Journal of Ethnopharmacology 210 (2018) 254–259

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Artemisinin permeability via Caco-2 cells increases after simulated digestion of *Artemisia annua* leaves

Matthew R. Desrosiers, Pamela J. Weathers*

Department of Biology and Biotechnology, Worcester Polytechnic Institute, 100 Institute Rd., Worcester, MA 01609, USA

ARTICLE INFO

Keywords:
Antiprotozoal
Drug transport
Essential oils
Flavonoids
Malaria
Terpenes

ABSTRACT

Ethnopharmacological relevance: *Artemisia annua* has been used for > 2000 yrs to treat fever and is more recently known for producing the important antimalarial drug, artemisinin.

Aim of the study: Artemisinin combination therapies (ACTs) are effective for treating malaria, but are often unavailable to those in need. Dried leaves of *A. annua* (DLA) have recently been studied as a cost effective alternative to traditional ACTs. DLA was shown to dramatically increase oral bioavailability compared to pure artemisinin, so more investigation into the mechanisms causing this increased bioavailability is needed.

Materials and methods: In this study, we used a simulated digestion system coupled with Caco-2 cell permeability assays to investigate the intestinal permeability of DLA compared to pure artemisinin. We also determined the effects of different phytochemicals (7 flavonoids, 3 monoterpenes, 2 phenolic acids, scopoletin and inulin) and the cytochrome P450 isoform CYP3A4 on artemisinin intestinal permeability.

Results: Artemisinin permeability, when delivered as digested DLA, significantly increased by 37% (P_{app} = 8.03 × 10^{-5} cm s^{-1}) compared to pure artemisinin (P_{app} = 5.03 × 10^{-5} cm s^{-1}). However, none of the phytochemicals tested or CYP3A4 had any significant effect on the intestinal permeability of artemisinin. We also showed that essential oil derived from *A. annua* negatively affected the intestinal permeability of artemisinin, but only after simulated digestion. Finally, we showed that *A. annua* essential oil reduced the transepithelial electrical resistance of Caco-2 monolayers, but only in the presence of bile. Although also reduced by essential oils, artemisinin P_{app} subsequently recovered in the presence of plant matrix.

Conclusions: These results shed light on the mechanisms by which DLA enhances the oral bioavailability of artemisinin.

1. Introduction

Malaria, a disease caused by parasites of the *Plasmodium* genus, remains a major global health problem across the developing world. There are over 3 billion people at risk of contracting malaria, about half the world's population, and each year there are over 400,000 deaths due to the disease (WHO, 2016). Although the vast majority of malaria infections can be treated effectively with artemisinin combination therapies (ACTs), there remains a large population, mostly in rural Africa, that does not have access or the financial resources to receive treatment. As a result, in 2015 about 90% of deaths due to malaria occurred in Sub-Saharan Africa. Of these deaths, about 70% were children under the age of 5 (WHO, 2016).

Artemisia annua L., the plant that naturally produces artemisinin (AN) in its glandular trichomes, has been used traditionally in China to treat malaria dating back as far as the second century BCE (Hsu, 2006). Recently, consumption of the dried leaves of *A. annua* (DLA) has been studied as a potential low-cost treatment option for people living in rural malaria endemic regions. In mouse studies, DLA was shown to be 5 times more effective than pure artemisinin at clearing *Plasmodium* parasites from the blood (Elfawal et al., 2012) and three times better at slowing the development of resistant parasites (Elfawal et al., 2015). In a small human trial in Kenya, patients treated twice daily for 6 days with tablets made from DLA achieved > 90% parasite clearance at 28 days with < 10% recrudescence, a result comparable with many ACTs (ICIPE, 2005). More recently, DLA successfully treated 18 patients who had ACT and i.v. artesunate resistant malaria (Daddy et al., 2017). Furthermore, when oral delivery of DLA was compared to oral delivery of pure artemisinin in mice, there was about 45 times more artemisinin found in the serum of mice given DLA (Weathers et al., 2011). The

Abbreviations: AA, artemisinic acid; AB, arteannuin B; ACT, artemisinin combination therapy; AN, artemisinin; DLA, dry leaf Artemisia; DLAS, DLA *A. annua* cultivar with ~1.4% artemisinin; DLAG, DLA glandless; GLS, glandless *A. annua* cultivar that contains no artemisinin; TEER, trans-epithelial electrical resistance; VD3, 1α,25-dihydroxyvitamin D3

* Corresponding author.
E-mail address: weathers@wpi.edu (P.J. Weathers).

http://dx.doi.org/10.1016/j.jep.2017.08.038
Received 8 May 2017; Received in revised form 27 July 2017; Accepted 28 August 2017
Available online 31 August 2017

mechanism causing this striking increase in drug bioavailability is yet to be fully determined. Our group recently showed through simulated digestion experiments that digestion of DLA results in about 4 times higher solubility of artemisinin in the resulting digestate and this was largely from essential oils found in the plant material (Desrosiers and Weathers, 2016). Artemisinin has very low aqueous solubility, so its increased solubility from DLA partially explains the 45-fold increase in serum concentration, however, there are likely other mechanisms in play.

One potential mechanism for the increased artemisinin bioavailability afforded by DLA is modulation of the intestinal permeability of artemisinin. Several phytochemicals found in *A. annua* have either increased the rate of transport of other drugs or inhibited key enzymes that mediate the first-pass metabolism of artemisinin. For example, De Magalhães et al. (2012) showed that tea infusions made from various *A. annua* cultivars inhibited CYP3A4, an enzyme present in the intestine involved in the metabolism of artemisinin (Svensson and Ashton, 1999). Quercetin, a flavonoid found in *A. annua*, also increased the absorption of doxorubiclin in rats (Choi et al., 2011), while tamarixetin, another flavonoid, increased absorption of fluvastatin in rats (Wang et al., 2014). Further, other studies showed that flavonoids found in *A. annua*, such as quercetin and rutin, inhibited CYP3A4 as well as other cytochrome P450 enzymes that mediate the metabolism of artemisinin (Wang et al., 2014).

In this study, we used the Caco-2 cell model of the intestinal epithelium to measure the intestinal permeability of artemisinin when delivered as pure drug or as DLA simulated digestate. This system has been used previously to show that artemisinin crosses the intestinal wall via a passive diffusion mechanism (Augustijns et al. 1996) however, no studies have been conducted using whole plant extracts or digestates on Caco-2 cell monolayers. Here we show that delivery as digested DLA increased the rate of artemisinin transport across the intestinal epithelium. We also tested a wide variety of phytochemicals found in *A. annua* for their effects on artemisinin permeability and tested the role of CYP3A4 in this process by upregulating its expression and activity in Caco-2 cells.

2. Materials and methods

2.1. Plant material

We used two *Artemisia annua* L. cultivars in these studies. The first, SAM (DLAS) (voucher MASS 317314), is a high artemisinin and flavonoid producing cultivar, about 1.4% and 0.3% (w/w) respectively, and was propagated clonally by rooted cuttings. DLAS was field grown in Stow, MA and harvested at the floral budding stage, dried and processed as detailed in Weathers et al. (2014). The second cultivar used, GLS (DLAG) (vouchers OR State Univ 171772 and 170353), is a mutant cultivar lacking glandular trichomes, producing no detectable artemisinin, with 25% of the flavonoids of DLAS, and negligible levels of essential oils as measured by our lab (Table S1) and others (Tellez et al., 1999). DLAG was grown in the lab under glass-filtered sunlight, harvested during the vegetative stage, dried and processed as DLAS.

2.2. Chemicals and reagents

All chemicals and reagents used were at least research grade from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. *A. annua* essential oils were purchased from Bella Mira (Mannford, OK, USA) or Jiangxi Jinyuan Natural Perfume Company (Ji'an, Jiangxi, China). Rutin, eupatorin, casticin, and isovitexin were purchased from ChromaDex (Irvine, CA, USA).

2.3. Caco-2 cell culture

The Caco-2 cell line was purchased from the American Type Culture Collection (ATCC: HTB-37) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) containing 4.5 g/L D-glucose, 110 mg/L sodium pyruvate, 20% fetal bovine serum (FBS) (Rocky Mountain Biologicals, Missoula, MT, USA), 1X GlutaMAX (Life Technologies, Carlsbad, CA, USA), and 1X penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) in a humidified incubator at 37 °C and 5% CO_2. Cells were harvested using TrypLE (Life Technologies, Carlsbad, CA, USA), resuspended in culture medium, and seeded at a density of 2.6×10^5 cells/cm^2 on 12 well polyethylene terephthalate transwell ThinCerts hanging well inserts (0.4 μm pore size, 1.13 cm^2 culture area) (Greiner Bio-One, Kremsmünster, Austria). Culture medium was changed every other day for 21–28 days and 24 h before performing permeability experiments. All cells used for permeability experiments were between passages 32 and 52.

2.4. Transepithelial electrical resistance and lucifer yellow assays

To ensure monolayer integrity throughout the experimental period, transepithelial electrical resistance (TEER) was measured before and after experiments using the EVOM2 epithelial voltmeter (World Precision Instruments, Sarasota, FL, USA). TEER values vary throughout the literature, so we set a TEER cutoff based on Lucifer yellow rejection. Lucifer yellow is a fluorescent dye that is only transported paracellularly and is thus used as a marker of Caco-2 tight junction integrity. To determine Lucifer yellow rejection rate, we performed a Lucifer yellow permeability assay using Caco-2 cells cultured in hanging wells. First, the TEER of Caco-2 monolayers was recorded and then 0.5 mL 100 μM Lucifer yellow in Hank's balanced salt solution (HBSS) was added to the apical side of the hanging wells. Hanging wells were then inserted into 12 well plates prefilled with 1.5 mL HBSS in each well and then stirred on a nutator (TCS Scientific, New Hope, PA, USA) at 24 RPM in a humidified, 37 °C incubator for 1 h. Afterwards samples were taken from the apical and basolateral sides of the hanging well and read on a fluorescent plate reader. The Lucifer yellow rejection value was calculated using the equation: LY % rejection = $100*(1\text{-}RFU_{basolateral}/RFU_{apical})$ where RFU is the relative fluorescent units recorded by the plate reader. It was determined that TEER values below 290 Ω*cm^2 had Lucifer yellow rejection values below 95% and as a result, wells with a TEER value below 290 Ω*cm^2 were not used for permeability assays.

2.5. Simulated digestion

Simulated digestion was performed using the method described in Weathers et al. (2014). Either 0.36 g DLA or 2 mg pure artemisinin was digested in a 50 mL conical tube. Digestions were run through oral, gastric, and intestinal stages of digestion and then filtered through Whatman #1 chromatography paper (0.16 mm thickness, porosity < 10 μm) to separate liquid and solid fractions. Only the liquid fraction of the digestate was used for permeability experiments.

2.6. CYP3A4 upregulation

Under normal culture conditions, the human cytochrome P450 isoform CYP3A4 is not expressed in Caco-2 cells. To better mimic the *in vivo* conditions, CYP3A4 expression was induced by adding 0.5 μM 1α,25-dihydroxyvitamin D_3 (VD3) to the culture media as described by Schmiedlin-Ren et al. (2001). We performed RNA isolation and qPCR for CYP3A4 on cells cultured in this VD3 media to confirm the upregulation of CYP3A4 transcription. RNA isolation was performed using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA), cDNA was prepared from total RNA according to the manufacturer's instructions. Using PerfeCta SYBR Green FastMix, low ROX (Quanta Biosciences, Beverly, MA, USA), qPCR was performed according to the manufacturer's instructions with an Applied Biosystems 7500 Real-Time PCR System