

1 **Characterization of the Intestinal Absorption of Morroniside from** 2 ***Cornus officinalis* Sieb. et Zucc via a Caco-2 Cell Monolayer Model**

3 Short title: Intestinal Absorption of Morroniside via Caco-2 Cell

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22

23 **Abstract**

24 Morroniside is biologically active polyphenols found in *Cornus officinalis* Sieb. et
25 *Zucc* (CO) which exhibits a broad spectrum of pharmacological activities, such as
26 protecting nerves, preventing diabetic liver damage and renal damage. However, little
27 data is available regarding its mechanism of intestinal absorption. Here, a human
28 intestinal epithelial cell model Caco-2 cell *in vitro* cultured was applied to study on
29 the absorption and transport of morroniside, the effects of time, donor concentration,
30 pH, temperature and inhibitors, the absorption and transport of morroniside were
31 investigated. The bidirectional permeability of morroniside from the apical (AP) to
32 the basolateral (BL) side and in the reverse direction was studied. When
33 administrated was set at three tested concentrations (5, 25 and 100 μ M), the P_{app} value
34 in AP-to-BL direction was ranged from 1.59 to 2.66 $\times 10^{-6}$ cm/s. In the reverse
35 direction, BL-to-AP, the value was ranged from 2.67 to 4.10 $\times 10^{-6}$ cm/s. The data
36 indicated that morroniside transport was both pH- and temperature- dependent. The
37 morroniside's permeability process affected by treatment with various inhibitors, such
38 as the multidrug resistance protein inhibitors MK571 and benzbromarone, the breast

39 cancer resistance protein inhibitor apigenin. It can be found that the mechanisms of
 40 intestinal absorption of morroniside may involve multiple transport pathways like
 41 passive diffusion as well as efflux protein-mediated active transport especially the
 42 multidrug resistance protein2 and breast cancer resistance protein. After CO was
 43 added, P_{app} AB increased significantly by about 125.26%, it can therefore be assumed
 44 that some ingredients in the crude material promote morroniside's absorption in the
 45 small intestine.

46 Keywords: Caco-2; Morroniside; multidrug resistance protein; absorption
 47 mechanism

48

49 **Introduction**

50 Traditional Chinese medicines (TCM) are natural therapeutic remedies that have been
 51 widely used for thousands of years (1). Morroniside (Fig 1), one of the most
 52 important iridoid glycosides, is the main active ingredient of *Cornus officinalis* Sieb.
 53 *et Zucc* (CO). It is a rich source of iridoid glycosides and has been used as a
 54 traditional Chinese medicinal herb for centuries (2). Various pharmacological studies
 55 have indicated that morroniside was effective in the treatment of Alzheimer's disease
 56 (3) for protecting nerves (4), preventing diabetic liver damage (5) and renal damage
 57 (6), having beneficial effects on lipid metabolism and inflammation (7) and anti-
 58 anaphylactic activity (8). Since morroniside and its correlative plant extracts have
 59 exhibited these pharmacological effects, it would be hopeful that morroniside to be
 60 developed to some promising preparations of herbal medicinal products.

61 Several studies have been conducted to determine the concentration of
 62 morroniside in biological matrices (9-11). The absolute oral bioavailability of
 63 morroniside was calculated to be 7.0, 6.1 and 3.6% for low, medium and high dosage,
 64 respectively (9, 12). It seemed like that the plasma levels of morroniside

65 administrated intravenously were much higher than those after oral administration. It
 66 is well known that oral administration is the main route for applying TCM and they
 67 should be absorbed in the gastrointestinal tract to exhibit pharmacologic effects (13).
 68 The intestinal absorption barrier is a major factor controlling the absorption and oral
 69 bioavailability of drugs(14-16) and it is here that the first steps of pharmacokinetics
 70 occur after oral intake. Therefore, exploration of the intestinal absorption mechanism
 71 of morroniside is necessary not only for the *in vivo* pharmacokinetics study but also to
 72 provide some key information for their effective delivery system.

73 The aim of present study was to further investigate the intestinal absorptive
 74 characteristics of morroniside using Caco-2 cells. This model is extensively used
 75 because of its morphological and functional similarities to the human small intestinal
 76 epithelium, and it has been recognized by the FDA as a viable model that replicates
 77 human intestinal absorption (17-19). The author aimed at further revealing the reason
 78 of low bioavailability of morroniside and providing a theoretical basis for the
 79 development of formulations.

80

Fig 1 The chemical structure of morroniside

Materials and Methods

Materials and Reagents

Transwell permeable polycarbonate inserts (0.4 μ m) and 12-well cell culture plates were obtained from Corning. (Cambridge, MA, USA). Caco-2 cell line was generously provided by Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco Laboratories (Life Technologies Inc., Grand Island, NY, USA). Hanks' balanced salt solution (HBSS, powder form) was obtained from Sigma Chemical Co. (Deisenhofen, Germany). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). 100 $\times$ nonessential amino acids, 100 $\times$ penicillin and streptomycin, 0.25% trypsin with ethylene- diaminetetraacetic acid (EDTA), 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and BSA were all purchased from Invitrogen Corp. (Carlsbad, CA). Verapamil, MK 571, indomethacin, benzbromarone, apigenin, sodium vanadate and cimetidine used in this study were obtained from Aladdin Industrial Inc.

97 (Shanghai, China). Morroniside (purity > 98.0%) and loganin (internal standard (IS),
98 purity > 98.0%) were obtained from the National Institute for the Control of
99 Pharmaceutical and Biological Products (Beijing, China). All other reagents (typically
100 analytical grade or better) were used as received.

101 *Herb extract preparation*

102 The extract was prepared according to reported methods (20). CO (100 g) crude
103 material was decocted with 1000 mL water for 2 h. Then, the filtrate was collected
104 and the residue was decocted with 1000 mL water for another 2 h. Finally, the two
105 batches of filtrates were combined and concentrated to 100 mL. The solution was
106 centrifuged to pellet any insoluble molecules then filter sterilized with a 0.22 µm
107 (PEM) syringe filter. The contents of morroniside in preparation turned out about
108 16.02mg/g in crude material after determined by a HPLC method(9).

109 *Caco-2 Cell Culture and Cytotoxicity Assay*

110 The conditions for Caco-2 cell culture have been described previously(21). In brief,
111 Cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The

112 culture medium (DMEM) was supplemented with 10% (v/v) fetal bovine serum, 1%
 113 nonessential amino acids, 1% penicillin, and streptomycin. The medium was changed
 114 every 2-3 days. When the cell monolayer reached 80-90% confluence, the cells were
 115 detached with a solution of trypsin (0.5 mg/ml) and EDTA (ethylene diamine
 116 tetraacetic acid, 0.2 mg/ml) (PAA) and reseeded at a density of 5×10^4 cells/cm².

117 For Cytotoxicity Assay, the cells were incubated in 96-well plate for 24 h,
 118 MTT solution was prepared at 1 mg/mL in PBS and was filtered through a 0.2 µm
 119 filter. Then, 20 µL of MTT was added into each well. Cells were incubated for 4 h at
 120 37 °C with 5% CO₂, 95% air and complete humidity. After 4 h, the MTT solution was
 121 removed and replaced with 150 µL of DMSO. The plate was further incubated for 5
 122 min at room temperature, and the optical density (OD) of the wells was determined
 123 using a plate reader at a test wavelength of 490 nm.

124 ***Transport of Analytes across the Caco-2 Monolayer***

125 An initial stock solution of morroniside in methanol was prepared. The stock solution
 126 was then diluted with HBSS. The transepithelial electrical resistance (TEER) value
 127 was measured with a Millipore ERS voltameter (Millipore, Massachusetts, USA) in

128 order to evaluate and determine the monolayer integrity. The monolayers were ready
129 for experiments from 19 to 22 days after seeding. Only monolayers that demonstrated
130 a TEER value above $400 \Omega \times \text{cm}^2$ were used for the experiment.

131 During incubation, the culture medium was refreshed every other day. The culture
132 medium was discarded, 500 μL of HBSS was added to each well and incubated for 20
133 min at 37°C . Afterward, each of the well was rinsed 2 times with HBSS at 37°C .

134 The test compounds were added to either the apical (AP, 0.5 mL) or basolateral side
135 (BL, 1.0 mL), while the receiving chamber contained the corresponding volume of
136 blank HBSS medium. Incubation was performed at 37°C for 180 min, with shaking at
137 50 rpm. To assess the drugs transport from AP to BL, at the incubation of 5, 15, 30,
138 45, 60, 90, 120 and 180 min, a 50 μL of the solution from BL or AP side was
139 collected, and replaced with an equal volume of HBSS. The effect on uptake was
140 investigated at 4°C and 37°C . One plate was placed in a cell incubator at 37°C ,
141 with 5% CO_2 , and the other was in a 4°C refrigerator. Caco-2 cells were seeded at 5
142 $\times 10^4$ cells cm^2 in a 12-well plate. After 19 day of incubation, the effect of
143 temperature on cellular uptake was investigated by 60 min of incubation with

144 morroniside at 25 µg/mL. To study the effect of pH on the morroniside transport, the
145 experiments were performed in HBSS buffer, which was adjusted to pH 6.5 or pH 7.4
146 on the apical side (0.5 mL) depending on the experiment, and to pH 7.4 on the
147 basolateral side (1.5 mL).

148 Efflux and influx transporters were investigated for their effects on the
149 transport flux of morroniside. Several ABC transporter inhibitors, one p-glycoprotein
150 inhibitor (100µM verapamil) (22), three multidrug resistance- related protein inhibitor
151 (100µM MK571, 50µM benzbromarone and 200µM indomethacin) (23-25), and one
152 breast cancer resistance protein inhibitor (25µM apigenin) (26) were used to
153 determine the transporters involved in the efflux transport of morroniside.; Inhibiting
154 the influx during morroniside transport across the Caco-2 cell monolayers was
155 investigated by adding 50µM sodium vanadate and cimetidine to evaluate the
156 selectivity of Na/K-ATPase (27).

157 ***Sample Processing***

158 Samples were treated with a protein precipitation method. After spiked with 4-fold
159 volume of acetonitrile containing 5% acetic acid and 70 ng/mL IS, the tubes were

vortexed for 1min. The tubes were centrifuged at 14,000rpm for 10min, and the supernatants were collected and filtered through a 0.22µm hydrophobic membrane. An aliquot of 2 µL was injected into the LC-MS/MS system for analysis.

UPLC-MS/MS analytical methods

An API 5500 triple-quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Canada) equipped with an electrospray ionization (ESI) source were used for chromatographic analyses. The separation was achieved with a ZORBAX Eclipse Plus C₁₈ column (50 mm × 2.1 mm, 1.8 µm). The mobile phase consisted of 0.1% acetic acid in aqueous solution as solvent A and 100% acetonitrile solution as solvent B, and the flow rate was 0.4 mL/min. The following gradient elution was used: 0-1.0 min 1% B; 1.0-2.2 min 1→29% B; 2.2-2.3 min 29→95% B; 2.3-3.3 min 95% B; 3.3-3.4 min 95-1% B, and followed by a 1.0 min re-equilibration to the initial condition. The column was maintained at 30°C and the autosampler tray at 30°C.

Analytical procedures were evaluated with negative electrospray ionization (ESI) mode. Typical parameters for the Turbo Ionspray source set were: ion spray source temperature at 550 °C, ionspray voltage (IS) at 5000V; Gas 1 and Gas 2

(nitrogen) at 50 psi; CUR at 50; CAD at -2.0. Other parameters were shown in Table

1.

Table 1 Optimized MRM parameters for the determination of morroniside and IS.

Compounds	Q1(Da)	Q3(Da)	DP(V)	CE(eV)	CXP(V)	Detected ion
Morroniside	451.0	243.0	-100	-30	-15	M ⁻
Loganin	435.2	227.0	-100	-23	-15	M ⁻

Statistical analysis

UPLC-MS/MS data acquisition was performed using Analyst 1.5.2 and MultiQuant

2.1.1 software (Applied Biosystems). All of the data shown are the mean (standard

deviation, n = 3. The data were analyzed by SPSS 20.0 Software. In the Caco-2 cell

model, rate of transport is obtained from amount transported versus time curve using

linear regression. The permeability of a compound is calculated using the following

equation: $P_{app} = (dQ/dt)/(A \cdot C_0)$

where, dQ/dt is the rate of drug transport, C₀ is the initial concentration of the

compound in the donor chamber and A represents the surface area of the cell

monolayer. The efflux ratio P was determined by calculating the ratio of P_{app} in the

secretory (BA) direction divided by that in the absorptive (AB) direction, according to

the following equation:

$$P = P_{app}^{BA} / P_{app}^{AB}$$

Authors had access to information that could identify individual participants during or

after data collection.

Results

Cytotoxicity in Caco-2 Cells

Viability of cells was directly measured using MTT test to evaluate the cytotoxicity of

morroniside toward Caco-2 cells prior to transport experiments. As shown in Fig 2,

An increase in the concentrations of morroniside was accompanied by a slight

decrease in the viability of Caco-2 cells, although the difference was not statistically

significant. Even at the highest concentration (200 μ M), the cell viability rates of

Caco-2 cells treated with the three compounds reduced only by 8%, indicating that in

our experimental design, morroniside was nontoxic to the growth of Caco-2 cells, as

was 0.63mg/mL CO (containing 25 μ M morroniside).

204 Fig 2 Cytotoxicity of morroniside in Caco-2 cells as evaluated by the MTT assay. Data are the mean
205 values $\pm$ standard variation of five replications. Different letters indicate a significant difference at $p <$
206 0.05.

207 *LC/MS/MS analysis to quantify morroniside*

208 The retention time for morroniside and IS were approximately 1.85 and 2.01 min,
209 respectively. As shown in Fig 3, any significant peaks interfering with morroniside or
210 IS were not observed in fresh blank HBSS. Representative chromatograms are
211 presented in Fig 3, including that of morroniside and IS in fresh blank HBSS and a
212 sample 5 minutes after transport experiments. Calibration curves, constructed using
213 linear least- squares regression, showed good linearity within a concentration range of
214 4-1000 ng/mL morroniside in HBSS. A typical calibration curve equation for
215 morroniside was $y=0.007x+0.0055$, where y represents the ratio of the morroniside
216 peak area to the loganin peak area (x is the concentration of morroniside). The
217 calibration curves displayed good linearity as indicated by high correlation
218 coefficients ($R^2=0.9997$). The lower limit of quantification (LLOQ) in the collected
219 samples was 4 ng/mL.

220 Fig 3 Representative multiple reaction monitoring (MRM) chromatograms of morroniside (1) and IS
221 (2) in fresh blank HBSS (A) and a sample (B) 5 minutes after transport experiments by LC/MS/MS
222 (7.81ng/mL for morroniside and 70ng/mL for IS)

223 *Uptake of morroniside by Caco-2 Cells*

224 Results of the cellular uptakes of different concentrations (5, 25 and 100μM) of
225 morroniside into Caco-2 cell monolayers from the AP compartment over 2 h were
226 shown in Fig 4. As shown in Fig 4, the uptakes of morroniside into Caco-2 cells
227 occurred quickly. P_{app}^{AB} of morroniside ranged from 1.59 at 100μM to 2.66×10^{-6}
228 cm/s at 5μM (AP to BL) and from 2.67 at 100μM to 4.10×10^{-6} cm/s at 5μM (BL to
229 AP)

230 Fig 4 Time-course of morroniside (5, 25 and 100μM) transport across the Caco-2 cell monolayers from
231 apical (AP) to basolateral (BL) and from BL to AP (n=3)

232 *Effect of pH and temperature on the permeation of morroniside*

233 The assay studying the AP to BL transport in HBSS with various pH values was
234 shown in Table 2. The P_{app}^{AB} values of morroniside at pH 7.4 were significantly

higher than those at pH 6.0 ($p < 0.05$), indicating an easier transport of morroniside at a higher pH (7.4) than at lower a pH (6.0).

Temperature plays an important role in the transport of drugs, it can not only affect the activity of the carrier protein, but also the state of drugs in solvent(28). As presented in Table 2, the morroniside transport across Caco-2 cell monolayers at low temperature (4°C) significantly reduced because P_{appAB} was decreased from $1.94 \times 10^{-6} \text{cm/s}$ to $0.6 \times 10^{-6} \text{cm/s}$.

Table 2 P_{appAB} of morroniside ($25 \mu\text{M}$) in human intestinal Caco-2 cells previously treated at different PH values and temperatures ($n = 3$, $p < 0.05$).

Conditions	P_{appAB}
4°C	$0.60 \pm 0.19^*$
37°C	1.94 ± 0.64
Ph 6.0	$1.85 \pm 0.60^{\Delta}$
Ph 7.4	0.59 ± 0.14

*Denote results significantly different from those of the 37°C experiments

$^{\Delta}$ Denote results significantly different from those of the Ph 7.4 experiments ($p < 0.05$).

Effects of Various Compounds on Morroniside Transport

With the participation of $50 \mu\text{M}$ of sodium vanadate or cimetidine, the uptake of morroniside did not significantly increase or decrease. These results indicated that the

250 influx transporters like OATs or Na⁺/K⁺ pump contributed little to the transport of
251 morroniside. (Table 3)

252 Table 3 Inhibitory effects of influx transporters on morroniside transport in Caco-2 cell monolayers.

Inhibitors	Transporter	P _{app} AB (×10 ⁻⁶ cm/s)	P _{app} BA (×10 ⁻⁶ cm/s)	P
Control		1.90±0.5	3.06±0.6	1.60
Sodium vanadate	Na ⁺ /K ⁺ pump	1.89±0.4	3.11±0.7	0.78
Cimetidine	OATs	1.82±0.5	3.1±0.7	1.23

253

254 MRP and BCRP are present in the intestinal tract of humans, which are responsible

255 for the transport and efflux of compounds or drugs (29). Fig 5 showed the P_{app}AB

256 values increased, but had no effect on the P_{app}BA, the data indicated that morroniside

257 was effluxed by MRP.

258 BCRP was found to be located at the apical side in the cell membrane (30).

259 Apigenin was used to determine the effect of BCRP on the transport of 25μM

260 morroniside. After addition of apigenin (25μM), the P_{app}AB of morroniside increased

261 significantly (p < 0.05), which resulted in a decrease in the efflux ratio by 72.05%

262 (Fig 5). It can therefore be assumed that morroniside is also a substrate of BCRP.

263

264 Fig 5 Inhibitory effects of efflux transporters on morroniside transport in human intestinal

265 Caco-2 cell monolayers: AP to BL (a) and from BL to AP (b) (n = 3)

266 *Denote results significantly different from those of the control experiments ($p < 0.05$).

267 **Denote results significantly different from those of the control experiments ($p < 0.001$)

268 **DISCUSSION**

269 To further study the transport characters of morroniside, we investigated the
 270 relationship of time and concentration to transport. The concentration of morroniside
 271 increased approximately linearly with time from 0 to 60 min. After 60 min, the
 272 concentration gradient between the two sides had greatly decreased, result in the
 273 curves turned to reach a platform (Fig 4). A possible reason is that the concentration
 274 gradient between the two sides had greatly decreased after 60 min, which resulted in
 275 much less transport and led to a platform. From the above results, it is concluded that
 276 passive transfer is the main transport way. In our previous work, loganin (31), a
 277 structural analog of morroniside, showed good intestinal permeability by using the
 278 human intestinal Caco-2 cells model. It seemed like the cell permeability of
 279 morroniside is far lower than loganin.

280 Our results showed that the P_{app} BA values of the morroniside was higher
281 than their P_{app} AB values ($p < 0.05$), indicating that some transporters might be
282 involved in the transport of morroniside in B-A direction.

283 Accounting for the limit of detection of the method, compounds which are
284 completely absorbed in the human intestine typically exhibit P_{app} values of $>70 \times 10^{-6}$
285 cm/s in the Caco-2 transwell system, whereas compounds with poor absorption
286 ($<20\%$) have P_{app} values of $<10 \times 10^{-6}$ cm/s(32). The P_{app} values of morroniside
287 determined was at the level of 10^{-6} which indicated poor intestinal absorption about
288 morroniside. It is also important to note that, strong metabolism may be regarded as
289 another major cause of the low oral bioavailability of morroniside *in vivo*. However,
290 further studies are required to clarify whether metabolism was the main factor in the
291 *in vivo* process of morroniside.

292 The data illustrating how pH and temperature effect permeation of
293 morroniside, show that morroniside transport is both pH-dependent and temperature-
294 dependent, these results indicate, that some transporters may be involved in the efflux
295 of morroniside. In fact, previous research indicated that both the influx (33) and efflux

296 transport like BCRP (34, 35) were activated at lower pH levels. It can therefore be
297 assumed that morroniside is also a substrate of BCRP. This could be indeed verified
298 in the next subsequent experiments.

299 To determine whether P-gp is involved in the transport of these compounds,
300 their transports were studied in the presence of verapamil, a known inhibitor for P-gp.
301 But the P_{app}^{AB} and P_{app}^{BA} of morroniside did not change significantly before and
302 after the addition of verapamil, which indicated that P-gp might not be involved in the
303 transport of morroniside. (Fig 5)

304 MRP has different isoforms, from which MRP2 is located at apical side in
305 the cell membrane and MRP3 was the main basolaterally localized MRP(36).
306 Indomethacin and benzbromarone were used as MRP2 and MRP3 inhibitors,
307 respectively. The results in Fig 5 showed that P_{app}^{AB} values caused a significant
308 increase in the AP to BL flux ($p < 0.001$) after Indomethacin was added. This in turn
309 indicated that morroniside is a substrate of MRP2. Co-treatment with 200 μ M
310 benzbromarone did not alter morroniside's P_{app}^{AB} or the P_{app}^{BA} values, and the
311 efflux ratio reduced only by 6.64%.

312 After 0.63mg/mL CO (containing 25 μ M morroniside) was added, $P_{app}AB$
 313 increased significantly by about 125.26%, and $P_{app}BA$ value was no significant
 314 difference compared with the control group. Many ingredients, especially iridoid
 315 glycosides are contained in CO. To our knowledge, the most abundant bioactive
 316 iridoid glycosides, which are widely distributed in CO, are loganin, morroniside and
 317 sweroside (37). Loganin, which was found to be a substrate of BCRP, in our previous
 318 study, may reduce efflux of morroniside and lead to a lower $P_{app}AB$ in the crude
 319 material group (31). But the compounds contained in CO are complex, and more
 320 research is needed to clarify the intestinal absorption characteristics of this herb
 321 preparation.

322 **Conclusions**

323 In the present study, morroniside was shown to be poor-absorbed compound in the
 324 Caco-2 cell monolayer model and the mechanisms were mainly by passive diffusion
 325 and with efflux protein-mediated active transport. Transporters, BCRP and MRPs
 326 (especially MRP2) are vital for morroniside transport in the intestine. These new
 327 findings provided useful information for predicting its oral bioavailability,

328 pharmacokinetics, and the clinical application as well as determination of the

329 bioactive substance basis of morroniside.

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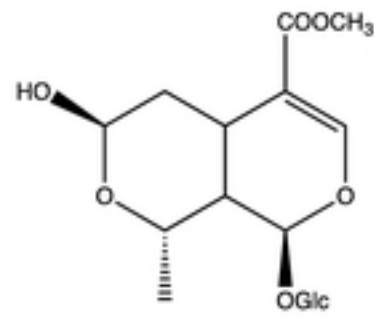


Fig 1 The chemical structure of morroniside

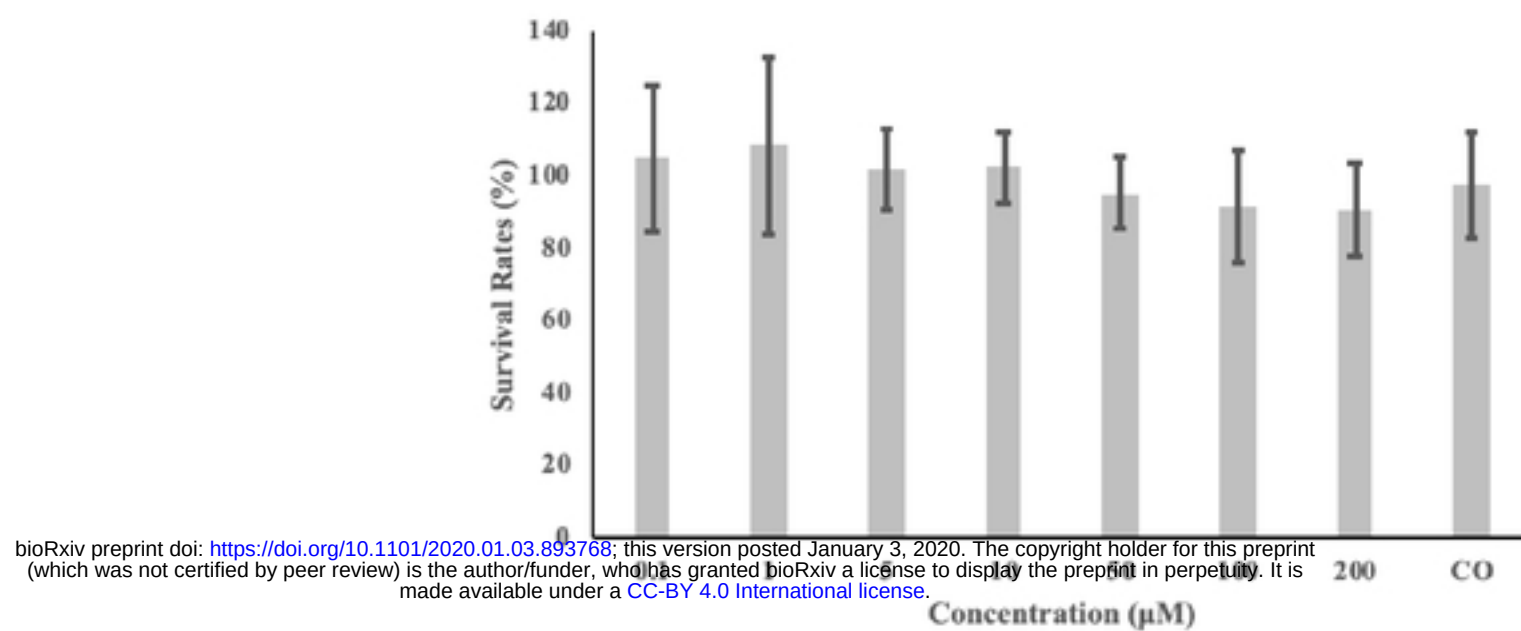


Fig 2 Cytotoxicity of morroniside in Caco-2 cells as evaluated by the MTT assay. Data are the mean values $\pm$ standard variation of five replications. Different letters indicate a significant difference at $p < 0.05$.

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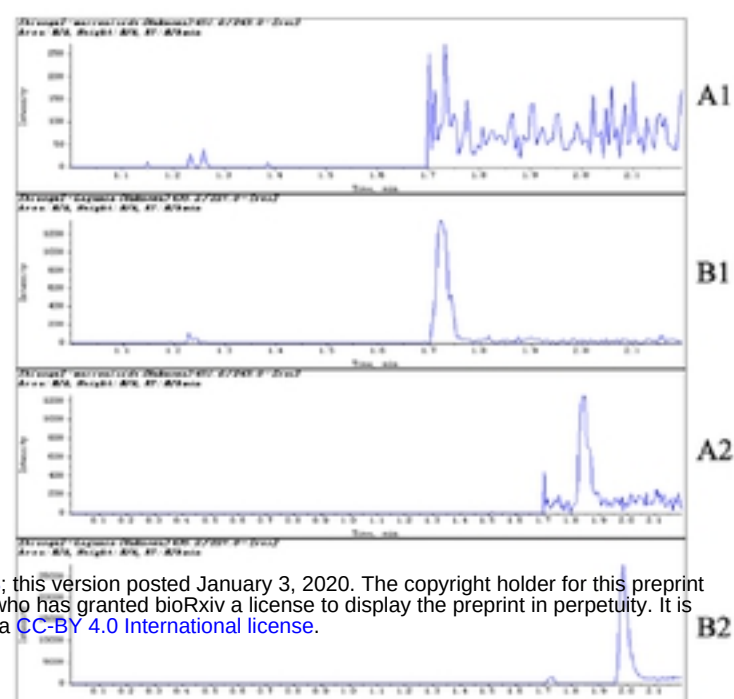
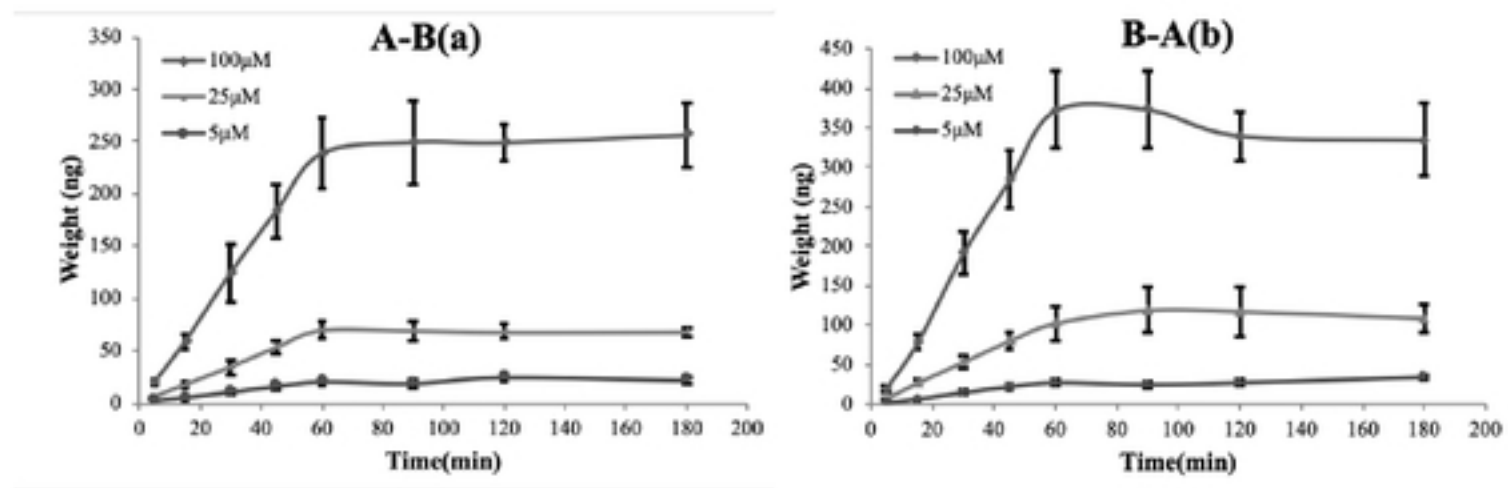


Fig 3 Representative multiple reaction monitoring (MRM) chromatograms of morroniside (1) and IS (2) in fresh blank HBSS (A) and a sample (B) 5 minutes after transport experiments by LC/MS/MS (7.81ng/mL for morroniside and 70ng/mL for IS)



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Fig 4 Time-course of monomelic (5, 25 and 100 μM) transport across the Caco-2 cell monolayers from apical (AP) to basolateral (BL) and from BL to AP (n=3)

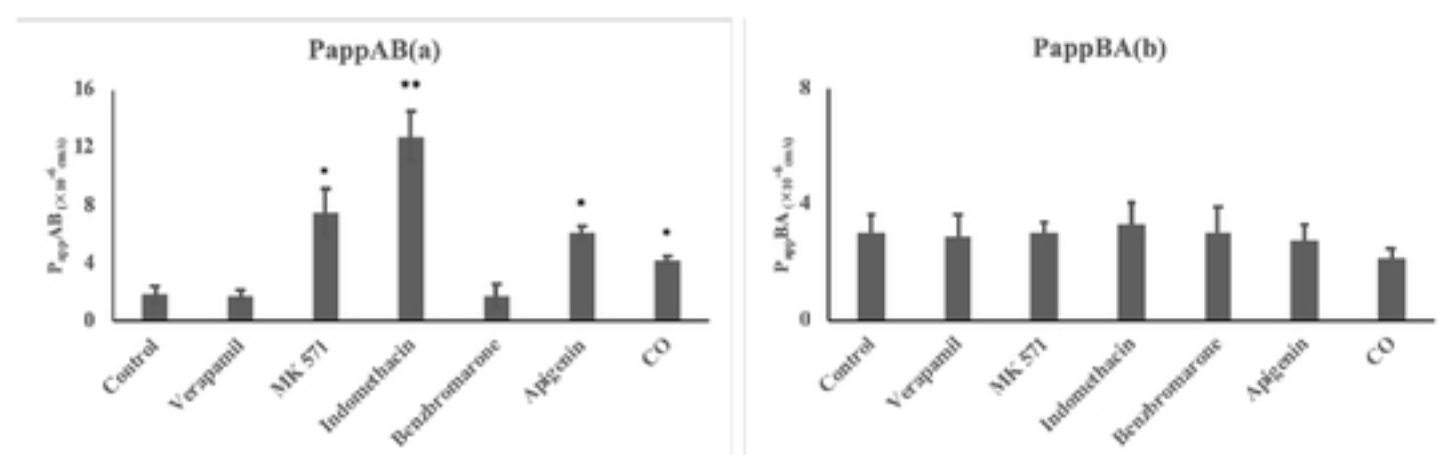


Fig 5 Inhibitory effects of efflux transporters on morroniside transport in human intestinal Caco-2 cell

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morroniside: AP to BL (a) and from BL to AP (b) (n = 3)

*Denote results significantly different from those of the control experiments (p < 0.05).

**Denote results significantly different from those of the control experiments (p < 0.001)