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The Influence of Aged Garlic Extract on the Uptake of Saquinavir and Darunavir into HepG2 Cells and Rat Liver Slices

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Summary: Bioavailability and therapeutic outcome of treatment with HIV-protease inhibitors depends on intestinal and hepatic transporter-enzyme interplay. Liver transport of HIV protease inhibitors (saquinavir, darunavir) was assessed in the presence of aged garlic extract, because the HIV-infected often consume garlic supplements together with prescribed therapy. The in vitro uptake of both drugs into HepG2 cells and precision cut rat liver slices significantly increased in the presence of Pgp and MRP-2 inhibitor ritonavir. The incubation medium containing aged garlic extract caused significant inhibition of saquinavir efflux from HepG2 cells and precision cut liver slices, while the activity of darunavir efflux transporters in both liver models significantly increased. Due to opposite in vitro interactions observed between aged garlic extract and HIV protease inhibitors, darunavir and saquinavir most probably bind to different binding sites on one or both efflux transporters. Based on this study, coadministration of investigated compounds with garlic supplements could result in significant in vivo modification of hepatic transport-enzyme interplay, possibly leading to further bioavailability change.

Keywords: HepG2 cells; rat liver slices; saquinavir; darunavir; aged garlic extract; efflux transporters

Introduction

Oral administration of drug combinations for HIV treatment is known as highly active antiretroviral therapy (HAART). Currently there are 24 drugs approved for HIV treatment with many more still in different stages of preclinical and clinical testing to address the ever-growing economic burden of HIV treatment and constant need for more tolerable drugs. Two-thirds of HIV infected use complementary and alternative medicine (CAM), vitamins and herbal preparations (garlic, ginseng, echinacea, aloa) to regain a feeling of self-control and overcome frequent adverse drug reactions. Nevertheless, drug-phytocchemical pharmacokinetic interactions that lead to significant changes of drug plasma profiles have been widely recognised as a result of altered activities of intestinal and hepatic transporter-enzyme interplay.

Garlic preparations are very common among the HIV infected, because they exert cholesterol-lowering effect and alleviate hypercholesterolemia caused by long-term HAART. However based on case-reports and clinical studies regarding concomitant consumption of HIV protease inhibitors and garlic supplements, significant pharmacokinetic changes and serious adverse reactions were reported. Namely, in the case of ritonavir (Rito) and garlic simultaneous application severe gastrointestinal toxicity in a patient starting ritonavir therapy was observed. Furthermore saquinavir (Saq) administration with garlic to ten healthy male volunteers resulted in more than 50% decrease of Saq AUC and cmax. Although Cyp3A4 inhibition (short-term usage) and induction (long-term usage) by garlic phytochemicals are recognised as reasons for the observed changes of Saq pharmacokinetic parameters, the exact mechanism behind this interaction is still unclear. Thus either metabolites of garlic phytochemical(s) or Saq metabolite responsible for the observed Cyp3A4 inhibition/induction remain to be identified. There are no reports on the effect of garlic preparations on intestinal or hepatic efflux transporters involved in the transport of HIV-protease inhibitors (HIV-PI). Therefore this aspect needs further evaluation. Namely, substances that induce CYP3A izoenzymes usually exert their action on Pgp too. In 2006 a novel HIV-PI darunavir (Prezista®) was approved by FDA which should be applied twice daily to treatment-ex-
Because Dar metabolism is mediated by Cyp3A4,8,9 interclinically relevant increase of systemic exposure to Dar.8) metabolism in the intestine and liver, which results in therapy must be boosted by Rito to inhibit Dar Cyp3A4 achieve effective plasma concentrations, darunavir (Dar) experienced or once daily to treatment-naive patients. To

To evaluate the influence of aged garlic extract (AGE), a water-ethanol standardized garlic supplement, on hepatic efflux transporters, the HepG2 hepatoma cell line and precision cut rat liver slices were used. Due to low gene expression encoding for cytochrome transcription factors, there is no or very little gene transcription of genes coding for Cyp1A1, 1A2, 3A and 2E1 enzymes in the HepG2 cell line.10) Studies for HepG2 transporters also indicate that this cell line expresses multidrug resistance associated protein 2 (MRP-2) and P-glycoprotein (Pgp) transporters in sufficient levels to allow qualitative evaluation of AGE effects on transporter (Pgp, MRP-2) activity.11) The results obtained with HepG2 cells were compared to the uptake of both HIV-PI in precision cut liver slices, because the magnitude of change determined in vitro with cancerous cell lines does not necessarily reflect the magnitude of response to the same stimuli for normal tissue.

Materials and Methods

Materials: Verapamil (Ver), MK-571, rhodamine 123 (Rho123), fluorescein (FLU), gentamicin, ampicillin, insulin, L-glutamine, Williams medium E, salts for incubation salines and all the necessary ingredients for cell cultivation were from Sigma Aldrich Chemie (Deisenhofen, Germany). ATP measuraments for cell viability assessment were performed with CellTiter-Glo® Luminiscent cell viability assay from Promega. Saquinavir (Saq), and ritonavir (Rito) were purchased from Sequoia Chemicals Inc. (Mission Viejo, CA, USA), lot number 5H04A. The AGE used in this study was standardized to 1.27 g/l S-allylcysteine content, the compound normally present in liquid nitrogen 3-times and by ultrasound sonification. After 10 min centrifugation at 16500 rpm at 4°C, 10 uL of supernatant were used for protein determination by Bradford method. The assay was performed according to the manufacturer’s instructions. The samples for HPLC analysis were prepared by precipitating 80 uL of supernatant with 80 uL of acetonitrile on ice for at least 10 min and then centrifuged again for 10 min at 12500 rpm and 4°C. Supernatant was used for HPLC analysis. The intracellular Saq or Dar concentrations determined by HPLC were normalized to the protein content measured by the Bradford method.

Reference experiments (Ref) are the uptake measurements of Saq/Dar alone without the addition of AGE or any other transporter modifying substances.

Impact of permeated AGE concentration (AGE-P) on the uptake of Saq and Dar into HepG2

The experiments were performed in the same manner as described in the previous section. To obtain physiologically more relevant concentration of AGE that reaches the liver after oral administration of garlic supplements and all potential AGE metabolites, which could be formed during intestinal metabolism, 2.5 mL of Ringer buffer pH 7.4 supplemented with 10 mM L-glucose and 10 mM L-glutamine containing 1 v/v% of AGE were applied to the mucosal side of rat duodenum mounted in side-by-side diffusion chambers and after 3 h of incubation, serosally applied 2.5 mL of Dulbecco’s modified Eagle’s medium were collected, containing all possible AGE metabolites and physiological AGE concentration (AGE P). It was then supplemented with FBS, L-glutamine, antibiotics as described in a) and with investigated substance and used in the uptake experiments.

In vitro experiments with rat duodenum and rat liver slices

The experiments conducted with rat intestinal and
hepatic tissue conform to the Law for the Protection of Animals (Republic of Slovenia) and were registered at the Veterinary Administration of the Republic of Slovenia. Rat small intestine and liver were obtained from male Wistar rats (250–320 g) fasted 18 hours prior to the experiments. After euthanasia and laparotomy, the intestine was rinsed with ice-cold 10 mM glucose Ringer solution. Duodenum, located 25 cm proximally from the pyloric sphincter was used for the experiments, because CYP expression in this segment is highest. The intestinal tissue was cut into 3 cm long segments, excluding visible Peyer’s patches. These intestinal segments were opened along the mesenteric border, stretched onto inserts with an exposed tissue area of 1 cm², then placed between two compartments of EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, USA) maintained at 37°C and continuously oxygenated and circulated by bubbling with carbogen (95% O₂, 5% CO₂). The liver was rinsed with Ringer buffer and preserved in ice-cold Williams medium E supplemented with L-glutamine (0.29 mg/mL), insulin (0.13 IU/mL), gentamicin (50 µg/mL) and ampicilin (100 µg/mL). Rat liver slices (250 µm thick) for the uptake experiments were prepared and incubated in the same medium as used for liver transportation. Saq and Dar were added to give final 20 µM. The influence of AGE (1 v/v%) and Rito (50 µM) was also evaluated in parallel experiments. After 1.5 h incubation, the liver was weighed (wet tissue weight), homogenized and the homogenate was precipitated with acetonitril (1:1). The supernatant was analyzed by HPLC. Measured intracellular Saq/Dar concentrations were normalized to the wet tissue weight.

Analysis of investigated substances

Concentrations of FLU, Rho(123) were measured with a fluorescence detector Tecan GENious (λex = 485 nm, λem = 535 nm). 100 µL of a sample were diluted with 100 µL of 0.025 M NaOH to measure the concentration of FLU or with 100 µL of 0.01 M HCl for Rho123 measurement.

Saquinavir and darunavir were analysed by HPLC (all components: binary pump, well-plate sampler, column thermostat and diode-array UV detector were Series 1100 from AgilentTechnologies, Waldbronn, Germany). Phenomenex Onyx Monolythic C18 50 × 4.6 mm column with guard column was used. Aqueous phosphate buffer (pH 4.6) and acetonitril (65:35 for saquinavir and 68:32 for darunavir) were used for the separation. The flow rate was adjusted to 2 ml/min for saquinavir and 2.2 ml/min for darunavir. Column temperature was 30°C and 39°C for saquinavir and darunavir, respectively. The retention times were 3.5 min for saquinavir and 2.1 min for darunavir and detection wavelengths were 240 nm and 266 nm, respectively.

Data analysis

Results in Tables and Figures are presented as means ± SD of 4 measurements in case of determining the uptake of drugs into HepG2 and liver slices. Data were evaluated statistically using SPSS 16.0 for Windows. When appropriate, F-test for testing the equality of variances and, afterwards, 2-tailed student t-test (α=0.05), were used. Otherwise, one way ANOVA, followed by Bonferroni post-hoc test was applied.

Results

Uptake of Pgp substrate Rho123 and MRP substrate FLU into HepG2 cells: Although there are numerous reports of sufficient Pgp and MRP expression in HepG2 cells, Rho123 (a Pgp substrate) and FLU (a MRP substrate) were utilized in the uptake experiments as positive controls to confirm the presence and adequate activity of both transporters before applying HIV-PI. The activity of Pgp and MRP were additionally evaluated by Pgp inhibitor Verapamil (Ver) and MRP inhibitor MK-571. The influence of 1 v/v% AGE on both...
transporters was also investigated and the results are presented in Figures 1 and 2 for Rho123 and FLU, respectively.

The uptake of Pgp substrate Rho123 and MRP substrate FLU increased significantly (p<0.05) in the presence of inhibitors Ver and MK-571 (Figs. 1 and 2). The Ver inhibitory impact on Pgp became significant after the 7th minute and continued till the end of the experiment, while significantly higher FLU intracellular accumulation in the presence of MRP MK-571 inhibitor was observed for all time points compared to reference (Ref) values. With AGE in the incubation medium there was a significant decrease (p<0.05) in Rho123 uptake, whereas that of FLU significantly increased (p<0.05) and was identical to the intracellular concentration profile obtained for FLU in the presence of MK-571.

Based on these experiments with positive controls and inhibitors, sufficient expression and activity of Pgp and MRP transporters were confirmed for the HepG2 cell line. In the presence of AGE, activities of Pgp increased, whereas that of MRP decreased.

**Uptake of Saq into HepG2 cells:** Saq uptake into HepG2 cells was monitored at different Saq donor concentrations as presented in Figure 3. Higher concentrations of Saq in donor media led to higher intracellular Saq concentrations normalized to protein content. At the lowest concentrations applied (2.5 μM), the velocity of Saq intracellular concentration increase was smallest (4.1×10^{-5} mmol/g min), whereas that determined for 20 μM was the highest (2.9×10^{-4} mmol/g min). Since the velocity of Saq cell uptake is proportional to passive diffusion and inversely proportional to efflux transporter activity, at 2.5 μM Saq the contribution of efflux transporters (Pgp and MRP-2) to Saq uptake would be greatest, and therefore this concentration should be the best to detect the impact of AGE on Saq uptake into HepG2 cells. However at higher concentrations, which could better reflect therapeutic Saq concentrations in the portal blood, the effects of tested inhibitors and AGE could be more relevant. Since there is no available literature data regarding portal blood Saq concentrations (only data about Saq plasma concentration after various peroral regimens with soft-gel capsules and ritonavir^{17,18}), 10 and 20 μM Saq concentrations were chosen to be tested in this regard. Therefore the influence of 1 v/v% AGE, AGE-P (concentration of AGE that permeated through the rat duodenum and also contains possible AGE metabolites), Ver, MK-571 and Rito on the HepG2 Saq uptake was evaluated at 3 donor concentrations. For better transparency of the results we compared intracellular Saq concentrations only at 3 time points (5, 10, 30 min). The results are given in Table 1.

At 2.5 μM Saq donor solution, the inhibitory effect of Ver and MK-571 was significant (p<0.05) at all three time points compared to the reference values. These results confirm that Saq transport in HepG2 cells is mediated by both, Pgp and MRP-2 transporters. The influence of Rito on Saq uptake was evaluated at 10 μM Saq, since Saq therapy is usually boosted with this Cyp3A4, Pgp and MRP-2 substrate/inhibitor^{16} to achieve higher Saq bioavailability. According to the results in Table 1, Rito induced significant increase (p<0.05) in Saq HepG2 cell retention due to Pgp and MRP-2 inhibition. LC-MS/MS analysis of samples from the reference experiment with HepG2 cells showed an absence of Saq metabolites and so the Rito-induced Saq uptake increase could only be due to the transporter and not to Cyp inhibition. The addition of 1 v/v% AGE to incubation media

**Table 1.** Influence of AGE and transporter inhibitors on Saq uptake into HepG2 cells. Intracellular Saq concentrations normalized to intracellular protein content (ICT c(Saq)/ICT c(protein)) for experiments with three (2.5, 10 and 20 μM) Saq donor concentrations (Ref) or in the presence of different modifying substances (MS): AGE, AGE-P, Verapamil (Ver), MK-571 and Ritonavir (Rito). The results are presented as means of 4 measurements ± SD

<table>
<thead>
<tr>
<th>Saq [μM]</th>
<th>MS [μM]</th>
<th>ICT c(saq)/ICT c(protein) [mmol/g] (×10^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>2.5</td>
<td>Ref</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>2.5</td>
<td>AGE</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>2.5</td>
<td>Ver [50]</td>
<td>11.5 ± 1.0</td>
</tr>
<tr>
<td>2.5</td>
<td>MK-571 [50]</td>
<td>8.7 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>Ref</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>AGE</td>
<td>31.7 ± 3.0</td>
</tr>
<tr>
<td>10</td>
<td>AGE-P</td>
<td>20.2 ± 2.1</td>
</tr>
<tr>
<td>10</td>
<td>Rito [50]</td>
<td>29.0 ± 0.8</td>
</tr>
<tr>
<td>20</td>
<td>Ref</td>
<td>34.1 ± 3.3</td>
</tr>
<tr>
<td>20</td>
<td>AGE</td>
<td>53.8 ± 5.1</td>
</tr>
</tbody>
</table>

*– change of intracellular Saq content normalized to protein concentration was statistically significant (p<0.05) (compared to reference values at the same time point).
Table 2. Influence of modifying substances and AGE on Dar uptake into HepG2 cells. Intracellular Dar concentrations normalized to intracellular protein content for experiments with 10 and 20 μM Dar donor concentrations (Ref) or in the presence of different modifying substances (MS): AGE, AGE-P, Verapamil (Ver) and Ritonavir (Rito). The results are given as means of 4 measurements with SD.

<table>
<thead>
<tr>
<th>Dar [μM]</th>
<th>MS [μM]</th>
<th>ICTc(Dar)/ICT c(protein) [mmol/g (×10⁻⁵)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>10</td>
<td>Ref</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>AGE</td>
<td>2.7 ± 0.0a</td>
</tr>
<tr>
<td>10</td>
<td>Ver [100]</td>
<td>7.1 ± 0.0a</td>
</tr>
<tr>
<td>10</td>
<td>Rito [50]</td>
<td>11.5 ± 0.8a</td>
</tr>
<tr>
<td>20</td>
<td>Ref</td>
<td>24.6 ± 0.2</td>
</tr>
<tr>
<td>20</td>
<td>AGE</td>
<td>24.2 ± 1.4</td>
</tr>
<tr>
<td>20</td>
<td>AGE-P</td>
<td>24.2 ± 0.8</td>
</tr>
</tbody>
</table>

- change of intracellular Dar content normalized to intracellular protein content.

Table 3. Influence of AGE and Rito on the uptake of Saq and Dar into rat liver slices. Intracellular concentration of Saq or Dar after 1.5 h incubation with rat liver slices normalized to the wet tissue weight. The intracellular concentration was monitored with Saq or Dar in incubation medium alone (Ref) or in the presence of AGE (1 v/v %) or Rito (50 μM). The data represent the average of 4 measurements with SD.

<table>
<thead>
<tr>
<th>ICT rat liver slices HIV-PI (×10⁻⁴ mol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref</td>
</tr>
<tr>
<td>Saq</td>
</tr>
<tr>
<td>Dar</td>
</tr>
</tbody>
</table>

- change of intracellular Saq/Dar content normalized to wet tissue weight was statistically significant (p<0.05) compared to appropriate reference value.

resulted in significantly higher (p<0.05) intracellular Saq concentrations for all donor solutions tested at all time points (except at 30 min for 20 μM Saq). Similar Saq intracellular concentration increase was observed also after the addition of AGE-P but with less pronounced effect than with AGE alone.

Uptake of Dar into HepG2 cells: Rapid in vivo Dar absorption159 and significantly lower food effect on Dar than on Saq bioavailability160 indicate, that Dar could be highly permeable. Therefore investigating lower (i.e. 2.5 μM) Dar concentrations would not be relevant when simulating physiological concentrations in portal blood. Namely after 400 mg Dar peroral administration Dar plasma concentrations reach maximal values after 3 h (ca 60 μM) and remain above 10 μM till the next dose.20

The uptake of Dar into HepG2 cells was therefore monitored at 10 and 20 μM Dar. Higher Dar concentrations were not applied because of toxicity (ATP vitality test indicated less than 90% of HepG2 cell vitality at higher concentrations). The impact of AGE, Ver (100 μM), Rito (50 μM) and AGE-P on Dar uptake into HepG2 cells is presented in Table 2.

Our results for 10 μM Dar presented in Table 2 show that Ver had no significant impact on Dar cell retention (Pgp efflux of Dar was not influenced by Ver at 10 and 30 min (p>0.05), although it was significantly inhibited for the first 5 min). A potent Pgp and MRP-2 inhibitor160 Rito significantly inhibited (p<0.05) efflux of Dar from HepG2 cells at all time points compared to the reference values. We concluded that in HepG2 cells Dar efflux is mediated mostly by MRP-2, since Ver did not influence intracellular Dar content. However, the contribution of Pgp could not be excluded. The presence of AGE at 10 μM Dar induced significant decrease (p<0.05) in intracellular Dar concentrations but at 20 μM Dar, AGE or AGE-P had no significant influence (p>0.05) on Dar concentrations inside HepG2 cells at any time point.

HIV-PI intracellular concentrations in rat liver slices: To ascertain whether the observed opposite AGE effect on Saq and Dar efflux transporters in HepG2 cells also occurs in normal liver tissue, three 1.5 h long parallel experiments were performed with precision cut rat liver slices as designated in Table 3. Saq or Dar was incubated at 20 μM concentration in all experiments and AGE and Rito were applied in 1 v/v % and 50 μM concentrations, respectively. Significant changes (p<0.05) of intracellular Saq or Dar concentrations compared to reference values (HIV-PI in Table 3) were observed in the presence of both substances. AGE and Rito inhibited efflux of Saq from liver slices causing significant increase (p<0.05) in intracellular Saq content, whereas in the case of Dar, its efflux significantly increased (p<0.05) in presence of AGE and decreased with Rito in the incubation medium, which is consistent with the results obtained on HepG2 cells for Dar.

Discussion

The uptake results obtained with Rho123 and FLU (Figs. 1 and 2) showed that the expression and activities of Pgp and MRP efflux transporters in HepG2 cell line used were sufficient to allow further assessment of both efflux transporter contribution to HIV-PI (Saq, Dar) transport from HepG2 cells and evaluate the impact of garlic phytochemicals in AGE on their activities. According to the results, Saq efflux from HepG2 cells was mediated by Pgp and MRP-2 (Table 1), whereas in the case of Dar, MRP-2 efflux is most probable (Table 2), which is in accordance with the data from Drewe et al.21 However, Pgp involvement in Dar transport from hepatocytes could not be excluded, since Rito, a potent MRP-2 and Pgp inhibitor used in the experiments, exerts higher affinity for Pgp than an unspecific inhibitor Ver (Table 2). Further support for the involvement of both transporters in Dar transport is given by a study by Fujimoto et
al. who found that Dar basolateral-to-apical transport through Caco-2 cell monolayers and MDR1 gene transfected LLC-PK1 cells was also mediated by both (Pgp and MRP-2) transporters.\(^{22}\)

The activities of Pgp and MRP-2 transporters were modulated by AGE and AGE-P (permeated AGE; described in the Materials and Method section). With positive controls (Rho123, FLU—Figs. 1 and 2) the activity of Pgp significantly increased, whereas that of MRP-2 significantly decreased in the presence of AGE. AGE effect on Pgp transporter is in accordance with previous in vitro studies using rat ileum and Caco-2 cell monolayers to determine apparent permeability of Pgp substrates in the presence of AGE. Namely the intestinal Pgp-mediated transport of all substrates (Rho123, digoxin, hydrochlorothiazide, glibenclamide and others) also increased. However in vitro intestinal MRP-2 transport of DNP-SG,\(^{12}\) atorvastatin, pravastatin, valsartan, losartan and furosemide through the rat duodenum after AGE addition increased,\(^{14}\) which is in contradiction with AGE effect on MRP-2 transporters observed in HepG2 cell line. Because MRP-2 transporters expressed in intestinal and hepatic tissues/cell lines are structurally identical, the observed discrepancies between AGE effect on intestinal, Caco-2 and HepG2 MRP-2 transporters could be caused only by altered MRP-2 regulation in liver (HepG2) compared to intestine and Caco-2 cells. In fact, clear distinctions regarding MRP-2 regulation in different organs (intestine, liver and kidney) have been observed also by others.\(^{23–25}\) In the case of hepatectomy,\(^{23}\) a diminished liver efflux by MRP-2 is compensated by increased intestinal MRP-2-mediated efflux, whereas in obstructive jaundice\(^{24}\) insufficient liver MRP-2 activity leads to increased intestinal Saq and MRP-2 upregulation in liver and kidney, respectively. Pregnancy has also been noted as a condition, during which MRP-2 protein content decreases in liver and remains unchanged in the intestine. Decreased MRP-2 levels in liver regeneration trigger a compensatory mechanism, causing significant increase in Pgp and MRP-1.\(^{25}\) Based on this data MRP-2 inhibition observed with AGE and FLU in HepG2 cells is most probably caused by distinct differences in intracellular pathways involved in the regulation of MRP-2 transporters in the liver and is different from that in intestine.

Liver transport of investigated HIV-PI changed in the presence of AGE and AGE-P. The uptake of Saq and Dar were significantly altered in both models used (HepG2 cells, rat liver slices). Intracellular Saq content in HepG2 and liver slices increased, whereas that of Dar decreased (Tables 1, 2 and 3). Both efflux proteins involved in Saq and Dar transport possess multiple binding/regulatory sites (Pgp four and MRP-2 two\(^{12}\)). Therefore substrate (i.e. HIV-PI) transport depends on allosteric modification inflicted by binding of other present modifying agents (e.g. garlic phytochemicals) to other binding/regulatory sites or in the case of competition for the same binding site, it depends on the affinity of both substances for the efflux protein. If garlic phytochemicals bind to the binding/regulatory site on efflux protein, which is not involved in the transport of HIV-PI studied, allosteric changes induced usually cause the transition of HIV-PI binding site from low to high affinity conformation (known as positive-cooperative effect) resulting in higher extrusion of the drug from the cells. This was observed with Dar, whereas in the case of Saq, it most probably competes with garlic phytochemicals for the same binding site. This resulted in lower Saq efflux from the HepG2 cells and rat hepatocytes. Based on the opposite effects of garlic phytochemicals on Pgp and MRP-2 transporters for Saq and Dar, it is clear that although these drugs belong to the same pharmacological group of compounds, differences in chemical structure are most probably responsible for binding of both drugs to different binding sites on efflux transporters. Therefore in polypharmaceutical patients, interactions that are recognized for Saq do not necessarily apply for the novel HIV-PI Dar. Therefore bioavailability changes, which could occur in vivo after concomitant consumption of Dar and garlic supplements, need further evaluation. Namely, in this study we evaluated only the activity of efflux transporters in the presence of AGE. However, liver Cyp3A4 metabolism is an important bioavailability limiting factor for Saq\(^{19}\) and also for Dar.\(^{8}\) Foster et al.\(^{26}\) reported significant inhibition of CYP 2C9*1, 2C19, 3A4, 3A5 and 3A7 isoenzymes in the presence of garlic and garlic supplements. Changed liver Saq and Dar transport from hepatocytes to bile and concomitant CYP3A4 inhibition could have profound effect on their plasma profiles. Saq bioavailability could increase, whereas that of Dar could decrease. However, efflux transporters and Cyp3A4 enzymes are also present in the gut, the first barrier that encounters ingested herbal preparation with all phytochemicals contained in the original preparation at even higher concentrations than those that are absorbed and reach the liver through the blood stream. Low intestinal Saq solubility prevents efflux transporters (Pgp, MRP-2) to be saturated and therefore the majority of Saq absorbed through the mucosal enterocyte membrane is effluxed back into the intestinal lumen leaving small amounts of Saq in the enterocytes available to be metabolized by Cyp3A4. Considering that in the presence of AGE, Pgp and MRP-2 intestinal transporters are activated\(^{1,2,14}\) and Cyp3A4 is inhibited, the amount of Saq permeating through the intestinal wall could be either unchanged due to counteracting garlic effects on efflux transporters and Cyp3A4, lower in the case of more pronounced efflux by Fgp and/or MRP-2 or higher in the case of more pronounced Cyp3A4 inhibition. Due to the complexity of intestinal and hepatic enzyme-transporter interplay in the presence of AGE or AGE-P, it is difficult to speculate
possible Saq bioavailability changes after concomitant garlic and Saq application. The modulated activity of intestinal and hepatic transporters (Pgp and MRP-2 activity) and Cyp3A4 inhibition by AGE coadministration could also alter Dar pharmacokinetic parameters and influence its bioavailability depending on the prevailing effect of garlic phytochemicals in intestine and liver (either CYP3A4 inhibition or Pgp and MRP-2 modulated activity).

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