Molecular and genetic association of interleukin-6 in tacrine-induced hepatotoxicity

Daniel F. Carr[a], Ana Alfirevic[a], Jonathan D. Tugwood[b], Bryan J. Barratt[c], James Sherwood[c], John Smith[c], Munir Pirmohamed[a] and B. Kevin Park[a]

Background Tacrine, an anticholinesterase used to treat Alzheimer's disease (AD), leads to an increase in serum alanine aminotransferase (ALT) levels. The factors determining individual susceptibility are largely unknown. The purpose of this study was to investigate genetic predisposition.

Methods Rats were administered single dose tacrine (3–40 mg/kg). After 6 and 24 h, hepatic gene expression was determined using the affymetrix rat U34A microarray. On the basis of the gene expression data, the *IL6* gene was identified as a potential candidate for tacrine transaminitis susceptibility. Sixty-nine patients with AD on tacrine with or without transaminitis were genotyped for 17 *IL6* polymorphisms.

Results Serum aspartate aminotransferase levels in rats increased after tacrine (40 mg/kg) administration. Forty-six and 29 genes showed significant upregulation at 6 and 24 h, respectively, after administration, including the IL-6-regulated acute phase response genes α2-macroglobulin, fibronectin and haptoglobin. Five of the 17 *IL6* polymorphisms studied in AD patients showed an association ($P<0.05$) with transaminitis [ALT > 2 × upper limit of normal (ULN)]. An association existed between maximum ALT and area under curve for ALT over 15 weeks and an intronic polymorphism ($P<0.01$) and a 3′-variable nucleotide tandem repeat ($P<0.05$). Multilocus haplotype analysis showed one haplotype (which included the −597A, −572G, −174G and variable nucleotide tandem repeat-D alleles) had a frequency of 0.1 in patients with ALT values > 2 × ULN, whereas it was absent in patients with ALT less than 2 × ULN ($P=0.0093$, $P_{corrected}=0.049$).

Conclusion The *IL6* genotype may act as a predisposing factor for tacrine transaminitis. This, however, requires further confirmatory functional studies. The role of acute dosing rodent models in identifying candidate genes associated with drug-induced liver injury in man deserves further study. *Pharmacogenetics and Genomics* 17:961–972 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Pharmacogenetics and Genomics 2007, **17**:961–972

Keywords: hepatotoxicity, interleukin 6, pharmacogenetics, tacrine

[a]Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, [b]Safety Assessment UK and [c]Research and Development Genetics, AstraZeneca, Alderley Park, Cheshire, UK

Correspondence to Munir Pirmohamed, Department of Pharmacology and Therapeutics, Sherrington Building, Ashton Street, Liverpool L69 3GE, UK
Tel: +44 151 794 5549; e-mail: munirp@liv.ac.uk

Received 23 October 2006 **Accepted** 14 July 2007

Introduction

The anticholinesterase inhibitor tacrine hydrochloride was used in the treatment of Alzheimer's disease (AD) [1,2]. Its use, however, was complicated by the occurrence of liver injury manifested as an elevation of serum alanine aminotransferase (ALT) levels (hereafter termed 'tacrine transaminitis'). Approximately 49% of AD patients receiving 40–160 mg of tacrine per day developed an asymptomatic elevation of ALT. Levels reached 3 × and 20 × upper limit of normal (ULN) in 25 and 2% of patients, respectively [3], usually within 4–12 weeks of the start of drug therapy [3]. In some cases, the ALT values resolve despite continuation of tacrine, which may be a form of adaptation, the mechanism of which is unknown. Some patients developed liver necrosis as judged by hepatic biopsy [3,4].

Single high-dose [5,6] and chronic dosing [7] studies have been used in rodents to determine the mechanisms of tacrine-induced transaminitis. Both dosing schedules are associated with mild transaminitis reminiscent of the situation observed in man. Hepatic gene expression profiling has also been investigated in an acute high-dose tacrine rodent model [8], but the findings were not extrapolated to man. A number of mechanisms of tacrine-induced hepatic injury have been suggested including hypoxia-reoxygenation injury [6], mitochondrial uncoupling/DNA depletion [7,9] and the bioactivation of tacrine to a chemically reactive quinone methide metabolite [10,11].

Individual predisposition to liver injury from various drugs has been postulated to be owing to genetic factors [12]. For tacrine, earlier work on genetic predisposition has focused on the bioinactivation pathway and, in particular, on the polymorphically expressed glutathione-S-transferase (GST) M1 and T1 genes. Although an association, however, was observed in one cohort [13], it was not replicated in our patient group [14,15].

1744-6872 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins

Candidate gene analysis is the most common and most efficient method currently available in identifying pharmacogenetic determinants of drug response. This strategy, however, relies on our knowledge of the mechanistic pathways involved in determining the response, which in most cases, is incomplete. To refine the process of identifying candidate genes for tacrine transaminitis, we have followed a two-stage strategy. First, gene expression changes were analysed in liver after acute administration of tacrine to a rat model to identify biologically plausible candidate genes. This provided an unbiased method to identify candidate pathways and genes. Second, to extrapolate from the rodent model to man, the importance of candidate genes so identified was tested using a previously defined DNA bank of AD patients receiving tacrine [15].

Experimental procedures

Materials

All chemicals and reagents were supplied by Sigma Chemical Company (Poole, UK) unless otherwise stated.

Microarray analysis

An in-house cDNA microarray database of male Sprague–Dawley rat hepatic gene expression data were used. A total of 48 animals were used in the study. All animal work was carried out under United States Department of Agriculture (USDA) license and Association for Assessment and Accreditation of Laboratory Animal care International (AAALAC) accreditation. A minimum of four animals (253–303 g in weight) were administered a single intraperitoneal dose of tacrine (3, 10, 30 or 40 mg/kg) with control groups ($n \geq 4$) receiving saline. A single dose of 40 mg/kg is comparable to that administered in earlier studies [5,6]. This dose is also the highest practicable for assessment of hepatotoxicity given that higher doses (50 mg/kg) can be lethal within 4 h due to acute cholinergic toxicity [6].

Total hepatic RNA isolation, probe preparation and GeneChip hybridization were carried out according to standard protocols (*http://www.affymetrix.com*). Expression profiles were generated from rat genome U34A arrays containing 8799 probe sets (Affymetrix Santa Clara, California, USA; three per animal) and normalized using the MAS5 algorithm [16].

Statistically significant gene expression changes between treated and control groups were identified by a volcano plot visualization method [17,18]. In brief, the volcano plot score is a bivariate parameter which establishes a cut-off value taking into account both the P value and the fold-change (from Student's t-test). It is expressed as $v = \log|\mathrm{FC}|{*}\log|P\ \text{value}|$. Outcomes with a volcano score of less than -0.339 (derived from threshold values of fold-change = 2, $P = 0.05$) were considered to be significant.

Patients

An AD patient cohort consisting of 69 patients (39 men, 30 women) was studied. All individuals (mean age 73 years, range 56–88 years) were of Caucasian origin and treated for mild to moderate AD with tacrine. All patients met the diagnostic criteria for AD [19]. Patients initially received 40 mg/day of tacrine; the dose was escalated to 80 mg and 120 mg/day at 6-week intervals. Medications known to affect the central nervous system or have cholinergic side effects were not allowed during the study. Pretreatment levels of ALT and aspartate aminotransferase (AST) were determined and monitored weekly for 18 weeks with a complete clinical laboratory assessment carried out every 6 weeks. Patients were withdrawn from the study if the serum ALT levels exceeded 3 × ULN. Transaminitis was defined as an elevation of ALT > 2 × ULN (70 IU/l) and was observed in 37 individuals (18 men, 19 women; mean age 71.9 years). The use of the DNA samples was approved by the Northern and Yorkshire Multi-Centre Research Ethics Committee.

DNA amplification and sequencing

M13-tagged forward and reverse primers (Sigma Genosys UK, Haverhill, Suffolk, UK) were designed to target *IL6* sequences to produce fragments less than 750 bp (Table 1). PCR reactions were undertaken using ReddyMix PCR master mix with 1.5 mmol/l $MgCl_2$ (Abgene, Epsom, UK) containing 200 mmol/l forward and reverse primers and 20 ng DNA. ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) was used to purify the products according to the manufacturer's instructions. Automated DNA sequencing of purified, M13-tagged PCR products was carried out using the MegaBACE DNA Analysis System (Amersham Biosciences, Little Chalfont, Bucks, UK). The genotype was analysed using the Mutation Surveyor v3.0 software (SoftGenetics, Pennsylvania, USA).

Genotyping of *IL6* –174G/C single nucleotide polymorphism

Genotyping of the *IL6* –174G/C single nucleotide polymorphism (SNP) (rs1800795) was performed by Taqman real-time PCR using an assay from Applied Biosystems (Foster City, California, USA). This consisted of forward (5′-GACGACCTAAGCTGCACTTTTC-3′) and reverse (5′-GGGCTGATTGGAAACCTTATTAAGATTG-3′) primers and allele-specific probes (CCTTTAGCCATCGCAAGAC and CCTTTAGCCATGGCAAGAC) labelled with VIC and 6-FAM dyes, respectively. PCR reactions (10 µl) consisted of 1 × Taqman Universal PCR master mix (Applied Biosystems), 1 × assay mix and 20 ng DNA. PCR was performed using an Applied Biosystems 7000 Real-Time PCR System (Applied

Table 1 Oligonucleotide primer sequences for human IL6 genotyping

Amplicon	Sense primer	Position (AF372214)	Antisense primer	Position (AF372214)
A	5′-#CATAGGAAGCCAAGTTGCTG-3′	7845–7864[a]	5′-*GCATGGCACTTTATTAGTATCAATG-3′	8594–8570[a]
B	5′-#CATTGATACTAATAAAGTGCCATGC-3′	8570–8594[a]	5′-*CATTCCTTGGCTCAGAGTCTCT-3′	9097–9076[a]
C	5′-#TTCAAAGAGCTAGCATGTATTGTGG-3′	9098–9122[a]	5′-*GTGCATAACATTTCAGGACCCGCC-3′	275–252
D	5′-#GAGAGCACTGGCAGCACAAG-3′	870–889	5′-*TCCTCTGACTCCATCGCAGCC-3′	1208–1188
E	5′-#CTTCGGTCCAGTTGCCTTCT-3′	1932–1951	5′-*TTTATGATTTGCCACTTTGGTG-3′	2597–2576
F	5′-#CATTCCAGCTAAGATTCATACCTCAG-3′	2546–2571	5′-*TCTTTGGAAGGTTCAGGTTGTT-3′	3249–3228
G	5′-#CTTCCAATCTGGATTCAATGAG-3′	3269–3290	5′-*CACCAGGCAAGTCTCCTAAGAG-3′	4012–3991
H	5′-#GCATCCAACTCCAGCCAGT-3′	4453–4471	5′-*TGAAATAATGCCTGGCACATAG-3′	5173–5152
I	5′-#GTGATGCTGCAGAATTCCAGGACC-3′	4902–4925	5′-*CATTAGACCACAAGCATCCTGA-3′	5399–5378
J	5′-#TGGAAGGGTCCTACTCAGAGCAGG-3′	5739–5762	5′-*CCCTCAGGCTGGACTGCA-3′	6040–6023
K	5′-#CATCATTAAATGTGTTGCATCC-3′	7063–7084	5′-*CAAGCGATTATCATGTCTCAGC-3′	7766–7745
	#M13 sense tag: 5′-ACTGTAAAACGACGGCCAGT-3′			
	*M13 antisense tag: 5′-CAGGAAACAGCTATGACC-3′			

Position in relation to *IL6* reference sequence is shown.
[a]Position relates to an alternative chromosome 7 reference sequence (accession no. AC073072).
Sense (#) and antisense (*) M13-tag positions are indicated.

Biosystems). The following cycling conditions were used: 95°C for 1 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. Allelic discrimination analysis and genotype calls were made with the ABI 7000 Sequence Detection System (Applied Biosystems).

Genotyping for the *IL6* variable nucleotide tandem repeat

The *IL6* variable nucleotide tandem repeat (VNTR) was analysed using a previously described PCR protocol [20]. In brief, the oligonucleotide primers (5′-ACTTTTGAGTGTGTCACG-3′ and 5′-TGACGTGATGGATGCAACAC-3′) were used with following cycling parameters: 94°C for 60 s, 60°C for 60 s, 72°C for 60 s and a final 7 min extension at 72°C. The products were resolved on a 3% agarose (Invitrogen Inc., Carlsbad, California, USA) gel.

Bioinformatic analysis

The genomic sequence contig for *IL6* was identified from the NCBI (National Center for Biotechnology Information) database (*http://www.ncbi.nlm.nih.gov*). Known SNPs were identified from the NCBI SNP database (*http://www.ncbi.nlm.nih.gov/SNP/*).

Statistical analysis

Single single nucleotide polymorphism analysis

All SNPs were analysed individually for association using 2×2 (allele-based) Fisher's exact test. Analysis was implemented in StatsDirect v2.4.5 (StatsDirect Ltd, Sale, UK). Analysis of polymorphisms was undertaken only when genotype was determined for > 80% patients in the cohort. The overall failure rate for genotyping was 8.44%.

Haplotype analysis

Haplotype analysis of all 17 polymorphisms was undertaken using the Haploview 3.2 software (*www.broad.mit.edu/mpg/haploview/*) to calculate maximum likelihood haplotype frequency estimates. When multiallelic variation was included, the alleles were defined as either the major allele or nonmajor allele.

Correction for multiple testing

Correction for multiple testing was applied by undertaking 1000 permutations of subject status (ALT > 2 × ULN versus controls) in the individual SNP and haplotype associations initially highlighted as being statistically significant ($P < 0.05$). Analysis was performed using the permutation test function with the Haploview 3.2 software.

Association of allelic variation with clinical alanine aminotransferase data

Patient serum ALT levels were expressed in two formats, the maximum level observed and the area under curve (AUC) for ALT levels over a period of 15 weeks. Both data were plotted against allelic variants. Statistical significance between genotypes was determined by one-way analysis of variance, implemented in Stata (StataCorp., College Station, Texas, USA).

Results

Serum transaminase levels in tacrine-treated rats

Serum AST levels 6 h after tacrine administration increased in a dose-dependent manner. Saline-treated control animals demonstrated mean AST levels of 188.8 ± 31.8 IU/l. The highest, and only statistically significant (405 ± 164.5 IU/l; $P = 0.04$ compared with the control group) increase in AST levels in 6 h posttreatment animals was observed in the 40 mg/kg group. AST levels at 24 h after tacrine treatment also showed an increase in a dose-dependent manner, although the highest observed value in the 40 mg/kg group (281.4 ± 363.2 IU/l) was not statistically different from that in control-treated animals ($P = 0.4$). A decrease was observed in mean AST levels between the 6 and 24 h

Table 2 Rat hepatic genes significantly upregulated or downregulated at 6 h following 40 mg/kg tacrine administration

Accession no.	Description	HUGO	Fold change	*P* value	Volcano score	Gene Ontology no.[a]/biological process
Upregulated genes						
AI639465	Tripartite motif protein 63	trim63	5.95	0.0194	−1.33	GO:0016874: ligase activity
AI639358	Unknown	–	5.88	0.0421	−1.06	–
AF091563	Olfactory receptor gene Olr1687	Olr1687	5.15	0.0009	−2.17	GO:0050911: detection of chemical stimulus during sensory perception of smell
M22670	Alpha2-macroglobulin	A2m	5.14	0.0381	−1.01	GO:0006953: acute-phase response
M64698	Inositol 1,4,5-triphosphate receptor 1	Itpr1	5.07	0.0116	−1.36	GO:0006816: calcium ion transport
X16038	Alkaline phosphatase	Alp	4.91	0.0308	−1.04	GO:0004035: alkaline phosphatase activity
E00717	Cytochrome P450, family 1, subfamily a, polypeptide 1	Cyp1a1	3.88	0.0340	−0.87	GO:0018894: dibenzo-*p*-dioxin metabolic process
U24174	Cyclin-dependent kinase inhibitor 1A	Cdkn1a	3.81	0.0303	−0.88	GO:0007050: cell cycle arrest
AI639033	Predicted: Rattus norvegicus dickkopf homolog 4	Dkk4	3.78	0.0045	−1.36	GO:0030178: negative regulation of Wnt receptor signalling pathway
AI638951	Unknown	–	3.78	0.0478	−0.76	–
M23889	Rat T-cell receptor active β-chain C-region mRNA, partial cds, clone TRB4	–	3.54	0.0268	−0.86	–
D00833	Glycine receptor, α1 subunit	Glra1	3.38	0.0061	−1.17	GO:0006820: anion transport
S96418	Activin receptor IIA	Acvr2a	3.26	0.0161	−0.92	GO:0006468: protein amino acid phosphorylation
M23881	Rat T-cell receptor active β-chain C-region mRNA, partial cds, clone TRB4	–	3.23	0.0184	−0.88	–
M32397	Acid phosphatase, prostate	Acpp	3.20	0.0037	−1.23	GO:0000074: regulation of progression through cell cycle
U48592	Interleukin 1 receptor accessory protein	Il1rap	3.13	0.0035	−1.22	GO:0019221: cytokine and chemokine mediated signaling pathway
M23566	Alpha2-macroglobulin	A2m	3.04	0.0101	−0.96	GO:0006953: acute-phase response
AI639051	Unknown	–	2.97	0.0046	−1.11	–
U12623	Cyclic nucleotide gated channel α4	Cnga4	2.92	0.0159	−0.84	GO:0007600: sensory perception
AI045002	Acyl-CoA synthetase long-chain family member 4	Acsl4	2.77	0.0017	−1.22	GO:0006631: fatty acid metabolism
AA866443	Protein, serine, 35	Prss35	2.69	0.0287	−0.66	GO:0006508: proteolysis
AF003904	Corticotropin releasing hormone binding protein	Crhbp	2.64	0.0039	−1.02	GO:0042445: hormone metabolism
V01543	Neuron specific gene family member 1	Nsg1	2.63	0.0428	−0.58	GO:0007268: synaptic transmission
AF007758	Synuclein, α	Snca	2.61	0.0420	−0.57	GO:0001963: synaptic transmission, dopaminergic
AI639137	Unknown	–	2.52	0.0232	−0.66	–
AF061945	Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 3A	Grin3a	2.43	0.0161	−0.69	GO:0016358: dendrite development
AI639526	Seminal vesicle protein, secretion 2	Svs2	2.37	0.0062	−0.83	GO:0007620: copulation
U02315	Neuregulin 1	Nrg1	2.34	0.0227	−0.61	GO:0030154: cell differentiation
D16309	Cyclin D3	Ccnd3	2.31	0.0050	−0.84	GO:0000074: regulation of progression through cell cycle
AJ011608	DNA primase, p49 subunit	Prim1	2.28	0.0008	−1.11	GO:0006260: DNA replication
D84486	Unknown	–	2.28	0.0040	−0.86	–
S75275	Similar to DEAD-box protein 4 (VASA homolog), mRNA	–	2.24	0.0216	−0.58	–
Z12152	Neurofilament protein middle (NF-M).	–	2.23	0.0455	−0.47	–
AF029310	Transient receptor potential cation channel, subfamily V, member 1	Trpv1	2.22	0.0230	−0.57	GO:0006816: calcium ion transport
AI639317	Unknown	–	2.21	0.0052	−0.79	–
AI180145	Protein tyrosine phosphatase, nonreceptor type 1	Ptpn1	2.20	0.0123	−0.65	GO:0006470: protein amino acid dephosphorylation
D10763	Erythropoietin	Epo	2.16	0.0207	−0.56	GO:0001666: response to hypoxia
U82612t	Fibronectin 1	Fn1	2.13	0.0281	−0.51	GO:0006953: acute-phase response
AA893857	Unknown	–	2.12	0.0180	−0.57	–
U52034	Placentae and embryos oncofetal gene	Pem	2.10	0.0096	−0.65	–
AA892168	Unknown	–	2.07	0.0473	−0.42	–
AI180410	Prolactin-like protein C 1	Prlpc1	2.04	0.0119	−0.60	–
U36771	Glycerol-3-phosphate acyltransferase, mitochondrial	Gpam	2.04	0.0476	−0.41	GO:0006631: fatty acid metabolism
X56541	Membrane-spanning proteoglycan NG2	Cspg4	2.03	0.0225	−0.51	GO:0030154: cell differentiation
U31367	Myelin and lymphocyte protein, T-cell differentiation protein	Mal	2.03	0.0196	−0.52	GO:0030154: cell differentiation
U24489	Tenascin XA	Tnxa	2.00	0.0095	−0.61	GO:0007517: muscle development
Downregulated genes						
AI639161	Unknown	–	2.00	0.0428	−0.41	–
AA875165	Unknown	–	2.04	0.0078	−0.65	–
AI229291	Purine rich element binding protein A	–	2.06	0.0127	−0.60	–
K00996	Rat cytochrome p-450e (phenobarbital-induced) mRNA	Cyp2b2	2.12	0.0179	−0.57	GO:0017144: drug metabolism
AF025670	Caspase 6	Casp6	2.16	0.0052	−0.76	GO:0006915: apoptosis

Table 2 (continued)

Accession no.	Description	HUGO	Fold change	*P* value	Volcano score	Gene Ontology no.[a]/biological process
AI234828	Partial mRNA for immunoglobulin α heavy chain	–	2.16	0.0149	−0.61	–
AA892861	Similar to RIKEN cDNA 2610528J11 (LOC362576), mRNA	–	2.39	0.0024	−0.99	–
AB010119	t-complex testis expressed 1	Tctex1	2.46	0.0022	−1.04	GO:0007017: microtubule-based process
D82071	Prostaglandin D2 synthase 2	Ptgds2	2.50	0.0440		GO:0001516: prostaglandin biosynthesis
D45920	130 kDa-Ins(1,4,5)P3 binding protein	–	2.53	0.0061	−0.89	–
M80367	Guanylate binding protein 2, interferon-inducible	Gbp2	2.53	0.0247	−0.65	GO:0006955: immune response
AI177256	Unknown	–	2.53	0.0025	−1.05	–
D14014	Cyclin D1	Ccnd1	2.84	0.0105	−0.90	GO:0000074: regulation of progression through cell cycle
rAA874906	Kelch-like 24	Klhl24	3.21	0.0051	−1.16	–
AA859631	Unknown	–	3.42	0.0217	−0.89	–
U83880	Glycerol-3-phosphate dehydrogenase 2	Gpd2	3.83	0.0494	−0.76	GO:0006094: gluconeogenesis

[a]Gene Ontology annotation provided by the Gene Ontology Consortium [21] and identified from the Rat Genome Database [22].

groups treated with either 30 or 40 mg/kg tacrine. These differences, however, were not statistically significant.

Tacrine modulation of hepatic gene expression

Using a simplified volcano plot for data visualization [18], significant changes in the regulation of some genes were observed in all dose groups. As mild transaminase elevation, however, was observed only at 40 mg/kg, gene changes specific to the 40 mg/kg dose group were analysed. A total of 46 gene probe sets were significantly upregulated at 6 h in the 40 mg/kg tacrine-treated animals (Table 2) [21,22], with a further 16 significantly downregulated. In addition, a similar number of significantly upregulated (29) and downregulated (9) genes were observed at 24 h (Table 3) [21,22]. No common genes existed that were significantly dysregulated in the 6 and 24 h datasets.

The highest fold increase at 6 h was observed with a tripartite motif protein 63 (*trim63*) transcript (5.95-fold, $P = 0.019$). Other notable genes significantly upregulated at 6 h included alkaline phosphatase (4.91-fold, $P = 0.031$) and cytochrome P450 (*CYP*) *1A1* (3.88-fold, $P = 0.034$). In addition, an increase was observed in two probe sets homologous for α2-macroglobulin (A2M) at 6 h, which showed 5.14 and 3.04-fold changes, respectively. Downregulated genes at 6 h included glycerol-3-phosphate dehydrogenase 2 (*Gpd2*) (3.83-fold, $P = 0.049$), cyclin D1 (*Ccnd1*) (2.84-fold, $P = 0.011$), caspase 6 (*casp6*) (2.26-fold, $P = 0.005$), fibronectin (*Fn*) (2.13-fold, $P = 0.018$) and *CYP2B19* (2.12-fold, $P = 0.018$) (Table 2).

The gene expression profile at 24 h differed from that observed at 6 h (Table 3). The highest upregulation was seen with the haptoglobin (*Hp*) (6.59-fold, $P = 0.022$), which codes for an acute phase protein (APP). Other notable upregulated genes included *CYP1B1* (4.78-fold, $P = 0.013$), nuclear receptor *Nr0b2* (commonly known as SHP-1) (2.43, $P = 0.0003$) and aminolevulinic acid synthase 1 (2.26-fold, $P = 0.043$). In the animals administered 40 mg/kg of tacrine, upregulation of the genes coding for known APPs was observed at both 6 h (*A2M* and *Fn*) (Table 2) and 24 h (*Hp*) (Table 3).

IL-6 is known to be a key regulator of these hepatic APPs [23]. Interestingly, however, hepatic *IL6* gene (accession no. M26744) expression in the 40 mg/kg-treated animals was not significantly dysregulated at either 6 h (1.49-fold increase, $P = 0.93$) or 24 h (3.01-fold increase, $P = 0.96$).

Interleukin-6 polymorphisms in patients with tacrine-induced transaminitis

On the basis that IL-6 is a key regulator of the genes known to code for hepatic APPs, which were shown to be significantly dysregulated in tacrine-treated animals, we went onto genotype a tacrine-treated AD patient cohort for 16 known *IL6* polymorphisms (Table 4). Four of these showed an initial association with transaminitis in a case–control analysis. These included one functional promoter region polymorphism (−597 G-A) [$P = 0.025$, odds ratio (OR) = 0.41 (2.44 reciprocal), 95% confidence interval (CI) = 0.18–0.95], and three intronic SNPs, rs2069832 [$P = 0.008$, OR = 0.34 (2.94), 95% CI = 0.14–0.79], rs2069833 [$P = 0.008$, OR = 0.34 (2.94), 95% CI = 0.14–0.79] and rs2066992 ($P = 0.0168$, OR = 9.77, 95% CI = 1.25–436.25). With all four SNPs, however, the corrected *P* value exceeded 0.05 when correction for multiple testing was applied.

Further analysis showed that two variant alleles (rs2066992 and the *IL6* VNTR) were associated with

Table 3 Rat hepatic genes significantly up or downregulated at 24 h following 40 mg/kg tacrine administration

Accession no.	Description	HUGO	Fold change	*P* value	Volcano score	Gene Ontology no./biological process[a]
Upregulated genes						
K01933	Haptoglobin	Hp	6.59	0.0220	−1.36	GO:0006953: acute-phase response
X52772	Synaptotagmin 1	Syt1	5.77	0.0112	−1.49	GO:0017156: calcium ion-dependent exocytosis
AF030088	Homer homolog 1 (Drosophila)	Homer1	5.52	0.0010	−2.23	GO:0007216: metabotropic glutamate receptor signaling pathway
AI176856	Cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1	4.78	0.0127	−1.29	GO:0006725: aromatic compound metabolism
AA875609	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a2	–	4.52	0.0058	−1.46	–
X95466	CPG2 protein	CPG2	4.41	0.0206	−1.09	–
AI639437	Unknown	–	4.36	0.0101	−1.28	–
U57097	Aortic preferentially expressed gene 1	Apeg1	4.10	0.0098	−1.23	GO:0042692: muscle cell differentiation
M27151	Myogenic factor 6	Myf6	3.96	0.0146	−1.10	GO:0030154: cell differentiation
U39571	Phosphatidylinositol 4-kinase	–	3.64	0.0434	−0.76	–
M22924	Rat IgE Fc receptor deleted β-subunit mRNA	–	3.62	0.0497	−0.73	–
AI638957	Unknown	–	3.42	0.0130	−1.01	–
AA799997	Similar to homoloc-13 (LOC360984), mRNA	–	3.39	0.0098	−1.06	–
X07314	Rat heart myosin light chain 2	Mlc2	3.33	0.0477	−0.69	–
AJ131848	Procollagen, type X, α1	Col10a1	3.29	0.0280	−0.80	GO:0051216: cartilage development
AA799729	Phosphodiesterase 4B	Pde4b	3.24	0.0024	−1.34	–
M63006	Adenylate cyclase activating polypeptide 1	Adcyap1	2.93	0.0181	−0.81	GO:0045786: negative regulation of progression through cell cycle
D84486	PMSG-induced ovarian mRNA	–	2.80	0.02574	−0.71	–
AF000901	Nucleoporin-like 1	Nupl1	2.78	0.0005	−1.48	GO:0006913: nucleocytoplasmic transport
D26499	Dynein-like protein 8	Dlp8	2.66	0.01601	−0.76	GO:0006508: proteolysis
AJ010351	CUG triplet repeat, RNA-binding protein 2	Cugbp2	2.59	0.03067	−0.63	GO:0006376: mRNA splice site selection
J03093	Odorant-binding protein 1F	Obp1F	2.77	0.0047	−1.03	–
AB019393	Myocilin	Myoc	2.53	0.0100	−0.81	GO:0009653: morphogenesis
D86580	Nuclear receptor subfamily 0, group B, member 2	Nr0b2	2.43	0.0003	−1.35	GO:0008203: cholesterol metabolism
AA875468	Putative jumanji gene	–	2.32	0.0384	−0.52	–
AA799666	Unknown	–	2.26	0.0325	−0.53	–
J03190	Aminolevulinic acid synthase 1	Alas1	2.26	0.0425	−0.49	GO:0006783: heme biosynthesis
AJ006295	Unknown	–	2.25	0.0002	−1.28	–
U69882	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	Kcnn2	2.14	0.04254	−0.45	GO:0006813: potassium ion transport
Downregulated genes						
X56326	Epsilon 1 globin	Hbe 1	2	0.0177	−0.53	GO:0015671: oxygen transport
AA894264	Similar to kIAA0367 (LOC293823), mRNA	–	2.12	0.0417	−0.45	–
AA800679	Guanine nucleotide binding protein-like 3 (nucleolar)	Gnl3	2.26	0.0043	−0.84	GO:0000074: regulation of progression through cell cycle
AF020758	Purinergic receptor P2X, ligand-gated ion channel, 2	P2rx2	2.39	0.0116	−0.73	GO:0007268: synaptic transmission
AI012942	Cell division cycle 25 homolog B	Cdc25b	2.40	0.0112	−0.74	GO:0007049: cell cycle
U08344	ATPase, Cu^{2+} transporting, β polypeptide	Atp7b	2.54	0.0263	−0.64	GO:0048511: rhythmic process
L19180	Protein tyrosine phosphatase, receptor type, D	Ptprd	2.65	0.0013	−1.23	GO:0006470: protein amino acid dephosphorylation
AF036959	Homeodomain interacting protein kinase 3	Hipk3	3.15	0.0004	−1.69	GO:0006468: protein amino acid phosphorylation
AI175764	Stearoyl-coenzyme A desaturase 1	Scd1	3.39	0.0137	−0.99	GO:0006633: fatty acid biosynthesis

[a]Gene Ontology annotation provided by the Gene Ontology Consortium [21] and identified from the Rat Genome Database [22].

Table 4 Association of IL6 allelic variants with tacrine-induced transaminitis

Accession no.	Alternative name	Region	Position (AF372214)	Major allele	Allelic frequency: Positive (ALT > 2 × ULN)	Allelic frequency: Negative (ALT < 2 × ULN)	χ^2	*P*-value	OR (95% CI)
rs2002792	–	5′ Upstream	8262[c]	G	35:31	33:25	0.186	0.720	0.86[1.16] (0.40–1.84)
rs940367	–	5′ Upstream	8842[c]	G	60:4	61:1	1.777	0.365	0.25[4.00] (0.01–2.60)
rs2069824	–	5′ Upstream	95	T	67:3	53:5	1.017	0.467	2.10 (0.39–14.10)
rs1800797	**–597**	**Promoter**	**1086**	**G**	**38:28**	**46:14**	**5.155**	**0.025**	**0.41[2.44] (0.18–0.95)**
rs1800796	–572	Promoter	1111	G	63:3	51:9	3.986	0.067	3.71 (0.86–22.16)
rs1800795	–174	Promoter	1509	G	48:22	49:13	1.847	0.236	0.58[1.72] (0.24–1.37)
rs2069832	**–**	**Intron 1**	**2298**	**G**	**39:29**	**48:12**	**7.509**	**0.008**	**0.34[2.94] (0.14–0.79)**
rs2069833	**–**	**Intron 1**	**2529**	**T**	**39:29**	**48:12**	**7.509**	**0.008**	**0.34[2.94] (0.14–0.79)**
rs2069835	–	Intron 1	2736	T	47:5	52:4	0.216	0.356	0.72[1.39] (0.11–2.20)
rs2069837	–	Intron 1	2892	A	58:6	58:2	1.873	0.275	0.33[3.03] (0.03–1.98)
rs2066992	**–**	**Intron 1**	**3114**	**G**	**51:1**	**47:9**	**6.424**	**0.0168**	**9.77 (1.25–436.25)**
rs1548216	–	Intron 2	3437	G	63:3	54:4	0.32	0.705	1.56 (0..25–11.05)
rs2069845	–	Intron 3	5014	A	37:27	44:16	3.293	0.090	0.50[2.00] (0.22–1.13)
rs2069860	D162V	Exon 4	5903	A	67:1	58:0	0.86	> 0.9999	n/a
rs13306435	D162E	Exon 4	5904	T	66:0	57:1	1.147	0.4677	n/a
VNTR[a]	**VNTR**	**3′ Downstream**	**6483–7092[b]**	**D**	**50:24:0**	**32:31:1**	**5.14**	**0.045**	**n/a**
rs1818879	–	3′ Downstream	7592	G	42:24	35:25	0.372	0.586	1.25 (0.57–2.73)

Three individuals were excluded due to insufficient genotype data. Statistically significant associations are highlighted in bold characters.
ALT, alanine aminotransferase; ULN, upper limit of normal; VNTR, variable nucleotide tandem repeat.
[a]VNTR allele is polyallelic (one negative individual possessed an A allele) so association analysed by 3 × 2 cross-tabulation.
[b]Position of 5′ nucleotide of primers.
[c]Position relates to an alternative chromosome 7 reference sequence (accession no. AC073072).

both maximum ALT and AUC of ALT (15 weeks). The maximum ALT values and the AUC for ALT (at 15 weeks) in patients carrying at least one 'D-allele' of the *IL6* VNTR polymorphism (Fig. 2) was significantly higher than for noncarriers ($P = 0.042$ and $P = 0.050$, respectively). Additionally, patients homozygous for the G allele for the rs2066992 SNP had greater ALT elevation in response to tacrine when compared with heterozygotes (GT) (Fig. 3). This was observed with both maximum ALT ($P = 0.019$) and AUC ($P = 0.009$) values.

Interleukin-6 haplotypes in patients with tacrine-induced transaminitis

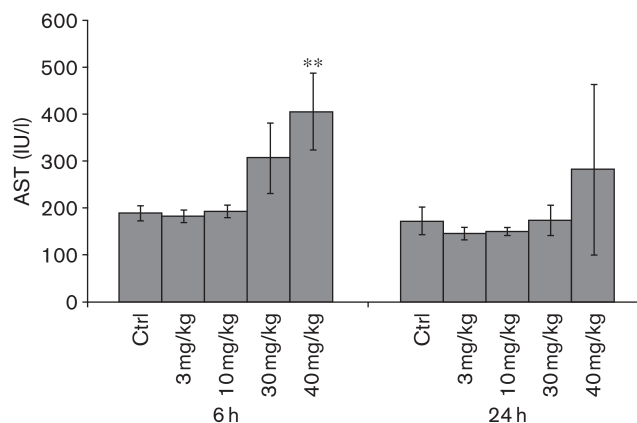
Multilocus analysis of all 16 *IL6* SNPs and the VNTR yielded 10 haplotypes with predicted frequencies > 2% in the tacrine-treated cohort (Fig. 4). One haplotype (haplotype 5) demonstrated an association with elevated serum transaminase levels. A haplotype frequency of 0.1 was observed in patients with ALT values > 2 × ULN, whereas it was absent in patients with ALT less than 2 × ULN ($P = 0.0093$, $P_{corrected} = 0.049$). Notably, this haplotype includes the –597A, –572G, –174G and VNTR-D alleles.

Discussion

Liver injury associated with tacrine is characterized by a mild elevation in transaminase levels, with occasional cases of more clinically frank liver damage [3,4,24]. Interestingly, earlier in-vivo rodent studies have also demonstrated an elevation of transaminases after single doses of 35–40 mg/kg tacrine [5,6]. This was confirmed in our study which showed a significant elevation of AST levels in rats administered 40 mg/kg tacrine (Fig. 1); the level at 6 h was 2.3 times higher than that observed in vehicle-treated animals. On the basis of body surface area, 40 mg/kg administered to a rat is equivalent to 240 mg/m^2. The maximum daily clinical dose is 160 mg or 98.79 mg/m^2 (i.e. 2.43-fold lower than the maximum rat dose given in this study). Although the elevation in transaminase levels in the rat is in accordance with tacrine transaminitis in man, the clear difference is that tacrine is used chronically in patients with AD, but was given as a single dose in this study. Nevertheless, liver enzyme elevation has also been observed when tacrine has been administered chronically at equivalent doses to rodents [7]. We therefore went onto analyse gene transcript changes in the livers of animals administered 40 mg/kg of tacrine.

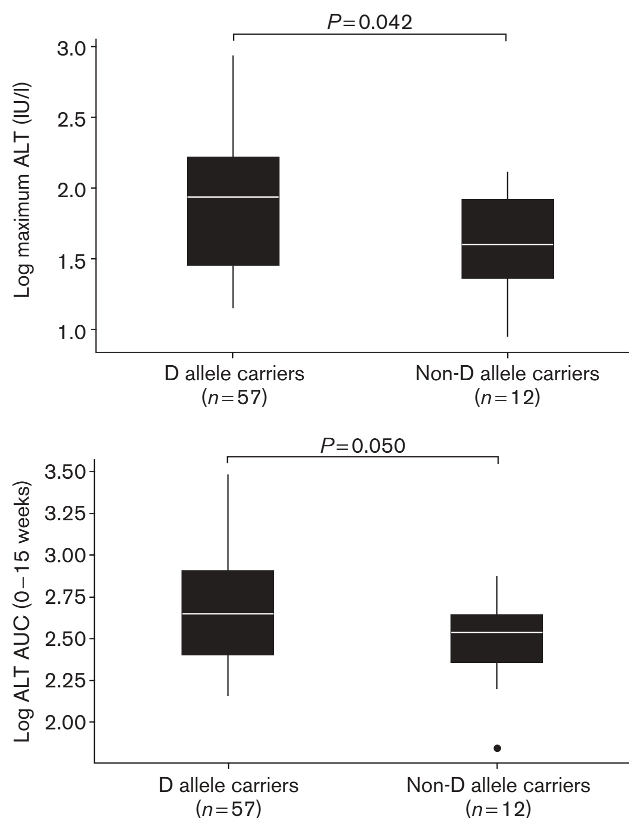
In analysing the gene expression changes, of particular interest to us were the coordinated changes that occurred in the genes encoding the well-characterized APPs, A2M, haptoglobin and fibronectin. These transcripts were significantly upregulated at 6 and 24 h in livers taken from animals administered 40 mg/kg tacrine (Tables 2 and 3). APPs fall into two major subtypes [25]. IL-6-like cytokines are the primary activators of type-2 APPs and initiate a synergistic response with IL1-type cytokines [25]. In the rat, A2M is commonly recognized as a type-2 APP while haptoglobin is a type-1 protein [26]. IL-6 plays a direct role in A2M transcriptional regulation in the rat [27], whereas stimulation of haptoglobin transcription has been shown to be both IL-6 and IL-1-dependent. Fibronectin is also defined as an APP; studies [28] have suggested that IL-6 which is produced during the acute

Fig. 1



Serum levels of transaminases in Sprague–Dawley rats administered tacrine. Aspartate aminotransferase levels are shown in animals receiving 0, 3, 30 and 40 mg/kg tacrine at 6 and 24 h postadministration are shown. Data represent mean ± SD ($n \geq 4$/group). $^{**}P<0.01$ compared with the equivalent saline-treated control group as determined by one-way ANOVA. ANOVA, analysis of variance; AST, aspartate aminotransferase.

Fig. 2



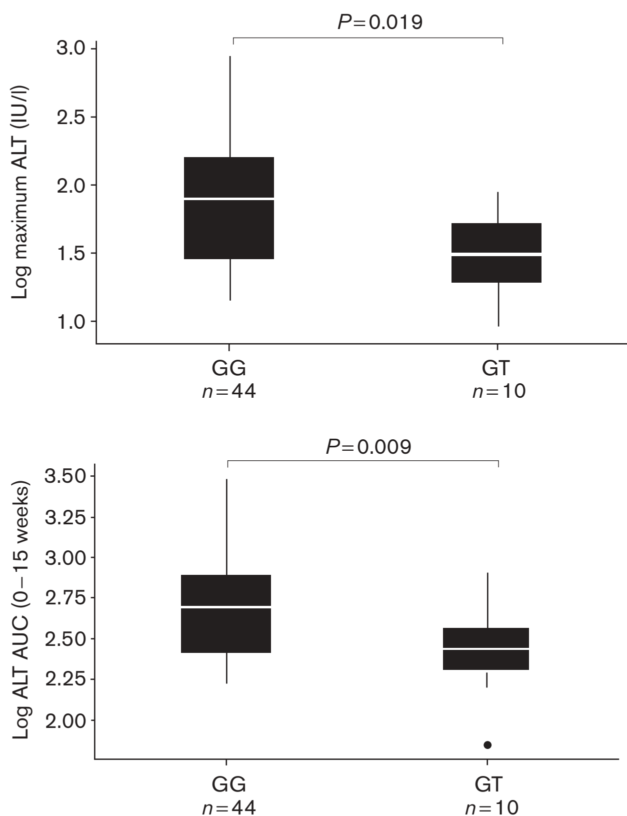
Association of *IL6* VNTR 'D-Allele' carriers with elevated ALT levels in AD patients receiving tacrine. (a) Maximum serum ALT levels in D-allele carriers and noncarriers. (b) AUC analysis of ALT levels monitored over a 15-week period. Mean value is indicated by the white line. Outlying data points are shown as black dots. Statistical significance was determined by one-way analysis of variance (ANOVA). AD, Alzheimer's disease; ALT, alanine aminotransferase; AUC, area under curve; VNTR, variable nucleotide tandem repeat.

phase induces an increase in fibronectin mRNA levels in liver fat-storing cells. Interestingly, however, we were unable to show a change in *IL6* transcript levels in our animal model. A possible explanation for this may be that any change in *IL6* transcript levels occurred outside the time window studied. For instance, in a mouse model of acetaminophen hepatotoxicity [29], *IL6* mRNA was elevated within 4 h but maximal serum ALT was not observed until 8 h suggesting that IL-6 expression may precede ALT elevation. In our tacrine-treated model, maximum ALT was observed at 6 h, and it is therefore possible that IL-6 expression may have returned to basal levels. An alternative explanation is that any changes in IL-6 levels were posttranscriptional and therefore not observed by microarray analysis. Indeed, in a hepatectomized rat model, hepatic *A2M* mRNA and protein elevation was detected at 12 h. Only a moderate increase in levels of hepatic *IL6* mRNA levels, however, were observed [30].

An option here would have been to measure IL-6 levels in rat serum. Our initial experiments, however, have suggested that commercially available rat IL-6 assays are not sensitive enough to detect small changes in IL-6 levels. Furthermore, Western blot analysis of liver protein expression has shown a trend towards an increase in IL-6 levels (data not shown), but again the usefulness of these data are limited by the sensitivity and specificity of the antirat antibodies available. The need for a more sensitive assay can perhaps be predicted from the rat hepatectomy model. In this model, severe impairment of rat liver function by 66% hepatectomy generated a small change in IL-6 mRNA levels and a maximal serum IL-6 of less than 200 pg/ml [31]. By contrast, our model leads to a maximal AST of 405 IU/l, which is mild by comparison. The ideal would have been to measure IL-6 levels in the AD patients at the time of their episode of transaminitis as a super-sensitive IL-6 assay is available, but we did not have access to any serum.

Given that (a) IL-6 is directly involved in the transcriptional control of a number of APPs [23], which were elevated in tacrine-treated animals (as discussed above), and (b) it has been identified as a key molecule in the response of the liver to injury [29,32,33], we hypothesized that the *IL6* gene was a plausible candidate for determining susceptibility to tacrine transaminitis. Our data suggest an association between tacrine transaminitis (ALT $> 2 \times$ ULN) and the possession of the *IL6* −597-A allele. This promoter polymorphism has previously been shown to alter *IL6* transcription [34]. Two other promoter region polymorphisms, however, also associated with *IL6*

Fig. 3



Association of *IL6* SNP, rs20266992, genotype with elevated transaminase levels in AD patients receiving tacrine. (a) Maximum serum ALT levels in D-allele carriers and noncarriers. (b) AUC representative of ALT levels monitored over a 15-week period. Mean value is indicated by the white line. Outlying data points are shown as black dots. Statistical significance was determined by one-way analysis of variance (ANOVA). AD, Alzheimer's disease; ALT, alanine aminotransferase; AUC, area under curve; SNP, single nucleotide polymorphism.

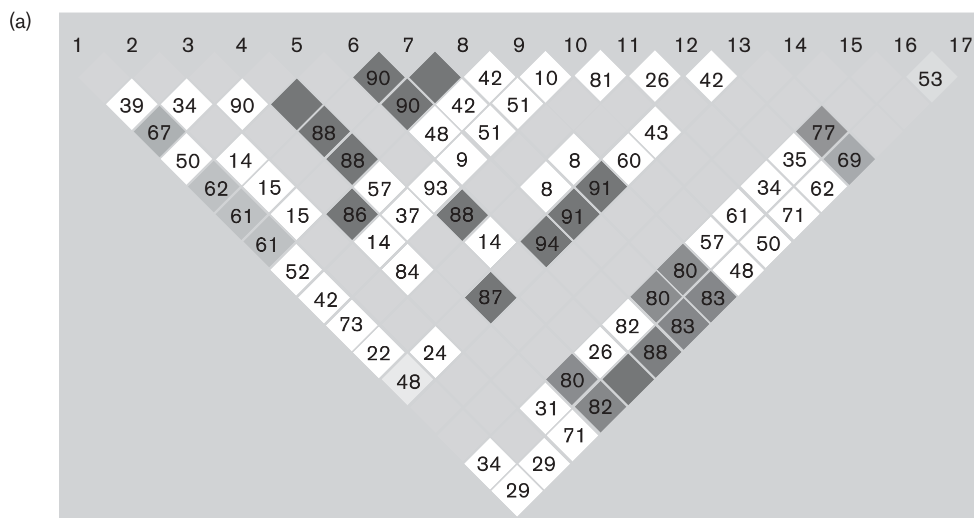
transcriptional regulation, −572G-C and −174G-C [34], did not show an association with tacrine transaminitis in our patients. Analysis of the 3′ VNTR in relation to the maximal ALT and AUC data suggest that the D-allele is a risk factor for tacrine transaminitis (Fig. 2). This was also observed with case–control analysis. The *IL6* VNTR D-allele is known to affect IL-6 serum levels [35].

An obvious limitation of our study is the small numbers and the large numbers of SNPs investigated raising the possibility of false-positive associations. Indeed, although four SNPs (rs1800797, rs2069832, rs2069833 and rs2066992) showed an association with transaminitis in the case–control analysis, the *P* value corrected for multiple testing was no longer significant. The same was true for the VNTR genotype. We, however, also undertook a quantitative analysis of the ALT values (both maximum and AUC over 15 weeks) for all SNPs as well as the VNTR. Of these, two allelic variants (rs2066992 and VNTR) demonstrated a significant association with both maximum ALT and AUC over 15 weeks (Figs 2 and 3, respectively). The likely role of *IL6* in predisposing to tacrine transaminitis was further emphasized by haplotype analysis, which showed an association ($P = 0.0093$, $P_{corrected} = 0.049$) with one *IL6* haplotype that possessed the −597A, −572G, −174G and the VNTR-D alleles. This haplotype was absent in patients with ALT less than $2 \times$ ULN in response to tacrine but found at a predicted frequency of 10.3% in patients with ALT $> 2 \times$ ULN. Interestingly, the presence of this specific combination of variants as part of a haplotype in *IL6* has a negative effect on *IL6* transcription [34]. This contrasts with the effect of the VNTR D-allele in isolation which has been shown to increase serum IL-6 levels in AD patients, but not in healthy volunteers [35]. It is likely that in a complex in-vivo situation the functional effect of the haplotype (Fig. 4) will overwhelm that of an individual allele. This is consistent with genetic studies of complex diseases [36] in which haplotype analysis is more likely to show an association with a modest genetic effect than individual polymorphisms.

Although we cannot exclude the possibility that the association with *IL6* is due to linkage disequilibrium with a neighbouring gene on chromosome 7, given the functional effect of the associated haplotype, we feel this may represent a causal association. Our finding is also biologically plausible given the known role of IL-6 in hepatic injury. For example, a number of studies have shown that IL-6 may have a protective role in hepatic injury mediated by a range of hepatotoxins including carbon tetrachloride [37], ethanol [32] and paracetamol [33]. The role of IL-6, however, varies with time: thus while short-term IL-6 exposure in the liver is protective, longer-term exposure can actually sensitize the liver to injury [38]. This is also consistent with the fact that IL-6 has previously been shown to be antiapoptotic in different acute liver injury models [39,40], but paradoxically, is proapoptotic in longer-term IL-6 exposure models [38]. To this end, it is interesting to note that apoptosis has been implicated in the mechanism of tacrine hepatotoxicity in both in-vivo [7] and in-vitro [41] studies. Taken together, our finding of the association of tacrine transaminitis with a functional haplotype that reduces IL-6 activity is consistent with the role of IL-6 in liver injury. Delineation of the exact mechanism of any protective effect with respect to tacrine will require further investigation. Ideally, we need to confirm this finding in an independent replication cohort; however, given that tacrine is no longer used in clinical practice, this will not be possible.

It is also important to consider the role IL-6 may have on the expression of drug metabolizing enzymes. It has

Fig. 4



(b)

Haplotype	Block	Frequency	Positive (ALT>2×ULN)	Negative (ALT<2×ULN)	χ^2	*P*-value	*P* corrected-value
1	GGTGGGGTTAGGAATGC	0.173	0.115	0.238	3.437	0.0638	0.365
2	CGTAGCACTAGGGATGD	0.166	0.188	0.142	0.501	0.4791	>1.0
3	GGTGGGGTTAGGAATAD	0.162	0.158	0.166	0.015	0.9029	>1.0
4	CGTAGGACTAGGGATGD	0.054	0.103	0.000	6.755	0.0093	0.049
5	CGTGCGGTTATGAATAC	0.038	0.015	0.064	2.074	0.1498	0.716
6	CGTGGGGTTAGGAATAD	0.031	0.046	0.016	0.938	0.3328	0.999
7	GCTGGGGTTAGGAATAD	0.031	0.044	0.016	0.854	0.3554	>1.0
8	GGCGGGGTCAGGAATGC	0.031	0.044	0.016	0.866	0.3521	>1.0
9	CGTGCGGTTATGAATGC	0.027	0.019	0.036	0.331	0.5648	>1.0
10	GGTGGGGTTGGGAATGC	0.022	0.025	0.020	0.045	0.8318	>1.0
11–15		0.078			n/a	>0.1	

Association of the *IL6* haplotype with tacrine-induced transaminitis. (a) represents pattern of LD, as represented in Haploview, for the 17 *IL6* polymorphisms analysed in the tacrine-treated AD cohort. White squares: D′ < 1 and logorithm of odds (LOD) < 2; Grey squares: D′ < 1 and LOD ≥ 2 and dark grey squares: D′ = 1, LOD ≥ 2. (b) represents case/control analysis of estimated haplotype frequencies. The VNTR (marker 17) is represented as biallelic with D representing a "D-allele" and C a "non-D-allele". Bold text indicates a statistically significant haplotype association (*P* corrected < 0.05).

previously been shown in a murine model that *CYP1A2* mRNA levels decrease in turpentine-treated animals in an IL-6-dependent manner [42]. CYP1A2 is responsible for oxidative metabolism of tacrine to its stable hydroxyl-tacrine metabolites [43]. No significant changes in *CYP1A2* expression, however, were observed in our model. Altered expression of other CYPs (*1A1, 2B2, 1B1*) was observed, though their role in tacrine metabolism is not defined.

A number of notable gene expression changes were observed but not investigated further in this study. Genes related to cell-cycle regulation (notably cyclins D1 and D3), apoptosis (caspase 6) and fatty acid metabolism (*Nr0b2*) could all potentially play a role in hepatotoxicity, but clearly some of these gene changes may also be related to liver regeneration which naturally occurs following injury. Alkaline phosphatase, a common marker of cholestatic liver injury was also upregulated. Given that tacrine toxicity, however, is characterized by transaminase elevation rather than cholestasis, the significance of this change is unclear.

In conclusion, the association between *IL6* allelic variants and transaminitis in tacrine-treated AD patients may be representative of a functional phenotype. We used a

rodent hepatic gene expression model to provide an unbiased approach to identifying potential pathways that may be important in predisposing to tacrine transaminitis. Although microarray technology is now widely used as a part of preclinical toxicology testing in the pharmaceutical industry, the relevance of the identified transcripts to the human situation is often a matter of conjecture. The novelty of our approach has been that we have been able to bridge between preclinical gene expression changes in a rodent model and functionally relevant SNPs and haplotype in a candidate gene, *IL6*. Our finding is biologically plausible given the known role of IL-6 in liver injury and protection. Our findings suggest that the role of acute dosing rodent models in identifying candidate genes associated with drug-induced liver injury in man deserves further study.

Acknowledgement

This study was funded entirely by AstraZeneca Pharmaceuticals UK Ltd.

References

1 Farlow M, Gracon SI, Hershey LA, Lewis KW, Sadowsky CH, Dolan-Ureno J. A controlled trial of tacrine in Alzheimer's disease. The Tacrine Study Group. *JAMA* 1992; **268**:2523–2529.

2 Gamzu ER, Thal LJ, Davis KL. Therapeutic trials using tacrine and other cholinesterase inhibitors. *Adv Neurol* 1990; **51**:241–245.

3 Watkins PB, Zimmerman HJ, Knapp MJ, Gracon SI, Lewis KW. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *JAMA* 1994; **271**:992–998.

4 Blackard WG Jr, Sood GK, Crowe DR, Fallon MB. Tacrine. A cause of fatal hepatotoxicity? *J Clin Gastroenterol* 1998; **26**:57–59.

5 Ma XC, Xin J, Wang HX, Zhang T, Tu ZH. Acute effects of huperzine A and tacrine on rat liver. *Acta Pharmacol Sin* 2003; **24**:247–250.

6 Stachlewitz RF, Arteel GE, Raleigh JA, Connor HD, Mason RP, Thurman RG. Development and characterization of a new model of tacrine-induced hepatotoxicity: role of the sympathetic nervous system and hypoxia-reoxygenation. *J Pharmacol Exp Ther* 1997; **282**:1591–1599.

7 Mansouri A, Haouzi D, Descatoire V, Demeilliers C, Sutton A, Vadrot N, *et al.* Tacrine inhibits topoisomerases and DNA synthesis to cause mitochondrial DNA depletion and apoptosis in mouse liver. *Hepatology* 2003; **38**: 715–725.

8 Jessen BA, Mullins JS, De Peyster A, Stevens GJ. Assessment of hepatocytes and liver slices as in vitro test systems to predict in vivo gene expression. *Toxicol Sci* 2003; **75**:208–222.

9 Berson A, Renault S, Letteron P, Robin MA, Fromenty B, Fau D, *et al.* Uncoupling of rat and human mitochondria: a possible explanation for tacrine-induced liver dysfunction. *Gastroenterology* 1996; **110**:1878–1890.

10 Madden S, Spaldin V, Hayes RN, Woolf TF, Pool WF, Park BK. Species variation in the bioactivation of tacrine by hepatic microsomes. *Xenobiotica* 1995; **25**:103–116.

11 Woolf TF, Pool WF, Bjorge SM, Chang T, Goel OP, Purchase CF 2nd, *et al.* Bioactivation and irreversible binding of the cognition activator tacrine using human and rat liver microsomal preparations. Species difference. *Drug Metab Dispos* 1993; **21**:874–882.

12 Larrey D, Pageaux GP. Genetic predisposition to drug-induced hepatotoxicity. *J Hepatol* 1997; **26** (**Suppl 2**):12–21.

13 Simon T, Becquemont L, Mary-Krause M, de Waziers I, Beaune P, Funck-Brentano C, *et al.* Combined glutathione-S-transferase M1 and T1 genetic polymorphism and tacrine hepatotoxicity. *Clin Pharmacol Ther* 2000; **67**:432–437.

14 De Sousa M, Pirmohamed M, Kitteringham NR, Woolf T, Park BK. No association between tacrine transaminitis and the glutathione transferase theta genotype in patients with Alzheimer's disease. *Pharmacogenetics* 1998; **8**:353–355.

15 Green VJ, Pirmohamed M, Kitteringham NR, Knapp MJ, Park BK. Glutathione S-transferase mu genotype (GSTM1*0) in Alzheimer's patients with tacrine transaminitis. *Br J Clin Pharmacol* 1995; **39**:411–415.

16 Affymetrix. MAS5, Statistical algorithms description document (available at *http://www.affymetrix.com*). 2002.

17 Hultin-Rosenberg L, Jagannathan S, Nilsson KC, Matis SA, Sjogren N, Huby RD, *et al.* Predictive models of hepatotoxicity using gene expression data from primary rat hepatocytes. *Xenobiotica* 2006; **36**:1122–1139.

18 Chen JJ, Wang SJ, Tsai CA, Lin CJ. Selection of differentially expressed genes in microarray data analysis. *Pharmacogenom J* 2007; **7**:212–220.

19 McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984; **34**: 939–944.

20 Bowcock AM, Ray A, Erlich H, Sehgal PB. Rapid detection and sequencing of alleles in the 3′ flanking region of the interleukin-6 gene. *Nucleic Acids Res* 1989; **17**:6855–6864.

21 Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000;**25**:25–29.

22 De la Cruz N, Bromberg S, Pasko D, Shimoyama M, Twigger S, Chen J, *et al.* The rat genome database (RGD): developments towards a phenome database. *Nucleic Acids Res* 2005; **33**:D485–D491.

23 Ramadori G, Christ B. Cytokines and the hepatic acute-phase response. *Semin Liver Dis* 1999; **19**:141–155.

24 Ames DJ, Bhathal PS, Davies BM, Fraser JR. Hepatotoxicity of tetrahydroaminoacridine. *Lancet* 1988; **1**:887.

25 Baumann H, Gauldie J. The acute phase response. *Immunol Today* 1994; **15**:74–80.

26 Moshage H. Cytokines and the hepatic acute phase response. *J Pathol* 1997; **181**:257–266.

27 Hattori M, Abraham LJ, Northemann W, Fey GH. Acute-phase reaction induces a specific complex between hepatic nuclear proteins and the interleukin 6 response element of the rat alpha 2-macroglobulin gene. *Proc Natl Acad Sci U S A* 1990; **87**:2364–2368.

28 Greenwel P, Iraburu MJ, Reyes-Romero M, Meraz-Cruz N, Casado E, Solis-Herruzo JA, *et al.* Induction of an acute phase response in rats stimulates the expression of alpha 1(I) procollagen messenger ribonucleic acid in their livers. Possible role of interleukin-6. *Lab Invest* 1995; **72**:83–91.

29 Masubuchi Y, Bourdi M, Reilly TP, Graf ML, George JW, Pohl LR. Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease. *Biochem Biophys Res Commun* 2003; **304**:207–212.

30 Scotte M, Masson S, Lyoumi S, Hiron M, Teniere P, Lebreton JP, *et al.* Cytokine gene expression in liver following minor or major hepatectomy in rat. *Cytokine* 1997; **9**:859–867.

31 Fulop AK, Pocsik E, Brozik M, Karabelyos C, Kiss A, Novak I, *et al.* Hepatic regeneration induces transient acute phase reaction: systemic elevation of acute phase reactants and soluble cytokine receptors. *Cell Biol Int* 2001; **25**:585–592.

32 El-Assal O, Hong F, Kim WH, Radaeva S, Gao B. IL-6-deficient mice are susceptible to ethanol-induced hepatic steatosis: IL-6 protects against ethanol-induced oxidative stress and mitochondrial permeability transition in the liver. *Cell Mol Immunol* 2004; **1**:205–211.

33 James LP, Lamps LW, McCullough S, Hinson JA. Interleukin 6 and hepatocyte regeneration in acetaminophen toxicity in the mouse. *Biochem Biophys Res Commun* 2003; **309**:857–863.

34 Terry CF, Loukaci V, Green FR. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. *J Biol Chem* 2000; **275**:18138–18144.

35 Licastro F, Grimaldi LM, Bonafe M, Martina C, Olivieri F, Cavallone L, *et al.* Interleukin-6 gene alleles affect the risk of Alzheimer's disease and levels of the cytokine in blood and brain. *Neurobiol Aging* 2003; **24**:921–926.

36 Tavtigian SV, Simard J, Teng DH, Abtin V, Baumgard M, Beck A, *et al.* A candidate prostate cancer susceptibility gene at chromosome 17p. *Nat Genet* 2001; **27**:172–180.

37 Kovalovich K, DeAngelis RA, Li W, Furth EE, Ciliberto G, Taub R. Increased toxin-induced liver injury and fibrosis in interleukin-6-deficient mice. *Hepatology* 2000; **31**:149–159.

38 Jin X, Zimmers TA, Perez EA, Pierce RH, Zhang Z, Koniaris LG. Paradoxical effects of short- and long-term interleukin-6 exposure on liver injury and repair. *Hepatology* 2006; **43**:474–484.

39 Hong F, Kim WH, Tian Z, Jaruga B, Ishac E, Shen X, *et al.* Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-x(L) proteins. *Oncogene* 2002; **21**:32–43.

40 Kovalovich K, Li W, DeAngelis R, Greenbaum LE, Ciliberto G, Taub R. Interleukin-6 protects against Fas-mediated death by establishing a critical level of anti-apoptotic hepatic proteins FLIP, Bcl-2, and Bcl-xL. *J Biol Chem* 2001; **276**:26605–26613.

41 Fariss MW, Mumaw VR, Walton LP. Tetrahydroaminoacridine-induced Apoptosis in rat hepatocytes. *Toxicol In Vitro* 1996; **10**:383–393.

42 Siewert E, Bort R, Kluge R, Heinrich PC, Castell J, Jover R. Hepatic cytochrome P450 down-regulation during aseptic inflammation in the mouse is interleukin 6 dependent. *Hepatology* 2000; **32**:49–55.

43 Spaldin V, Madden S, Adams DA, Edwards RJ, Davies DS, Park BK. Determination of human hepatic cytochrome P4501A2 activity in vitro use of tacrine as an isoenzyme-specific probe. *Drug Metab Dispos* 1995; **23**:929–934.