induction potency, the CYP3A4 mRNA induction level by 10 μM rifamycins was measured in human cryopreserved hepatocytes reverse transfected with siAADAC (Fig. 8). It was confirmed that the transfection of siAADAC revealed significant decreases of AADAC mRNA and flutamide hydrolase activity by 89.9% and 50.5% (lot 82), and by 78.0% and 51.2% (lot 100), respectively (Fig. 8A and B). The CYP3A4 mRNA expression in human hepatocytes (lot 82) with siScramble (negative control) was induced 4.4-fold, 3.8-fold, and 5.6-fold by 10 μM rifampicin, rifabutin, and rifapentine, respectively. In contrast, in human hepatocytes with the decreased AADAC expression, the CYP3A4 mRNA expression was significantly induced to 5.5-fold, 5.3-fold, and 6.2-fold, respectively, compared to those with siScramble. Similarly, the CYP3A4 mRNA expression in human hepatocytes (lot 100) with siScramble was induced 27.9-fold, 20.0-fold, and 44.7-fold by 10 μM rifampicin, rifabutin, and rifapentine, respectively. In contrast, in human hepatocytes with decreased AADAC expression, the CYP3A4 mRNA expression was significantly induced to 87.1-fold, 27.2-fold, and 57.8-fold, respectively compared to those with siScramble (Fig. 8C). These results supported that AADAC enzyme activity leads to the decreased levels of PXR-target genes by rifamycins.

4. Discussion

Rifamycins such as rifampicin, rifabutin, and rifapentine are the most important first-line antituberculosis drugs, and they are sometimes used for other diseases such as chronic staphylococcal infections and AIDS [3,4]. Rifamycins are well-known to induce various drug-metabolizing enzymes including CYP3A4 [5], and drug–drug interactions are sometimes caused by co-administration with rifamycins [8]. Moreover, hepatotoxicity was also reported to be one of their side effects [7]. It has been reported that rifamycins are mainly metabolized into a 25-deacetyl form [3], although rifabutin is also metabolized to hydroxyl forms [9]. However, the enzyme responsible for rifamycin deacetylation in human was unknown. In this study, we found that human AADAC is the responsible enzyme for the deacetylation of rifamycins.

Recombinant AADAC showed rifamycins deacetylase activity with similar kinetic parameters (K_m and K_i values) to those of HLM (Fig. 2 and Table 1). For both HLM and recombinant AADAC, the rifampicin and rifapentine deacetylase activities followed the substrate inhibition kinetics, whereas the rifabutin deacetylase activity followed Michaelis–Menten kinetics (Fig. 2). These similarities between recombinant AADAC and HLM supported that AADAC is the principal enzyme responsible for rifamycin deacetylation. The three rifamycins have similar structures (Fig. 1), with the exception that rifampicin and rifapentine have a

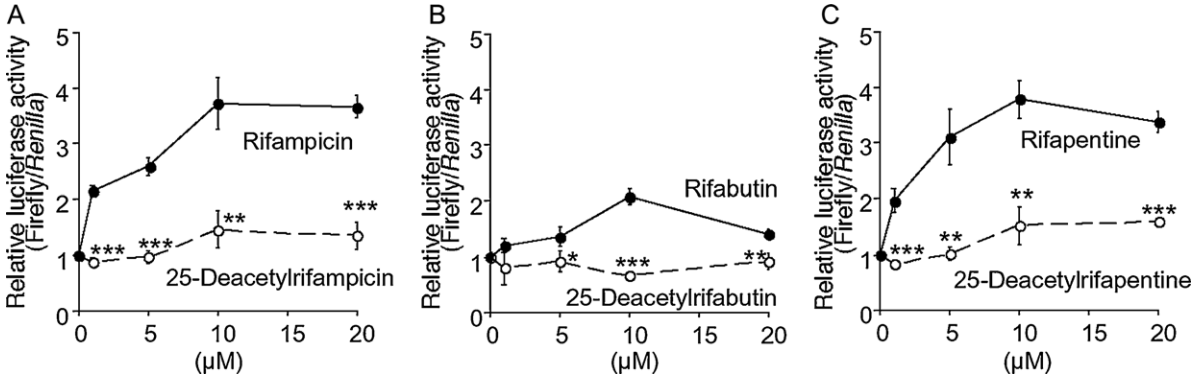

Fig. 7. Transactivation of CYP3A4 promoter constructs by rifampicin/25-deaccetylrifampicin (A), rifabutin/25-deacetylrifabutin (B), and rifapentine/25-deacetylrifapentine (C). HepG2 cells were transfected with pCYP3A4-362-7.7K and phRL-TK plasmid using Tfx-20 reagent. The relative luciferase activities were normalized with the *Renilla* luciferase activities. Each column represents the mean ± S.D. of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with rifamycins.

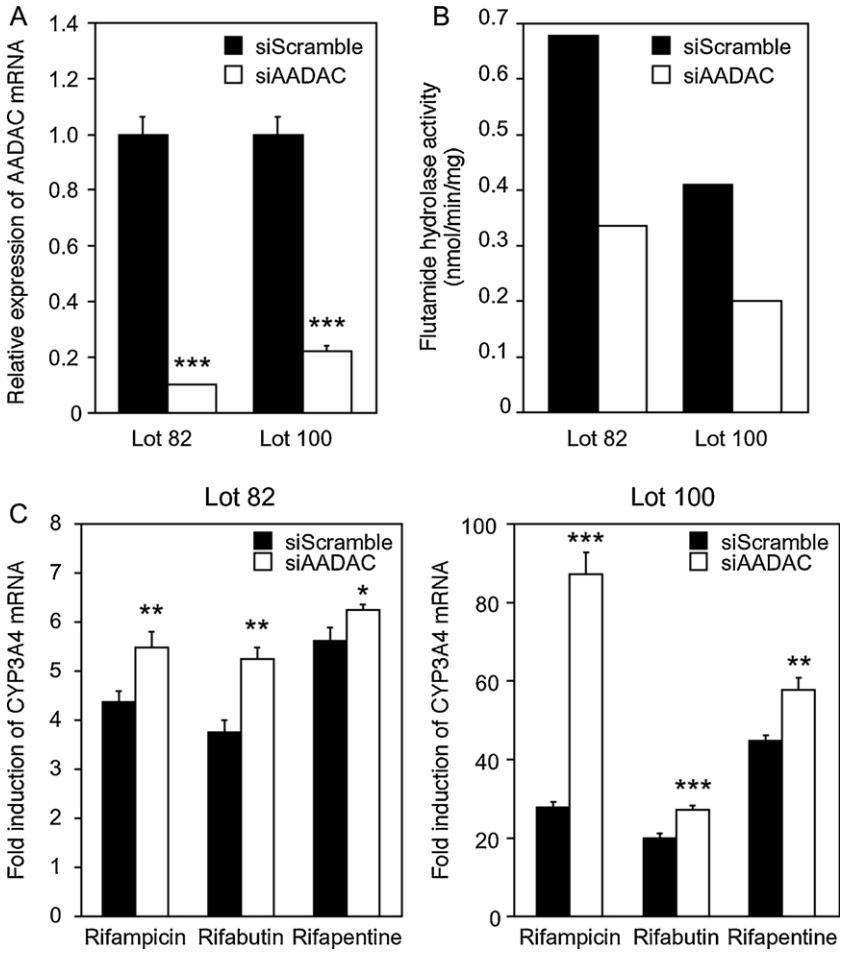

Fig. 8. Effects of siAADAC on AADAC mRNA expression level (A), flutamide hydrolase activity (500 μM) (B) and rifamycins-induction potency to CYP3A4 mRNA (C) in human cryopreserved hepatocytes (lots 82 and 100). Human cryopreserved hepatocytes reverse transfected with siAADAC (white bar) or siScramble (black bar) were treated with 10 μM rifamycins for 48 h. The AADAC and CYP3A4 mRNA expression level were quantified by real-time RT-PCR and normalized by human glyceraldehyde 3-phosphate dehydrogenase mRNA. Data are mean ± S.D. of triplicate determinations (A and C) or mean of duplicate determination (B). $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ compared with siScramble transfected cells.

piperazinyl iminomethyl group, while rifabutin does not contain this group. The less flexible ring structure of rifabutin may lead to steric hindrance in the substrate recognition of AADAC resulting in the differences in kinetic patterns among the rifamycins.

In contrast to AADAC, recombinant CES1A1 and CES2, which are major esterases responsible for the hydrolysis of a variety of drugs, showed no activities of rifamycin deacetylation (data not shown). This result is consistent with the fact that the activities were not detected in HLC (data not shown), in which CES1A1 and CES2 are expressed [26,27]. Thus, although it is obvious that CES enzymes are not involved in the rifamycin deacetylation, other esterases responsible for the drug metabolism are also known to be expressed in HLM. For example, ChE is involved in the hydrolysis of succinylcholine and CPT-11 [28,29] and PON is involved in the hydrolysis of simvastatin and lovastatin [30]. Therefore, it was confirmed by the inhibition analysis using various chemical inhibitors that AADAC is the principal enzyme responsible for rifamycin deacetylation (Fig. 3). The deacetylase activities of all rifamycins investigated in this study were potently inhibited by 10 μM DFP and 10 μM eserin, but were slightly inhibited by 1 mM NaF and 10 μM DTNB. ChE enzyme activity was generally inhibited by both eserin and NaF [19,20], suggesting that ChE is not responsible for rifamycins deacetylation. This viewpoint was supported by the fact that the rifabutin deacetylase activity was not detected in human serum expressing ChE [9,31]. DTNB is known as an inhibitor of arylesterase, PON that requires calcium ion to exert its activities and stabilities [32]. Our previous study demonstrated that pilocarpine hydrolysis, which is catalyzed by PON1, was not detected unless calcium chloride was added to the reaction mixture [33]. In addition, PON is also expressed in human serum [32]. From these backgrounds, ChE and PON could be excluded from the candidate esterases responsible for rifamycin deacetylation. Until now, the inhibitors specific for AADAC have not been found. In addition, the antibody against human AADAC for inhibition study was not commercially available. However, the inhibitory characteristics of rifamycin deacetylation by the recombinant AADAC were quite similar to those in HLM (Fig. 3). Our previous study demonstrated that flutamide hydrolysis, which is specifically catalyzed by AADAC, in HLM was potently inhibited by DFP and eserin, but moderately inhibited by BNPP and PMSF [14]. These findings also supported that human AADAC is a principal enzyme responsible for rifamycin deacetylation in human liver.

In patients receiving rifampicin, hepatotoxicity has been occasionally reported as a side effect [7]. Rifampicin can cause hepatocellular damage, and its hepatotoxicity has been postulated to occur as a part of a systemic allergic reaction [34,35]. The rifamycins investigated in this study showed cytotoxicity against HepG2 cells. Among them, rifabutin and rifapentine showed potent toxicity against HepG2 cells, but rifampicin showed less toxicity

(Fig. 5). There is no report about differences in the frequency of rifamycins-induced hepatotoxicity in human. In general, rifamycins are used in combination with other antitubercular agents such as isoniazid and pyrazinamide. Therefore, it may be difficult to compare the frequency of hepatotoxicity occurrence induced by rifamycins themselves in the clinical setting. On the other hand, 25-deacetylrifamycins showed no or less cytotoxicity against HepG2 cells compared with the parent rifamycins (Fig. 5). Although the permeability between rifamycins and their 25-deacetylmetabolites into the cells may be different, it was considered that human AADAC plays an important role in the protection against rifamycins-induced cytotoxicity. In fact, the rifamycins-induced cytotoxicity was significantly diminished by AADAC overexpression in HepG2 cells (Fig. 6). The estimated maximum therapeutic concentrations of rifamycins in human liver were as follows: rifampicin (600 mg twice weekly), 9.1 μM; rifabutin (300 mg twice weekly), 1.4 μM; rifapentine (600 mg once weekly), 12 μM [3], which values are lower than the concentrations used in the present study (10–100 or 200 μM). Thus, some mechanisms as well as direct cytotoxicity may be involved in the rifamycins-induced hepatotoxicity. In addition, HepG2 cells infected with AdAADAC showed higher AADAC enzyme activity than human hepatocytes (Fig. 4A: HepG2 cells infected with AdAADAC at MOI 20 versus human hepatocytes). Further studies will be needed to clarify whether AADAC plays a role in the detoxification of rifamycins.

Rifamycins require attention in the clinical setting because of their induction potencies [5]. Co-administration of rifamycins with drugs metabolized by enzymes induced by rifamycins sometimes causes drug–drug interactions [6,36]. Rifamycins are well-known inducers of CYP3A4 in human [5]. Adachi et al. [37] reported that the administration of 25-deacetylrifampicin to rats had no inductive effect on the activity of rat hepatic UDP-glucuronocyl transferase to bilirubin, although an inductive effect was observed by rifampicin. There is no report about the induction potencies of 25-deacetylrifabutin and 25-deacetylrifapentine. However, this study with luciferase assay demonstrated that 25-deacetylrifamycins showed no or less induction potency (Fig. 7). In addition, human hepatocytes isolated from 2 individuals (lot 82 and lot 100) were used to investigate the involvement of endogenous AADAC in the induction potency of CYP3A4 (Fig. 8). The induction rate of CYP3A4 mRNA by rifamycins was quite different between them, probably due to differences in the expression levels of PXR, retinoid X receptor, and other related factors. The induction rates of CYP3A4 mRNA by rifamycins in human hepatocytes with the decreased AADAC expression were higher than those in control human hepatocytes (Fig. 8). Thus, AADAC may play an important role in the induction rate of PXR-target genes by rifamycins.

In conclusion, we found that human AADAC is the principal enzyme in the rifamycin deacetylation. AADAC would play important roles in the induction rates of drug-metabolizing enzymes by rifamycins and the protective effect against rifamycins-induced hepatotoxicity. This study could provide useful information about human AADAC in drug metabolism.

Acknowledgments

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