Tacrine-induced liver damage: an analysis of 19 candidate genes

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Objectives **Tacrine, the first acetylcholinesterase inhibitor used in the treatment of Alzheimer's disease, is associated with transaminase elevation in up to 50% of patients. The mechanism of tacrine-induced liver damage is not fully understood, but earlier studies have suggested that genetic factors may play a role. Our aim was to investigate whether single-nucleotide polymorphisms (SNPs) in 19 candidate genes were associated with tacrine-induced liver damage.**

Methods **Sixty-nine patients of Caucasian origin treated with tacrine for Alzheimer's disease were investigated by genotyping 241 SNPs in 19 candidate genes potentially related to hepatotoxicity. The association with *ABCB4* [which encodes MultiDrug Resistance Protein 3 (MDR3)] was explored in transepithelial transport studies using the *ABCB4*-transfected pig kidney epithelial cell line (LLC-PK1).**

Results **The strongest association between alanine aminotransferase levels and three SNPs within ATP-binding cassette, subfamily B (MDR/TAP), member 4 (*ABCB4*) (uncorrected $P = 0.0005$) was not significant after adjusting for multiple testing. No association was demonstrated with ATP-binding cassette, subfamily B (MDR/TAP), member 1 (*ABCB1*) or carnitine O-octanoyltransferase (CROT) which are located adjacent to *ABCB4*. Using the transepithelial transport system we failed to show a difference in tacrine accumulation between *ABCB4*-transfected and parental cell lines. The association with *ABCB4* warrants further testing using either another population and/or functional studies.**
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Introduction

Tacrine (tetrahydroaminoacridine) was the first acetylcholinesterase inhibitor used for the symptomatic treatment of Alzheimer's disease (AD). Its use, however, was hampered by an elevation of liver transaminases [alanine aminotransferase (ALT) and aspartate aminotransferase] in up to 50% of patients [1–4]. The ALT elevations were transient in nature, but sometimes led to transaminase levels that were 20 times the upper limit of normal (ULN) [1]. The high rate of liver injury, together with relatively poor efficacy, has led to the demise of the drug. Nevertheless, it represents a good paradigm with which to study the mechanisms involved in drug-induced liver injury (DILI).

The chemical, molecular and genetic mechanisms of tacrine-induced liver injury have been examined, and several hypotheses have been generated. For example, Mansouri *et al.* [5] used a mouse model to show that tacrine can cause mitochondrial injury leading to mitochondrial DNA depletion and apoptosis of hepatocytes. Similar effects have also been detected in rat and human cells [6–8]. An alternative mechanism was proposed by Stachlewitz and colleagues [9] who suggested that inhibition of acetylcholine breakdown by tacrine led to increased sympathetic activity in the liver that resulted in vasoconstriction, hypoxia and liver injury. Work in our laboratory has shown that tacrine undergoes metabolism to a number of stable metabolites but, in addition, may also form a reactive quinonemethide metabolite, which could bind to liver proteins and elicit dysfunction of hepatocytes either directly or through an immune-mediated mechanism [10,11].

A great deal of interest exists currently in determining the genetic factors that predispose to DILI (*http://dilin.dcri.duke.edu/*; *http://www.diligen.org/*). Genetic associations have already been observed for hepatic injury caused by a number of drugs including the anti-Parkinson drug tolcapone [12], the antidiabetic drug troglitazone

[13], the antituberculosis drug isoniazid [14] and the nonsteroidal anti-inflammatory drug diclofenac [15,16]. The data for tacrine, however, are contradictory. An association of tacrine-induced liver injury with glutathione-S-transferase mu (GSTM1) and glutathione-S-transferase theta (GSTT1) null variants was demonstrated in a cohort of French patients [17], but was not seen in our patients [18,19].

Given the importance of DILI in clinical practice and for the pharmaceutical industry, our aim was to identify genetic predisposing factors using a candidate gene approach utilizing tacrine-induced liver damage in phenotypically well-characterized AD patients as a paradigm. We genotyped 241 single-nucleotide polymorphisms (SNPs) selected from 19 candidate genes in 69 AD patients displaying a range of liver enzyme levels. Functional studies were then performed on multidrug resistance protein 3 (MDR3), the product of ATP-binding cassette, subfamily B (MDR/TAP), member 4 (*ABCB4*) which had demonstrated the strongest association with raised liver enzyme levels.

Patients and methods

Materials

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) [1–^{3}H] D-mannitol and [1–^{14}C] D-mannitol were purchased from Sigma-Aldrich Ltd (Poole, Dorset, UK). [1,2–^{3}H]-Tacrine was purchased from Moravek Biochemicals (Brea, California, USA), whereas [^{3}H(G)]-digoxin was obtained from American Radiolabeled Chemicals Inc. (St Louis, Missouri, USA).

Patients

A group of 69 patients (39 men, 30 women) of Caucasian origin treated with tacrine for mild-to-moderate AD were studied (mean age 73 years, range: 56–88). Patients were otherwise healthy with absence of significant cardiac or cerebrovascular disease, diabetes mellitus, liver disease or renal insufficiency. All patients met the criteria for a diagnosis of AD [20] and had symptoms for at least 1 year. Patients were given 40 mg/day of tacrine with dose escalation to 80 and 120 mg/day at 6-week intervals, if tolerated. ALT levels were measured before treatment, and then weekly for 18 weeks. ALT levels in the patients ranged from 5 to 44 U/l before tacrine administration (mean = 17.2, median = 16 U/l), and ranged from 9 to 859 U/l at 15 weeks (mean = 125, median = 73 U/l) after tacrine administration. In 25 patients (36%) the ALT levels were three times above the ULN, whereas four patients (6%) had ALT elevations exceeding 10 times the ULN. The mean time to onset of maximum ALT was 8 weeks. A complete clinical laboratory assessment was performed every 6 weeks. Tacrine treatment was discontinued if serum ALT levels exceeded three times the ULN. Medications with known central nervous system effects or with cholinergic side effects were not allowed during the trial. Written informed consent was obtained from patients or their legal representatives. The use of the DNA samples was approved by the Northern and Yorkshire Multicentre Research Committee.

Candidate gene selection and genotyping

Candidate gene selection was based on the clinical, pharmacological and molecular knowledge of hepatotoxicity in general, but emphasis was placed on the postulated mechanisms of tacrine-induced toxicity in humans and in animal species (Table 1). Specifically, the genes involved in (a) metabolism and transport of drugs and their regulation, (b) hepatotoxicity as determined in transgenic studies, (c) oxidative stress response, (d) inflammation and apoptosis, (e) encoding tacrine drug targets or implicated in AD and (f) familial forms of liver disease, were included. Well-characterized SNPs were selected from several sources: the literature, the HapMap project (*http://www.hapmap.org/*), the Applied Biosystems (ABI) database (*https://products.appliedbiosystems.com*), dbSNP (*http://www.ncbi.nlm.nih.gov/entrez*) and an in-house SNP database at AstraZeneca. For the 19 genes analysed (Table 1), linkage disequilibrium (LD) maps were generated from HapMap genotyping data for the CEPH population [Utah residents with ancestry from northern and western Europe, (CEU)] using Haploview (*http://www.broad.mit.edu/mpg/haploview/*) [21]. The selection of multiple SNPs with a pair-wise LD correlation coefficient of $r^2 > 0.9$ in CEU HapMap data were avoided.

The carnitine octanoyl transferase gene (*CROT*) was selected after the initial genotype analysis showed *ABCB4* to be most strongly associated, which is in LD with *CROT*. SNP selection for *CROT* was as above. A list of all the SNPs and the ABI codes for the SNPs studied can be provided on request.

ABI TaqMan assays were used for genotyping, if available, while all the other SNPs were genotyped by sequencing as described earlier [22]. Briefly, PCR products containing the SNP of interest were generated using AmpliTaq Gold DNA polymerase (Applied Biosystems, Warrington, UK) and touchdown cycling conditions (initial denaturation for 10 min at 95°C, followed by 13 cycles of denaturation at 95°C for 15 s, annealing at 63°C for 45 s and extension at 72°C for 4.5 min, gradually reaching an annealing temperature of 56°C in decrements of half a degree per cycle). A further 20 cycles were performed with an annealing temperature of 56°C. The PCR products were run on a 4% agarose gel to check the quality of amplification. Unincorporated nucleotides and primers were removed by treatment with ExoSAP-IT (Amersham Biosciences UK Ltd, Amersham, Buckinghamshire, UK) prior to sequencing. PCR products were sequenced on an ABI 3730 × l 96-capillary DNA analyser using BigDye Terminator Chemistry (Applied Biosys-

Table 1 Candidate genes investigated for an association with tacrine-induced liver injury

HUGO gene symbol	Gene	Function related to tacrine or hepatotoxicity
ABCB1	P-glycoprotein or MDR1	Inhibition of transporters can induce hepatotoxicity
ABCB4	MDR3	Implicated in progressive familial intrahepatic cholestasis (PFIC 3)
ABCB11	Bile salt export pump BSEP	Implicated in progressive familial intrahepatic cholestasis (PFIC 2)
ACHE	Acetylcholinesterase	Target for tacrine action
AHR	Arylhydrocarbon receptor	Involved in regulation of drug-metabolizing enzymes involved in tacrine metabolism
APOE	Apolipoprotein E	Involved in Alzheimer's disease (AD) and hyperlipidaemia
ATP8B1	ATPase class I type B	Implicated in Byler's disease (PFIC 1)
BCHE	Butyrylcholinesterase	Implicated in late-onset AD
CYP1A2	Cytochrome P450 1A2	Involved in tacrine metabolism
HTR2C	Serotonin receptor	Implicated in AD
KEAP1	Kelch-like associated protein	Represses NRF2 function
KRT8	Keratin 8	Implicated in liver disease
KRT18	Keratin 18	Implicated in liver disease
LTA	Lymphotoxin α	Implicated in the pathogenesis of liver injury
NQO1	NADPH dehydrogenase quinone 1	Implicated in liver injury with other toxicants such as bromobenzene
NR1H4	Farnesoid-x activated receptor, nuclear receptor subfamily	Regulation of metabolizing enzymes and transporters. Bile acid homeostasis.
NFE2L2	Nuclear factor erythroid 2-like 2, NRF2	Essential for the expression of detoxifying enzymes and oxidative stress-inducible genes
SLC22A1	Organic cation transporter, OCT1	Involved in the disposition of cationic compounds
TNF	TNF-α	Implicated in the pathogenesis of liver injury

Gene symbol, full name of the gene and a brief function are presented.

tems). Sequence analysis and genotype calling was performed using SeqMan II, Lasergene software version 5.06 (DNASTAR Inc., Madison, Wisconsin, USA), Consed and the Mutation Surveyor software (Softgenetics Inc., State College, Pennsylvania, USA).

TaqMan SNP genotyping assays with FAM or VIC reporter dyes at the 5′ end of each MGB probe and a nonfluorescent quencher at the 3′ end were used (Applied Biosystems). Primer concentrations were 18 μmol/l with a probe concentration of 4 μmol/l in a 20 × mix. Real-time PCR to determine allelic discrimination was performed on an ABI PRISM 7900 Sequence Detection System (ABI).

Tissue culture

The LLC-PK1 parental cell line and multidrug resistance 3 (*ABCB4*)-transfected LLC-PK1 pig kidney epithelial cells were a kind gift from Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The cells were cultured in M199 medium (Sigma-Aldrich) supplemented with 10% foetal calf serum, 50 units of penicillin and 50 μg of streptomycin/ml (both from Gibco/Invitrogen Ltd, Paisley, UK) at 37°C in 5% CO_2. We used 200 μg/ml of geneticin (G418) (Sigma-Aldrich) to maintain selective pressure on *ABCB4*-transfected cells. The cells were trypsinized and subcultured every 3–4 days.

3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay of drug-induced toxicity

The cytotoxicity of tacrine, digoxin, verapamil and PSC833 towards *ABCB4*-transfected and parent LLC-PK1 cell lines was assessed by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 2×10^5 cells/well were exposed to different concentrations of the drugs (0.5–100 μmol/l/l) in 96-well culture plates for 4 days at 37°C in 5% CO_2. The cells were then exposed to 20 μl of MTT solution (5 mg/ml, Sigma) for 4 h, then solubilized with 100 μl of 10% sodium dodecyl sulphate in 0.01 mol/l HCl and incubated at 37°C overnight. The absorbance was measured at a wavelength of 570 nm with background subtraction at 690 nm using a plate reader (MRX Dynatech Laboratories, Billingshurst, UK).

Drug transport studies

For the transport studies, cells were seeded onto 6-well polycarbonate Transwell filter membranes (pore size 3.0 μm, diameter 24.5 mm) (Transwell plate, Costar UK Ltd, High Wycombe, Buckinghamshire, UK) at a density of $1.5–2 \times 10^6$ cells/filter. The cells were grown in complete M199 medium (in the absence of G418) for 3 days. The medium was changed 1 day after seeding. One hour before the start of the experiment, the medium on both sides of the monolayer was replaced with serum-free media. Transendothelial electrical resistance was measured with the Millicell-ERS ohm meter (Millipore Corporation, Billerica, Massachusetts, USA). The level of acceptable resistance was 200 Ω above background. The experiment was started by replacing the medium in either the apical or the basolateral side with medium containing [^{3}H]-labelled digoxin as a positive control or [^{3}H]-labelled tacrine (0.25 μCi/ml). The reference drug for paracellular transport ([^{14}C]-mannitol at 0.025 μCi/ml) was included in each experiment. The cells were incubated at 37°C in 5% CO_2 and 50 μl aliquots were taken from both compartments. Aliquots for digoxin measurements were taken at 1-h intervals for 4 h, while tacrine was measured at 0.5-h intervals for 2 h because of

the rapid cell uptake. The appearance of the radioactive drug in the opposite compartment was measured in triplicate and presented as the fraction of total radioactivity added at the beginning of the experiment. The concentration of the radioactive drugs was measured by liquid scintillation using a Beckman counter after addition of 4 ml of scintillation fluid (Ultima gold, Packard Instruments, USA).

Drug accumulation experiments

Digoxin and tacrine accumulation was measured in the LLC-PK1 parental cell line and in the *ABCB4*-transfected LLC-PK1 cell lines by seeding 1×10^6 cells in each well of a 12-well plate. The cells were incubated in serum-free media for 24 h at 37°C in 5% CO_2 prior to the experiment. The medium was replaced by 1 ml of medium containing 0.25 μCi/ml of [^{3}H]-digoxin or [^{3}H]-tacrine in the absence and presence of the inhibitors PSC833 (5 and 10 μmol/l) and verapamil (20 μmol/l). After a 2-h incubation, the medium was removed, and the cells were washed twice with 1 ml of ice-cold Hanks balanced solution (Sigma). The cells were lysed using 100 μl of ice-cold tap water and 100 μl of 0.5 mol/l NaOH, then scraped and removed from the wells. Radioactivity was determined by liquid scintillation counting. Experiments were done in quadruplicate.

Determination of multidrug resistance protein 3 mRNA levels

Total RNA was extracted from 1×10^4 *ABCB4*-transfected and parental LLC-PK1 cells using the Dynabeads Oligo(dT)25 method (Invitrogen). Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturers protocol. The measurement of *MDR3* mRNA expression in both cell lines was performed using the TaqMan expression assay (AoD: HS 00251620_ml, ABI) and 7900 Sequence Detection System (ABI).

Statistical analysis

One-way analysis of variance was performed for two quantitative measures of ALT levels: the maximum ALT level seen during treatment with tacrine and area under the curve (AUC) over a 15-week period. Both measures were log-transformed and standardized. ALT AUC was calculated between 0 and 105 days of treatment using the trapezoid sum method. If ALT was not observed at day 0 or 105 these values were obtained by linear interpolation between the nearest preceding and subsequent observations. If a participant had no observations ≥ 105 days, the ALT value at day 0 was used at day 105. Correction for multiple testing was performed with 10 000 permutations using the permtest function in Stata (part of the permutation package available at *http://www-gene.cimr.cam.ac.uk/clayton/software/*). The adjusted *P* value was obtained by comparing the original test statistic with the maximum permuted test statistic over all SNPs in each permutation. Analysis of variance was also used for the statistical analysis of functional studies using *ABCB4*-transfected cells.

Results

Association analysis

We analysed two quantitative measures of ALT levels, the maximum ALT and the AUC over a 15-week period of treatment with tacrine. A strong correlation ($r^2 = 0.89$) exists between Max ALT and AUC. The 15-week period was selected because the ALT increase in the majority of patients occurs between 4 and 12 weeks after administration of tacrine and resolves within 2 weeks of drug discontinuation [1].

A number of SNPs in different genes showed a nominally significant difference in the allelic and/or genotype frequencies between AD patients who developed tacrine-induced liver damage compared with those who tolerated the drug. A summary of the statistical analysis is shown in Table 2. A list of all SNPs studied is available as supplementary data. Polymorphisms in *ABCB4* showed the strongest association with either the maximal ALT or AUC for ALT. Polymorphisms in *AHR*, *ATP8B1* and *APOE* showed a weaker association with ALT ($P < 0.02$). A further group of SNPs showed an association at $P < 0.05$. After correction for multiple testing using permutation analysis, none of the associations, however, remained significant.

The highest significance level was observed for three SNPs (rs2097937, rs9655950 and rs31675) in *ABCB4*

Table 2 Association of tacrine-induced liver damage with single nucleotide polymorphisms in a number of candidate genes

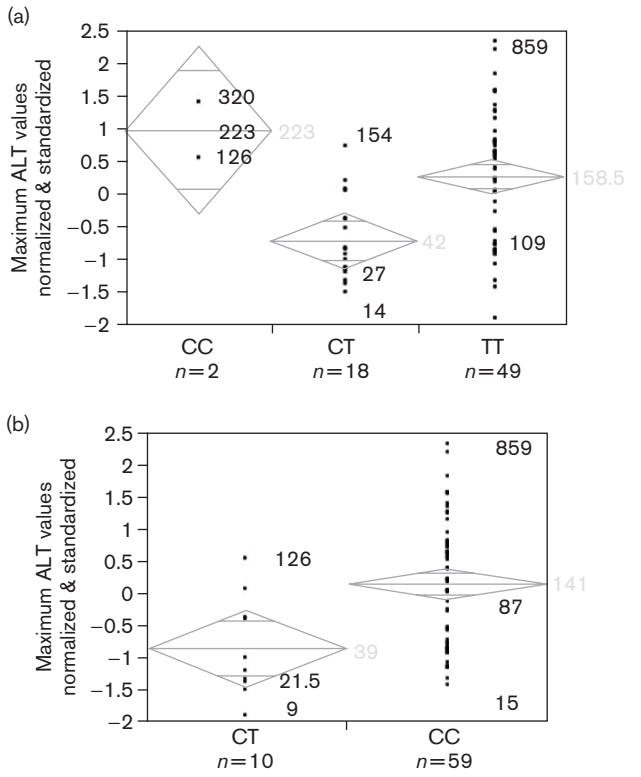
Gene	Single-nucleotide polymorphism	Hardy–Weinberg Equilibrium *P* value	*P* value for maximal ALT or AUC for ALT
ABCB4	rs9655950	1	0.0005
ABCB4	rs2097937	0.7	0.0007
ABCB4	rs31675	1	0.0028
ATP8B1	rs4940996	0.2	0.0111
ABCB4	rs2373593	0.4	0.0131
APOE	rs429358	<0.01	0.0147
ABCB4	rs31662	0.5	0.0157
ABCB4	rs31659	0.5	0.0157
CROT	rs31651	0.5	0.0157
ATP8B1	rs319409	0.5	0.0162
TNF	rs3093661	1	0.0211
CROT	rs2072207	1	0.0232
SLC22A1	rs3798168	1	0.0237
CROT	rs802054	1	0.0260

Maximum ALT and the area under the curve for ALT measurements over a 15-week period were used as markers of tacrine-induced liver injury. The initial analysis using one-way ANOVA demonstrated that tacrine-induced liver injury in patients with Alzheimer's disease was associated with a number of SNPs. The strongest association was observed with the three SNPs in *ABCB4*. Even the lowest *P* value, however, did not withstand correction for multiple testing using permutation analysis ($P = 0.115$). An interesting observation is that some SNPs in *APOE* and all SNPs in *HTR2C* (data not shown) demonstrated Hardy–Weinberg disequilibrium possibly because they are associated with Alzheimer's disease.

ALT, alanine aminotransferase; ANOVA, analysis of variance; AUC, area under the curve; SNP, single-nucleotide polymorphism.

(Table 2 and Fig. 1), which encodes the multidrug-resistant transporter MDR3. The *P* value corrected for multiple testing ($P = 0.1154$) represented the proportion of permuted data sets in which the most significant *P* value for any SNP was less than the most significant *P* value in the original data set. It is interesting to note that the lowest ALT values were detected in heterozygous (CT) individuals for the SNP rs9655950 (Fig. 1a), although there were only two individuals homozygous for the rare allele. In a case control analysis, we found a lower frequency of the rare rs9655950 C allele in patients with ALT levels exceeding three times ULN than in patients with normal ALT values (6% vs. 30%, respectively) ($P = 0.04$, OR = 0.22, 95% confidence interval 0.04–0.95). The SNP rs2097937, which is in strong LD with rs9655950, also showed a similar result (data not shown). Lower ALT values were also detected in rs31675 heterozygous (CT) individuals, but there were no individuals who were homozygous for the rare allele (Fig. 1b).

Fig. 1



Genotypic association of tacrine-induced liver damage with two SNPs in *ABCB4*, rs9655950 (a), rs31675 (b), determined by one-way analysis of variance. The mean values of the maximum ALT measurements are presented with confidence intervals for each genotype group, shown as diamond and digits. Furthermore, the minimum, median and maximum values of the maximum ALT measurements are provided for each genotype group. CC (homozygous, rare allele), CT (heterozygotes) and TT (homozygous, common allele) are shown on the *x*-axis. ALT, alanine aminotransferase; SNP, single-nucleotide polymorphism.

ABCB4 is located on chromosome 7q21.1, adjacent to *ABCB1*, which encodes P-glycoprotein or MDR1. An LD map generated from our data showed two distinct regions of *ABCB4* (Fig. 2), one of which is in tight LD with *ABCB1*, whereas the other is in LD with neighbouring *CROT*.

Although *CROT* was not initially included into our candidate gene list, we decided to genotype eight SNPs in *CROT* (of which six were in tight LD with *ABCB4*) to assess whether the associated SNPs in *ABCB4* were possible causative variants, or were merely in LD with functional *CROT* SNPs. This showed a borderline association of three SNPs in *CROT* and ALT elevation. The level of significance, however, was lower than that for the SNPs in *ABCB4* (Fig. 3). From this, we concluded that *ABCB4* was more likely to be driving the association, although an association with *CROT* could not be excluded.

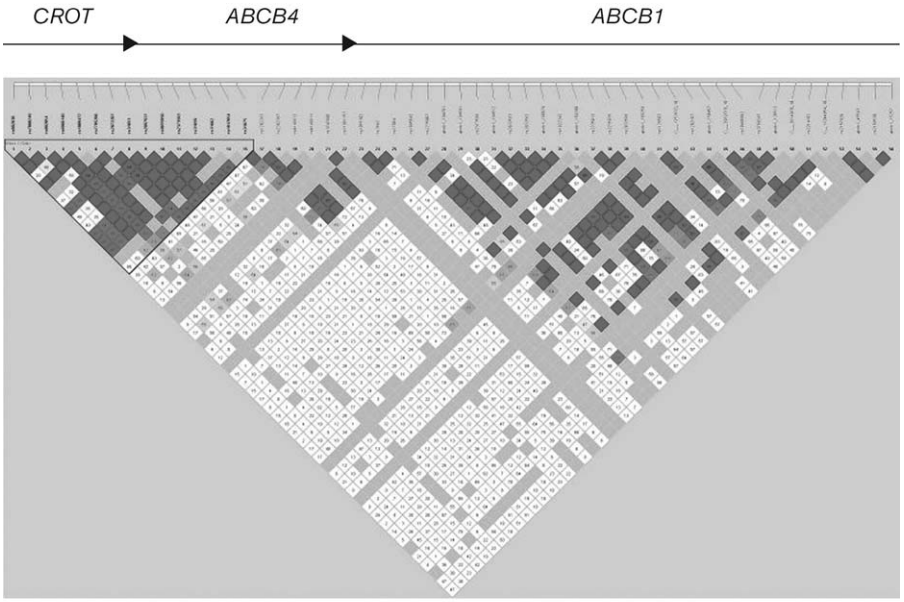
Functional studies with *ABCB4*-transfected cells

Given the strongest association with *ABCB4* SNPs in our data set, we undertook functional studies to determine whether tacrine was either a substrate and/or an inhibitor of MDR3. Initial studies confirmed that *ABCB4*-transfected cells overexpressed the gene product in comparison to the parental cell line (Fig. 4). MDR3 expression, however, varied according to cell passage number in that it showed a decrease with an increase in number of passages. Thus, all experiments were performed within the first six passages. Cytotoxicity testing using the MTT assay showed that tacrine concentrations up to 150 μmol/l exhibited no significant toxicity (data not shown), and thus we chose to use a concentration range between 50 and 100 μmol/l for the transport studies.

The transport of tacrine by MDR3 was assessed in comparison with digoxin, a known MDR3 substrate [23], whereas establishing the integrity of the monolayer using [^{14}C]-mannitol. The directional transport of digoxin to the apical compartment was increased in the *ABCB4*-transfected cells compared with the parental cell line. Tacrine transport, however, did not show any difference regardless of the cell line used. Digoxin cell accumulation was lower in the *ABCB4*-transfected cell line compared with the parental cell line ($P = 0.03$), whereas tacrine showed similar cell accumulation in both cell lines ($P = 0.23$) (Fig. 4).

The active transport of drugs by MDR3 through monolayers of LLC-PK1-derived cell lines can be inhibited by verapamil and PSC833 [23]. Using these inhibitors, we were able to demonstrate partial reversal of digoxin accumulation in the *ABCB4*-transfected cells by verapamil (20 μmol/l) (Fig. 4) and PSC833 (5 μmol/l), with the latter being a more potent inhibitor. No reversal of digoxin accumulation was seen when tacrine was used

Fig. 2

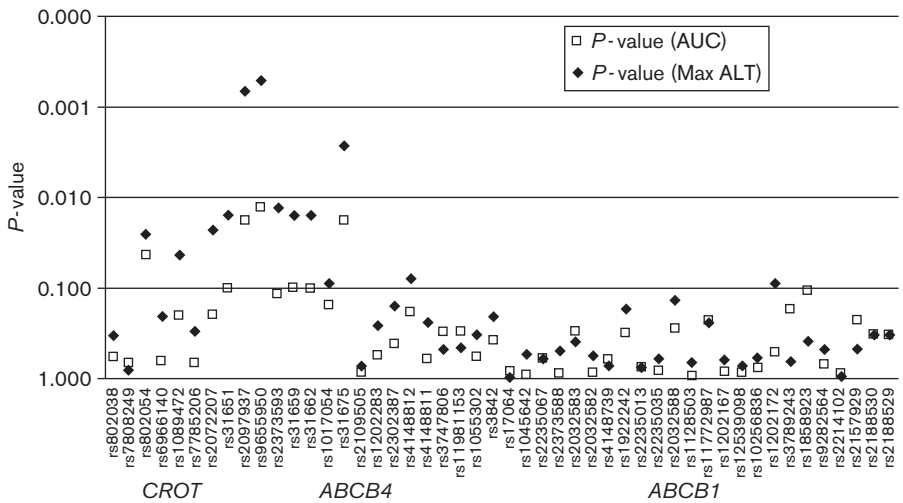


Key to the color scheme used on the map:

	D'<1	D'>1
LOD<2	White	Grey
LOD>2	Grey	Dark grey

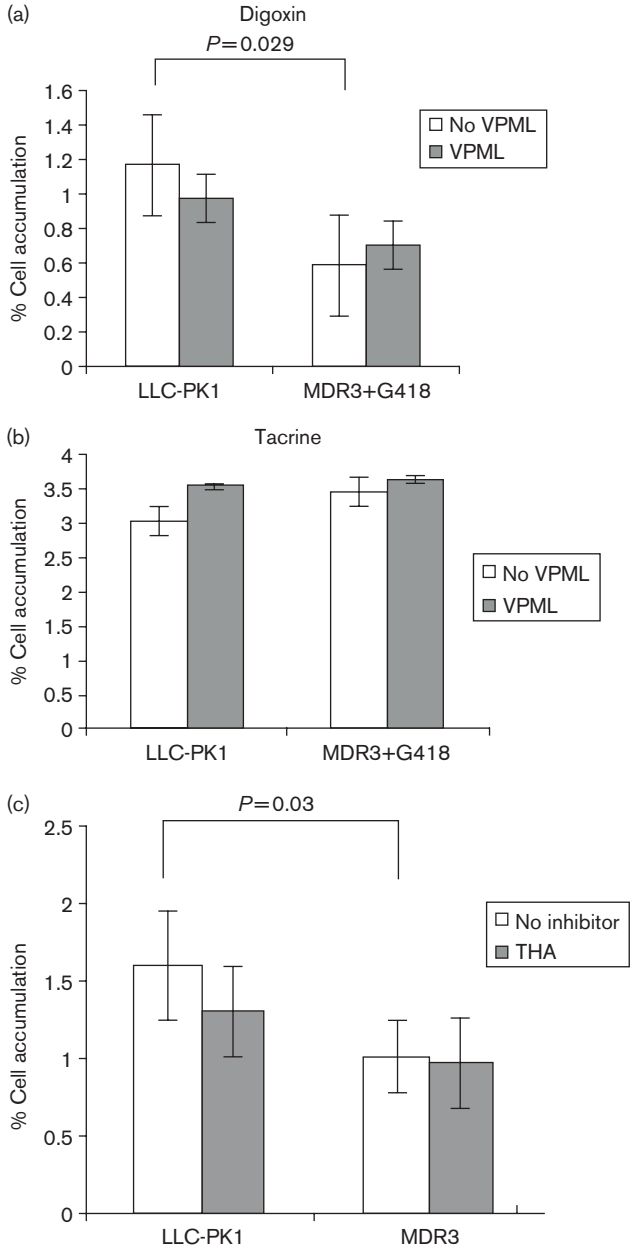
LD map of *ABCB4* together with the two neighbouring genes, *ABCB1* and *CROT*. This was constructed using Haploview software from our genotyping data. The standard colour scheme has been used (see key). Two separate regions of high LD are seen in *ABCB4*. LD, linkage disequilibrium.

Fig. 3



Genotype *P* values for *CROT*, *ABCB4* and *ABCB1* in patients with tacrine-induced transaminitis. *P* values for the maximal ALT and AUC over the 15-week period are shown in relation to SNPs in *ABCB4* (from rs2097937 to rs11981153) and the two neighbouring genes. The most significant values can be seen for three SNPs in *ABCB4* (rs2097937, rs9655950 and rs31675). ALT, alanine aminotransferase; AUC, area under the curve; SNP, single-nucleotide polymorphism.

Fig. 4



Effect of MDR3 on digoxin (a) and tacrine (b) cellular accumulation (c). Effect of tacrine on digoxin cellular accumulation. (a) MDR3 and its effect on digoxin accumulation. *MDR3* gene transfected cells showed lower digoxin accumulation than the parental LLC-PK1 cells ($P=0.029$). Only partial reversal of accumulation in the presence of verapamil is detected. (b) No difference was found in tacrine accumulation between MDR3 transfected and parental cell lines ($P=0.23$). (c) Cellular digoxin accumulation was significantly lower in MDR3 expressing cells in the absence of an inhibitor ($P=0.03$). Tacrine did not have any effect on digoxin accumulation. The data represent the summary of six independent experiments.

as a potential inhibitor. In addition, tacrine did not accumulate in the *ABCB4*-transfected cell lines and this was unaffected by either verapamil or PSC833 (Fig. 4).

Discussion

DILI is the most common cause of acute liver failure and the foremost reason for drug withdrawal postlicensing [24]. In this study, we have used tacrine, an anti-AD drug, to identify genetic factors predisposing to liver injury. Tacrine is well known to cause liver injury. Approximately 25% of patients on tacrine develop ALT elevation to $3\times$ ULN, and in 2% of patients, ALT values exceeded $20\times$ ULN [1].

DILI is thought to have a genetic basis, as demonstrated in a number of studies [12,13,15,16]. The molecular basis of tacrine toxicity has not been fully elucidated, although various hypotheses have been advanced. In an attempt to identify the genetic factors associated with tacrine-induced liver damage, we have genotyped our patients for 241 SNPs in 19 genes. We related the frequencies of SNPs to ALT levels, both maximal ALT and the AUC of ALT over 15 weeks of treatment with tacrine. The advantage of using this approach is two-fold: (a) it avoids the use of arbitrary cut-off levels to define normality; and (b) the use of quantitative variables, as opposed to categorical variables, may improve statistical power.

Associations at the $P<0.05$ level were identified with a number of genes (Table 2). The strongest association was seen with SNPs in *ABCB4* that encodes MDR3, an ATP-binding cassette (ABC) protein which is a transmembrane drug transporter involved in biliary secretion of phosphatidylcholine [25] and a number of drugs including digoxin [23]. Furthermore, genetic variation in *ABCB4* has been associated with cholestatic syndromes [26–33] and recently, with cholestatic and hepatocellular drug-induced liver toxicity [34]. The three SNPs showing the strongest associations were located in the introns and the 3′-flanking region. The functional effect of these SNPs has not been evaluated, but potentially they could affect expression of MDR3 – this requires further investigation.

It is interesting to note that for the two SNPs, rs9655950 and rs2097937, which are in strong LD, the lowest ALT levels were detected in heterozygotes and not in homozygotes for the rare allele. This may have been a chance finding particularly as there were only two patients who were homozygous for the rare allele. In contrast, heterozygous advantage, which is a well-known phenomenon in disease genetics (for example, cystic fibrosis and cholera, sickle cell disease and malaria, and Tay-Sachs disease and tuberculosis, all show heterozygous advantage) [35–37], cannot be entirely excluded, but is difficult to explain for *ABCB4* in relation to ALT levels.

To control the type 1 error, we undertook correction for multiple testing, which left us with no SNPs showing

significance $P < 0.05$. Owing to the limited statistical power of our sample to detect all but the strongest genetic effects, this was not surprising, especially as in complex traits, such as DILI, multiple SNPs or haplotypes are likely to have small effects which contribute to the overall phenotype. The fact that DILI, however, is a relatively uncommon condition makes it difficult to obtain large numbers of affected patients. As such, the genetic results of our study are 'hypothesis-generating' as opposed to firmly establishing any associations. Replication of the results in an independent cohort would be necessary to prove the association using a genetic approach. Such a cohort, however, is unavailable in the UK due to discontinuation of tacrine use.

Therefore, we undertook an alternative approach to determine whether MDR3 was important in the pathogenesis of tacrine-induced liver damage. Using cell lines transfected with wild-type *ABCB4*, our aim was to determine whether tacrine was either a substrate and/or inhibitor of MDR3. Our data show that tacrine is not a substrate for MDR3, unlike digoxin, which was used as a positive control. Our findings are consistent with the fact that tacrine is not a substrate for MDR1 either, given the overlapping substrate specificities of the two ABC transporters [38]. In addition, tacrine did not inhibit either accumulation or transport of digoxin.

If tacrine is neither a substrate nor an inhibitor of MDR3, does the potential association of MDR3 and ALT levels have any biologically plausible explanations? Several possible interpretations are found. First, we cannot exclude the possibility that the SNPs we have identified are in LD with nonsynonymous exonic SNPs in *ABCB4*. Indeed, resequencing of the *ABCB4* exonic regions has recently been undertaken in 36 patients with drug-induced liver injury [34]. Two *ABCB4* nonsynonymous SNPs (I764L and L1082Q) were specific for drug-induced cholestasis and hepatocellular injury, respectively. Given the low allelic frequencies of both polymorphisms in patients with hepatotoxicity (0.02 and 0.04), however, it would be difficult to confirm these findings in our relatively small patient group. In addition, further investigation would be required to establish whether these SNPs alter the function and substrate specificity of MDR3, as has been shown previously with the related transporter MDR1 [39].

Second, MDR3 also functions as a phosphatidylcholine flippase, translocating phosphatidylcholine from the inner to the outer membrane [25]. Interestingly, phosphatidylcholine has been used in the prevention of liver damage associated with ethanol toxicity in several animal species [40–42]. Phosphatidylcholine has also been used in conjunction with tacrine to both increase efficacy and prevent an increase in transaminase levels, although the efficacy for both has not been proven [43–46]. One study has suggested that tacrine changes membrane fluidity without affecting lipid peroxidation, and this may have a role in the hepatic injury [47]. Taking this together with the fact that tacrine can lead to hepatocyte cell death [7,48], and MDR3 has a multifunctional role which can give rise to at least three diseases [49,50], we cannot exclude a complex interaction between MDR3 and tacrine which ultimately contributes to the rise in transaminases.

Third, the SNPs identified in *ABCB4* may be in LD with SNPs in neighbouring genes. The association with SNPs in the adjoining *ABCB1* was nonsignificant. *CROT*, however, the gene encoding carnitine octanoyl transferase, is located on 3′ of *ABCB4*. Its main role is to catalyze the reversible transfer of fatty acyl groups between CoA and carnitine, which allows the transport of medium and long chain acyl-CoA out of the mammalian peroxisome to the cytosol and mitochondria [51]. It is interesting to note that carnitine has been implicated in valproate hepatotoxicity [52], the milder versions of which are characterized by a rise in transaminases [53]. We therefore genotyped our patients for variants in *CROT* – however, only a weak association was found with tacrine transaminitis, and although we cannot exclude a causal link, this seems unlikely and the association most likely reflects the strong LD with *ABCB4*.

In our study, hepatic injury was defined biochemically by an increase in ALT. It is usual to define hepatotoxicity on the basis of a rise in ALT, although there is debate as to the most appropriate cut-off between normality and abnormality. For example, many studies have used values of more than $3 \times$ ULN as being clinically relevant [4]. It, however, has also been argued that the $3 \times$ ULN cut-off does not reflect genuine toxicity, and a greater than $10 \times$ ULN has been suggested as a cut-off [54]. To avoid a biased approach and clinical misclassification of patients, we analysed the transaminase levels as a continuous variable in our patients, utilizing maximal ALT values and the AUC of ALT over a 15-week period to the genotypes. In that way patients and controls are grouped according to their genotypes and ALT values were attributed to each genotype allowing a nonbiased analysis.

In conclusion, our data suggest, but do not prove, an association between tacrine-induced transaminitis and genetic variants in *ABCB4*, which encodes the phosphatidylcholine transporter MDR3. Tacrine, however, does not seem to be either a substrate or an inhibitor of MDR3. The mechanism by which tacrine interacts with MDR3, phosphatidylcholine transport and possibly with membrane fluidity requires further investigation. Our study also highlights the limitations of genetic studies of rare adverse events in the postgenome era where genotyping strategies that evaluate multiple genes or whole genomes will have limited statistical power.

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