ANTICANCER RESEARCH *37*: 3483-3492 (2017)
doi:10.21873/anticanres.11716

Distribution and Metabolism of Lipocurc™ (Liposomal Curcumin) in Dog and Human Blood Cells: Species Selectivity and Pharmacokinetic Relevance

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Abstract. *Background/Aim: The aim of this study was to investigate the distribution of curcumin (in the form of Lipocurc™) and its major metabolite tetrahydrocurcumin (THC) in Beagle dog and human red blood cells, peripheral blood mononuclear cells (PBMC) and hepatocytes. Materials and Methods: Lipocurc™ was used as the source of curcumin for the cell distribution assays. In vitro findings with red blood cells were also compared to in vivo pharmacokinetic data available from preclinical studies in dogs and phase I clinical studies in humans. Results: High levels of curcumin were measured in PBMCs (625.5 ng/g w.w. cell pellet or 7,297 pg/10^6 cells in dog and 353.7 ng/g w.w. cell pellet or 6,809 pg/10^6 cells in human) and in hepatocytes (414.5 ng/g w.w. cell pellet or 14,005 pg/10^6 cells in dog and 813.5 ng/g w.w. cell pellet or 13,780 pg/10^6 cells in human). Lower curcumin levels were measured in red blood cells (dog: 78.4 ng/g w.w. cell pellet or 7.2 pg/10^6 cells, human: 201.5 ng/g w.w. cell pellet or 18.6 pg/10^6 cells). A decrease in the medium concentration of curcumin was observed in red blood cells and hepatocytes, but not in PBMCs. Red blood cell levels of THC were ~5-fold higher in dog compared to human and similar between dog and human for hepatocytes and PBMCs. The ratio of THC to curcumin found in the red blood cell medium following incubation was 6.3 for dog compared to 0.006 for human, while for PBMCs and hepatocytes the ratio of THC to curcumin in the medium did not display such marked species differences. Conclusion: There was an excellent correlation between the in vitro disposition of curcumin and THC following incubation with red blood cells and in vivo plasma levels of curcumin and THC in dog and human following intravenous infusion. The disposition of curcumin in blood cells is, therefore, species-dependent and of pharmacokinetic relevance.*

Curcumin (diferuloylmethane) is being extensively investigated for its anticancer, anti-inflammatory and antioxidant properties with promising results. Clinical studies with curcumin have indicated that it has limited, albeit, significant anticancer activity against pancreatic, colorectal cancer and leukemia and alters the regulation of oncogenes (1-4). Anti-inflammatory activity for curcumin has been observed in a number of conditions such as inflammatory bowel disease, pancreatitis, arthritis and chronic anterior uveitis (5). Challenging the therapeutic potential of curcumin, is its limited exposure following both oral and parenteral routes of administration. Following oral administration of large doses of curcumin, poor solubility and gut metabolism, in particular by gut microorganisms *via* the reductase pathway (6) are limitations for the amount of curcumin entering the blood (7, 8). Curcumin (Figure 1) is a chemically unstable molecule in alkaline buffer (higher pH) (9), breaking down into the predicted major product trans-6-(4'-hydroxy-3'methoxyphenyl)-2.4-dioxo-5-hexenal. The presence of albumin increases the chemical stability of curcumin (10). Following bolus intravenous administration, curcumin is rapidly metabolized by reductases, to the reduced forms dihydrocurcumin, tetrahydrocurcumin (THC)

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Key Words: Curcumin, tetrahydrocurcumin, blood cells, distribution, metabolism.

and hexahydrocurcumin (11). Curcumin and the reduced forms of curcumin can be further processed into alcohols and conjugated by either glucuronidation or sulfation (11). Pharmacokinetic studies of curcumin revealed plasma half-life tissue half-life and mean residence time in rats, Beagle dogs and humans that ranged from 6-42 min following either bolus intravenous administration or the termination of intravenous infusion (12-16). The proposed working hypothesis is that hepatic metabolism is a key pharmacological deactivation step (11, 17). Thus, the short plasma half-life of curcumin is considered to be due to extensive and rapid phase I and phase II metabolism by the liver and to some extent in other tissues (11, 14, 17, 18-21).

Given its instability and sensitivity to metabolic degradation, efforts have been made to modify how curcumin is administered in order to improve its limited bioavailability and prolong its presence in the plasma, thereby optimizing its therapeutic potential. One such approach has been the application of nano-technology to curcumin. Many nano-curcumin preparations have been described in the literature which prolong the plasma half-life of curcumin and increase its bioavailability following oral administration (7, 8, 13). Lipocurc™ (liposomal curcumin) is a nanoformulation that was developed for parenteral administration. The pharmacokinetics and safety of intravenously administered Lipocurc™ have been extensively studied in preclinical animal models and in Phase I clinical studies in man. The safety, pharmacokinetics, excretion and tissue distribution of curcumin and its metabolite tetrahydrocurcumin (THC) (Figure 1) were evaluated in Beagle dogs following intravenous infusion of Lipocurc™ at a dose of 10 mg/kg over a 2-h and 8-h infusion time (14, 22). Consistent with its metabolic instability, the plasma-half-lives of curcumin following infusion were 24 min and 42 min, respectively, and considered to be a consequence of hepatic and renal clearance. In these infusion studies, both curcumin and THC were extensively distributed in tissues, with the lung and liver having the highest levels. Tissue levels of curcumin were considerably higher following the 8 h infusion of curcumin, despite much lower plasma curcumin concentrations. In Phase 1 clinical studies, infusion of Lipocurc™ was well tolerated with limited toxicity but was very rapidly cleared from the plasma following cessation of infusion (23). Nonetheless, Lipocurc™ together with other nano-curcumin preparations have demonstrated antitumor activity in animal models (24, 25).

Preclinical and clinical studies of curcumin have also revealed marked species differences of the plasma disposition of curcumin and THC during intravenous infusion of curcumin in Beagle dogs and humans. Plasma levels of THC were considerably higher than curcumin in Beagle dogs while the plasma levels of curcumin were much higher than THC in humans (14, 16). However, in both species curcumin and THC were rapidly cleared from the plasma following cessation of infusion.

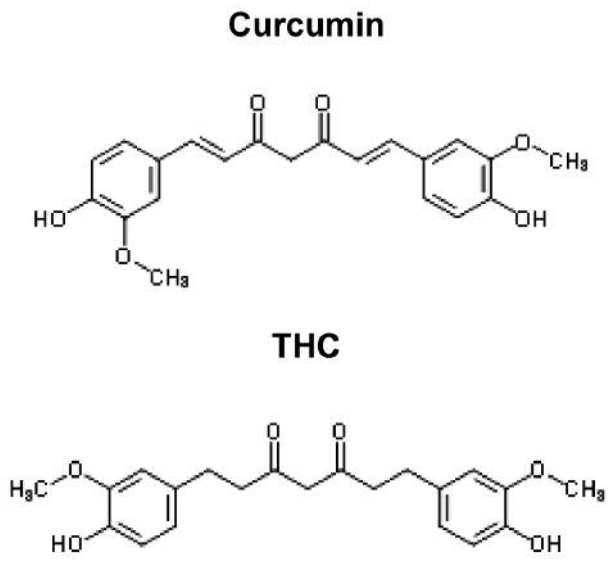

Figure 1. *Chemical structures of curcumin and tetrahydrocurcumin (THC).*

To further understand the mechanism(s) underlying the species differences for the plasma disposition and elimination of curcumin and THC, we evaluated the potential for curcumin to be distributed and metabolized by blood cells. Curcumin is known to interact with red blood cells and regulate their morphology and physiological activity (23, 26, 27), however the impact of curcumin's interaction with red and white blood cells, on the plasma pharmacokinetics of curcumin is not known. We have extended these studies to investigate the cellular distribution and metabolism of curcumin in the form of Lipocurc™ in blood cells (erythrocytes and peripheral blood mononuclear cells (PBMCs)). For comparison, the cellular distribution of curcumin was also investigated in cryopreserved hepatocytes from Beagle dogs and humans because the liver has been proposed as a key metabolic site for curcumin. The cellular distribution of the dihydrocurcumin reductase product of curcumin, THC, was also investigated to assess the level of reductase activity in blood cells and hepatocytes.

Materials and Methods

Liposomal Curcumin (Lipocurc™). Lipocurc™ (liposomal curcumin for intravenous administration was obtained from Polymun Scientific (Klosterneuburg, Austria). Lipocurc™ contained curcumin 6.0 mg/mL, DMPC (14:0-1,2-dimyristoyl-sn-glycero-3-phosphocholine) 72 mg/ml and DMPG (14:0-1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol) 8.0 mg/mL. The average liposome size and distribution were a Z-average of 117 nm and a Zeta potential of -36 mV at pH 5.0. Upon receipt, Lipocurc™ was stored frozen at –10 to –25˚C and protected from light.

Preparation of incubation medium for cell studies. Kreb's Henseleit incubation medium (KHIM) was prepared by adding a premeasured amount of modified Kreb's Henseleit powder containing glucose (Sigma-Aldrich, Oakville, Ontario, Canada) to 1 l of Sterile Water for Injection (USP) and was supplemented with 2.4 g of $NaHCO_3$, 0.15 g of $CaCl_2$ and 4.8 g of HEPES buffer. The pH was adjusted to 7.4 by the drop wise addition of NaOH (8 N) and stored frozen at –20˚C. Plasma protein supplemented KHIM was prepared by combining 6.25 mL of dog plasma (obtained from whole blood using lithium or sodium heparin as the anticoagulant and stored frozen, BioreclamationIVT, Westbury, NY) with 3.75 mL of KHIM. Plasma protein supplemented KHIM was maintained at 2-8˚C prior to use and frozen for subsequent experiments if not used.

Preparation of cells for distribution studies. Cryopreserved human hepatocytes were obtained from Sekisui XenoTech, LLC (Kansas City, KS, USA). Human hepatocytes were obtained from a male donor, who died of anoxia and was screened negative for human immunodeficiency virus, hepatitis B surface antigen and antibodies to hepatitis C and positive for cytomegalovirus. The minimum yield per vial was 6.0×10^6 hepatocytes with a reported viability of 82.8% upon thawing. Cryopreserved male Beagle dog hepatocytes were obtained from Sekisui XenoTech, LLC (Kansas City, KS, USA). The minimum yield per vial was set at 3.5×10^6 cells with a reported viability of 81.4% upon thawing. Hepatocytes were thawed and prepared according to the manufacturer's instructions prior to use. Briefly, tube A containing supplemental DMEM (Dulbecco's Modified Eagle's Medium, Sekisui XenoTech, Kansas City, KS, USA) and isotonic Percoll™ and tube B containing Supplemental DMEM were thawed in a 37±1˚C water bath before use for 15-20 minutes. Vials containing cryopreserved hepatocytes were removed from their liquid nitrogen storage compartment and placed in a 37±1˚C water bath for approximately 80 sec until the hepatocyte pellet separated from the wall of the vial and moved freely. The pellets from each vial were transferred into tube A and each vial was rinsed with 1.5 ml of media from tube B. The contents from tube A were gently inverted until all the ice had melted. Tube A was centrifuged at 100 × *g* for 5 min and the supernatant removed and discarded. Following centrifugation, the pellet was gently resuspended in KHIM at a minimum volume based on the minimum yield of hepatocytes per vial. A 50 µl volume of the cell suspension was removed, diluted 10-fold with KHIM and placed into a counting tube containing Trypan blue for determination of viable cells by the Trypan blue exclusion method with the number of cells counted determined using a hemocytometer. The hepatocyte suspensions were maintained at 2-8˚C and supplemented with O_2 prior to use within 2 h following preparation.

Dog red blood cells were obtained from male colony Beagle Dogs at Nucro-Technics(Scarborough, Ontario, Canada). Blood was collected into K_2-EDTA containing tubes and fractionated into red and white blood cells on a sucrose gradient (Histopaque-1077™ , Sigma-Aldrich, Oakville, Ontario, Canada) as follows. Briefly, whole blood was diluted to 50% at room temperature using 0.9% w/v NaCl. The diluted blood was carefully layered onto the sucrose gradient (3 volumes diluted blood to 2 volumes of gradient medium) and centrifuged at room temperature for 30 min at 377 × *g*. PBMCs were collected at the interface of the sucrose gradient and placed into a collection tube. The remaining supernatant was removed to isolate the red blood cell pellet.

Approximately 1.5 ml of freshly isolated Beagle Dog red blood cells and the collected PBMCs were resuspended in a total volume 10 ml with ice-cold KHIM. The cell suspensions were then centrifuged at 2000 rpm for 20 min at 4˚C, the supernatant was discarded and the pellet gently resuspended in 3 mL of ice-cold KHIM for red blood cells and 1.2 ml for PBMCs. The viability of the PBMCs was determined by the trypan blue exclusion method and the number of cells was determined using a hemocytometer.

Human blood cells were obtained from fresh blood taken with consent from a male donor and used within 2 h following isolation for experimental trial #1. The isolation of red and white blood cells from fresh human blood was the same as described for the isolation of blood cells from fresh dog blood. Packed human red blood cells, isolated using K_2-EDTA as the anticoagulant, were obtained fresh from Bioreclamation IVT (Westbury, New York, USA) and used prior to the date of expiry for experimental trials #2 and #3. Approximately 1.5 ml of packed human red blood cells were resuspended in a total volume 10 mL with ice-cold KHIM. The cell suspension was then centrifuged at 2000 rpm for 20 min at 4˚C, the supernatant discarded and the pellet gently resuspended in 3 ml of ice-cold KHIM for red blood cell.

Cryopreserved human PBMC were obtained from Bioreclamation IVT (Westbury, New York, USA) and Dr. Richard Greil, University Medical Hematology Clinic, Internal Oncology (Salzburg, Austria) and used in experimental trials #1 and #2, respectively. Freshly prepared human white blood cells were used in trial #3. Prepared hepatocytes, red blood cells, white blood cells and human blood were maintained at 2-8˚C and supplemented with O_2 and used within 2 h following preparation for cell distribution assays. Curcumin-d6 and THC-d6 were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). All other reagents used in this study were of reagent grade purity or higher and obtained from approved commercial vendors.

Stock solutions and dilutions of ipocurc™ . Lipocurc™ was used as the source of curcumin for the cell distribution assays. Lipocurc™ was diluted 100-fold into plasma protein supplemented KHIM to produce a curcumin concentration of 162 µM. A final 10 µM working solution concentration of curcumin was produced by adding 61.7 µl of stock A to 938.3 µl of plasma protein supplemented KHIM. This working solution was directly added to the cell distribution assay tubes.

Cell distribution studies. The distribution of curcumin in the form of Lipocurc™ was conducted in 1.5 ml conical microfuge tubes in triplicate and was based on the method of Duan *et al*. (28). The assay volume consisted of 300 µl of human plasma protein supplemented KHIM, 100 µl of cell suspension and 100 µl of plasma protein supplemented KHIM containing Lipocurc™ for a total assay volume of 500 µl, a plasma protein content of 50% v/v and nominal concentration of curcumin of ~2 µM. For whole blood experiments, to a 400 µl of whole blood was added 100 µl of plasma protein supplemented KHIM containing Lipocurc™ for a total assay volume of 500 µl. Initially, tubes containing protein supplemented KHIM and the cell suspensions were prewarmed to 37˚ in a water bath calibrated with a thermometer to 37˚C and flooded with O_2. Following 3 min of incubation, 100 µl of Lipocurc™ (liposomal curcumin) was added to the tubes. Incubations were carried out at 37˚C for 15 min. Following incubation, the tubes were placed in a water bath at 2-8˚C for ~5 min. The tubes were then centrifuged at 6,000 × *g* for 3 min. An aliquot of the supernatant/plasma was taken from individual tubes for analysis of curcumin and THC levels. The remainder of the supernatant/plasma

from these tubes was carefully removed by aspiration. The pellets were resuspended in 1 mL of ice-cold KHIM and subsequently centrifuged at 6,000 × *g* for 3 min. The supernatant was removed and the pellets resuspended in 100 μL of ice-cold KHIM. To the resuspended cells was added a volume of ice-cold extraction medium either of composition 80% acetonitrile, 18% methanol and 2% formic acid (v/v/v/) or 98% acetonitrile, 2% formic acid (v/v). Hepatocytes and white blood cell pellets were extracted with 300 μl of extraction medium and, while 600 μl was used for the extraction from red blood cell pellets. The extracted material was mixed by vortexing followed by ~5 sec of sonication and was incubated in dark for ~30 min at 2-8˚C. Subsequently, the extracted material was centrifuged at 6,000 × *g* for 3 min. The supernatant were stored at –80˚C prior to analysis. In addition a 100 μl sample of the Lipocurc™ solution that was added to the assay tubes was extracted for analysis to determine the total amount of curcumin added to the assay tubes.

For determination of the wet weight of the cell pellet, 100 μl aliquot of cells was pipetted into pre-weighed microfuge tubes and diluted with 0.9 ml of KHIM. The cell suspension was centrifuged at 6,000 × *g* for 3 min, the supernatant carefully removed and the wet weight of the cell pellet determined. Pellet weights ranged from 27.9-55.8 mg for red blood cells, 12.8-19.5 mg for PBMCs and 17.6-19.4 mg for hepatocytes. Viable cell numbers per assay tube for PBMCs and hepatocytes were determined from the number of viable cells determined to be in the suspension, while for red blood cells, the red blood cell number in each assay was estimated based on the reported number of red blood cells per ml of blood ($4.6\text{-}6.2\times10^9$ cells/ml), the weight of the packed cells and knowledge of the hematocrit (0.45).

Sample analysis. The levels of curcumin and, for THC using published methodology (29), were determined in samples by LC-MS/MS. In brief, calibration samples (CS) were prepared in precipitation solvent (acetonitrile/methanol/formic acid (80/18/2 v/v/v) or aetonitrile/formic acid (98/2 v/v) in an ice/water bath and with the protection from ambient light for constructing the standard curves. The CS concentrations were 0, 2, 5, 10, 50, 250, 750 and 1,500 ng/ml each for curcumin and THC. Frozen samples were thawed in an ice/water bath under the protection from ambient light with aluminium foil. Once thawed, the samples were centrifuged at 13000 rpm for 5 min at 4˚C. Then, 200 μL of the supernatant was aliquoted and mixed with 200 μl of working internal standard solution (WIS, 250 ng/ml of curcumin-d6 and 250 ng/ml of THC-d6 in precipitation solvent) or precipitation solvent (for control blanks). Then, the samples were dried down under nitrogen flow (15 psi, 40˚C). The residues were reconstituted with 200 μl of reconstitution solution (acetonitrile/H_2O, 60/40, v/v). The reconstituted samples were transferred to the autosampler vials for injection. An Agilent 1290 liquid chromatography system coupled with an Agilent 6410 Triple Quad LC/MS were used for the LC-MS/MS analysis. A 20 μL aliquot of the extracted sample was injected onto a Durashell C18 column (4.6×50 mm, 5 μm) maintained at 30˚C for isocratic separation. For the analysis of curcumin, the mobile phase (MP) used was acetonitrile/H_2O, 40/60, v/v) with 0.1% (v/v) of formic acid while for the analysis of THC, the MP was acetonitrile/H2O, 60/40, v/v) with 0.2% (v/v) of ammonium hydroxide. The flow rate used was 0.7 ml/min. The MS detection was in the negative mode using the mass transitions of 367→134, 371→235, 373→134, and 377→238 for curcumin, THC, and their internal standards, respectively. Curcumin and its internal standard were eluted at 2.0 min. THC and its internal standard were eluted at 1.0 min. The quantitation was based analyte/IS peak area ratios using quadratic calibration model with a weighting factor of 1/×2.

Data analysis and statistics. Data were presented as the group mean±standard error of the mean (SE). For the purpose of statistics, two group comparisons were performed using either a two tailed Student's *t*-test for normal data or a Student's *t*-test and Mann-Whitney Rank Sum Test for data that were not normal. In either case the statistical significance of the data was assessed at the $p<0.05$ level of significance.

Results

Cell viability was assessed to determine the number of viable cells prior to cell distribution studies. The viability of the PBMCs either freshly isolated or cryopreserved and prepared according to the manufacturer's instructions from cryopreservation ranged from 82-95%. The number of viable cells used in the assay was on average 2.2×10^6 cells per assay. Cyropreserved hepatocytes prepared according to the manufacturer's instructions were used at a concentration of 1.3×10^6 viable cells per assay and had a viability ranging from 60-70%. Cell pellet weights ranged from 27.9-55.8 mg for red blood cells, 12.8-19.5 mg for PBMCs and 17.6-19.4 mg for hepatocytes.

Curcumin and THC were measured in the dog and human cell pellets and medium collected from cells incubated with ~2 μM curcumin (as Lipocurc™) at 37˚C for 15 min in 50% plasma supplemented medium and the results expressed as ng/g w.w. of cell pellet are presented in Tables I to IV. A number of sources of fresh human red blood cells and either cryopreserved or freshly isolated human PBMCs were investigated to determine the variability of cellular distribution, while for dog, the cells were freshly isolated in-house. In general, the results were fairly consistent amongst the different cellular preparations. The incubation of curcumin with dog red blood cells in 50% dog plasma-supplemented medium resulted in a slight degree of hemolysis, which was neither noted with human red blood cells nor when 2 μM of curcumin was incubated with whole blood. In general the levels of curcumin and THC were highest in hepatocytes and PBMCs and lower in red blood cells. Species differences between the distribution of curcumin and THC in blood cells from dog and human were noted. Curcumin levels tended to be significantly higher in human red blood cells ($p=0.001$, two-tailed Student's *t*-test based on ranks) and hepatocytes ($p<0.001$, two-tailed Student's *t*-test) compared to dog, while curcumin levels in PBMCs were higher in dog compared to human PBMCs ($p=0.039$, two tailed Student's *t*-test based on ranks). In contrast, THC levels were significantly higher in dog red blood cells and PBMCs compared to human ($p<0.001$, two tailed Student's *t*-test based on ranks), but significantly lower in dog hepatocytes compared to human hepatocytes ($p<0.001$, two tailed Student's *t*-test).

The medium concentrations of curcumin decreased over the 15-min incubation with red blood cells and hepatoyctes, but not with PBMCs. The decrease of curcumin concentration in red blood cells displayed a species difference in that it was 92% for dog and 68% for human while for hepatocytes the decrease of curcumin in the medium was 48% for dog and 26% for human. The most striking species difference was observed for the levels of THC in the red blood cell medium which amounted to 446 ng/ml THC for dog and 1.3 ng/mL for human red blood cells, resulting in THC/curcumin concentration ratios of 6.3 in dog and 0.006 in human for a 15 min incubation. In contrast, the incubation medium concentrations of THC and the ratios of THC/curcumin for PBMCs and hepatocytes were in a similar range for human and dog.

Cell distribution levels of curcumin and THC were also calculated on a per 10^6 cell basis. The estimated levels of curcumin and THC per 10^6 viable cells (PBMCs and hepatocytes) and calculated cell number (red blood cells) are presented in Table V. The levels of curcumin and THC on a per cell basis were considerably higher for PBMCs and hepatocytes compared to red blood cells consistent with the different volume of the cell types. Interestingly, on a per cell basis, the cellular distribution of curcumin was ~2-fold higher for human compared to dog red blood cells and similar between the two species for PBMCs and hepatocyes. THC levels were ~3-4-fold higher in the dog compared to human for red blood cells and PBMCs and similar for dog and human in hepatocytes.

Given the marked species differences between dog and human for the blood cell distribution and apparent metabolism of curcumin to THC, we tried to relate the *in vitro* findings with red blood cells to *in vivo* pharmacokinetic data available from preclinical studies in dogs and phase I clinical studies in humans. Thus, the data available for similar doses of curcumin (as Lipocurc™) infused over 2 h in dogs (14) and humans (16) included the maximum plasma concentrations during infusion (C_{max}) and area under the curve of the plasma concentration time curve (AUC) which included the infusion period and up to 15 min following the termination of infusion and the 15 min post-dose plasma levels for curcumin and THC. The results of the comparison are shown in Figures 2-4. There was an excellent relationship between the *in vitro* red blood cell data and *in vivo* pharmacokinetic data.

Table I. *Comparison of curcumin levels in Beagle dog cells and medium following incubation with curcumin for 15 min.*

Experimental trial	Cell (ng/g w.w.)	Initial medium concentration (ng/ml)	Incubation medium concentration (ng/ml)
Red blood cells			
Trial-1	51.6±2.1	883±61	70±5
Trial-2	52.3±2.5		
Trial-3	140.0±0.8		
Across trials	78.4±13.5		
PBMCs			
Trial-1	885.2±1.1	689	618±18
Trial-2	419.8±18.5		
Across trials	625.5±104.4		
Hepatocytes			
Trial-1	414.5±22.0	726	380

Values are the mean±SE of n=3-4 for individual determinations and n=3-0 for across trial values and medium concentrations.

Table II. *Comparison of curcumin levels in human cells and medium following incubation with urcumin for 15 min.*

Experimental trial and source of cells	Cell (ng/g w.w.)	Initial medium (ng/ml)	Incubation medium (ng/ml)
Red blood cells			
Trial-1	145.7±4.8	699±76	223±63
Trial-2	294.4±32.8		
Trial-3	150.6±43.1		
Across Trials	201.5±25.6		
White blood cells			
Trial-1	574.5±39.2	770±109	825±53
Trial-2	122.3±24.7		
Trial-3	220.6±10.1		
Across trials	353.7±62.7		
Hepatocytes			
Trial-1	831.5±21.9	810	602

Values are the mean±SE of n=3-4 for individual determinations and n=3-10 for across trials values and medium concentrations.

Discussion

The distribution of curcumin into Beagle dog and human blood cells and hepatocytes was investigated at a single nominal concentration of 2 μM (range=1.8-2.4 μM or 662-883 ng/ml) curcumin (as Lipocurc™), since the maximum plasma concentration of curcumin observed in intravenous infusion studies with Lipocurc™ in dogs and phase I clinical studies ranged from 0.4-4.0 μM (14, 16). In order to restrict the distribution studies to certain blood cell types, preparations of red blood cells and PBMCs were purchased or prepared by fractionation of blood. The distribution of curcumin into hepatocytes was evaluated for comparison with blood cell distribution and metabolism. In order to maintain as physiologic an environment as possible and to limit the hydrolytic degradation of curcumin, the incubation

Table III. *Comparison of THC levels and the ratio of THC to curcumin in Beagle dog cells and medium following incubation with curcumin for 15 min.*

Experimental trial	Cell (ng/g w.w.)	Following incubation medium concentration (ng/ml)	THC/curcumin cell	THC/Curcumin medium
Red blood cells				
Trial-1	97.1±6.3	446±100	1.895±0.181	6.326
Trial-2	94.6±5.3		1.955±0.245	
Trial-3	125.1±9.0		0.897±0.080	
Across trials	107.5±6.0		1.619±0.178	
PBMCs				
Trial-1	101.2±3.6	5.3±0.3	0.114±0.004	0.009
Trial-2	94.5±5.3		0.225 ±0.009	
Across trials	97.9±3.3		0.170±0.025	
Hepatocytes				
Trial-1	71.6±3.4	14.6±0.4	0.173±0.011	0.038

Values are the mean±SE of n=3-4 for individual determinations and n=3-10 for across trial values.

Table IV. *Comparison of THC levels and the ratio of THC to curcumin in human cells and medium following incubation with curcumin for 15 min.*

Experimental trial	Cell (ng/g w.w.)	Following incubation medium concentration (ng/ml)	THC/Curcumin cell	THC/Curcumin medium
Red blood cells				
Trial-1	40.2±0.3	1.3±1.3	0.277±0.009	0.006
Trial-2	17.1±9.9		0.061±0.050	
Trial-3	18.7±0.8		0.138±0.034	
Across trials	24.0±4.5		0.148±0.031	
PBMCs				
Trial-1	23.08±0.75	14.2±5.3	0.041±0.003	0.024
Trial-2	23.94±0.72		0.218±0.037	
Trial-3	42.04±0.97		0.192±0.012	
Across Trials	27.7±0.5		0.130±0.026	
Hepatocytes				
Trial-1	230.9±15.3	27.9	0.278±0.018	0.046

Values are the mean±SE of n=3-4 for individual determinations and n=3-10 for across trial values.

was performed in the presence of 50% plasma, the maximum amount of plasma that could be introduced into a HEPES buffer at pH of 7.4 without changing the pH levels of the medium (28). Identical assay conditions as the ones used in the experiments presented here were used to successfully predict the *in vivo* distribution of hepatitis C antiviral development candidates (28). The incubation time chosen, 15 min, represents a time following infusion of curcumin (as Lipocurc™) in both dogs and humans during which the levels of curcumin decrease rapidly (14, 16).

Several key and novel observations were made in this study, which included the blood cell distribution and metabolism of curcumin (as Lipocurc™), factors that can impact the pharmacokinetics of curcumin. Lipocurc™ rapidly distributed curcumin into red blood cells and PBMC from both dog and human. When expressed as concentrations per cell number, the levels of curcumin were several hundred folds higher in PBMCs compared to red blood cells. Furthermore, the measurement of THC in red blood cells and PBMC suggest that there is metabolism of curcumin in both cell types and therefore curcumin must have entered the cells to be metabolized. This observation is consistent with previous studies where THC was measured in the plasma of whole blood incubated with liposomal

Table V. *Comparison of the estimated curcumin and THC levels per cell in Beagle dog and human cells.*

Species	(pg/10^6 cells)
Curcumin, Red blood cells	
Dog	7.2
Human	18.6
Curcumin, PBMCs	
Dog	7,297
Human	6,809
Curcumin, Hepatocytes	
Dog	14,005
Human	13,780
THC, Red blood cells	
Dog	9.9
Human	2.2
THC, PBMCs	
Dog	1,288
Human	455
THC, Hepatocytes	
Dog	2,428
Human	3,828

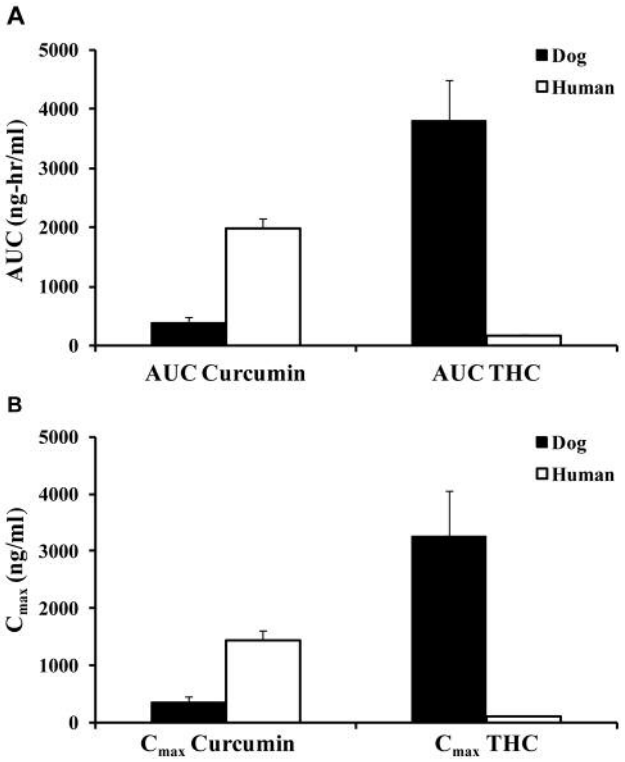

Figure 2. *Comparison of the $AUC_{0\text{-}Tlast}$ and C_{max} between dog and human for curcumin and THC. A) The AUC (area under the curve up until the last measurable plasma concentration time point) and the B) C_{max} (maximum plasma concentration are shown for curcumin and THC in dog and human following a 2-h intravenous infusion. The data is the mean±the standard error of determinations from 4 Beagle dogs and 6 patients both of which underwent infusion with Lipocurc™ at doses of 200 mg/m^2 (10 mg/kg) (14) and 240 mg/m^2 (16), respectively.*

curcumin (23). Thus, it can be assumed that curcumin is likely absorbed as Lipocurc™ onto the cell membrane and then diffuses out of the cell membrane into the intracellular compartment in blood cells. With respect to PBMCs, the average level of curcumin associated with dog and human PBMC was 6,809 and 7,297 pg/10^6 cells, respectively which is equivalent to 18.5 and 19.8 pmol/10^6 cells of curcumin, respectively. These values can be compared to studies in the literature using various cell lines. When curcumin, in various formulations, including lipid formulations, was incubated with lymphocytes and lymphocytic cell lines for up to 8 h, at a normalized curcumin concentration of 1 μM, the amount of curcumin incorporated into the cells measured using laser confocal microscopy florescence ranged from 22.6-44.2 pmol/10^6 cells, values that are in good agreement with those obtained in this study (30, 31). In light of the short incubation times used in this study, it is likely that curcumin associates very rapidly with PBMC. Furthermore, confocal microscopy revealed both a plasma membrane and intracellular location of curcumin (31). The excellent agreement between the results obtained in this study and those from the literature suggests that carry-over of curcumin from the incubation medium to the cell pellet is unlikely to have been a significant source of the total curcumin measured in the cell pellet; this would also apply to the measurement of curcumin in the red blood cell and hepatocyte cell pellets as well.

For comparison with blood cells, the distribution and apparent metabolism of curcumin was investigated in dog and human hepatocytes. Curcumin was also distributed into hepatocytes at levels that were higher than red blood cells and in a similar range to PBMCs when expressed on a ng/g w.w. cell pellet basis. When expressed on a per cell basis, curcumin levels in hepatocytes were two-fold higher than PBMCs, which could be due to rapid uptake in hepaocytes which are rich in membrane transport proteins on the cell surface (28).

One of the most surprising observations made from this study was the apparent metabolism of curcumin in particular by red blood cells as measured by a decrease in the medium concentrations of curcumin and appearance of THC. Furthermore, there was significant species dependence for the red blood cell metabolism of curcumin. The largest difference between dog and human red blood cells were the levels of THC found in the incubation medium of dog red blood cells which were considerably higher than for human red blood cells. On a ng/g w.w. of cell pellet, dog red blood cells and PBMCs also contained higher levels of THC compared to

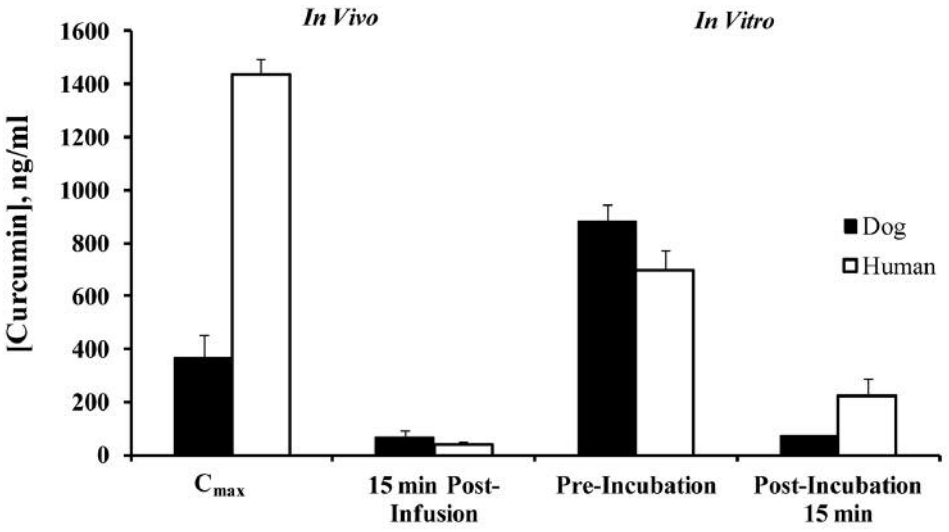

Figure 3. *Comparison of the changes in plasma concentrations of curcumin post infusion and medium concentrations of curcumin post-incubation with red blood cells between dog and human for curcumin and THC. For the in vivo data, the data were obtained from the references indicated in the legend to Figure 2; the in vitro data were obtained from Table I.*

human cells, whereas human red blood cells contained higher levels of curcumin but PBMCs had lower levels. Human and dog hepatocytes also displayed species differences with higher levels of curcumin and THC observed in human hepatocytes compared to dog hepatocytes.

The metabolism of curcumin by red blood cells was rapid as 92% and 68% of the curcumin disappeared from the medium in 15 min upon incubation with dog and human red blood cells, compared to a 48% and 26% loss of curcumin from the medium of dog and human hepatocytes, respectively. It is unlikely that the "chemical" stability of curcumin in the incubation medium contributed to its loss from the medium as there was no remarkable loss of curcumin from the medium following incubation with PBMCs and as previously mentioned the presence of protein in the incubation medium stabilizes the chemical "instability" of curcumin (9, 26). Clearly, curcumin did produce some hemolysis of red blood cells at the concentration employed consistent with the sensitivity of the Beagle dog to the hemolytic effects of curcumin based on in-house toxicological studies. However, the hemolysis likely did not impact metabolism, as metabolism also occurred in dog whole blood where hemolysis was not observed and whole human blood (23 and results not shown). Thus, it is apparent that human, and in particular dog red blood cells, can both incorporate curcumin and metabolize it to THC and likely other metabolites. Given the large amount of THC produced by dog red blood cells, reductase activity must be present in red blood cells that would participate in the production of THC. While the nature of this enzyme(s) is not known at the present time, the enzyme might be similar to the dihydrocurcumin reductase found in gut microorganisms (6). Another plausible candidate might be Cytochrome b5 reductase (both soluble and particulate) (32) which *in silico* has been shown to preferentially bind curcumin (27). Furthermore, cytochrome b_5 reductase has also been shown to effectively reduce small molecules such as dichlrophenolindophenol (32).

Some of the key observations made in this *in vitro* study may provide a mechanistic interpretation to explain observations made from *in vivo* pharmacokinetic and tissue distribution studies in Beagle dogs (14, 22). The excellent correlation observed between the *in vitro* disposition of curcumin and THC following incubation of human and dog red blood cells with curcumin and the *in vivo* levels of curcumin and THC in clinical studies provides strong evidence to suggest that a "blood-based metabolism" of curcumin plays an important role in the overall pharmacokinetics of curcumin following the intravenous infusion of Lipocurc™. Furthermore, upon intravenous infusion of curcumin in Beagle dogs at a dose of 10 mg/kg over period of either two or eight h, steady-state levels of curcumin were not observed which would be consistent with a "blood based metabolism" as well as organ based metabolism of curcumin as potential competing mechanisms impacting the attainment of steady-state levels of curcumin during infusion. The red blood cell metabolism of curcumin provides an effective and rapid mechanism for elimination of curcumin from the plasma in addition to phase I and phase II metabolism and in general, the metabolism of curcumin

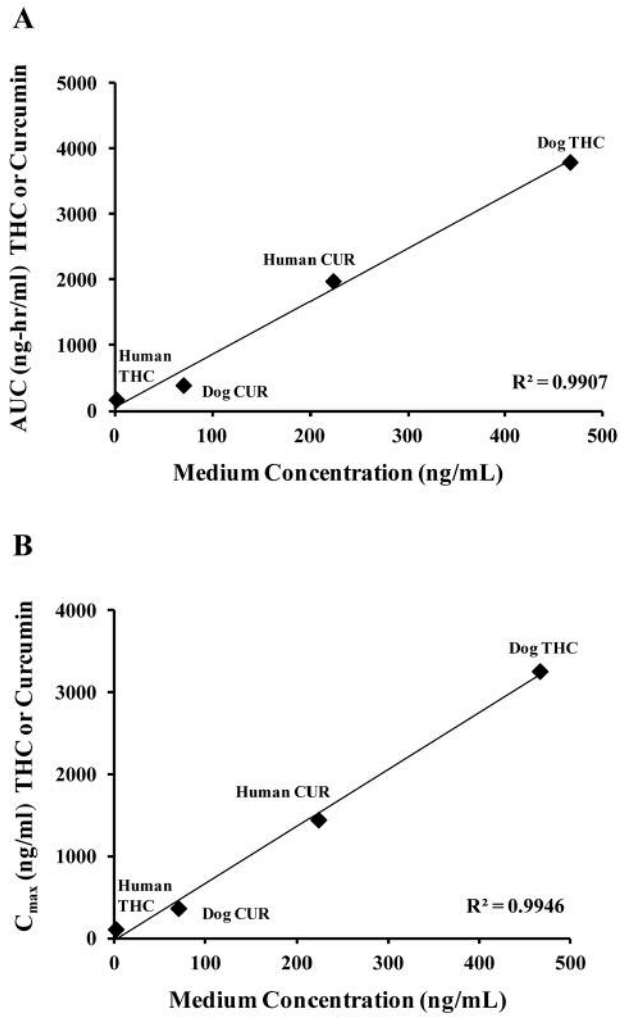

Figure 4. *Correlation between curcumin and THC levels from in vitro experiments with red blood cells and in Vivo clinical studies. The correlation between in vivo (AUC and C_{max} for 2 h infusion) and in vitro data (medium concentrations following incubation of curcumin with red blood cells for 15 min.) are shown above in A) AUC vs. medium concentration and B) C_{max} versus medium concentration. The in vivo data are from the references cited in Figure 2 and the in vitro data are taken from the preceding tables.*

by red blood cells may, in part, provide an explanation for the extremely short plasma half-lives of curcumin observed following intravenous infusion in several species (12-16). Furthermore, the large differences in THC plasma levels in dogs compared to humans are likely largely due to a blood cell-based metabolic source for THC in dogs, while the source of plasma THC in humans likely comes from tissue (*i.e.* liver) sources.

In contrast to metabolism, curcumin was clearly shown to distribute into red blood cells and given their large presence in the blood, they may serve as efficient vehicles to distribute curcumin to tissues. This would explain why infusion of a 10 mg/kg dose of curcumin over either 2 h or 8 h in Beagle dogs resulted in generally higher tissues levels of curcumin for the 8 h infusion despite average plasma levels of curcumin being ~13-fold higher during the 2-h infusion (22).

In summary, in Beagle dog and human blood cells, curcumin in the form of Lipocurc™ was distributed into red blood cells and PBMCs and to a higher concentration in PBMCs compared to red blood cells, particularly when expressed on a per cell basis. PBMCs may be able to concentrate curcumin, potentially enhancing the therapeutic potential of curcumin in the treatment of tumors of lymphocytic origin. Curcumin metabolism to THC was observed across all blood cell types and there were significant species differences between the disposition and metabolism of curcumin between dog and human blood cells. Blood cells from dogs, particularly red blood cells, appear to possess a higher reductase activity compared to human blood cells resulting in a greater conversion of curcumin to THC and in general greater degree of overall metabolism of curcumin. There was a very good correlation between the species differences of red blood cell metabolism of curcumin to THC and *in vivo* plasma levels of curcumin and THC from clinical studies. Thus, curcumin's distribution into, and metabolism by, red blood cells significantly impacts the pharmacokinetics and potentially the tissue distribution of curcumin.

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Received April 21, 2017
Revised May 16, 2017
Accepted May 22, 2017