Uptake and Metabolism of Ginsenoside Rh2 and Its Aglycon Protopanaxadiol by Caco-2 Cells

Hai-Tang XIE,[a] Guang-Ji WANG,*,[a] Miao CHEN,[a] Xi-ling JIANG,[a] Hao LI,[a] Hua LV,[a] Chen-Rong HUANG,[a] Rui WANG,[a] and Michael ROBERTS[b]

[a] *Key Laboratory of Pharmacokinetics, China Pharmaceutical University; 24 Tong Jia Xing Street, Nanjing, Jiangsu Province, China: and* [b] *School of Medicine, Princess Alexandra Hospital, University of Queensland; 210009 Australia.*
Received March 8, 2004; accepted August 27, 2004

The uptake and metabolism profiles of ginsenoside Rh2 and its aglycon protopanaxadiol (ppd) were studied in the human epithelial Caco-2 cell line. High-performance liquid chromatography-mass spectrometry was applied to determine Rh2 and its aglycon ppd concentration in the cells at different pH, temperature, concentration levels and in the presence or absence of inhibitors. Rh2 uptake was time and concentration dependent, and its uptake rates were reduced by metabolic inhibitors and influenced by low temperature, thus indicating that the absorption process was energy-dependent. Drug uptake was maximal when the extracellular pH was 7.0 for Rh2 and 8.0 for ppd. Rh2 kinetic analysis showed that a non-saturable component (K_d 0.17 nmol·h^{-1}·mg^{-1} protein) and an active transport system with a K_m of 3.95 μmol·l^{-1} and a V_{max} of 4.78 nmol·h^{-1}·mg^{-1}protein were responsible for the drug uptake. Kinetic analysis of ppd showed a non-saturable component (K_d 0.78 nmol·h^{-1}·mg^{-1} protein). It was suggested that active extrusion of P-glycoprotein and drug degradation in the intestine may influence Rh2 bioavailability.

Key words Caco-2 cell; ginsenoside Rh2; protopanaxadiol; uptake; metabolism; high-performance liquid chromatography-mass spectrometry (HPLC-MS)

Ginseng is derived from the root of *Panax ginseng* C. A. MEYER, and is widely used therapeutically and as a health tonic. The main active components of *Panax ginseng* are ginsenosides, which have been shown to possess a variety of beneficial effects, including anti-inflammatory, antioxidant, and anticancer effects.[1)] Ginsenoside Rh2 (Fig. 1) is primarily found in Korean red ginseng root (steamed root of ginseng) and has been reported[2)] to alter cancer cell proliferation and perturb normal cell cycle events. It can also exhibit antiallergic activity originating from cell membrane-stabilizing activity and anti-inflammatory activity by the inhibition of nitric oxide and prostaglandin E_2 production.[3)] Orally administered natural glycosides inevitably come into contact with microflora and enzymes in the alimentary tract and may be transformed before they are absorbed from the gastrointestinal tract.[4)] Ota[5)] reported that the B16 melanoma cell could degrade Rh2 to its aglycon protopanaxadiol (ppd) (Fig. 1), and the pharmacological effects of ppd are similar or stronger than those of Rh2. Thus, knowledge of the absorption and metabolism profiles and kinetics of ginsenoside Rh2 is of great importance in understanding its biological effects.

The Caco-2 cell line exhibits a well-differentiated brush border on the apical surface and at tight junctions, and expresses typical small-intestinal microvillus hydrolases and various cytochrome P450 isoforms, as well as P-glycoprotein (P-gp) and phase II enzymes such as UDP-glucuronosyltransferases, sulfotransferases and glutathione-S-transferases. This cell line has proven to be the most popular *in vitro* model to elucidate pathways of drug transport, to determine the optimal physicochemical characteristics for passive diffusion of drugs, and to study pre-systemic drug metabolism.[6,7)] In this study, we examined the cellular uptake and metabolism of Rh2 and ppd in Caco-2 cells which possess intestinal enterocyte-like properties when cultured. To our knowledge, this is the first report on the uptake of ginsenosides in Caco-2 cells.

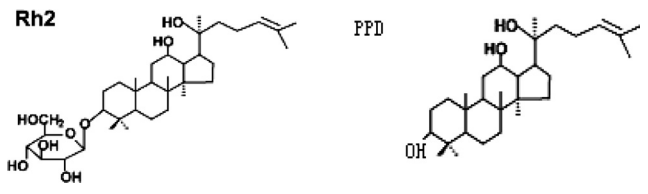

Fig. 1 Chemical Structures of Rh2 and ppd

MATERIALS AND METHODS

Materials The human colon adenocarcinoma cell line Caco-2 was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's Modified Eagle's medium and non-essential amino acids (NEAA) L-glutamine and penicillin/streptomycin were obtained from GIBCO Co. (U.S.A.). Fetal bovine serum (FBS) was obtained from Hyclone (Hyclone, Logan, UT, U.S.A.). All other cell culture materials were obtained from Costar (Cambridge, MA, U.S.A.). Hank's balanced salt solution (HBSS) was prepared in our laboratory. The reference standards of ginsenoside Rh2 and ppd were obtained from Bai Qiu-En Medical University (Jilin, Changchung, China) and the internal standard digitoxin (No: 51201) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The inhibitors verapamil, cyclosporine, 2,4-dinitrophenol and sodium azide were purchased from Sigma. Purified water from a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used throughout. Acetonitrile (Tedia Company, U.S.A.) and methanol (Merck, Germany) were of high-performance liquid chromatography (HPLC) grade, and commercially available ammonium chloride (NH_4Cl) was of analytical grade.

* To whom correspondence should be addressed. e-mail: xiehaitang@hotmail.com

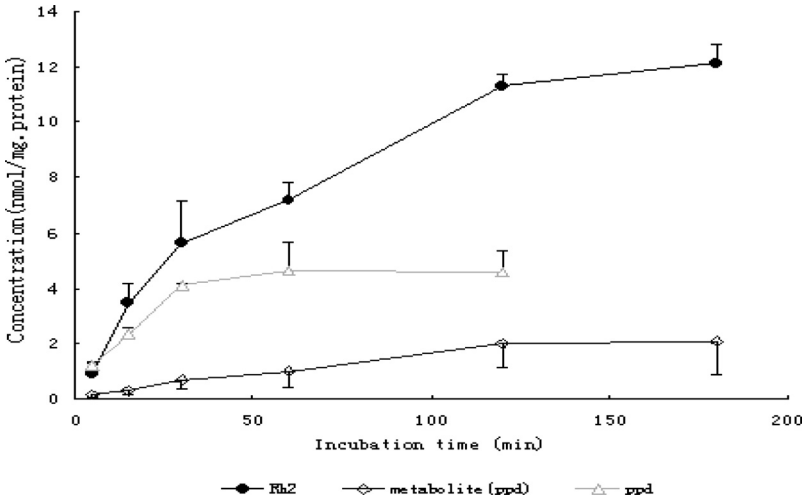

Fig. 2. Deglycosylation of Rh2 by Caco-2 Cells Incubated in HBSS Buffer (pH 7.4) at 37 °C (n=4)

Cell Culture and Experiments The cell medium was changed every other day and maintained in a humidified atmosphere of 5% CO_2 at 37 °C supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, and the pH was buffered to 7.4 using sodium bicarbonate. All cells used in this study were between passages 30 to 60, and the experiments were generally performed after cells reached confluence. Uptake of Rh2 by cultured monolayers of Caco-2 cells was examined according to methods described previously.[8] Experiments were performed with confluent epithelial monolayers grown on 24-well plates. The culture medium was renewed 2 h before the start of the uptake experiment. Cultured cells were washed three times with 1 ml aliquots of HBSS at 37 °C, and then preincubated in HBSS for 10 min prior to the addition of drugs. Uptake was initiated by adding 1 ml HBSS containing Rh2 (6 μmol·l^{-1}) or ppd (6 μmol·l^{-1}) alone or with P-gp (cyclosporine A, 10 μmol·l^{-1}; verapamil, 100 μmol·l^{-1}) or metabolic inhibitors 2,4-dinitrophenol and sodium azide. At the due time, solutions were removed by suction and the monolayers were washed three times with 1 ml aliquot of 4 °C HBSS. Cells were lysed by three freeze-thaw cycles. Protein concentrations were determined by the Coomassie Brilliant Blue method. Rh2 and ppd were assayed by HPLC-mass spectrometry (MS) and expressed per milligram of protein.

HPLC-MS Assay All analytical procedures were performed on a Shimadzu 2010 LC-MS, with an ESI probe (Q-array-Octapole-Quadrupole mass analyzer), a Shimadzu 10 ADvp Pump, and a Shimadzu 10 ATvp Autosampler. Separation was carried out by an ODS column (5 μ, 2.1×150 mm, Metachem, U.S.A.) with a C_{18} guard column (Security Guard, Phenomenex U.S.A.). The mobile phase was acetonitrile: 500 μmol·l^{-1} of ammonium chloride aqueous solution at the flow rate of 0.2 ml/min (gradient separation, with a ratio of 68 : 32 at 4 min and 95 : 5 thereafter). The following optimized MS conditions were selected: gas flow 4.5 l/min; CDL (Curve Dissolution Line) voltage fixed as in tuning; CDL temperature 250 °C; and block temperature 200 °C. LC-MS Solution (version 2.02) was used for data processing.

Statictics Data is presented as the mean±standard deviation. Student's t test was used for statistical analysis, and statistical significance was defined as $p<0.05$ or $p<0.01$.

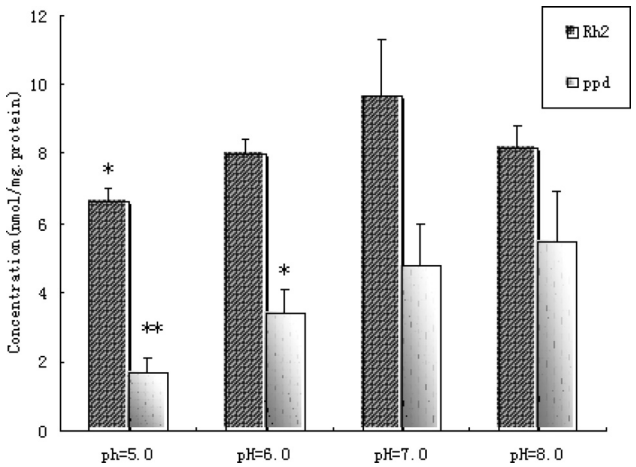

Fig. 3. pH Dependent Rh2 or ppd Uptake by Caco-2 Cells Incubated in HBSS Buffer at 37 °C for 1 h (n=4)

RESULTS

Time Course for Rh2 Uptake (Effect of Incubation Time) Caco-2 cells were incubated with Rh2 or ppd (6 μmol·l^{-1}) in 1 ml HBSS in each well over a 180 or 120 min time course, respectively. As shown in Fig. 2, the cellular uptake of Rh2 and ppd was linear for the first 30 min, and a maximum was reached at 120—180 min for Rh2 or 30 min for ppd. The metabolite ppd from Rh2 was increased in a time-dependent manner, reaching a plateau at 60 to 120 min as shown in Fig. 2.

pH Dependence of Uptake The effect of varying the extracellular pH from 5.0 to 8.0 on the rate of 6 μM·l^{-1} Rh2 or ppd uptake was measured at 37 °C after a 1 h incubation period.

As shown in Fig. 3, Rh2 and ppd uptake was influenced by the pH of extacellular buffer, and was maximal at pH 7.0 for Rh2 and at 8.0 for ppd. The uptake or drug accumulation in the Caco-2 cells at these two pH levels served as the control groups for Rh2 and ppd respectively. (* means $p<0.05$,

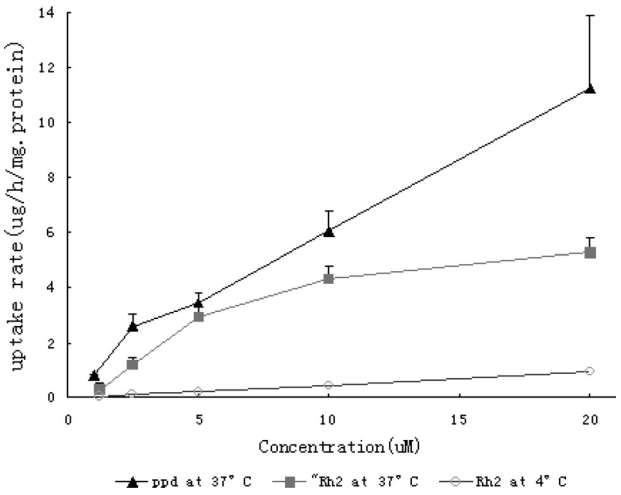

Fig. 4. Concentration Dependent Uptake of Rh2 and ppd Incubated in HBSS Buffer (pH 7.4) at 37 °C

** means $p<0.01$). Rh2 uptake was markedly decreased at pH 5.0 which showed statictical difference compared to that at pH 7.0; ppd uptake was gradually increased within the pH range from 5.0 to 8.0, and was higest at pH 8.0.

Concentration Dependence of Rh2 and Uptake To examine the kinetics of Rh2 and ppd transport by Caco-2 cells, initial rates of drug uptake were measured at pH 7.4 over the concentration range of 1 to 20 μmol in HBSS at 37 °C. The uptake rate was enhanced and tended towards saturation as the Rh2 concentration increased. Drug uptake was linear over the concentration range when the cells were incubated at 4 °C for Rh2. However, the uptake rate was concentration-dependent and not saturable over the concentration range from 1 to 20 $\mu mol \cdot l^{-1}$ for ppd when the cells were incubated at 37 °C (Fig. 4).

For active transport, the uptake rate can be described[7] by determination of relative rates of uptake and efflux. This allows for identification of the rate-limitating step of transcellular transport, where v is the uptake rate, V_{max} is the maximal uptake rate, K_m is the half-maximal uptake or transport concentration, C is the concentration of the compound, and K_d is the coefficient for nonmediated and passive uptake.

$$v=\frac{V_{max} \cdot C}{K_m+C}+K_d \cdot C$$

The kinetic parameters for the Rh2 transport carrier were: V_{max} of 4.78 $nmol \cdot h^{-1} \cdot mg^{-1}$protein, K_m of 3.95 $\mu mol \cdot l^{-1}$, and K_d of 0.17 $nmol \cdot h^{-1} \cdot mg^{-1}$ protein. The ppd kinetic analysis showed that this was a non-saturable component (K_d 0.78 $nmol \cdot h^{-1} \cdot mg^{-1}$ protein).

P-gp and Metabolic Inhibitors on the Uptake of Rh2 The Rh2 uptake rate was examined by co-administration with P-gp or metabolic inhibitors incubated in HBSS buffer (pH 7.4) at 37 °C and compared with Rh2 feeding alone (see Table 1).

The uptake of Rh2 increased markedly in the presence of verapamil ($p<0.05$), and was significantly enhanced with co-administration of cyclosporine, 2,4-dinitrophenol or sodium azide ($p<0.01$) as compared with Rh2 given alone.

Table 1. Metabolic and P-gp Inhibitors on the Uptake of Rh2 ($n=6$)

Compound	Rh2 Uptake Rate ($nmol \cdot h^{-1} \cdot mg^{-1}$ protein)
Control (Rh2 6 $\mu mol \cdot l^{-1}$)	5.84±0.95
Verapamil (100 $\mu mol \cdot l^{-1}$)	7.35±1.16*
Cyclosporine (10 $\mu mol \cdot l^{-1}$)	15.13± 0.37**
2,4-Dinitrophenol (0.5 $mmol \cdot l^{-1}$)	16.78±1.19**
Sodium azide (10 $mmol \cdot l^{-1}$)	13.37±2.79**

* $p<0.05$; ** $p<0.01$.

DISCUSSION

The uptake of Rh2 was time, pH and concentration dependent and could be degradated to ppd. Kinetic studies of Rh2 at 4 °C and ppd at 37 °C demonstrated first-order velocity. In the upake experiment conducted in HBSS buffer (pH 7.4) at 37 °C, Rh2 exhibited a two-order velocity process. Caco-2 cells incorporated ppd more quickly than Rh2 suggesting their uptake and release rates were different. Studies of the absorption and kinetics of the cellular uptake of Rh2 and ppd are essential to elucidate the mechanism of pharmacodynamic effects, and to subsequently determine formulation strategies.

The human colon adenocarcinoma cell line Caco-2 has ouabain-sensitive Na^+-K^+-ATPase[9] and a major phase I drug metabolizing enzyme in the villi of small intestine enterocytes, which is the primary site of absorption for orally administered drugs. Experiments with the Caco-2 cell line have provided strong evidence[10] that inhibition of the intestinal multidrug efflux pump, P-gp is another means by which oral drug bioavailability could be enhanced. The chemically pure compounds from medicinal plants may modulate activity in multidrug-resistant cells.[11] These results also demonstrated that cyclosporine A and verapamil could enhance Rh2 and ppd uptake in the Caco-2 cells. Meanwhile, in the presence of metabolic inhibitors (2,4-dinitrophenol and sodium azide), the uptake amount of Rh2 increased approximately 2-3-fold, indicating that the energy-dependent P-gp pump had been suppressed.

In conclusion, our data suggest that Rh2 might be one of the substrates of P-gp and that Rh2 degradation in the intestine may influence its oral bioavailability.

Acknowledgement This work was supported by the National High Technology Foundation of China ("863" Project) for preclinical pharmacokinetic studies (2003AA2Z3471).

REFERENCES

1) Kiefer D., Pantuso T., *Am. Fam. Physician*, **68**, 1539—1542 (2003).
2) Lee K. Y., Park J. A., Chung E., Lee Y. H., Kim S. I., Lee S. K., *Cancer Lett.*, **110**, 93—200 (1996).
3) Park E. K., Choo M. K., Kim E. J., *Biol. Pharm. Bull.*, **26**, 1581—1584 (2003).
4) Bae E. A., Han M. J., Choo M. K., *Biol. Pharm. Bull.*, **25**, 58—63 (2002).
5) Ota T., Maeda M., Odashima S., *J. Pharm. Sci.*, **80**, 1141—1146 (1991).
6) Meunier V., Bourrie M., Berger Y., Fabre G., *Cell Biol. Toxicol.*, **11**, 187—194 (1995).
7) Dantzig A. H., Bergin L., *Biochim. Biophys. Acta*, **1027**, 211—217 (1990).

8) Pan G. Y., Wang G. J., Liu X. D., Fawcett J. P., Xie Y. Y., *Pharmacol. Toxicol.*, **91**, 193—197 (2002).
9) Ramond M. J., Martinot-Peignoux M., Erlinger S., *Biol. Cell.*, **54**, 89—92 (1985).
10) Wacher V. J., Salphati L., Benet L. Z., *Adv. Drug Deliv. Rev.*, **46**, 89—102 (2001).
11) Efferth T., Davey M., Olbrich A., Rucker G., Gebhart E., Davey R., *Blood Cells Mol. Dis.*, **28**, 160—168 (2002).