

# Subcellular localization of flavonol aglycone in hepatocytes visualized by confocal laser scanning fluorescence microscope

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Received: 22 December 2008 / Accepted: 15 June 2009 / Published online: 1 July 2009  
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**Abstract** Flavonoids are widely distributed in the plant kingdom and show various biological activities. The bioavailability of flavonoids in biological samples has conventionally been quantified by high-performance liquid chromatography and mass spectrometry, but with these analytical techniques it is difficult to estimate the subcellular localization of flavonoids in intact cells. In this study, we attempted to examine the localization of flavonoids in cultured cells using a confocal laser scanning fluorescence microscope and mouse hepatoma Hepa-1c1c7 cells. Five flavonol aglycones showed autofluorescence in the cells under the conditions (Ex. 488 nm to Em. 515–535 nm), whereas three flavonol glycosides and eight compounds belonging to other flavonoid subclasses, i.e., flavones, flavanones, and catechins, did not. The autofluorescence of galangin and kaempferol appeared stronger in the nucleus than cytoplasm, suggesting that they are incorporated into the cells and accumulated in the nucleus. The proposed method provided evidence that flavonol aglycones

are incorporated into, and accumulated in the nucleus of, hepatocytes.

**Keywords** Cellular uptake · Flavonoid · Fluorescence microscope · Hepatocytes · Kaempferol

## Introduction

Flavonoids are widely distributed in the plant kingdom including edible plants such as vegetables and fruits. They are divided into seven subclasses; flavone, flavonol, flavanone, catechin, anthocyan, isoflavone and chalcone, according to their basic skeletal structure. Flavonoids have various beneficial effects such as anti-cancer, anti-obesity, anti-inflammatory and anti-oxidative activities (Ahn et al. 2008; Boots et al. 2008; Chu et al. 2007; Hsu and Yen 2006; Kuzuhara et al. 2008; Murakami et al. 2008). To evaluate the cellular absorption and metabolism of flavonoids, human colon adenocarcinoma Caco-2 cells were used as an intestinal model. It was reported that flavonoids are incorporated into Caco-2 cells by passive diffusion (Walgren et al. 1998) and/or an active transporter, sodium-dependent glucose transporter 1 (Walgren et al. 2000b), and excreted through an efflux transporter such as multidrug resistance-associated protein-2 (Walgren et al. 2000a). In addition, the bioavailability of flavonoids has also been investigated in animals and human subjects. In mucosa and

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the liver, flavonoids undergo glucuronidation, sulfation and methylation, and the metabolites produced are detected in plasma, urine and the liver (Mullen et al. 2006; Natsume et al. 2003). Minute amounts of intact flavonoids are also detected in these tissues (de Boer et al. 2005; Urpi-Sarda et al. 2008).

To determine the physiological concentrations and chemical structure of flavonoid metabolites in vivo and in cultured cells, high-performance liquid chromatography (HPLC) or HPLC combined with mass spectrometry (MS) is usually used (de Boer et al. 2005; Urpi-Sarda et al. 2008; Wang et al. 2003). To examine the subcellular localization of flavonoids, it is necessary to prepare the subcellular fractions from tissues or cultured cells. Moreover, cross contamination can occur among the fractions. In this study, we attempted to visualize the incorporation and subcellular localization of flavonoids in mouse hepatoma Hepa-1c1c7 cells with a fluorescence microscope.

## Materials and methods

### Materials

[<sup>3</sup>H]-Kaempferol was obtained from Moravek Biochemicals, Inc. (Brea, CA), flavone, morin and naringenin from Nacalai tesque (Kyoto, Japan), isorhamnetin, kaempferol-3-glucoside, kaempferol-3-rutinoside, quercetin, apigenin, luteolin, galangin and eriodictyol from Extrasynthèse (Genay, France), kaempferol and rutin from Wako Pure Chemical Industries, Ltd (Osaka, Japan), and (–)-epigallocatechin (EGC) and (–)-Epigallocatechin gallate (EGCG) from Kurita Kogyo (Tokyo, Japan). (–)-Epigallocatechin 3-(3'-O-methyl) gallate (Met-EGCG) was generously provided by Dr. Ryoyasu Saijo. Propidium iodide was purchased from Molecular Probes, Inc. (Carlsbad, CA).

### Cell culture

The mouse hepatoma cell line, Hepa-1c1c7 cells were grown and maintained at 37 °C in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Sigma, St. Louis, MO), 4 mM L-glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin under a humidified atmosphere containing 5% CO<sub>2</sub>.

### Uptake of [<sup>3</sup>H]-kaempferol

Hepa-1c1c7 cells were seeded onto 24-well culture plates at a density of  $2 \times 10^5$  cells/well, incubated for 24 h, and then treated with [<sup>3</sup>H]-kaempferol at 1, 10 and 20  $\mu$ M in Krebs–Ringer HEPES buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM CaCl<sub>2</sub>, and 1.3 mM MgSO<sub>4</sub>) for 70 min. The cells were washed three times with ice-cold phosphate-buffered saline and lysed by adding 250  $\mu$ L of 0.05 N NaOH for 4 h. The lysate was transferred to a vial with scintillation cocktail, and the radioactivity incorporated into the cells was measured by a liquid scintillation counter.

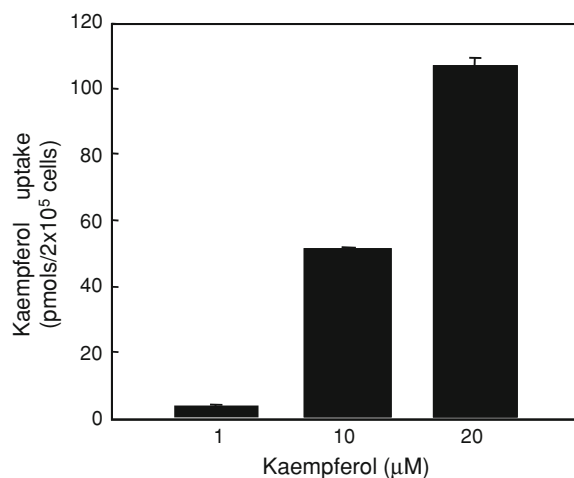
### Fluorescence microscopic analysis

Hepa-1c1c7 cells were seeded onto a glass-bottomed culture dish (MatTek Corp., Ashland, MA) and incubated with fresh  $\alpha$ -MEM supplemented with 5% fetal bovine serum (biowest, Nuaille, France) for 24 h prior to treatment. The cells were treated with flavonoids in the medium. The concentration and incubation time are given in the figure captions. After the incubation, the cells were fixed with 4% paraformaldehyde and 0.2% picric acid in 100 mM sodium phosphate buffer (pH 7.2) overnight. Autofluorescence from the flavonoid was observed under a confocal laser scanning fluorescence microscope (LSM 510 invert, Carl Zeiss, Jena, Germany) with excitation by an argon laser at 488 nm and a 515–535 nm band pass filter. The nucleus was counterstained with propidium iodide (PI; Molecular Probes, Carlsbad, CA) according to the manufacturer's directions, and its fluorescence was monitored at 543 nm (HeNe excitation) with a 560–615 nm band pass filter.

## Results

### Incorporation of [<sup>3</sup>H]-kaempferol into Hepa-1c1c7 cells

To investigate the uptake of flavonoids by hepatocytes, [<sup>3</sup>H]-kaempferol was employed as a model compound and treated to Hepa-1c1c7 cells. The radioactivity, which is reflecting the amount of kaempferol in the cells, increased in a dose-



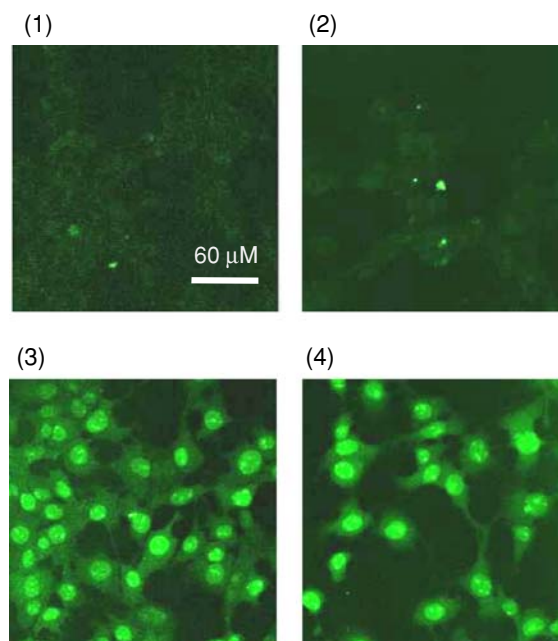
**Fig. 1** Determination of cellular uptake of [<sup>3</sup>H]-kaempferol in Hepa-1c1c7 cells. The cells were treated with [<sup>3</sup>H]-kaempferol at 1, 10 and 20 μM for 70 min. Cell lysate was transferred to vials with scintillation cocktail, and the radioactivity incorporated into the cells was measured by a liquid scintillation counter. Data are represented as the mean ± SE (*n* = 3)

dependent manner (Fig. 1), though percent uptake was almost the same (data not shown). This result indicated that kaempferol was incorporated into Hepa-1c1c7 cells without saturation under the experimental conditions.

#### Detection of flavonol aglycones in hepatocytes by fluorescence microscopy

Next, we examined the autofluorescence from kaempferol in Hepa-1c1c7 cells under the confocal scanning microscope. Green autofluorescence was detected at over 50 μM of kaempferol (Fig. 2). When the time-dependent uptake of kaempferol at 50 μM was also monitored, the intensity of fluorescence increased time-dependently (Fig. 3). After 15 min, apparent fluorescence was observed to the cells. Taken together, the fluorescence microscope is able to monitor the uptake of kaempferol in Hepa-1c1c7 cells.

We applied this method to the uptake of other flavonoids. When the cells were treated with each flavonoid at 50 μM for 70 min, four flavonol aglycones, namely galangin, isorhamnetin, morin, and quercetin, showed green autofluorescence in the cells [Fig. 4(1)–(4)]. In contrast, three flavonol glycosides, i.e., kaempferol-3-glucoside, kaempferol-3-rutinoside



**Fig. 2** Cellular uptake of kaempferol visualized by a fluorescence microscope in Hepa-1c1c7 cells. The cells were seeded onto the glass bottom dishes and treated with kaempferol at (1) 0 μM, (2) 10 μM, (3) 50 μM and (4) 100 μM for 70 min. Green fluorescence was observed under a confocal microscope (Ex. 488 nm to Em. 515–535 nm). (Color figure online)

and rutin, did not show autofluorescence [Fig. 4(5)–(7)]. We further examined 8 aglycones; flavone, apigenin, luteolin, naringenin, eriodictyol, EGC, EGCG and Met-EGCG, belonging to other flavonoid subclasses, but did not detect any autofluorescence (data not shown). These results indicated that the uptake of flavonol aglycones in Hepa-1c1c7 cells can be observed with a confocal microscope.

#### Accumulation of kaempferol and galangin in the nucleus

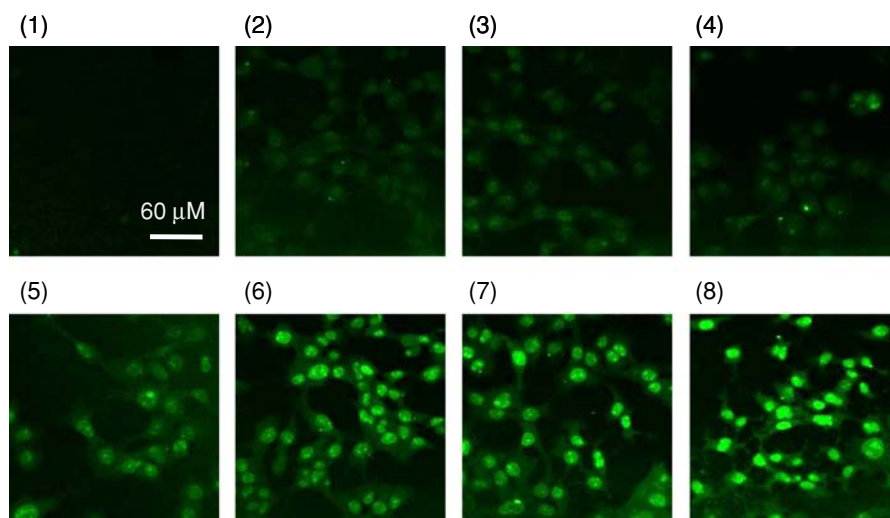
To determine the subcellular localization of flavonols in intact mouse hepatoma Hepa-1c1c7 cells, we stained the nucleus with PI and found red fluorescence in the nuclear compartment (Fig. 5). This red fluorescence overlapped with the green fluorescence from flavonol because yellow fluorescence was observed in merged images. Therefore, kaempferol and galangin were incorporated into Hepa-1c1c7 cells, and accumulated in the nucleus.

## Discussion

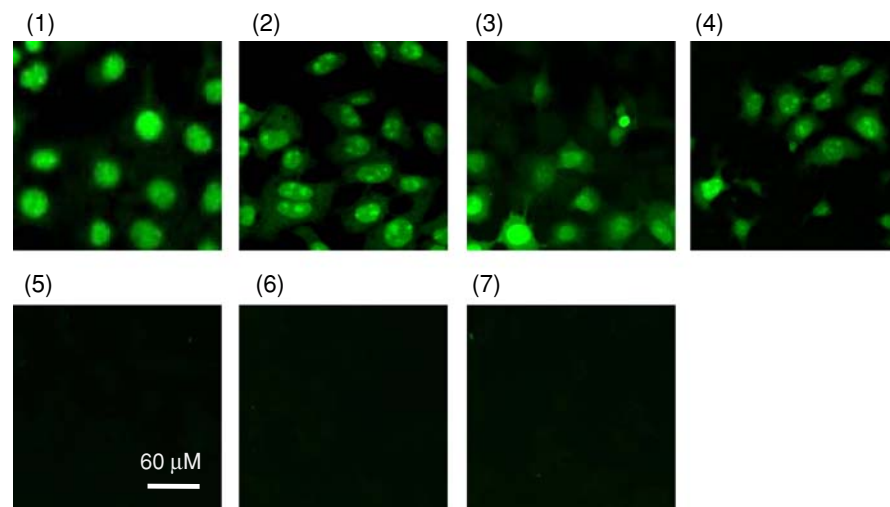
In the present study, we demonstrated that flavonol aglycones specifically accumulated into the nucleus of Hepa-1c1c7 cells using a confocal laser scanning fluorescent microscope (Figs. 2, 3, 4, 5). It is likely that flavonol aglycones are easily cross cellular and nuclear membranes through the passive diffusion (Walgren et al. 1998) but not endocytosis. Previously, it was reported that quercetin but not its conjugated form emitted fluorescence in Caco-2 cells by a fluorescence microscope (Walgren et al. 2000b) but this report did not address the subcellular localization. Thus, this is the first report demonstrating the nuclear accumulation of flavonol aglycones detected

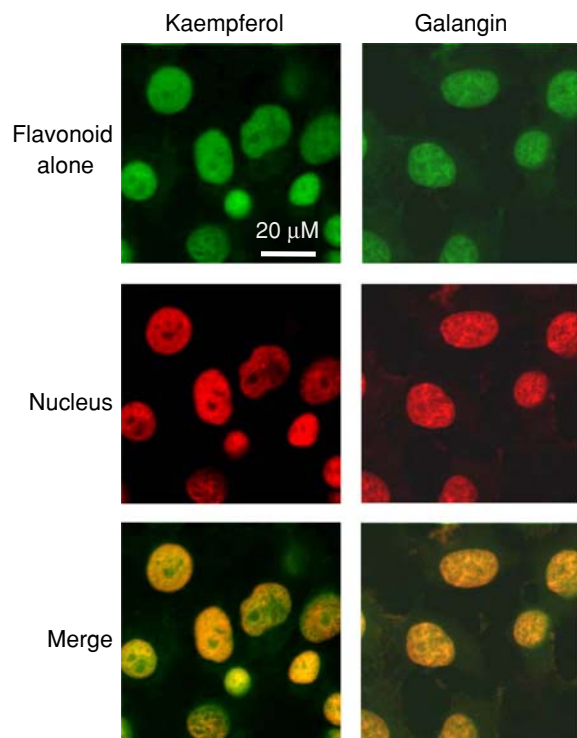
with a fluorescence microscope. This method is superior to HPLC and LC/MS because it does not require intracellular fractionation and extraction of the compound from the cells, although fluorescence microscopy has a limitation to detect the compound belonging to flavonol subclass and it can not quantify the compound. Sensitivity of this method was lower than that of the method using radioactive compounds, such as [<sup>3</sup>H]-kaempferol (Fig. 1), since the former method can detect incorporated flavonol aglycone itself whereas the later one can detected sum of the aglycone and its metabolites. This indicates that sensitivity of our developed method would be depending on the cell types because of the different metabolizing activities of flavonoids.

**Fig. 3** Time-dependent cellular uptake of kaempferol in Hepa-1c1c7 cells. The cells on the glass bottom dishes were treated with kaempferol at 50  $\mu$ M for (1) 0 min, (2) 1 min, (3) 5 min, (4) 10 min, (5) 15 min, (6) 30 min, (7) 45 min, and (8) 70 min. Green fluorescence was observed under a confocal microscope. (Color figure online)



**Fig. 4** Cellular uptake of various flavonoids visualized by fluorescence microscopy. Hepa-1c1c7 cells were seeded onto glass bottom dishes and treated with (1) galangin, (2) isorhamnetin, (3) morin, (4) quercetin, (5) kaempferol-3-glucoside, (6) kaempferol-3-rutioside, or (7) rutin at 50  $\mu$ M for 70 min. Green fluorescence was measured under a confocal microscope. (Color figure online)





**Fig. 5** Subcellular localization of kaempferol and galangin in Hepa-1c1c7 cells. The cells were seeded onto glass bottom dishes, treated with kaempferol (*left*) or galangin (*right*) at 50  $\mu$ M for 70 min, and stained with propidium iodide. *Green fluorescence* (Ex. 488 nm to Em. 515–535 nm) from flavonol (*top*) and *red fluorescence* (Ex. 543 nm to Em. 560–615) from PI (*middle*) were monitored under a confocal microscope, and the images were merged (*bottom*). (Color figure online)

As to the relationship between chemical structure and autofluorescence, a hydroxyl group at the C3-position in the flavonol skeleton is important because no fluorescence was observed in the cells treated with compounds belonging to the flavone, flavanone and catechin subclasses. Even flavonol lost its autofluorescence when a sugar moiety was bound to the C3-position (Fig. 4). It is suggested that a substituent functional group introduced to the C3-position by metabolism also leads to the loss of the autofluorescence in the cells. Further study is needed to clarify the structure-fluorescence relationship.

Flavonoids have been detected *in vivo* not only in the intestine but also in various tissues and organs including the liver by HPLC and LC/MS (de Boer et al. 2005; Urpi-Sarda et al. 2008; Wang et al. 2003). The liver plays an important role in the metabolism of flavonoids because aglycone and its metabolites are

detected in this organ (de Boer et al. 2005; Vaidyanathan and Walle 2002). In the present study, we quantified the uptake of flavonoid into hepatocytes using [ $^3$ H]-kaempferol, and found that the amount of radioactivity in the cells increased dose-dependently with a constant absorption ratio. This result indicates that a certain amount of flavonoid was incorporated into the cells. In previous reports, the absorption of flavonoids was increased by lipids (Azuma et al. 2003) and their excretion was reduced by an inhibitor of P-glycoprotein (Tian et al. 2006; Walgren et al. 2000a), indicating that these chemicals increase the bioavailability of flavonoids. It is worth investigating the subcellular localization of flavonols using fluorescence microscopy after pretreatment with these chemicals. Our developed method could be applied also to other type of cells, because it was reported that quercetin emitted autofluorescence in Caco-2 cells, a model of intestinal epithelial cells (Walgren et al. 2000a). Flavonol aglycones would be incorporated not only into hepatocytes but also into other types of cells in the same manner as they do into hepatocytes, whereas flavonol glycosides and metabolites are considered to be incorporated into the cells in different manners because their hydrophobicity is different from that of aglycone. Moreover, the metabolizing activity of the cells will influence the distribution of flavonols. Further study is needed to clarify subcellular localization of flavonoids in other types of cells.

Previously, it was reported that flavonols were excreted from the cells through an efflux transporter existing on the plasma membrane (Brand et al. 2008; Ofer et al. 2005). This result and our findings indicated that flavonol could be shuttled between cytoplasm and nucleus in the cells and gradually accumulate into the nucleus, because they might have a greater affinity to cellular components of nuclei, such as nucleic acid, than to those of cytoplasm. Therefore, flavonol might be able to be localized in both nuclei and cytoplasm, and shows various physiological functions. It is known that antioxidant activity is one of the typical functions of flavonoids. It is reported that flavonoids are able to interact with isolated DNA *in vitro*, and to protect it from damage induced by oxidative stress (Cai et al. 1997; Furukawa et al. 2003). It was also reported that flavonoids induced the oxidative breakage of cellular DNA in the presence of copper ions in cancer cells as a

preventive effect on cancer promotion (Hadi et al. 2007). Taken together, these results and the findings of our study suggest that flavonol aglycones accumulate in the nucleus of hepatocytes and act to prevent DNA damage caused by oxidative stress and chemical carcinogens. Other reports demonstrated that flavonoid shows harmful effects through DNA damage under certain cellular conditions (Hodek et al. 2006; Sahu and Gray 1997). Further study is needed to investigate the relationship between sub-cellular localization of flavonoid and its beneficial and/or harmful function in various types of the cells.

**Acknowledgments** Part of this work was supported by Grant-in-Aid for the Japan Society for the Promotion of Science Fellows (to R.M.) and Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan.

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