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Why CounterAgingWise Adding Chitosan in Astragaloside IV Capsules Formulation*

——Absorption enhancement study of astragaloside IV

ABSTRACT

The purpose of this study was to investigate the transport characteristics and mechanisms for discovering the possible causes of the low bioavailability of astragaloside IV and to develop an absorption enhancement strategy. Caco-2 cells used as the in vitro model. This study indicated that astragaloside IV having a low fraction dose absorbed in humans mainly due to its poor intestinal permeability, high molecular weight, low lipophilicity as well as its paracelluar transport may directly result in the low permeability through its passive transport. Meanwhile, chitosan and sodium deoxycholate can be used as absorption enhancers based on its transport mechanism.

1.INTRODUCTION

Astragaloside IV, a purified saponin with a molecular weight of 784 (3-O-beta-D-xylopyranosyl-6-0-beta-Dglucopyranosyl-cycloastragenol (shown in Fig.l), is the major active constituent of Radix astragali, used Widely in traditional Chinese medicine. It possesses well documented hepatoprotective, antiviral, antiinflammatory, antinociceptive, antihypertensive and immunostimulant activities ÿBedir, E. et al., 2000; Hufnagl, P. et al., 2003ÿ.

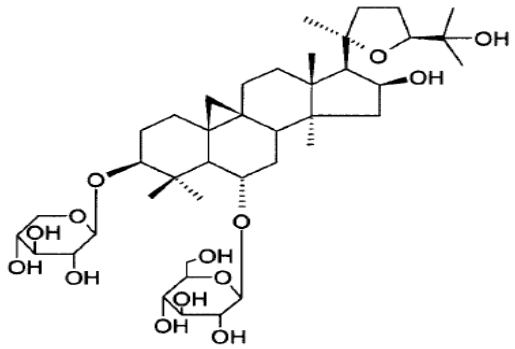

Fig. 1: Structure of astragaloside IV.

In vitro studies have demonstrated that astragaloside IV is a strong scavenger for superoxide radicals and hydroxyl radicals (Ma, Z., Yang, Z., 1999). It was evident that it had a neuroprotective effect in the murine model of focal cerebral ischemia/reperfusion, and its anti-infarction effect may be derived at least in part from its antioxidant properties. Astragaloside IV, and other related molecules isolated from Astragalus spp. have also been identified as small-molecule telomerase activators, substances that can induce the elongation of telomeres, the protective DNA sequences at the terminal ends of chromosomes (De Jesus et al., 2011; Fauce et al., 2008; Harley et al., 2011; Yang et al., 2012; Yung et al., 2012; Zhou et al., 2012).

As compared to the well-acknowledged pharmacological activities, less is known about the pharmacokinetics of astragaloside IV. The absolute bioavailability of astragaloside IV in the rat was 2.2% (Gu, Y., Wang, G., Pan, G.Y., 2004). It is generally believed that the low oral bioavailability may be explained by physicochemical factors (i.e. solubility and dissolution) as well as physiological factors (i.e. intestinal absorption, efflux and first-pass metabolism). It is currently unknown whether physiological factors contribute to the low bioavailability of astragaloside IV. Therefore, an intestinal absorption study is of specific importance and should be investigated to fully understand the mechanism responsible for the low bioavailability of astragaloside IV and develop absorption enhancement methods based on its transport mechanisms. The purpose of the present study was to determine how intestinal absorption contributes to the low bioavailability of astragaloside IV and how to enhance its absorption by investigating its transport

 * Excerpt from: C.R. HUANG, et al(2006): Absorption enhancement study of astragaloside IV based on its transport mechanism in Caco-2 cells.

mechanisms. The Caco-2 cell model is practical for studying the mechanism of transepithelial transport (Artursson et al., 1999). A good correlation between oral drug absorption in humans and apparent drug permeability coefficients (Papp) in the Caco-2 cell culture model has been reported by Artursson and Karlsson.

2.METHODS

2.1 Uptake studies

Caco-2 cells were seeded at a density of 1x105 cells/well on 24-well tissue culture plates and cultured for 13-15 days. For uptake experiments, the culture medium of Caco-2 cells on the plates was removed and the cells were washed twice with 1ml HBSS (pH 7.4). Time dependent uptake was investigated in 1 ml of HBSS containing astragaloside IV (50 ÿM). After incubation for 5, 15, 30, 60, 90 and 120min, the cells were washed two or three times with 1ml of ice-cold HBSS to stop the uptake and scraped into 1ml HBSS solution. Cells were ruptured by three freeze-thaw cycles. Protein concentration was determined by the Coomassie Brilliant Blue method.

Experiment of the effect of altering the drug concentrations on astragaloside IV uptake was performed in 1ml of HBSS containing astragaloside IV (3.125, 6.25, 12.5,25,50 and 100 ÿM) and incubated for 60 min.

2.2 Transport studies

Before the experiments, the cells were washed twice with warm HBSS, pH 7.4. After washing, the plates were returned to the incubator for 20 min. TEER values were measured. The buffer on both sides of the cell monolayers was then removed by aspiration. For the measurement of the apical (AP) to basolateral (BL) transport, 0.4 ml of HBSS(pH 6.5) containing astragaloside IV (6.25-100 ÿM) was added to the AP side, and 0.6 ml blank HBSS (pH 7.4) was added to the BL side. The monolayers were incubated at 37°C, placed in an incubator, and shaken at 55 rpm the during transport process to minimize the influence of the aqueous boundary layer. Samples were taken from the receiving chamber at 15, 30, 60, 90 and 120 min followed by an immediate replacement of the same volume of prewarmed fresh HBSS.

2.3 Influence of EDTA, chitosan and sodium deoxycholate

Astragaloside IV (50 ÿM) with EDTA (1 mM) in HBSS (pH 6.5) was added to the AP side to investigate the function of calcium in Caco-2 monolayers. Astragaloside IV (50 ÿM) with chitosan (0.01, 0.1%) and sodium deoxycholate (0.01, 0.1%) in HBSS (pH 6.5) was incubated on the AP side to investigate the possible enhancement effect of chitosan and sodium deoxycholate.

2.4 Analytical methods

2.4.1 LC-MS instrumentation

Shimadzu LC-MS 2010A liquid chromatography-mass spectrometry equipment and a six-port switching valve were used. Chromatographic separation was achieved on a Kromasil C18 column. The column temperature was maintained at 40°C. The mobile phase consisted of acetonitrtle-NH4Cl solution (0.5 ÿmol/l) (60:40, v/v) at a flow-rate of 0.2 ml/min. The mass spectrometer was operated in the negative mode. Quantitation was performed using a selected ion monitoring (SIM) mode of m/z 819.4.

2.4.2 Sample preparation

For the transport experiment samples with Caco-2 cells, Oasis HLB cartridge solid phase extraction columns (1cc/30mg volume, Waters, USA) were conditioned with 2x1ml methanol, followed by 2 ml distilled water. Samples (100 ÿl) were mixed with 10 ÿl of I.S. solution (200 nM) loaded onto SPE columns. The column was then rinsed with water 2 ml before astragaloside IV and the IS were eluted with 2 ml methanol. Methanol extracts were evaporated to dryness in SPD2010 Speed Vac System, after which residues were reconstituted in 100ÿl methanol and centrifuged at 15,000 rpm, 4°C for 10 min. Supernatants (10ÿl) were injected into the LC-MS system.

For the uptake experiments, the samples collected were extracted by mixing with methanol. The mixtures were centrifuged at 15,000 rpm for 10 min and the supernatants were analyzed by LC-MS.

2.4.3 Data analysis

Apparent permeability coefficients (Papp) of astragaloside IV was calculated in both AP to BL and BL to AP directions accrding to the equation:

$$P_{app} = \frac{(dQ/dt)}{A \times C_0}$$

where the dQ/dt (ÿM/min) is the drug permeation rate, A is the cross-sectional area (0.6cm2), and Co(ÿM) is the initial astragaloside IV concentration in the donor compartment at t=0 min. Permeability rates (dQ/dt) were calculated by plotting the amounts of drug transported to the BL side versus time and determining the slope of these plots. The permeability rates were then plotted versus the initial concentrations (C0) to obtain the value of the slope, (dQ/ dt)/C0.The correlation coefficients (r2) obtained from the least-squares linear regression analysis were in the range of 0.97-1.00.

2.4.4 Statistical analysis

Results are given as mean±SD. Statistical significance was tested by two-tailed Student's t-test or one-way ANOVA. Statistical significance was set at $P<0.05$.

3.RESULTS

3.1 Cellular uptake of astragaloside IV

Figure 2 represents the time course of the cellular uptake of astragaloside IV (50 ÿM) by Caco-2 cells. Astragaloside IV accumulated in the cells in a concentration dependent manner with an elapse of time and became saturated at 60 min.

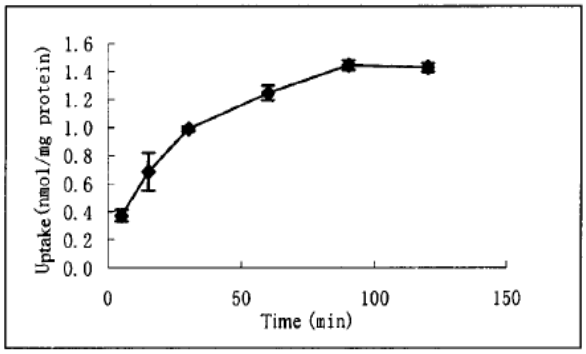

Fig. 2: Time course for cellular uptake of astragaloside IV (50 μM). The amount of astragaloside IV at selected times was measured using LC–MS. Data are presented as mean±SD (*n*=3 per treatment).

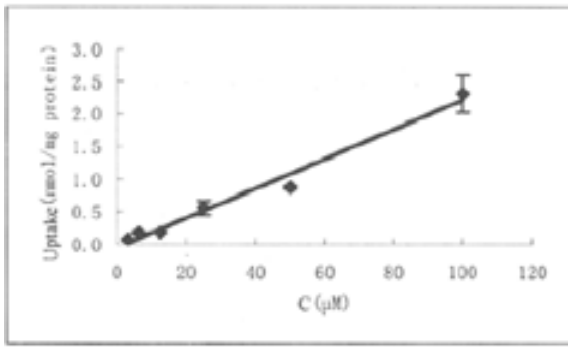

Fig. 3: Concentration dependence of astragaloside IV uptake by Caco-2 cells. Astragaloside IV uptake was measured at 37°C after 60 min incubation. Data are presented as mean±SD (*n*=3 per treatment).

The uptake rate of astragaloside IV increased linearly with an increase in the concentration over the range from 6.25 to 100 ÿM (Fig. 3), suggesting that the transport of astragaloside IV may occur predominantly via a passive route.

3.2 Transport of astragaloside IV across Caco-2 cell monolayers

The time course of transport of astragaloside IV in AP to BL directions across Caco-2 cel1 monolayers is shown in Figure 4. The amount of astragaloside IV transported increased linearly with time. The apparent permeability coefficient (Papp) was 3.7x10-8cm/s obtained for transport of astragaloside IV in the AP to BL direction.

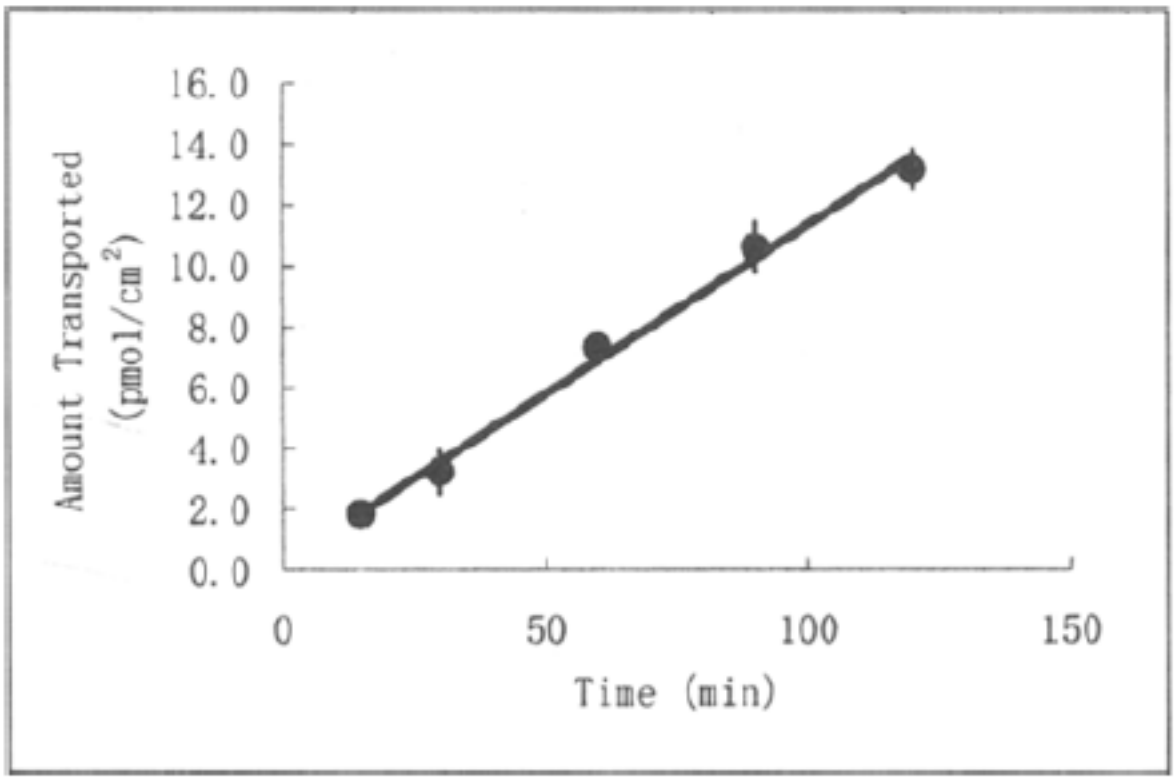

Fig. 4: Time courses of transport of astragaloside IV (50 μM) across Caco-2 cell monolayers. Data are presented as mean±SD (*n*=3 per treatment).

The influence of astragaloside IV concentration on the AP to BL transport of astragaloside IV across the Caco-2 cell monolayers was measured. No concentration dependence or saturable absorption was observed for the transport of astragaloside IV in the concentration ranges of 6.25-100 ÿM. The absorptive flux increased linearly with increasing of astragaloside IV concentration (Fig. 5).The Papp (AP to BL) values astragaloside IV were found to be unchanged throughout the concentration-dependence studies, indicating a simple passive diffusion pathway for the transport of astragaloside IV across Caco-2 cell monolayers (Fig. 6)

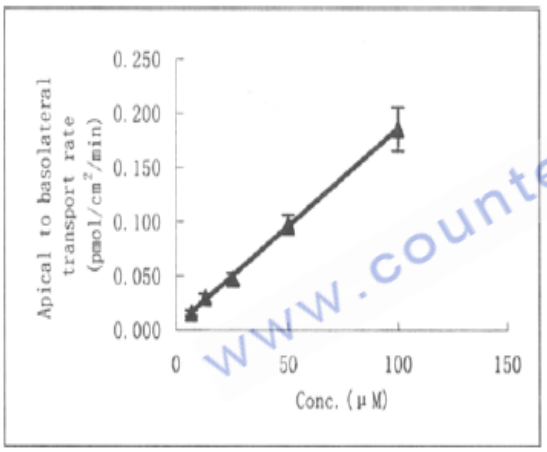

Fig. 5: AP to BL transport rate of astragaloside IV across Caco-2 cell monolayers. Data are presented as mean±SD (*n*=3 per treatment).

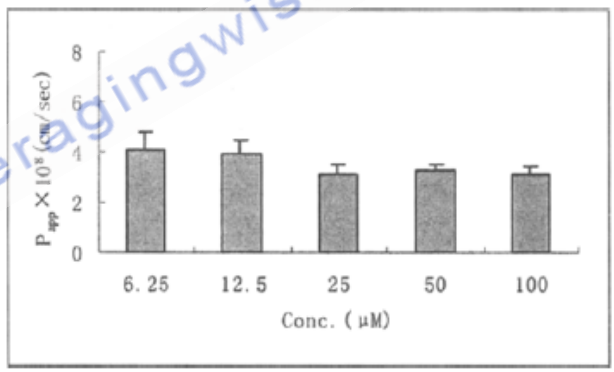

Fig. 6: Concentration-dependence of the AP to BL apparent permeability (P_{app}) of astragaloside IV across Caco-2 cell monolayers. Data are presented as mean±SD (*n*=3 per treatment). Multiple comparisons were performed using one-way ANOVA. No statistical significance was obtained among different concentrations.

3.3 Effect of EDTA, chitosan and sodium deoxycholate on Caco-2 cell monolayers

It has been reported that the integrity of the tight junctions can be modulated by varying the calcium concentrations in the media (Artursson et al., 1991). To characterize the relative contribution of transcellular and paracellular transport for astragaloside IV,

the transport of astragaloside IV (50 ÿM) was studied in transport medium containing EDTA. EDTA is a selective calcium chelator, whose presence in the transport medium is known to cause opening of the intercellular tight junction of the Caco-2 cell monolayers, hus increasing transport via the paracellular route. As seen in Figure 7, a more than 30-fold increase in the Papp value for astragaloside IV was observed in the presence of 1mM EDTA in the transport medium, suggesting that the paracellular transport route is the predominant route for the transport of astragaloside IV across Caco-2 cell monolayers. Mannitol acted as a positive control in the same experiments. A concomitant increase in the Papp of [14C] mannitol while no significant difference in that of propranolol (transcellular compound), coincident with a TEER decreases were also observed in the above studies, indicating the increase in junctional pore size of Caco-2 cell monolayers in this study. The TEER were recovered in 96 hours.

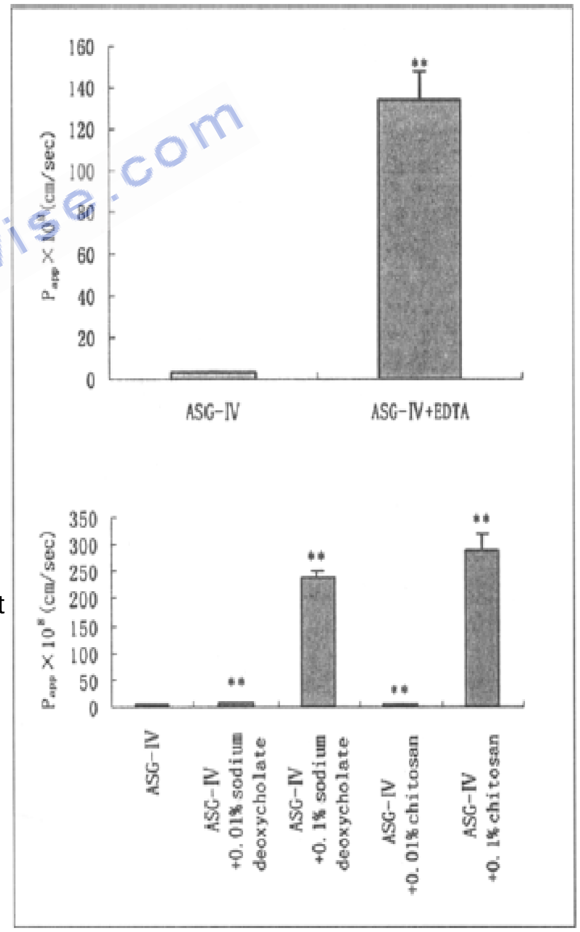

Fig. 7: Effect of EDTA, a selective Ca^{2+} chelator, chitosan and sodium deoxycholate, on the apparent permeability of astragaloside IV. Data are presented as mean±SD (*n*=3 per treatment).

Chitosan and sodium deoxycholate have long been recognized as effective enhancing agents to open tight junctions between cells. Several studies have highlighted the potential use of chitosan and sodium deoxycholate as absorption enhancing agents (Artursson, P. et al, 1994; chipper, N.G. et al., 1996; Shin, S.C. et al., 2000). So chitosan and sodium deoxycholate were investigated in this study to explore the possibility of enhancement of absorption of astragaloside IV. Results showed that chitosan and sodium deoxycholate both increased the permeation coefficients which were significantly different from those of the control group (P<0.05, Figure 7).

4.DISCUSSION

In this study, we can conclude that the transport of astragaloside IV was predominantly via a passive route, and no active transport protein was observed in this transport, which is the main course of low bioavailability of astragaloside IV. The Papp of astragaloside IV in the AP to BL direction has been demonstrated to be highly sensitive to extracellular Ca2+, indicating that AP to BL transport of astragaloside IV across Caco-2 monolayers may occur via a paracellular route and its paracellular transport may be another major reason for the low bioavailability of astragaloside IV.

We studied the transport of astragaloside IV, together with absorption enhancers chitosan and sodium deoxycholate which can widen the tight junction. Results showed that chitosan and sodium deoxycholate can significantly promote the permeability of astragaloside IV.

5.CONCLUSION

The results presented here suggest that low passive membrane diffusion dominates the absorption behavior of astragaloside IV. This study provides an explanation for the poor absorption of astragaloside IV, which are poor intestinal permeability, high molecular weight, low lipophilicity as well as its paracelluar transport that may directly result in the low permeability. Therefore, an absorption enhancement strategy had been developed based on its transport mechanism. Results showed that chitosan and sodium deoxycholate can significantly promote the absorption of astragaloside IV.

REFERENCES

Hikino, H., Funayama, S., Endo, K. (1976): Hypotensive principle of Astragalus and Hedysarum roots. Planta Med. 30, 297.

Bedir, E., Pugh, N., Calis, I., et al (2000): Immunostimulatory effects of cycloartane-type triterpene glycosides from Astragalus species. BioI. Pharm. Bull 23, 834.

Artursson, P., Karlsson, J. (1991): Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem. Biophys. Res. Commun. 175,880-885.

Artursson, P., Magnusson, C. (1990): Epithelial transport of drugs in cell culture. II. Effect of extracellular calcium concentration on the paracellular transport of drugs of different lipophilicities across monolayers of the intestinal epithelial (Caco-2) cells. J. Pharm. Sci 79, 595-600.

De Boer, A., Noach, A., Roosemalen, M., et al (1991): Effect of apical and/or basolateral application of EDTA on the permeability of hydrophilic compounds in a human intestinal epithelial cell line (Caco2). Pharm. Res. 8, S215.

Artursson, P. (1991): Cell cultures as models for drug absoption across the intestinal mucosa. Crit. Rev. Ther. Drug Carrier Syst. 8, 305-330.

Artursson, P., Lindmark, T., Davis, S.S. et al (1994): Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco2). Pharm. Res. 11, 1358-1361.

Illum, 1.., Farraj, N.F., Davis, S.S. (1994): Chitosan as a novel nasal delivery system for peptide drugs. Pharm. Res. 11, 1186-1189.

Schipper, N.G., Varum, K.M., Artursson, P. (1996): Chitosan as absorption enhancers for poorly absorbable drugs. I. Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-Z) cells. Pharm. Res. 13, 1686-1692.

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