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Intestinal permeability of antivirus constituents from the fruits of *Eucalyptus globulus* Labill. in Caco-2 Cell Model

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Abstract—The uptake and transepithelial transport of the three main constituents macrocarpal A (M-A), macrocarpal B (M-B), and cypellocarpa C (Cy-C) from the fruits of *Eucalyptus globulus* Labill. were investigated. Monolayers of the human intestinal epithelial cancer cell line Caco-2 were incubated with M-A, M-B, and Cy-C to model its intestinal absorption and transport, respectively. The determination of compounds was performed by HPLC. The apparent permeability coefficients (P_{app}) for M-A, M-B, and Cy-C in the apical-to-basolateral direction of a Caco-2 monolayer were $(1.70 \pm 0.06) \times 10^{-6}$, $(1.99 \pm 0.10) \times 10^{-6}$, and $(6.08 \pm 0.41) \times 10^{-6}$ cm/s, respectively. In the presence of iodoacetamide, the P_{app} of Cy-C were both reducted in apical-to-basolateral and basolateral-to-apical directions. M-A and M-B appear to accumulate in the epithelial cells. The intestinal absorption of M-A, M-B, and Cy-C was passive diffusion as the dominating process and Cy-C was partly ATP-dependent.

Eucalyptus globulus Labill. (Myrtaceae) is a fast-growing species native to Australia and is widely distributed in southern China. The fruits of *E. globulus* are known to be a rich source of secondary compounds with a variety of biological activities, in which macrocarpal A (M-A), macrocarpal B (M-B), and cypellocarpa C (Cy-C, Camaldulenside) are characteristic chemical constituents. The M-A and M-B (see structure in Fig. 1) are unique phloroglucinol-sesquiterpene coupled compounds and have attracted the most attention. Various studies have identified that M-A and M-B have strong antibacterial activity against cariogenic and periodontopathic bacteria with the MIC of 0.39–1.56 and 0.39–3.13 μg/ml, respectively.[1] The M-A and M-B exhibited inhibitory effect against HIV-RTase activity with IC_{50} of 10 and 5.3 μM,[2] respectively. The Cy-C is phenol glycosides acylated with (+)-oleuropeic acid (see structure in Fig. 1), and showed potent in vitro antitumor-promoting activity in a short-term bioassay by evaluating the inhibitory effect on Epstein–Barr virus early antigen activation induced by 12-*O*-tetradecanoyl phorbol 13-acetate (63.4% inhibition at 500 mol ratio/TPA), and also suppressed an in vivo two-stage carcinogenesis induced with nitric oxide and TPA on mouse skin remarkably, reducing the percentage of tumor-carrying mice to 30% after 10 weeks at a concentration of 50 mol ratio/TPA.[3]

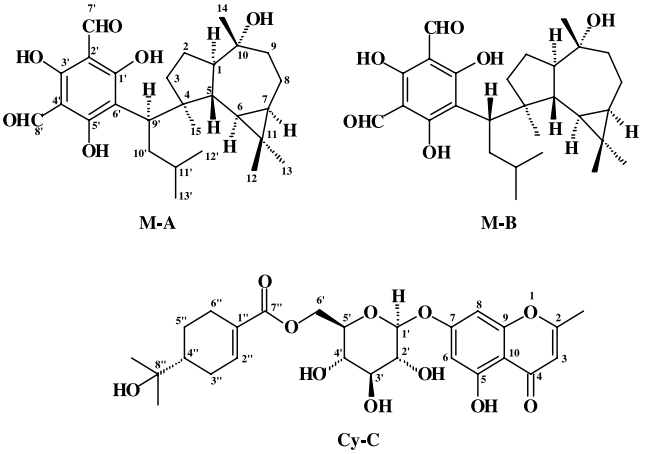

Figure 1. Chemical structures of M-A, M-B and Cy-C.

Keywords: *Eucalyptus globulus*; Macrocarpal A; Macrocarpal B; Cypellocarpa C; Intestinal permeability; Caco-2.
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It is important to research their absorption in developing new drugs per os utilizing these activities. If those compounds with antibacterial and anti-HIV activities are of good membrane permeability, their absorptions are usually good. There are no reports about the membrane permeability and pharmacokinetics of related natural products so far.

In the present study, we investigated the intestinal permeability of M-A, M-B, and Cy-C using human Caco-2 cell monolayers as a model system for intestinal absorption. This model provided important predictive information regarding the oral bioavailability of M-A, M-B, and Cy-C.

Isolation of M-A, M-B, and Cy-C. The 95% aqueous ethanolic extract of the fruits of *E. globulus* was divided into cyclohexane-, ethyl acetate-, *n*-butanol-, and water-soluble fractions. The ethyl acetate-soluble fraction was further subjected to chromatographic techniques on silica gel to afford M-A,[4] M-B[5], and Cy-C,[6] respectively, reported for the first time from the fruits of *E. globulus*.

Analysis method. A rapid, simple, and reliable HPLC method has been established for the analysis of the M-A, M-B, and Cy-C in the Caco-2 cell cultures.[7] The apical chamber cultures, basolateral chamber cultures, and Caco-2 cells were obtained, respectively, in transport experiments.[8] These samples were frozen at −20 °C immediately after the operation and were freeze-dried. These dried samples were then treated with equal volume (w/v) of methanol to precipitate and remove proteins, followed by centrifugation at 13,000*g* for 10 min. The supernatant solution was filtered through a 0.45 μm filter. A 20.0 μL aliquot was injected into the HPLC system. The calibration curves for M-A, M-B, and Cy-C were constructed by plotting concentration (y, nmol) versus peak area (x). The linear equation and range were $y = 0.0009x - 0.0411$ ($r = 0.9995$) and $2.00 \sim 4.00 \times 10^{-2}$ nmol for M-A; $y = 0.006x + 0.0316$ ($r = 0.9992$) and $2.00 \sim 4.00 \times 10^{-2}$ nmol for M-B; and $y = 0.0012x + 0.0329$ ($r = 0.9995$) and $2.00 \sim 4.00 \times 10^{-2}$ nmol for Cy-C, respectively.

Transport of M-A, M-B, and Cy-C through the Caco-2 monolayer. Under the conditions of the experiments,[8] the human intestinal permeability of M-A, M-B, and Cy-C was evaluated using the Caco-2 cell model. The transport rates of three compounds across the monolayer at different concentrations were linear up to 100 μM (see Figs. 2 and 3). The results of P_{app} in the apical-to-basolateral (AP → BL) and basolateral-to-apical (BL → AP) directions are shown in Table 1. These values are about 10 times less than drugs such as propranolol, which is often used as reference standard of high permeability and the main mechanism of intestinal absorption by passive diffusion, and about 10 times higher than drugs such as atenolol, which is often used as reference standard of poor permeability and the main mechanism of intestinal absorption by passive diffusion. Under the current condition, the P_{app} value for propranolol was 2.18×10^{-5} cm/s and for atenolol was 2.77×10^{-7} cm/s. In addition, the transport rates of the

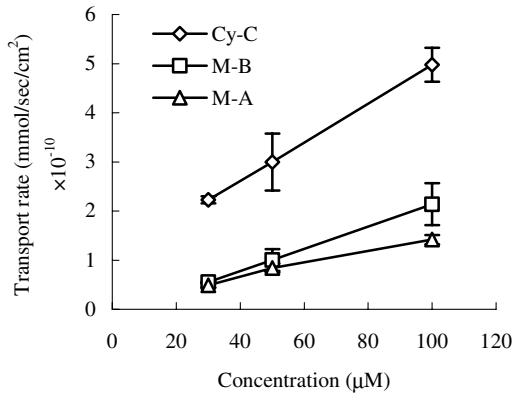

Figure 2. The apical-to-basolateral transport rate of the compounds at different concentrations. The incubation time was up to 90 min and all experiments were carried out in triplicate. Data are means ± SD.

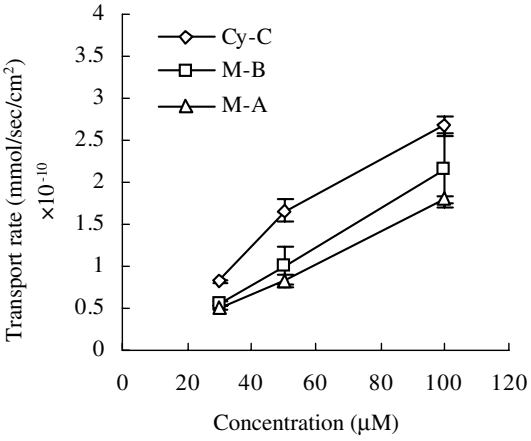

Figure 3. The basolateral-to-apical transport rate of the compounds at different concentrations. The incubation time was up to 90 min and all experiments were carried out in triplicate. Data are the means ± SD.

three compounds were shown with similarly linear increasing trend and unsaturation (see Figs. 2 and 3), but P_{app} values were shown as it appears unchanged (see Table 1) with increasing concentration. From kinetics curve presented in Figure. 4, it is evident that the transport of three compounds from AP → BL increased approximately linearly with the incubation time. During the incubation up to 180 min, the transport trends of M-A and M-B was similar with, but less than that of Cy-C. The increasing order of the transport was shown as M-B < M-A < Cy-C. Since the ratio of $P_{app\ AP \rightarrow BL}$ to $P_{app\ BL \rightarrow AP}$ for M-A and M-B (see Table 1) were essentially equal, there was no indication of efflux or active transport. In the Caco-2 monolayer cell model, it can be assumed that the drugs are transported exclusively by the passive transcellular route since the surface area of the brush border membranes is >1000-fold larger than the paracellular surface area.[9] While the ratio of $P_{app\ AP \rightarrow BL}$ to $P_{app\ BL \rightarrow AP}$ for Cy-C was 2.10, it indicated partly involved active transport.

Table 1. P_{app} values of M-A, M-B and Cy-C from two directions at different concentrations

Concentrations (μM)		$P_{app\ AP\ \rightarrow BL}$ ($\times 10^{-6}$ cm/s)	$P_{app\ BL\ \rightarrow AP}$ ($\times 10^{-6}$ cm/s)	$P_{app\ AP\ \rightarrow BL}/P_{app\ BL\ \rightarrow AP}$
M-A	30	1.66 ± 0.08	1.63 ± 0.11	1.02
	50	1.64 ± 0.10	1.68 ± 0.04	0.98
	100	1.80 ± 0.26	1.42 ± 0.06	1.27
	Average	1.70 ± 0.06	1.58 ± 0.11	1.08
M-B	30	2.29 ± 0.15	1.84 ± 0.04	1.25
	50	1.71 ± 0.11	2.00 ± 0.32	0.85
	100	1.64 ± 0.19	2.14 ± 0.32	0.77
	Average	1.88 ± 0.28	1.99 ± 0.10	0.96
Cy-C	30	7.40 ± 0.25	2.66 ± 0.00	2.74
	50	5.99 ± 1.16	3.32 ± 0.29	1.80
	100	4.82 ± 0.37	2.71 ± 0.41	1.81
	Average	6.08 ± 0.41	2.90 ± 0.15	2.10

The incubation time was up to 90 min and all experiments were carried out in triplicate. Data are means ± SD.

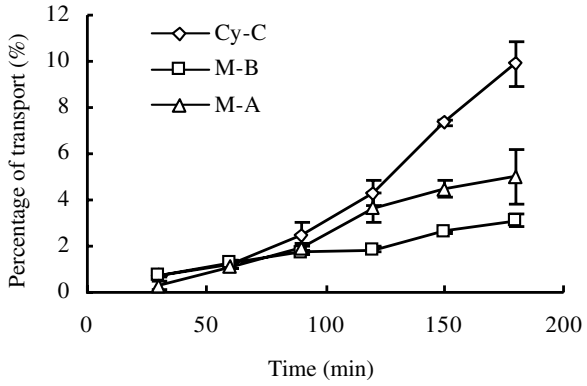

Figure 4. Kinetics curves of M-A, M-B and Cy-C transport in Caco-2 monolayer from apical to basolateral direction at 50 μM. All experiments were carried out in triplicate. Data are the means ± SD.

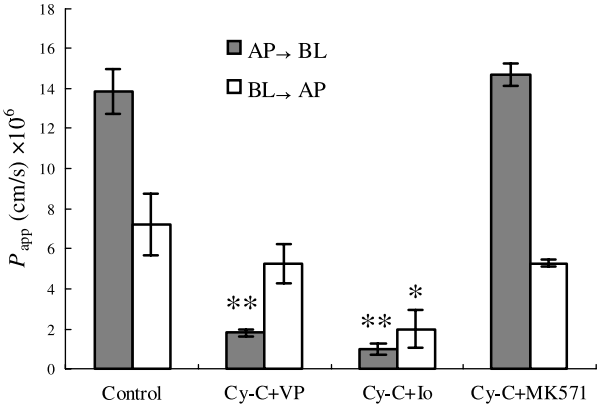

Figure 5. P_{app} values of Cy-C transport in Caco-2 monolayer from two directions at 50 μM in the presence of iodoacetamide (Io, 5 mM), verapamil (VP, 50 μM) and MK571 (50 μM). All experiments were carried out in triplicate. Data are the means ± SD. The columns with asterisk are significantly different from the control. $^*p < 0.05$, $^{**}p < 0.01$.

Transport in the presence of iodoacetamide, verapamil, and MK571. To further investigate the possible involvement of transport mechanism in this process, the experiment was repeated in the presence of iodoacetamide, which is often used as reference standard to inhibit ATP-dependent transport.[10] A stock solution of iodoacetamide was prepared in DMSO and added to the transport medium HBSS, which was given to both the apical and the basolateral chambers of the Transwell-system to achieve a final concentration of 5 mM, and the Cy-C (50 μM; the final concentration of DMSO was 0.5%) was added to one side. From results shown in Figure. 5, treatment of the Caco-2 monolayer with iodoacetamide markedly reduced both the apical and basolateral concentrations of the Cy-C from the basolateral-to-apical and the apical-to-basolateral directions of transport, which were from 1.38×10^{-5} to 8.97×10^{-7} cm/s for $P_{app\ AP\ \rightarrow BL}$ and from 7.2×10^{-6} to 2.46×10^{-6} cm/s for $P_{app\ BL\ \rightarrow AP}$, respectively. The difference of the former was much larger than the latter. This shows that ATP is involved in the transport of Cy-C toward the both apical and basolateral sides of the Caco-2 monolayer.

Verapamil inhibits ATP-driven active transport of drugs. Caco-2 monolayer was treated with verapamil at 50 μM by the similar procedures described above. P_{app} Values of Cy-C transport were reduced about 10-fold on $P_{app\ AP\ \rightarrow BL}$ value from 1.38×10^{-5} to 1.79×10^{-6} cm/s, but $P_{app\ BL\ \rightarrow AP}$ value was essentially unchanged (see Fig. 5). This shows that ATP is involved in the transport of Cy-C toward the basolateral side from apical side of the Caco-2 monolayer.

MK-571 is a well-known inhibitor of MRPs including MRP2.[11] It is interesting to note that treatment of the Caco-2 monolayer with 50 μM MK-571 did not affect the transport of Cy-C toward the both apical and basolateral side. This shows that MRP2 pump is not involved in the transport of Cy-C and suggests that Cy-C haply be incapable of multidrug resistance (MDR).

Based on the above experiments, results, it is suggested that the transport of Cy-C from apical side to basolateral side in the Caco-2 monolayer was partly ATP-dependent except for passive diffusion.

In general, completely absorbed drugs were found to have high permeability coefficients ($P_{app} > 1 \times 10^{-6}$cm/s), whereas incompletely absorbed drugs had low permeability coefficients ($P_{app} < 1 \times 10^{-7}$ cm/s) in the Caco-2

monolayers.[12] From above results, it is sturdily noted that M-A, M-B, and Cy-C were estimated to be moderately absorbed drugs.

Physicochemical characters and permeability. Physicochemical characters, such as logD, PSA (polar surface area), etc., are generally utilized for the prediction of the permeability of compounds. The molecular weights (M_W) of M-A, M-B, and Cy-C are 472.68, 472.68, and 520.58, respectively. logD values at pH 7.4 of M-A, M-B, and Cy-C were 1.32, 1.32, and 1.71, respectively, calculated with Pallas 3.3.2.6 (CompuDrug International, Inc.). According to a reported model for estimation of the permeability of a compound through Caco-2 cells by passive diffusion,[13] using M_W and logD, the $P_{app\ AP\rightarrow BL}$ prediction results of M-A and M-B both ranged from 1×10^{-6} to 3×10^{-6} cm/s approximately. This accorded with the corresponding experimental values in Table 1 and showed that M-A and M-B were transported by passive diffusion. The $P_{app\ AP\rightarrow BL}$ prediction range of Cy-C was less than 2.0×10^{-6} cm/s, while the corresponding experimental value in the Table 1 was far more than 2.0×10^{-6} cm/s. So it indicated that not only passive diffusion but also active transport was involved in the permeation of Cy-C. These results confirmed the conclusions from the transport experiments.

PSA values of M-A, M-B, and Cy-C are 115.06, 115.06 and 172.21 Å^2, respectively, calculated with Pallas 3.3.2.6. It was proposed that PSA $\leqslant$ 61 Å^2, 140 Å^2 > PSA > 61 Å^2 and PSA $\geqslant$ 140 Å^2 represent poor, moderate, and good absorption, respectively.[14] According to the above criteria, the permeability of M-A and M-B should be moderate and that of Cy-C should be poor. In fact, they were all moderate, and the permeability of Cy-C was even higher than that of M-A and M-B.

Thus, we saw that the prediction approaches could be used for the passive diffusion compounds only and be not suitable for the active transport compounds such as Cy-C. Therefore, experiments at cell level, for example, Caco-2 model are essential for the investigation of the real absorption of the candidate compounds.

Intracellular accumulation and recovery rate of M-A, M-B, and Cy-C. The determination of the M-A, M-B, and Cy-C accumulated in Caco-2 cell was performed by HPLC method.[7] To obtain basic profiles of uptake of M-A, M-B, and Cy-C by Caco-2 cells, M-A, M-B, and Cy-C were incubated, respectively, with Caco-2 monolayer for various incubation times of 30, 60, 90, 120, 150, and 180 min. At various time points, the intracellular accumulation rate (%) is shown in Table 2. These results provided strong evidence that M-A and M-B had directly accumulated in the Caco-2 cells. In contrast, it is interesting to note that Cy-C could not be accumulated. Whitley et al.[15] showed that polyphenol ellagic acid is extensively binding to protein and DNA, and accumulated in the Caco-2 cells to be preventive of esophageal cancer in animals both at the initiation and promotion stages. The M-A and M-B with two phenolic hydroxyl and two aldehyde group in aromatic moiety of the structure are also probably extensively binding to protein and DNA as a result of accumulation in the Caco-2 cells. The M-A and M-B are a pair of 9′*S* and 9′*R* diastereomers (see Fig. 1), showing the same high accumulation in the Caco-2 cells, indicating facile absorptive transport across the apical membrane. Thus, M-A and M-B appear to accumulate selectively in the epithelial cells of the aerodigestive tract, where their cancer preventive effects may be displayed. In contrast, the Cy-C only with a phenolic hydroxyl of aromatic moiety, a γ-pyrone group, and a carboxylic ester group in molecular structure is poorly accumulated in the Caco-2 cells.

Table 2. The intracellular accumulation rate (%) of M-A, M-B and Cy-C in the Caco-2 cells for incubation interval[a]

Incubation time (min)	Accumulation rate (%)		
	M-A	M-B	Cy-C
30	36.80 ± 1.99	41.19 ± 1.72	1.42 ± 0.53
60	40.79 ± 2.49	50.56 ± 1.71	0.83 ± 0.09
90	44.21 ± 2.11	46.98 ± 5.78	0.81 ± 0.03
120	50.42 ± 1.39	63.25 ± 0.41	0.69 ± 0.10
150	50.57 ± 2.95	53.21 ± 4.17	0.81 ± 0.07
180	56.67 ± 3.71	49.26 ± 4.02	0.89 ± 0.23

[a] All values were determined at 50 μM for each compound. Data are the means ± SD. All experiments were carried out in triplicate.

During the incubation interval mentioned above, total recovery rate (%) from the intracellular, basolateral, and apical side is accordingly shown in Table 3. From the results of Table 3, it is evident that the total recovery rates of each compound at different time points were essentially same and the total recovery rate within 30–180 min was 60.84–76.41% for M-A, 49.99–68.72% for M-B, and 52.45–56.83% for Cy-C.

The Caco-2 cells showed a characteristic pattern of M-A and M-B accumulation in direct uptake of M-A and M-B. This result is consistent with the idea that the intestinal wall has a unique ability to absorb orally administered M-A and M-B. Like the polyphenol ellagic acid, M-A and M-B may bind with cellular DNA and protein leading to the highly limited transcellular absorption. In the case of Cy-C, the Caco-2 cell transport experiment showed large P_{app} value and uptake studies showed poor accumulation in the cells, indicating facile absorptive transport across the membrane and this did not seem to be the case with the DNA

Table 3. The total recovery rate (%) of M-A, M-B and Cy-C in the Caco-2 cell cultures for incubation interval[a]

Incubation time (min)	Total recovery rate (%)		
	M-A	M-B	Cy-C
30	76.41 ± 3.82	49.99 ± 1.63	56.83 ± 1.02
60	63.15 ± 1.09	56.63 ± 0.71	53.14 ± 0.34
90	60.84 ± 1.12	64.67 ± 6.79	52.82 ± 0.77
120	69.76 ± 0.50	68.72 ± 0.54	52.74 ± 0.82
150	68.49 ± 2.34	59.47 ± 4.18	54.61 ± 0.25
180	75.65 ± 4.58	55.54 ± 3.90	52.45 ± 2.84

[a] All values were determined at 50 μM for each compound. Data are the means ± SD. All experiments were carried out in triplicate.

and protein binding and it is delivered directly into the blood circulation. The portion released into the blood circulation will be the subject of future reports.

Acknowledgments

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7. *HPLC analysis*. The analysis was carried out using a Varian 230 pump and Varian 330 photodiode array detector and a Dikma Diamonsil™ C_{18} column (250×4.6 mm, i.d. 5 μm). For Cy-C, the mobile phase was methanol/water (65:35) at a flow rate of 1.0 mL/min and an aliquot (20 μL) of sample was injected into the HPLC. The analysis was carried out at room temperature and detection wavelength was at 254 nm. For M-A and M-B, the mobile phase was methanol/water/glacial acetic acid (100:4:1), detection wavelength was at 270 nm, and other conditions were same as those of Cy-C. Quantitative determination of M-A, M-B, and Cy-C was performed based on the regression equations obtained from calibration curves.
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