Pulmonary Administration of Water-soluble Curcumin Complex Reduces ALI Severity

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ONLINE DATA SUPPLEMENT

ONLINE SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell Culture

Calu-3 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium and nutrient mixture F12 (DMEM:F12) with 1% (v/v) non-essential amino acids, 1% (v/v) penicillin-streptomycin and 10% fetal bovine serum (FBS). Cultures were maintained at 37°C in a humidified incubator with 95% air/5% CO_2. For the transport experiments, Calu-3 cells (passage 26-36) were seeded at 5×10^5 cells/cm^2 on Transwell inserts with polyester membranes (area, 0.33 μm^2; pore size, 0.4 μm) (Corning Life Sciences; Lowell, MA). An air-liquid interface culture (ALC) was then created by aspirating the medium in the apical compartment after overnight culture. The apical side of the membrane was washed with HBSS to remove unattached cells and the medium in the basolateral compartment was replaced with fresh medium.

Investigational Curcumin Formulation

Hydroxypropyl-γ-cyclodextrin was dissolved to a concentration of 112 g/l in 0.18 mol/l sodium hydroxide solution. Curcumin (Curcumin C3 Complex; Sabinsa Corporation) was added to a concentration of 15 g/l. The solution was agitated and after complete dissolution of curcumin the pH was adjusted to pH 6.0 with a mixture of hydrochloric and citric acids. The solution was sterile filtered and filled aseptically into sterile vials, then capped and sealed. The recovered CDC solution contained 12 g/l curcumin and 93 g/l cyclodextrin in 20 mM sodium citrate, 100 mM NaCl solution. Endotoxin content was less than 1.8 IU/ml as measured by the Limulus amebocyte lysate gel clot method. The CDC solution was stored at 2-8°C protected from light. The cyclodextrin vehicle was prepared in the same way but without the addition of curcumin.

Curcumin Transport across the Calu-3 Cell Monolayer

On day 6 of ALC culture, the medium in the basolateral compartment was removed and the apical and basolateral sides of the cell monolayers on the membrane inserts were washed twice with an HBSS transport buffer (HBSS with 10 mM HEPES and 25 mM D-glucose, pH 7.4). The Calu-3 cell monolayers on the polyester membranes were then pre-equilibrated with HBSS transport buffer in the apical and basolateral compartments for 20 min at 37°C. The integrity of the cell monolayers was examined by transepithelial electrical resistance (TEER) measurements using the Millicell ERS (Millipore; Billerica, MA). The TEER values of the cell monolayers were corrected by subtracting the blank TEER values without the cells in the inserts and the TEER values ($\Omega \cdot cm^2$) for the 24-well inserts were obtained by using the area of the membranes (0.33 μm^2). Cell monolayers with TEER values ~ 350 $\Omega \cdot cm^2$ were used for the Lucifer Yellow (LY) and curcumin transport experiments. LY permeability served as a further check on integrity of the cell monolayers. LY (1 mM in HBSS transport buffer) was added to the donor compartment and apical-to-basolateral (AP→BL) or basolateral-to-apical (BL→AP) transport was measured during 180 min incubation on rocking platform shakers at 37°C in a 5% CO_2 incubator. LY in the samples and the standard was measured by a plate reader (BioTEK; Winooski, VT) set for 485 nm (excitation)/540 nm (emission).

For CDC transport experiments, CDC stock solutions were diluted with HBSS transport buffer to concentrations of 50, 100, and 200 μM (donor concentrations). Curcumin solution at each concentration was added to the donor compartment (apical for AP→BL; basolateral for BL→AP transport) with the corresponding curcumin-free HBSS buffer in the receiver compartment. Cells were incubated for 180 min with shaking and samples were collected from the receiver compartment at 0, 5, 10, 30, 60, 90, 120, 150, and 180 min. Samples were also

collected from the donor compartment at 180 min. The donor and receiver sides of the inserts were washed twice with HBSS transport buffer and these washings were also included in the mass calculations. Fluorescence of curcumin in the standard and sample solutions was measured in 96-well optical bottom plates using a plate reader (BioTEK Synergy) at 485 nm (EX)/540 nm (EM). For AP→BL or BL→AP transport, the transcellular permeability coefficient, P_{eff} (cm/sec) was calculated by dividing the AP→BL or BL→AP mass transport rate (*dM/dt*) by the product of insert area, A (0.33 cm^2) and initial donor concentration of curcumin, C_o, as shown in the following equation:

$$P_{\text{eff}} = \frac{\frac{\mathrm{d}M}{\mathrm{d}t}}{A \cdot \mathrm{C_o}}$$

Following each transport experiment, TEER was measured and the cell monolayers were examined by light contrast inverted microscopy to confirm that integrity of the cell monolayers remained unchanged.

To compare effects on transport and monolayer integrity of the uncomplexed curcumin powder with those of CDC, curcumin stock solutions were prepared by dissolving the uncomplexed curcumin powder in DMSO (DMSO-C) or absolute ethanol (EtOH-C). These stock solutions were then diluted with HBSS transport buffer to make donor solutions and these solutions were used for transport experiments as described.

Cellular Curcumin Binding

After the transport assays, the inserts and attached cells were washed twice with cold HBSS transport buffer to remove unbound curcumin. The cells were then incubated with Hoechst 33342 solution in HBSS in 24-well inserts for 30 min at 37°C in a 5% CO_2 incubator. Media in the apical compartment was 100 µl of 10 µg/ml Hoechst 33342 (Invitrogen; Carlsbad, CA) in

HBSS and that in the basolateral compartment was 600 µl of dye-free HBSS transport buffer. Following incubation, the inserts were washed with HBSS transport buffer and examined with the 10 × objective of a Nikon TE2000 fluorescence microscope equipped with a XF93 triple pass filter set (Omega Optical; Brattleboro, VT). Cell-associated curcumin could be detected with the fluorescein isothiocyanate (FITC) channel of the filter set while the nuclei were visualized with the diamidino-2-phenylindole (DAPI) channel. Inserts from all experimental conditions were examined for curcumin association and intactness of the cell monolayers.

Other inserts from each experimental condition were used for quantitative determination of curcumin cell-associated mass. These inserts were incubated with 1% Triton X-100 (100 µl in the apical compartment; 600 µl in the basolateral compartment) for 1.5 hours at 37°C in a 95% air/5% CO_2 incubator. The concentration of curcumin extracted into the Triton X-100 medium was measured with a plate reader at 485 nm (EX)/540 nm (EM) and cell-bound curcumin mass per cell was calculated from the cell numbers counted with a hemocytometer following detachment by trypsinization.

Wet:Dry Weight Ratio

The superior lobe of the right lung was excised and the wet weight was recorded. The tissue was then placed in an incubator at 60°C for 24 hours, after which the dry weight was recorded and the ratio of wet to dry weight was calculated.

Lung Histology

For general morphology, sections from formalin-fixed, paraffin-embedded lung tissues were stained with hematoxylin and eosin. For fluorescence analysis, lung tissues were infused with a solution of 50% Tissue-Tek optimum cutting temperature (O.C.T.) compound (Sakura; Torrance, CA) and 50% PBS. The lungs were then frozen and sectioned.

Canine Toxicology and Pharmacokinetics

CDC (34.8 mM in physiological saline) was administered intravenously (IV) to beagle dogs (n = 1/group) at doses of 1 or 4 mg/kg daily or at a dose of 10 mg/kg twice a day. Dosing was continued for 14 days. The first dose was infused over the course of 5 min, subsequent doses over the course of 3 min. On days 1, 7, and 14 blood was drawn at specified intervals following infusion and concentrations of curcumin and its metabolites were determined by mass spectrometry. Blood for a complete cell count and determination of clinical chemistry parameters was drawn on days 2, 7, and 14. Two to four hours after the last infusion the animals were sacrificed and an autopsy was performed.

Supplemental Results

In Vivo Pharmacokinetics of Curcumin and its Metabolites

Preliminary studies on IV curcumin pharmacokinetics were conducted in beagle dogs. The CDC formulation was given at doses of 1 mg/kg and 4 mg/kg once daily and at 10 mg/kg twice daily for 14 days. Serial blood samples were drawn in EDTA anticoagulant before and 5 min, 15 min, 30 min, 60 min, 2 and 4 hours following the morning dose on days 1, 7 and 14. Blood samples were kept on ice bath and plasma was separated within 20 min. Plasma concentrations of curcumin and its major circulating metabolites, tetrahydrocurcumin and tetrahydrocurcumin sulfate, were analyzed by liquid chromatography-mass spectrometry (LC-MS).

Briefly, 200 µl of dog plasma was combined with 50 µl methanol, 1 ml of 1M ammonium acetate pH 5, 3 ml of 90% ethyl acetate/10% propanol, and 20 µl of internal standard (1 ng/µl in methanol) in a 13 × 100 mm glass tube. Tubes were capped and shaken for 30 minutes, then centrifuged at 1500 × g for 10 minutes. The organic layer was transferred to clean

12 × 75 mm glass tubes and evaporated to dryness under nitrogen, then reconstituted in 50 µl methanol. Two-µl aliquots of each sample were injected onto an LC-MS. Chromatography was performed on an Agilent 1100 Series HPLC system equipped with a Zorbax SB-C18 column (150 × 2.1 mm i.d., 5 µm particle size). The mobile phase consisting of 1 mM ammonium acetate, pH 4.5 (A) and acetonitrile (B) was pumped at a flow rate of 0.3 ml/min according to the following gradient: 75% A and 25% B (0 min) → 45% A and 55% B (5 min) → 30% A and 70% B (7.5 min) and stopped at 15 min; there was a post-run equilibration of 4 min. Detection of the analytes was performed on an Agilent G1956B Series or G1946B MSD run in negative electrospray ionization (ESI^-) mode, with a drying gas temperature of 350°C and flow rate of 12 l/min, and a nebulizer pressure of 35 psi. Ions monitored included m/z 253 (chrysin, IS), m/z 367 (curcumin), m/z 371 (tetrahydrocurcumin), m/z 467 (tetrahydrocurcumin sulfate), and m/z 543 (curcumin glucuronide) (1). The fragmentor was optimized to 120 V (for curcumin) and 160 V (for chrysin), while the capillary voltage was optimized to 3600 V. Curcumin glucuronide was synthesized enzymatically from curcumin and UDP-glucuronide by using rat liver microsomes and curcumin sulphate and THC sulphate were synthesized as described before (1).

No changes in the kinetics of plasma curcumin or its major metabolite, tetrahydrocurcumin, were observed over the 2 weeks of daily curcumin treatment; hence the mean plasma concentration-time data collected on days 1, 7 and 14 are presented. The half-life of curcumin in beagle dogs following IV CDC infusion was approximately 7 min independent of dose (Figure E1). The metabolite tetrahydrocurcumin (THC) appeared almost immediately after infusion of curcumin, then declined at a rate similar to that of the parent compound (Figure E1). Little or no THC sulfate was detectable on day 1, but a prominent mass ion peak appeared at 30

min after infusion on days 7 and 14 (Figure E2). On day 14 this compound was the major curcumin metabolite present at times ≥ 15 min following infusion (Figure E3).

These results indicate that curcumin is rapidly cleared from the bloodstream following IV administration of CDC. This calls into question the feasibility of systemic CDC administration in pulmonary therapy.

Toxicology

Complete blood cell count and clinical chemistry parameters including biomarkers of muscle/heart, liver, kidney and pancreas function (Table E1) were determined on days 0 (prior to initial CDC administration) and 13. Autopsy was carried out 2-4 hours after the last injection. Histopathological examination of tissue biopsies was carried out by a veterinary pathologist. Biopsies were taken from buccal mucosa, esophagus, small intestine, large intestine, heart, lung, liver, gall bladder, pancreas, kidney, urinary bladder, testicle/ovary, skeletal muscle and bone marrow, including a smear from bone marrow.

No adverse effects were observed in any dog. Hematologic and clinical chemistry parameters were normal throughout dosing, except that blood glucose levels were slightly below the reference range both before and during treatment (Table E1). No abnormalities were observed at autopsy except a parasitic lung infection believed to have been present prior to the study. No curcumin was detected in tissue samples. Systemically administered CDC thus appears safe in this small preclinical study, supporting the expectation of safety following delivery directly to the lung in pulmonary disease.

Supplemental Reference

1. Ireson C, Orr S, Jones DJ, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer S, Jukes R, Williams M, Steward WP, and Gescher A. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. Cancer Res 2001; 61:1058–1064.

Supplemental Figure Legends

Figure E1. Time Course of Curcumin and Tetrahydrocurcumin (THC) Plasma Concentrations Following Intravenous Administration of CDC. CDC was administered at a dose of 10 mg/kg and blood was drawn for determination of plasma curcumin and THC concentrations at the indicated intervals following infusion. Values are the mean of measurements on days 1, 7, and 14, as no between-day differences in pharmacokinetics of these compounds was observed.

Figure E2. Time Course of Tetrahydrocurcumin Sulfate (THC-S) Plasma Concentration Following CDC Infusion. Doses of 10 mg/kg were administered intravenously twice daily for 14 days. On days 1, 7, and 14 blood was drawn at indicated intervals following infusion and plasma concentrations of THC-S were determined.

Figure E3. Comparative Plasma Concentration-Time Profile of Curcumin and its Major Circulating Metabolites after Repeated CDC Administration. CDC (10 mg/kg) was

administered intravenously twice daily for 14 days. On day 14, blood was drawn at indicated intervals following infusion and curcumin, THC, and THC-S were determined.

Table E1. Laboratory Parameters of Dogs Receiving Different Dosages of CDC.

Values are shown before (Day 0) and after (Day 13) administration of CDC at the indicated daily doses for 14 days. 20 mg/kg was given as two daily infusions.

		1 mg/kg		4 mg/kg		20 mg/kg		Reference
		Day 0	Day 13	Day 0	Day 13	Day 0	Day 13	values
WBC	/dl	7700	7470	7070	7550	7180	10500	
HCT	%	47	46	50	49	47	45	
PLT	/dl	269000	307000	137000	179000	327000	391000	
Poly	%	32	42	55	65	53	66	
ANC	/dl	2464	3137	3888	4907	3805	6930	
Ly	%	52	42	31	22	29	23	
Mo	%	2	6	8	7	10	10	
Eo	%	14	10	6	6	8	1	
Glucose	mg/dl	54	45	58	63	58	51	65-130
BUN	mg/dl	11	18	16	15	17	26	6-29
Crea	mg/dl	0.9	0.9	0.9	0.9	0.8	0.8	0.6-1.6
Na	mEq/l	149	147	149	149	148	149	140-158
K	mEq/l	5.3	5.3	5.1	4.5	4.9	5.2	4.0-5.7
Na/K		28	28		33	30	29	27-40
Cl	mEq/l	108	110	108	108	111	111	100-115
CO_2	mEq/l	26	26	25	23	22	25	18-26
Anion gap		20	16	21	23	20	18	13-25
Ca	mg/dl	10.8	10.8	10.9	11	10.1	10.1	8.0-12.0
P	mg/dl	6.0	6.3	6.4	6.1	3.7	5.3	3.0-7.0
Osmol		294	292	296	294	294	299	270-310
Total prot	g/dl	5.9	5.8	6.5	5.9	6.1	6.5	5.4-7.6
Albumin	g/dl	3.3	3.3	3.6	3.4	3.2	3.4	2.3-4.0
Globulin	g/dl	2.6	2.5	2.9	2.5	2.9	3.1	2.7-4.4
Alb/Glob		1.3	1.3	1.2	1.4	1.1	1.1	0.6-1.2
Bil	mg/dl	0.1	0.1	0.1	0.2	0.1	0.1	0.0-0.5
ALP	U/l	120	102	88	75	49	57	10-84
GGT	U/l	1	0	2	0	0	2	0-10
ALT	U/l	31	31	48	55	42	51	5-65
AST	U/l	48	51	56	56	52	53	16-60
CK	U/l	324	355	319	309	300	331	50-300
Chol	mg/dl	200	183	188	178	143	143	150-275
Amylase	U/l	887	752	588	565	1130	1189	300-1500
Lipase	U/l	288	223	455	299	224	231	0-425

Figure E1

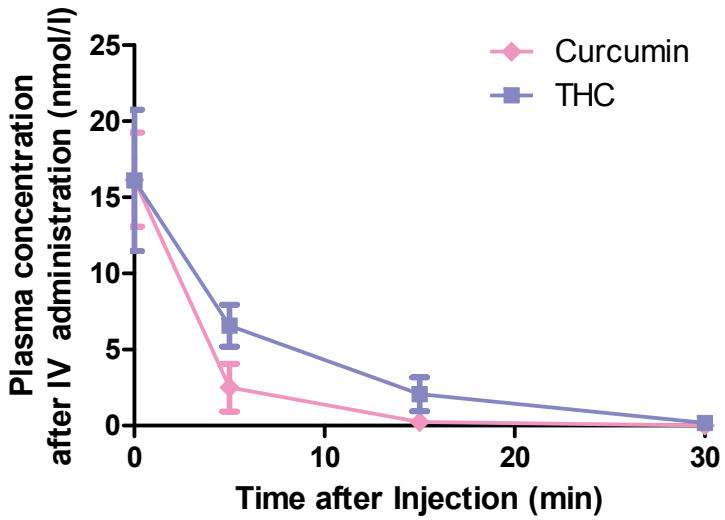

Plasma concentration after IV administration (nmol/l)
25
20
15
10
5
0
0
10
20
30
Time after Injection (min)
Curcumin
THC

Figure E2

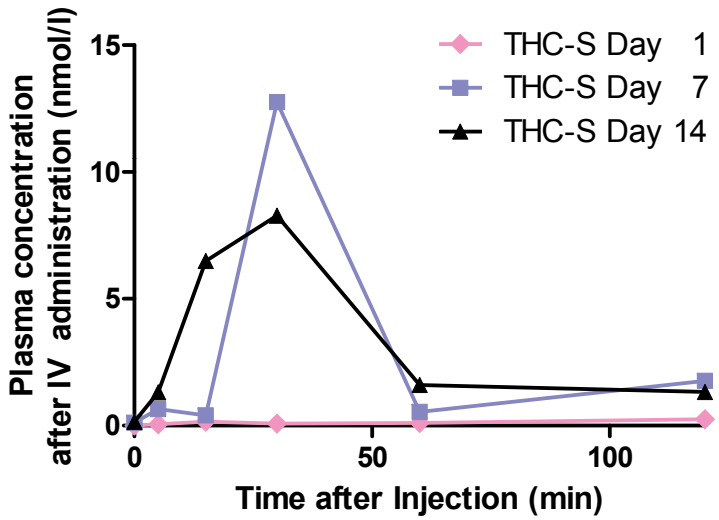
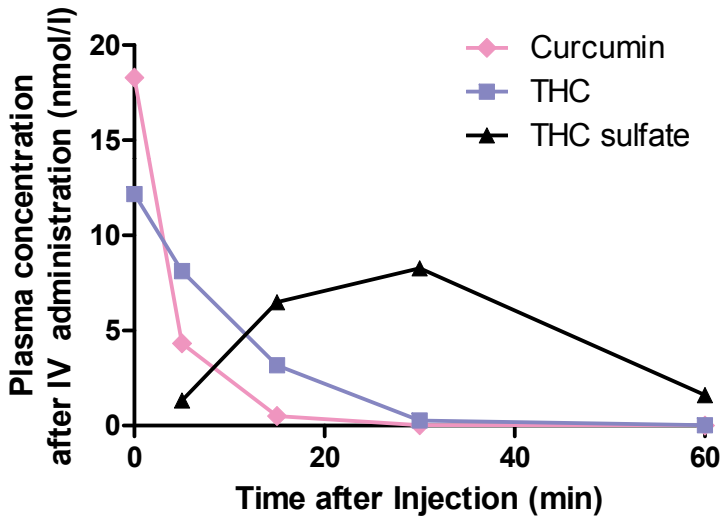

Plasma concentration after IV administration (nmol/l)
15
10
5
0
0
50
100
Time after Injection (min)
THC-S Day 1
THC-S Day 7
THC-S Day 14

Figure E3


Plasma concentration after IV administration (nmol/l)
20
15
10
5
0
0
20
40
60
Time after Injection (min)
Curcumin
THC
THC sulfate