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Transport of Natural-Colored Ingredients, Anthocyanin and Dehydrozingerone, in Epithelial Cells

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Abstract

Anthocyanins (ACNs) and dehydrozingerone (DHZ) are purple and yellow natural pigment substances found in purple sweet potato and ginger, respectively. The mechanisms of intestinal absorption of these substances are ambiguous or have not been studied. The purpose of this study was to clarify the absorption of these substances using Caco-2 and T84 cell lines as in vitro models of intestinal absorption. For the intracellular uptake experiment, cells were cultivated on plastic dishes. For bidirectional permeability experiment, Transwell method was used. The uptake of ANCs by Caco-2 and T84 reached a maximum at 0.5 to 1 min in both pH 6.5 and 7.4, and decreased until 20 min. The uptake amounts were slightly higher in pH 6.5 than pH 7.4 in Caco-2, while no difference was observed in T84. The uptake of DHZ increased rapidly from 1 to 2 min, and then gradually increased thereafter with no pH dependency. Transepithelial transport of ANCs across Caco-2 and T84 were bidirectional, with similar rates of absorptive and secretory transports, suggesting no active transporter involvement in the ANCs transport. The transepithelial transport of DHZ in Caco-2 exhibited secretory-directed, with a secretory rate of approximately 7 at the lowest concentration (1.0 μg/mL). However, secretory-directed transport was not observed at higher concentrations. In T84, secretory-directed transport with secretory rate of approximately 3 at 10.0 μg/mL, but not at higher concentration. Both intracellular uptake and transcellular transport of ANCs were minimal, while DHZ exhibited significantly greater abundance. It can be inferred that no transporters mediating both absorptive and secretory directions participate in the intestinal absorptive process for ANCs. For DHZ, a secretory transporter may be involved in the absorptive process at lower concentrations; however, the contribution of the secretory transporter is minimal, and a considerable amount of DHZ is suggested to be absorbed from intestine.

Keywords: Anthocyanins; Dehydrozingerone; Transport; Caco-2; T84; Intestine; Absorption

Introduction

Food is one of the most important factors affecting our health. Natural-colored foods have been shown to promote human health and are attracting increasing attention. These foods contain many vitamins, nutrients, and beneficial substances that contribute to our well-being. In recent years, pigment substances in colored foods have been attracting attention, and have been isolated from plants and studied for their biological activity.

Anthocyanins (ACNs) are a class of natural pigments found in various plants, fruits, and vegetables. These compounds are responsible for the vibrant red, purple, and blue colors observed in many plant tissues, and play crucial roles in plant growth and development. They are a class of water-soluble pigments that belong to the larger group of flavonoids. They are widely distributed in nature and are especially abundant in berries, grapes, cherries, pomegranates, and dark-colored fruits. ANCs exhibit a variety of physiological functions such as antioxidative [1], antimutagenic [2], and anticarcinogenic activities [3, 4]. Research has also explored their potential therapeutic applications in various health conditions, including cardiovascular diseases [5], diabetes [6], and retinopathy [7]. ANCs have been isolated from a newly bred sweet potato variety, *Ipomoea batatas* cv. Okiyumemurasaki, and their direct absorption after administration is expected to promote our good health [8].

Dehydrozingerone (DHZ) is a light yellowish, pungent, and water-soluble compound found in ginger rhizomes (*Zingiber officinale* Roscoe, Zingiberaceae) [9]. It belongs to the class of phenolic compounds and possesses a unique chemical structure similar to a half-analogue of curcumin, an active component of turmeric present in the rhizomes of *Curcuma longa L.* (Zingiberaceae).

DHZ has also demonstrated a wide range of bioactivity, including synergistic antifungal activities [10] and potential chemotherapeutic effects on colon cancer.

Various in-vitro experimental systems, as well as in-vivo systems in animals and humans, are employed to elucidate the mechanism of small intestinal absorption of drugs. Among these in-vitro experiments, the Caco-2 cell monolayer experiment using Transwell is the most commonly used approach. Caco-2 cells have microvilli that are connected by tight junctions, possessing microvilli, resembling simple columnar intestinal cells when cultured on plastic dishes or nitrocellulose filters [11]. These cells express transporters that contribute to both absorptive and secretory processes, and are thus widely used as a standard permeability-screening assay to predict drug absorption in humans [12, 13].

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The T84 cell line, originally isolated from a patient with colorectal adenocarcinoma, has been extensively studied to understand various aspects of intestinal epithelial cell biology and functions. It was demonstrated that T84 cells grew to confluence as a monolayer with the basolateral membrane attached to the surface of the culture dish, and showed the existence of a microvillus-studded apical membrane facing the media. T84 cells also grow as polarized monolayers and display morphology similar to that of undifferentiated crypt cells rather than villus tip cells of the small intestine. They have been used extensively as a model system for studying epithelial electrolyte transport and its regulation by various hormones and neurotransmitters [14, 15].

As described above, both ACNs and DHZ have demonstrated bioactivity in vitro and potential health and medical benefits. These substances are contained in food and ingested orally, but the mechanisms of absorption from the small intestine remain to be elucidated. Therefore, the objective of this study was to investigate the absorption of ACN and DHZ using Caco-2 and T84 cell systems.

Materials and Methods

Natural-colored ingredients

ANCs were extracted and isolated from sweet potatoes (*Ipomoea batatas* cv. Okiyumemurasaki) as previously described [8]. Dehydrozingerone was purchased from Abbexa LTD (Cambridge, UK).

Culture of Caco-2 and T84 cells

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 units/mL penicillin G, and 100 μg/mL streptomycin, as previously described. Caco-2 cells were seeded onto six-well plastic dishes at 1.0×10^6 cells/well and onto inserts of 12-well Transwell (3401, Corning-Costar) at 1.26×10^5 cells/insert and cultured for 14 and 21 days, respectively [16-18].

T84 cells were grown in a 1: 1 mixture of Dulbecco's modified Eagle's medium with 4.5 g/L of D-glucose and Ham's F12 Nutrient mixture containing 5% of bovine serum. T84 cells were seeded onto six-well plastic dishes at 4.4×10^6 cells/well insert of 12-well Transwell (3401, Corning-Costar) at 0.565×10^6 cells/insert and cultured for 7-9 and 12-14 days, respectively [19].

The transepithelial electrical resistance of the monolayer of Caco-2 and T84 cells at the time of transepithelial transport experiment was 250~350 and 550~700 Ω cm^2, respectively.

Uptake experiment

The transepithelial transport study with Caco-2 or T84 cells grown on 6-well plastic dish was performed as previously described. The incubation buffer used was Hanks' balanced salt solution (HBSS) (0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 25 mM D-glucose, and 10 mM Hepes; pH: 7.4 or 6.5; osmolarity: 315 mOsm/kg).

The culture medium was removed, and the cells were preincubated at 37°C for 15 min in 1.5 mL of incubation buffer at pH 7.4. After preincubation, the buffer was aspirated, and the cells were incubated with 1.0 mL of incubation buffer (37°C; pH: 7.4 or 6.5) containing ANC or DHZ at 37°C. At the designated times, the incubation buffer was aspirated, and the cell surface was quickly washed three times with ice-cold incubation buffer. The cells were scraped with a scraper, 1 mL of incubation buffer was added, and cells were sonicated with an ultrasonic homogenizer (Smurt NR-50M, Microtec Co., Ltd, Japan). The suspension solutions were centrifuged at 15,780 *g* for 10 min to obtain the supernatant. The supernatant was subsequently filtrated through a membrane filter (Cosmonice Filter [W] [0.45 μm], Nacalai Tesque, Inc., Kyoto, Japan), and the filtrate was subjected to the HPCL system.

Transepithelial transport experiment

The transepithelial transport study with Caco-2 or T84 cells cultured on polycarbonate filters of 12-well Transwell was performed as previously described. The same buffer as mentioned above was used.

The volumes of apical and basolateral compartments were 0.5 and 1.5 mL, respectively. To measure apical-to-basolateral (absorptive) or basolateral-to-apical (secretory) flux, a test compound was included in the apical or basolateral side, respectively. At the designated time, 0.5 mL of the basolateral or 0.2 mL of the apical side solution was withdrawn and replaced with an equal volume of HBSS. The experiments were conducted to maintain the buffer at 37°C using hotplate.

Analytical methods

Samples from transepithelial transport experiment by Caco-2 and T84 cells were appropriately diluted in HBSS when necessary and subjected to high-performance liquid chromatography (HPLC) analysis.

The HPLC system consisted of a constant-flow pump (LC-20AD; Shimadzu Co., Kyoto, Japan), a UV detector (SPD-20A; Shimadzu Co.), a system controller (CBM-20A; Shimadzu Co.), and an automatic sample injector (SIL-20A; Shimadzu Co.). A Cosmocil 5C18 MS-II column was used as the analytical column (150 mm height × 4.6 mm I.D; Nacalai Tesque, Kyoto, Japan).

For quantification of ANC, HPLC analysis was performed using gradient elution. A binary mobile phase composed of solvent A (water with 0.1% trifluoroacetic acid) and solvent B (acetonitrile with 0.1% trifluoroacetic acid) was used. The mobile phase composition was [95% of A] and [5 of B] at 0 min, [50% of A] and [50 of B] at 5 min, and [35% of A] and [65 of B] at 15 min. The flow rate was set at 1.0 mL/min and the column temperature was maintained at 40°C. Detection was conducted using UV detection at 520 nm. Peaks between 10 and 15 min were analyzed since ACNs are mixtures of several derivatives.

For quantification of DHZ, HPLC analysis was performed. The mobile phase consisted of water: methanol (35: 65, [v/v]). The flow rate was set at 1.0 mL/min and the column temperature was maintained at 40°C. Detection was conducted using UV detection at 254 nm. The peak at 2.7 min was analyzed as DHZ.

Data analysis

The uptake of the test ingredients by the cells was estimated in terms of the amounts (ng) taken up per well (9.6 cm^2). Transepithelial transport was estimated by the amount (ng) of the test ingredient transported across the cell monolayer per well. The transepithelial permeability coefficient (ng/cm^2/min) was obtained from the slope of the linear portion of the permeation against time (min) plots and divided by the area of Transwell (1.12 cm^2). The secretory ratio was calculated from the permeability coefficient of absorptive transport divided by that of secretory transport. All data are expressed as means ± SEM, and statistical analysis was performed using Student's t-test. A difference of $P<0.05$ between means was considered significant.

Results

Uptakes of ACNs and DHZ by Caco-2 cells and T84 cells, respectively

We compared the uptakes ANCs (Figure 1) and DHZ (Figure 2) by Caco-2 and T84 cells at different pH levels. The uptake of ANCs by Caco-2 and T84 cells was highest at 0.5 to 1 min in both pH 6.5 and 7.4. In Caco-2 cells, the uptake amounts were higher at 1 min in pH 6.5 than in pH 7.4, while no difference was observed between pH 6.5 and pH 7.4 in T84 cells. Cell-to-medium ratios at 20 min were approximately 0.16 and 0.11 μL/cm^2 in Caco-2 and T84 cells, respectively. pH dependency was observed.

The uptake of DHZ by Caco-2 and T84 cells was remarkably similar in both pH 6.5 and 7.4. The uptake of DHZ increased rapidly from 1 to 2 min and then increased gradually. Cell-to-medium ratios at 20 min were approximately 3.5 and 1.7 μL/cm^2 in Caco-2 and T84 cells, respectively. No pH dependency was observed.

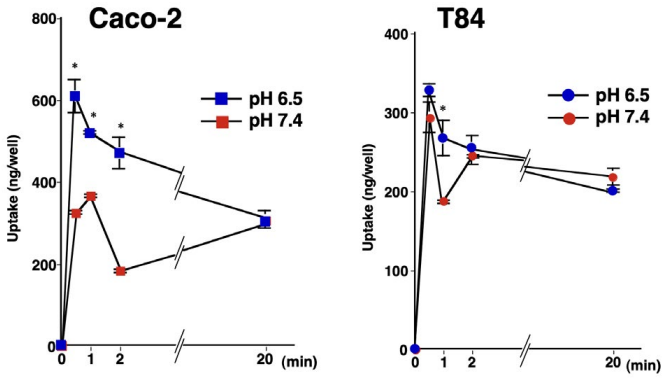

Figure 1: Uptake of anthocyanins in Caco-2 and T84 cell monolayers cultured on plastic dish.

The experimental solution was adjusted to pH 7.4 (■●) or pH 6.5 (■●) and the temperature was maintained at 37°C.

The initial concentration of anthocyanins was 200 μg/mL.

Each point represents the Mean ± SEM of three experiments.

* P<0.05 vs corresponding pH 7.4.

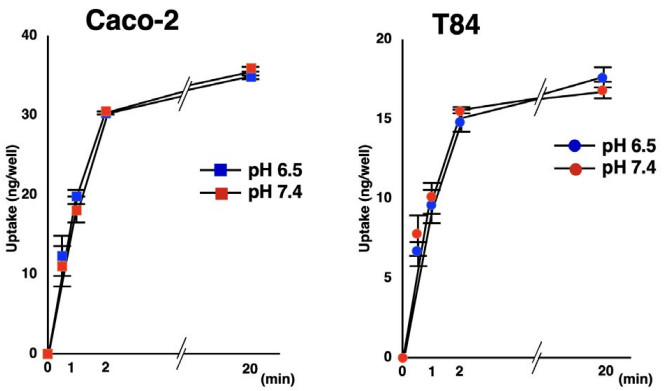

Figure 2: Uptake of dehydrozingerone in Caco-2 and T84 cell monolayers cultured on plastic dish.

The experimental solution was adjusted to pH 7.4 (■●) or pH 6.5 (■●) and the temperature was maintained at 37°C.

The initial concentration of dehydrozingerone was 10 μg/mL.

Each point represents the Mean ± SEM of three experiments.

Transepithelial transport of ANCs across Caco-2- and T84-cell monolayers

To determine if the transport of ANCs across Caco-2 cells and T84 cells was unidirectional, transepithelial fluxes were measured by adding ANCs to either the apical or basolateral side of the cell monolayers and monitoring their appearance on the opposite side (Figure 3).

In Caco-2 cells, at pH 6.5, transport of ANCs in the absorptive and secretory directions was comparable (Figure 3A). The permeability coefficients for both directions were nearly identical, with a calculated secretory ratio of 0.92. At pH 7.4, transport of ACNs in both directions was also comparable, with a calculated secretory ratio of 1.09. However, when comparing the permeability coefficients at pH 6.5 and 7.4, the value at pH 6.5 was approximately three times higher (Table 1).

Transport of ACNs in T84 cells was remarkably similar to those in Caco-2 cells (Figure 3B). But the secretory ratios were 1.36 and 1.75 at pH 6.5 and pH 7.4, respectively, with statically significant differences between absorptive and secretory permeability coefficients. The permeability coefficient at pH 6.5 was approximately 2.5 times higher than that at pH 7.4 (Table 1). No directionality preference was observed; however, pH dependence was evident in the transport of ACNs in both cells.

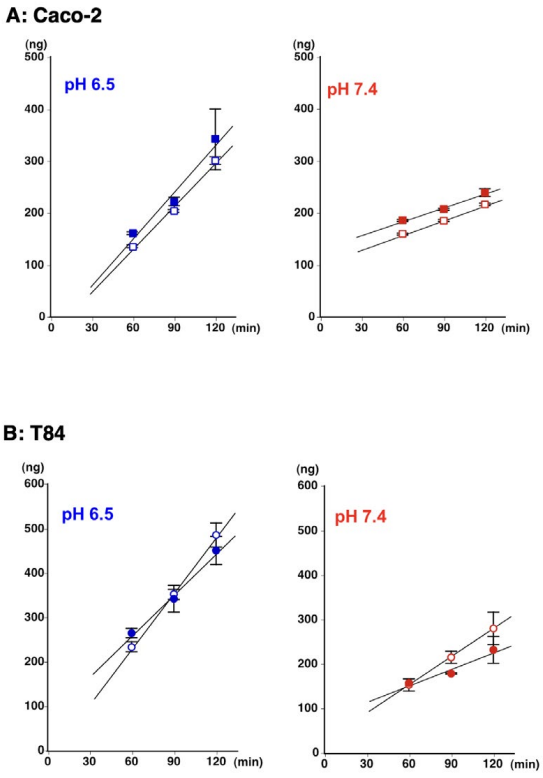

Figure 3: Transepithelial transport of anthocyanins across Caco-2 (A) and T84 (B) cell monolayers.

Transport of anthocyanins in the apical-to-basolateral (absorptive) direction (closed symbols) and in the basolateral-to-apical (secretory) direction (open symbols).

The experimental solution was adjusted to pH 6.5 or 7.4 and the temperature was maintained at 37°C.

The initial concentrations of anthocyanins were 500 μg/mL and 200 μg/mL in Caco-2 and T84 cells, respectively.

Each point represents the Mean ± SEM of three experiments.

Table 1: Transepithelial permeability coefficients and secretory ratio of anthocyanin's across Caco-2 and T84 Cell monolayers.

	pH 6.5			pH 7.4		
	Permeation Coefficient		Secretory rate	Permeation Coefficient		Secretory rate
	Absorptive	Secretory		Absorptive	Secretory	
Caco-2	2.69 ± 0.88	2.46 ± 0.13	0.92	0.70 ± 0.04	0.85 ± 0.04	1.09
T84	2.75 ± 0.43	3.75 ± 0.49	1.36	1.13 ± 0.34	1.89 ± 0.33	1.75

Permeability Coefficient: ng/cm^2/min

Secretory rate = Secretory permeability coefficient / Absorptive permeability coefficient

Mean ± SEM (n=3)

* p<0.05 vs Absorptive permeability coefficient (t-test)

Transepithelial transport of DHZ Across Caco-2- and T84-cell monolayers

Transepithelial transport experiments were conducted to investigate the transport of DHZ across Caco- 2 cells and T84 cells (Figure 4).

In Caco-2 cells, secretory transport of DHZ was significantly faster than absorptive transport at 1.0 μg/mL, while transports in both directions were nearly equal at 10 and 50 μg/mL, with slightly faster secretory transport (Figure 4A). The secretory ratio calculated from the absorptive and secretory permeability coefficients at 1.0 μg/mL were 7.21, indicating secretory-directed transport. The secretory ratios were 1.30 and 1.52 at 10 and 50 μg/mL, respectively (Table 2).

In T84 cells, secretory transport of DHZ was significantly faster than absorptive transport at 10 μg/mL, with a secretory ratio of 3.3. Transports in both directions were almost comparable at 5 and 50 μg/mL, with significantly but slightly faster secretory transport and secretory ratios of 1.32 and 1.60, respectively (Figure 4B, Table 2).

Discussion

In this study, we used Caco-2 cells and T84 cells, which are commonly used to assessing drug absorption in humans, to investigate intracellular uptake and transepithelial transport of ANCs and DHZ. These pigment ingredients are found in food and are believed to provide health benefits upon ingestion, and we aimed to gain insights into their oral absorption.

For ANCs, since both intracellular uptake and transepithelial transport were only trace (see below for a quantitative comparison with DHZ), the initial concentration was set so high as 500 μg/mL that it could quantitate. Therefore, in all experiments, only one concentration was employed in the experiments. Moreover, since ANCs change their ionization state and color under weakly acidic to neutral pH conditions, experiments were conducted at two pH levels.

In terms of intracellular uptake, relatively high uptake was initially observed, especially in Caco-2 cells at lower pH. However, the amount of uptake decreased over time. Although this phenomenon resembles that of uptake involving active transporters, the markedly low uptake amount of ANCs makes it highly unlikely that absorptive pH-dependent active transporter are involved.

The equivalent uptake amounts of ANCs by both Caco-2 and T84 cells, despite the pH difference, at the 20-minute endpoint suggests that the ANCs are taken up in their ionized state at lower pH, which is the more easily absorbed form. They then equilibrated at neutral intracellular pH, resulting in similar uptake levels.

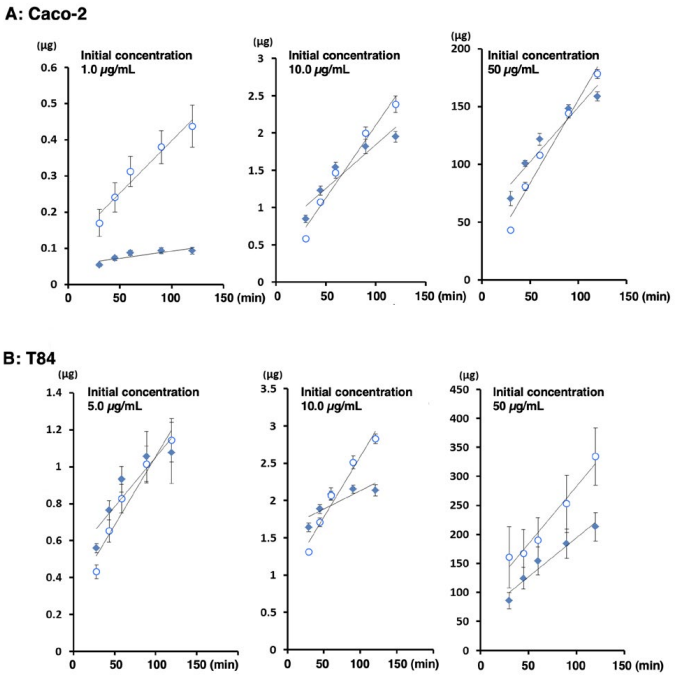

Figure 4: Transepithelial transport of dehydrozingerone across Caco-2 (A) and T84 (B) cell monolayers.

Transport of dehydrozingerone in the apical-to-basolateral (absorptive) direction (closed symbols) and in the basolateral-to-apical (secretory) direction (open symbols).

The experimental solution was adjusted to pH 7.4 and the temperature was maintained at 37°C.

Each point represents the Mean ± SEM of three experiments.

In transcellular transport, there was no directionality observed, but the amount transported at lower pH was higher than at higher pH. This further supports the absence of an absorptive transporter and suggests that the differences in ionization due to pH may play a role.

Although ANCs have been reported to have a variety of beneficial biological activities, this study reveals that their uptake and absorptive transepithelial transport are minimal, suggesting a very low bioavailability in humans. Previous reports have also shown low bioavailability for ANCs, ranging from 2% to less than 1%, and thus, only small amount of ANCs would enter the bloodstream and reach target organs [20].

While ANCs have been reported to have beneficial biological activity, their actions may not be manifested in vivo unless a sufficient amount of absorbed ANCs from food is delivered to the sites of action. This study, along with previous reports, underscores the enigma of how ANCs, with their low absorption, still exert bioactivity.

The low bioavailability of ANCs was calculated by targeting only the parent compound. However, if one considers not only the parent compound, but also Phase I and Phase II metabolites, complex products, and microbially generated metabolites, the bioavailability of the total would likely be higher than previously thought [21]. While ANCs themselves are not readily absorbed, their interactions with foods and other nutrients could improve their stability and absorption. Furthermore, orally ingested ANCs may provide health benefits through potential mechanisms, such as metabolism by intestinal bacteria resulting in enhanced absorption of their metabolites and subsequent biological activity in cells [22-24]. The antioxidant activity

Table 2: Transepithelial permeability coefficients and secretory ratio of dehydrozingerone across Caco-2 and T84 Cell monolayers.

	1 or 5 † μg/mL			10 μg/mL			50 μg/mL		
	Permeation Coefficient		Secretory rate	Permeation Coefficient		Secretory rate	Permeation Coefficient		Secretory rate
	Absorptive	Secretory		Absorptive	Secretory		Absorptive	Secretory	
Caco-2	0.36 ± 0.05	2.58 ± 0.40	7.21	15.43 ± 4.95	20.07 ± 2.67	1.3	84.73 ± 3.66	128.75 ± 3.75	1.52
T84	7.94 ± 1.48	9.70 ± 1.04	1.32	4.40 ± 0.24	14.55 ± 0.38	3.3	94.20 ± 9.29	150.54 ± 7.50	1.6

Permeability coefficient: ng/cm^2/min

Secretory rate = Secretory permeability coefficient / Absorptive permeability coefficient

Mean ± SEM (n=3)

* $p<0.05$ vs Absorptive permeability coefficient (t-test)

† Caco-2: 1 μg/mL, T84: 5 μg/mL

of ANCs is so high [25] that it is conceivable that even small amounts absorbed or reached may reduce oxidative stress in the body and provide sufficient health benefits.

In contrast, little is known about the absorption of DHZ. Published studies are currently unavailable to the best of our knowledge. As for intracellular uptake of DHZ, the uptake gradually increased over time in both cell types, suggesting no involvement of any specific absorptive transporters.

Transepithelial transport of DHZ showed significant secretory-directed transport at low concentrations in Caco-2 cells, and higher concentrations in both Caco-2 and in T84 cells showed a trend towards secretion-directed transport as well. Thus, DHZ transport was secretion-directed; however, this does not necessarily indicate that DHZ is not absorbed from the small intestine.

We have previously reported on the small-intestinal transport mechanism of grepafloxacin (GPFX), a quinolone antimicrobial agent, in Caco-2 cells and rat small-intestinal tissue [26, 27]. Although the transport of GPFX showed secretory-directed transport behavior mediated by secretory transporters like MRP2 and P-glycoprotein, with a secretory ratio of 2 in both in-vitro experimental systems, GPFX is a highly absorbable drug in humans (absolute oral bioavailability is approximately 70%). Therefore, secretory-directed transport does not imply lack of absorption.

Rather, it is more appropriate to consider the permeability coefficient to determine whether the drug is absorbed or not, and if so, whether the absorption rate is high or not. This is because in in-vitro experiments, the drug is tested on the basolateral (i.e., blood) side under conditions of unrealistically high drug concentrations.

In Caco-2 cells, at an initial concentration of 1 μg/mL of DHZ, the permeability coefficient rates (permeability coefficient divided by initial concentration: μL/cm^2/min) were 0.36 in the absorptive direction and 2.58 in the secretory direction, resulting in a considerably high secretory ratio of 7.2, suggesting the involvement of secretory transporter. At 10 μg/mL, the permeability coefficient rates for absorptive and secretory directions were 1.54 and 2.01, respectively, and at 50 μg/mL, 1.69 and 2.57, indicating statistically significant but only slight secretory-directed transport. These results indicate that the loss of secretory-directed transport at higher concentrations is due to saturation of the secretory transporter, and also suggest that the involvement of secretory transporter in net transcellular transport of DHZ is negligible.

Based on our previously reported results on GPFX transport, the permeability coefficient rates were calculated to be comparable to the values obtained in this study. The values of absorptive and secretory directions were 1.4 and 2.8, respectively. The DHZ values in this study are relatively close to these values, suggesting that DHZ is well absorbed in humans and is delivered to the target organs in sufficient amounts.

In conclusion, the uptake and transcellular transport of two bioactive food-derived natural-colored ingredients in small intestinal model cells were revealed. Intracellular uptake and transcellular permeation of ANCs in Caco-2 and T84 cells were minimal. In general, for a drug to be biologically active, it must be absorbed from the small intestine and distributed to various tissues. However, based on the results of this study, small intestinal absorption of ANCs in humans is estimated to be low. ANCs may enter the body as metabolites or undergo molecular modifications, which may contribute to biological activity; however, details are still unknown.

In contrast, DHZ was taken up by Caco-2 and T84 cells in sufficient amounts, but it appears that absorption transporters were not involved. As for transcellular permeation, secretory-directed transport was observed at low concentrations, suggesting the involvement of secretory transporter. However, saturation was observed at higher concentrations, suggesting limited capacity of the transcellular permeation. These results suggest that DHZ can be absorbed from the small intestine in humans in sufficient amounts without the involvement of transporters.

Declarations

• Ethics approval and consent to participate

Not Applicable.

• Consent for publication

Not Applicable.

• Availability of data and materials

Not Applicable.

• Competing interests

Not Applicable.

• Acknowledgements

Not Applicable.

List of abbreviations

ANCs	anthocyanins
DHZ	dehydrozingerone
GPFX	grepafloxacin
HBSS	Hanks' balanced salt solution
HPLC	high performance liquid chromatography

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