Evaluation of Liposomal Curcumin Cytochrome P450 Metabolism

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Abstract. *Background: Curcumin (diferuloylmethane) is a commonly used spice and nutritional supplement that has demonstrated potential anti-tumor and anti-inflammatory activity. There is limited information regarding curcumin metabolism and the potential for drug-drug interactions. The objective of this study was to characterize the hepatic metabolism of synthetic curcumin used in the liposomal curcumin formulation. Materials and Methods: High-throughput cytochrome P450 (CYP450) metabolism inhibition assays were conducted in vitro evaluating CYP450 3A4, 2C8, 2C9, and 2D6. An ex vivo model of cryopreserved human hepatocytes was used to evaluate the CYP450 metabolism induction potential of curcumin for CYP P450 3A4, 2C8/2C9, and 2D6. Results: In the in vitro CYP450 inhibition studies, curcumin at any concentration did not inhibit CYP450 3A4 or CYP450 2D6 activity. At a curcumin concentration of 58.3 μM, 10.5% and 22.5% inhibition of CYP450 2C9 and CYP450 2C8 activity, respectively, was observed. In the ex vivo hepatocyte inductions studies, minimal to no induction of CYP450 3A4, CYP450 2C8/2C9 or CYP450 2D6 was observed. Rifampicin did not induce the metabolism of curcumin and curcumin did not induce its own metabolism. Conclusion: There is low potential for CYP450 mediated drug interactions at physiologic serum concentrations of liposomal curcumin. Based on preliminary data, liposomal curcumin will not interact with other chemotherapy agents that are metabolized and/or eliminated via the primary drug metabolizing CYP450 pathways.*

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Curcumin is one of the most frequently evaluated natural products for the prevention and treatment of cancer. It has known antiproliferative activity in numerous cancer cell types. It has demonstrated anticancer activity as a single agent and in combination with other chemotherapeutic agents in animal models of cancer, as well as chemopreventative activity in animal models (1-3). In preliminary clinical studies, there were patients with reported stable disease following oral administration of curcumin despite detection of minimal curcumin plasma concentrations (4-7). However, two major hurdles remain for curcumin in the treatment of cancer: its aqueous insolubility and lack of bioavailability. In theory, if an agent with low bioavailability has activity, improving bioavailability would increase the activity of the agent. This is supported by preliminary studies of an intravenous liposomal curcumin formulation, where impressive results in animal models were observed (8). To date, the metabolic pathway of curcumin has not been elucidated. After successfully increasing bioavailability via intravenous administration, it is important to rule out the potential for increased drug interactions through hepatic cytochrome P450 (CYP450) drug metabolism. For example, curcumin has the potential to be a useful therapy for numerous cancer types and will likely be used in combination with other chemotherapy agents that are known substrates of the CYP450 pathways (9). However, potential drug-drug interactions of natural products with other chemotherapy agents could have severe consequences, ranging from toxic side effects to therapeutic failure common and frequently reported due to enzyme induction or

Table I. *Inhibitors used in the in vitro inhibition studies.*

CYP 450 inhibition	Fluorometric substrate	Excitation/ emission wavelength	CYP inhibited	Inhibitor concentration	Reaction time
Ketoconazole	DBF	485/528	3A4	0 to 7.5 μM	30 minutes
Quercetin	DBF	485/528	2C8	0 to 10 μM	60 minutes
Quinidine	AMMC	360/460	2D6	0 to 0.75 μM	45 minutes
Sulfaphenazole	DBF	485/528	2C9	0 to 15 μM	60 minutes

inhibition. The primary objectives of these *in vitro* and *ex vivo* metabolism studies were to investigate the potential drug-drug interactions mediated through inhibition and/or induction of CYP3A4, CYP2C8/2C9, and CYP2D6 and associated with synthetic curcumin (diferuloylmethane), the most active component of turmeric (*Curcuma longa*).

Materials and Methods

Chemicals. The chemicals/regents used were of the highest analytical grade available. Synthetic curcumin (diferuloylmethane) ((1E, 6E)-1. 7-Bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (99.2% purity) was provided by Sami Labs Limited (Karnataka, India). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cytochrome P450 enzyme microsomes. CYP450 3A4, 2C8, and 2C9 isoenzyme microsomes were obtained from BD Biosciences (Gentest™) Discovery Labware (Woburn, MA, USA). Each isoenzyme was packaged in 0.5 mL aliquots, the stated total protein content is 5.8 mg/mL in 100 mM potassium phosphate (pH 7.4) and corresponding CYP450 content is 1000 pmol/mL. These products are stable for two years; they were stored at –80˚C, and utilized in accordance with provided Material Safety Data Sheets (MSDS) and technical bulletins.

High throughput CYP450 inhibition assays. The assay protocol was adapted from a validated High Throughput Method for Measuring CYP450 Inhibition (version 4.2, 2000) method from BD Gentest (Woburn, MA, USA) (10). Briefly, the positive controls (quercetin, sulfaphenazole, quinidine and ketoconazole), test compound (curcumin), and substrates (dibenzylfluorescein and 3-[2-(*N*,*N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin iodide (AMMC) were dissolved in acetonitrile and working solutions were made by dilution in 0.5 M potassium phosphate buffer, pH 7.4. The common solutions, cofactors stocks, enzyme/substrate mixes, and positive control solutions were all prepared as outlined as recommended in manufacturer methods. Each reaction well had final cofactor concentrations of 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.4 Units/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium ion. The assay reactions were carried out at 200 μL total volume in 96-well titer plates. After the addition of the appropriate enzyme, substrate (DBF at 100 μM or AMMC at 500 μM) and inhibitor positive control (Table I) or curcumin at a maximum concentration of 58.3 μM was added to the reaction mixture and serially diluted 1 to 3 for eight wells (range 58.3 μM down to 0.01 μM). The concentration of 58.3 μM was chosen based on previous *in vitro* growth inhibition studies of pancreatic cancer cell lines that demonstrated it achieved maximum anticancer activity (1). The reactions incubated at 37˚C for 30 to 60 min and were stopped with the addition of 75 μL of a 2M NaOH solution or 80:20 acetonitrile:tris base solution (CYP450 2D6 only). In each reaction well there were 4, 2, or 0.5 pmol of CYP450 2C8, 2C9 or 3A4, respectively, present. CYP450 inhibition was calculated and reported as IC_{50} values. These data were produced by comparing the metabolism in assay reactions containing varied concentrations in the presence and absence of the known inhibitor. The amount of product metabolized for the control comparison reactions was determined *via* fluorescence emission detection at 528 nm (excitation 485 nm) of fluorescein (metabolite product of DBF metabolism by CYP450) or at 460 nm (excitation 360) of 3-[2-(*N*, *N*-diethyl-*N*-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin (AMHC) (metabolite product of AMMC). The relative amount of substrate metabolized was determined with FL600 Dual-Band plate reader from BioTek Instruments, Inc. (Winooski, VT, USA).

Hepatic metabolism induction assay

Human hepatocytes. Cryopreserved human hepatocytes were obtained from BD Biosciences (Gentest™) Discovery Labware. Hepatocytes were re-plated using supplemented Hepatozyme SFM media (Gibco™ Invitrogen Corporation, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA) and 250 μM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). Hepatocytes were then maintained in unsupplemented Hepatozyme SFM media for the seven to ten days duration of the study. Primary hepatocyte cells were plated into separate 6-well collagen I-coated plates for the CYP450 induction and substrate experiments, respectively. The cultures were prepared in quadruplicate for each experimental time point. The cells were handled according to the supplier's instructions and were thawed, isolated, and plated according to the supplier's protocol. The cells were incubated for at least 48 hours at 37˚C (5% CO_2) to allow stabilization after shipping before use in any experimentation.

An *ex vivo* model of cryopreserved human hepatocytes was used to evaluate the ability of curcumin to induce CYP450 metabolism for CYP P450 3A4, 2C8/2C9, and 2D6. A known substrate for each isoenzyme was selected including diclofenac (CYP450 2C8/2C9), dextromethorphan (CYP450 2D6), or docetaxel (CYP450 3A4). The experiment was set up in quadruplicate comparing known substrate alone to account for intrinsic metabolism of the known substrate, using a control inducer, rifampicin 25 μM (Sigma-Aldrich), or curcumin 20 μM as the test inducer and known substrate. The 20 μM concentration of curcumin was the maximum concentration tested due to limited solubility of curcumin in the media necessary for maintaining viability of human hepatocytes but 20 μM above what has currently been detected in plasma for oral curcumin

Table II. *Curcumin CYP450 Inhibition determination.*

	Percentage metabolism inhibited			
Substrate tested	3A4	2C8	2C9	2D6
Control inhibitor*	90.1%	58.6%	45%	89.3%
Curcumin	0.5%	22.5%	10.5%	1.7%

*See Table I for appropriate fluorometric substrate.

studies in humans and above the plasma concentrations detected of liposomal curcumin in animal studies (Data not shown). The hepatocytes were treated for a total of 72 hours with either the control inducer rifampicin or curcumin with media changes every 24 hours. After 72 hours the drug, rifampicin or curcumin, were removed and the appropriate concentration substrate specific to the CYP450 isoenzyme of interest was added or test substrate curcumin was added. Time points were taken at time zero, 2.5 and 24 hours and absorbance read at the appropriate wavelength for the test substrate to determine if curcumin was an inducer or if its metabolism was induced by the control inducer rifampicin.

When curcumin was evaluated to determine if it was a CYP450 substrate, the curcumin concentration was determined by HPLC as previously described (11). Briefly, curcumin was isolated from samples by liquid/liquid extraction with ethyl acetate: methanol (95:5). Liquid chromatographic separation was achieved by isocratic elution on a Waters Nova-Pak C18, 3.9×150 mm, 4 μm particle size packing analytical column at PDA absorbance at a wavelength of 430 nm. The internal standard (IS), estradiol acetate, was positively identified from other peaks using PDA absorbance at a wavelength of 280 nm. The mobile phase consisted of a composition of acetonitrile: methanol: diH_2O: acetic acid (41:23:36:1), and measured pH of 3.3 Retention time for curcumin was 6.525±0.2 minutes.

Table III. *Curcumin CYP450 induction determination.*

	Relative % Increase in substrate metabolism	
Cytochrome P450	Inducer rifampin	Inducer curcumin
3A4 (2 h)	120.7%	39.1%
3A4 (24 h)	32.8%	18.1%
2C8/2C9 (2 h)	15.2%	0%
2C8/2C9 (24 h)	55.9%	0%
2D6 (2 h)	3.8%	0%
2D6 (24 h)	25%	4.6%

Table IV. *Common anticancer therapy and CYP450 metabolism.*

Drug	Substrate	Inhibitor	Inducer
Cyclophosphamide	2B6, 2C19		
Ifosfamide	2B6		
Paclitaxel	2C8/2C9, 3A4		2C8/2C9
Tamoxifen	2C9, 2D6, 3A4		
Docetaxel	3A4		
Irinotecan	3A4		
Vincristine	3A4		
Interferon	1A2	1A2	
Imatinib	3A4	3A4	
Doxorubicin	3A4	2D6	
Gemcitabine	None		
Carboplatin	None		

Results

High throughput CYP450 inhibition assays. Curcumin achieved minimal inhibition at all concentratiosn in the CYP450 3A4 or 2D6 *in vitro* studies (Table II). Only 10.5% and 22.5% inhibition of CYP450 2C9 and CYP450 2C8 was achieved at curcumin concentration of 58.3 μM, respectively. The curcumin concentration required to achieve 50% inhibition of CYP450 activity (IC_{50}) was 276.8 μM for CYP450 2C9 and 129.7 μM for CYP450 2C8.

Hepatic metabolism induction assay. The induction assays revealed an initial increase in substrate metabolism for CYP3A4 and no induction of metabolism for CYP2D6 or CYP2C8/2C9 (Table III). At two hours, the relative increase in substrate metabolism when compared to non-induced metabolism of substrate was 39.1% for CYP3A4, however, at 24 hours, the relative metabolism of substrate was 18.1%, suggesting there is a possible concentration-dependent induction for CYP3A4. Curcumin degrades rapidly at physiological temperature (37˚C) and pH (7.2). A 5.8% increase in curcumin metabolism resulted when hepatocytes were induced with rifampicin, indicating that curcumin metabolism is not significantly induced by rifampicin. Since minimal increase of 3.59% in curcumin metabolism occurred when curcumin was used as the inducer, curcumin does not appear to induce its own metabolism.

Discussion

Since minimal plasma concentrations have been achieved, no drug interactions via hepatic metabolism have been reported in peer reviewed literature with oral administration of curcumin. However, significant curcumin concentrations have been achieved with the administration of liposomal curcumin in pre-clinical pharmacokinetic studies in dogs and rats (Data not shown). Curcumin, has exhibited potential for drug interactions in previous *in vitro* studies (12, 13). In this study, curcumin demonstrated the potential to induce the CYP450 3A4 metabolism pathway at high concentration. This may contribute to the poor bioavailability of curcumin due to the prevalent expression and activity of CYP450 3A4

in the intestinal tract walls. It is currently unknown as to the role intestinal metabolism plays in combination with hepatic metabolism since in humans it has not been possible as of yet to uncouple and study these functions separately (14). With plasma concentrations in the nanomolar range for oral curcumin, hepatic CYPs would be unaffected. In contrast, the concentrations of curcumin in the intestinal tract after eight to 12 gram daily oral doses is likely to be in the micromolar range. Based upon this study, micromolar concentrations would be associated with induction of CYP3A4 thus decreasing bioavailability. Similarly, administering liposomal curcumin intravenously with concentrations in the micromolar range being achieved, the possible hepatic limited CYP450 3A4 drug interactions should be considered in development of combination chemotherapy regimens.

CYP450-mediated drug interactions can result in decreased activity or increased toxicity of one or both agents used in combination and have to be carefully evaluated in the cancer population. Since the majority of cytotoxic chemotherapy agents administered in the oncology setting have a narrow therapeutic index are, it will be important to evaluate the potential effect of liposomal curcumin in combination with other therapies in the context of CYP450 drug interactions through preclinical *in vivo* and/or *ex vivo* model experiments.

Both the positive control rifampicin and curcumin induced the CYP3A4 pathway in the *ex vivo* hepatocyte model. The drug interactions with rifampicin observed in clinical practice are significant and close monitoring for drug interaction in patients on rifampicin is generally recommended. Hence, patients receiving liposomal curcumin should be monitored closely for drug interaction with substrates of the CYP450 3A4 pathway (Table IV). Because of the short term, concentration dependent induction of CYP450 3A4 observed in the *ex vivo* model, the clinical significance of this potential interaction with other drugs is unknown and warrants further *in vivo* evaluation.

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