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Transport rates of dietary phytochemicals in cell monolayers is inversely correlated with absorption kinetics in humans

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ABSTRACT

Dietary phytochemicals promote health and reduce the risk of chronic disease. The Phytochemical Absorption Prediction Model (PCAP) predicts the time required for phytochemicals to reach maximal plasma concentrations (T_{max}) in humans based on their lipophilicity and molecular mass. Cell-based transport models have been used to quantify transport rate and efficiency of pharmaceuticals and phytochemicals, however these parameters have not previously been related to the human absorption T_{max}. Caco-2/HT29-MTX-E12 monolayers were used to characterise transport of phytochemical standards and extracts and to establish a relationship between the *in vitro* permeability (P_{app}) of standards and their *in vivo* T_{max} predicted from the PCAP model. Lipophilic compounds transported through the cell monolayer at relatively faster rates (higher P_{app}) than hydrophilic compounds, whilst having slower predicted *in vivo* absorption rates (longer T_{max}). The results infer differences between *in vitro* (cell monolayer) and *in vivo* (human gastrointestinal tract) absorption kinetics of phytochemicals.

1. Introduction

Diets rich in fruits and vegetables promote health and are associated with a reduced risk of chronic diseases including obesity (Jarzab & Kukula-Koch, 2017), diabetes (Stravodimos et al., 2017), cancer (Key, 2011), cardiovascular (Dauchet, Amouyel, & Dallongeville, 2009) and neurodegenerative diseases (D'Onofrio et al., 2017). The protection provided by these plant-based foods has been attributed to their high levels of phytochemicals that help to regulate oxidative stress and inflammation (OSI) (Del Rio et al., 2013; Schinella, Tournier, Prieto, de Buschiazzo, & Ríos, 2002). Elevated OSI is persistent in individuals suffering from chronic diseases (Calder et al., 2009) and also associated with normal activities including exercise (van der Merwe & Bloomer, 2016) and meal digestion (Burton-Freeman, 2010) in healthy individuals.

Uptake of phytochemicals into circulation and their absorption by target cells is necessary for providing biological benefits (Lee, 2013). However, phytochemicals are recognised by the body as xenobiotics resulting in their low bioavailability and transient presence (Holst & Williamson, 2008). Depending on their chemical structures and dietary intake forms, the time required for phytochemicals to reach maximal plasma concentrations (T_{max}) can be 1–2 h (h) or 15–33 h post consumption and are completely cleared over the next few hours or days, respectively (Gustin et al., 2004; Stalmach, Troufflard, Serafini, & Crozier, 2009). Therefore, T_{max} is important to define the optimal temporal window to observe the bioefficacy of phytochemicals. For example, a strawberry drink with T_{max} of the associated phytochemicals of 1–2 h was reported to significantly attenuate the OSI induced by a high-fat meal when the drink was consumed 2 h before the meal and not with the meal or after the meal (Huang, Park, Edirisinghe, & Burton-Freeman, 2016; Sandhu et al., 2016).

Following ingestion, absorption of some phytochemicals into the blood stream occurs in the small intestine via passive diffusion or carrier mediated routes (Donovan, Manach, Faulks, & Kroon, 2006). Uptake routes of passive diffusion are dependent on physicochemical properties of phytochemicals such as lipophilicity and molecular mass. Lipophilic compounds diffuse through the lipid core of the membrane (transcellular diffusion) whereas hydrophilic compounds pass through the water-filled junctions between adjacent cells (paracellular diffusion) (Artursson, Palm, & Luthman, 2001). The molecular mass of phytochemicals also affects passive absorption as large molecules are limited to diffusing through the membrane by their size (Lipinski, Lombardo, Dominy, & Feeney, 1997). Alternatively, phytochemicals can be transported across the intestinal epithelium by active carrier-

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mediated pathway, however the mechanisms and extent of transport via this pathway is largely unknown (Day, Gee, DuPont, Johnson, & Williamson, 2003; Kottra & Daniel, 2007).

A model to predict passive absorption of phytochemicals in humans was recently reported (Selby-Pham, Miller, Howell, Dunshea, & Bennett, 2017). This model, referred to as the phytochemical absorption prediction (PCAP) model, permits direct calculation of phytochemicals' T_{max} based on their lipophilicity descriptor log P and molecular mass. The PCAP model is applicable for phytochemicals that are passively absorbed in the small intestine and does not account for absorption pathways involving either chemical modification by brush border enzymes, nor fermentation by the gut microflora. Further, the PCAP model can be applied to individual or mixtures of phytochemicals as isolated compounds or as present in native matrices of vegetables and whole fruits (Selby-Pham, Miller, et al., 2017). The PCAP model is unique in its ability to account for the interaction of phytochemicals with macronutrients such as protein and fibre, and to predict phytochemical absorption kinetics following oral intake in humans.

The human colorectal adenocarcinoma cell line, Caco-2 has been widely used to study intestinal passive absorption of drugs (Artursson, 1990; Hubatsch, Ragnarsson, & Artursson, 2007) and phytochemicals (Boyer, Brown, & Liu, 2004; Liu, Glahn, & Liu, 2004). The apparent permeability (P_{app}) of compounds across the Caco-2 monolayer has been positively correlated with their bioavailability in human *in vivo,* as indicated by % absorption i.e., the fraction of compounds absorbed after oral administration in comparison to the intake dose (Artursson & Karlsson, 1991; Cheng, Li, & Uss, 2008). Additionally, P_{app} of several drug compounds and phenolic acids across the Caco-2 monolayer via the transcellular diffusion pathway were dependent on their lipophilicity descriptor log D and molecular mass (Farrell, Poquet, Dew, Barber, & Williamson, 2012). This relationship was successfully developed into a predictive model showing that the transcellular P_{app} of compounds decreased with increasing molecular mass and reducing lipophilicity. However, the relationship between *in vitro* P_{app} and *in vivo* T_{max} has not been previously investigated.

The aim of this study was to use the co-cultured monolayers of the Caco-2 cells and the mucus-producing human colorectal adenocarcinoma HT29-MTX-E12 cells as an improved model of the human small intestinal epithelium (Walter, Janich, Roessler, Hilfinger, & Amidon, 1996), to determine apparent permeability (P_{app}) of pure standards and phytochemical extracts across the co-cultured cell monolayer. Further, this study then investigated the relationship of respective measures of *in vitro* P_{app} and *in vivo* T_{max} predicted by the PCAP model.

2. Materials and methods

2.1. Cell culture and materials

Human colorectal adenocarcinoma Caco-2 (ATCC no. HTB-37) and HT29-MTX-E12 (ATCC no. HTB-38) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and Sigma-Aldrich (St Louise, MO, USA), respectively. Dulbecco's modified eagle's medium (DMEM) with high glucose (4500 mg/mL), heat-inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), Hank's balanced salt solutions (HBSS) and penicillin/streptomycin were from Gibco-Life Technology (Rockville, MD, USA). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (St Louise, MO, USA) and gradient grade methanol was from Merck (Darmstadt, Germany). Dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) and phenazine methosulfate (PMS) reagents were from Promega (Madision, WI, USA). Transwell® 24-well plates including permeable supports with polycarbonate membranes (0.33 cm^2 growth area) were purchased from Corning (Sydney, Australia) and 96-well plates were from Nunc (Roskilde, Denmark).

Caco-2 and HT29-MTX-E12 cells were cultured separately in DMEM supplemented with 10% FBS, 1% w/v penicillin/streptomycin and 1% w/v NEAA. All cells were grown at 37 °C/5% CO_2 in a humidified atmosphere. Cells grown in Transwell® at passage 5–20 were used for the experiment.

Standards for cell transport experiment covering a dynamic range of log P from −3.5 to 6.7 included ascorbic acid, gallic acid, thiamine HCl, curcumin (Sigma-Aldrich, St Louise, MO, USA) and bromophenol blue (Bio-Rad Laboratories, Richmond, CA, USA).

Two groups of plant extracts were used in this study. Group one, referred to as 'project extracts' included ten vegetable extracts made from fresh materials purchased from local retailers (Woolworths, Werribee, VIC, Australia): broccoli (*Brassica oleracea* var. *italica*), carrot (*Daucus carota* ssp. *sativus*), red cabbage (*Brassica oleracea* var. *italica*), red sweet potato (*Impomoea batatas*), rhubarb (*Rheum rhabarbarum*), squash (*Cucurbita pepo* var. *ovifera*), eggplant (*Solanum melongena*), white zucchini (*Cucurbita pepo* var. *melopepo*), kale (*Brassica oleracea* var. *acephala*) and Vietnamese coriander (*Persicaria odorata*). Vegetables were subjected to Stage 1 of a three-stage generic processing as described previously (Bennett & Muench, 2011). Group two, referred to as 'reference extracts' included seven commercial extracts: blueberry powder (Super Sprout, Campbellfield, Victoria, Australia), cacao powder (TRU-RA Cacao, Big Tree Farm, Ashland, OR, USA), grape seed and grape skin extracts (Grapex, Tarac Technologies, Nuriootpa, South Australia, Australia), green tea powder (Absolute Green, DeDu Pty Ltd., Ermington, NSW, Australia), olive leaf powder (Austral herbs, Uralla, NSW, Australia) and tomato powder (Herbies's Spices, Rozelle, NSW, Australia). Characterisation of the project and reference extracts was performed including total phenolics content and proximate analyses with results reported elsewhere (Selby-Pham, Howell, et al., submitted for publication).

2.2. Determination of standards and plant extracts toxicity

Prior to the cell monolayer transport experiments, cell viability in response to standards and plant extracts was analysed to ensure that the concentrations of standards and plant extracts to be used in the cell monolayer transport experiment were nontoxic for the cells. Cell viability was evaluated by MTS assay using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madision, WI, USA) according to manufacturer's directions. Briefly, $1x10^4$ Caco-2/HT29-MTX-E12 (ratio 9:1) cells per well were grown in a flat bottom 96 well plate for 7 days. On day 7, standards and plant extracts were prepared in 1% DMSO/HBSS at concentrations of 0.05–1 mg/mL and centrifuged at 15,000*g* for 5 min (Model 5417R, Eppendorf AG, Hamburg, Germany) to obtain the supernatants. Growth media was removed and replaced with the supernatants of standards and plant extracts prior to incubation at 37 °C/5% CO_2 for 60 min. The cells were then washed once in HBSS and assayed using 100 µL HBSS with 20 µL MTS/PMS reagents. Absorbance at 492 nm was monitored for 1–4 h of 37 °C/5% CO_2 incubation as an indicator of cell viability. Confluency of the cell monolayers was also monitored prior to, 24 h and 48 h following the transport experiments by measuring the transepithelial electrical resistance (TEER) using a Millicell-ERS Volt-ohm meter (Millipore, Bedford, MA, USA). Concentrations of standards and plant extracts resulting in cell viability > 90% were chosen for the cell monolayer transport experiment. Accordingly, for the cell monolayer transport experiment, the chosen concentrations of standards were 0.09 mg/mL ascorbic acid, 0.09 mg/mL gallic acid, 0.13 mg/mL thiamine hydrochloride, 0.07 mg/mL curcumin and 0.07 mg/mL bromophenol blue. The chosen concentrations of project extracts and reference extracts were 1 mg/mL, except for grape seed extract at 0.5 mg/mL. All standards and extracts were prepared in 1% DMSO/HBSS and centrifuged at 15,000*g* for 5 min to obtain the supernatants to be used in the cell monolayer experiment.

2.3. Cell monolayer transport of standards and plant extracts

Prior to seeding, Caco-2 and HT29-MTX-E12 cells were mixed to