The intestinal absorption characteristics of anthraquinones emodin and chrysophanol were observed by measuring the intracellular accumulation across Caco-2 cells by the reverse-phase high performance liquid chromatography. The intracellular accumulation of chrysophanol was much greater than that of emodin, the maximum absorption of emodin and chrysophanol being $414.02 \pm 15.28$ and $105.56 \pm 11.57$ nmol/l-mg-protein, respectively. The absorption of each anthraquinone was significantly lower at $4$ °C than that of $37$ °C. The effects of the transport inhibitors, verapamil, cyclosporine and phloridzin, on the intracellular accumulation were also examined. Verapamil and cyclosporine increased the absorption of emodin and chrysophanol, while phloridzin inhibited their absorption, all in a dose-dependent manner. These results suggest that the absorption characteristics of emodin and chrysophanol were closely related to their special structure with the hydroxy groups. It is also likely that a specific transport system mediated the intracellular accumulation of emodin and chrysophanol across the Caco-2 cells.

Key words: emodin; chrysophanol; Caco-2 cell; intestinal absorption

Polyphenols, a group of complex naturally occurring compounds, are widely distributed throughout the plant kingdom and are thus readily consumed by humans. As a member of the polyphenol family, dietary anthraquinones have received considerable attention as potential protectors against a variety of human diseases, in particular cardiovascular disease and cancer. Epidemiological evidence has long suggested that dietary anthraquinones, which are abundant in fruits and vegetables, can reduce the risk of cancer. Such diets are well known to contain a variety of chemicals that can affect the carcinogenic process in many ways. A large number of mechanisms of action have been investigated, including antioxidative properties and effects on enzymes and signal transduction pathways. There has been increased interest in recent years in these compounds from both consumers and food manufacturers. Humans are always exposed directly or indirectly to anthraquinones in medicinal and industrial applications.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) and chrysophanol (1,8-dihydroxy-3-methylanthraquinone), the active components of such herbal laxatives as aloe, senna, cascara sagrada, and rhubarb, belong to the anthraquinone family. These compounds have a variety of biological effects. Emodin inhibits nuclear transcription factor-κB activation and induces free radical production in human mononuclear cells. Chrysophanol inhibits the replication of poliovirus types 2 and 3 (Picornaviridae) in vitro, which is related to its special structure with two hydrophobic positions on the chrysophanol molecule (C-6 and the methyl group attached to C-3). These biological effects of emodin and chrysophanol are regarded as the underlying causes for the antiviral, anticancer, and vasorelaxant activities of hydroxyanthraquinones.

The main problem concerning anthraquinones is our limited understanding of their absorption characteristics and metabolic pathways which makes it difficult to understand the reasons for their poor bioavailability. In part, the observed variability is a result of the complexity of the metabolism in an in vivo system which involves limited absorption as well as extensive metabolism and degradation with significant losses attributable to some enzymes and intestinal microflora. An additional factor is the relative absence of a molecular-specific methodology for analyzing the various forms of anthraquinones in this complex system.
Some transport proteins play an important role in drug accumulation and transport in the human intestines. P-glycoprotein (P-gp) and multidrug-resistant proteins (MRPs) are constitutively expressed and abundant in the apical membrane of many epithelial and endothelial barriers. The polyphenols, quercetin, kaempferol andisorhamnetin, are substrates of P-glycoprotein, and the P-glycoprotein type of efflux pump might limit the bioavailability of Ginkgo flavonoids.\(^\text{12-14}\) It has been reported that some natural components and their glucosides are substrates of the sodium glucose cotransporters (SGLT\(_1\)) expressed in a number of vectors including Xenopus oocytes and Chinese hamster ovary cells. Evidence has been accumulated that dietary polyphenols can be absorbed by the intestinal epithelium via interaction with glucose transport proteins.\(^\text{15}\)

The human colon adenocarcinoma cell line, Caco-2, has been used as a model to study the intestinal absorption or secretion of various drugs.\(^\text{16}\) This cell line spontaneously differentiates during culture into polarized cells with many enterocyte-like properties of transport-related epithelia. Caco-2 cells retain various transporters expressed in the intestines such as P-glycoprotein, MRPs and SGLT\(_1\).\(^\text{17}\) This model has been used in a number of studies to characterize the intestinal transport mechanism of certain compounds. Caco-2 cells have been widely used in studies to determine the transport kinetics, absorption characteristic and metabolism of dietary polyphenols.

We used the human Caco-2 cell model in this study together with a molecular-specific analysis, to better understand the mechanisms governing anthraquinone absorption. In particular, we focused on the absorption characteristics of emodin and chrysophanol and investigated the effects of certain cellular transporters on this process.

Materials and Methods

**Chemicals.** Emodin, chrysophanol and phloridzin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with the highest purity available (98%, as determined by HPLC). Emodin and chrysophanol were dissolved in DMSO (0.1% \(v/v\) final concentration) due to their hydropobicity and poor solubility in water. Stock solutions of emodin and chrysophanol in DMSO were diluted with a transport buffer before their use in the experiments. Verapamil and cyclosporine were obtained from Sigma (St. Louis, MO, USA). Stock solutions of emodin, chrysophanol and verapamil were wrapped in aluminum foil for protection against light and stored at 4 °C when not being used. All chemicals and reagents used were of analytical grade or HPLC grade.

**Cell culture.** Caco-2 cells, which were obtained from the American Type Culture Collection (Manassas, VA, USA), were maintained in plastic culture flasks (Corning Costar, Cambridge, MA, USA). The Caco-2 cells were cultured in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 1% non-essential amino acids, 1% \(L\)-glutamine, 20% fetal bovine serum, 100 U/ml of penicillin and 0.1 mg/ml of streptomycin, and were grown in a humidified atmosphere of 5% CO\(_2\) in air at 37 °C. The cells were subcultured at 80% confluence.\(^\text{18}\)

**Cellular uptake studies.** All the cellular uptake studies on emodin and chrysophanol used Caco-2 cells seeded at a density of \(6 \times 10^5\) cells/cm\(^2\) on six-well plastic plates (Corning Costar). The culture medium was replaced three times per week, and the cells were used 14 to 21 days after seeding. A fresh culture medium was applied 24 h before the uptake experiments. One hour before the uptake experiments, the culture medium was removed, the cells were quickly rinsed twice with warm PBS and then preincubated at 37 °C for 30 min with 1 ml of an incubation medium. The incubation medium used for the uptake study was a modified Hank’s balanced salt solution (HBSS) at pH 7.4 containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), 0.4 mM KH\(_2\)PO\(_4\), 0.3 mM NaH\(_2\)PO\(_4\), and 10 mM HEPES/Tris. After removing the medium, 1 ml incubation medium containing emodin or chrysophanol at various concentrations was added to evaluate the uptake characteristics at different times and temperatures (37 °C and 4 °C). To investigate the role of transporters, Caco-2 cells were incubated with emodin or chrysophanol for 10 min at 37 °C in the presence of different concentrations of a P-glycoprotein inhibitor (verapamil), MRP inhibitor (cyclosporine) and SGLT\(_1\) inhibitor (phloridzin) to measure the intracellular accumulation of the drugs. At the end of the incubation period, the drug-containing medium was removed, and the cells were washed three times with a cold incubation medium prior to cell lysis.\(^\text{19,20}\)

**Analytical procedures.** To determine the intracellular emodin and chrysophanol concentration, a cell lysate was obtained by subjecting the drug-containing cells to three freeze-thaw cycles in liquid nitrogen. Finally, the absorbed substrate was extracted from the cell lysate with methanol and analyzed with a reverse-phase HPLC system (Waters Corp., Milford, MA, USA), using a 2996 photodiode array detector and a symmetry C\(_{18}\) column (250 × 4.6 mm, 5 \(\mu\)m). The mobile phase was 0.1% acetic acid/acetonitrile (15:85, \(v/v\)), the column temperature and flow rate were 25 °C and 1.0 ml/min, respectively, and the wavelength of the detector was 254 nm. The protein concentration was measured by the method of Bradford, with bovine serum albumin used as a standard.\(^\text{21}\)

**Data analysis.** Student’s \(t\)-test was used for a statistical analysis, \(P\) values \(<0.05\) being considered significant.
Fig. 1. Chromatograms of Emodin and Chrysophanol.

The respective retention times of emodin and chrysophanol were 5.7 and 7.6 min by reverse-phase HPLC with a mobile phase consisting of 0.1% acetic acid/acetonitrile (15:85, v/v).

Fig. 2. Absorption Characteristics of Emodin in the Caco-2 Cells.
A. Time-dependent intracellular accumulation of emodin at 4 °C and 37 °C; B. Dose-dependent absorption of emodin at 37 °C for 10 min. Each value is the mean with SD of five determinations.
Results

HPLC analysis to detect the cellular absorption of emodin and chrysophanol

A liquid chromatogram of pure emodin and chrysophanol absorbed across the Caco-2 cells is presented in Fig. 1. The retention times were 5.7 min and 7.6 min, respectively.

Absorption characteristics of emodin and chrysophanol across Caco-2 cells

To examine the time-course characteristics of anthraquinone absorption, we incubated Caco-2 cells with 50 μM emodin or chrysophanol for different times. To reveal the possible carrier-mediated transport of emodin and chrysophanol, the experiments were performed in parallel at 37°C and 4°C. The dose dependence of the uptake was examined by incubating the cells with various concentrations of emodin or chrysophanol.

The intracellular accumulation of emodin increased rapidly during the first 10 min and reached 108.56 ± 11.57 nmol/l/mg protein at 37°C. Thereafter, the level decreased gradually (Fig. 2A). After 120 min of treatment, the intracellular concentration of emodin was only about a third of the maximal concentration measured at 10 min, which indicated the prevalence of efflux over influx or that the compound had been metabolized. The initial rate of the accumulation of emodin was about two times lower at 4°C than that at 37°C. When the total uptake measured at 37°C is corrected from the passive uptake measured at 4°C, the curve obtained, which appears to be saturated, corresponds to carrier-mediated transport.

Fig. 3. Absorption Characteristics of Chrysophanol in the Caco-2 Cells. 
A, Time-dependent intracellular accumulation of chrysophanol at 4°C and 37°C; B, Dose-dependent absorption of chrysophanol at 37°C for 10 min. Each value is the mean with SD of five determinations.
The dose-dependent absorption of emodin was evaluated at 37°C (Fig. 2B). When the concentration of emodin was increased from 2 μM to 50 μM, the intracellular emodin concentration increased almost linearly. However, saturation was apparent when the concentration of emodin was increased from 50 μM to 200 μM.

The uptake of chrysophanol by the Caco-2 cells was determined under similar conditions. The time-course characteristics of chrysophanol uptake were similar to those obtained for emodin, with a maximal amount of intracellular chrysophanol of \(414.02 \pm 15.28\) nmol/l·mg-protein after 10 min at 37°C (Fig. 3A). In contrast to the emodin concentration curve, the chrysophanol uptake continued to increase when the concentration of chrysophanol was increased from 2 μM to 200 μM, and no saturation was apparent (Fig. 3B). The maximal intracellular accumulation of chrysophanol was nearly four-fold higher than that of emodin. The intracellular accumulation of chrysophanol was higher than that of emodin, indicating that the drug absorption characteristics were closely related their special structures.

Effects of inhibitors on the absorption of emodin and chrysophanol

P-glycoprotein has been shown to inhibit the cellular uptake of hundreds of drugs. This efflux pump is thought to have evolved to protect sensitive areas of the body from the xenotoxins present in our diets. Naturally derived drugs make up a high proportion of the substrate of P-glycoprotein. Inhibition experiments have investigated the intracellular accumulation of emodin and chrysophanol in the absence and presence of verapamil and cyclosporine, inhibitors of P-glycoprotein and various MRPs. As one of the competitive inhibitors of SGLT₁, phloridzin can reduce the uptake of flavonoids.22) There is a similar structure between flavonoids and anthraquinones. To test the hypothesis that emodin and chrysophanol are substrates of SGLT₁, we examined the uptake of emodin and chrysophanol in the presence of the SGLT₁ competitive inhibitor, phloridzin.

These inhibitors all had a dramatic effect on the intracellular concentration of emodin and chrysophanol. Verapamil and cyclosporine increased the uptake of emodin and chrysophanol in a dose-dependent manner; phloridzin, however, reduced their absorption in a dose-dependent manner (Fig. 4). The cellular accumulation of emodin was respectively increased 1.3-fold and 1.1-fold (n = 5, \(P < 0.05\)) in the presence of 50 μM verapamil and 50 μM cyclosporine (Fig. 5A). The cellular uptake of emodin was reduced by approximately 34% in the

![Fig. 4. Dose-Dependent Effects of Verapamil, Cyclosporine and Phloridzin on the Absorption of Emodin and Chrysophanol in the Caco-2 Cells.](image-url)
presence of 50 μM phloridzin (Fig. 5A), being 72.22 ± 6.46 nmol/l-mg-protein in the presence of phloridzin versus 109.79 ± 10.57 nmol/l-mg-protein for the control (absence of an inhibitor; n = 5, P < 0.05). With 50 μM chrysophanol, the cellular accumulation of chrysophanol was respectively increased 1.6-fold and 1.5-fold (n = 5, P < 0.05) in the presence of 50 μM verapamil and 50 μM cyclosporine. The cellular uptake of chrysophanol was reduced by approximately 41% in the presence of phloridzin, being 246.72 ± 14.84 nmol/l-mg-protein for phloridzin versus 419.03 ± 28.96 nmol/l-mg-protein for the control (absence of an inhibitor; n = 5, P < 0.05) (Fig. 5B).

**Discussion**

Epidemiological studies have shown that the consumption of a polyphenol-rich diet is associated with a decrease in the incidence of hormone-related cancers such as breast and prostate cancer. Anthraquinones are widely available as dietary supplements in pharmacies and health food stores. We used human Caco-2 cells, a widely accepted model of human intestinal absorption, to examine the relative absorption of dietary anthraquinones and to gain insight into the mechanisms governing their absorption.

In the first part of this study, the absorption of emodin and chrysophanol by Caco-2 cells was evaluated. The intracellular accumulation of chrysophanol was much greater than that of emodin, and interesting differences were also apparent between the dose-response absorption data for emodin and chrysophanol. Clearly, saturation occurred with emodin, whereas a linear response was apparent from 2 to 200 μM chrysophanol.
Information regarding the functional characteristics of drug transporters is important for improving drug delivery and drug design by engineering the interaction of a drug for specific transporter proteins. The fact that the intracellular accumulation of emodin and chrysophanol was significantly reduced at 4 °C suggests the involvement of a carrier-mediated process. These results indicate that the emodin and chrysophanol uptake at 37 °C probably occurred by both passive and carrier-mediated processes. Furthermore, the absorption of emodin and chrysophanol dramatically decreased after 10 minutes, so it is possible that emodin and chrysophanol were pumped out of the cells by P-glycoprotein and MRP or metabolized in the Caco-2 cells by a phase II metabolic enzyme such as glucuronyl-, sulfate-, or glutathione-transferase, each of which may play an important role in the drug accumulation, transport and phase II metabolism in human intestines.

Our results also show that verapamil and cyclosporine could inhibit the transport of emodin and chrysophanol by reducing the efflux and increasing the cellular accumulation of these compounds. The results suggest that P-glycoprotein or MRP, localized in the apical or basolateral membrane, was capable of effluxing emodin and chrysophanol, effectively opposing absorption and intracellular accumulation. Intestinal P-glycoprotein and MRP can reduce the absorption of emodin and chrysophanol, thereby decreasing their toxicity. When emodin (or chrysophanol) is co-administered with other P-glycoprotein or MRP inhibitors, drug-drug interaction may occur, making the toxicity of emodin and chrysophanol increase unpredictably. The competitive inhibition of SGLT1 by phloridzin has been well documented.

We have demonstrated that phloridzin can inhibit the cellular accumulation of emodin and chrysophanol across the apical membrane of Caco-2 cells. It has been suggested that partial intestinal absorption of emodin and chrysophanol may occur, making the toxicity of emodin and chrysophanol increase unpredictably. The competitive inhibition of SGLT1 by phloridzin has been well documented. We have demonstrated that phloridzin can inhibit the cellular accumulation of emodin and chrysophanol across the apical membrane of Caco-2 cells. It has been suggested that partial intestinal absorption of emodin and chrysophanol may be through transport by SGLT1. It is possible that the absorption of emodin and chrysophanol, crossing the apical membrane in Caco-2 cells, may be partially governed by a balance between transport by SGLT1 and efflux by P-glycoprotein or MRP. This balance may favor efflux by P-glycoprotein and MRP for emodin and chrysophanol, or it may also be shifted to favor influx by SGLT1. This possibility, which is of great relevance to new drug development, will require further studies. Studies are therefore needed to elucidate the mechanism for the intestinal absorption of emodin and chrysophanol. A more detailed understanding of transporter functions will enable the development of highly efficient drugs with tailored pharmacokinetic profiles; moreover, approaches that intentionally exploit positive drug-drug interaction may become more important in the future.

In conclusion, this study shows that the inequality of structure between emodin and chrysophanol with different numbers and positions of hydroxyl groups may result in the distinction of their intracellular accumulation. Furthermore, a specific transport system mediates emodin and chrysophanol across the apical membrane in Caco-2 cells. Thus, strategic application of intestinal P-glycoprotein or an MRP inhibitor and an SGLT1 stimulator may improve the oral therapeutic effect of both emodin and chrysophanol.

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