Combination Effects of Quercetin, Resveratrol and Curcumin on In Vitro Intestinal Absorption

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Abstract

Objective: Quercetin, resveratrol and curcumin are plant derived natural products that are rapidly gaining popularity as supplements for a wide assortment of conditions including cardiovascular disease, cancer, asthma, diabetes, neurodegeneration, aging and stress. Unfortunately the therapeutic potential of these compounds is limited by their poor intestinal and intracellular bioavailability. Therefore this study sought to examine how combinations of quercetin, resveratrol, and curcumin, with and without piperine, 200 nM, affected an in vitro permeability model using apical-to-basal permeability across intact caco-2 monolayers. Quercetin, resveratrol and curcumin were applied apically alone or in combination at 50 ?M and measured in the basal chamber at 30 min.

Results: Resveratrol received the greatest enhancement in permeability when combined with other agents: quercetin (310%), curcumin (300%), quercetin and curcumin (323%, 350% with piperine). Curcumin also demonstrated increased permeability when combined with quercetin alone (147%) and both quercetin and resveratrol (188%), addition of piperine resulted in a 229% increase in permeability. Quercetin permeability was not significantly affected using any combination, but showed maximal permeability when combined with resveratrol and the lowest permeability when combined with resveratrol, curcumin and piperine together.

Conclusion: Combination of quercetin, resveratrol and curcumin may improve intestinal absorption of resveratrol and curcumin without affecting quercetin absorption. These data highlight the need for further research and suggest that developing combination therapies may improve intestinal absorption of these constituents. Our study also demonstrates that the apical-to-basal permeability across intact caco-2 monolayer model is a viable model to investigate absorption of natural compounds.

INTRODUCTION

Quercetin, resveratrol and curcumin are increasingly being researched due to their apparent therapeutic activity in numerous disease states. Quercetin, a ubiquitous flavonol found in most land-plants including many foods and dietary supplements, has demonstrated beneficial effects in inflammatory and immune conditions including cardiovascular disease, cancer, asthma/allergies and stress. Resveratrol, a naturally produced stilbenoid found in Japanese knotweed (Fallopia japonica) and more famously in the skin of red grapes (Vitis vinifera), has displayed antioxidant, anti-inflammatory and anticarcinogenic effects and may be beneficial in preventing complications associated with diabetes, atherosclerosis, aging and metabolic disease. Resveratrol appears to
work through the activation of SIRT1 (silent mating-type information regulation 2 homolog 1) or AMPK (5'-adenosine monophosphate-activated kinase) pathways. Finally, curcumin, a natural phenolic compound (diarylheptanoid) found in turmeric (Curcuma longa), is a well-studied inhibitor of the inhibitory kappa B alpha kinase (I?B? kinase), a key activator of nuclear-factor ?B (NF-?B). Blocking this central mediator makes curcumin therapeutically active in various inflammatory disease states including arthritis, cardiovascular disease, metabolic disease, neurodegeneration and tumorigenesis.

Unfortunately, despite the impressive therapeutic potential of these compounds, they have also demonstrated poor bioavailability when given orally. This feature limits their use as supplements because large doses are required to achieve therapeutic intracellular levels. Curcumin bioavailability has been improved by delivering it in liposomal preparations or by coadministering it with piperine. Piperine is a piperidine alkaloid found in the fruit of the black pepper (Piper nigrum), and is responsible for the pungency of black pepper spice. Piperine’s effect on curcumin bioavailability is believed to be due to its inhibition of phase II glucuronidation; piperine may also improve intestinal epithelial uptake by inhibiting P-glycoprotein-1 (P-gp; a broad-acting xenobiotic efflux transporter). Interestingly, there is also evidence to suggest quercetin is also a P-gp inhibitor for some compounds; in fact, many herbs/constituents have demonstrated modulation of this efflux pump.

Quercetin, resveratrol and curcumin, despite their various botanical origins, all share similar biosynthesis pathways originating with 4-hydroxycinnamic acid of the shikimate pathway – a major synthesis pathway utilized by plants to synthesize aromatic/phenolic secondary compounds and amino acids. The molecular topography of these three compounds also display similar features which suggests they may share targets for absorption or efflux and may alter each other’s uptake (Figure 1). Therefore, we hypothesized that concomitant exposure of all three compounds will increase the apical-to-basal absorption of these constituents.

Figure 1: Molecular structures of resveratrol, quercetin and curcumin demonstrating the relative similarities of placement of hydroxyl groups along unsaturated carbon chains and the presence of several phenolic groups.
MATERIALS AND METHODS

REAGENTS:

Cell culture reagents: Eagle’s minimum essential media (EMEM), Hank’s balanced salt solution (HBSS), Pen-strep and trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25%) were purchased from Mediatech (Corning, VA, USA); fetal bovine serum (FBS) was purchased from Atlanta Biologicals (GA, USA). High performance liquid chromatography (HPLC) reagents: acetic acid, sodium acetate, methanol and acetonitrile were purchased from Sigma-Aldrich (MO, USA). Samples of quercetin, resveratrol and curcumin and a blend of curcumin and piperine (PC) were gifts from Gaia Herbs (NC, USA). Additional standards of quercetin, resveratrol, curcumin and piperine were purchased from Sigma-Aldrich.

Stocks of quercetin, resveratrol, curcumin and piperine were prepared in methanol at 1 mg/mL. Stocks were stored at ?20°C and monitored regularly for stability. The PC blend was prepared as a 1 mg/mL stock solution and assayed to contain 148 ?M curcumin and 0.6 ?M piperine.

CELL CULTURE:

Caco-2 cells obtained from the American Type Culture Collection (ATCC, Virginia, USA) were cultured in EMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained in 75-cm² tissue culture flasks and grown in a 5% CO₂ incubator at 37°C and saturating humidity with media changes every 2–3 days. Cells were passaged by rinsing with PBS and detachment with 0.25% trypsin and 0.5 mM EDTA.

IN VITRO ABSORPTION:

For in vitro absorption studies cells were harvested and seeded in 24-well plates fitted with polycarbonate Transwell® inserts (6.5 mm i.d., 0.4-m pore size, 0.3 cm² of growth area, Corning Costar Co., NY) at a density of 75,000 cells/insert. Cells in the inserts were grown for 21 days and the transepithelial electrical resistance (TEER) measured regularly to monitor the monolayer integrity. Only wells that had a TEER value greater than 400 ??(cm²) were used for absorption experiments (TEER = (R_{monolayer} ? R_{blank}) × Area).

Inserts with intact monolayers were rinsed with HBSS and transferred to a clean 24-well plate and allowed to equilibrate in HBSS for 1 h. After equilibration TEER was verified and HBSS on the apical side was replaced with HBSS containing the treatments alone or in combination (quercetin, resveratrol and curcumin at 50 ?M and piperine at 200 nM). After 30 min 700 ?L of HBSS was removed from the basal compartment and immediately combined with an equal volume of 10 mM acetic acid in an HPLC sample vial. Samples were then immediately analyzed by HPLC.

HPLC:

Quantitation of quercetin, resveratrol and curcumin was accomplished using an Alliance HPLC system with 2998 photodiode array (Waters Corporation, MA, USA) equipped with an Agilent Eclipse XDB-C18 column (4.6 × 50 mm, 3.5 ?m) at 35°C. Mobile phase consisted of A: 0.1 M acetate buffer (pH 4.0) and B: acetonitrile. Flow rate was 0.6 mL/min and gradient conditions were 0 min, 75% A; 8 min, 55% A; 18 min, 55% A followed
by 5 min of equilibration at 75% A. Detection was done using the PDA at 306 nm for resveratrol, 364 nm for quercetin and 428 nm for curcumin.

**DATA ANALYSIS:**

The apparent permeability coefficient ($P_{app}$, cm/s) for each compound/combination was calculated using the following equation:

\[
\frac{dC_b}{dT} = \frac{J}{A}
\]

where $(dC_b/dT)$ is the rate of appearance of the constituent on the basal side (nmol/s), $C_a$ is the concentration of the constituent on the apical side (50 ?M) and $A$ is the area of the monolayer (0.3 cm$^2$). The apparent permeability coefficient is property of individual compounds which describes its ability to pass through a cell monolayer under the experimental conditions.

Each treatment or treatment combination was repeated three times in separate wells, using new caco-2 monolayers for each experiment. $P_{app}$ values ($n = 3$) between constituent alone and their combinations were compared using the Student’s $t$-test (two-tailed, unpaired), statistical significance was assumed at a level of $? = 0.05$.

**RESULTS**

HPLC ANALYSIS: Quercetin, resveratrol and total curcumanoids were well resolved using this method and quantitation was linear in the range tested (0.15–110 ?M) (**Figure 2**). Quercetin had a retention time of approximately 4.7 min, resveratrol 3.6 min and curcumin displayed the typical three curcuminoids: bisdemethoxycurcumin (11.4 min), demethoxycurcumin (12.1 min) and curcumin (12.9 min) (**Figure 2**). Only curcumin was detected in the basal chamber in the absorption experiments. Piperine, when included, co-eluted with demethoxycurcumin at 12 min and was detected in the basal chamber, but was not quantitated.
Figure 2: Representative chromatographs and standard curves of quercetin (364 nm), resveratrol (306 nm) and curcumin (428 nm). Seven point standard curves were calculated based on the average peak area ± SD (n = 3) at each concentration (0.15–110 ?M); curcuminoid standard curve was calculated from the total peak area of the three major curcuminoids.

Caco-2 monolayer permeability:

Each of the three compounds tested showed consistent transport across caco-2 monolayers. When applied singly, quercetin showed the highest apparent permeability ($P_{\text{app}} \times 10^{7.6}$ cm/s) (10.09 ± 0.77) followed by resveratrol (7.77 ± 1.10) and then curcumin (0.99 ± 0.11) (Figure 3). In combination, both resveratrol and curcumin achieved significantly higher permeability. Quercetin did not receive any significant benefit from combination, but showed the highest permeability when paired with resveratrol (11.90 ± 0.52) and the lowest when combined with resveratrol, curcumin and piperine (8.89 ± 0.40; Figure 3).
Figure 3: Apical-to-basal permeability of (A) quercetin (Q), (B) resveratrol (R) or (C) curcumin (C), alone or in combination across intact caco-2 monolayers. Compounds were applied apically at 50 ?M (piperine (P) at 200 nM) and measured in the basal compartment at 30 min. Data is expressed as the average $P_{app} \times 10^{26}$ (cm/s) of three separate experiments ± SD. Bars labeled with different letters are significantly different from each other, $P <0.05$.

Resveratrol, when combined with quercetin and curcumin, showed a significant increase in $P_{app}$ (7.70 ± 1.10 to 25.09 ± 0.48), inclusion of piperine resulted in a further increase to 27.22 ± 0.89. In order to determine if either quercetin or curcumin were responsible for this effect, they were applied singly with resveratrol; however both caused a similar increase in $P_{aap}$ (24.92 ± 0.68 and 24.45 ± 0.69, respectively) (Figure 3). Curcumin demonstrated a similar pattern to resveratrol when applied in combination, though the magnitude was not as great. When paired with quercetin or resveratrol alone, $P_{app}$ increased moderately but not significantly (1.46 ± 0.15 and 1.25 ± 0.07, respectively). When all three were combined there was a significant increase in $P_{app}$ (1.85 ± 0.13), the addition of piperine increased the $P_{app}$ to 2.26 ± 0.21. Interestingly, the greatest effect was observed with curcumin and piperine alone which resulted in a $P_{app}$ of 2.33 ± 0.12 (Figure 3). Together this suggests an additive effect of resveratrol and quercetin on curcumin absorption and that quercetin and resveratrol absorption are also affected by piperine and may compete with curcumin in these pathways.

**DISCUSSION**

The transepithelial permeability across intact monolayers using caco-2 cells is a well validated and accepted method to model intestinal absorption. It is commonly employed as a preliminary screen to determine transport barriers in the development of pharmaceuticals and recently has been employed to investigate the absorption of natural compounds. The current study demonstrates that this model may also provide a cost-effective method to screen absorption characteristics of naturally derived compounds singly or in combination therapies and to provide preliminary data to support *in vivo* studies.

The main limiting factors of this experiment were 1) poor aqueous solubility of the test substances, and 2) poor aqueous stability of quercetin at physiological pH. In order to prevent precipitation of the test substances alone or in combination, a concentration of 50 ?M was chosen. This concentration was stable over the time-frame of the experiment, including analysis time (data not shown). In order to overcome the instability of quercetin in aqueous solutions at pH of 7.4, the treatments were prepared immediately before the experiment and the exposures were limited to 30 min. After 30 min the samples were immediately acidified which stabilized the quercetin for analysis (Figure 4). An aqueous concentration (50 ?M) was chosen in favor of higher concentrations in non-aqueous solvents (DMSO, dimethylformamide, etc.) in order to better approximate oral ingestion of these supplements (i.e. capsule taken with water).
Figure 4: Stabilization of quercetin using acetic acid. Quercetin (25 μM) is rapidly degraded in aqueous solutions at pH 7.4 (green line). In order to preserve quercetin for analysis, samples were acidified using 10 mM acetic acid (final concentration 5 mM, red line). Data is expressed as the ratio of the quercetin peak area at the indicated times over the initial area ($t_n/t_0$) ± SD; $n = 3$.

This study demonstrates that applying quercetin, resveratrol and curcumin in combination can significantly increase apical-to-basal uptake of curcumin and resveratrol. Although quercetin absorption did not significantly increase from these combinations, it did not decrease either – no significant change from quercetin alone was observed when combined with the other compounds. It is worth mentioning, however, that quercetin showed the lowest absorption in the PQRC combination suggesting piperine may adversely affect quercetin permeability. In contrast, adding piperine appears to have a positive effect on apical-to-basal permeability, with resveratrol and curcumin. This latter observation is consistent with a previous study that showed improved bioavailability of curcumin when applied with piperine. Resveratrol absorption has been shown to be primarily passive, but suffers from extensive sulfate and glucuronidate conjugation reactions. The increases in resveratrol permeability observed in this study may be due to inhibition of these conjugation reactions and blockade of resveratrol apical export. Regardless of the combination, resveratrol demonstrated a significant increase in permeability and there were no differences observed between combinations; this may suggest that there is overlap in the mechanisms and at the concentrations used the effect was saturated.

Quercetin is known to affect the pharmacokinetics of various compounds by modulating transport and metabolism, some of the observed effects include: inhibition of CYP3A4 (IC$_{50}$ 2 μM), inhibition of phenol sulfotransferase (SULT 1A1) (IC$_{50}$ 13 nM) and inhibition the P-gp efflux pump. These activities could explain increased permeability of curcumin and resveratrol in the presence of quercetin.

Curcumin has also demonstrated inhibition of P-gp and CYP3A4; however, resveratrol does not appear to be
exported by P-gp, rather it is exported by the breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2). Recent evidence also suggests that curcumin inhibits BCRP, which may explain the improved permeability of resveratrol observed with curcumin in the current study.

Similarly, piperine has demonstrated various activities which may contribute to its effect on permeability. Like quercetin and curcumin, piperine inhibits CYP3A4 and P-gp. Piperine also inhibits glucuronidation and may affect MDR1 and BCRP. These activities have been attributed to its effect on curcumin permeability, but this data shows that it may also contribute to absorption of resveratrol since there was a modest rise in $P_{aap}$ when piperine was included.

As mentioned previously, these compounds are products of the same biosynthetic pathway (shikimate) and share several structural elements (Figure 1). It is not surprising therefore that the literature suggests that these compounds utilize and modulate similar pathways for membrane transport and metabolism. Collectively they may cause a substantial effect on P-gp, BCRP, CYP3A4 and other enzymes of xenobiotic metabolism. It is likely that the concerted inhibition, competition or modulation of membrane transporter and biotransformation enzymes of quercetin, resveratrol, curcumin and piperine results in the improved acute permeability of resveratrol and curcumin observed in the current study.

**CONCLUSIONS**

Quercetin, resveratrol and curcumin may provide substantial therapeutic benefit especially in aging and chronic inflammatory diseases. This underscores the importance of investigating the bioavailability and efficacy of these compounds in order to maximize their therapeutic potential. These data suggest that delivering these compounds in combination may improve the acute bioavailability of curcumin and resveratrol compared to supplementation with single compounds, allowing for lower overall doses and simpler treatment protocols using combination therapies.

The current study was able to show improved permeability of these compounds when applied in combination; however, future studies are needed to investigate the specific mechanisms including the use of inhibitors for export pathways such as P-gp, MRP2 and BCRP. Additionally, long-term or consistent exposure to these combinations is likely to affect absorption considering that previous studies have demonstrated changes in expression of metabolic and transport enzymes with longer exposures (i.e. UGT1A1, CYP3A4, P-gp). Further studies on the long-term modulation of biotransformation pathways of these combinations are warranted as well as *in vivo* studies using combination therapies.

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**DISCLOSURE OF INTERESTS**
The authors disclose no conflict of interest in this study and are in no way affiliated with Gaia Herbs.

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