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Research Paper

Fluorocurcumins as Cyclooxygenase-2 Inhibitor: Molecular Docking, Pharmacokinetics and Tissue Distribution in Mice

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Purpose. The purpose of the current study was to assess the effect of newly synthesized Curcumin analogs on COX-2 protein by molecular docking studies and by assessments of the effect of one such analog (CDF) on nuclear factor NF-κB and PGE_2. In addition, we have determined the pharmacokinetics and tissue distribution of CDF in mice compared to Curcumin.
Methods. Molecular docking on COX-2 protein was assessed by standard computer modeling studies. PGE_2 assay in conditioned media was done utilizing high sensitivity immunoassay kit following manufacturer's instructions, while NF-κB was done by routine EMSA. Serum pharmacokinetics and tissue distribution studies were carried out using the validated high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods.
Results. The molecular docking showed that fluorocurcumin analogs do not introduce any major steric changes compared to the parent Curcumin molecule, which was consistent with down-regulation of NF-κB and reduced PGE_2 levels in cells treated with CDF. Pharmacokinetic parameters revealed that CDF had better retention and bioavailability and that the concentration of CDF in the pancreas tissue was 10-fold higher compared to Curcumin.
Conclusion. Our observations clearly suggest that the bioavailability of CDF is much superior compared to Curcumin, suggesting that CDF would be clinically useful.

KEY WORDS: COX-2; curcumin; fluorocurcumin; pharmacokinetics.

INTRODUCTION

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States; an estimated 37,000 new cases and almost equal number of deaths occurred in 2008 (1). The high mortality rate is in large part due to the high incidence of metastatic disease at initial diagnosis, the aggressive nature of the tumor, and the lack of effective systemic therapies. As a result, the disease-free survival time even after complete resection of the tumor and adjuvant administration of gemcitabine is less than a year (2). Since the administration of cytotoxic therapeutic agents has been found to be inadequate, there is a clear need for the development of new and novel chemotherapeutics, as well as targeted agents for the treatment of pancreatic cancer, because there is no curative therapy for this deadly disease.

There have been many reports linking inflammatory mediators including cytokines (e.g. TNF-alpha, IL-6, IL-8 and interferon-gamma (3–5)), transcription factors like NF-κB (6), and pro-inflammatory enzymes like Cyclooxygenase, as well as lipooxygenase isoforms (7,8), with the development and progression of pancreatic cancer. The expression of COX-2 has been found to be increased in a variety of malignancies including pancreatic cancer (8–11). It has been well-established that COX-2-mediated synthesis of prostaglandins (PGE_2) favors the growth of tumor cells by stimulating proliferation and angiogenesis (12). The COX-2 expression is regulated in part by transcriptional mechanism mediated by the transcription factor NF-κB, suggesting that inactivation of NF-κB pathway could also inhibit pancreatic cancer progression (13). This requirement is amicably met by Curcumin (diferuloylmethane) which is a naturally occurring active polyphenolic yellow pigment obtainable from the rhizomes of perennial herb *Curcuma longa* (14–23). In addition, the biological effects of Curcumin appear to be pleiotropic (15), suggesting the importance of Curcumin as a preventive and/or therapeutic agent against human malignancies. Most importantly, Curcumin has been reported to be very safe because it does not cause any adverse effects, even up to doses as high as

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8 gm per day in humans, and no development of resistance against Curcumin has been reported (24). However, the bioavailability of Curcumin is a major concern limiting its therapeutic utility, since as much as 75% of Curcumin gets excreted in the feces, indicating its poor absorption in the gut (25). Piperine, a known inhibitor of hepatic and intestinal glucuronidation has been shown to increase the bioavailability of Curcumin (26,27). In addition, different drug delivery systems, including liposomes, micelles, phospholipid complexes, and nanoparticles, have also been employed to improve Curcumin's bioavailability with disappointing and unacceptable results (27–32).

Since the chemical structure of Curcumin plays a crucial role in its biological activity, it is anticipated that enhanced absorption of Curcumin without loss in its activity can be achieved by preparing its appropriate analogs (33). Further studies have reported that cyclopentanone and cyclohexanone analogs have antibacterial properties indicating that of heteroaryl, and long chain substituents may enhance the activity of these compounds (34,35). More recently, pyrazolic and isoxaxolic analogs of Curcumin have also been prepared and evaluated for their neuroprotective activity (36). Another strategy employed to improve biological activity of Curcumin is through metal complexation (37), and some enhancement in anticancer activity has been reported by Kuttan *et al.* (38).

In our group, we have addressed the problem of slowing down the rapid metabolism of Curcumin by preparing its Knoevenagel condensates and their metal complexes, which were found to be more potent as anticancer agents than Curcumin, suggesting that such an approach may yield desirable analogs (39). Recently, we have explored the effects of introducing bioisosteric fluoro substitution in the Knoevenagel condensates and their corresponding Schiff bases (40) with the anticipation that the higher metabolic stability of C–F bond (than C–H or C–OH bonds) would slow down the metabolic breakdown of Curcumin, yielding an improved pharmacokinetic profile. We also found that amongst such analogs, CDF was superior in inhibiting the proteosome and cell growth and in inducing apoptosis (40). Based on these encouraging results, we have conducted studies to confirm the superiority of the new analog in the inactivation of NF-κB and one of its downstream targets, COX-2, through molecular modeling and corresponding bioassays. Most importantly, here we report, for the first time, the pharmacokinetic and tissue distribution of CDF in mice compared to Curcumin which clearly show that CDF is highly bioavailable, and especially accumulates in the pancreas, suggesting that CDF would be a better anti-tumor agent for the prevention and/or treatment of pancreatic cancer as well as other human malignancies.

EXPERIMENTAL DESIGN, MATERIALS AND METHODS

Cell Culture and Reagents

The human pancreatic carcinoma cell lines BxPC-3 and MIA PaCa-2 were obtained from American Type Culture Collection (Manassas, VA). The cell lines were maintained in continuous exponential growth by twice-a-week passaging in Dulbecco-modified Eagle's medium (DMEM; Life Technologies, Inc., Gaithesburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 10 mg/ml streptomycin. CDF and Curcumin were dissolved in DMSO at 10 mM/L concentration, aliquoted and stored at −20ºC and diluted to the desired concentration before use by media.

Materials

Curcumin and other chemicals required for synthesis of its analogs were obtained from Sigma-Aldrich (St. Louis, MO). Fluorocurcumin analogs were synthesized by a method that was described earlier by our laboratory (40).

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from treated samples, and EMSA was performed by incubating 10μg of nuclear extract with IRDye^{TM}–700 labeled NF-κB oligonucleotide as described earlier by Banerjee *et al.* (41). The DNA-protein complex formed was visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

PGE_2 Immunoassay for Quantitation of Prostaglandin E_2

For determination of PGE_2 levels, the BxPC-3 and MIAPaCa-2 cells were either untreated or treated with CDF (1 or 4 μM) and/or curcumin (1 or 4 μM) for 24 h. The conditioned medium was collected and analyzed for PGE_2 concentration according to manufacturer's protocol using PGE_2 high sensitivity immunoassay kit (R & D Systems, Minneapolis, MN). The optical density was measured at 450 nm and concentration of PGE_2 was calculated from the standard curve. Results are expressed as PGE_2 in pg/10^6 cells. Statistical comparison between treatment groups and untreated control was assessed by paired *t*-test.

Docking Studies

All calculations were performed using Auto Dock 3.05 software. The crystal structure of COX-2 protein was obtained from PDB ID (**6COX**). The active site of the enzyme was defined to include residues ALA562, GLU 346, GLN 350 within 0.65A radius to any of the inhibitor atoms. The Auto Dock 3.05 program, which is an automated docking program, was used to dock all seven fluorocurcumin analogs, as well as parent Curcumin molecule in the active site of the COX-2 enzyme. For each compound, the most stable docking model was selected based upon conformation of best scored predicted by the Auto Dock scoring function. The compounds were energy minimized with a MMFF94 force-field until the gradient convergence value of 0.05 kcal/mol was reached using distance-dependence dielectric function (ε=4r).

Experimental Animals

Seven-to-eight-week-old female ICR-SCID mice were purchased from Taconic Farms (Germantown, NY). The mice were housed and maintained under sterile conditions and were used in accordance with Animal Care and Use Guidelines of Wayne State University. Mice received Lab Diet 5021 (Purina Mills, Inc., Richmond, IN).

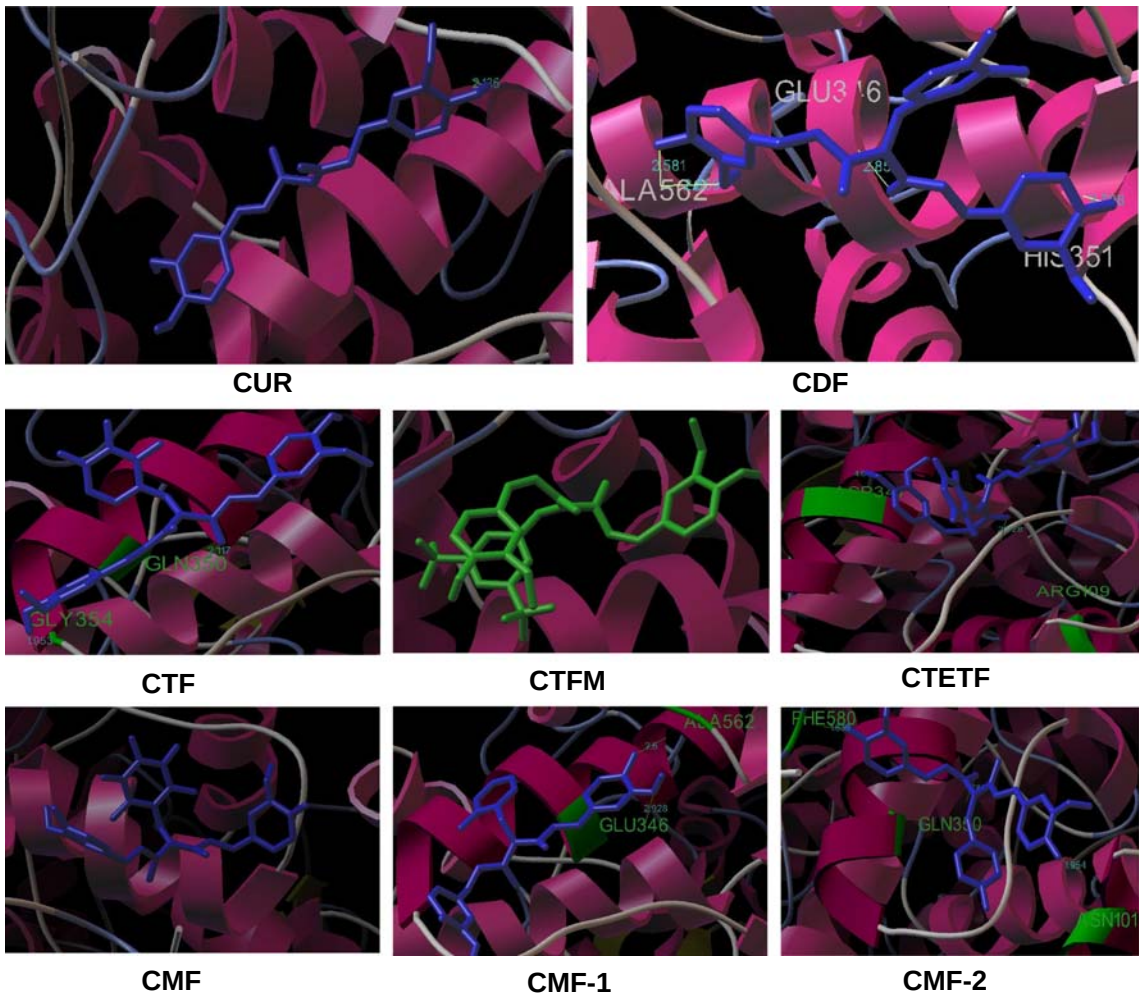

Fig. 1. Binding of fluorocurcumin analogs into the active site of COX-2 as assessed by computer modeling studies.

Pharmacokinetics and Tissue Distribution Studies

The pharmacokinetics and tissue distribution of Curcumin and CDF were examined in female mice. The mice were randomly divided into two groups, each with 18 mice. One group was given a single dose of Curcumin (250 mg/kg) diluted in 0.1 ml volume of sesame oil by intragastric intubation, and the other was similarly administered with a single dose of CDF (250 mg/kg). Blood and tissue samples were harvested before initiation of treatment (0 h) and at 1, 2,

Table I. Docking Results and Consensus Scores of Fluorocurcumin Analogs

Molecules	Docking Energy (kcal/mol)	Binding Energy (kcal/mol)	No. of H Bonds	H-bonding Residues	Log P
CUR	−7.78	−5.71	1	ALA 562	6.330
CDF	−9.93	−7.91	4	GLU 346 PHE 580 ASN 101 GLN 350	4.321
CTF	−10.31	−7.83	2	GYL 354 GLN 360	4.280
CTFM	−9.5	−6.15	–	–	4.160
CTETF	−9.27	−7.36	2	ALA 562 GLN 192	4.700
CPF	−10.61	−8.56	–	–	4.606
CMF-1	−6.84	−2.61	2	ASP 347 AGN 109	5.102
CMF-2	−9.63	−7.17	2	ALA 562 GLU 346	5.288

4, 6, 8, 12, 16, and 24 h following the intragastric administration. At each time-point, two mice were euthanized and ~200 μL blood was collected by cardiac puncture, and tissues (i.e., liver, lung, kidney, heart, pancreas, and colon) were harvested, washed free of blood with PBS, blotted dry, weighed, and stored at −80°C until analysis. The collected blood samples were allowed to clot and were centrifuged, and serum was separated and stored at −80°C until analysis.

Bioanalytical Assay

Curcumin and CDF in mouse serum and tissue samples were determined using the validated high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods as described below. The tissue samples were homogenized in 5 volumes of ice-cold 0.9% saline using VDI 12 homogenizer (VWR, USA). A 100 μL aliquot of serum or 250 μL aliquot of tissue homogenate was spiked with 500 μL (for serum) or 1 mL (for tissue) ethyl acetate (containing zileuton as internal standard 50 ng/mL). The mixture was vortex-mixed and centrifuged at 14,000 rpm for 5 min, and the top layer was transferred and evaporated to dryness under a stream of nitrogen in a water bath at 50°C±5°C. The residue was reconstituted in 100 μL of methanol/water containing 0.45% formic acid (70:30, v/v), and the mixture was centrifuged at 14,000 rpm for 5 min. One-hundred micro liters of the supernatant were injected into the HPLC and separated on a Waters XTerra MS column (2.1×50 mm, 3.5 μm i.d.) with a mobile phase consisting of methanol/water containing 0.45% formic acid (70:30, v/v) at a flow rate of 0.2 mL/min. The column effluent was monitored using a Waters Quattro Micro™ triple quadruple mass-spectrometric detector equipped with electrospray ionization source (Milford, MA, USA). Curcumin and CDF were monitored in the negative ionization mode at the transition of m/z, 367.1→148.8 and 491.1→216.9, respectively. The internal standard zileuton was monitored in the positive mode at the transition of m/z, 237.1→160.8. The calibration curves for Curcumin and CDF were constructed over the concentration range of 5 to 2,000 and 5 to 10,000 ng/mL, respectively, for the serum and tissue samples. The intra- and inter-day precision and accuracies of the assay was <15%.

Pharmacokinetic Data Analysis

Serum pharmacokinetic parameters were estimated using non-compartmental analysis with WinNonlin version 5.2 (Pharsight Corporation, Cary, NC). The maximum serum concentration (C_{max}) and the time of occurrence for maximum concentration (T_{max}) were obtained by visual inspection of the serum concentration-time curve after the drug administration. The total area under the serum concentration-time curve from time zero to the last measurable time point (AUC_{last}) was calculated using the linear and logarithmic trapezoidal method for ascending and descending serum concentrations, respectively.

RESULTS AND DISCUSSION

Since antioxidant property of Curcumin is a very crucial descriptor of most of its biological activities, we initially

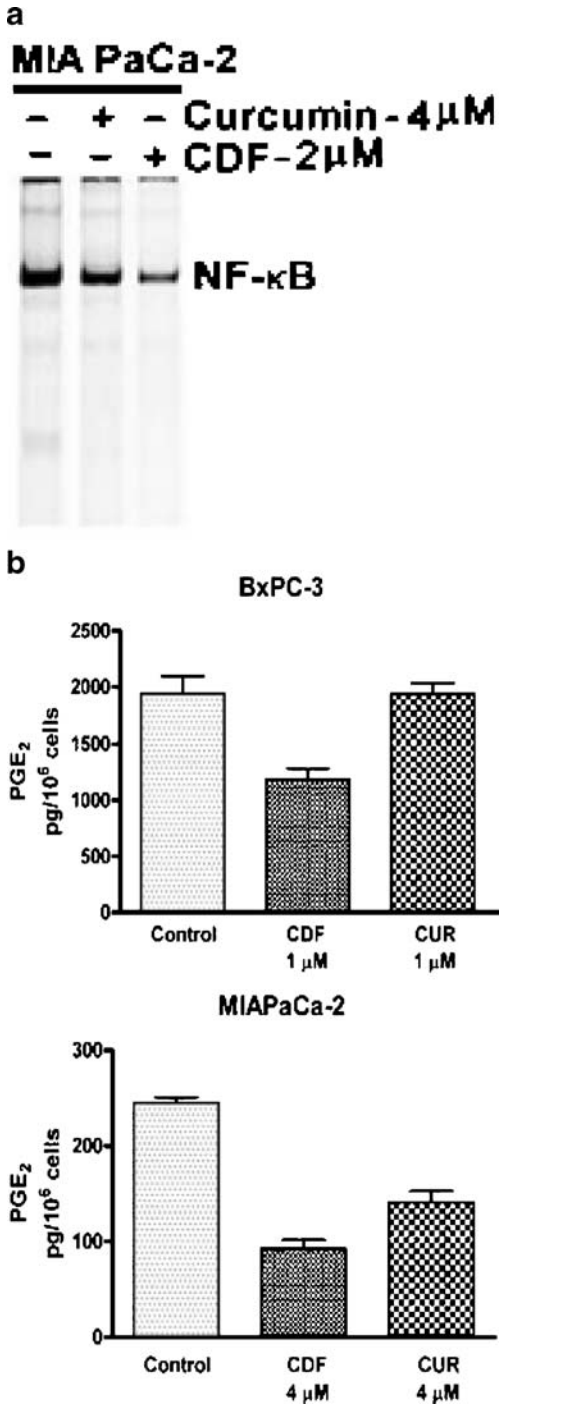

Fig. 2. (A) Electrophoretic mobility shift assay for NF-κB DNA binding activity in MIAPaCa-2 cells exposed to Curcumin and CDF at indicated concentrations; (B) PGE_2 activity in conditioned medium derived from CDF- and Curcumin-treated BxPC-3 and MIAPaCa-2 pancreatic cancer cells. A significant reduction in PGE_2 level was observed in BxPC-3 cells treated with CDF (P=0.0268), but no significant change in PGE_2 level was noted when cells were treated with Curcumin (P=0.9628). In MIAPaCa-2 cells, a substantial reduction in PGE_2 level was observed with both CDF (P=0.0024), and Curcumin (P=0.0076), but the effect was still better with CDF compared to Curcumin.

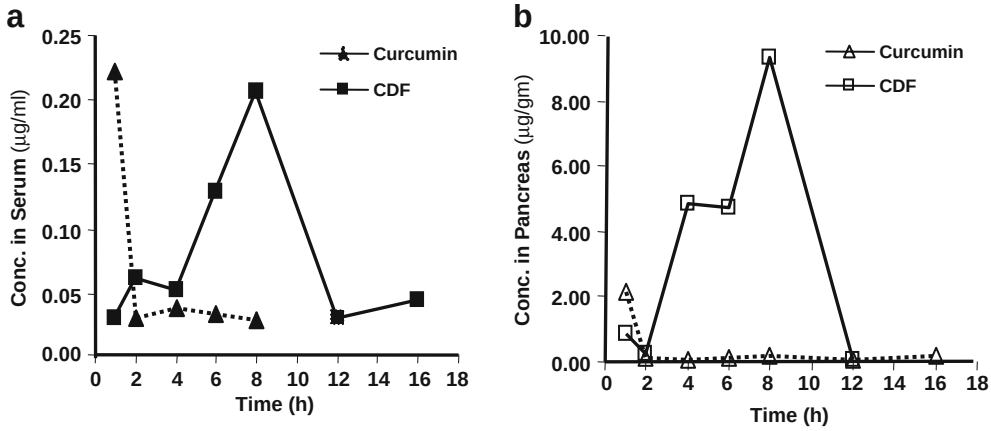

Fig. 3. Concentration *vs.* time profiling of Curcumin and CDF in mice serum (A) and pancreas (B) following single intragastric administration (250 mg/kg) in mice. Each point represents the mean concentration from two mice.

evaluated the antioxidant potential of all fluorocurcumin analogs synthesized presently using DPPH radical scavenging assay, which demonstrated significant antioxidant activity for CDF compound showing the lowest IC_{50} value (0.05 µM) (data not shown), which was lower than parent Curcumin molecule (35 µM). These results are consistent with published data by Sompran *et al.* (42). Since CDF also exhibited low IC_{50} value for the anti-proliferative activity against COX-2 positive BxPC-3 pancreatic cancer cells by MTT assay in our earlier study (40), we were prompted to perform molecular docking studies to understand ligand-protein interactions and COX-2 selectivity of the new analogs. All fluorocurcumin analogs were found to dock into the active site of COX-2, confirming that fluoro substitution does not introduce any major steric changes in the parent Curcumin molecule except to allow more hydrogen bonding interactions (Fig. 1 and Table I). The binding energies of these analogs were in the range −2.6 to −8.56 kcal/mol compared to Curcumin's −5.71 kcal/mol. The lower interaction energy observed for CDF analog rationalizes the tighter binding of this compound in the active site of COX-2 than other analogs. In our docking experiments, Curcumin showed only one H-bonding interaction with ALA562. On the other hand the most potent fluoro analog, viz. CDF exhibits four H-bonding interactions involving residues GLU 346, PHE 580, ASN101 and GLN 350, respectively. All other compounds (except CTFM and CPF) exhibited a maximum of two H-bonding interactions, wherein the residue ALA562 is being common with parent Curcumin. Favorable van der Waals interactions between styryl carbon atoms and the hydrophobic residues such as GLU 346(3.01A), SER 353(3.13A), between methoxy group of CDF and HIS 351, ALA 582 contributed to stabilize the ligand-enzyme complexes (Fig. 1). The lower liposolubility observed for the CDF analog suggests that it should have slower metabolism with enhanced pharmacokinetic profile, which was further confirmed as presented below.

Since we found that CDF docks into the active site of COX-2, which is transcriptionally regulated by NF-κB, we believed that similar to Curcumin, CDF may also have an effect on the nuclear transcription factor NF-κB and also COX-2 activity, which was assessed by measuring the effects of CDF on NF-κB DNA-binding activity in MIAPaCa-2 cells, and PGE_2 production in both MIAPaCa-2 and BxPC-3 pancreatic cancer cells. The efficacy of CDF was compared with Curcumin. PGE_2 assay was performed using COX-2 over-expressing BxPC-3 and MIAPaCa-2 cells using CDF (1 or 4 µM) compared to similar concentration of Curcumin (1 or 4 µM). As widely reported, Curcumin caused down-regulation of NF-κB, but the effect was more pronounced with a low concentration of CDF. Additionally, we found that both drugs caused a significant decrease in the PGE_2 levels in MIAPaCa-2 cells with a p value of 0.0076 with Curcumin and 0.0024 with CDF (Fig. 2B). However, in BxPC-3 cells, we found a significant decrease in PGE_2 level only in CDF-

Table II. Comparative Pharmacokinetic Analysis of Curcumin and CDF in Serum and Pancreas Following a Single Intragastric Administration (250 mg/kg) in Mice. Data are Expressed as the Mean from Two Mice

	Serum		Pancreas	
	Curcumin	CDF	Curcumin	CDF
T_{max} (h)	1.0	8.0	1.0	8.0
C_{max} (µg/mL for serum, µg/g for pancreas)	0.22	0.21	2.15	9.35
T_{last} (h)	8.0	16.0	16.0	12.0
C_{last} (µg/mL for serum, µg/g for pancreas)	0.03	0.04	0.20	0.04
AUC_{last} (h*µg/mL for serum, h*µg/g for pancreas)	0.44	1.22	3.46	36.56

C_{max} maximum serum concentration, T_{max} the time to achieve maximum concentration, C_{last} last measurable concentration, T_{last} the time for the last measurable concentration, AUC_{last} total area under the serum concentration-time curve from time zero to the last measurable time point

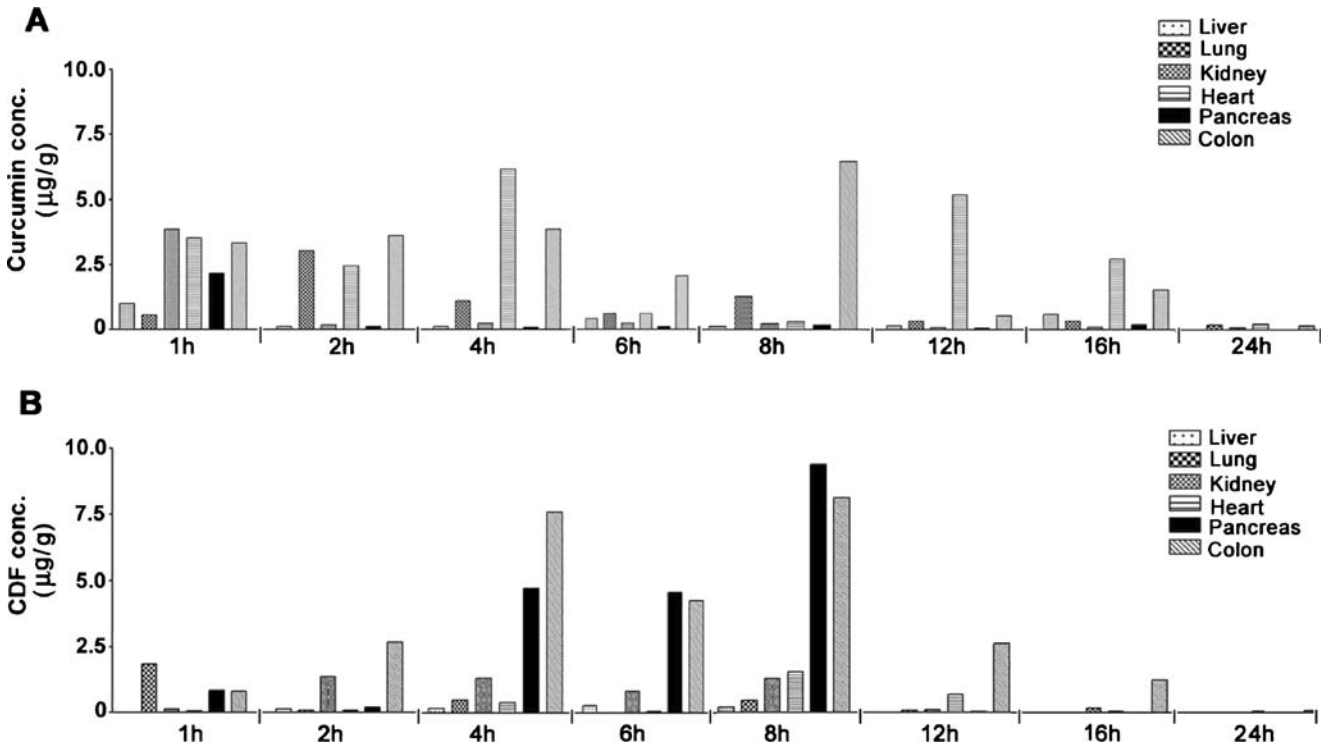

Fig. 4. Concentration *vs.* time profiling of Curcumin (A) and CDF (B) in mouse tissues following single intragastric administration (250 mg/kg) in mice. Each point represents the mean concentration from two mice.

treated cells ($p=0.0268$), but such results were not found with Curcumin treatment (Fig. 2B; $p=0.9628$). These results clearly suggest that CDF is a better target of COX-2, resulting in a greater inhibition of PGE_2 production in both the cell lines relative to Curcumin.

The concentration and time profiles of Curcumin and CDF in serum and pancreas tissue following a single dose oral administration (250 mg/kg) in female ICR-SCID mice are depicted in Fig. 3A and 3B. Pharmacokinetic parameters for Curcumin and CDF are summarized in Table II. Following a single oral dosing of 250 mg/kg in mice, Curcumin achieved the maximum serum concentration (C_{max}) of 0.22 μg/mL at 1 h, after which serum concentration of Curcumin declined rapidly and was undetectable after 8 h (below the lower limit of 5 ng/mL) (Fig. 2A). The AUC_{last} was estimated as 0.44 μg/mL*h (Table II). Compared to Curcumin, CDF achieved a similar C_{max} (0.21 μg/mL) with a relatively slow oral absorption with T_{max} of 8 h; however, CDF had 2.7-fold higher systemic drug level than Curcumin (AUC_{last}, 1.22 *vs.* 0.44 μg/mL*h; Table II).

The distribution of Curcumin and CDF following single dose administration of 250 mg/kg body weight in mice is presented in Fig. 4A and 4B. As shown, both Curcumin and CDF were detectable in all tissues tested, including liver, lung, kidney, heart, pancreas, and colon. However, Curcumin and CDF were detectable at high concentrations in colon after oral administration. Interestingly, Curcumin was found to be present mainly in heart and lung, while CDF accumulated preferentially in the pancreas (Figs. 3B and 4B). Moreover, consistent with serum concentration *vs*. time profile, Curcumin and CDF also achieved the maximum concentration in pancreas at 1 and 8 h, respectively, after oral administration (Fig. 3B and 4B). The C_{max} and AUC_{last} of CDF in pancreas were 4.3- and 10.6-fold those for Curcumin, respectively (Table II), suggesting that CDF has a better bioavailability profile, especially in pancreas tissue. This observation further suggests that CDF would show better anti-tumor activity against pancreatic cancer, which is being currently tested in our laboratory.

Curcumin has been demonstrated to have a low oral bioavailability in animals and humans perhaps because of its rapid secretion as conjugates (28). Consistent with previous findings, we also observed very low serum levels of Curcumin after oral administration in mice. Following a single oral dose of 250 mg/kg in mice, Curcumin achieved the C_{max} of 0.22 μg/mL at 1 h, after which Curcumin serum concentration declined rapidly and was undetectable after 8 h (Fig. 3A and 4A). It is important to note that our results were similar for the two animals. Collectively, our results are also consistent with a previously reported mouse study in which oral administration of 1 g/kg body weight of Curcumin resulted in a C_{max} of 0.22 μg/mL at 1 h, and the serum concentrations then declined below the detection limit by 6 h (43). It has been postulated that poor water solubility and extensive first-pass intestinal and hepatic metabolism are attributable, for a large part, to the low oral bioavailability of Curcumin (43,44). In contrast, CDF, which is an analog of Curcumin, exhibited enhanced bioavailability, which could be due in part to its low excretion rate as conjugates, although further in-depth investigations are needed in this area. Interestingly, oral administration of CDF produced 2.7-fold more increase in systemic drug level than Curcumin (AUC_{last}, 1.22 *vs.*

0.44 μg/mL*h; Table II), although the underlying mechanisms by which CDF shows superior bioavailability need further investigations.

Consistent with our objective, improved water solubility of CDF may account, at least in part, for its enhanced oral bioavailability. The water solubility of CDF was determined to be 8.4-fold higher than Curcumin (data not shown). Both Curcumin and CDF were found to be present, albeit in different concentrations, in almost all tissues tested, including liver, lung, kidney, heart, pancreas and colon. Of interest, CDF was accumulated preferably in pancreas (Figs. 3B and 4B). The C_{max} (at 8 h) of CDF achieved in pancreas was 44.5-fold higher than in serum (Figs. 3 and 4, and Table II). Furthermore, relative to Curcumin, the concentration of CDF in pancreas was 10.6-fold higher (Table II). The mechanism for this observed preferential accumulation of CDF in pancreas remains to be determined. However, due to its high accumulation in pancreas, we speculate that CDF may be a good drug candidate for either inclusion in the prevention strategy of pancreatic cancer or for the treatment of pancreatic cancer in conjunction with cytotoxic agents similar to those reported for Curcumin (45–48). Therefore, CDF could become an ideal chemosensitizer in restituting sensitivity of pancreatic cancer to cytotoxic drugs, which must be tested in future studies for optimizing a successful treatment regimen for human pancreatic cancer.

In conclusion, here we have presented evidence in support of a superior, sustainable, and biologically active analog of Curcumin that could prove highly valuable in the future, not only in chemoprevention research in populations at risk of developing cancer, but also in an effective arsenal for the treatment of pancreatic and other human cancers either alone or in combination with conventional therapeutics. Moreover, in view of its preferential accumulation in the pancreas and its ability to down-regulate NF-κB, as well as reduce the levels of PGE_2 concentration, it could further be exploited for molecular-targeted therapy against COX-2 over-expressing tumors including pancreatic cancer.

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