The peroxisome proliferator-activated receptor α agonist, AZD4619, induces alanine aminotransferase-1 gene and protein expression in human, but not in rat hepatocytes: Correlation with serum ALT levels

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Abstract. Alanine aminotransferase (ALT) in serum is the standard biomarker for liver injury. We have previously described a clinical trial with a novel selective peroxisome proliferator-activated receptor α (PPARα) agonist (AZD4619), which unexpectedly caused increased serum levels of ALT in treated individuals without any other evidence of liver injury. We pinpointed a plausible mechanism through which AZD4619 could increase serum ALT levels; namely through the PPARα-specific activation of the human ALT1 gene at the transcriptional level. In the present study, we present data from the preceding rat toxicity study, demonstrating that AZD4619 had no effect on rat serum ALT activity levels, and further experiments were performed to elucidate the mechanisms responsible for this species-related difference. Our results revealed that AZD4619 increased ALT1 protein expression in a dose-dependent manner in human, but not in rat primary hepatocytes. Cloning of the human and rat ALT1 promoters into luciferase vectors confirmed that AZD4619 induced only the human, but not the rat ALT1 gene promoter in a dose-dependent manner. In PPARα-GAL4 reporter gene assays, AZD4619 was >100-fold more potent on the human vs. rat PPARα levels, explaining the differences in induction of the ALT1 gene between the species at the concentration range tested. These data demonstrate the usefulness of the human and rat ALT1 reporter gene assays for testing future drug candidates at the preclinical stage. In drug discovery projects, these assays elucidate whether elevations in ALT levels observed *in vivo* or in the clinic are due to metabolic effects rather than a toxic event in the liver.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Dex, dexamethasone; FA, fenofibric acid; LBD, ligand-binding domain; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element

Key words: alanine aminotransferase, peroxisome proliferator-activated receptor α, liver, dexamethasone, PPAR response element

Introduction

Drug-induced liver injury is a serious issue for patients and physicians, and hence an area of intense investigations for pharmaceutical companies. Since its introduction 60 years ago, alanine aminotransferase (ALT) activity in serum still remains the gold standard biomarker of liver injury both preclinically and clinically (1). Although, the protein expression of ALT is mainly observed in the liver, the ALT enzyme is expressed in many other organs and tissues, such as the kidneys, heart, skeletal muscle and pancreas (2,3). Furthermore, the two genes, glutamic-pyruvate transaminase 1 and 2 (*GPT1* and *GPT2*), located on chromosomes 8 and 16, encode the human ALT proteins, ALT1 and ALT2, and their protein products show similar enzymatic activities (4,5). ALT1 is the dominant isoform expressed in the liver and constitutes the majority of the basal ALT activity of normal human serum (6,7). Of note, several reports and a large amount of empirical data from the pharmaceutical industry have shown that increases in serum ALT activity can occur without apparent liver damage, as detected in histopathological assessments. For example, the exposure of rats to dexamethasone has been shown to increase both liver and serum ALT activity >4-fold, without any histopatological evidence of hepatocellular damage (8,9). Likewise, it has been demonstrated that fibrates, which are peroxisome proliferator-activated receptor (PPAR)α agonists, have been shown to increase ALT activity in the serum of rats and humans (10,11). In humans, fibrates have been shown to cause transient elevations in serum aspartate aminotransferase (AST) and ALT levels in approximately 10% of patients, without any other evidence of hepatotoxicity (11). It has also been demonstrated that the gene expression of AST and ALT in human hepatoma cells and primary human hepatocytes increases following treatment with fibrates, thus suggesting an alternative 'non-toxic' mechanism for the elevation of AST and ALT enzyme levels in serum (12). The hypothesis states that moderate increases (2-4-fold) in AST and ALT activity in serum could in some cases, be due to the induction of the expression of respective genes, resulting in elevated protein expression levels in the liver. The transport of the increased amounts of protein from the liver into the serum is a poorly understood process; however, it is believed to be due to normal hepatocyte turnover.

In a previous study, we described a clinical trial of a selective PPARα agonist; AZD4619, in which moderately increased levels of serum ALT and AST activity were observed in human volunteers, without any other signs of hepatic injury (13). We identified a functional PPAR response element (PPRE) in the proximal ALT1 promoter and concluded that PPAR agonists are inducers of the human ALT1 gene. In the present study, we demonstrate that treatment with AZD4619 induces serum ALT activity in humans, but not in rats, and provide a hypothesis to explain this observed species-related difference. We also suggest that future drug candidates be screened for their capacity to induce the human and rat ALT1 gene, which will help drug projects in early discovery in removing compounds with this liability, as even a benign increase in serum ALT levels would hinder the progression of the compound to further clinical development.

Materials and methods

Compounds. AZD4619, potassium salt with a molecular mass of 554.7 g/mol had a purity of 99.5% and was dissolved in tap water (*in vivo*, rat study). AZD4619 has a high binding affinity for albumin of 99.97%. Fenofibric acid and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fenofibric acid and dexamethasone have a molecular weight of 318.7 and 392.5 g/mol, respectively. All compounds were dissolved in dimetyl sulfoxide (DMSO) on the same day of treatment of the cell cultures.

Rat study. Four groups of Wistar rats (Taconic M&B A/S, Denmark), each consisting of 10 males and 10 females, were administered AZD4619 orally by gavage once a day for 32 days. The dose levels were 5, 30, 180 and 1,000 μmol/kg (3, 15, 93, 517 mg/kg), respectively. One control group was included in the study and was administered an appropriate amount of tap water. The animals were approximately 8 weeks old when the dosing started and the weight ranges were 180-320 g for the males and 130-210 g for the females. The animals had free access to food (RM1.E.SQC low protein food for rats and mice; Special Diets Services Ltd., England) and to municipal tap water for human consumption. The systemic exposure to AZD4619 was investigated by analyzing the concentration of the test compound in blood plasma after a single oral dose or after 32 days of repeated oral dosing. Blood chemistry measurements were analysed by using Cobas Integra 400, (Tegimenta Ltd., Roche Diagnostics Instrument Center, Basel, Switzerland) with appropriate test kits. The animals were euthanised on the day after final day of dose administration. The study was conducted within the framework of the Swedish National Animal Welfare Act including a review and approval of the study protocol by the local laboratory animal ethics committee. It was also conducted in compliance with the Organisation for Economic Co-operation and development (OECD) Good Laboratory Practice.

Clinical trial. The study was a phase I study, to assess the safety, tolerability, effect on lipids and pharmacokinetics of repeated oral doses of AZD4619. A total of 109 subjects was enrolled in order to procure 40 randomised subjects aged between 20 and 29 years, inclusively. The subjects were divided into 2 groups with 20 subjects in each. In the first group, through random selection, 15 subjects were administered AZD4619 (5 mg daily) and 5 subjects were administered the placebo. In the second group, through random selection, 15 subjects were administered AZD4619 (0.5 mg daily) and 5 subjects the placebo. The dosing of AZD4619 occurred from days 1-21 and the follow-up serum measurements proceeded for 31 days. Additional information about the clinical trial of AZD4619 has been published previously (13).

Cell culture. Cryopreserved male human hepatocytes from one donor (Lot GIU) were obtained from In Vitro Technologies (Baltimore MD, USA) and cryopreserved male rat hepatocytes (Lot Rs704) were obtained from Life Technologies/Gibco/Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, 1 g/l glucose) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, 1% non-essential amino acids (Gibco/Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 95% air, 5% CO_2 at 37˚C. The hepatoma cell lines, HuH-7 and MH1C1, were maintained using the culture conditions described above. The human osteosarcoma U2OS cells were cultivated in DMEM supplemented with resin-charcoal-stripped FBS for the GAL4 binding assays. All cell lines were from ATCC (Manassas, VA, USA).

Analysis of protein expression. Primary human hepatocytes (GIU) and primary rat hepatocytes (Rs704) were seeded out in collagen-coated 6-well plates (500,000 cells/well) and treated with 0.5% DMSO as a control, or with 10, 100, 1,000 and 10,0000 nM AZD4619 and 250 μM fenofibric acid for 48 h. After washing the cells in phosphate-buffered saline (PBS), they were lysed with RIPA lysis buffer [1X PBS, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors (Complete, Roche Diagnostics, Basel, Switzerland)] and cell debris was removed by centrifugation. The protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). A total of 5 μg of the human and 10 μg of the rat whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, performed under reducing conditions on 4-12% Nupage Novex Bis-Tris gels with MES-SDS running buffer (both from Invitrogen/Thermo Fisher Scientific, Inc). The resolved proteins were transferred onto a nitrocellulose sheet and subjected to Ponceau staining (Sigma-Aldrich). The staining intensity was consistently identical in all lanes. The filters were subsequently incubated for 2 h with either a rabbit polyclonal antibody against human (h)ALT1 (cat no. Ab1399, 0.1 μg/ml; Abcam, Cambridge, UK) or a goat polyclonal antibody against rat (r)ALT1 (cat. no. sc-47024, 0.2 μg/ml; Santa Cruz Biotechnology, CA, USA). The filters incubated with primary antibody were then probed with the corresponding secondary antibodies to IgG (goat α rabbit, cat. no. 31464, 1:30,000 dilution; Pierce, Thermo Fisher Scientific, Inc.; or rabbit α goat, cat. no. PO449, 1:10,000 dilution; Dako, Glostrup, Denmark), conjugated to horseradish peroxidase (HRP). The chemiluminescent HRP substrate (Immobilon Western, Millipore, Billerica, MA, USA) was used according to the manufacturer's instructions and chemiluminescence was detected using a Fuji Film Las-3000 mini image analyser (Science Imaging Scandinavia, Nacka, Sweden). The resulting bands were compared with the size of known molecular markers (MagicMark XP; Invitrogen/Thermo Fisher Scientific, Inc.).

Promoter constructs. The human ALT1 promoter construct has been described elsewhere (13). To obtain the rat ALT1 promoter construct, approximately 2,000 bp of the proximal ALT1 promoter were amplified from rat genomic DNA using the primers gpt1f (5'-atccacttcagcacataccc) and gpt1r (5'-tgggaa tgggaaaatctgcg). The PCR-product was subcloned into the PCR 2.1 vector (Invitrogen/Thermo Fisher Scientific, Inc.) and further introduced into the multiple cloning site (MCS) of the pGL3b vector (Promega, Madison, WI, USA) using the *Kpn*I and *Xho*I restriction sites. The DNA used in the transfection experiments was purified using the column-based Endofree Plasmid Maxi kit (Qiagen, Valencia, CA, USA). The glucocorticoid receptor (GR) constructs were a kind gift from Dr Ann-Charlotte Wikström, Karolinska Institutet, Stockholm, Sweden.

Transfection and luciferase assay. The HuH-7 and MH1C1 cells ($5x10^6$) were seeded in a Petri dish with culture medium and incubated at 37˚C for 24 h. After washing with PBS, the cells were incubated at 37˚C for 4 h with 16 μg of plasmid DNA in transfection medium (DMEM medium only) and 40 μl Lipofectamine™ 2000, according to the manufacturer's instructions (Invitrogen/Thermo Fisher Scientific, Inc.). A total of 100,000 cells/well was seeded in a 24-well plate with full growth culture medium (containing 10% FBS) and incubated overnight at 37˚C. The culture medium was removed and replaced with the compounds to be tested (AZD4619, fenofibric acid or dexamethasone). The concentration of the vehicle (DMSO) was >0.1% in both the control and treated cells. Every control and treatment was run in 4 replicates on the same plate (n=4). The cells were incubated 24 h at 37˚C and subsequently lysed (Promega lysis buffer 1X with the addition of 1% Triton X-100). After 20 min on ice, the cell lysates were transferred onto a non-transparent 96-multiwell plate and the luciferase activity was measured using a luminometer (Luminoskan Ascent, Thermo Electron Corp./ Thermo Fisher Scientific Inc.).

PPARα-GAL4 assay. The U2OS cells were transiently transfected via electroporation with a luciferase reporter gene under control of the yeast UAS sequence and a mouse, rat, monkey or human PPARα construct, where the PPARα ligand binding domain had been cloned in frame downstream of the DNA binding domain from yeast GAL4 (14). The cells were seeded in 96-well plates and incubated for 3 h at 37˚C, 5% CO_2, prior to the addition of the compounds and additional incubation for 40 h. The signal was developed using the SteadyLiye kit (Perkin-Elmer Inc., Waltham, MA, USA) and resulting luminiscence was measured after 10 min of incubation at room temperature in a Victor plate reader (Wallach, FI; Perkin-Elmer Inc.). The concentration response curves and half maximal effective concentration (EC_{50}) values were generated using Xlfit (ID Business Solutions, Ltd., Guildford, UK).

In silico analysis. Approximately 2 kb of the ALT1 promoter sequences corresponding to *Homo sapiens* (NC_000008 REGION: 145698231.145700231) and *Rattus norvegicus* (NC_005106 REGION: 114744054.114746054) were analysed using the online tools, ECRbrowser (15) and zPicture (16). The EMBL-EBI T-Coffee multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/tcoffee/) was used for alignment of the protein sequences for *Homo sapiens* (NP_001001928.1), *Macaca mulatta* (NP_001028201.1) and *Rattus norvegicus* (NP_037328.1) PPARα.

Table I. Serum ALT, AST and concentrations following the repeated oral administration of AZD4619 (0.5 or 5 mg) or the placebo to human volunteers.

Plasma parameter	Placebo	0.5 mg dose	5 mg dose
ALT, U/l			
Pre-dose	26±16	19±7	21±7
ALT, U/l			
At day 31, follow-up	28±14	23±13	41±26[a]
AST, U/l			
Pre-dose	26±7	22±4	23±5
AST, U/l			
At day 31, follow-up	32±12	26±5	39±30
Triglycerides, mmol/l			
Pre-dose	0.84±0.28	0.88±0.48	0.78±0.23
Triglycerides, mmol/l			
At day 21, follow-up	1.05±0.64	0.63±0.23	0.54±0.15
Plasma			
Cmax, μM	n/a	0.05±0.02	0.40±0.20
Plasma exposure			
AUC, 0-24 h μM x h	n/a	n/a	0.83±0.40

Plasma Cmax values, drug exposure and triglycerides were measured on the last day of dosing (day 21) and aminotransferase levels were measured at follow-up (day 31). n/a, not applicable due to the fact that most plasma sample concentrations were below the limit of quantification (LOQ) or no data. Values represent the means ± SD. [a]P<0.05.

Statistical analysis. In order to establish differences in variables measured over time from the clinical data, a paired t-test was used to calculate significant changes. In the animal study, a Pairwise Shirley's test was used. In addition, one-way Anova with Dunnett's test was used to analyse differences in the transfection experiments between the control and treatment groups. Statistical differences were tested at the confidence level of 95% and are represented in bar diagrams using asterisks to indicate P-values <0.05. Bar diagrams represent the mean values ± SD.

Results

AZD4619 causes elevations in ALT levels in the phase I clinical trial. AZD4619 was administered orally at 0.5 and 5 mg daily to healthy individuals for 21 days. The results from this study revealed an increase in aminotransferase levels in 1 out of 15 subjects in the 0.5 mg dose group and 5 out of 15 subjects in the 5 mg dose group. AZD4619 was rapidly absorbed both after single and repeated dosing, and the Cmax was generally reached within 1 h. The pharmacokinetic steady state was achieved within 4 days and the steady state Cmax at day 21 was on average 0.40 μM (AUC 0-24 h: 0.83 μM x h) for the 5 mg dose (Table I). In addition, a decrease in serum

Table II. Serum ALT, AST and AZD4619 concentrations in rats after 32 days of daily oral administration.

	Gender	Control	3 mg/kg	15 mg/kg	93 mg/kg	517 mg/kg
ALT (U/l)	M	79±18	76±14	71±10	67±6	81±20
	F	55±21	47±10	51±6	56±8	52±6
AST (U/l)	M	87±15	93±15	93±15	93±23	103±15[a]
	F	110±48	87±15	87±14	88±10	84±10
Triglycerides, mmol/l	M	2.30±0.76	2.40±0.67	2.03±0.67	1.87±0.48	0.94±0.22[c]
	F	1.39±0.62	1.24±0.39	0.90±0.37	1.20±0.56	0.78±0.22[b]
Cmax, μM	M	n/a	0.64±0.15	5.84±4.48	26.0±5.6	536±91
	F	n/a	1.35±0.56	11.9±5.0	65.3±36.9	742±357
Exposure	M	n/a	3.31 (2.64-4.95)	15.5 (10.8-20.7)	157 (81-278)	1,870 (1,020-3,040)
AUC (0-24 h)	F	n/a	6.16 (3.71-10.1)	32.1 (23.9-38.9)	149 (112-190)	1,730 (921-3,410)
μM x h						

[a]Values represent the means ± SD. [a]P<0.05, [b]P<0.001, [c]P<0.01 compared to the control group (0 mg/kg). M, 4 male rats, F, 4 female rats.

triglyceride (TG) levels, which is a pharmacological marker of PPARα activation, was observed in both the 0.5 and 5 mg treatment groups compared to the pre-dose sample [TG decreased by about 30% (P<0.01) from day 1 to day 21].

AZD4619 does not cause elevations in ALT levels in the 1-month rat study. During the preclinical toxicological evaluations of AZD4619, 4 groups of rats were administered AZD4619 orally by gavage once a day for 1 month. A control group was also included in the study and received tap water without the compound. The dose levels for the treatment group were 3, 15, 93 and 517 mg/kg bodyweight. At day 32, the compound concentration in the plasma ranged between 0.64 to 742 μM for the low- to high-dose groups, respectively (Table II). Blood chemical analysis did not reveal any elevations in ALT levels in any of the treatment groups, and only a marginal elevation of AST levels was observed in the male high-dose group. In addition, serum TG levels decreased only in the high-dose group (517 mg/kg) from 2.30 to 0.94 mmol/l (P<0.001) in males and from 1.39 to 0.78 mmol/l (P<0.01) in females. As expected, histopathological analysis demonstrated that AZD4619 had a clear-cut effect on the liver, comprising a diffuse hepatocyte hypertrophy and diffuse eosinophilic cytoplasm. The changes occurred in a dose-dependent manner and closely correlated with the observed increase in liver weights (data not shown). These diffuse liver changes were considered to be a known effect of PPARα agonists mediated by the proliferation of peroxisomes in rodent hepatocytes.

AZD4619 induces ALT1 expression in human, but not in rat hepatocytes. To elucidate the mechanisms responsible for the increase in ALT levels by AZD4619 in humans, but not in rats, primary human and rat hepatocytes were treated with AZD4619. Cell extracts from treated human and rat cells were subjected to western blot analysis and the results revealed that the expression of the dominant liver ALT isoform (ALT1) was increased in human hepatocytes following treatment with AZD4619 (10-10 000 nM) compared to the cells treated with the vehicle (Fig. 1A). To study the species-specific responses of AZD4619 on the ALT1 gene, promoter constructs of approximately 2 kb of either the human or the rat gene promoter were generated and subsequently transfected into human (HuH-7) or rat hepatoma cells (MH1C1), respectively. Treatment of the human cells which were transfected with the human ALT1 construct with AZD4619 resulted in a statistically significant increase in reporter gene activity at 100-1000 nM (Fig. 1B). By contrast, as shown by western blot analysis, there were no changes in rat ALT1 protein expression at equivalent concentrations of AZD4619 (Fig. 2A). This was also true for the rat MH1C1 cells transfected with the rat ALT1 promoter construct. AZD4619 did not have the capacity to provoke any change in rat ALT1 promoter activity in the concentrations tested (10-10 ,000 nM) (Fig. 2B). The PPARα agonist, fenofibric acid, was used as a positive control in both protein and promoter assays, which also demonstrated the expected responses in the systems.

AZD4619 displays specificity for human PPARα in reporter gene assays. To elucidate the mechanisms responsible for the induction of human ALT1 and not rat ALT1 levels by AZD4619 in the concentration range tested, we investigated receptor selectivity by the compound. AZD4619 was found to be a highly specific and potent agonist of human PPARα with a calculated EC_{50} value of 0.10 μM in reporter gene assays. However, the potency of AZD4619 on rat PPARα was >100-fold lower, with an EC_{50} value of 10.3 μM (Table III). The evaluation of fenofibric acid in the same assay resulted in an EC_{50} value of 13.1 and 17.0 μM for human and rat PPARα, respectively (data not shown). The amino acid compositions for human and rat PPARα were compared in a multiple sequence alignment and several amino acids deviated between the human and rat ligand-binding domains (Fig. 3A).

Functional PPRE in the human ALT1 promoter is not conserved in the rat. To further investigate the reason for the differential responses by AZD4619 on the human and rat ALT1 genes, approximately 2 kb of the human and rat promoters were compared using pairwise sequence alignment. The functional PPRE at -574 in the human promoter was found within a 400 bp evolutionary conserved region where the nucleotide sequences

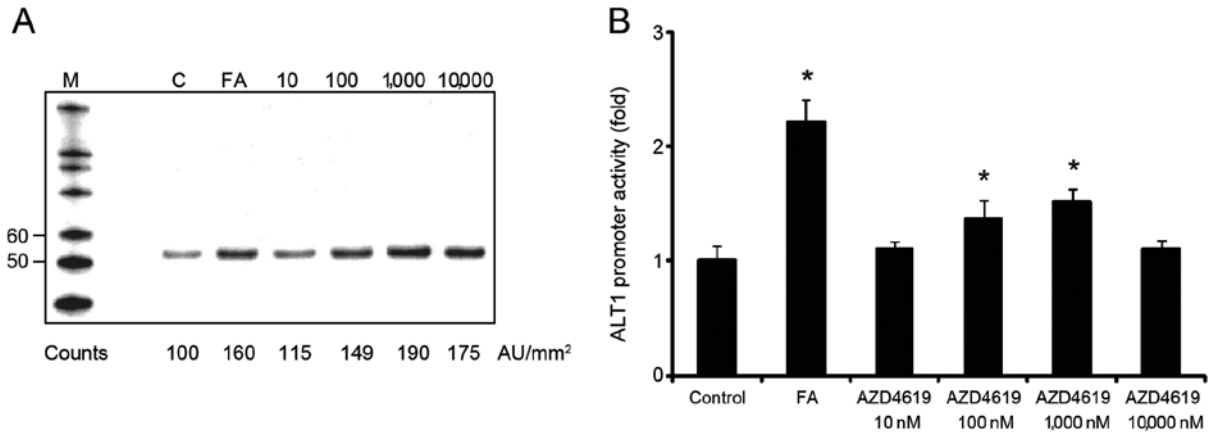

Figure 1. (A) Western blot analysis of human primary hepatocytes treated with FA, 250 μM and AZD4619 (10-10,000 nM). (B) Human hepatoma cells, HuH-7 cells transfected with the human ALT1 reporter construct. Cells were treated with AZD4619 (10-10,000 nM) for 24 h. FA (250 μM) was used as a positive control in all transfection experiments with ALT-1 promoter activity. Diagram shows representative data from 3 experiments. FA, fenofibric acid; ALT1, alanine aminotransferase 1. *P<0.05 compared to control.

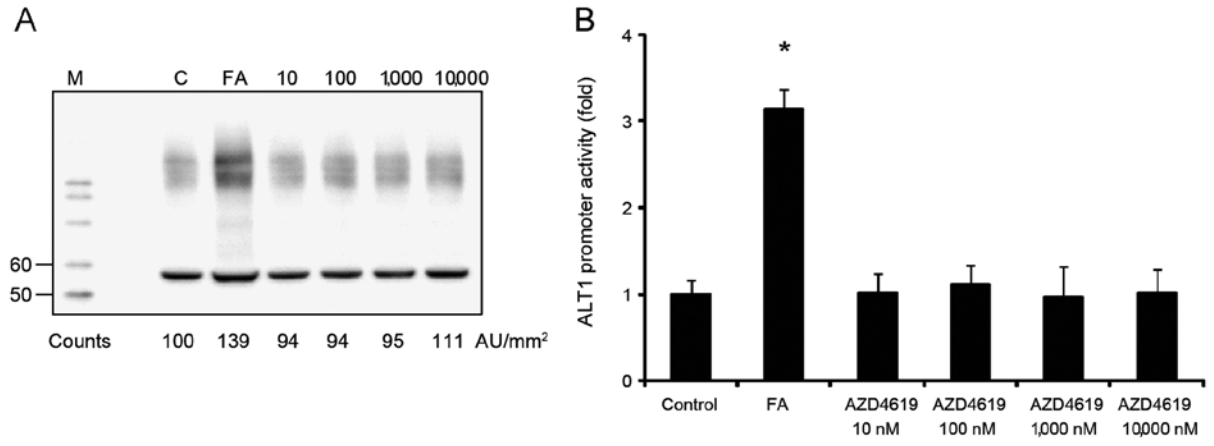

Figure 2. (A) Western blot analysis of rat primary hepatocytes treated with FA, 250 μM and AZD4619 (10-10,000 nM). (B) Rat hepatoma cells (MH1C1) were transfected with the rat ALT1 reporter construct. Cells were treated with AZD4619 (10, 100, 1,000 and 10,000 nM) for 24 h. FA (250 μM) was used as a positive control in all transfection experiments with ALT-1 promoter activity. Diagram shows representative data from three experiments. FA, fenofibric acid; ALT1, alanine aminotransferase 1.*P<0.05 compared to control.

Table III. EC_{50} values (μM) for AZD4619 in PPARα-GAL4 reporter gene assay.

PPARα	EC_{50}
Human	0.10
Monkey	0.47
Rat	10.3
Mouse	6.2

EC_{50}, half maximal effective concentration; PPARα, peroxisome proliferator-activated receptor α.

matched by 67.5% between the two species. However, the core sequence of the human PPRE at -574 was not identical in the rat promoter (Fig. 3B).

Glucocorticoids also show the capacity to induce the activation of the ALT1 promoter. To determine whether another compound known to induce moderate serum ALT activity in rats and humans would cause the activation of the ALT1 promoter, transient transfections with ALT1 promoter constructs and treatment with dexamethasone was performed. The human hepatoma cell line, HuH-7, and the rat MH1C1 cells were transfected with the promoter constructs for human or rat ALT1, respectively, and dexamethasone treatment increased the promoter activity in both species (Fig. 4A and B). Co-transfection of the HuH-7 cells with a vector expressing the human GR (hGR) increased the ALT1 promoter more than dexamethasone treatment alone, and the combination of hGR and dexamethasone in the HuH-7 cells gave rise to an additional effect on the promoter (Fig. 4A). In the rat cells, transfection with the rat GR (rGR) did not affect the ALT1 expression levels, neither alone nor in combination with dexamethasone (Fig. 4B).

Discussion

A novel PPAR agonist (AZD4619) did not increase serum ALT levels in a one month rat toxicological study, but unexpectedly

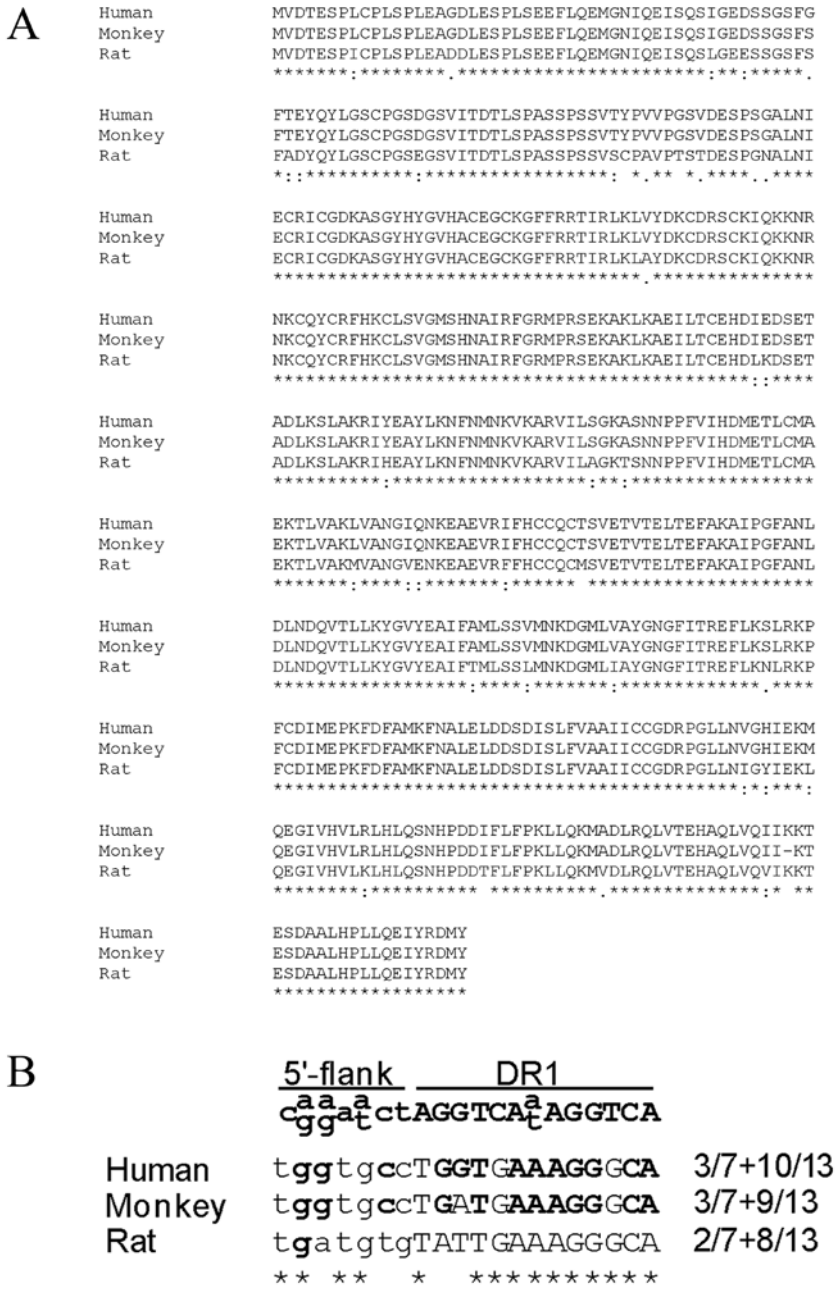

Figure 3. (A) Comparison of human (top lines), monkey (middle lines) and rat (bottom lines) PPARα protein sequences. Below the alignment is a key denoting conserved sequence (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations ( ). The ligand-binding domain is located between amino acids 201-467. (B) Comparison of human, monkey and rat PPRE at -574 in the ALT1 gene promoter. The consensus PPRE is depicted above the aligned sequence. Nucleotides matching the consensus PPRE are highlighted in bold. Nucleotides conserved between human and rat, are indicated by an asterix below the alignment. PPARα, peroxisome proliferator-activated receptor α; PPRE, PPAR response element; ALT1, alanine aminotransferase 1.

did so in the first human clinical trial (13). To elucidate the mechanisms responsible for this discrepancy, we investigated the effects of AZD4619 on ALT1 gene and protein expression and compared the response between human and rat species. AZD4619 was tested *in vitro* at concentrations ranging from its human receptor potency up to >100-fold higher concentrations (0.01 to 10 μM). Still, there was no increase in rat ALT1 levels, neither in ALT1 reporter gene assay nor in ALT1 protein expression assessed by western blot analysis in primary rat hepatocytes. Treatment with AZD4619 at the same concentrations (0.01 to 10 μM) in human hepatocytes gave rise to statistically significant elevations, both in ALT1 promoter gene activity and protein expression. In the human subjects in which plasma ALT1 levels were elevated, the plasma concentrations of AZD4619 were in the same concentration range (Cmax, 0.40 μM) as where the *in vitro* assays detected increased ALT1 promoter activity and protein expression (0.1-1 μM). In the *in vitro* assays, compounds dissolved in cell medium (containing FBS, about 23 mg/l albumin) are protein bound to albumin (99.97%), as in *in vivo* conditions. In the rat *in vivo* study, the concentration of AZD4619 in rat plasma ranged from 0.64 up to 742 μM between the dose groups. Hence, the plasma levels of AZD4619 in the high-dose group were >6,000-fold higher than the human EC_{50} value in the PPAR binding assay, without any effects on serum ALT-levels *in vivo*. Furthermore, the lowest plasma

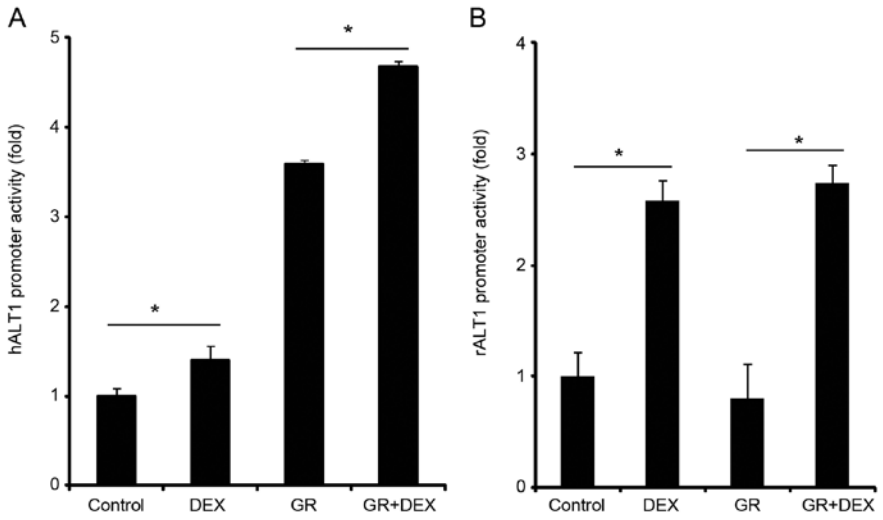

Figure 4. Effects of glucocorticoids on the ALT1 promoter in human and rat hepatoma cells. (A) HuH-7 cells were transfected with human ALT1 reporter plasmid in the presence or absence of hGR, followed by treatment with dexamethasone. (B) The rat cell line MH1C1 was transfected with rat ALT1 reporter plasmid in the presence or absence of rGR, followed by treatment with dexamethasone. hGR, human glucocorticoid receptor; rGR, rat glucocorticoid receptor; ALT1, alanine aminotransferase 1. *P<0.05.

concentration where a pharmacological effect of AZD4619 was observed (i.e., decrease in circulating triglycerides) in humans, occurred already in the 0.5 mg dose group (Cmax, 0.05 μM) (Table I). On the other hand, in rats, decreases in circulating triglycerides occurred first in the high-dose group, 517 mg/kg (Cmax >536 μM). These data point to a clear species-related difference in the ability of AZD4619 to activate PPARα.

To mechanistically understand the difference between human and rat species in response to AZD4619, PPARα-GAL4 assays were performed. The PPAR reporter gene assays demonstrated that AZD4619 is a very human-specific PPARα ligand activating human PPAR at a 100-fold lower concentration compared to rat PPARα, which might fully or partly explain the lack of plasma ALT elevations in the rat. The ligand-binding domains (LBDs) of human and rat PPARα are 93% identical, key amino acids yet to be confirmed are likely contributing to this species selective activation. Furthermore, analysis of the genomic region in the rat corresponding to the functional human -574 PPRE denoted a difference in the core PPRE between human and rat, which might be sufficient to make the rat PPRE substantially less functional. In a comparison of the two sites with the PPRE consensus described by Juge-Aubry *et al* (17), the human site matched by 3/7+10/13, whereas the rat site only corresponded by 2/7+8/13. However, fenofibric acid induced both the human and rat ALT1 promoter genes and increased ALT1 protein expression *in vitro*. The EC_{50} for fenofibric acid in the PPARα-GAL4 assay was similar for both species (13 and 17 μM) (data not shown). These results indicate that the PPRE in the rat is functional and that the species-selective activation of the human vs. the rat ALT1 gene by AZD4619 can be explained by selected amino acid differences in the respective LBD. The support for species-selective PPARα activation comes from studies with another PPAR agonist, Wy14643, shown to be at least 38-fold more rodent selective compared to humans in a time-resolved fluoresence resonance energy transfer (TR-FRET) binding assay (18). Even though the PPRE in the ALT1 promoter was not identical in the human and rat, the surrounding region was evolutionary conserved between the two species, implying its importance. Another explanation for the induction of fenofibric acid on the rat ALT1 gene might be that the effect is mediated through an unidentified unique rat PPRE distinct from the -574 PPRE in the ALT1 promoter (13).

It has been reported in the literature that fenofibrate induces the rat ALT gene even though no exact mechanism has been established (10). Male rats treated with fenofibrate at 180 and 1,000 mg/kg for 13 weeks were shown to have increased ALT levels in serum, whereas AST levels increased already at 30 mg/kg fenofibrate dose (10). Both AST and ALT have been reported to increase mildly in some patients who receive fenofibrate (11,19) and *in vitro* studies have shown that human hepatocytes respond to fenofibrate by a PPARα-dependent increase in aminotransferases (12,20). In humans receiving the dose of 5 mg/kg fenofibrate, approximately 9% develop mild serum aminotransferase increases without any reports on hepatic pathologies (11). The transient and small elevation of aminotransferases in clinical trials and the historical absence of liver injury using fenofibrates, have been suggested to be due to the induction of the ALT and AST genes, rather than damage to hepatic cells (12). Knowledge gained from the application of genome-wide approaches and 'omics' technologies has given way to a more complex and interconnected view of the importance of liver transaminases in the regulation of systemic metabolic function (21).

Dexamethasone is another drug that is capable of inducing ALT expression in the livers of rats and contributes to increased serum ALT levels without involving hepatic cell death (8,22,23). We previously found a consensus binding site for glucocorticoids (GRE, -1253), highly conserved between the human and rat ALT1 promoter, which may explain the promoting effect of glucocorticoids on ALT1 expression in the liver and serum (13). In the present study, we confirmed that dexamethasone increased the transcription of both the rat and the human ALT1 reporter genes (Fig. 4).

Although not shown in this study, preclinical toxicological testing of AZD4619 in cynomolgus monkeys treated for 28 days

with the same doses as the rats (0, 3, 15, 93 and 517 mg/kg) resulted in a weak serum ALT increase (2-fold) in females and a trend towards increased serum ALT levels in males in the mid- and high-dose groups. Of note, no histopathological changes were detected in any organs with known ALT expression such as the liver, skeletal muscle, heart, pancreas and kidneys (data not shown). The magnitudes of the observed transcriptional changes for ALT1 by AZD4619 in cynomolgus monkeys are very much in line with the changes in human hepatocytes reported herein. In the PPARα-GAL4 reporter assay comparing different species, human and cynomolgus monkey PPARα exhibited EC_{50} values similar to each other compared to human and rat/mouse PPARα following treatment with AZD4619 (Table III). Furthermore, an *in silico* comparison of the cynomolgus PPARα LBD with the human PPAR LBD revealed that they are identical, apart from one amino acid deletion in the monkey compared to the human LBD (Fig. 3A). In addition, the PPRE in the monkey ALT1 gene only deviates one nucleotide from the human PPRE (Fig. 3B), which would explain an induction by PPARα agonists in this species.

To summarise, AZD4619 induces the human ALT1 gene and protein expression in hepatocytes, but has no effect on rat ALT1 gene and protein expression in the concentrations tested. The most likely reason for this difference is the high species (>100-fold) selectivity of AZD4619 for the human PPARα. In the *in vivo* study, serum ALT was increased in the human clinical trial, an effect not observed in the preceding rat toxicity study. Discrepancies of ALT1 induction between species might result in problematic drug development, where drugs designed for humans, give rise to findings not detected in pre-clinical toxicity testing: mechanistic data to provide context to these findings is therefore key in considering the clinical risk assessment. These data highlight the importance of translational assessment for hepatoxicity testing and assessment of candidate compounds in humanised model systems is recommended; such systems could include assay of ALT1 gene induction to reveal if drug candidates result in species specific effects.

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