Effects of two functionally important *SLCO1B1* gene polymorphisms on pharmacokinetics of atorvastatin

Tausif Ahmed Rajput[1], Abdul Khaliq Naveed[2], Zia-Ur-Rehman Farooqi[3] and Shakir Khan[4]

[1]Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad, Pakistan
[2]Biochemistry, Islamic International Medical College-TRUST, Riphah International University, Islamabad, Pakistan
[3]Department of Medical Technology, Shifa Tameer-e-Millat University, Islamabad, Pakistan
[4]Department of Biochemistry, Margalla Institute of Health Sciences, Rawalpindi, Pakistan

Abstract: Organic anion transporter polypeptide 1B1 (OATP1B1) encoded by (*SLCO1B1*) gene, an uptake transporter involved in the transport of drugs and endogenous compounds and located in hepatocyte sinusoidal membrane. Objective of study was to investigate the effects of two functionally significant SNPs (388A>G and 521T>C) and their respective genotypes of *SLCO1B1* gene encoding OATP1B1 on the pharmacokinetics of atorvastatin. A total of 100 subjects divided into 6 groups as per their genotype profile were recruited. A single dose of 80mg atorvastatin was orally administered and plasma concentration measured up to 48 hours. The 388A>G and 521T>C genotypes were significantly associated with each other when compared for AUC and C_{max} but exhibited no significant variations in T_{max} and $t_{1/2}$. 521 SNP is rather more strongly associated with altered pharmacokinetics of atorvastatin when compared with the 388 SNP, though the homozygous bi-allelic variant of 388 SNP also exhibited a fairly significant variation along with homozygous bi-allelic variant of 521 SNP. The inter-individual variation in pharmacokinetics can be explained by *SLCO1B1* polymorphism.

Keywords: OATP1B1, Pharmacokinetics, Single nucleotide polymorphism, *SLCO1B1*.

INTRODUCTION

A large number of studies have demonstrated and anonymously rated statins as the best lipid lowering agents because of their tested pivotal role in the prevention of cardiovascular diseases irrespective of their fundamental factors such as age, gender or baseline lipid profile (Strandberg *et al.*, 2001, Strandberg *et al.*, 2008). Statins exert their action by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are widely used in the treatment and prevention of hypercholesterolemia therefore decreasing the risk of cardiovascular morbidity and mortality. Atorvastatin is one of the most widely used statins in the treatment and prevention of hypercholesterolemia and is very well absorbed following its oral administration. It undergoes first pass effect metabolism parting its oral bioavailability to 15% (Lennernas, 2003) and exhibits a half-life of 14 hours. The acid form of atorvastatin undergoes CoA dependent/acyl glucuronide intermediate pathway (Prueksaritanont *et al.*, 2002) resulting in its conversion to a more lipophilic lactone. Both acid and lactone forms of atorvastatin are metabolized mainly be CYP3A4 and to a lesser extent by CYP2C8 (Jacobsen *et al.*, 2000, Neuvonen *et al.*, 2006). The lactone form is non-enzymatic ally hydrolyzed to its respective acid forms. Both 2-hydroxyatorvastatin and 4-hydroxyatorvastatin are active compounds and directly responsible for the inhibition of HMG-CoA (Lennernas, 2003). The transport of atorvastatin acid throughout its biotransformation at varying specificities is attributed to the *OATP1B1*, OATP2B1 and efflux transporters (Wu *et al.*, 2000, Chen *et al.*, 2005, Hirano *et al.*, 2005, Grube *et al.*, 2006).

Organic anion transporting polypeptide 1B1 (OATP1B1) has been referred as the most significant determinant of intestinal absorption and hepatobiliary clearance of hydrophilic statins and is an important member of the solute carrier (SLC) super family (Zair *et al.*, 2008). Their substantial importance as uptake transporters expressed on the sinusoidal membrane of hepatocytes and play a pivotal role in the hepatic uptake of endogenous compounds and several important drugs such as statins, rifampicin, methotrexate, benzyl penicillin and fexofenadine etc. (Niemi, 2007, Seithel *et al.*, 2008). These are sodium independent uptake transporters and function through electro-neutral exchange and coupling of neutralizing anions with organic compounds which result in their cellular uptake (Niemi, 2007). Several sequence variations have been discovered in the *SLCO1B1* gene (Pasanen *et al.*, 2008) out of which two SNPs, namely; 388A>G and 521 T>C have been discussed in many studies as they had proved to be the most prevalent and functionally important for the proper transport function of *OATP1B1* transporter (Niemi, 2007, Morimoto *et al.*, 2004, Zimmerman *et al.*, 2013, Anne *et al.*, 2013, Zhu *et al.*, 2013, Zhou *et al.*, 2013b, Zhou *et al.*, 2013a, Shitara *et al.*, 2013, Ulvestad *et al.*, 2013). The 521T>C and 388A>G SNPs cause an amino acid change at positions 174 (from valine to alanine) and position 130 (from asparginine to aspartic acid) respectively. Both SNPs

**Corresponding author:* e-mail: chairperson.scps@stmu.edu.pk

521T>C and 388A>G have been reported to exist in perfect linkage disequilibrium (LD) (Niemi *et al.*, 2004). Various studies have reported altered plasma concentration and inter-individual variability in therapeutic responses of statins such as simvastatin (Zhou *et al.*, 2013b), pitavastatin (Zhu *et al.*, 2013, Zhou *et al.*, 2013a), atorvastatin (Ulvestad *et al.*, 2013, Shabana *et al.*, 2013), cerivastatin (Tamraz *et al.*, 2013) and linked them to the two functionally important SNPs (388A>G and 521T>C) and their respective genotypes. The inter-individual variability in drug plasma concentration and therapeutic response is hence attributed to the reduced activity of OATP1B1 encoded by the *SLCO1B1* gene (Tirona *et al.*, 2001, Kameyama *et al.*, 2005). We, therefore wanted to investigate the effects of two functionally important *SLCO1B1* SNPs (388A>G and 521T>C) and their respective genotypes on the pharmacokinetics of atorvastatin in Pakistani subjects.

MATERIALS AND METHODS

Subjects

It was a quasi-experimental study in which a total of 100 Pakistani subjects were scrutinized for their genetic profile followed by their subdivision into respective six genotypic groups (388AA, 388AG, 388GG, 521TT, 521TC and 521CC). The study sought its approval from Ethics Committee of Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, National University of Sciences and Technology (NUST), Islamabad, Pakistan vide project no. 02/CREAM-A-2 dated; 17th Sep 2012. All the subjects were enrolled after asserting their health condition from previous medical history and physical examination. Participation in the study was at their free will and only after explaining the study purpose, procedure and protocol.

DNA extraction & genotyping

5 ml blood sample was collected from each participant in ethylenediaminetetraacetic acid (EDTA) tubes and stored at -20°C. Genomic DNA was extracted using standard/ conventional kit method (QIAamp DNA kit, Qiagen, Hilden, Germany) according to the protocol provided (Qaigen, 2007) and analyzed on 1% agarose gel followed by its storage at 4°C.

Two SNP variations of *OATP1B1* –encoding gene *SLCO1B1* 388A>G rs2306283 and 521T>C rs4149056 were investigated. Genotyping of DNA samples was performed after obtaining the reference sequences from National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) database (http://www.ncbi.nlm.nih.gov/); using one step Tetra Amplification Refractory Mutation System (T. ARMS) Polymerase Chain Reaction (PCR) assay with some modifications on Applied Biosystems, Inc., 96 well Thermal Cycler (ABI Veriti, Foster City, CA 94404, USA). Primer templates were obtained by using the online software tool specifically designed for T-ARMS primer and accessible on the Cedar Genetics website http://cedar.genetics.soton.ac.uk/public_html/primer1.html (Ye *et al.*, 2001) (table 1). The cocktail for PCR amplification are presented in table 2.

PCR Cycling conditions

PCR machine was preheated to 95°C (hot start) and incubated for 5min at this temperature (long denaturation). This was followed by 40 cycles of 1min at 95°C (template denaturation), 1 min at 60°C (primer annealing) and 1 min at 72°C (primer extension). PCR protocol was finalized by the last long extension at 72°C for 10min. The protocol for both the 388A>G and 521T>C SNPs were same except for the annealing temperature of 55°C for 521T>C during the 40 cycles.

The amplified PCR product was analyzed by using agarose gel (2% w/v) electrophoresis. 5µl ethidium bromide (10mg/ml) and 0.25% bromophenol blue solutions for loading samples into the wells. Electrophoresis was performed at 100 volts for 30 minutes followed by amplified product's detection on ENDURO^{TM} GDS (Labnet International, Inc., NJ 08837, USA) Gel Documentation System.

Quantification of Plasma Atorvastatin concentration

Venous blood sample was drawn by venipuncture and an indwelling cannula was placed for convenient and repeated sampling. 5ml blood was taken in serum gel separator tubes after a single oral dose of 80mg atorvastatin at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 8, 12, 24, 36 and 48 hours. Samples were centrifuged and plasma stored at -80°C until further analysis for pharmacokinetic profiling.

Plasma concentration of atorvastatin was quantified by High Pressure Liquid Chromatography (HPLC) using modified methods described by (Bahrami *et al.*, 2005, Zarghi *et al.*, 2005). Gradient HPLC system (Perkin-Elmer, Series 200, USA) coupled with an auto sampler, column oven, vacuum degasser and UV-Vis spectrophotometric detector at wavelength of 247nm was used. Chromatographic separation of atorvastatin was achieved by using C18-RT analytical column by Merck (150 x 4.6 ID. 5µm) and a guard column by Perkin-Elmer (1 cm x 4.0mm ID., 5µm particle size). All analysis was performed at room temperature while column was maintained at 62°C in column oven throughout the analysis (Bahrami *et al.*, 2005). Mobile phase was freshly prepared daily and degassed by ultrasonic water bath and HPLC built-in degasser and was directed to the waste bottle and never recirculated into the system. Progesterone was used as an internal standard. The detailed programmable parameters of HPLC are illustrated in table 3. Samples were prepared by reconstituting 500µl of plasma with 20µl of internal

standard (progesterone) 4µl/ml, 750µl acetonitrile and 200 µl saturated NaCl solution, vortex mixing for 30 seconds followed by 10 minutes centrifugation at 4000 rpm. 20µl of supernatant was used for injection into the HPLC system.

Chromatographic data was interpreted and chromatograms were obtained by Perkin-Elmer Total Chrom Navigator Workstation software (Perkin-Elmer Inc.,) version 6.3.0.0445. Plasma concentrations were interpreted as a function of time and area of atorvastatin exhibited peaks.

Pharmacokinetics

All pharmacokinetic parameters were calculated as a function of plasma concentration and time using one compartment model and characterized as peak plasma concentration (C_{max}), time to reach maximum concentration (T_{max}), elimination half-life ($t_{1/2}$) and area under the curve (AUC). Area under the curve (AUC) was calculated by using a linear and log-linear trapezoidal and polyexponential rule from t=0 to the last quantifiable data point in time.

STATISTICAL ANALYSIS

Results are presented as mean ± SEM and percentage differences. Pharmacokinetic data variables were computed by APO, MW PHARM software, version 3.60 (Mediware, Holland) using single compartment model. Only maximum concentration (C_{max}) and time to reach maximum concentration (t_{max}) were calculated manually from the atorvastatin plasma concentrations obtained by HPLC. All the data was then inferred for statistical significance by SPSS version 20.0. Statistical comparisons between pharmacokinetic variables and subjects of various groups was done by analysis of variance (ANOVA) followed by post-hoc tukey test for pairwise comparisons. Levene's test of equality was used for validating the homogeneity of variance. All testing was performed at a confidence interval (CI) of 95% and results inferred statistically significant with a p-value of less than 0.05.

RESULTS

A complete pharmacokinetic profile of all the six genotypic groups affiliated with the two SNPs were performed and presented in table 4. There was an insignificant rise of 39.64 % ($p>0.05$) in Area under the curve (AUC) between group 1 (AA) and group 2 (AG) but a significant difference of 75.79% ($p<0.05$) between control (AA) and group 3 (GG). Group 2 (AG) lied moderately between both the group 1 and 3 and showed no significance with either of them, but in case of maximum drug concentration (C_{max}), group 2 (AG) demonstrated a significant difference of 40.4% ($P<0.05$) with group 3 (GG) and no difference with group 1 (AA). Time to reach maximum concentration (T_{max}) and elimination half-life ($t_{1/2}$) were not much affected by the genotypic variations within the groups and therefore showed no significant variations ($p>0.05$) (table 5).

In contrast to the 3 genotypes of 388 SNP, the later 3 genotype groups of the 521 SNP showed greater and significant variations in pharmacokinetic profiles (table 4). Each group 4 (TT), 5 (TC) and 6 (CC) was significantly associated with each other. AUC was presented with 83.27% ($p<0.05$) and 151.16% ($p<0.05$) significant difference when compared group 4 (TT) with 5 (TC) and 4 (TT) with 6 (TC) respectively. Similarly, a 37.04% ($p<0.05$) significant difference was also present between group 5 (TC) and 6 (CC). C_{max} exhibited the same pattern as AUC when compared among groups 4 (TT), 5 (TC) and 6 (CC). There was a significant difference of 41.64% ($p<0.05$), 29.91% ($p<0.05$) and 59.10% ($p<0.05$) between groups 4 (TT) and 5 (TC), 5 (TC) and 6 (CC) and, 4 (TT) and 6 (CC) respectively.

The 521 T>C SNP seems to have a greater effect and therefore strongly related with the pharmacokinetics of atorvastatin in general comparison with the 388 A>G SNP. The fact was augmented and strengthened when 388 variant genotype groups 2 (AG) and 3 (GG) were compared with the 521 variant genotype groups 5 (TC) and 6 (CC) (table 4). There was a 115.5% ($p<0.05$) difference between the AUC of heterozygous groups 2 (AG) and 5 (TC) of both the SNPs. However, their C_{max} was not significantly affected despite a difference of 30.04% ($p>0.05$). A difference of 111.53% ($p<0.05$) in AUC and 85.55% ($p<0.05$) in C_{max} was observed when bi-allelic variant group 6 (CC) was compared with group 2 (AG). This behavior was also observed when both the bi-allelic variant groups 3 (GG) and 6 (CC) were compared with each other. There was a difference of 68.03% ($p< 0.05$) and 32.13% ($p<0.05$) in AUC and C_{max} between both the groups respectively.

DISCUSSION

In the light of studies conducted in different populations over the last few years (Pasanen *et al.*, 2006, Kalliokoski and Niemi, 2009, Takane, 2011, Trdan Lusin *et al.*, 2012, Hua *et al.*, 2012), it is becoming strongly evident that the *SLCO1B1* SNPs, particularly the 388A>G and 521T>C have a substantial role in defining *OATP1B1* activity and as a result its effect on drug disposition. In this study, we tried to relate the possible genetic variants (genotypes) of *SLCO1B1* with the pharmacokinetic profile of atorvastatin in Pakistani subjects in an attempt to develop accurate and clear picture of a relation between pharmacokinetics and OATP1B1 activity in our population.

The main pharmacokinetic effects of both the SNPs were only observed in attenuating the area under the curve (AUC) and maximum plasma concentration (C_{max}).

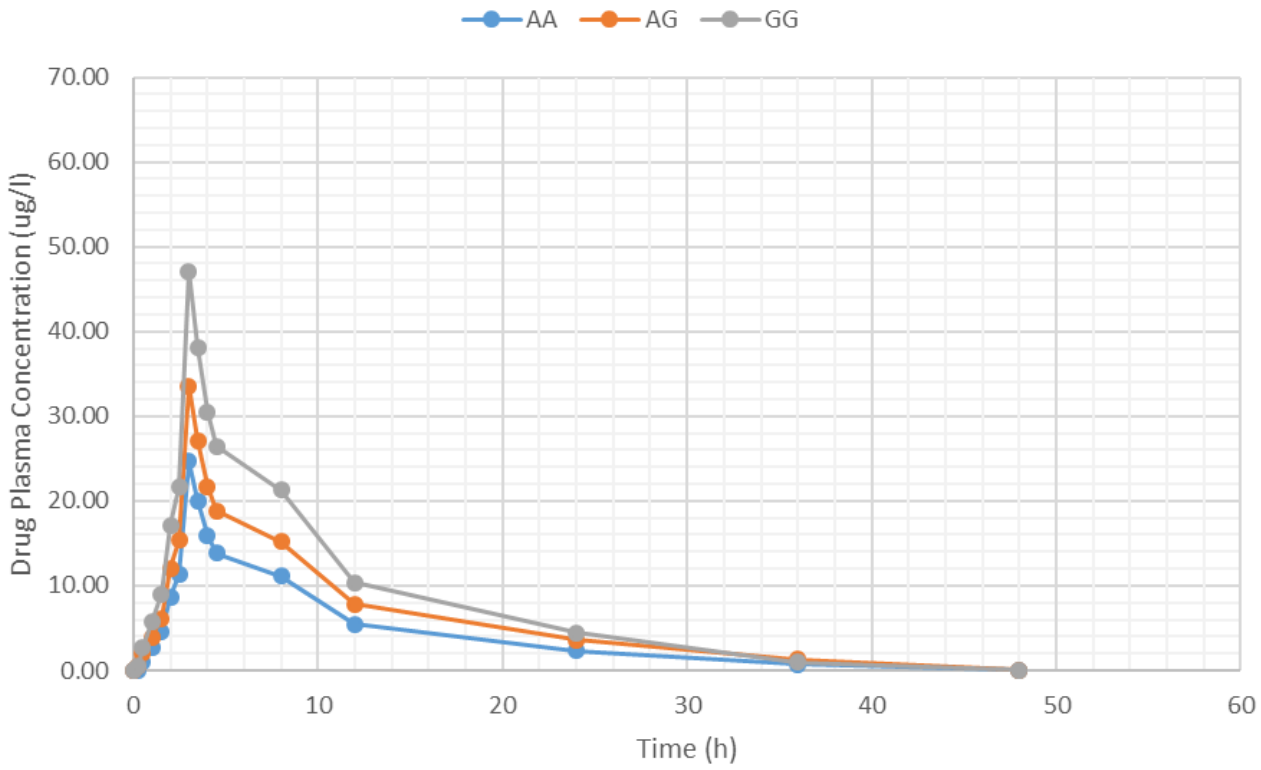

Fig. 1: Variation in AUC and C_{max} among the three genotypes of 388A>G SNP.

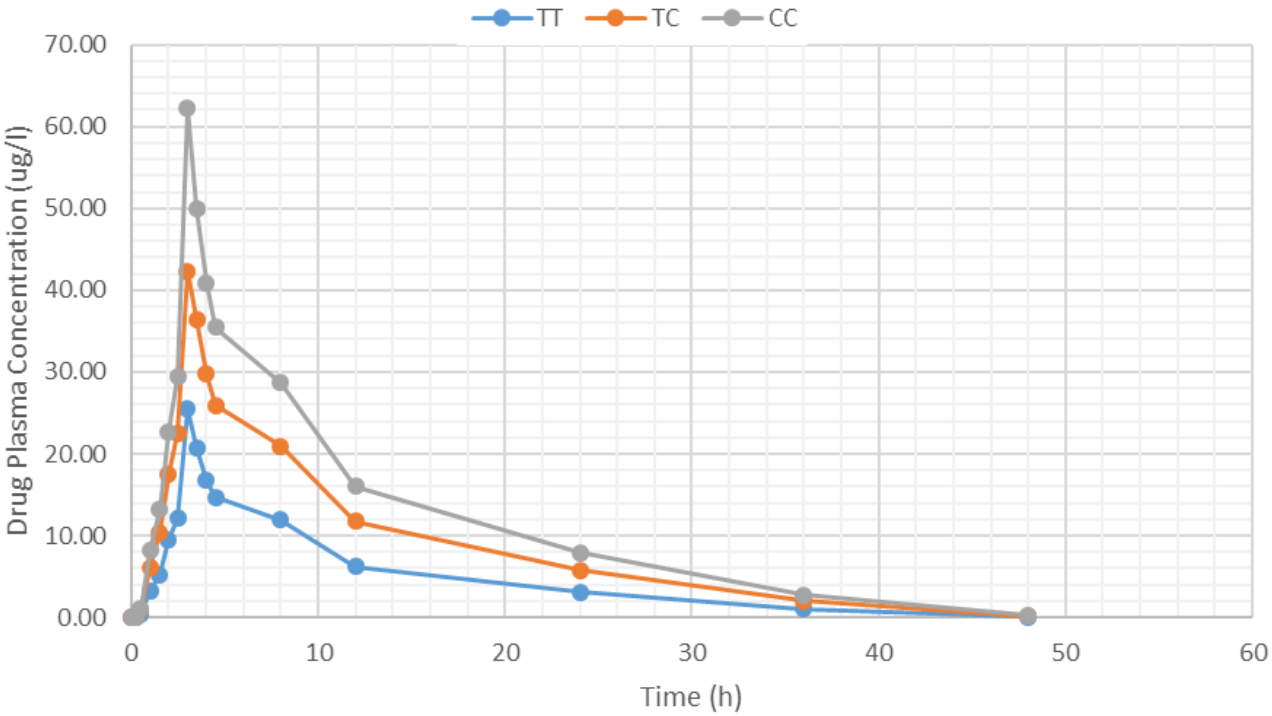

Fig. 2: Variation in AUC and C_{max} among the three genotypes of 521T>C SNP.

Table 1: T. ARMS PCR primer templates

Region		Primer Sequence *SLCO1B1* 388A>G	Primer Sequence *SLCO1B1* 521T>C
Forward	Outer	5´-AATTGACAGAAAGTACTCTGGTAA TTTG-3´	5´-CACCATATTGTCAAAGTTTGCAA AGTGA-3´
	Inner	5´-TTACAGGTATTCTAAAGAAACTAATA GCG-3´	5´-ATCTGGGTCATACATGTGGATAT AGGT-3´
Reverse	Outer	5´-ATTTTGCTAATGAATATCACAACAA TTT-3´	5´-TTCAAAAGTAGACAAAGGGAAA GTGATCA-3´
	Inner	5´-GTCGATGTTGAATTTTCTGATGACTT-3´	5´-ATTCCACGAAGCATATTACCCAT GACCG-3´

Table 2: Cocktail for PCR amplification

Constituents	*SLCO1B1* 388A>G	*SLCO1B1* 521T>C
Template	50 ng	50 ng
10X PCR buffer	1X	1X
$MgCl_2$ (25 mM)	3 mM	5 mM
dNTP's	0.2 mM	0.2 mM
Outer F primer (10 pmol/µl)	1 pmol	1 pmol
Specific F Primer (10 pmol/µl)	1 pmol	1 pmol
Outer R primer (10 pmol/µl)	1 pmol	1 pmol
Specific R primer (10 pmol/µl)	1 pmol	1 pmol
Taq DNA Polymerase (2 U/µl)	10 U	10 U
dH_2O (nuclease free)	Upto 50µl	Upto 50µl
Total reaction mixture (µl)	50	50

Table 3: HPLC Programed parameters

Programmable parameters	Values
Mobile phase flow rate (ml/min)	1.5
Mobile phase composition	A: 60 % acetonitrile B: 40 % 0.05 M Sodium Phosphate Buffer
Degasser	On
Auto-sampler	On
Column Oven	62°C
Total Run time (min)	7
Data acquisition time (min)	7
Sampling interval (min)	5
Washing time (min)	4.5
UV detector wavelength (nm)	247
Sample injection volume (µl)	20
Pressure range (psi)	1100-2800
Internal standard (IS)	Progesterone

Other pharmacokinetic parameters, especially T_{max} and elimination half-life ($t_{1/2}$) remained unchanged regardless of the genetic changes. This can be explained by the high hepatic extraction ratios and increase in oral bioavailability (Niemi *et al.*, 2005). The allelic genotypes of both the SNPs exhibited a positive relation with increased AUC and C_{max} but, the 521 T>C SNP took a lead by establishing a relatively more profound effect. Even the heterozygous genotype (TC) exhibited a significant difference both in AUC (115.55%) and C_{max} (76.88%) when compared to the 388 wild (AA) genotype, while the homozygous bi-allelic genotype (CC) demonstrated the maximum effect with 195.40% increase in AUC and 152.40% increase in C_{max} when compared with the same. Similar significant ratios were maintained throughout their comparisons with other 388 heterozygous (AG) and bi-allelic (GG) genotype variants (table 5). In continuance to these findings, the inter-genotype (TT with TC, TC with CC and TT with CC) significant difference of 521 T>C SNP was obvious, while in case of 388 SNP genotypes, significance was observed only between AA and AG (table 5). Significant effect of these SNPs on two most vital pharmacokinetic parameters within the genotypes can be observed in fig. 1 and 2. The significant difference increase in AUC and C_{max} among both the SNPs is evident by comparing the Pharmacokinetic time-concentration graph (fig. 1 and 2) along the concentration axis.

Table 4: Pharmacokinetic profile of atorvastatin among 6 genotypic groups

Groups Disposition		388 A>G (n=51)			521 T>C (n=49)		
		1 (AA)	2 (AG)	3 (GG)	4 (TT)	5 (TC)	6 (CC)
Pharmacokinetic Profile	AUC [h.µg/l]	196.39±18.72	274.25±24.16	345.24±30.01	230.98±21.74	423.33±39.35	580.14±53.94
	C_{max} [µg/l]	25.4±1.59	34.55±2.17	48.52±3.05	26.22±1.60	44.93±2.62	64.11±4.05
	T_{max} [h]	3.147±0.07	3.147±0.07	3.088±0.05	3.176±0.07	3.187±0.06	3.156±0.07
	$t_{1/2}$ [h]	6.526±1.02	6.733± 0.88	5.408±0.57	6.141±1.10	6.255±1.10	6.352±1.11
	AUC_p [h.µg/l]	179.83±15.98	258.91±20.87	336.75±28.88	217.84±17.69	399.13±32.03	545.91±43.86
	CL [l/h]	0.46±0.04	0.33±0.03	0.25±0.02	0.40±0.04	0.22±0.02	0.16±0.01
	V_d [l]	3.71±0.40	2.83±0.27	1.85±0.15	2.84±0.27	1.58±0.15	1.18±0.11
	k [l/h]	0.16±0.02	0.14±0.02	0.15±0.02	0.17±0.03	0.17±0.03	0.17±0.03
	MRT [h]	9.41±1.47	9.71±1.26	7.80±0.82	8.86±1.59	9.02±1.58	9.16±1.61
	MRT+MIT [h]	12.70±1.18	13.13±0.97	11.45±0.63	13.20±1.28	13.38±1.25	13.28±1.29
	MIT [h]	3.29±0.40	3.42±0.38	3.64±0.38	4.14±0.34	4.18±0.38	4.12±0.35
	ka [l/h]	0.50±0.07	0.42±0.05	0.37±0.05	0.44±0.12	0.40±0.10	0.46±0.13
	$t_{1/2\ a}$ [h]	1.91±0.25	2.13±0.25	2.32±0.25	2.47±0.24	2.54±0.26	2.45z0.24
	t_0 [h]	0.53±0.08	0.34±0.06	0.30±0.03	0.57±0.08	0.51±0.08	0.58±0.08

Values are expressed as Mean ± SEM. Groups in white and grey shade are genetically non-variant and variant respectively.

Table 5: Pairwise group comparison of pharmacokinetic profile

PK	Groups	1 (AA)	2 (AG)	3 (GG)	4 (TT)	5 (TC)	6 (CC)
AUC [h.µg/l]	1 (AA)		39.64	75.79	17.61	115.55	195.40
	2 (AG)	0.564		25.88	15.77	54.35	111.53
	3 (GG)	0.025*	0.658		33.09	22.60	68.03
	4 (TT)	0.977	0.940	0.156		83.27	151.16
	5 (TC)	0.000*	0.028*	0.577	0.002*		37.04
	6 (CC)	0.000*	0.000*	0.000*	0.000*	0.020*	
$52C_{max}$ [µg/l]	1 (AA)		36.02	91.02	3.22	76.88	152.40
	2 (AG)	0.150		40.4	24.16	30.04	85.55
	3 (GG)	0.000*	0.004*		45.96	7.39	32.13
	4 (TT)	1.000	0.233	0.000*		71.35	144.50
	5 (TC)	0.000*	0.078	0.932	0.000*		42.68
	6 (CC)	0.000*	0.000*	0.001*	0.000*	0.000*	
T_{max} [h]	1 (AA)		0	1.87	0.921	1.27	0.28
	2 (AG)	1.000		1.87	0.92	1.27	0.28
	3 (GG)	0.989	0.989		2.849	3.2	2.20
	4 (TT)	1.000	1.000	0.937		0.34	0.629
	5 (TC)	0.998	0.998	0.906	1.000		0.97
	6 (CC)	1.000	1.000	0.981	1.000	1.000	
$t_{1/2}$ [h]	1 (AA)		3.17	17.1	5.89	4.15	2.66
	2 (AG)	1.000		19.6	8.79	7.09	5.65
	3 (GG)	0.966	0.930		13.55	15.66	17.45
	4 (TT)	1.000	0.998	0.995		1.856	3.435
	5 (TC)	1.000	0.999	0.991	1.000		1.55
	6 (CC)	1.000	1.000	0.985	1.000	1.000	

*p<0.05 (confidence interval 95 %). Data presented as % age difference and p-values above and below the diagonal respectively.

This probably reflects the major role of 521 SNP on transporter activity of *OATP1B1*. One most recent study (Nies *et al.*, 2013) has held the 388A>G SNP responsible for the expression of OATP on the hepatocytes besides its effect on AUC and C_{max}, therefore rendering both the 521 and 388 SNPs equally important in their role towards drug disposition hence directly effecting atorvastatin pharmacokinetics.

The liver is not only a site of therapeutic action for atorvastatin but is also responsible for its elimination. The therapeutic effect of atorvastatin is therefore dependent on its transport to the HMG-CoA reductase in the hepatocytes, which is medicated by OATP1B1. Thus reduced OATP1B1 activity can lead to decreased uptake of atorvastatin in to the liver hence resulting in attenuated cholesterol lowering efficacy and increase concentration

of drug in peripheral blood owing to an increased risk of myopathy (Pasanen *et al.*, 2007, Neuvonen *et al.*, 2006). The pharmacokinetic data produced from this study clearly indicates an increased concentration of atorvastatin in the peripheral blood circulation. This evidently suggests a reduced hepatic uptake of atorvastatin in subjects with the homozygous and heterozygous allelic variants of both 388A>G and 521T>C SNPs. This could result in reduced therapeutic efficacy of atorvastatin and increase in the risk of associated adverse effects. Further clinical studies are prerequisite to characterize the effects of two functionally important *SLCO1B1* polymorphic variants (388A>G and 521T>C) on the lipid lowering efficacy and tolerability of atorvastatin.

In conclusion, the two functionally important SNPs of *SLCO1B1* gene encoding hepatic uptake transporter OATP1B1 have a marked significant effect on the plasma concentration of atorvastatin hence effecting its therapeutic efficacy. This genetic variability in OATP1B1 function can exert a clinically significant impact on the risk to benefit ratio of atorvastatin therapy.

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