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

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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 Cmax but exhibited no significant variations in T max and t1/2. 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 (Pruckasritanont 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, ritampicin, 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, Shibata 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 asparagine to aspartic acid) respectively. Both SNPs
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 (Qiagen, 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™ 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-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 İD. 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 (Cmax), time to reach maximum concentration (Tmax), elimination half-life (t1/2) 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 (Medware, Holland) using single compartment model. Only maximum concentration (Cmax) and time to reach maximum concentration (tmax) 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 (Cmax), 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 (Tmax) and elimination half-life (t1/2) 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). 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 Cmax 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 Cmax, was observed when both the 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 Cmax between both the groups respectively.

DISCUSSION
In the light of studies conducted in different populations over the last few years (Pasane et al., 2006, Kalliokoski and Niemi, 2009, Takane, 2011, Trdan Lusin 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 (Cmax).
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.
Other pharmacokinetic parameters, especially $T_{\text{max}}$ 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 $C_{\text{max}}$ 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 $C_{\text{max}}$ (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 $C_{\text{max}}$ 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 $C_{\text{max}}$ among both the SNPs is evident by comparing the Pharmacokinetic time-concentration graph (fig. 1 and 2) along the concentration axis.
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Table 4: Pharmacokinetic profile of atorvastatin among 6 genotypic groups

<table>
<thead>
<tr>
<th>Groups Disposition</th>
<th>388 A&gt;G (n=51)</th>
<th>521 T&gt;C (n=49)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (AA)</td>
<td>2 (AG)</td>
</tr>
<tr>
<td>AUC [h.µg/l]</td>
<td>196.39±18.72</td>
<td>274.25±24.16</td>
</tr>
<tr>
<td>C max [µg/l]</td>
<td>25.4±1.59</td>
<td>34.55±2.17</td>
</tr>
<tr>
<td>T max [h]</td>
<td>3.147±0.07</td>
<td>3.147±0.07</td>
</tr>
<tr>
<td>t 1/2 [h]</td>
<td>6.526±1.02</td>
<td>6.733±0.88</td>
</tr>
<tr>
<td>AUC 0 [h.µg/l]</td>
<td>179.83±15.98</td>
<td>258.91±20.87</td>
</tr>
<tr>
<td>CL [l/h]</td>
<td>0.46±0.04</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>k [l/h]</td>
<td>0.16±0.02</td>
<td>0.14±0.02</td>
</tr>
<tr>
<td>MRT [h]</td>
<td>9.41±1.47</td>
<td>9.71±1.26</td>
</tr>
<tr>
<td>MIT [h]</td>
<td>3.29±0.40</td>
<td>3.42±0.38</td>
</tr>
<tr>
<td>t 1/2 a [h]</td>
<td>1.91±0.25</td>
<td>2.13±0.25</td>
</tr>
<tr>
<td>t 0 [h]</td>
<td>0.53±0.08</td>
<td>0.34±0.06</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>PK</th>
<th>Groups</th>
<th>1 (AA)</th>
<th>2 (AG)</th>
<th>3 (GG)</th>
<th>4 (TT)</th>
<th>5 (TC)</th>
<th>6 (CC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC [h.µg/l]</td>
<td></td>
<td>39.64</td>
<td>25.88</td>
<td>33.09</td>
<td>22.60</td>
<td>68.03</td>
<td></td>
</tr>
<tr>
<td>52C max [µg/l]</td>
<td></td>
<td>15.77</td>
<td>17.16</td>
<td>28.87</td>
<td>30.04</td>
<td>39.12</td>
<td></td>
</tr>
<tr>
<td>T max [h]</td>
<td></td>
<td>0.92</td>
<td>1.27</td>
<td>0.92</td>
<td>0.92</td>
<td>1.27</td>
<td></td>
</tr>
<tr>
<td>t 1/2 [h]</td>
<td></td>
<td>2.67</td>
<td>3.14</td>
<td>2.32</td>
<td>2.67</td>
<td>3.14</td>
<td></td>
</tr>
</tbody>
</table>

*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.
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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