Regular Article

Ex Vivo and In Vivo Investigations of the Effects of Extracts of Vernonia amygdalina, Carica papaya and Tapinanthus sessilifolius on Digoxin Transport and Pharmacokinetics: Assessing the Significance on Rat Intestinal P-glycoprotein Efflux

Enoche Florence OGA, Shuichi SEKINE and Toshiharu HORIE*
Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

Summary: Vernonia amygdalina (VA), Carica papaya (CP), and Tapinanthus sessilifolius (ML) are widely used in some countries as medicinal herbs to treat ailments including malaria, cancer, and diabetes. We previously reported the inhibitory effects of these herbs on permeability glycoprotein (P-gp) in Caco-2 cell monolayers. This study used ex vivo and in vivo models to investigate the likelihood of P-gp-mediated herb-drug interactions occurring. The study utilized excised rat intestinal tissues mounted in Ussing chambers to predict changes in drug absorption and an in vivo study in rats using digoxin as the P-gp substrate. Apparent permeability values and pharmacokinetic parameters of digoxin were compared to determine if co-administration of digoxin with ML, CP, or VA modulated the activity of P-gp. When VA was co-administered, the total area under the plasma concentration-time curve was significantly higher (2.1-fold) than when digoxin was administered alone. Co-administration of ML, VA, and CP significantly increased the mean digoxin apparent permeability in the mucosal-to-serosal direction by 7.8, 43.3, and 54.5%, respectively, in comparison to when digoxin was administered alone. These findings suggest that VA increases intestinal absorption of digoxin in vivo by inhibiting P-gp and may also modulate the pharmacokinetic disposition of other p-gp substrate drugs.

Keywords: drug disposition; herb-drug interaction; intestinal absorption; P-glycoprotein; pharmacokinetics

Introduction

Herb-drug interactions (HDIs) can occur when herbal and conventional medicines are co-administered, and the possible changes in pharmacokinetic disposition as well as the pharmacodynamic impact of such interactions need to be investigated.1,2) Several HDIs have been reported including the influences of St. John’s wort, ginseng, gingko, and milk thistle when they are co-administered with conventional medicines.3,4) The majority of these HDIs interfere with cytochrome P450 enzymes.5) Although reports concerning the influence of HDIs on xenobiotic transporters are limited, the impact of HDIs on permeability glycoprotein (P-gp) is slowly gaining interest.6–8) P-gp is an efflux protein that pumps its substrates out of cells in an ATP-dependent manner, thereby reducing the intracellular accumulation of these substrates.9) P-gp is expressed on the apical membrane of epithelial cells where it extrudes several orally administered drugs into the intestinal lumen and thereby interferes with drug disposition.10,11) Inhibition of P-gp is of great clinical interest as this can enhance the oral bioavailability of specific drugs and reverse multidrug resistance (MDR). However, the mechanisms and potential influence of HDIs on P-gp are not fully understood.

Caco-2 cells are widely used to predict drug absorption patterns because they retain several morphological and functional properties of enterocytes.12) However, drug transporter expression and the tightness of tight junctions may differ between Caco-2 cells and the human intestine, which may affect drug dissolution.13) Attempts have been made to develop in situ and ex vivo models that more closely replicate intestinal absorption in humans; however, the data obtained from studies using such models may be limited.14) Consequently, further studies using animal models to predict drug absorption in humans are needed. In vivo models using rats are beneficial because mesenteric blood circulation is intact, and the mucosal layer and other factors that can influence drug dissolu-
tion are present. In addition, rats are more comparable to humans than other animal models in terms of paracellular space and metabolism.\textsuperscript{25}

The likelihood of HDIs occurring can be studied by monitoring drug absorption when co-administered with various herbs. Sections of intestinal mucosa mounted on Ussing chambers are useful tools for analyzing such changes in drug absorption, and the results obtained using this model are reported to have more merits than those obtained using Caco-2 cells or parallel artificial membrane permeability assay models. Furthermore, the expression levels of drug transporters and drug-metabolizing enzymes in the Ussing chamber system are comparable to those in vivo and the morphological structure of the intestine is preserved.\textsuperscript{16,17} This study utilized excised rat ileal segments in which P-gp is highly expressed on the apical side of the epithelial cells.\textsuperscript{18,19} The ileum was specifically chosen as the level of absorptive-directed transport is higher in this segment than in other intestinal segments.\textsuperscript{20}

Rats are a valuable animal model to assess pharmacokinetic drug interactions and data obtained from studies using rats has been used to justify the clinical use of specific drugs.\textsuperscript{21,22} Digoxin is a well-known probe drug that is used to investigate intestinal P-gp. It was used in this study because it is clinically significant and selectively transported by P-gp, not by other xenobiotic transporters.\textsuperscript{23,24}

\textit{Vernonia amygdalina} Delile (VA), \textit{Carica papaya} L. (CP), and \textit{Tapinanthus sessilifolius} Blume (ML) are members of the Asteraceae, Caricaceae, and Loranthaceae families, respectively. Leaves of these herbs are commonly used to treat a variety of ailments including malaria, cancer, and diabetes, and they also have nutritional value in the diet. As these herbs are commonly co-administered with conventional drugs, HDIs can potentially occur. The present study was designed to further investigate the inhibitory effects of these herbs on P-gp using the Ussing chamber model and \textit{in vivo} pharmacokinetic studies in rats, to confirm that HDIs occur with these herbs, and to provide further insight into when these P-gp-mediated HDIs may be clinically relevant.

\textbf{Materials and Methods}

\textbf{Reagents and chemicals:} Unlabeled digoxin was purchased from Sigma-Aldrich Co. (St. Louis, MO). Cyclosporin A (CysA) and Verapamil (VER) were from Wako Pure Chem. Industries Ltd. (Osaka, Japan). \textit{3H}-digoxin (40 Ci/mmol) was purchased from PerkinElmer (Boston, MA). All other chemicals and reagents were of analytical grade.

\textbf{Herbs:} The aqueous herbal extracts investigated in this study were from bitter leaf (VA), pawpaw leaves (CP), and a species of mistletoe leaves (ML). The plant parts were collected from their natural habitat in various locations near Jos, Nigeria, and were dried in the shade for several days at room temperature. The identity of the plants was confirmed at the Foresty Research Institute of Nigeria, Ibadan, where voucher specimens were deposited. Leaves were blended using an Ace homogenizer (Nipponseiki Co. Ltd., Tokyo, Japan) and extracts were prepared by decocction for 30 min at 40°C using 100 g of leaves per 100 ml distilled water before being allowed to steep for 6 h. This was followed by filtration with \textit{Advantec} No. 2 qualitative filter paper (Toyo Roshi Kaisha Ltd., Tokyo, Japan) before extracts were concentrated by evaporation at 40°C \textit{in vacuo} using an Eyela CVE-200D centrifugal vaporizer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Samples were stored at 4°C until use.

\textbf{Animals:} Healthy male Sprague Dawley (SD) rats (220–300 g) obtained from Japan SLC Inc. (Shizuoka, Japan) were maintained in the housing facility in a temperature-controlled environment with a 12 h light/12 h dark cycle and received a standard diet with water. The animals were housed in this facility for at least one week before experiments were initiated to ensure proper acclimatization. Prior to each experiment, the rats were fasted overnight but had free access to water and were randomly assigned to different groups. The study was approved by the Chiba University Animal Ethics Committee. The research adhered to the “Principles of Laboratory Animal Care.”\textsuperscript{25}

\textbf{Transport studies across the rat ileum in Ussing chambers:} After anaesthetization with diethyl ether and laparotomy through a midline abdominal incision, the small intestine was immediately excised and immersed in ice-cold, freshly prepared standard Krebs-Ringer bicarbonate buffer (KRBB) [115 mM NaCl, 25 mM NaHCO\textsubscript{3}, 2.4 mM K\textsubscript{2}HPO\textsubscript{4}, 1.2 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 0.4 mM KH\textsubscript{2}PO\textsubscript{4} maintained at pH 7.4] bubbled with carbogen (95% O\textsubscript{2}, 5% CO\textsubscript{2}). Ilea that had been cut 25 cm proximally from the caecum were used. The tissue was rinsed with ice-cold standard KRBB to remove the luminal contents and cut into 1.7 cm long segments, excluding visible Peyer’s patches. The ileum segments were opened along the mesenteric border and stretched onto pins on one half of the chamber with an exposed tissue area of 0.64 cm\textsuperscript{2} placed between two Navicyte side-by-side chambers (Harvard Apparatus, Holliston, MA) clamped together. Each compartment was filled with 5 ml of KRBB supplemented with 10 mM D-glucose or 10 mM mannitol on the serosal and mucosal side of the ileal tissue, respectively. The tissue was kept at 37°C during the experiments using a circulating water bath attached to a heat block. The incubation buffer inside the diffusion chambers was oxygenated and circulated by bubbling with carbogen through a gas manifold. After pre-incubation for 40–50 min with the test inhibitor agent diluted in KRBB (final concentrations of 200 µM VER; 20 µM CysA; 10 mg/ml of each of the herbal extracts), digoxin was added to a final concentration of 10 µM (7 nmol of \textit{3H}-digoxin/mol of unlabeled digoxin) to the incubation buffer on the mucosal side of the chamber. At time intervals of 15, 30, 60, 90, 105, and 120 min after the introduction of digoxin, 1 ml aliquots were withdrawn from the serosal chamber and were replaced with the appropriate volume of drug-free buffer to maintain a constant volume in the chamber. The amount of permeated digoxin was assayed using liquid scintillation counting. The amount of \textit{3H}-digoxin transported was quantified by mixing the 1 ml sample with 2 ml of Clear-sol I scintillation cocktail (Nacalai Tesque, Kyoto, Japan) and radioactivity was measured with a LSC-6100 liquid scintillation counter (Aloka, Tokyo, Japan). Ileal tissue integrity and viability were observed throughout each study by measuring the transepithelial electrical resistance (TEER), using a Millicell ERS-2 (Millipore, Billerica, MA).

\textbf{In vivo pharmacokinetic studies:} The rats were randomly assigned into five different groups each containing 5–9 rats. One group served as the control and these rats were pre-treated with 0.5% sodium carboxymethylcellulose 2 h before receiving 25.6 µmol/kg digoxin by oral gavage (0.17 µmol of \textit{3H}-digoxin/mol of unlabeled digoxin) in 0.2 ml of vehicle. For the groups pre-treated with herbal extracts, the rats were administered a dose of 90 mg/kg (suspected in the vehicle) by oral gavage 2 h prior to receiving digoxin. The VER control group received 25 mg/kg p.o. VER 2 h before receiving digoxin. A heating lamp was used to main-
tain blood temperature, increase circulation, and improve blood collection. Blood samples were taken from the saphenous vein by venipuncture and transferred into heparinized tubes at 15, 30, 45, 60, 90, 120, and 180 min after oral administration of 25.6 μmol/kg digoxin. Blood samples were stored at −20°C before ³H activity was quantified using liquid scintillation after mixing blood with the scintillation cocktail as described above. The rats were sacrificed with an anesthetic overdose on completion of each experiment.

Data and statistical analysis: The apparent permeability coefficient (Papp) was calculated as: Papp = (dQ/dt)/(C × A), dQ/ dt represents the rate of appearance of digoxin in the receiver chamber, A represents the exposed membrane surface area of the ileal tissue (0.64 cm²), and C is the initial digoxin concentration on the donor side. All data are expressed as mean ± standard error of mean (SEM) of at least three replicates for Ussing experiments and 5–9 replicates for in vivo experiments. Statistical significance was assessed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA) considering values of *p < 0.05, **p < 0.01, ***p < 0.001 to be significant using one-way analysis of variance with Dunnett’s post-hoc test for multiple comparisons.

A series of pharmacokinetic parameters was determined based on the individual plasma concentration-time data. Peak digoxin concentrations (Cmax; in ng/ml) were taken directly from the data. Concentration-time data. Peak digoxin post-hoc test for multiple comparisons.

Results

Tissue integrity and viability: The electrophysiological parameter TEER of the ileal segments was monitored throughout the experiments to assess the maintenance of tissue viability and integrity. The values were measured before and during the course of each permeation experiment. The TEER ranged from 55 to 75 Ω·cm² prior to the introduction of the test agents. Any tissue segment that showed a deviation of ±15% from this range was excluded from the experiments. The TEER values were stable with no significant variation following introduction of the test agents. Tissue viability in the Ussing chamber decreases over time; therefore, sampling times did not exceed 120 min.

Mucosal-to-serosal transport across the rat ileal tissue: The time-course of the percentage of digoxin transported in the absorptive direction (mucosal-to-serosal) in the presence or absence of the test agents and the control inhibitors (VER and CysA) is illustrated in Figure 1. Digoxin was used as the model P-gp substrate as it is clinically relevant. The mean apparent permeability values obtained 120 min after the introduction of digoxin are shown in Figure 2 in comparison to the control (digoxin alone). The increase in Papp was highest following treatment with the positive control inhibitor, VER, where Papp was significantly 56% higher than in the control. In comparison to the control, Papp was significantly higher, by 54.5, 43.3, 16.2 and 7.8% following treatment with CP, VA, CysA, and ML, respectively.

In vivo pharmacoKinetic study: To determine whether the inhibition of p-gp observed in vitro (in Caco-2 cells) and ex vivo (in Ussing chambers) also occurs in vivo, pharmacokinetic parameters of digoxin with or without co-administration of the herbal extracts were determined in male SD rats. The mean (±SEM) plasma digoxin concentration versus time profiles after a single dose of oral digoxin was administered alone or was co-administered with ML, CP, VA, or VER are illustrated in Figures 3A–3D. The pharmacokinetic parameters are summarized in Table 1.

ML administration non-significantly increased the AUC₀₋₅ of digoxin by 1.2- and 1.4-fold, respectively, compared to the control group (Fig. 3A). Cmax was 1.2-fold higher in the ML-treated group than in the control group. T₁/₂ increased from 2.0 h to 2.7 h following ML administration. Figure 3B depicts the influence of CP on digoxin pharmacokinetics. Co-administration of CP non-significantly increased the AUC₀₋₅ of digoxin by 1.1- and 1.3-fold, respectively, compared to the control group. Cmax was 1.2-fold higher in the CP-treated group than in the
control group. T1/2 increased from 2.0 to 2.7 h following CP administration. The effects of VA on digoxin pharmacokinetics were detected within the first hour following digoxin administration. Co-administration of VA increased the AUC(0–3) and AUC(0–∞) of digoxin by 1.9- and 2.1-fold, respectively, compared to the control group. Cmax was 1.7-fold higher in the VA-treated group than in the control group. VA administration did not significantly affect clearance or T1/2.

VER inhibits P-gp and thereby reduces digoxin transport. VER was used as a positive control in this study. Figure 3D illustrates the impact of VER on the pharmacokinetics of digoxin. As expected following administration of a P-gp inhibitor, VER significantly increased both the AUC(0–3) and AUC(0–∞) of digoxin by 1.5-fold compared to the control group. VER administration did not significantly affect T1/2, although there was a non-significant reduction in the clearance compared to the control group. Cmax was significantly higher (1.3-fold) in the VER-treated group than in the control group. The absorption rate constant (Ka) increased 1.2-, 1.3-, and 1.4-fold following administration of ML, CP, and VA, respectively, compared to the control group. Therefore, of the herbs studied, VA exerted its inhibitory effect on digoxin transport in the shortest time after administration.

Discussion

The efflux transporter P-gp is highly expressed in the intestinal tract where it extrudes drugs, toxins, and xenobiotics. The pharmacokinetic changes arising from concomitant administration of drugs with P-gp modulators may cause beneficial or detrimental drug interactions. Therefore, drugs and herbs that interfere with P-gp can be clinically important in modifying the bioavailability, pharmacokinetic disposition, and safety profiles of substrate drugs.

The herbs CP, VA, and ML, are popular ethnomedicines with clinical significance that are widely used in certain countries and continents. VA and ML are commonly used in tropical Africa. Other species of Vernonia are medicinally used in the Americas and parts of Asia. Although CP originated from Central America and its fruit is mainly consumed there, several studies report that its leaves are used as a herbal medicine in vast regions of the world. These herbs function as anthelmintics and are used to treat cancer, malaria, diabetes mellitus, fevers, allergies and gastrointestinal disorders. Multi-center clinical trials of VA have been initiated.

Our previous study examined the influence of seven herbs on the bidirectional transport of digoxin using a Caco-2 cell monolayer.
The transport of digoxin in the apical-to-basolateral direction was significantly enhanced by VA, CP, and ML, as well as the P-gp inhibitor VER. Furthermore, the basolateral-to-apical transport of digoxin was significantly inhibited by these herbs. These results suggested that ML, CP, and VA inhibit P-gp. A cell viability assay indicated that these in vitro effects were not due to cytotoxicity. The present study was designed to further investigate these findings by examining the effects of VP, VA, and ML on the modulation of P-gp in ex vivo and in vivo models using digoxin as a clinically relevant substrate.

The present study used crude herbal extracts, rather than single components isolated from the herbs, because patients generally take whole herbal products, usually containing several constituents. Consequently, crude extracts may contain additional components that can affect P-gp, besides their pharmacologically active components. Studies on drug disposition are complicated by the overlapping tissue distributions of CYP3A4 and P-gp, and the broad spectrum of their interacting substrates. Therefore, digoxin was chosen as the substrate in this study because it selectively interacts with P-gp but negligibly with CYP3A4.

In the Ussing chamber study, CP significantly increased the mean apparent permeability (p < 0.001), comparable with VER. However, the AUC did not significantly increase following oral administration of CP in rats. Ussing chambers containing rat intestinal segments provide a simple method to investigate permeability, which highly correlates with absorption properties in humans. However, despite the merits of Ussing chambers, some drawbacks have been reported. For example, Ussing chambers may underestimate drug transport because the drug needs to cross the entire intestinal wall, which might be rate-limiting, whereas this is not necessary in vivo. Furthermore, only rat ilea were used in Ussing chambers in this study and regional differences in intestinal absorption were not investigated. Also, in the rat model, tissue viability is not a limitation, as may be the case in an Ussing system. These factors may underlie the difference in the effects of CP observed in the two systems. Importantly, although the mucosal architecture is intact in the Ussing system, it may not behave exactly as the intact gut does.

Although medicinal herbs are efficacious in the treatment of various ailments, the lack of standard prescribed dosages is a major concern. This may lead to the consumption of large quantities of herbs, resulting in their bioaccumulation and complications in humans. However, non-standardized doses of up to 6 cups (2 cups thrice) of aqueous extracts (approximately 20–40 g of plant material) are consumed daily by humans. We converted the estimated human dose to the equivalent dose in rats according to body surface area. Therefore, 90 mg/kg of all the studied herbs were administered to rats in this study.

The effects of these herbs on the function of P-gp in vivo were investigated using male SD rats. Oral administration of VA enhanced the AUC and $C_{\text{max}}$ of digoxin. The $T_{1/2}$ of digoxin was similar to that previously reported. $T_{1/2}$ did not significantly change when VA or VER were co-administered, which suggests that they increased the intestinal absorption of digoxin but did not affect its systemic elimination. The herbs affected digoxin transport within 1 h of administration. This suggests that digoxin absorption may be enhanced due to inhibition of intestinal P-gp. This is in agreement with the findings of the Ussing chamber study. No significant change in the total clearance of digoxin on co-administration with the herbs was observed. Organ blood flow, extraction ratio, and transporters influence the clearance and disposition of drugs. The in vivo study suggests that VA increases digoxin absorption more potently than VER; co-administration of VA or VER increased AUC$_{0-\infty}$ 2.1- and 1.5-fold, respectively, compared to the control. This effect of VA is of particular importance because in addition to its potent medicinal value, it is also a common vegetable in the African diet. There is no known study reporting that VA may modulate the pharmacokinetics of drugs that are P-gp substrates. Hence, this finding may be of particular clinical relevance as it indicates a food-drug interaction may also occur. Digoxin is a substrate of P-gp, and this interaction results in the extrusion of digoxin from enterocytes throughout the intestinal mucosa into the gut lumen. Consequently, the P-gp inhibitor VER reduced the intestinal secretion of digoxin. VA inhibited the secretion of digoxin more than VER. It is thought that these herbs and P-gp compete for binding to digoxin and this underlies the changes in digoxin disposition observed following its co-administration with herbs.

Extracts of VA have very high concentrations of flavonoids. There is increasing evidence that flavonoids can inhibit P-gp. Flavonoids obtained from the diet or supplements can alter P-gp levels and thereby affect the oral availability of drugs that are transported by P-gp. The flavonoids kaempferide, quercetin, apigenin, naringenin, genistein, and rutin are reported to affect P-gp activity through various mechanisms including direct interaction with the vicinal ATP-binding sites, the steroid-binding site, or the substrate-binding domains, or via a heterotrophic allosteric mechanism. Similarly, the in vivo absorption of nitrofurantoin is enhanced following administration of the flavonoid chrysin due to modulation of transporter function. Several other flavonoids including silymarin, morin, biochanin A, and phloretin modulate P-gp. Therefore, the high level of flavonoids in the herbal extracts may be responsible for the inhibition of P-gp. The bioactive phytochemicals in VA include flavonoids (luteolin, luteolin-7-O-β-glucoronoside, and luteolin-7-O-β-glucoside), bitter sesquiterpene lactones, tannins, and steroid glucosides. Because of the common backbone flavone structure of luteolin and other flavonoids which have shown P-gp inhibition, the observed effect may be partly contributed by luteolin. CP is rich in carpaine, pseudocarpaine, dehydrocarpaine I and II, choline and carposide alongside flavonoids (quercetin and kaempferol) that are found in the leaves. Hydrolysable tannins, terpenes, saponins, and flavonoids are abundant in mistletoe leaves.

ML is a species of mistletoe. Other mistletoe species including Viscum album have been reported to potently inhibit P-gp. In our previous research using Caco-2 cells, treatment with 2–20 mg/ml ML significantly inhibited P-gp, with similar potency to treatment with 100 µM VER. The reason why ML did not inhibit P-gp as potently in the present study may be because the expression of drug transporters and permeability varies between Caco-2 cells and the human intestine. $C_{\text{max}}$, AUC$_{0-\infty}$ of digoxin were increased in the presence of VA in comparison to the control. In addition, the systemic clearance of digoxin was reduced following treatment with ML and VER; however, this was not statistically significant. These results are consistent with a study investigating the HDI potential of curcumin when co-administered with celioprolol and midazolam. A similar study reported that co-administration of VER decreases the clearance of digoxin.

Inhibition of P-gp by VA increased the digoxin plasma concentration after a single oral dose, which is in agreement with the
previous in vitro results. This effect was observed within the first hour after administration, suggesting that VA mainly affects the absorption of digoxin. The findings suggest that VA could have beneficial roles, especially as an adjuvant in cancer chemotherapy and diabetes treatment. Complementary and alternative forms of treatment are utilized by many patients in addition to well-established conventional treatments for several medical ailments. Thus, it is important to investigate the likelihood of changes in drug disposition when conventional and alternative medicines are co-administered. The majority of anticancer agents are P-gp substrates that many cancers are resistant to; therefore co-administration may increase the oral bioavailability of these agents or promote MDR reversal. The hypoglycaemic agents are co-administered, such as digoxin, as this may result in toxic responses. When herbs are used as adjuvants in cancer therapy they can provide support to patients being treated with conventional medicines by alleviating side-effects and improving self-defense and quality of life.\(^{53,59}\) P-gp inhibitors, including tariquidar, GF120918, and bircodar, are used as adjuvants in cancer therapy.\(^{59,60}\) Dose adjustments may be necessary in clinical situations and caution is recommended. Our findings indicate that HDIs need to be investigated further to obtain a more detailed understanding of their impact in human subjects and to ensure appropriate clinical intervention when they are co-administered with conventional P-gp substrates.

In conclusion, the observed changes in the pharmacokinetics of digoxin and the absorption profile from the Ussing chamber study indicate that VA co-administration poses a more clinically significant risk for P-gp-mediated HDIs than CP or ML. The in vitro, ex vivo, and in vivo studies in rats confirm that VA inhibits P-gp. Importantly, significant in vitro P-gp inhibition may not be replicated in animal models.

References


