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Resveratrol in combination with other dietary polyphenols concomitantly enhances antiproliferation and UGT1A1 induction in Caco-2 cells

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Abstract

Aims—The only FDA approved medication for colorectal cancer (CRC) prevention is celecoxib. Its adverse effects underline the need for safer drugs. Polyphenols like resveratrol are in clinical trials for this purpose. This study aimed at examining effects of resveratrol alone and in combination with curcumin or chrysin on UGT induction in Caco-2 cells. Phytochemical combinations were selected using drug combination analyses of various anti-proliferation ratios of resveratrol + curcumin and resveratrol + chrysin.

Main methods—Cell proliferation and UGT1A1 induction assays were carried out with individual polyphenols and combinations. Cell viability was determined with AlamarBlue assays. UGT1A1 mRNA was quantified via real time RT-PCR. UGT activity was determined with 4-methylumbelliferone (4MU) glucuronidation.

Key findings—Cell proliferation IC₅₀ estimates ($\pm$ SE) for resveratrol, curcumin and chrysin were 20.8 ± 1.2 , 20.1 ± 1.1 and 16.3 ± 1.3 μ M respectively. Combination anti-proliferative effects showed additivity for resveratrol + chrysin and resveratrol + curcumin. Resveratrol at its IC₅₀ mediated a four-fold induction of UGT1A1 mRNA in a concentration independent manner. Chrysin at its IC₅₀ induced UGT1A1 expression seven-fold while Curcumin at its IC₉₀ mediated a two-fold induction. The 20 μ M:40 μ M resveratrol + curcumin and 20 μ M:32 μ M resveratrol + chrysin combinations mediated the greatest increases in mRNA expression (12 and 22 fold respectively). Significant increase in 4-MU glucuronidation was observed with combinations exhibiting maximal mRNA induction.

Significance—Phytochemical combinations can offer greater chemoprevention than single agents. These chemicals might offer safer options than present synthetic therapeutics for CRC prevention.

Keywords

UGT; glucuronidation; resveratrol; phytochemical combinations; colorectal cancer

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Introduction

Celecoxib is the only FDA approved drug for CRC prevention. Its adverse effects underline the need for safer alternatives. The dietary polyphenol *trans*-3,5,4'-trihydroxystilbene (resveratrol; RES) is in clinical trials for CRC [Identifiers NCT00433576, NCT00256334 at ClinicalTrials.gov]. RES efficacy against all stages of carcinogenesis has been shown *in vitro* and *in vivo*. Various mechanisms are suggested to explain its biological effects (Goswami and Das 2009, Kundu and Surh 2008). One such mechanism, induction of 'phase II' enzymes, is expected to increase detoxification of dietary carcinogens, thus decreasing cancer risk. Although modulation (by induction) of phase II enzymes such as glutathione S-transferases and uridine diphosphoglucuronosyl transferases (UGTs) by RES has been reported in the rat liver (Hebbar, et al. 2005), tissue-specific UGT expression, specific UGT isozyme modulation, and mechanisms for UGT transcriptional modulation by phytochemicals are not well understood. Glucuronidation is a predominant pathway for metabolism of RES and other phytochemicals. Low bioavailability of these dietary polyphenols has been attributed to extensive conjugation (Walle, et al. 2007). Glucuronidation is also a major pathway for inactivation of numerous carcinogens. UGT induction may thus cause decreased phytochemical availability but increased carcinogen detoxification.

Some reports on UGT induction by RES have focused on the liver (in rodents) (Hebbar, et al. 2005, Szaefer, et al. 2004) and hepatic cell lines (Lancon, et al. 2007). Phytochemicals can accumulate in the small intestine and colon at levels greater than in plasma (Sale, et al. 2005, Lopez-Lazaro 2008, Howells, et al. 2007, Patel, et al. 2010). RES is a substrate for intestinal UGT isoforms such as UGT1A1 (with minor contributions by UGT1A7, 1A8 and 1A10). It therefore becomes important to study intestinal UGT induction by RES, especially given the known differences in tissue-specific UGT regulation.

The effects of combinations of phytochemicals on cell proliferation and other markers of chemoprevention and carcinogenesis have been reported (Aftab and Vieira 2010, Majumdar, et al. 2009, Patel and Majumdar 2009, Svehlikova, et al. 2004). UGT induction by phytochemical combinations has not been studied. Potentiating effects on multiple mechanisms such as cell growth and enzyme induction might be expected upon combining chemicals known to act *via* overlapping or unique pathways. Gaining knowledge of additive versus synergistic effect requires rationally designed experiments based on pharmacologic effects of single agents instead of empirical combinations.

This study presents effects of RES alone and combined with select phytochemicals on two distinct but interrelated stages of carcinogenesis in the human intestinal adenocarcinoma cell line (Caco-2) - inhibition of cancer cell proliferation (progression) and extrahepatic UGT induction (initiation). Caco-2 cells were used because as human intestinal cancer cells, they are a standard cellular model for CRC. Caco-2 cells are also known to express human UGTs, the proteins evaluated in this study. Curcumin and chrysin were combined with RES because: i) these polyphenols belong to three unique chemical classes, ii) each phytochemical exhibited strong chemopreventive potential in CRC models (Johnson and Mukhtar 2007, Miyamoto, et al. 2006, Wolter, et al. 2004), iii) each phytochemical is a known UGT substrate (Hoehle, et al. 2007, Aumont, et al. 2001, Galijatovic, et al. 1999) and iv) each phytochemical alone is known to modulate UGT transcription *via* unique and/or overlapping pathways with conflicting reports on modulation by either induction or inhibition (Table 1).

Materials and Methods

Chemical and Reagents

RES (trans-RES, purity > 99%) and Curcumin were purchased from Cayman Chemical Company (Ann Arbor, MI). Chrysin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO), lidocaine was purchased from Spectrum Chemicals (New Brunswick, NJ), *tert*-butyl hydroquinone was purchased from TCI Americas (Portland, OR), 4-methylumbelliferone (4-MU) sodium salt was from MP Biomedicals (Solon, OH), 4-methylumbelliferone-O- β -D-glucuronide (4MUG) was from EMD Biosciences (La Jolla, CA). Complete, Mini, EDTA-free protease inhibitor cocktail Tablets were from Roche Applied Science (Indianapolis, IN). Caco-2 cells were purchased from the American Tissue Culture Collection (Manassas, VA), Cell culture reagents Eagle's Minimum Essential Medium (EMEM), penicillin/streptomycin and 1X Trypsin EDTA solution were obtained from Mediatech (Manassas, VA), and fetal bovine serum was obtained from Thermo Scientific (Pittsburgh, PA). Primers and Probes for gene expression assays as well as the TaqMan® GAPDH control reagents (human) were purchased from Applied Biosystems (Foster City, CA). All other chemicals and reagents were of analytical grade.

Cell Culture and Cell Proliferation Assays

Caco-2 cells were cultured in EMEM containing 10% fetal bovine serum albumin and 5% penicillin/streptomycin. Cells were incubated in 10 cm plates in a humidified atmosphere at 37°C with 5% CO₂ and allowed to grow to 80% confluency before passaging.

For cell proliferation assays, a cell viability assay based on the alamarBlue assay (Invitrogen) was used. All compounds were dissolved in DMSO, with 0.1% v/v final DMSO concentration. Caco-2 cells were seeded in 96 well plates at a density of 3×10^3 cells/cm² and allowed to attach overnight. The next day, the media was replaced with fresh medium containing varying concentrations of test compounds (1 – 100 μ M). For proliferation assays using polyphenol combinations, IC₅₀s for the individual polyphenols were initially obtained using 1 – 100 μ M concentrations. Combinations of polyphenol IC₅₀ ratios (1:1, 1:2 and 2:1 i.e. 20 μ M + 16 μ M, 20 μ M + 32 μ M and 40 μ M + 16 μ M [RES + chrysin] or 20 μ M + 20 μ M, 20 μ M + 40 μ M and 40 μ M + 20 μ M [RES + Curc]) were used to treat the cells for a total of 72 h. Preliminary studies had checked cell viability over this time period. For Curc, proliferation IC₉₀ ratios were additionally tested. AlamarBlue® reagent was added to the wells 12 h prior to reading the 72-h fluorescence level. All emitted fluorescence from viable cells was read on a SpectraMax M2 spectrophotometer with SoftMax Pro software (Molecular Devices, Sunnyvale, CA). Calculations for IC₅₀ and IC₉₀ estimates were conducted with GraphPad Prism for Windows (version 4.03; GraphPad Software Inc., San Diego, CA). All experiments were conducted in 4 replicates.

UGT Induction Studies and Protein Extract Preparation

Nine-day post-confluent Caco-2 cells were utilized for UGT induction experiments as differentiated Caco-2 cells are known to exhibit high expression of UGTs (Siissalo, et al. 2008). Caco-2 cells were seeded at a density of 8×10^4 cells/cm². Cells were cultured for 9 days with media changes every other day. Cells were then treated with varying combinations of polyphenols based on the IC₅₀ or IC₉₀ ratios from cytotoxicity assays.

All treatments were carried out in triplicate for 72 h with fresh phytochemical media changes every 24h. Control experiments were set up in treatment free media with 0.1% DMSO. After 72 h, each set of treated and control cells was washed with Hanks balanced salt solution and harvested using Trypsin-EDTA. Trypsinized cells were centrifuged at 3500

rpm for 5 minutes at 4°C to remove cellular debris and the cell pellets were collected for RNA or protein extraction.

For protein extraction, cell pellets were initially homogenized in 100 mM Tris-HCl buffer (containing 0.25M sucrose and protease inhibitor cocktail) by triple passage through a 27-gauge hypodermic needle and then ultrasonicated with a Vibra-cell™ VCX130 sonicator (Sonics and Materials, Newton, CT) at 50% amplitude using 5 bursts of 20 seconds each with a one minute resting pulse between bursts. Protein concentration of cell homogenates was determined by the Bradford method. Cell homogenates were stored at -80°C until further use for glucuronidation assays (described below).

Total RNA Isolation and Gene Expression Assays

Total RNA from treated and control Caco-2 cell pellets was isolated using the Promega SV total RNA isolation kit. RNA concentration and purity was determined based on the ratio of absorbance at 260 and 280nm. Gene expression and quantification of the target UGT mRNA was carried out using the TaqMan real-time reverse transcription polymerase chain reaction (RT-PCR) assay. TaqMan probes containing 6-carboxyfluorescein (FAM) at the 5'-end and 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end were used. RT-PCR analysis was performed in 25 -50 µl of TaqMan One-Step RT-PCR Master Mix Reagent containing 200 nM forward primer, 200 nM reverse primer, 100 nM TaqMan probes and 20 ng of total RNA. The primer and probe sequences used for the gene expression assays are as follows: UGT1A1 -Forward - 5'GGTGACTGTCCAGGACCTATTGA-3', Reverse primer - 5'-TAGTGGATTTTGGTGAAGGCAGTT-3' and Taqman Probe - 5'-ATTACCCTAGGCCCATCATGCCCAATATG-3'.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) - Forward - 5'-CATGGGTGTGAACCATGAGAA-3', Reverse - 5'-GGTCATGAGTCCTTCCACGAT-3' and Probe - 5'-AACAGCCTCAAGATCATCAGCAATGCCT-3'. Amplification and detection were performed on the ABI Prism 7700 Sequence Detection System using the following profile: 1 cycle of 48°C for 30 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Control reactions included water instead of RNA as template and target mRNA levels were normalized to the human endogenous control GAPDH mRNA levels. Expression levels in treated cells were compared to untreated Caco-2 cells using the comparative cycle threshold (Ct) method.

Glucuronidation Assays

Glucuronidation assays were conducted with cell homogenates collected from UGT induction studies. Incubations were conducted under initial rate conditions. The rate of formation of 4MUG from 4-MU was measured. Briefly, the assay comprised incubation at 37°C for 30 minutes of a 50 µl reaction mixture containing the substrate (0-1000 µM 4-MU), 1mg/ml Caco-2 cell lysate, 5 mM MgCl₂, 10 µg/ml alamethicin and 1 mM saccharolactone in 100 mM Tris Buffer (pH 7.4 at 37°C). The reaction mixture was pre-warmed for 3 min at 37°C and the reaction was initiated by the addition of the cofactor UDPGA (5 mM). The reaction was quenched with an equal volume of ice-cold methanol containing the internal standard, lidocaine (final concentration 200 µM). The quenched reaction mixtures were centrifuged for 10 minutes at 14,000 rpm to precipitate proteins and 10 µl supernatant was analyzed with HPLC. A modification of the HPLC assays described by Fujiwara et al (Fujiwara, et al. 2009) and Narayanan et al (Narayanan, et al. 2000) was used to detect 4-MUG. All reactions were conducted in triplicate, with negative control (no UDPGA).

The HPLC system (HP 1100 series; Agilent Technologies, Santa Clara, CA) consisted of a solvent delivery quaternary pump, an autosampler, a diode array detector with UV detection

set at a $\lambda_{\max}$ of 220 nm, and a Zorbax Rx-C18 column (4.6 × 250 mm, 5 μ particle size; Agilent Tech., Santa Clara, CA). Integration was carried out with the software ChemStation version for LC Rev.A.08.01 (Agilent Technologies, Palo Alto, CA). The isocratic mobile phase comprising 20 mM potassium phosphate buffer (pH 3.1) and methanol (70: 30) was set at a flow rate of 1.3 ml/min, at a column temperature of 35°C. The areas of 4MUG peaks formed were normalized to that of the internal standard, lidocaine. Quantification of formed 4MUG was against a calibration curve of authentic standards.

Data Analysis for Enzyme Kinetics

Prior to nonlinear regression analysis, all data were transformed, and Eadie-Hofstee curves were plotted and checked for linearity. The Michaelis-Menten model was used as the simplest model to fit the data. To determine Michaelis-Menten parameter estimates, the following equation was used:

$$v = V_{\max} \cdot [S] / K_m + [S]$$

where v is the rate of the reaction, $V_{\max}$ is the maximum velocity estimate, $[S]$ is the substrate concentration, and K_m is the Michaelis-Menten constant. Nonlinear regression was performed with GraphPad Prism for Windows (version 4.03; GraphPad Software Inc., San Diego, CA).

Drug Combination Analysis

The assessment of an interaction between two agonist drugs uses the concept of dose equivalence, the same concept used in isobolographic analysis. For two drugs, here denoted A and B, we obtained the dose of drug A that was equally effective to drug B. That equivalent, denoted $Beq(A)$, was then added to the actual dose of drug B whose dose-effect relation provided the expected (additive) effect. When the potency ratio was constant, i.e., dose A/dose B = R, then the drug B-equivalent of dose a of drug A was a/R , and this led to the expected combination effect Eab . The experimentally obtained combination effects were then compared with each calculated Eab value (Student's t -test) to determine differences indicative of either sub-additive or synergistic interactions (Tallarida 2006).

Statistical Analysis

Statistical comparison of the combination antiproliferative effects was performed with GraphPad InStat using a one way ANOVA followed by a post hoc Tukey's multiple comparison analysis ($p < 0.05$). For UGT1A1 induction, comparisons between treatment and control groups were conducted with a one way ANOVA followed by post hoc Student Newman Keuls multiple comparison analysis. For glucuronidation assays, the Michaelis-Menten parameter estimates were compared with a two sided Student's t test assuming normal distribution, with significance set at $p < 0.01$.

Results

RES, Curcumin (Curc) and chrysin were initially characterized individually for their inhibition of Caco-2 cell growth. Figure 1 depicts dose response curves generated from fitting the sigmoid four parameter logistic model to data obtained using a 0-100 μ M polyphenol concentration range. The IC_{50} values reported here are specific for the concentration range studied (1 – 100 μ M) and estimated values $\pm$ SE were 20.8 ± 1.2 (95% C.I 15.3 – 28.5), 20.1 ± 1.1 (95% C.I 15.1 – 26.8), and 16.3 ± 1.3 (95% C.I 10.2 – 25.8) μ M for RES, Curc and chrysin respectively. The shape of the curves indicates a complete/full antagonistic activity for Curc and a partial response for RES and chrysin. Maximal anti-

proliferation (as percent of control growth) at 100 μ M RES, Curc, or chrysin was 45%, 100%, and 80% respectively. Thus, a '50% effect' with IC₅₀ concentrations for RES, Curc, or chrysin alone was 22.5%, 50%, and 40% inhibition of cell growth.

Caco-2 cells were treated with varying concentrations of RES (0.5, 1, 2 and 5 fold antiproliferation IC₅₀) to probe for UGT1A1 induction. Figure 2 depicts the UGT1A1 mRNA induction obtained with various RES concentrations. The induction did not appear to be concentration dependent. The highest (4-fold) induction was seen with 20 μ M RES. At the 40 and 100 μ M concentrations, RES caused a 2-3 fold induction of UGT1A1 mRNA. Induction observed at these three concentrations was significantly different from untreated cells ($p < 0.001$).

The combination of RES with chrysin was evaluated next for two separate endpoints: Caco-2 cell antiproliferation and UGT1A1 induction (Figure 3). For antiproliferation assays, combinations of RES + chrysin (at their IC₅₀ values) tested were - RES:chrysin 20 μ M:16 μ M (1:1), 20 μ M:32 μ M (1:2), and 40 μ M:16 μ M (2:1). Figure 3A depicts these results. RES or chrysin alone at their IC₅₀ exhibited modest cell growth inhibition which was potentiated with all RES + chrysin combinations (Figure 3A). All RES + chrysin combinations were significantly more cell growth inhibitory than RES alone ($p < 0.001$). The effects of the 1:2 and 2:1 RES + chrysin combinations were significantly different from either RES or chrysin alone ($p < 0.001$) (Figure 3A). Analysis of log dose-effect data for the RES + chrysin combinations (Figure 3B) revealed good linear fits for the individual compounds with similar slopes. Reconstruction to parallelism yielded a potency ratio R of 2.66 (Figure 3B). In other words, 2.66 times the concentration of chrysin is the required dose of RES (when each is alone). The set of calculated additive effects did not differ significantly from the experimentally observed effects for the three combination ratios tested ($p < 0.05$; table 2).

Figure 3C depicts the data obtained for RES + chrysin UGT1A1 induction. Chrysin caused a 7 fold induction of UGT1A1 mRNA at its antiproliferation IC₅₀ (16 μ M). The 20 μ M:32 μ M and 40 μ M:16 μ M RES + chrysin combinations caused a statistically significant increase in UGT1A1 mRNA expression compared with untreated cells, RES and chrysin alone ($p < 0.001$) (22 and 17 fold respectively; Figure 3C). *Tert*-butyl hydroquinone (TBHQ) was used as a positive control.

Next, RES in combination with Curc was evaluated for Caco-2 cell antiproliferation and UGT1A1 induction (Figure 4). Depicted in Figure 4A is observed antiproliferation upon the following treatments: RES:Curc 20 μ M:20 μ M (1:1), 20 μ M: 40 μ M (1:2), and 40 μ M:20 μ M (2:1). All combinations caused significantly higher antiproliferation than either polyphenol alone ($p < 0.001$; Figure 4A). RES + Curc at the 20 μ M: 40 μ M ratio caused significantly greater cell growth inhibition than the other ratios. In order to confirm results with IC₅₀ ratios, we additionally evaluated RES + Curc at RES IC₅₀ and Curc IC₉₀ (Figure 4B). RES: Curc 20 μ M: 40 μ M, 20 μ M: 80 μ M, as well as 40 μ M: 40 μ M ratios all significantly inhibited cell growth compared with either polyphenol alone ($p < 0.001$). All RES + Curc ratios evaluated were found to be additive in anti-proliferative activity (Table 2).

UGT1A1 induction with RES + Curc is shown in Figure 4C. Preliminary studies with Curc alone showed lack of UGT1A1 induction with 20 μ M Curc, therefore IC₉₀ (40 μ M) Curc was utilized for these studies. Curc (40 μ M) alone mediated a statistically significant two fold induction of UGT1A1 mRNA transcription ($p < 0.05$; Figure 4C). The four-fold RES mediated UGT1A1 induction was potentiated with the 20 μ M: 40 μ M RES + Curc combination which showed a 12 fold increase in mRNA expression compared with untreated cells ($p < 0.001$). Induction at the 40 μ M: 40 μ M RES + Curc combination was

similar to that seen with RES alone. The 72h treatments with 20 μ M: 80 μ M RES + Curc combination was cytotoxic to the post confluent cells (all cells died with this treatment) and UGT mRNA expression was undetectable with this combination (Figure 4C).

Finally we examined UGT catalytic activity by evaluating 4-MU glucuronidation. Single point velocities were obtained using 1 mM 4-MU as a first step. These velocities were not significantly increased in any treatment group except 20 μ M: 40 μ M RES + Curc and 20 μ M: 32 μ M RES + chrysin combinations (data not shown). These combinations were therefore used to conduct full glucuronidation assays using a 0-1000 μ M 4-MU concentration range. Figure 5 depicts 4MUG formation velocity in Caco-2 cells treated with 20 μ M RES, 40 μ M Curc, 16 μ M chrysin, 20 μ M: 40 μ M RES + Curc, 20 μ M: 32 μ M RES + chrysin combinations and TBHQ. The $V_{\max}$ estimates in the treated cells were significantly different from that that in the untreated cells ($p < 0.01$) (Table 3). The greatest activity (besides the positive control) was seen with 20 μ M: 40 μ M RES + Curc, 20 μ M: 32 μ M RES + chrysin combinations. The K_m estimates in treated cells were not significantly different from control cells except 20 μ M: 40 μ M RES + Curc. However, the trend of increasing K_m estimates with increasing $V_{\max}$ resulted in similar $V_{\max}/K_m$ ratios among the groups (Table 3).

Discussion

With respect to CRC, target tissue (intestinal) modulation of phase II enzymes such as the UGTs by dietary polyphenolic compounds is poorly understood. The ability of RES to modulate intestinal UGTs (and other detoxifying enzymes) has not been extensively studied. Further, UGT induction due to RES in combination with other phytochemicals has not been evaluated to date. Phytochemical combinations are of great interest as they better mimic dietary exposure, and may achieve more effective chemoprevention than a single agent. While RES and Curc have been used in combination to achieve various end points such as enhanced antioxidant, anti-inflammatory or cell growth inhibitory effects (Aftab and Vieira 2010, Majumdar, et al. 2009, Harasstani, et al. 2010), combinations of RES and chrysin have not been evaluated. The novelty of this study lies in the use of rational phytochemical combinations to achieve both cytotoxicity and UGT induction. The cell growth inhibitory potential of dietary polyphenols used in this study has been reported in several human tumorigenic cell lines (Surh and Chun 2007, Ulrich, et al. 2005, Wang, et al. 2004). Several combination studies carried out did not clearly explain the rationale for selecting concentrations of each phytochemical in a combination, or simply combined similar molar concentrations (Aftab and Vieira 2010, Majumdar, et al. 2009, Svehlikova, et al. 2004, van der Logt, et al. 2003). The goal of our studies was to utilize a rational method (such as use of IC_{50} ratios of combinations) of combining phytochemicals to achieve greater efficacy. The choice of combinations (phytochemicals as well as concentrations) was based on several considerations: i) unique and overlapping UGT induction mechanisms (see Table 1), ii) physiologically relevant and reportedly high concentrations achievable in the intestine (Shehzad, et al. 2010, Alfaras, et al. 2010) iii) concentrations mediating anti-proliferation in Caco-2 cells (an anticancer effect) and iv) log dose – effect analysis of varying ratios of phytochemicals. Isobolographic methods to design combination studies are well known, but have not been extensively applied in the study of phytochemicals. The study by Harrastani et al used various phytochemical IC_{50} combinations and found that these combinations mediated synergistic inhibition of secretion of pro-inflammatory mediators in lipopolysaccharide induced RAW 264.7 cells. We report here the use of this approach – not employed, to date - for cell growth inhibition and subsequent UGT induction.

Modeling the data obtained from anti-proliferation studies with RES, Curc and chrysin yielded IC_{50} estimates for Caco-2 cell growth inhibition (Figure 1). Curc mediated a

complete inhibition of cell growth within the concentration range used while RES and chrysin caused only about 50 – 70 percent cell growth inhibition at these concentrations, reflecting partial antagonism. The potency of chrysin against cell growth has been reported for the human squamous carcinoma cell line SCC-9 and the breast cancer cell line MCF-7 (Walle, et al. 2007) but not for the Caco-2 cell line. For Curc, IC₅₀ estimates ranging between 10.26 μ M and 13.31 μ M have been reported for SW480, HT-29, and HCT116 - three independent human colorectal cancer cell lines (Cen, et al. 2009) and 12 μ M in MCF-7 cells (Gupta, et al. 2006). For RES, an IC₅₀ of 25 μ M was reported for Caco-2 cells (Chabert, et al. 2006). Our IC₅₀ estimates are similar to most of these reports albeit in different cell lines.

Induction of UGT1A1 by RES in Caco-2 cells has not been previously reported. In the present work, we show a concentration independent induction of UGT1A1 expression by RES (Figure 2) with the greatest fold induction seen at its IC₅₀ for antiproliferation (20 μ M). RES has been shown to be a modest inducer of UGT enzymes in rat liver (Hebbar, et al. 2005) and concentration independent RES mediated UGT1A1 and 2B7 induction has been shown in HepG2 cells (Lancon, et al. 2007). The 3-5 fold induction of UGT1A1 mRNA we report with RES is similar to that reported in HepG2 cells (10). RES is reportedly a ligand of Aryl hydrocarbon receptor (AhR) and an activator of Nrf-2, a master regulator of phase II gene expression (Table 1). RES is also reported to activate the pregnane and xenobiotic receptor (PXR) in the nucleus, another pathway for UGT transcriptional regulation (Saracino and Lampe 2007). Future studies in our laboratory will focus on mechanisms underlying UGT induction with phytochemicals.

The combination of RES with chrysin was additive in Caco-2 antiproliferative effect and UGT1A1 induction (Figures 3A, 3B; Table 2). Anti-proliferative additivity with chrysin can be explained by overlapping pathways involving signaling proteins such as, p38, Jnk1, Akt, protein kinase C, PI3K. Most of these proteins are located upstream of the nuclear factor erythroid 2-related factor 2 (Nrf-2) which also mediates control of UGT transcription and expression (Saracino and Lampe 2007). Chrysin alone induced UGT1A1 to significantly higher levels compared with control (Figure 3C) and this was not surprising since chrysin is known to be a potent inducer of UGT1A1 in HepG2 and Caco-2 cells at concentrations similar to those used in the present study (Galijatovic, et al. 2000, Walle, et al. 2000). Maximal induction of UGT1A1 mRNA expression was observed at the 20 μ M:32 μ M RES + chrysin combination (Figure 3C). If chrysin-mediated UGT1A1 induction is indeed concentration dependent as has been previously reported (Smith, et al. 2005) then one could postulate that the induction seen with this combination is additive rather than synergistic. Chrysin is reported to be an AhR agonist, but its induction of the UGT1A1 gene has been shown to be dependent on additional cellular factors (Bonzo, et al. 2007). Since chrysin also reportedly modulates Nrf-2, it is possible that the additivity in UGT1A1 induction seen with RES and chrysin combinations occurs *via* overlapping transcriptional mechanisms, and this possibility requires further evaluation.

With RES + Curc, all combinations exhibited additivity with respect to Caco-2 inhibition of cell growth (Figures 4A, 4B). Studies were conducted with both IC₅₀ and IC₉₀ ratios of Curc in order to confirm results at various concentrations of the phytochemical. As with chrysin, the observed additivity can be explained by overlapping pathways involving signaling proteins modulated by both RES and Curc. Preliminary studies showed a lack of UGT induction with Curc IC₅₀ concentrations, therefore subsequent UGT1A1 induction studies were conducted at IC₉₀ concentrations of Curc. Curc at its IC₉₀ (40 μ M) induced UGT1A1 mRNA expression modestly (2 fold; Figure 4C). Our results are in line with the study by Naganuma et al (Naganuma, et al. 2006) where they reported Curc-mediated induction of UGT1A1 and 1A6 in Caco-2 cells at similar concentration as those used in the present

study. Van der Logt et al (van der Logt, et al. 2003) reported an enhancement of mouse ugt enzyme activity in small and large intestine of mice fed Curc for three weeks with no significant effects in the liver, underlining the tissue specific nature of phytochemical UGT induction. For RES + Curc, UGT1A1 induction observed with the 20 μ M: 40 μ M combination (12 fold) was synergistic in nature compared to RES alone (4 fold) or Curc alone (2 fold; Fig. 4C). These results are interesting in light of possible anti-tumor growth activity of phytochemicals including Curc. Possible mechanisms of UGT induction *via* Curc include binding to AhR, modulating Nrf-2, and activating PXR (Table 1) and as with chrysin, the enhanced UGT induction effected by RES + Curc combinations is possibly due to an overlap in these transcriptional mechanisms.

We did not see as great an increase in UGT catalytic activity with the various combinations as for UGT1A1 mRNA expression (Figure 5, Table 3). It was however interesting to see that those combinations with the highest levels of UGT1A1 mRNA expression also mediated significant increases in 4MUG formation V_{max} when compared with untreated cells. 4MU glucuronidation is mediated by other UGT isoforms besides UGT1A1 (Uchaipichat, et al. 2004). Previous work by Hanioka et al with β -naphthoflavone in HepG2 cells (Hanioka, et al. 2006) showed a marked induction of UGT1A1 (but no induction of UGT1A6 and 1A9) which was reflected in the total UGT activity for 4-MU glucuronidation. A comparison of the V_{max}/K_m ratios for 4MUG formation (Table 3) shows no marked differences in the apparent intrinsic clearance values across all treatment groups versus control cells despite significant increase in V_{max} estimates. The observed shift in K_m values with increasing V_{max} values obviously affected the calculated intrinsic clearance values. This shift in K_m may be due to the contribution of other low affinity UGTs induced by these phytochemicals in addition to UGT1A1. Future studies will examine the effect of phytochemicals on induction of UGT family members besides UGT1A1, utilizing UGT-specific substrates.

Phytochemical potentiation of UGT activity has been tested *in vivo* (Tobin, et al. 2006) on the assumption that enhanced UGT activity would lead to increased detoxification of dietary carcinogens and xenobiotics. The flipside to this increased UGT expression would be autoinduction where the phytochemicals themselves are metabolized and rapidly cleared. It remains to be studied whether autoinduction of phytochemical glucuronidation leads to time-dependent negative feedback, returning induced UGT levels to baseline. Thus, evaluation of UGT induction as a mechanism of anticancer activity of phytochemicals needs to carefully balance detoxification of carcinogens with deactivation of 'anticancer' phytochemicals.

Conclusions

We have shown for the first time that certain combinations of dietary phytochemicals when used in rational combinations are able to interact either additively or synergistically to mediate both inhibition of cell viability and induction of UGT1A1 expression. This synergy can be further exploited to design chemical combinations to help achieve better CRC chemoprevention. The only FDA approved drug for CRC prevention is currently celecoxib. Whether celecoxib, a COX2 inhibitor, induces UGTs is unknown. Dietary phytochemicals offer a safe CRC preventive alternative, and additionally offer a safe choice of combination with drugs like celecoxib for targeting CRC via various pathways.

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Figure 1. Caco-2 cell cytotoxicity dose response curves for 1 – 100 μ M resveratrol (RES), curcumin (Curc) and chrysin. Data are expressed as Mean $\pm$ SE, n = 4. Representative fitted lines are depicted; IC₅₀ estimates were obtained by fitting the model to actual data replicates.

Figure 2. Induction of UGT 1A1 mRNA in post confluent Caco-2 cells treated with varying concentrations of RES. Data are expressed as mean + SD, n = 6. ***significantly different from control ($p < 0.001$).

A)

B)

Figure 3.

Studies with RES + chrysin combinations. A) Caco-2 cell cytotoxicity observed upon 72 h treatment with IC₅₀ combinations of RES + chrysin. RES + chrysin 1:1 is 20 uM RES + 16 uM chrysin, 1:2 is 20 uM RES + 32 uM chrysin, and 2:1 is 40 uM RES + 16 uM chrysin. Data expressed as mean + SD, n = 4 *** greater than RES alone (p < 0.001); +++ greater than either RES alone and chrysin alone (p < 0.001) using a one way ANOVA and post hoc Tukey's multiple comparison analysis.

B) Dose-effect data for RES and chrysin plotted as the magnitude of the percent reduction in growth against log dose.

C) Induction of UGT 1A1 mRNA in post confluent Caco-2 cells treated for 72h with IC₅₀ combinations of RES + chrysin. Data are expressed as mean + SD, n = 3.*** Groups significantly different from control (p < 0.001). +++different from RES alone or chrysin alone (p < 0.001) using a one way ANOVA followed by a *post hoc* Student-Newman-Keuls multiple comparison analysis.

A)

B)

Figure 4.

Studies with RES + Curc combinations. A) Caco-2 cell growth inhibition observed upon 72 h treatment with IC₅₀ combinations of RES + Curc where RES + Curc 1:1 is 20 uM RES + 20 uM Curc, 1:2 is 20 uM RES + 40 uM Curc, and 2:1 is 40 uM RES + 20 uM Curc. *** greater than RES alone and Curcumin alone ($p < 0.001$); +++ greater than 1:1 RES + Curc

and 2:1 Res + Curc ($p < 0.001$) using a one way ANOVA and post hoc Tukey's multiple comparison analysis.

B) Cell growth inhibition observed with RES + Curc IC_{90} combinations where RES IC_{50} and Curc IC_{90} concentrations were used to determine ratios and RES + Curc 1:1 is 20 μ M RES + 40 μ M Curc, 1:2 is 20 μ M RES + 80 μ M Curc, and 2:1 is 40 μ M RES + 40 μ M Curc. Data expressed as mean + SD, $n = 4$. *** greater than RES alone ($p < 0.001$). C) Induction of UGT 1A1 mRNA in post confluent Caco-2 cells treated for 72h with RES IC_{50} + Curc IC_{90} combinations. ***significantly different from control ($p < 0.001$) * ($p < 0.05$), +++different from RES alone and Curc alone ($p < 0.001$) N.D = not detectable

Figure 5. Michaelis-Menten curves for 4-MUG formation in control and treated Caco-2 whole cell homogenates. 4-MU glucuronidation assays were conducted using a substrate concentration range of 0-1000uM. Experiments were conducted in triplicate and data are expressed as Mean $\pm$ SE. Representative fitted lines are depicted; kinetics estimates were obtained by fitting the model to actual data replicates.

Table I

Dietary phytochemicals used and their reported modulatory effects on UGT expression and associated transcriptional pathways

Phytochemical (Class)	Major human UGT(s) responsible for auto glucuronidation	Known UGT transcriptional modulation	Effects on UGT transcriptional regulators
Curcumin (Curcuminoid)	UGT1A1, 1A3, 1A7, 1A8, 1A10, 2B7 (Hoehle, et al. 2007)	Induced rat intestinal <i>ugts</i> (van der Logt, et al. 2003), Induced UGT1A1 and 1A6 expression in Caco-2 cells (Naganuma, et al. 2006)	AhR agonist (Ciolino, et al. 1998) Binds as an agonist but exhibits antagonist activity in the presence of stronger ligands (Garg, et al. 2008, Nishiumi, et al. 2007) Nrf2 agonist (Garg, et al. 2008) PXR agonist (Saracino and Lampe 2007)
Chrysin (Flavonoid)	UGT1A1, 1A7, 1A8, 1A9, 1A10 (Basu, et al. 2004)	Induced human UGT1As in Caco-2 and HepG2 cell lines (Galijatovic, et al. 2000, Walle, et al. 2000) Induced UGT1A1 in human hepatocytes and HepG2 cells (Smith, et al. 2005)	AhR agonist (Bonzo, et al. 2007)
Trans-resveratrol (Stilbenoid)	UGT1A1, 1A9 (Aumont, et al. 2001, Brill, et al. 2006)	Induced human UGT1A1 and UGT2B7 in HepG2 cells (Lancon, et al. 2007) Induced mouse <i>ugt</i> and rat <i>Ugt1a1</i> , <i>1a6</i> , and <i>1a7</i> (Hebbar, et al. 2005, Szafer, et al. 2004)	AhR antagonist (Casper, et al. 1999) AhR agonist (Lee and Safe 2001) Nrf2 activator (agonist) (Rubiolo, et al. 2008) PXR agonist (Saracino and Lampe 2007)

Table II

Log Dose – Effect analysis of the percent reduction in cell growth mediated by RES + Chrysin and RES + Curc combinations

Combinations	Additive (Theoretical) ^a	Observed (Experimental) ^a
RES + Chrysin IC₅₀		
1:1 [20 µM + 16 µM]	50.6 +/- 4.8	43 +/- 1.5
1:2 [20 µM + 32 µM]	62.1 +/- 4.7	51.0 +/- 1.4
2:1 [40 µM + 16 µM]	56.6 +/- 4.5	52.0 +/- 2.8
RES + Curc IC₅₀		
1:1 [20 µM + 20 µM]	65.2 +/- 5.9	74.4 +/- 2.0
1:2 [20 µM + 40 µM]	78.8 +/- 7.0	94.7 +/- 0.85
2:1 [40 µM + 20 µM]	70.7 +/- 6.3	71.2 +/- 0.84
RES + Curc IC₉₀		
1:1 [20 µM + 40 µM]	79	89
1:2 [20 µM + 80 µM]	100	90
2:1 [40 µM + 40 µM]	85	85

^aThe additive and observed values were calculated as described under Methods. Values for RES + chrysin IC₅₀ and RES + Curc IC₅₀ are percent reduction in cell growth ± SD. Values for RES + Curc IC₉₀ are reported as percent reduction in cell growth as the data could only be analyzed graphically, hence associated error could not be calculated. No significant difference was seen between calculated additive and actual observed effects of RES + chrysin and RES + Curc IC₅₀ (p < 0.05, student's t-test).

Table III

Kinetic parameter estimates for formation of 4-MUG in phytochemical-treated and control Caco-2 cells

Estimate	Control	RES 20 μM	RES + Curc (20μM + 40μM)	RES + Chrysin (20μM + 32μM)	TBHQ	Chrysin 16 μM	Curcumin 40 μM
V_{max}	2.86 ± 0.07	3.35 ± 0.13 ^a	5.6 ± 0.22 ^a	5.37 ± 0.26 ^a	6.0 ± 0.35 ^a	2.1 ± 0.18 ^a	4.32 ±0.23 ^a
K_m	110 ± 9.5	126.6 ± 16.9	223.8 ± 25.5 ^a	172 ± 27	184.3 ± 33.7	116.6 ± 37	191 ± 31.7
V_{max}/K_m	0.026	0.026	0.025	0.031	0.032	0.018	0.023
R^2	0.99	0.97	0.98	0.96	0.95	0.83	0.96

Data are expressed as estimate ± SE, n = 3. Estimate units are as follows: V_{max} = nmol/min/mg; K_m = μM; V_{max}/K_m = ml/min/mg.

^a Estimates significantly different from control. Statistical comparison of the parameter estimates was performed using a two sided Student's *t* test assuming normal distribution, for which a *p* value of 0.01 was considered significant.