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Research Paper

A novel method using confocal laser scanning microscopy for sensitive measurement of P-glycoprotein-mediated transport activity in Caco-2 cells

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Abstract

Objectives The aim of this study was to use time-lapse confocal laser scanning microscopy to establish a more sensitive and specific method for evaluating P-glycoprotein activity in Caco-2 cells.

Methods The change in the fluorescence of residual rhodamine 123 at the apical and central regions of Caco-2 cells was measured in the presence of digoxin or St John's wort by using time-lapse confocal laser scanning microscopy. The data were compared with measurements made using conventional techniques, a fluorescence microplate reader and a fluorescence microscope.

Key findings The percentage decrease of rhodamine 123 caused by 10 μM digoxin or 0.1 μg/ml St John's wort was significantly larger in the apical region of the Caco-2 cell than in the central region or in the whole cell. The digoxin-induced inhibition in the apical region as measured by time-lapse confocal laser scanning microscopy was greater than that measured in the whole cell by a microplate reader or a fluorescence microscope.

Conclusions The assay of residual rhodamine 123 in the apical region of Caco-2 cells by confocal laser scanning microscopy was more sensitive than the conventional methods using a microplate reader or fluorescence microscopy. It will be a valuable screening tool for studying both the inhibition and induction of P-glycoprotein activity.

Keywords Caco-2 cells; confocal laser scanning microscopy; digoxin; P-glycoprotein; St John's wort

Introduction

The 170-kDa membrane transport protein P-glycoprotein (P-gp) has attracted attention as a contributing factor to a wide range of drug–drug interactions. This transporter is an ATP-dependent drug efflux pump that is constitutively expressed in several human tissues, including the intestinal epithelia and the blood–brain barrier endothelia. P-gp is widely expressed in the epithelial cells of the liver, kidney, testes and lymphocytes, as well as in cancer cells.[1–3] Numerous drugs have been identified as P-gp substrates, inhibitors or inducers. Digoxin, a well-characterized P-gp substrate, contributes to clinical drug–drug interactions with increased exposure when co-administered with known P-gp modulators such as quinidine, itraconazole and rifampicin.[4–11] This highlights the role of intestinal efflux in digoxin absorption.

In recent years, the potential clinical implications of drug–food interactions have gained recognition. For example, St John's wort is an inducer of cytochrome P450 3A4 and is known to modulate P-gp activity.[12–14] Extracts of St John's wort are increasingly being used as natural medicines for the treatment of mild to moderate depression. In Europe and the United States of America millions of doses of St John's wort are taken daily, both through medical prescriptions and as over-the-counter self-medication. As the use of this herb has become more common, interactions between St John's wort and co-administered medications have been reported.[15,16] Acute heart transplant rejection due to decreased

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ciclosporin plasma levels (greater than 50% reduction) with co-administration of St John's wort has been reported in several case studies and similar findings have been seen in kidney transplant patients.[17–19] Moreover, St John's wort (300 mg taken three-times daily for 14 days) significantly reduced the digoxin area under the curve and maximum serum concentration.[20]

Recently, numerous supplements including St John's wort have appeared on the market. It is not yet well understood whether any of these supplements or foods have the potential to influence P-gp. Many supplements and foods are thought to act more gradually than medicines and their influence on P-gp is thought to be weaker. However, when multiple supplements and foods with only a weak influence on P-gp are ingested at the same time, there is a possibility of a strong influence on P-gp. Additionally, in high-risk groups – such as senior citizens who suffer from renal damage, hepatic toxicity, arterial hypertension, or cancer – even a weak effect may increase the danger of an interaction.[21,22] Therefore, a highly sensitive tool for detecting even a weak influence on P-gp is necessary.

The rapid, sensitive and convenient assessment of the interaction characteristics of a drug or food is crucial for the anticipation of potentially serious drug interactions or therapy failures. Rhodamine 123 and Caco-2 cells are frequently used to measure such interactions. Caco-2 cells, which originate from human colon adenocarcinoma cells, have been used widely as a cell-based model to study transport and absorption across human intestinal epithelia. Rhodamine 123 is a lipophilic cation that is subject to P-gp-dependent transport. It has been used widely as an in-vitro and in-vivo marker of P-gp function.[23–26]

Generally, a microplate reader is used to detect the fluorescence of rhodamine 123 in Caco-2 cells; the fluorescence value represents the total residual rhodamine 123 in the whole cell. Alternatively, some investigators have measured the efflux of rhodamine 123 by using fluorescence microscopy, which also measures the cell's total rhodamine 123 efflux.[27,28] Both types of assay include excretion not only through the P-gp transporter but also through the other efflux transporters.

However, P-gp is located on the brush-border membrane (apical membrane) of Caco-2 cells and more sensitive detection of changes in P-gp function may be possible by measuring the level of rhodamine 123 only under the apical membrane. This study aimed to develop a method for measuring P-gp activity with a higher sensitivity than that of the methods currently available. Using confocal laser scanning microscope image analysis, the dynamics of intracellular rhodamine 123 were analysed three-dimensionally over time.

Materials and Methods

Materials

Digoxin was obtained from Sigma-Aldrich (St Louis, MO, USA). St John's wort (containing not less than 0.3% hypericin-like substances as measured by spectrophotometry and 3.0% hyperforin as measured by high-performance liquid chromatography) was obtained from Indena (Milan, Italy). Rhodamine 123 (Molecular Probes, Eugene, OR, USA) was used as the P-gp substrate.

Cell culture of Caco-2 cells

The human colon adenocarcinoma cell line (Caco-2) was purchased from DS Pharma Biomedical (Osaka, Japan) and cells were cultured in minimal essential medium with GlutaMAX (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% nonessential amino acids in an atmosphere of 5% CO_2 and 95% air at 37°C. Cells were subcultured at approximately 80% confluence by using 0.02% EDTA and 0.25% trypsin. The cells were used for the experiment at seven days after seeding and between passages 47 and 52.

Measurement of rhodamine 123 by time-lapse confocal laser scanning microscopy

Transport measurement was performed in a Transwell chamber (BD Biosciences, San Jose, CA, USA). Caco-2 cells were seeded at a density of 3×10^4 cells per filter on polyethylene terephthalate filters (BD Biosciences, San Jose, CA, USA; pore size 0.4 μm) in cell culture inserts (0.3 cm^2 effective growth area). All experiments were performed at 37°C in Hanks' balanced salt solution (HBSS (in mM): 137 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 0.4 $MgSO_4$, 0.5 $MgCl_2$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 4.2 $NaHCO_3$, 25 HEPES and 5.6 glucose, pH 7.4).

The cell culture insert was placed on a glass-bottomed dish and the Caco-2 cells were incubated with 3 μM rhodamine 123 for 90 min at 37°C. The cells were rinsed three times with HBSS. The dish containing the cell culture insert was wrapped with parafilm around the edges to prevent water vapour loss and then placed onto the stage (37°C) of a time-lapse confocal laser scanning microscope (C1si; Nikon, Tokyo, Japan). An argon laser (Melles Eriot 85BCD020053; Albuquerque, NM, USA) emitted an excitation wavelength of 488 nm. Emitted light was collected with a 515-nm long-pass dichroic reflector and a 515-nm long-pass emission filter through a Plan Fluor objective (× 60; numerical aperture = 0.7). The cell setup was equilibrated for 10 min at 37°C to stabilize the cells and pericellular environment. After this period, digoxin, St John's wort or HBSS (vehicle) was added to the cells (final concentration: digoxin 10 μM; St John's wort 0.1 μg/ml). Quantification of the intracellular concentration of rhodamine 123 in the Caco-2 cells was performed using fluorescent images at 0, 10, 15, 20, 30, 60 and 90 min with a chronologic measurement computer EZ-C1 system program (Nikon, Tokyo, Japan).

Twenty cell-slice images were captured along the z-axis at equal intervals from the apical brush-border membrane to the basal membrane, with a 12-bit dynamic range. Approximately 75 cells were shown in each cell-slice image. One typical cell was chosen and analysed in each Transwell chamber. An image under the apical membrane and an image of the central region of the cell (the brightest part) were immediately analysed. The total residual amount of rhodamine 123 was calculated by summing the fluorescence intensities of each of the 20 image slices. The fluorescence intensity at the time of administration of a drug or vehicle was defined as 100%.

Measurement of rhodamine 123 by a microplate reader[29]

Caco-2 cells were seeded into 24-well plates (Iwaki, Tokyo, Japan) at a density of 5×10^4 cells per well in 1 ml per well of

culture medium. Cells were washed three times with warm HBSS before the addition of rhodamine 123.

Caco-2 cells were incubated in fresh HBSS containing 10 μM rhodamine 123 for 60 min (loading time). After loading, the HBSS was immediately removed from the wells and the cells were quickly washed three times with HBSS. For the efflux study, loaded cells were incubated with fresh warm HBSS with or without 10 μM digoxin for the indicated times (10, 30, 60 and 90 min) at 37°C. Incubation was stopped by aspiration of the HBSS from the wells, followed by three washes with ice-cold phosphate-buffered saline (PBS (in mM): 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4 and 8.1 $NaHPO_4$, pH 7.4).

After the uptake or efflux experiments, cells were solubilized with 1 ml 0.3 M NaOH, and 500-μl portions were neutralized by 500 μl 0.3 M HCl. Samples (200 μl) of the neutralized solution were transferred to 96-well plates (Iwaki, Tokyo, Japan) and the fluorescence intensity of rhodamine 123 was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm by using a fluorescence multiwell plate reader (CytoFluor Series 4000; Life Technologies, Carlsbad, CA, USA). Protein content was determined by the method of Lowry *et al.*[30] using bovine serum albumin as the standard.

Measurement of rhodamine 123 by fluorescence microscopy[27]

Caco-2 cells were seeded into 96-well plates (Iwaki, Tokyo, Japan) at a density of 2×10^4 cells per well, each well containing 0.25 ml culture medium. Cells were washed three times with warm HBSS.

In the efflux experiments, Caco-2 cells were incubated for 90 min in fresh HBSS containing 3 μM rhodamine 123. After loading, the HBSS was immediately removed from the wells and cells were quickly washed three times with HBSS. Efflux experiments were started by the addition of fresh warm HBSS with or without 10 μM digoxin.

The Caco-2 cells were then analysed by fluorescence microscopy (BIOREVO BZ-9000; Keyence, Osaka, Japan) at 10, 30, 60, and 90 min. Rhodamine 123 was visualized using a BZ filter GFP-BP (excitation wavelength 470/40 nm; emission wavelength 535/50 nm). Images were captured using a CCD camera system and the BZ-II software (Keyence, Osaka, Japan).

Statistical analysis

All values represent the mean ± standard error. The fluorescence intensity at 10 min was defined as 100%. The data were analysed using a two-way analysis of variance followed by a Bonferroni post-test when the analysis of variance results were significant ($P < 0.05$). Statistical analyses were performed using the GraphPad Prism 4.03 software (GraphPad Software, La Jolla, CA, USA).

Results

Time-lapse confocal laser scanning microscopy was used to measure the decrease over time in the fluorescence of residual rhodamine 123 in the whole cell (Figure 1a), the apical region

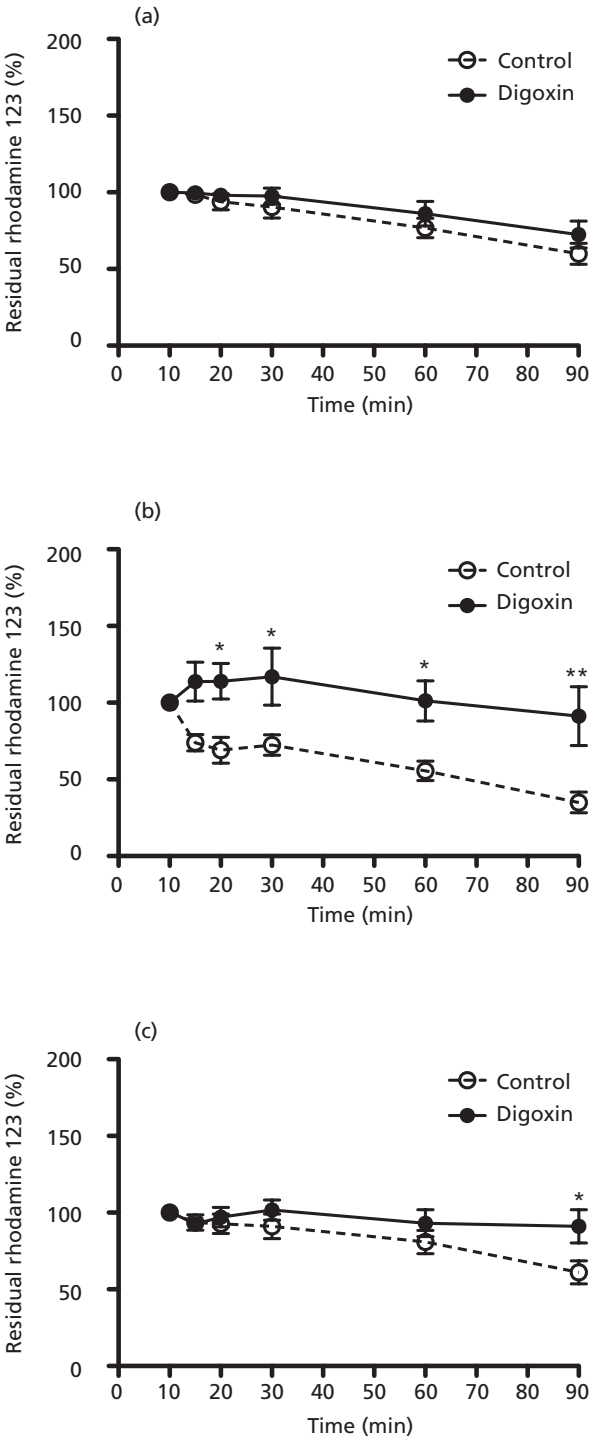

Figure 1 Influence of 10 μM digoxin on the time-dependent change in residual rhodamine 123 in Caco-2 cells as measured by time-lapse confocal laser scanning microscopy. (a) The total residual amount of rhodamine 123 in the whole cell. The residual amount of rhodamine 123 in (b) the apical region and (c) the central region. The y-axis of each panel represents the percentage of fluorescence value at 10 min. Each point and bar represents the mean ± SE ($n = 8$). *$P < 0.05$, **$P < 0.01$, the control group compared with the digoxin group.

(Figure 1b) and central region (Figure 1c) of control cells and cells treated with digoxin. Generally, the fluorescence decreased with time. The fluorescence decreases in the whole cell and in the central region were mild and smaller than those in the apical region. Digoxin tended to cause a decrease in residual rhodamine 123 in the whole cell, but the effect was not significant (Figure 1a). Meanwhile the fluorescence decrease in the apical region was abolished by digoxin (Figure 1b) after 20 min. In the central cellular region, a significant difference between the control and digoxin groups was observed only after the 90-min mark (Figure 1c).

The decrease over time in the fluorescence of residual rhodamine 123 in the whole cell (Figure 2a), apical region (Figure 2b) and central region (Figure 2c) of Caco-2 cells in the presence or absence of St John's wort was also measured with time-lapse confocal laser scanning microscopy. In the apical region, there was a significant difference in fluorescence between the control and St John's wort, starting at 30 min (Figure 2b). In the whole cell and the central region, the respective decrease in fluorescence was mild; no significant difference between the control and St John's wort was observed (Figure 2a and c).

The decrease in fluorescence measured by a microplate reader, a fluorescence microscope and a time-lapse confocal laser scanning microscope is shown in Figure 3a–3c, respectively. The microplate reader and the fluorescence microscope measured the fluorescence level in the whole cell; in both cases the decrease in residual rhodamine 123 was significantly inhibited by digoxin only at the 90-min mark. In contrast, time-lapse confocal laser scanning microscopy showed digoxin-induced inhibition of the fluorescence decrease in the apical region starting at the 30-min mark. Thus, the confocal laser scanning microscope was shown to be more sensitive than the microplate reader and the fluorescence microscope. In addition, fluorescence fading of rhodamine 123 was not observed during this experiment, which lasted for 90 min.

Discussion

The fluorescent dye rhodamine 123 has been used extensively as a probe to assess P-gp activity in rodent and tissue culture models. Indeed, previously we have examined the effects of seven dietary ingredients on P-gp-mediated transport by measuring the accumulation and efflux of rhodamine 123, and we found that capsaicin, piperine, and sesamin significantly inhibited P-gp function.[31] However, other compounds, such as curcumin and taurine did not show significant effects, which could be partially due to the low sensitivity of microplate readers. When multiple dietary ingredients with only a weak influence on P-gp are co-administered, a strong influence on P-gp is possible.

In this study, we developed a more sensitive method to assess P-gp activity using time-lapse confocal laser scanning microscopy. The decrease in the intensity of rhodamine 123 fluorescence differed between the apical and central regions of Caco-2 cells. In the apical region, the fluorescence intensity rapidly decreased at early time points and then slowly decreased from 30 to 90 min. By contrast, fluorescence intensity in the central region showed a slow decline from the start; the total intensity of fluorescence in whole cells showed a similar pattern. These findings indicated that specific measurement of fluorescence in the apical region could be more sensitive than measurement of overall fluorescence.

Digoxin, a potent substrate of P-gp, immediately and completely inhibited the rhodamine 123 efflux rate (measured by the decline in fluorescence) in the apical region of Caco-2 cells. This suggested that the spontaneous decrease of fluorescence in the apical region was based on the excretion of rhodamine 123 by P-gp. In the central region, however, a significant difference in the fluorescence decrease between the control and digoxin groups was observed only after 90 min. Overall, the fluorescence value measured in the apical region could be considered to be a better index of P-gp-mediated transport.

Recently, Aller *et al*.[32] reported the X-ray structure of mouse P-gp and suggested that the substrate-binding cavity (~6000 Å) within the lipid bilayer of the membrane was made up of mostly hydrophobic and aromatic residues, allowing access to hydrophobic compounds from the inner membrane. It is generally believed that the binding of substrates to the P-gp transmembranes on the inner leaflet of the membrane bilayer initiates the transport cycle. This is followed by ATP binding and hydrolysis to efflux the substrate and return the pump conformation to its original state. Therefore, the partitioning into the lipid membrane is considered to be the rate-limiting step for the interaction of a substrate with P-gp. Evidence suggests that a drug with a high log P (the octanol-water partition coefficients) will accumulate to a high concentration within the cytoplasmic membrane and will favour binding to P-gp with a low K_m (the Michaelis-Menten constant) value, whereas a drug with low partitioning will have a lower membrane concentration and a high K_m value.[33,34] In this study, the relatively fast apical efflux rates of rhodamine 123 observed with digoxin were probably due to the rapid efflux of digoxin available in the close vicinity of P-gp. However, the slow efflux rate seen in whole cells may have been due to the associated rate-limiting diffusion of digoxin into the inner leaflet from the cytosol.

Additionally, we compared the time-lapse confocal laser scanning microscopy technique with assays using a fluorescence microplate reader and a fluorescence microscope in terms of their respective sensitivities in detecting P-gp inhibition. With the microplate reader and fluorescence microscope, the decrease in residual rhodamine 123 in Caco-2 cells was significantly inhibited by digoxin only at 90 min. By contrast, time-lapse confocal laser scanning microscopy revealed a digoxin-induced inhibition of the fluorescence decrease in the apical region from 20 min onward. Taken together, these results indicated that the measurement of rhodamine 123 in the apical region of Caco-2 cells provided a more sensitive and more rapid evaluation of P-gp function than the measurement of total rhodamine 123 distributed over the whole cell.

Caco-2 cells express several transporters, such as multidrug resistance-associated protein 2 (MRP2), organic anion-transporting polypeptide B (OATP-B), the peptide transporter, and P-gp, in the apical membrane.[35–41] Few studies have been published on the passive transport of drugs.[42] It has been reported that P-gp is localized preferentially to the apical borders of intestinal villus enterocytes.[43,44] Hunter *et al*.[41] reported functional expression of P-gp in the apical

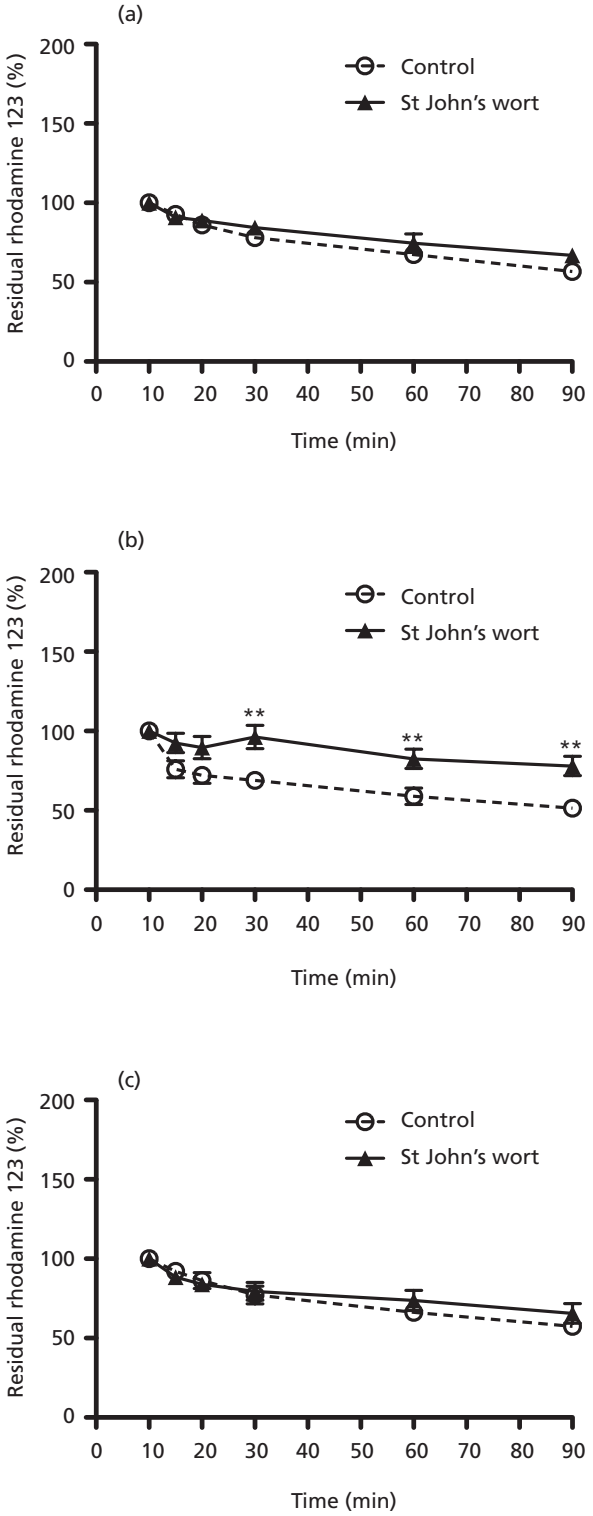

Figure 2 Influence of 0.1 μg/ml St John's wort on the time-dependent change in residual rhodamine 123 in Caco-2 cells as measured by time-lapse confocal laser scanning microscopy. (a) The total residual amount of rhodamine 123 in the whole cell. The residual amount of rhodamine 123 in (b) the apical region and (c) the central region. The y-axis of each panel represents the percentage of the fluorescence value at 10 min. Each point and bar represents the mean ± SE ($n = 8$). $^{**}P < 0.01$, the control group compared with the St John's wort group.

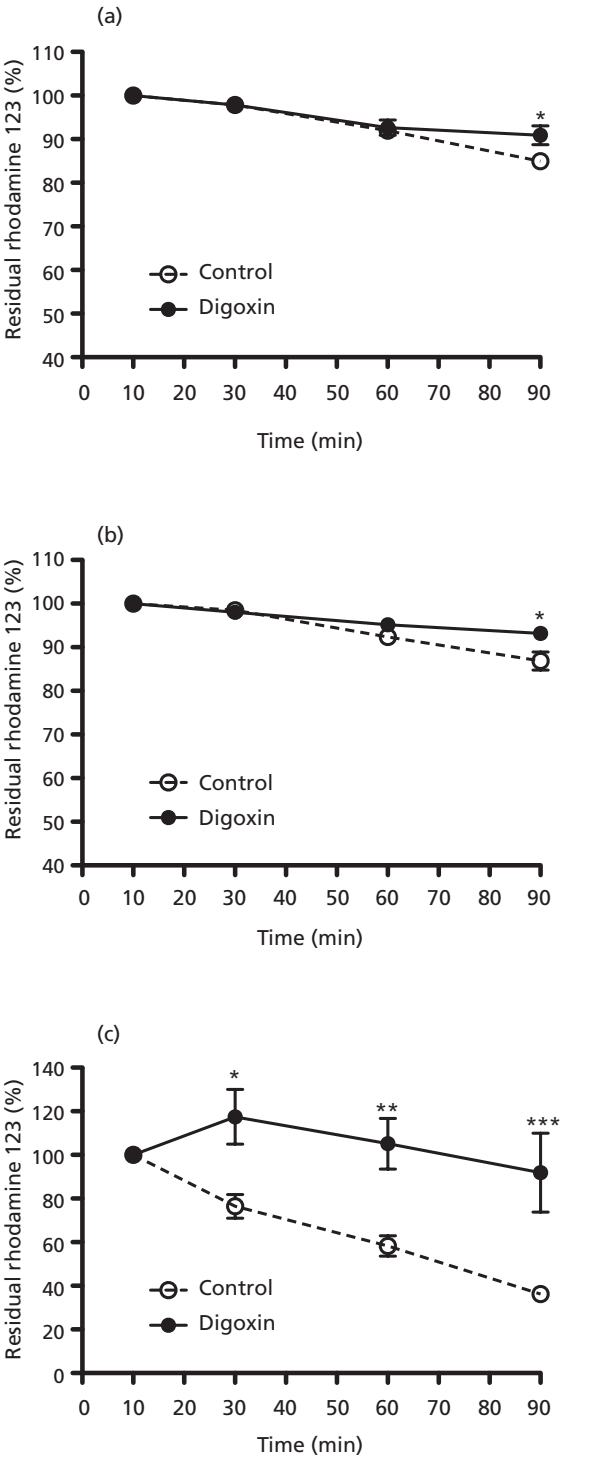

Figure 3 Comparison of 10 μM digoxin-induced inhibition of the time-dependent decrease of residual rhodamine 123 in Caco-2 cells as measured by three techniques. (a) The amount of rhodamine 123 in whole cells as measured by a microplate reader. (b) The amount of rhodamine 123 in whole cells as measured by a fluorescence microscope. (c) The amount of rhodamine 123 in the apical region of the cells as measured by time-lapse confocal laser scanning microscopy. The y-axis of each panel represents the percentage of the fluorescence value at 10 min. Each point and bar represents the mean ± SE ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, the control group compared with the digoxin group.

membranes of human intestinal Caco-2 cells. Additionally, rhodamine 123 is a specific substrate of P-gp. Therefore, the change in the level of rhodamine 123 in the apical region of Caco-2 cells will reflect the function of P-gp. The control in Figure 3c and the controls in Figure 3a and b are shown to differ. Figure 3c shows the apical region, whereas Figure 3a and b show whole cells. Thus, P-gp was expressed at the apical membrane and the decrease in fluorescence in the apical region was more sensitive to P-gp activity than the fluorescence decrease in the whole cell.

In our experiments, the decrease in rhodamine 123 transport in the apical region of the Caco-2 cells was completely inhibited by digoxin or by St John's wort. Perloff *et al*.[14,27] reported little or no inhibition by digoxin and only 40% inhibition by St John's wort (300 μg/ml). Hellum and Nilsen[45] reported that St John's wort decreased the basolateral-apical and apparent permeability value of digoxin (80–800 μg/ml). Hughes and Crowe[46] reported the bidirectional transport of 20 μM digoxin through the Caco-2 sub-clone with high P-gp expression, by HPLC. In our experiment, the concentrations of digoxin or St John's wort were lower than those of these earlier reports and our method detected the inhibition of P-gp activity with high sensitivity. Therefore, our method was more sensitive than other traditional assay systems. In addition, these traditional methods generally use Caco-2 cells after 21 days of seeding, whereas the current technique can use the cells after only seven days (when the cells reach confluence). Thus, our assay is more sensitive and more rapid than the conventional assays.[45–48]

Recently, polypharmacy (e.g. antiretroviral therapy, immunosuppressant treatment and cancer chemotherapy) has become a common practice for drug therapies, thus a highly sensitive assay of P-gp is necessary. The assay presented here appeared to be a highly sensitive tool for the detection of P-gp by local measurement in cells. In particular, this system may be suitable for the detection of mild influences of food ingredients and may provide a better understanding of drug–food interactions, which are increasingly being recognized as having potential clinical implications.[49,50] However, it is not clear whether many foods influence P-gp (with some exceptions, such as quercetin, grapefruit juice, and curcumin). [51–56] This new assay can thus contribute to generating an extensive database of valuable information on potential P-gp-related interactions between drugs, drug candidates and food ingredients.

Conclusions

Our assay for evaluating P-gp activity in Caco-2 cells by using time-lapse confocal laser scanning microscopy to measure residual rhodamine 123 fluorescence (specifically in the apical region of cells) was shown to be more sensitive and more rapid than traditional assays using a microplate reader or fluorescence microscope. The decrease in total rhodamine 123 fluorescence in whole cells was not significantly influenced by digoxin at 60 min. However, the fluorescence intensity in the apical region measured using time-lapse confocal laser scanning microscopy was significantly altered by digoxin and St John's wort at 30 min. We predict that this assay will be a valuable screening tool for studying both the inhibition and induction of P-gp activity and may also be useful for predicting P-gp-mediated alterations in the intestinal absorption of drugs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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