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Transport of *Schisandra chinensis* extract and its biologically-active constituents across Caco-2 cell monolayers – an in-vitro model of intestinal transport

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Abstract

We have determined the intestinal transport of *Schisandra chinensis* extract and its lignans (gomisin A, gomisin N and schisandrin C) in the Caco-2 cell monolayer model. The transport across monolayers was examined for 2 h in absorptive and secretory directions. Quantitation of lignans was performed by HPLC. Out of the three lignans, gomisin A exhibited bi-directional transport, with P_{app} values in the range of $25–29 \times 10^{-6}\,cm\,s^{-1}$, indicating a passive diffusion. Gomisin N, mixture and *Schisandra* extract displayed a higher transport in the secretory direction with efflux ratios in the range of 2.2–5.2. The efflux was decreased in the presence of inhibitors of multidrug resistance protein (MRP) transporter (MK-571) and P-glycoprotein (verapamil) indicating a possible involvement of an efflux pump and MRP in the transport of *Schisandra* lignans. Poor transport of schisandrin C was observed which could not be quantitated. The permeability of gomisin A in the isolated form was significantly different compared with the mixture or extract.

Introduction

Traditional Chinese Medicines (TCMs) are essential components of alternative medicine. However, the increased and indiscriminate use of natural products, including TCMs has been associated with adverse effects such as drug–drug interactions (Kaplowitz 1997). Often, these drug–drug interactions are reported at intestinal level for the drugs whose transport is mediated by P-glycoprotein (P-gp) and multidrug resistance protein (MRP) transporters (Pedersen 1985; Ito et al 1993; Saitoh & Aungst 1995; Greiner et al 1999; Johne et al 1999; Yu 1999; Mols et al 2005; El-Sheikh et al 2007). Such adverse reactions have caused serious concerns in recent years and a detailed evaluation of dietary supplements, botanicals or herbs that are consumed daily along with conventional medications has become necessary. As part of our continuous efforts to study the medicinal plants/dietary supplements for their intestinal transport and bioavailability, we selected *Schisandra chinensis*, a popular medicinal herb native to northern China, Japan, Russia and Korea for this study. Dried fruits of *S. chinensis*, also known as wu wei zi (five taste berry), have been used in TCM for several thousands of years in China as a health food product. In Japan *Schisandra* fruit is widely used as a component of Kampo medicines, such as sho-seiryu-to, ninjin-eiyou-to etc. *Schisandra* fruit is currently used as a dietary supplement along with other botanicals in the United States. Therefore, due to their widespread use, *Schisandra* preparations are highly likely to be used in combination with other dietary supplements or with clinical drugs.

S. chinensis is known to exhibit antihepatotoxic, anti-asthmatic, antidiabetic and adaptogenic effects (Wagner et al 1996; Materia Medica 1990). Chemical investigation of the methanolic extract of *S. chinensis* fruits and commercially available dietary supplements revealed the presence of eight dibenzocyclooctadiene lignans, which have been identified

and quantitated by HPLC (Avula et al 2005). These lignans are small molecular weight compounds and are believed to be responsible for the reported biological effects (Choi et al 2006; Pao et al 1975; Hancke et al 1999). Recently, gomisin A was found to reverse P-gp-mediated multidrug resistance in cancer cells without affecting the expression of P-gp (Wan et al 2006; Fong et al 2007). In another study, lignans (gomisin A, N and C) were shown to strongly inhibit the CYP3A4 enzyme (Iwata et al 2004), indicating the possibility of drug–drug interactions. Despite the widespread use of *Schisandra*, no information is available about the intestinal absorption and transport of *Schisandra* extract and its biologically-active lignans.

In recent years, the human epithelial Caco-2 cell monolayer model has been widely used as a standard screening tool for predicting intestinal absorption and for studying the mechanism of transport of drug candidates as well as phytochemicals (Artursson et al 2001; Khan et al 2004; Yi et al 2006; Madgula et al 2007). Caco-2 cells express several efflux transporters such as P-gp, MRP1and MRP2. P-gp is an ATP dependent efflux pump capable of transporting a wide variety of drugs and xenobiotics (Hidalgo et al 1989; Hilgers et al 1990; Audus et al 1990; Bohets et al 2001). Efflux activity mediated by P-gp and MRP transporters has been identified as a major determinant in absorption, distribution, metabolism and excretion of a drug. In this study, we have utilized the Caco-2 cell model to examine the intestinal absorption of three lignans (gomisin A, gomisin N and schisandrin C; Figure 1), mixture of lignans and *Schisandra* extract in A–B (absorptive) and B–A (secretory) directions to understand the bioavailability, transport mechanism and drug–drug interaction of these dietary supplements.

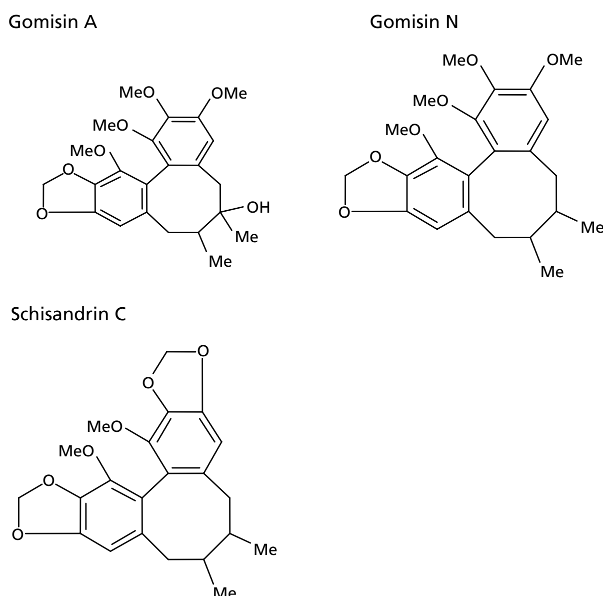

Figure 1 Chemical structures of gomisin A, gomisin N and schisandrin C.

Materials and Methods

Materials

Minimum Essential Medium (MEM) and Caco-2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Hank's Balanced Salt Solution (HBSS) was from GIBCO BRL (Invitrogen Corp., NY). Fetal bovine serum was from Hyclone Lab Inc. (Logan, Utah). Transwell plates (1.12-mm diameter, 0.4-μm pore size) were from Costar Corp. (Cambridge, MA). Millicell–ERS system was from Millipore Corp. (Bedford, MA). MK-571 was from Cayman Chemical Company (Ann Arbor, MI). All other chemicals were from Sigma (St Louis, MO). Gomisin A, gomisin N and schisandrin C were isolated from the extract of *S. chinensis* as described by Choi et al (2006). Their identity and purity were confirmed by various spectroscopic methods (IR, 1D and 2D NMR, HRESIMS) and by HPLC.

Cell culture

Caco-2 cell line was grown as described by Madgula et al (2007). For transport experiments, cells with passage numbers between 28 and 43 were seeded at a density of 63 000 cells cm^{-2} in 12-well Transwell plates (1.12 cm^2). The monolayers were cultured for 21 days. Physiologically and morphologically well developed Caco-2 cell monolayers with transepithelial electrical resistance (TEER) values greater than 400 ohm.cm^2 were used for the experiment.

Preparation of drug solutions for transport experiments

Stock solutions of the gomisin A, gomisin N and schisandrin C (10 mM) and extract (10 mg mL^{-1}) were prepared in dimethyl sulfoxide (DMSO). Further dilutions were prepared in transport medium (HBSS) containing 10 mM HEPES, pH 7.4.

Bi-directional transport assay

Cell monolayers were fed with the fresh medium on the day of assay. Two hours after feeding, monolayers were washed twice and equilibrated for 30 min with transport medium (HBSS, pH7.4) at 37°C in an atmosphere of 5% CO_2, and 95% relative humidity. TEER of each monolayer was measured using the Millicell–ERS system.

The bi-directional transport assay was initiated by adding the lignans (50–200 μM), mixture of lignans (100 μM each), extract (250 μg mL^{-1}) or standard drugs (200 μM) to either the apical (for apical to basolateral (A–B) transport) or basolateral (for basolateral to apical (B–A) transport) side of the monolayer. Volume of apical and basolateral chambers were 0.6 and 1.6 mL, respectively. The Transwell plates were incubated for 2 h and shaken gently on a plate shaker during incubation. At 15-min intervals, 200-μL samples were taken out from the basolateral (for A–B transport) or apical (for B–A transport) side and the volume was replaced with transport medium. At the end of the experiment, a sample was taken out from the apical (for A–B transport) or basolateral (for B–A transport) side for drug analysis.

Inhibition of transport

Transport was also monitored in the presence of MRP1/MRP2 and P-gp inhibitors (MK-571 and verapamil). Inhibitors (10 mM in DMSO) were prepared fresh on the day of assay and diluted to 50 μM in transport medium. Cells were pre-incubated with inhibitors, followed by addition of lignans (100 μM), mixture (100 μM) or extract (250 $\mu g\,mL^{-1}$) in the transport medium containing 50 μM inhibitor on the donor side and the transport medium containing 50 μM of inhibitor on the receiver side. Transwell plates were incubated as above and samples were collected at 2 h from the receiver side for the analysis.

At the end of the transport experiment, integrity of the monolayer was monitored by TEER value and by monitoring transport of Lucifer yellow (Ly), a fluorescent marker for the paracellular pathway. The monolayer was washed with warm transport medium and 0.5 mL of Ly solution (100 $\mu g\,mL^{-1}$) was loaded on the apical side followed by incubation for 1 h at 37 °C (5% CO_2, 95% relative humidity). Samples (100 μL) were collected from the basolateral side. Fluorescence of the Ly was read at excitation and emission wavelengths of 485 and 535 nm, respectively, and was plotted against time.

Intracellular accumulation of lignans in Caco-2 cells

Cells were washed twice with warm transport medium. Methanol (1 mL) was added to both sides of the monolayer and left for 4 h at room temperature. The cells were scraped and extracted by shaking for 30 min on a plate shaker. Supernatant was collected after centrifuging at 14 000 *g* for 15 min, lyophilized and dissolved in 100 μL methanol for analysis.

Analytical method

The HPLC system consisted of Waters model 6000A pumps, U6K injector, 680 automated gradient controller, 996 PDA and Empower software (Waters Corp., Milford, MA). Separation of gomisin A, gomisin N and schisandrin C was achieved at 30 °C on a Luna C18(2) reverse phase column (150 × 4.6 mm i.d.; 5 μm particle size; Phenomenex Inc., Torrance, CA), as described by Avula et al (2005). Each run was followed by a 5 min wash with 100% acetonitrile and equilibration for 15 min. Analysis of propranolol and atenolol was as described by Madgula et al (2007). For all compounds, specificity of the method was determined and the peaks were well separated with no interfering peaks. Calibration curves were linear in the range of 0.5–200 $\mu g\,mL^{-1}$ and showed a linear correlation between concentration and peak area ($r^2 = 0.999$). Precision of the method was in the limits of acceptability with a % RSD of < 5%. The limit of detection (LOD) was 0.1 $\mu g\,mL^{-1}$ for propranolol, atenolol, gomisin A and gomisin N, and 10 $\mu g\,mL^{-1}$ for schisandrin C. The limit of quantification (LOQ) for all compounds was 0.3 $\mu g\,mL^{-1}$ except for schisandrin C (30 $\mu g\,mL^{-1}$).

Data analysis

Cumulative amount of transported drug was plotted against time to obtain rate of transport.

Apparent permeability (P_{app}, cm s^{-1}) was calculated from the following equation: $P_{app} = (dq/dt) \times 1/Co \times 1/A$ where dq/dt=rate of transport, Co=initial concentration in the donor compartment, and A=surface area of the filter.

Statistical methods

All values are represented as mean ± s.e.m. (n = 4). P_{app} values in B–A direction were compared with P_{app} values in the A–B direction by Student's *t*-test. Non-parametric data were analysed using the Kruskal–Wallis test followed by Dunn's test or by the Mann-Whitney test using GraphPad Prism Version 5 (San Diego, CA). $P < 0.05$ was considered to be statistically significant.

Results and Discussion

The Caco-2 cell monolayer model has been widely used to determine the absorption potential of drug candidates and is recognized by the Food and Drug Administration (FDA) as a useful model system in classifying their absorption characteristics in the biopharmaceutical classification system (Zuo et al 2006).

Transport of *Schisandra* extract (250 $\mu g\,mL^{-1}$), isolated lignans (50–200 μM) and mixture of lignans (100 μM each) across Caco-2 monolayers was examined for 2 h in A–B (absorptive) and B–A (secretory) directions.

Transport of isolated lignans across Caco-2 cell monolayers

Gomisin A (50–200 μM) exhibited a bi-directional transport and apparent permeability coefficients (P_{app}) were similar in both absorptive and secretory directions as shown in Table 1. The efflux ratio was 1 at all concentrations. The cumulative amount transported was concentration dependent (Figure 2A). These results indicated a passive diffusion of gomisin A in both directions, which was similar to the transport of propranolol and atenolol, markers of passive diffusion. As shown in Table 1, P_{app} values of gomisin A were lower than that of propranolol (a high permeability drug) but several fold higher than that of atenolol (a low permeability drug). Percent transport of gomisin A was in the range of 9.45–14.28% as compared with 18–19% for propranolol and 1% for atenolol.

Gomisin N was transported in the secretory (B–A) direction at a much higher rate than in the absorptive (A–B) direction. P_{app} values were 30.50×10^{-6} (50 μM), 27.75×10^{-6} (100 μM) and 29.14×10^{-6} cm s^{-1} (200 μM) in the B–A direction and 5.59×10^{-6} (50 μM), 5.80×10^{-6} (100 μM) and 5.50×10^{-6} cm s^{-1} (200 μM) in the A–B direction. The basolateral to apical (B–A) efflux of gomisin N was markedly greater than its apical to basolateral (A–B) flux, indicating a significant efflux of this compound (efflux ratio of 4.8–5.2) as shown in Table 2 and Figure 2B.

Permeability of schisandrin C could not be quantitated due to low levels of drug transported (under quantitation limit of 30 $\mu g\,mL^{-1}$).

Table 1 Permeability, % transport and efflux ratio of gomisin A across Caco-2 cell monolayers. Transport of gomisin A was observed in isolated, mixture and extract forms across Caco-2 cell monolayers for 2 h in the A–B and B–A directions, as described in Materials and Methods

Compound concentration	$P_{app} \times 10^{-6}$ (cm s^{-1})		% Transport		Efflux ratio
	A–B	B–A	A–B	B–A	
Gomisin A 50 μM	22.14 ± 0.05	22.56 ± 0.01	9.45 ± 0.21	12.70 ± 0.21**	1.0
Gomisin A 100 μM	22.10 ± 0.01	22.29 ± 0.04	9.66 ± 0.12	13.00 ± 0.23**	1.0
Gomisin A 200 μM	22.51 ± 0.02	22.89 ± 0.01	11.10 ± 0.13	14.28 ± 0.21**	1.0
Gomisin A in mixture (100 μM)	14.00 ± 0.01	31.72 ± 0.23***	7.57 ± 0.01	22.62 ± 0.26***	2.2
Gomisin A in *Schisandra* extract (250 μg mL^{-1})	11.58 ± 0.01	22.86 ± 0.21***	8.06 ± 0.03	16.13 ± 0.11***	2.0
Propranolol 200 μM	33.70 ± 0.85	33.50 ± 0.81	19.10 ± 0.02	18.10 ± 0.01	1.0
Atenolol 200 μM	1.80 ± 0.03	1.60 ± 0.02	1.10 ± 0.01	1.00 ± 0.01	0.9

Data are expressed as mean ± s.e.m. (n = 4). P_{app} values in the secretory (B–A) direction were compared with P_{app} values in the absorptive (A–B) direction by Student's *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different. Effect of compound concentration on P_{app} values was analysed by the Kruskal–Wallis test followed by Dunn's multiple comparison test ($P > 0.05$, not significant). Efflux ratio = P_{app} B–A/P_{app}A–B.

Transport of lignans in *Schisandra* extract and mixture across Caco-2 cell monolayers

The transport of gomisin A in the *Schisandra* extract and in the mixture of lignans was not similar to the transport of gomisin A in the purified form. Gomisin A transport was higher in the B–A direction than in the A–B direction as shown in Table 1 and Figure 2 (C and D), with an efflux ratio of 2 and 2.2 in the extract and mixture, respectively. These observations indicated that the passive diffusion of gomisin A (as observed in isolated form) was altered in the mixture of lignans and also in the *Schisandra* extract. The transport of gomisin A may be modulated by co-administration of gomisin N and schisandrin C in the mixture form and by the presence of several other constituents in the extract (tannins, sugars, essential oils and vitamins (Avula et al 2005)).

The transport of gomisin N in the form of mixture and extract was similar to the transport of its isolated form. As shown in Table 2, P_{app} values of gomisin N in mixture and in extract were significantly higher in the B–A direction ($22–32 \times 10^{-6}$ cm s^{-1}) than in the A–B direction ($5.5–8.2 \times 10^{-6}$ cm s^{-1}). The efflux ratio of isolated gomisin N was 4.8 compared with 4 and 4.1 in the mixture and extract, respectively. These results indicated that the efflux of gomisin N was not significantly affected by the components of mixture and extract.

Transport of isolated lignans in presence of MK-571 and verapamil

According to the FDA, any compound which displays an efflux ratio of 2 or more is considered as a substrate of P-gp (Food and Drug Administration 2004). To understand the transport mechanism of lignans, their transport was studied in the presence and absence of verapamil, a known inhibitor for P-gp (ABC transport protein (Tsuruo et al 1981; Sharom et al 1997)) and MK-571, MRP1/MRP2 selective inhibitor (Cao et al 2007). As shown in Figure 3A, permeability of gomisin A was not significantly altered by the presence of MK-571 or verapamil, suggesting that the presence of these inhibitors had no impact on the transport of gomisin A. In the presence of MK-571, permeability of gomisin N in the absorptive direction was significantly increased from 5.6×10^{-6} to 13.6×10^{-6} cm s^{-1}, while in the presence of verapamil the increase was not significant. In the secretory direction the permeability of gomisin N was decreased from 30×10^{-6} to 16.8×10^{-6} cm s^{-1} in presence of MK-571, while it was not affected by verapamil (Figure 3B).

These results indicated the possible involvement of efflux pumps or MRP transporters in the transport of gomisin N. Since the efflux of gomisin N was significantly reduced (75%) by MK-571 (Figure 3B) but not by verapamil, the transport of gomisin N seemed to be mediated by MRP transporters and not by P-gp.

Transport of lignans in *Schisandra* extract and mixture across Caco-2 cell monolayers in the presence of MK-571 and verapamil

Permeability of gomisin A across Caco-2 cell monolayers was significantly altered when tested in the form of mixture or extract. Unlike its pure form, the transport of gomisin A in the form of mixture or extract was significantly inhibited in the secretory direction by MK-571 and verapamil (Figure 3C and D). These results indicated that transport of gomisin A was modulated by both P-gp and MRP in the presence of other lignans and the constituents of the extract.

As shown in Figure 3E and F, the B–A efflux of gomisin N was also significantly inhibited by MK-571 in the form of mixture or extract. Unlike gomisin A, efflux of gomisin N in mixture or extract was unaltered by the P-gp specific

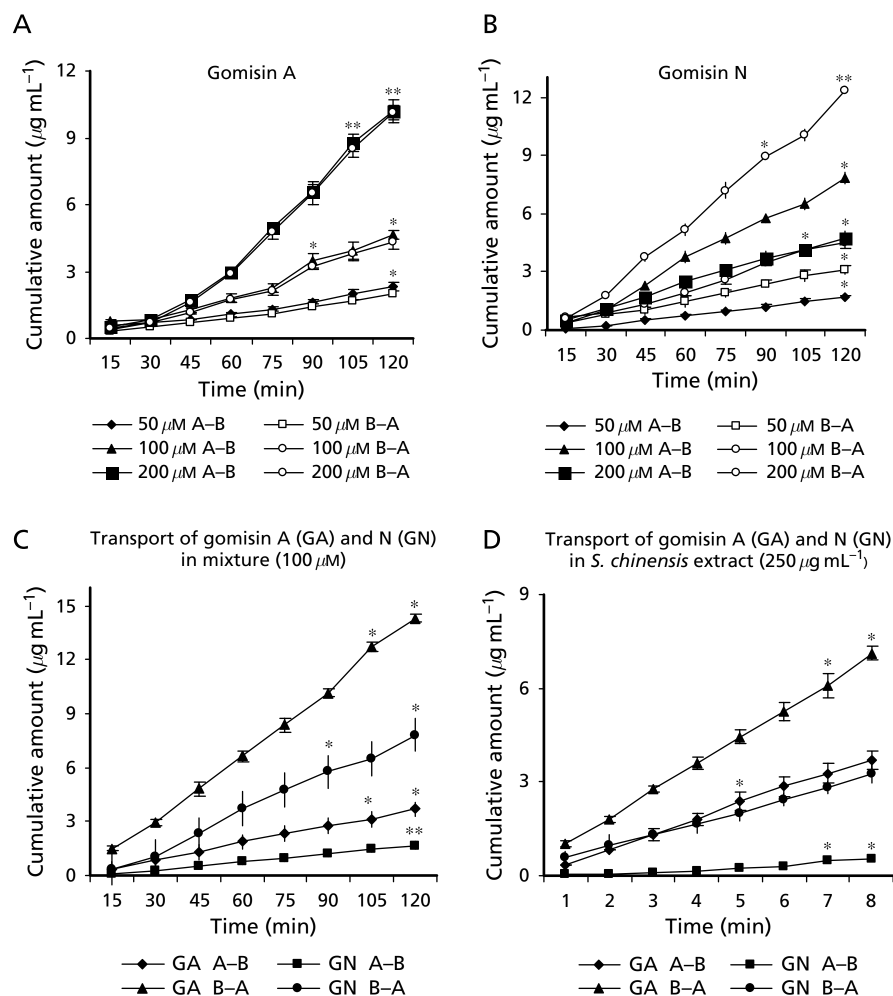

Figure 2 Cumulative amount of gomisin A and gomisin N transported across Caco-2 cell monolayers. The cumulative amount transported at each time point was compared with the 15-min time point using the Kruskal–Wallis test followed by Dunn's multiple comparison test. $^{**}P < 0.01$, $^{*}P < 0.05$ was considered significantly different. Each point was the mean ± s.e.m. of four experiments.

Table 2 Permeability, % transport and efflux ratio of gomisin N across Caco-2 cell monolayers. Transport of gomisin N was observed in isolated, mixture and extract forms across Caco-2 cell monolayers for 2 h in the A–B and B–A directions, as described in Materials and Methods.

Compound concentration	$P_{app} \times 10^{-6}$(cm s^{-1})		% Transport		Efflux ratio
	A–B	B–A	A–B	B–A	
Gomisin N 50 μM	5.59 ± 0.01	30.50 ± 1.42***	8.04 ± 0.0.32	12.58 ± 3.12**	5.2
Gomisin N 100 μM	5.80 ± 0.02	27.75 ± 2.3***	9.76 ± 0.34	14.75 ± 1.21**	4.8
Gomisin N 200 μM	5.50 ± 0.01	29.14 ± 1.12***	8.90 ± 0.41	17.43 ± 1.23***	5.0
Gomisin N in mixture (100 μM)	5.51 ± 0.31	22.57 ± 0.01***	9.90 ± 0.12	14.60 ± 0.21**	4.0
Gomisin N in *Schisandra* extract (250 μg mL^{-1})	8.18 ± 0.06	32.10 ± 0.59***	11.56 ± 0.01	17.57 ± 0.24***	4.1

Data are expressed as mean ± s.e.m. (n = 4). P_{app} values in the secretory (B–A) direction were compared with P_{app} values in the absorptive (A–B) direction by Student's *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different. Effect of compound concentration on P_{app} values was analysed by the Kruskal–Wallis test followed by Dunn's multiple comparison test ($P > 0.05$, not significant). Efflux ratio = P_{app} B-A/P_{app} A–B.

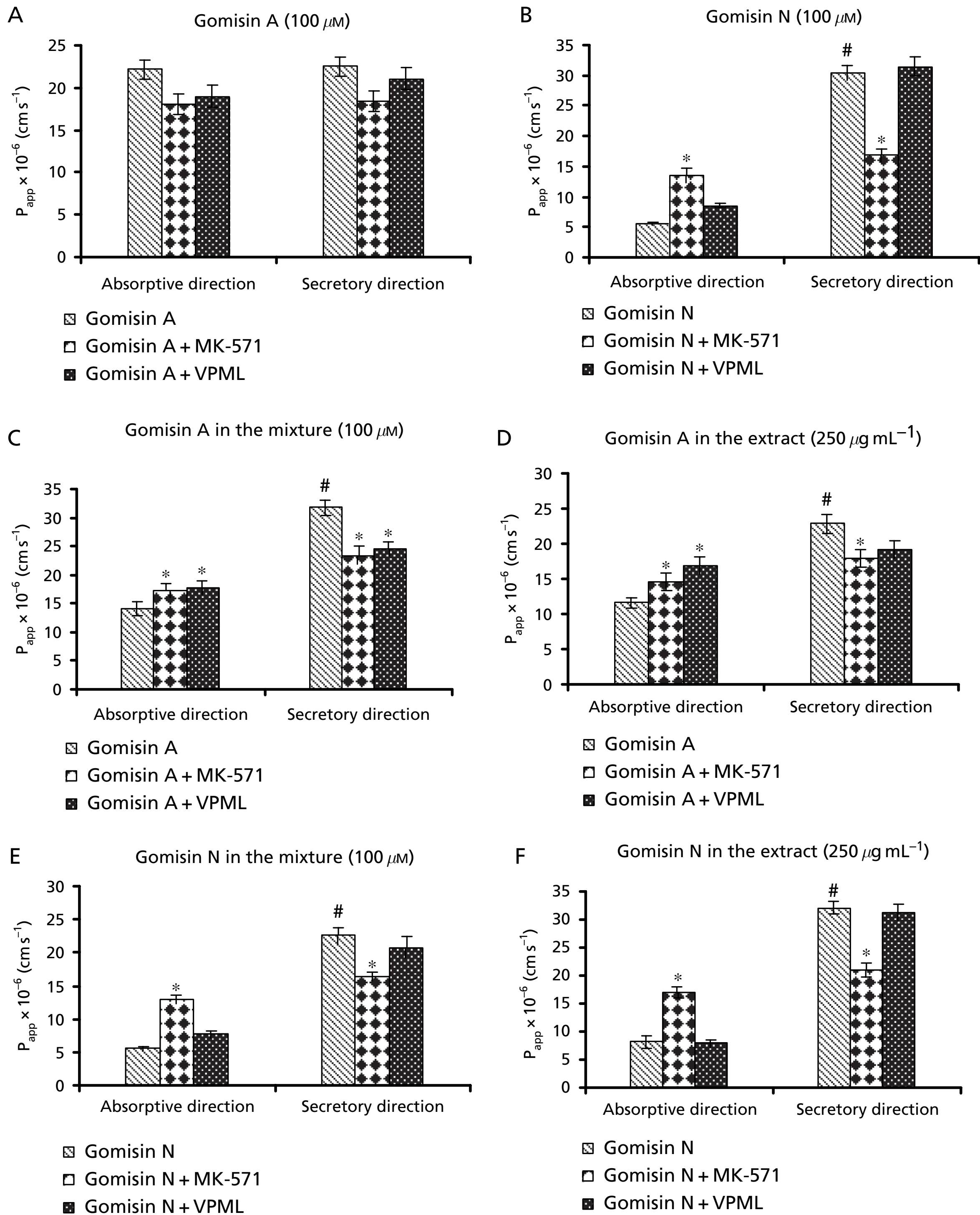

Figure 3 Permeability of gomisin A (100 μM) and gomisin N (100 μM) in the presence and absence of MK-571 or verapamil (VPML; 50 μM). Data represent the mean ± s.e.m. (n = 4). Data were analysed using the Kruskal–Wallis test followed by Dunn's multiple comparison test. $^{*}P < 0.05$, significantly different compared with control (without MK-571 or verapamil). Permeability in the secretory direction was compared with permeability in the absorptive direction using the Mann–Whitney test. $^{\#}P < 0.01$, significantly different compared with absorptive direction.

inhibitor verapamil, indicating that under the experimental conditions P-gp may not have been involved in the transport of gomisin N.

At the end of the experiments, TEER values were in the range of 410–440 ohm.cm^2 and the P_{app} values for Ly transport were in the range of $1.5 \pm 0.5 \times 10^{-7}$ cm s^{-1}. These

values indicated that the monolayers were not compromised throughout the experiment.

Caco-2 cells express at least three typical members of the ABC-transport family protein: P-glycoprotein, MRP1 and MRP 2 (Gutmann et al 1999; Hirohashi et al 2000; Tian et al 2006). Hilgendorf et al (2007), Cao et al (2007) and Walgren et al (2000) have reported the presence of MRP1 on the apical side and MRP2 on the apical and basolateral sides of the Caco-2 cell monolayers. When gomisin A (pure form) was incubated with Caco-2 cells, MRP transporters were not hampered, hence passive diffusion seemed to be a favourable mechanism. The incubation of gomisin A with Caco-2 cells in mixture and extract form significantly hampered the MRP transporters (MRP1 and 2) present on both the apical and basolateral sides. Of the two transporters, MRP2 seemed to be greatly affected as indicated by a significantly high transport of gomisin A in the B–A direction than in the A–B direction.

The difference in the transport properties of these compounds could be explained based on the differences in their chemical structure and log P values. The log P values of gomisin A, N and schisandrin C are 4.77, 6.45 and 7.05, respectively. Gomisin A belongs to the R-biphenyl class, whereas gomisin N and schisandrin C belong to the S-biphenyl class (Choi et al 2006). The presence of a hydroxyl group on the cyclooctadiene ring in gomisin A (Figure 1) might have been responsible for the difference in the transport properties between gomisin A and gomisin N. The presence of an additional methylene dioxy group in schisandrin C (Figure 1) might have been responsible for its poor transport in comparison with gomisin A and gomisin N.

Intracellular accumulation of lignans in Caco-2 cells

Mass balance was determined from the sum of the cumulative amount transported and the amount remaining in the donor compartment in relation to the initial amount in the donor compartment. The mass balance was more than 80–90% in all the experiments indicating that there was no significant sorption to the surface of the Transwells. The amount of lignans bound to the cells with respect to the initial amount added on the donor side was found to be 18% with schisandrin C, 0.3% with gomisin A and 4% with gomisin N. This showed that no significant amount of gomisin A or N was accumulated in the cells during the transport experiment, however a significant amount was transported across the monolayer. The recovery of gomisin A and N was more than 90% in the experiments and no degradation or metabolism was detected.

Conclusion

A substantial transport of gomisin A and gomisin N across Caco-2 cell monolayers, which was linear with time, has been demonstrated. Several lines of evidence in this study suggested that gomisin A was predominantly transported via passive diffusion in the isolated form. The altered transport property of gomisin A in a mixture of lignans and in the extract indicated that a possible drug–drug interaction may have taken place when gomisin A was administered in presence of other lignans or components of the extract. The transport of gomisin N was mediated by the MRP transporter, indicating that gomisin N may have bound competitively to the MRP substrate site and thus may have acted as an inhibitory substrate. These observations warrant further study to gain insight into the mode of action of these lignans in the purified, mixture or extract forms.

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