Phytomedicine 20 (2013) 461–468

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Use of the Combination Index to determine interactions between plant-derived phenolic acids on hepatotoxicity endpoints in human and rat hepatoma cells

Yitong Liu[a,c], Thomas J. Flynn[a,*], Martine S. Ferguson[b], Erica M. Hoagland[a,1]

[a] Division of Toxicology, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Laurel, MD 20708, United States
[b] Division of Public Health and Biostatistics, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD 20740, United States
[c] Oak Ridge Institute for Science and Education, Oak Ridge, TN 37831, United States

ARTICLE INFO

Keywords:
Hepatotoxicity
Dietary phenolic acids
Mixtures
Interactions
Species differences

ABSTRACT

The beneficial or adverse effects of isolated phytochemicals are not always concordant with effects of the botanical dietary supplements from which they were derived. This disparity could be due to interactions between the various phytochemicals present in the whole plant. The phenolic acids, rosmarinic acid (RA), caffeic acid (CA) and ferulic acid (FA) are widely present in foods and dietary supplements, and they are assumed to exert various beneficial biological effects. However, there is little data on the potential biological interactions of these three phenolic acids which commonly occur together and are linked metabolically. In the present study, liver toxicity of the three phenolic acids was assessed on the three compounds singly and in various binary and one ternary combinations. A series of *in vitro* endpoints relevant to liver toxicity were evaluated in both a human (HepG2/C3A) and rat (MH1C1) hepatocyte cell line. The Combination Index (CI) was calculated for each endpoint from both the concentration responses of the single compounds and the responses of the various binary and ternary mixtures. Both synergistic and antagonistic interactions were observed for some endpoints and some combinations of test agents. Interactions were most prevalent in measures of oxidative stress and cytochrome P450 activities in both cell types. There was only a 53% concordance between the rat and human cells which may be suggestive of species differences. The data suggest an approach for better characterizing the beneficial or adverse effects of complex botanical products through evaluation of interactions between individual phytochemical components.

Published by Elsevier GmbH.

Introduction

Drugs that produce overtly similar effects individually will sometimes show exaggerated or diminished effects when used concurrently (Tallarida 2001). Similar interactions can take place between drugs and dietary chemicals (Nahrstedt and Butterweck 2010). Botanicals used as both phytomedicines and as dietary supplements are complex mixtures of phytochemicals. However, safety and efficacy studies on these products often focus on single chemical components tested individually. Effects observed in studies on individual components often do not mimic those observed with the intact plant (Liu 2004; De Kok et al. 2008). Therefore, a rapid and quantitative assessment is necessary to identify potential synergistic or antagonistic effects from simple additive effects.

The Combination Index (CI), also called the Interaction Index, is widely used to assess both beneficial and adverse interactions between pharmaceuticals (Zhao et al. 2004). The CI has also been used to assess the interactive toxicities of environmental chemicals (McDermott et al. 2008). Khafif et al. (1998) used the CI to quantitate chemopreventive synergism between botanical phenolics in cell cycle blockade using human oral epithelial cells. Saw et al. (2011) used the CI to demonstrate synergism between phytochemical indoles and isothiocyanates in the induction of the antioxidant response in HepG2-C8 cells. The aforementioned studies on food-related chemicals focused on identifying beneficial interactions between phytochemicals. However, there appear to be no literature reports where the CI has been used to assess potential harmful interactive effects between natural compounds occurring together in botanical products that are consumed as foods or as dietary supplements.

The liver plays a central role in the disposition and detoxication of xenobiotic and natural chemicals including drugs, dietary

Abbreviations: RA, rosmarinic acid; CA, caffeic acid; FA, ferulic acid; CI, Combination Index; CYP1A, cytochromes P4501A1 and P4501A2; CYP2B/3A, cytochromes P4502B and P4503A.

* Corresponding author at: U.S. Food and Drug Administration, MOD-1 Laboratories, 8301 Muirkirk Road, Laurel, MD 20708, United States. Tel.: +1 301 210 6377; fax: +1 301 210 4699.
E-mail address: thomas.flynn@fda.hhs.gov (T.J. Flynn).
[1] Current address: Allen County Kentucky School, Scottsville, KY 42164, United States.

0944-7113/$ – see front matter. Published by Elsevier GmbH.
http://dx.doi.org/10.1016/j.phymed.2012.12.013

supplements, food additives, and food contaminants. This role of liver makes it a primary target of toxicity following oral exposure to a wide variety of chemicals. Therefore, identification of potential synergistic interactions that can alter liver function and, possibly, lead to liver toxicity is an important public health concern.

Rosmarinic acid (RA), caffeic acid (CA) and ferulic acid (FA) are common components of various foods such as thyme, rosemary, and basil, and of botanical dietary supplements either as the single compound or as a component of a botanical extract. There is evidence for the metabolic conversion of RA to CA and FA *in vivo* in both rats (Nakazawa and Ohsawa 1998) and humans (Baba et al. 2005). The reported *in vivo* biological activities of all three phenolic acids are similar and include antioxidant effects (Petersen and Simmonds 2003; Maurya and Devasagayam 2010; Srinivasan et al. 2005).

An *in vivo* study in rats (Nahrstedt and Butterweck 2010) evaluated pharmacological interactive effects (synergism) between individual phytochemicals isolated from St. John's Wort (*Hypericum perforatum L.*). However, animal studies are time consuming and resource intensive, especially when looking for potential interactions following exposures to multiple agents. Therefore, validated *in vitro* cell culture systems are desirable for studying pharmacological interactions such as synergism or antagonism. Due to the limited lifespan, high cost, and significant batch-to-batch variability of fresh hepatocytes, liver hepatoma cell lines are commonly used to evaluate hepatotoxicity *in vitro* because of their availability and stable phenotype. HepG2/C3A cells, a clonal derivative of HepG2, have many desirable properties including strong contact inhibition of growth, high albumin production, low production of alpha-fetoprotein, measurable basal and inducible monooxygenase activities, and the ability to grow in glucose deficient medium (Kelly 1994). MH1C1 rat hepatoma cells show many similarities to cultured rat hepatocytes in the expression of biotransformation activities (Donato et al. 1994), albumin synthesis (Richardson et al. 1969), and growth in low glucose medium (Schamhart et al. 1979).

The current study was designed to investigate the ability of rapid, cell-based assays to identify potential synergistic and antagonistic interactions between phytochemicals that could alter the liver toxicity profiles of the individual compounds. Three phenolic acids, rosmarinic acid, caffeic acid and ferulic acid were selected as model phytochemicals. Studies were conducted in both human (HepG2/C3A) and rat (MH1C1) hepatocyte cell lines to assess species differences. Seven endpoints were evaluated which cover a variety of biological mechanisms relevant to hepatotoxicity including oxidative stress, mitochondrial membrane permeability, cellular neutral and polar lipid accumulation, CYP1A, 2B, and 3A activities, and cytolethality (Flynn and Ferguson 2008). Biological activity was evaluated for the compounds individually and in several binary and one ternary combination. Significant interactions, antagonistic or synergistic, were identified using the Combination Index along with appropriate confidence intervals.

Materials and methods

Materials

The human and rat hepatoma cell lines HepG2/C3A cells (CRL-10741) and MH1C1 cells (CCL-144) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Cell culture medium and medium supplements were obtained from Life Technologies/Gibco (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). Rosmarinic acid was purchased from INDOFINE (Hillsborough, NJ). Caffeic acid, ferulic acid, DMSO, and assay substrates (dihydrodichlorofluorescein, rhodamine 123, nile red, 7-ethoxyresorufin, 7-benzyloxyresorufin, resorufin, H33258, salicylamide, dicumarol) were purchased from Sigma (St. Louis, MO).

Cell culture

HepG2/C3A and MH1C1 cells were both cultured in Dulbecco's minimal essential medium (DMEM) with low glucose, pyruvate, Glutamax™ and pyridoxine. This basal medium was supplemented with MEM non-essential amino acids (1% final concentration), HEPES (10 mM final concentration) and fetal bovine serum (10% final concentration). No antibiotics were added to the medium. Cells were plated onto the inner 60 wells of 96-well tissue culture plates at a density of 6×10^4 cells/cm^2 for HepG2/C3A cells and 12×10^4 cells/cm^2 for MH1C1 cells. Cells were propagated and used up to a maximum of 5 passages at which point propagation began anew from a fresh vial of cryopreserved stock. Plates were incubated at 37 °C in 5% CO_2. Under these conditions, cells reached confluence in 5–6 days. Cells were treated with test compounds for 72 h beginning on culture day 8.

Treatment with phenolic acids

In the concentration dependence study, the three phenolic acids were dissolved in DMSO and then diluted in culture medium to a series of concentrations (125, 250, 500, 1000 μM) with 0.5% DMSO final concentration. Vehicle only (DMSO) was used as the control. Each agent was tested in three concurrent plates with six replicates for 72 h. In the interaction study, the three phenolic acids were tested in different binary combinations (1:3, 1:1, 3:1) and the single ternary combination (1:1:1) with the total final phenolic concentration of 500 μM to demonstrate potential interactive effects.

Endpoint assays

Endpoint assays were conducted essentially as described in Liu et al. (2011).

Statistical analysis

The Combination Index (CI) (Zhao et al. 2004) between two drugs A and B is:

$$\mathrm{CI} = \left(\frac{C_{A,X}}{\mathrm{IC}_{X,A}}\right) + \left(\frac{C_{B,X}}{\mathrm{IC}_{X,B}}\right)$$

where $C_{A,X}$ and $C_{B,X}$ are the concentrations of drug A and drug B used in combination to achieve X% drug effect. $\mathrm{IC}_{X,A}$ and $\mathrm{IC}_{X,B}$ are the concentrations for single agents to achieve the same effect. A CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively.

$\mathrm{IC}_{X,A}$ and $\mathrm{IC}_{X,B}$ were determined by fitting the commonly-used log-logistic concentration response curve (Seefeldt et al. 1995), a variant of the Hill equation, to the single agent data:

$$y = C + \frac{D - C}{1 + \exp[b(\log(x) - \log(I_{50}))]} \quad (1)$$

where x is the compound concentration, C is the mean response at very high doses, D is the mean response at control, b is the slope (positive if responses decreases with dose or negative if response increases with concentration) and I_{50} is the concentration that causes 50% of the response. D was set to 100 as all responses were standardized to percent of control. The parameters were estimated via nonlinear modeling using PROC NLIN in SAS (SAS® 9.2