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# Molecular mechanisms of the naringin low uptake by intestinal Caco-2 cells

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Naringin, the main flavanone of grapefruit, was reported to display numerous biological effects: antioxidant, hypocholesteremic, anti-atherogenic and favoring drug absorption. Naringin absorption mechanisms were studied in Caco-2 cells (TC7 clone). We investigated the possible involvement of several membrane transporters implicated in polyphenolic compounds intestinal transport (sodium-dependent glucose transporter 1, monocarboxylate transporter, multidrug-associated resistance proteins 1 and 2, and P-glycoprotein). Naringin was poorly absorbed by Caco-2 cells, according to its low value of apparent permeability coefficient ($P_{app}$ = 8.1 ± 0.9 × $10^{-8}$ cm/s). In the presence of verapamil, a specific inhibitor of P-glycoprotein, cellular uptake was increased by almost threefold after 5 min, and $P_{app}$ was doubled after 30 min. Our results indicated the involvement of P-glycoprotein, an ATP-driven efflux pump, capable of transporting naringin from the Caco-2 cell to the apical side. This phenomenon could explain, at least in part, the low absorption of this flavanone at the upper intestinal level.

**Keywords:** Naringin / Caco-2/TC7 cells / Intestinal absorption / P-glycoprotein

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## 1 Introduction

Naringin (NAR) belongs to the large class of flavonoids and more specifically to the flavanone subgroup. Its structure contains the aglycone moiety named naringenin, which is linked to a dioside, the neohesperidoside. This conjugated form confers to NAR its bitter taste, whereas the conjugation with rutinoside (named narirutin) is tasteless. NAR occurs in high amounts in grapefruit (53 mg/100 g edible portion, and 34 mg/100 mL in grapefruit juice) and in sour oranges (25 mg/100 g edible portion); its content is ten times less in orange [1] (http://www.nalusda.gov/fnic/foodcomp). Numerous biological effects of NAR were described [2]. As the major phenolic compound of grapefruit juice, NAR was reported to be responsible for a better drug absorption at intestinal level [3]. NAR was supposed to be a substrate of P-glycoprotein (P-gp), an ATP-binding cassette (ABC) transporter, modulating transport of drugs such as taxol, paclitaxel [4], vinblastine [5], and talinolol [6]. Like most of flavonoid structures, NAR can act as an antioxidant either directly through its phenolic moiety [7–9], or undirectly by increasing antioxidant enzyme gene expressions [10] or by sparing vitamin E [11], which is a strong lipophilic antioxidant. Besides these properties, NAR was reported to display other health beneficial roles by preventing hypercholesteremia [12, 13] or reducing atherosclerosis risk [14]. Such activities depend on the bioavailability of NAR. Compared to isoflavones and quercetin glucosides, NAR appeared to be poorly absorbed [15]. Its bioavailability appeared higher than anthocyanins and procyanidins (oligomers of flavanols), but data on NAR absorption remain scarce [15]. Absorption studies indicated that NAR is absorbed at the colon level after its deglycosylation by microflora [16, 17]. In addition, 3-phenylpropionic acid was detected from NAR degradation in an *in vitro* mixed-culture model of the human colonic microflora [17], as well as in a pig cecum model [18]. However, other data suggest that for high ingested doses, intact NAR could also reach the systemic circulation [19, 20].

The aim of the present study was to investigate the molecular mechanisms involved in the uptake and transport of NAR by enterocytes. Caco-2/TC7 cell monolayers, recognized as a model of the intestinal barrier, were used [21]. The effect of verapamil (VERAP), an inhibitor of P-gp

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**Abbreviations: ABC**, ATP-binding cassette; **AP**, apical; **BL**, basolateral; **MCT**, monocarboxylic acid transporter; **MRP**, multidrug resistance-associated protein; **NAR**, naringin; **P-COUM**, *p*-coumaric acid; **P-gp**, P-glycoprotein; **PHLO**, phloridzin; **SGLT1**, sodium-dependent glucose transporter 1; **VERAP**, verapamil

function, was studied. Besides VERAP, other inhibitors were assayed to specify the involvement of other transporters, known to play a role in the uptake of polyphenols [sodium-dependent glucose transporter 1 (SGLT1), monocarboxylic acid transporter (MCT), multidrug resistance-associated proteins (MRPs)].

## 2 Material and methods

### 2.1 Chemicals

NAR, naringenin, MES, VERAP hydrochloride, *p*-coumaric acid (P-COUM), phloridzin (PHLO) and rutin were purchased from Sigma (Saint Quentin Fallavier, France). MK-571 was obtained from VWR (Fontenay Sous Bois, France).

### 2.2 Cell culture

All cell culture reagents were from Invitrogen (Cergy-Pontoise, France), except fetal calf serum, which was from Sigma. Caco-2 cells (TC7 clone) were a kind gift from Monique Rousset (INSERM U505, Paris). Cells (passage 51–87) were grown in culture medium composed of DMEM supplemented with 20% fetal calf serum, 1% non-essential amino acids, 100 U/mL penicillin and 0.1 mg/mL streptomycin, in humidified atmosphere of 10% $CO_2$ at 37°C.

### 2.3 Uptake studies

For uptake experiments, cells were seeded at a density of 10000 cells/cm$^2$ in six-well plastic dishes (BD Falcon, Le Pont De Claix, France) and medium was renewed every 2 days. On day 14 post-seeding, cells were washed once with PBS and allowed to incubate at 37°C for 30 min in glucose-free DMEM, with or without putative transport inhibitors (VERAP, PHLO, MK-571, P-COUM). (All inhibitors were in a higher, but non-toxic, concentration than NAR, data not shown). Medium was then aspired, and replaced by 2 mL of a solution containing various concentrations of NAR in glucose-free DMEM, (with or without transport inhibitors) and incubated between 15 s and 60 min at 37°C. To investigate a possible effect of pH on NAR uptake, medium was acidified by adding 50 mM MES (pH 6).

### 2.4 Transport studies

For transport experiments, 250000 cells were seeded in 23.1-mm i.d. inserts (polyethylene terephtalate membrane, 1-μm pore size, BD Falcon) and grown for 21 days, allowing the formation of a well-differentiated enterocytes monolayer. The basolateral (BL) and apical (AP) compartments contained 3.0 and 2.0 mL culture medium, respectively, and were cultured as previously decribed [22]. On the day of experiment, cells were washed with warm PBS, and pre-incubated with or without 750 μM VERAP in glucose-free DMEM for 30 min. Medium was removed, and cells were incubated with 90 μM NAR for 30 min at 37°C in the presence or absence of 750 μM VERAP.

### 2.5 Extraction procedure of NAR

Cell content was extracted as follow: immediately after the end of incubation, cells were rinsed three times with ice-cold PBS, scraped in 1 mL methanol containing 50 μl of a solution or rutin (0.02 mg/mL as an internal standard), transferred to a 2-mL plastic tube and vortexed for 10 s. The cell suspension was pelleted by centrifugation at 14000 g for 10 min, 4°C, and the supernatant was transferred to a new tube. Pellet was extracted a second time with 1 mL methanol, and the combined supernatants dried under nitrogen at 40°C, resuspended in 100 μl 15% ACN, and vortexed thoroughly twice for 10 s. After centrifugation (20 min at $14000 \times g$, 4°C), the clear supernatant was placed in a glass vial for HPLC analysis.

To determine NAR concentration in AP and BL compartments, whole medium (2 or 3 mL) was collected, and NAR was extracted by SPE using Oasis HLB 3cc (Waters, Saint Quentin en Yvelines, France) cartridges washed with 1 mL methanol and equilibrated with 1 mL water. The whole sample was loaded, the cartridge was washed with 1 mL water and eluted with 2 mL methanol. The eluate was dried under a nitrogen stream at 40°C, and resuspended in 100 μl of 15% ACN for HPLC analysis.

Stability of NAR in culture medium was checked at different experimental times, and extraction yields for the cellular and SPE procedures were 100% and 98%, respectively, as recently reported by Georgé *et al.* [23].

### 2.6 $P_{app}$ calculation

The AP to BL (or *vice versa*) permeability (apparent permeability coefficient: $P_{app}$) of NAR was calculated according to the following equation: $P_{app} = dQ/dt \times V/A.C_0$, where dQ/dt is the rate of appearance of NAR on the BL side (μM/s), V is the volume of the receiving chamber (2 or 3 mL), $C_0$ is the initial concentration on the AP side (μM), and A is the surface area of the monolayer (4.2 cm$^2$). $P_{app}$ is expressed in cm/s [24].

 

### 2.7 HPLC analysis of NAR

Samples were analyzed by reverse-phase HPLC using a Waters 2690 Alliance system. Separation was achieved using a monolithic type column Chromolith Performance RP18 E 100 × 4.6 mm (VWR) maintained at 37°C. Solvents A (5% ACN, 0.006% TFA) and B (100% ACN) were run at a flow rate of 2 mL/min using the following gradient: solvent B increased from 5% to 30% over a 12-min period followed by an increase up to 100% over 1 min.

Sample injection volume was 80 µl, and the eluent was monitored at 280 nm with a Waters 2996 diode array detector. Calibration curve used for quantification was linear between 10 to 1500 ng, and its slope was 785.31 µVs/ng.

The accuracy for the measurement of NAR was assessed: inter-day and intra-day standard relative deviations ($n = 6$), were 3.4 and 3.1%, respectively.

### 2.8 Protein determination

Cells were rinsed three times with ice-cold PBS, and scraped in 1 mL PBS. The suspension was transferred into a plastic tube and sonicated 2 × 15 s on ice. Protein concentration of the lysate was obtained using the Uptima (Interchim, Montluçon, France) bicinchoninic acid assay according to the manufacturer instructions, with BSA as a standard [25].

### 2.9 Statistical analysis

Data, expressed as means ± SEM, were analyzed with the StatView 5.0 software (SAS Institute, Cary, NC). Differences between two groups were tested by the non-parametric Mann-Whitney test, and were considered significant if $p < 0.05$.

## 3 Results

### 3.1 NAR uptake

Cells (Caco-2/TC7, D14) accumulated NAR when incubated at 37°C with 90 µM NAR prepared in DMEM without glucose (Fig. 1). NAR appeared to be rapidly incorporated during the first 15 min and a plateau was reached after 60 min, with a maximum at 111 pmol/mg proteins.

### 3.2 Influence of P-gp on NAR absorption

The possibility that NAR was a substrate of P-gp was tested by performing an uptake experiment in the presence of a well-known specific P-gp inhibitor, VERAP. No difference in 90 µM NAR absorption was observed for a short incubation time of 15 s (Fig. 2A). However, absorption was enhanced by twofold after 90 s and by close to threefold after 5 min when co-administred with 750 µM VERAP. In addition, the effect of VERAP appeared to be dose dependent on NAR absorption as shown in Fig. 2B.

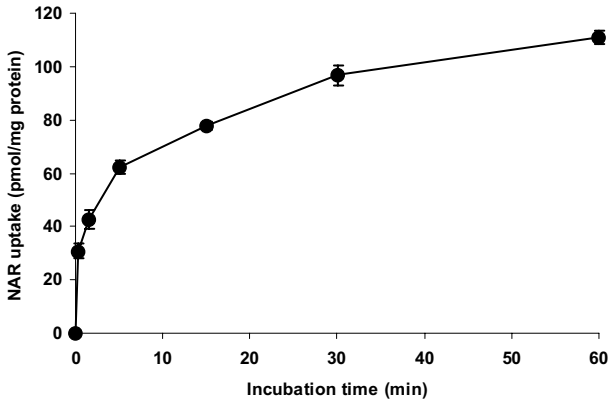


**Figure 1.** Time course of NAR accumulation in Caco-2/TC7 cells. Values are expressed as means ± SEM of three independent experiments.

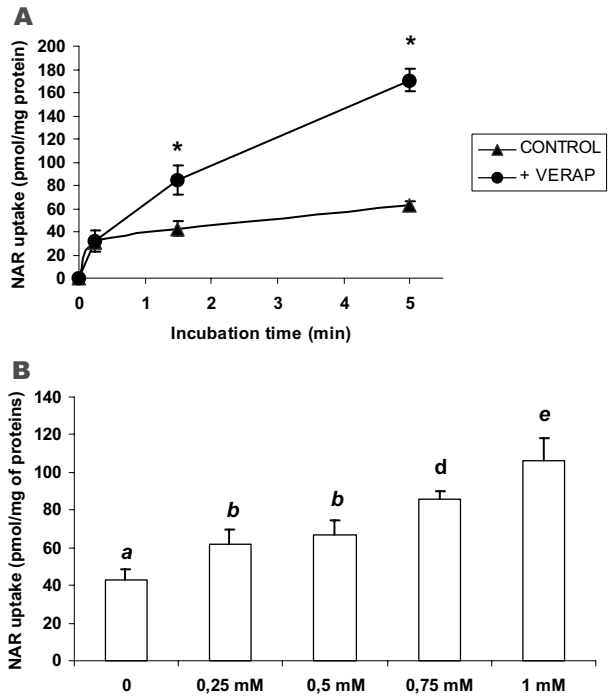


**Figure 2.** Effect of P-gp inhibition on NAR cellular uptake. (A) Effect of co-incubation with 750 µM VERAP on 90 µM NAR accumulation (means ± SEM). * indicates a statistical significant difference with the corresponding incubation time ($p < 0.05$, $n = 3$). (B) Effect of increasing doses of VERAP on NAR accumulation after 90 s. Data with different letters display significant statistical differences ($p < 0.05$, $n = 3$).

 

### 3.3 Transport studies

AP → BL (in the presence or in the absence of VERAP) and BL → AP transport of 90 μM NAR were examined (Table 1). AP → BL $P_{app}$ ($8.1 \pm 0.9 \times 10^{-8}$ cm/s) was significantly lower than BL → AP $P_{app}$ ($10.4 \pm 0.6 \times 10^{-8}$ cm/s) confirming the existence of a favored NAR efflux. Moreover, $P_{app}$ values obtained for the AP → BL transport of NAR in the presence of 750 μM VERAP were also significantly higher ($19.5 \pm 3.0 \times 10^{-8}$ cm/s).

**Table 1.** Apparent permeability coefficient ($P_{app}$) values for NAR (90 μM) in different conditions after a 30-min incubation, in the presence or the absence of 750 μM VERAP. Data are given as means ± SEM of $n = 3$ independent experiments.

| Direction of transport | $P_{app}$ value ($\times 10^{-8}$ cm/s) |
|---|---|
| AP → BL | 8.1 ± 0.9 |
| AP → BL (+VERAP) | 19.5 ± 3.0[a)] |
| BL → AP | 10.4 ± 0.6[a)] |

a) Significant statistical difference with AP → BL value.

### 3.4 Effects of polyphenols transporter inhibitors on NAR uptake

Initial absorption rate (Vi) *versus* NAR concentration curve displayed a saturable profile up to 180 μM and was followed by a linear part (Fig. 3). We decided to investigate which membrane transporter could be involved in the NAR uptake. To specify the role of a carrier involving $H^+$ co-transport, an acid pH was tested. PHLO (1 mM) was used to inhibit SGLT1, *P-COUM* (1 mM) for MCT, VERAP (0.75 mM) for P-gp and MK-571 (0.2 mM) for MRP 1 and 2. Results are reported in Fig. 4: acid conditions did not favor a better uptake and, none of the inhibitors tested were able to significantly modify 90 μM NAR uptake after a 90-s incubation, except VERAP.

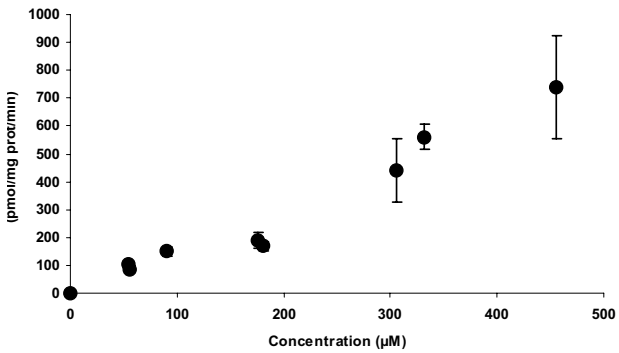


**Figure 3.** Effect of NAR concentration on initial absorption rate ($t = 15$ s). Values are expressed as means ± SEM of three to six independent experiments.

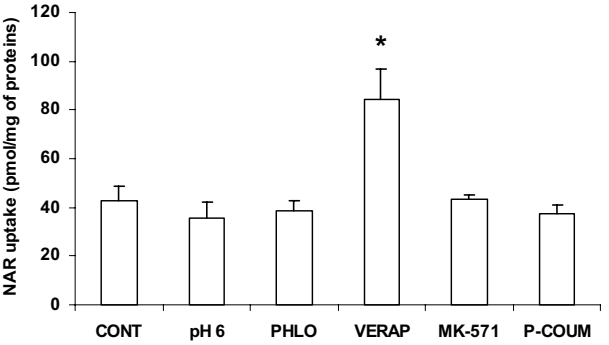


**Figure 4.** Effect of various transport inhibitors on NAR accumulation. Cells were pre-incubated for 30 min with inhibitors, followed by co-incubation with 90 μM NAR for 90 s at 37°C. Concentrations used for PHLO, VERAP, MK-571, and P-COUM were 1 mM, 0.75 mM, 0.2 mM and 1 mM, respectively. Values are expressed as means ± SEM, * indicates a significant statistical difference with the control ($p < 0.05$, $n = 3$).

## 4 Discussion

Our studies on the intestinal absorption of NAR were performed with a Caco-2/TC7 cell line, which has been characterized for its ability to transport and metabolize xenobiotics as well as nutrients (reviewed in [26]). Our results revealed the presence of NAR in the intestinal cell. Nevertheless, this uptake was very low (Fig. 1), and the $P_{app}$ value obtained for the AP → BL transport (Table 1) is much lower than those reported for other flavonoid aglycones: chrysin (86-fold) [27] and quercetin (73-fold) [24]. Our value was similar to those obtained for glucoside forms such as quercetin- 3,4′-diglucoside ($P_{app} = 9 \times 10^{-8}$ cm/s) and quercetin-4′-glucoside ($2 \times 10^{-8}$ cm/s).

In our experiments, neither conjugated forms of naringenin nor naringenin itself have been detected in the culture medium or in the cells, whereas in similar conditions a conjugated form of naringenin has been characterized (data not shown). Our results are consistent with data obtained by Day *et al.* [28, 29] showing that the rhamnoglucosides of naringenin and quercetin could not be hydrolyzed by enterocytic enzymes (cytosolic β-glucosidase and lactase phlorizin hydrolase). Rhamnoglucosidic forms were suggested to be absorbed after a deglycosylation step by the colon microflora [30]. In plasma and urine, only naringenin and its conjugates (sulfates, glucuronides, glucuronide sulfates) were found [16, 31–33] after consumption of NAR. Only two papers reported the presence of native NAR in human urine [19] and in rat plasma [20]. However, the doses given were particularly high, suggesting that for such amounts of NAR, a passive transport across the small intestine epithelium could occur. Our results (Fig. 3) appeared to be in agreement with those observations. In fact, the linear curve for concentrations higher than 200 μM could suggest a passive transport.

For lower NAR concentrations, the existence of a specific transporter could be proposed. Several membrane transporters that have been described, such as polyphenol carriers, have been investigated in the present study (Fig. 4). We first looked at the SGLT1, which is responsible of the absorption of quercetin-4'-glucoside [34] and PHLO [35], although the role of SGLT1 in quercetin-3-glucoside absorption still remains controversial [36, 37]. To investigate the possible involvement of SGLT1, our experiments were performed in glucose-free conditions, which could mask the transport by SGLT1, and we used a SGLT1 inhibitor, PHLO. Under such conditions, NAR uptake was not modified, suggesting that this rhamnoglucoside conjugate is not a substrate of SGLT1. MCT is another transmembrane protein that is involved in the absorption of several hydroxycinnamic acids [38, 39] as well as epicatechin gallate [35]. The substrates of MCT are co-transported with a proton, so transport via MCT is favored in acidic conditions. Experiments performed at pH 6 and likewise, in the presence of 1 mM P-COUM (a substrate of MCT) did not affect NAR cellular absorption, as was expected according to its structure. MRP 1 and 2 are members of the ABC transporters family expressed in the intestine [40], and act by exporting drugs or xenobiotics (especially their conjugates) out of the cells. Several polyphenolic compounds have been reported to be transported by MRP 1 and 2: epicatechin [41], PHLO [42], epicatechin-3-gallate [35] and quercetin-4′-glucoside [34]. In our experiments, the presence of MK-571, a specific MRP 1–2 inhibitor, did not lead to any change in the NAR uptake, thus showing that NAR is not a substrate for MRP 1–2. P-gp is an ATP-driven efflux pump capable of transporting a wide variety of structurally diverse compounds from the cell interior into the extracellular space [43, 44]. Uptake experiments performed in the presence of VERAP, a P-gp inhibitor (Fig. 2), enhanced the cellular NAR concentration, demonstrating the involvement of P-gp in the efflux of NAR out of the enterocyte. These results are similar to the observations of Tsai [45], who showed that intravenously given NAR could cross the rat blood-brain barrier (where P-gp is also highly expressed) when co-administered with cyclosporin A, another P-gp inhibitor. Such an efflux observed with NAR at intestine level supports that this flavanone is probably absorbed at the colon level after its deglycosylation, as previously described [16].

As reported recently by Halliwell *et al.* [46], most of the biological effects of poorly absorbed flavonoids such as NAR probably occurs along the digestive tract, from stomach to colon. From the results of this study, we conclude that, despite its very weak transport across the intestinal epithelium, NAR is absorded by intestinal cells and could act at this level. For example, NAR could modulate cholesterol metabolism (by a possible inhibition of intestinal acyl-coA : cholesterol acyltransferase and 3-hydroxy-3-methyl-glutaryl-CoA reductase), but it could also have adverse effects by increasing xenobiotics absorption through inhibition of P-gp.

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