Original Article

Evidence-Based P-Glycoprotein Inhibition by Green Tea Extract Enhanced the Oral Bioavailability of Atorvastatin: From Animal and Human Experimental Studies

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Abstract

Background: The study aimed to explore the beneficial effects of green tea extract (GTE) on the permeability and absorption kinetics of atorvastatin in rats and healthy human volunteers. **Methods:** Wistar rats for both *in situ* and *in vivo* studies. In *in situ Single pass intestinal perfusion* study, three groups ($n = 6$), wherein Group 1 perfused with atorvastatin as control, Group 2 coperfused with verapamil, and Group 3 coperfused with GTE then the effective permeability of atorvastatin was determined. In *in vivo* study, three groups ($n = 6$), wherein Group 1 is treated with atorvastatin as control, Group 2 pre-treatment with verapamil for 7 days and Group 3 pretreatment with GTE for 7 days and on 8th day atorvastatin was repeated and subjected to pharmacokinetic study. These results were confirmed on 24 healthy human volunteers, the randomized crossover trial was carried with atorvastatin for 11 days to check the bioavailability of atorvastatin by pre-treatment with GTE. Blood samples collected between 0.5 and 24 h on day-1, following administration of atorvastatin. Blood sampling was repeated using similarly specified time intervals on day-11, after treating human volunteers with GTE capsule 400 mg for 10 days. **Results:** Effective permeability of atorvastatin has been increased by GTE in *in situ* studies. The clearance of atorvastatin was decreased by 17.5% ($P < 0.001$), and C_{max} was increased many folds significantly in *in vivo* studies. A significant increase in serum concentrations of atorvastatin was observed from 1st h. C_{max}, bioavailability were increased by 14.5% ($P < 0.05$), and 22.7% ($P < 0.001$), respectively, in human volunteers. **Conclusion:** Increased bioavailability of atorvastatin is due to the P-gp inhibition by GTE, leads to the reduced dose. Further anti-hyperlipidemic activity of the GTE enables the dyslipidemic patients to take this herbal product safely.

Keywords: Atorvastatin, bioavailability, green tea extract, hyperlipidemia, pharmacokinetics

Introduction

Atorvastatin, an HMG-CoA reductase inhibitor used widely to treat the hyperlipidemia, acts by inhibiting the rate-limiting step in cholesterol synthesis in the liver. Bioavailability of the drug is approximately12%; extensive first-pass metabolism is the major reason for its poor bioavailability.[1] Many efforts are under progress to improve the bioavailability of atorvastatin by pharmaceutical or pharmacological means. Atorvastatin is a Class II drug i.e., low soluble and highly permeable, and may be well absorbed in the duodenum and proximal jejunum, where P-glycoprotein (P-gp) expression is less.[2] However, due to the solubility, the absorption site is shifted more toward the distal intestine, where P-gp effect may be pronounced. The saturation of P-gp by providing high drug concentrations at the site of absorption or P-gp inhibition using P-gp modulators may significantly improve the pharmacokinetics of this drug.[3] In these two procedures, the P-gp inhibition by P-gp modulators is very compatible and more advantageous. Atorvastatin is an antihyperlipidemic drug having pharmacoresistance. Transport of atorvastatin by P-gp in the intestinal membrane is the major determinant in the development of its pharmacoresistance.[4] P-gp, an efflux Transporter, is located on the apical membrane of intestinal epithelial cells, oriented such that P-gp substrates

Access this article online

Quick Response Code:

Website: www.jnsbm.org

DOI: 10.4103/jnsbm.JNSBM_201_19

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Submitted: 13-Nov-2019 **Revised:** 15-Jan-2020
Accepted: 27-Jan-2020 **Published:** 22-Jul-2020

How to cite this article: Danaboina KK, Neerati P. Evidence-based P-glycoprotein inhibition by green tea extract enhanced the oral bioavailability of atorvastatin: From animal and human experimental studies. J Nat Sc Biol Med 2020;11:105-10.

are secreted from the epithelial cell into the intestinal lumen. P-gp mediated efflux has the potential to decrease intestinal drug absorption. The inhibition of intestinal P-gp may have clinically significant effects on the plasma concentration of atorvastatin. To test this hypothesis, these studies were conducted to examine the effects of inhibition of P-gp by green tea extract (GTE) on the pharmacokinetics of atorvastatin.

The current study was aimed at improving the bioavailability of atorvastatin by GTE pretreatment. Cytochrome P450 metabolizing enzyme (CYP 3A4) is the major P450 isoform that is responsible for the metabolism of atorvastatin.[5] and it is also a P-gp substrate.[6] Efflux of the drug by the P-gp into the lumen from the absorption site might be the reason probably for the low bioavailability of the atorvastatin.[7] Drugs are repeatedly taken up and pumped out of the enterocytes by P-gp, thus increasing the probability of drugs being metabolized.[8] CYP3A enzymes account for 80% of the total of CYP enzymes in the small intestine.[9] The amount of CYP3A enzymes in the intestine equals 80%–100% of that found in the liver.[10] The inhibition of P-gp would also result in the reduced metabolism of drugs by CYP enzymes.[8] GTE, obtained from the leaves of *Camelia sinensis* was employed in the study as a P-gp inhibitor. The effect of green tea polyphenol on cellular uptake of 99 mTc-tetrofosmin *in vitro* confirms that it can act as a P-gp modulator.[11] *In vivo* studies reported that the GTE could stop or slows the function of the P-gp. The anti hyperlipdemic property of GTE was the reason for selecting this herbal product as P-gp inhibitor. How come this extract could influence the pharmacokinetics of atorvastatin is the main objective to be evaluated here.

Twenty-four healthy volunteers were selected for the study, maintained on a standardized diet throughout the study period. The serum atorvastatin concentrations were determined using high performance liquid chromatographic (HPLC) and test results were compared with that of the baseline. Here, every volunteer serves as their own control.

Methods

Drugs and chemicals

Atorvastatin, naproxen, and verapamil are gift samples from the Matrix Laboratories Ltd., (Hyderabad, India). Propranolol is a kind gift sample from Aurobindo pharma (Hyderabad, India); phenol red was purchased from Himedia (Mumbai, India). Atorvastatin 40 mg tablets, manufactured by Lupin Pharmaceuticals, Mumbai, were purchased from a local medical shop. GTE capsules 400 mg and pure GTE samples were purchased from the Zenith Pharmaceuticals (Bangalore, India). All solvents used for the HPLC were of analytical grade (Merck, India).

Single pass intestinal perfusion (SPIP)

In *in situ* experiments male Wistar rats (weighing 250–350 g) are procured from the central animal house, Kakatiya University, Warangal.[12] The animals were placed in polypropylene cages, 4 per cage, with free access to standard laboratory diet and water. They were kept at 25°C ± 1°C and 45%–55% RH with a 12 h light/dark cycle. The *in situ* experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC/17/UCPSc/KU/2013), Kakatiya University, Warangal.

Protocol

Following an overnight fasting, rats were divided into three groups ($n = 6$). The rats were treated as follows.

- Group 1: Perfusion of atorvastatin (30 µM) + Propranolol (100 µM) + Phenol red (50 mg/L)
- Group 2: Co-perfusion of atorvastatin (30 µM) + Verapamil (200 µM) + Propranolol (100 µM) + Phenol red (50 mg/L)
- Group 3: Co-perfusion of atorvastatin (30 µM) + GTE (10 mg/mL) + Propranolol (100 µM) + Phenol red (50 mg/L).

The perfusate was collected every 10 min. At the end of the perfusion, the length of the segment was measured following the last collection. The animal was sacrificed by injecting a saturated solution of KCl (10%). Samples were stored at −20°C until analysis, and perfusate concentrations of atorvastatin were quantified using reverse-phase HPLC (RP-HPLC).

Stability test

The stability of atorvastatin and propranolol were tested by their incubation in the perfusion solution containing Phosphate buffer saline (pH-7.4) and phenol red at room temperature for 6 h. samples were taken at 1, 2, 4, and 6 h. Then, the samples were analyzed by RP-HPLC. Drugs were found to be stable in perfusion samples for 6 h at room temperature. The samples were stored at −20°C for short-term stability experiment. There was no sign of the degradation of drugs and no sign of interaction of drugs with phenol red.[13]

Effective permeability coefficient

It is the quantitative estimate of the rate of passage of a solute across a membrane. It is calculated from the steady-state concentration of compounds in the collected perfusate which is considered to be attainable when the concentration level of phenol red is stable. The steady-state effective permeability is calculated using the following equation as the buffer solution is perfused from an entrance in one end of the intestinal segment.[13]

$$P_{eff} = - Q \times \ln (C_{out} (corr)/C_{in})/2 \pi rL.$$

Where,

- P_{eff} = Effective permeability coefficient
- Q = Perfusion flow rate
- C_{out} (corr) = Corrected outlet drug concentration
- C_{in} = Inlet drug concentration
- r = Radius of small intestine
- L = length of the perfused intestinal segment.

In vivo study in rats

Animals

For *in vivo* experiments, male Wistar rats (weighing 250–300 g) are procured from the central animal house, Kakatiya University,

Warangal. The animals were placed in polypropylene cages, 4 per cage, with free access to standard laboratory diet and water. They were kept at 25°C ± 1°C and 45%–55% RH with a 12 h light/dark cycle. The *in vivo* experimental protocol was approved by the Institutional animal ethical committee (Reference number: IAEC/17/a/UCPSc/KU/2013) Kakatiya University, Warangal.

Protocol

Following an overnight fasting, rats were divided into three groups ($n = 6$). The rats were treated as follows:

- Group 1: Atorvastatin (20 mg/kg; po) serving as control
- Group 2: Verapamil (25 mg/kg; po) for 7 days followed by atorvastatin (20 mg/kg; po) on the 8th day
- Group 3: GTE (10 mg/kg; po) for 7 days followed by atorvastatin (10 mg/kg; po) on the 8th day.

Blood samples were collected from retro-orbital plexus of the rats,[14] at 0,0.5,1,2,4,8,12 and 24 h time points into Eppendorf tubes the serum was separated by centrifugation using Biofuge 13 (Heraeus Instruments, Germany) at 3000 g/15 min separate serum and store at −20°C until further analysis.

Study with human volunteers

Twenty-four healthy volunteers, in the age group of 23–27, were selected for the study. Mean of height, weight, and body mass index of the volunteers were recorded as 175 cm, 68 kg, and 22.2, respectively. It was ensured that they are not suffering from any kind of illness and are not on any drug therapy were included and those volunteers with concomitant diseases and use of other drugs and alcoholics, smokers were excluded in the study. All the volunteers were maintained on a standardized diet (Breakfast: Lunch: Dinner timings are 7 a.m.: 1 p.m.: 8 p.m. respectively) 1 week before the study, and the same is continued throughout the study period. The study was approved by the Human Ethical Committee (HEC/23/A2/UCPSC/KU-2013) and an informed consent form was taken from each volunteer and ICH-GCP guidelines are followed.

Collection of blood samples

Following administration of atorvastatin 40 mg, blood samples (1 ml) were collected from the volunteers at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h. Volunteers were treated with GTE 400 mg capsules for the next 10 consecutive days, 1 capsule/day. During the treatment period, volunteers receive only GTE. On the 11th day of study, the following administration of GTE along with the atorvastatin 40 mg, blood samples (1 ml) were collected once again, at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h. Blood samples were allowed to clot for 10 min at room temperature and were centrifuged at 10,000 rpm for 10 min to obtain the serum, and the samples were stored at − 80°C until further analysis.

Preparation of test samples

A volume of 0.2 ml of serum and a volume of 50 µl of internal standard (Naproxen at a concentration of 400 ng/ml) is pipette out into a microcentrifuge tube of 2 ml volume and vertexed for 30 s. A volume of 0.6 ml of absolute ethanol was added for the protein precipitation and vertexes for 2 min, liquid-liquid extraction of the drug was done by adding 0.5 ml of dichloromethane to the above solution and vertexed for 5 min and centrifuged for 5 min at 10,000 rpm and the supernatant is collected and dried in the vacuumed evaporator and then reconstituted with the 0.5 ml of mobile phase and the resultant solution is vertexes for 2 min and centrifuged for 5 min at 10,000 rpm an aliquot of 20 µl of supernatant was loaded and injected into the HPLC.

The determination of atorvastatin concentration by high-performance liquid chromatographic

Atorvastatin concentrations in the serum samples were determined using a previously reported method[15] on HPLC, Shimadzu (Kyoto, Japan). C18 column (Merck, India) with dimensions 250 mm × 4.6 mm and 5 µm particle size was used. A volume of 20 µl of the sample was injected into the column. A mixture of methanol and water adjusted to pH 3.0 with trifluoroacetic acid was used as a mobile phase in the ratio of 68:32. The flow rate was set at 1.5 ml/min and chromatograms were recorded at 241 nm. The retention time of atorvastatin is 7.6 min and naproxen is 6 min. The total run time was set for 10 min.

Stability tests

All the samples were stable for 6 months when stored at −80°C. Stock solutions of the drugs were stable for 1 month, when stored at 2°C–8°C. Extracted serum samples were stable for 72 h, when stored at room temperature.

Data and statistical analysis

All the data were expressed as mean ± standard error of mean (SEM). Pharmacokinetic parameters were calculated using KINETICA 5 software (A product of Adept Scientific Ltd., UK). The significance of the difference was determined using paired *t*-test. The values were considered as statistically significant at $P < 0.05$.

Results

Intestinal permeability co-efficient of atorvastatin in the absence and presence of P-gp inhibitors such as verapamil and GTE were found to be $0.251 \pm 0.011 \times 10^{-4}$ cm/sec, $0.345 \pm 0.061 \times 10^{-4}$ cm/s, and $0.409 \pm 0.094 \times 10^{-4}$ cm/s, respectively. P-gp inhibitors such as Verapamil (200 µM) and GTE (10 mg/kg), co-perfused with atorvastatin (30 µM)) resulted in significant increase in intestinal permeability by 1.6-fold from 0.251 ± 0.011 to $0.409 \pm 0.094 \times 10^{-4}$ cm/s, 2.1-fold from 0.251 ± 0.011 to $0.52\,7 \pm 0.0067 \times 10^{-4}$ cm/s, respectively [Figure 1]. Intestinal permeability coefficient of propranolol in the absence and presence of P-gp inhibitors such as verapamil and GTE were found to be statistically insignificant. Propranolol is highly permeable marker was shown to have no interaction with P-gp.

Pharmacokinetics of atorvastatin in the absence and presence of verapamil and GTE time and plasma concentrations of atorvastatin (20 mg/kg; po) in the absence and presence of

verapamil (25 mg/kg, po) and GTE (10 mg/kg, po) in rats are shown in Table 1. Plasma concentration versus time curve of atorvastatin in the absence and presence of verapamil and GTE are depicted in Figure 2. Pharmacokinetic parameters of atorvastatin in rats were mentioned in Table 1. Atorvastatin is rapidly absorbed, and a maximum plasma concentration level was reached within 1–2 h of administration. After an initial absorption phase, plasma concentrations of atorvastatin declined gradually. However, in the presence of verapamil and GTE atorvastatin concentrations increased gradually even after the initial absorption phase, this leads to pronounced alteration in pharmacokinetics. The overall AUC was raised from (control to GTE treatment) 2.581 ± 0.357 to 12.516 ± 2.772 and 14.6 ± 2.13, and the reduction atorvastatin clearance from 1200.7 ± 76 to 233.4 ± 66.37 and 206.4 ± 29.7 significant increase in C_{max} from 0.036 ± 0.018 to 1.524 ± 0.225 and 1.907 ± 0.088 with verapamil and GTE [Table 2].

Results were given as mean ± SEM Serum concentrations of atorvastatin, when treated alone and its combination with GTE. Mean pharmacokinetic parameters of atorvastatin alone and its combination in human volunteers with GTE were given [Table 3]. Serum atorvastatin concentrations were increased significantly, from the 1st h. C_{max}, bioavailability were increased by 14.5% ($P < 0.05$) and 22.7% ($P < 0.001$), respectively. T_{max} was unchanged and observed to be at 1st h in all the patients before and after green tea treatment.

Discussion

Atorvastatin belongs to the biopharmaceutics classification system (BCS) Class II has low solubility and high permeability as a result of which atorvastatin is well absorbed in the duodenum and proximal jejunum. However, due to the low solubility, the absorption site is shifted more toward the distal jejunum and ileum. The Food and Drug Administration recognized the *in situ* single-pass intestinal perfusion (SPIP) technique as a useful model to classify a compound's absorption characteristics in the BCS.[16] These techniques maintain an intact blood supply to the intestine and can be used to estimate the impact of clearance pathways, such as enzymes and transporters, that are present in the gut. In addition, it was reported that oral drug absorption in rats and humans is very similar. Thus, it is likely that the intestinal perfusion conducted in rats may give a better prediction of the fraction of oral dose absorbed in humans than in *in vitro* models.[16] In the present study, *in situ* SPIP was performed in the ileum segment of rats to investigate the functional role of P-gp in intestinal permeability of atorvastatin. The effective

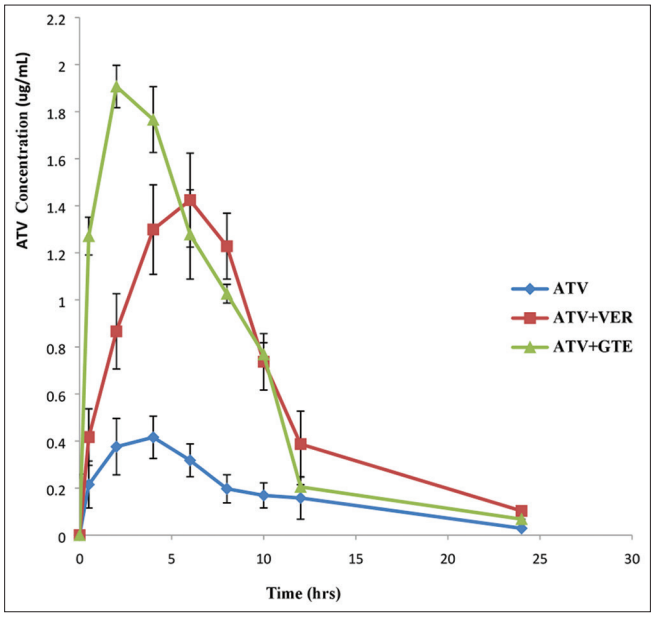

Figure 1: Plasma concentrations time curves of atorvastatin following oral administration of VER 25 mg/kg and GTE 10 mg/kg in male Wistar rats. ATV: Atorvastatin (20 mg/kg), VER: Verapamil (25 mg/kg), GTE: Green tea extract (10 mg/kg)

Table 1: Mean serum concentration of atorvastatin in presence and absence of green tea extract

Time (h)	ATV	ATV + GTE
0	0	0
0.5	10.2±0.46	10.4±0.67
1	11.0±0.61	12.6±0.72*
2	8.0±0.30	10.9±0.48**
3	6.8±0.25	9.4±0.20**
4	5.8±0.12	8.3±0.22**
6	5.6±0.17	7.2±0.26*
8	4.6±0.31	5.8±0.24*
12	3.5±0.12	3.6±0.08
24	3.1±0.19	3.2±0.15

Values are expressed as mean±SEM. **Significant at $P<0.01$, *Significant at $P<0.05$. ATV: Atorvastatin (20 mg/kg), VER: Verapamil (25 mg/kg), GTE: Green tea extract (10 mg/kg), SEM: Standard error of mean

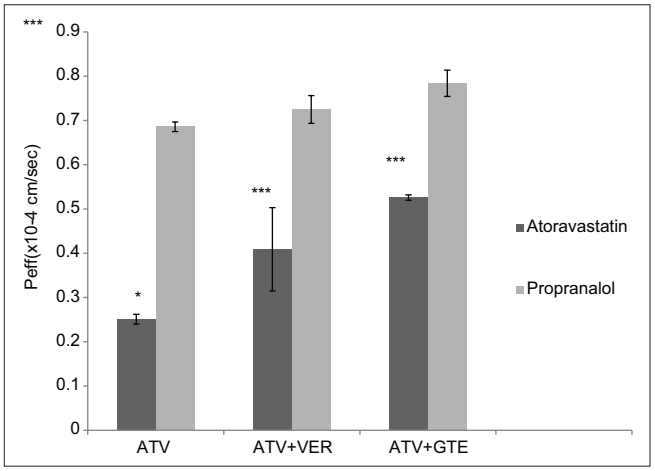

Figure 2: Effective permeability of atorvastatin in presence of verapamil and GTE. Statistical data represented as mean ± standard deviation ($n = 6$). ***$P < 0.001$. Statistically significant. $P < 0.05$. *Significant difference *($P < 0.05$) in comparison with the control. ATV: Atorvastatin (20 mg/kg), VER: Verapamil (25 mg/kg), GTE: Green tea extract (10 mg/kg)

Table 2: Pharmacokinetic parameters of of atorvastatin in control and green tea Extract pretreated group rats 10 mg/kg (po)

Pharmacokinetic parameters	Atorvastatin 20 mg/kg; po		
	ATV	ATR + VER	ATR + GTE
C_{max} (ng/ml)	0.336±0.01	1.524±0.225***	1.907±0.225***
T_{max} (h)	2	3	2
$T_{1/2}$(h)	10.85±3.20	5.28±0.42*	4.37±0.63*
K_{el} (h^{-1})	0.085±0.008	0.084±0.008	
AUC_{0-t} (h.ng/ml)	2.581±0.34	12.516±2.77*	14.62±2.13**
$AUC_{0-\infty}$ (h.ng/ml)	15.633±1.71	103.48±29.08*	91.50±18.86*
CL/F (ml/h/kg)	1200.75±0.92	233.46±1.12***	206.48±29.79***
VZ (ml)	18808.8±15.06	1778.83±115.93***	1303.72±155.93***
Vss (ml)	15329.90±16.06	2242.98±165.930***	1420.21±125.44***

Data values are presented as mean±SD (n=6). *Significant difference (P<0.05) in comparison with the control, **P<0.01, ***P<0.001. ATV: Atorvastatin (20 mg/kg), VER: Verapamil (25 mg/kg), GTE: Green tea extract (10 mg/kg), SD: Standard deviation, AUC: Area under the curve

Table 3: Mean pharmacokinetic parameters of atorvastatin (40 mg/kg) in presence and absence of green tea extract in healthy human volunteers

Pharmacokinetic parameter	ATV	ATV + GTE
C_{max} (ng/ml)	11.0±0.61	12.6±0.72*
T_{max} (h)	1	1
$AUC_{(0-12\ h)}$ (ng h/ml)	69.9±0.52	85.8±0.56***
AUC_{tot} (ng h/ml)	117.1±1.58	120±0.57
$AUMC_{(0-12\ hr)}$ (ng h^2/ml)	351.2±2.49	428.9±3.05***
$AUMC_{tot}$ (ng h^2/ml)	1060±7.50	1675±7509***
$T_{1/2}$ (h)	7.9±0.62	9.8±0.33***
MRT (h)	9.1±0.08	13.8±0.45***
CL (l/h)	344.2±1.09	333.7±4.53*
V_d (l)	460±95.94	314±35.66***

Values are expressed as mean±SEM. ***Significant at P<0.001; *Significant at P<0.05; Compared to atorvastatin baseline values. Statistical analysis was performed using paired t-test. ATV: Atorvastatin (40 mg/kg), VER: Verapamil (25 mg/kg), GTE: Green tea extract (10 mg/kg), SEM: Standard error of mean

permeability of atorvastatin (30 μM) was significantly increased by co-perfusion with verapamil (200 μM) and GTE 10 mg/kg indicates the inhibition of P-gp at the absorption site. It has been previously reported that verapamil is a P-gp inhibitor and improves the intestinal permeability of paclitaxol (P-gp substrate) by blocking the P-gp mediated efflux.[17] As the same way, atorvastatin intestinal permeability was increased with verapamil and GTE. These results were indicated that P-gp limits the intestinal permeability of atorvastatin by extruding it back to the intestine. Phenol red was used as a nonabsorbable marker in *in situ* technique. Phenol red concentration in the outlet perfusate was used to indicate the intestinal mucosa integrity during perfusion. Propranolol was used as a passive permeable marker and as an indicator of major changes in mesenteric blood flow. The permeability values of propranolol (highly permeable marker) in the absence and presence of verapamil and GTE were found to be statistically insignificant. Therefore indicating that changes in atorvastatin permeability in the presence of P-gp inhibitors such as verapamil and GTE is not due to compromise in membrane integrity. Rat is the best Fa, human (fraction of the dose absorbed in humans) predictor animal models for passive permeability and further, there exists a similar level of P-gp expression and overlapped substrate specificity with quantitatively same affinity for a large number of P-gp substrates in rat mdr1a and human MDR1.[18] Therefore, pharmacokinetic studies in rat provide more meaningful forecast on human absorption of P-gp substrates.[19] The selection of doses of atorvastatin and verapamil was based on the pharmacokinetic properties of the study and GTE dose was decided based on the presence of catechins content in extract. Verapamil and GTE expedited the peak plasma concentration and AUC with a concomitant decrease in plasma clearance. Reduction in atorvastatin plasma clearance may due to inhibition of P-gp. Further clinical studies required to reduce the dose of atorvastatin by using above mentioned P-gp inhibitors. GTE shows more significant values than verapamil. Apart from these natural P-gp inhibitors such as GTE produce less side effects than verapamil and also it produced a pronounced effect. In addition, clinical trials proved that green tea has lot of beneficial effects along with an anti-hyperlipidemia activity.[20] Green tea reduces the cholesterol levels in serum in hyperlipidemic patients, and my study suggests that a combination of atorvastatin and green tea, combination produce more beneficial effect in hyperlipidemic patients. The increased bioavailability of atorvastatin might be attributed to the P-gp inhibition by GTE but not because of the CYP3A4 inhibition, green tea (*Camelia sinensis*) extract does not alter Cytochrome P450 3A4 activity in healthy human volunteers.[21] Grapefruit juice increased the serum concentrations of atorvastatin and increased the bioavailability by CYP3A4 inhibition.[22] Drug interactions are the main possible reason for the exacerbation of serious adverse effects of statins.[23] Drugs are repeatedly taken up and pumped out of the enterocytes by P-gp, thus increasing the probability of drugs being metabolized by CYP enzymes in enterocytes. In the intestinal tract, P450s are present in the crypt cells, but the highest concentration

is in the enterocytes at the tips of the villi and can account for first-pass metabolism of many drugs.[24] According to the earlier evidence, GTE is a P-gp inhibitor but not the CYP3A4 inhibitor. Increased bioavailability of atorvastatin in the present study might be due to the P-gp inhibition in the small intestine and reduced metabolism in the enterocytes, but not in the liver. Further lipid-lowering property of the GTE could be beneficial to the patients when co-administered with atorvastatin. Various experiments that were conducted in animals demonstrated that the hypocholesterolemic effect of green tea is due to decreased absorption of cholesterol and increased excretion of cholesterol.[25-27] The studies in humans reported that for each one cup of green tea, the TC fell by 0.015 mmol/L; consumption of 10 cups of green tea/day reduced TC by 2%.[28]

Conclusions

GTE capsules increased the bioavailability of atorvastatin may be due to inhibition of P-gp, and also, the dose of atorvastatin can be reduced to get the therapeutic benefit. In all the types of therapeutic benefits of atorvastatin, the dose of atorvastatin can be reduced when being given along with GTE. Further studies on a large number of patient populations are recommended to correlate the results of the present study and to outweigh the risk-benefit ratio.

Acknowledgment

PN is thankful to the tart-up research funds of University College of Pharmaceutical Sciences at Kakatiya University, Warangal, AP, India, for the submitted work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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