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Distribution of Curcumin and THC in Peripheral Blood Mononuclear Cells Isolated from Healthy Individuals and Patients with Chronic Lymphocytic Leukemia

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Abstract. *Background/Aim: Curcumin is being widely investigated for its anticancer properties and studies in the literature suggest that curcumin distributes to a higher degree in tumor versus non-tumor cells. In the current study, we report on investigation of the distribution of curcumin and metabolism to THC in PBMC from healthy individuals and chronic lymphocytic leukemia (CLL) patients following exposure to Lipocurc™ (liposomal curcumin). Materials and Methods: The time and temperature-dependent distribution of liposomal curcumin and metabolism to tetrahydrocurcumin (THC) were measured in vitro in human peripheral blood mononuclear cells (PBMC) obtained from healthy individuals, $PBMC_{HI}$ (cryopreserved and freshly isolated PBMC) and CLL patients (cryopreserved PBMC) with lymphocyte counts ranging from 17-58×10^6 cells/ml ($PBMC_{CLL,Grp\ 1}$) and >150×10^6 cells/ml ($PBMC_{CLL,Grp\ 2}$). PBMC were incubated in plasma protein supplemented media with Lipocurc™ for 2-16 min at 37˚C and 4˚C and the cell and medium levels of curcumin determined by LC-MS/MS. Results: PBMC from CLL patients displayed a 2.2-2.6-fold higher distribution of curcumin compared to $PBMC_{HI}$. Curcumin distribution into PBMCCLL, Grp 1/Grp 2 ranged from 384.75 - 574.50 ng/g w.w. of cell pellet and was greater compared to $PBMC_{HI}$ that ranged from 122.27-220.59 ng/g w.w. of cell pellet following incubation for up to 15-16 min at 37˚C. The distribution of curcumin into $PBMC_{CLL,Grp\ 2}$ was time-dependent in comparison to $PBMC_{HI}$ which did not display a time-dependence and there was no temperature-dependence for curcumin distribution in either cell type. Curcumin was metabolized to THC in PBMC. The metabolism of curcumin to THC was not markedly different between $PBMC_{HI}$ (range=23.94-42.04 ng/g w.w. cell pellet) and $PBMC_{CLL,Grp\ 1/Grp\ 2}$ (range=23.08-48.22 ng/g. w.w. cell pellet). However, a significantly greater time and temperature-dependence was noted for THC in $PBMC_{CLL,Grp\ 2}$ compared to $PBMC_{HI}$. Conclusion: Curcumin distribution into PBMC from CLL patients was higher compared to PBMC from healthy individuals, while metabolism to THC was similar. The potential for a greater distribution of curcumin into PBMC from CLL patients may be of therapeutic benefit.*

Curcumin (diferuloylmethane) is being extensively investigated for its anticancer properties with several studies indicating its potential for therapeutic benefit. Clinical studies with curcumin have indicated that it has limited, but significant anticancer activity in part as a consequence of its poor oral availability. Significant anticancer effects upon oral administration of curcumin powder have been observed in pancreatic and colorectal cancer and leukemia as well as a variety of high risk and pre-malignant lesions (1-3) including altering the regulation of oncogenes (4). In studies in mice, curcumin enhanced the cytotoxicity of antigen-specific CD8 T-cells (5) and prevented the loss of T-cells, expanded central member T cell populations, reversed the type 2 immune bias and attenuated the tumor-induced inhibition of T-cell proliferation in tumor bearing hosts (6).

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Nano-curcumin preparations offer improved bioavailability (7-11) of curcumin and potentially better treatment outcomes in cancer patients. Lipocurc™ (liposomal curcumin) has been shown to suppress pancreatic tumor growth in a mouse xenograft model (10). Meriva®, a patented formulation of curcumin with soy lecithin produced a decreased absolute lymphocyte count in a subset of lymphocytic leukemia patients accompanied by an increase in CD4 and CD8 T-lymphocytes and NK cells (7).

The potential therapeutic application of curcumin in chronic lymphocytic leukemia (CLL) B cells and as a potential maintenance therapy for CLL has been reviewed (12, 13). In addition to its anti-inflammatory and chemosensitizing properties, curcumin treatment was shown to overcome stromal protection and induce apoptosis in CLL cells by inhibiting prosurvival pathways and suppressing the expression of anti-apoptic proteins while also producing a synergistic activity with the green tea extract epigallocatechin-3 gallate (14). Curcumin has also been found to induce apoptosis in CLL cells possibly due to inhibition of constitutively produced NF-κβ (15). Furthermore, curcumin was found to suppress the induction of nitric oxide synthetase by lipopolysaccharide by suppressing the activity of NF-κβ (16).

Paramount to the activity of curcumin in immune cells is the association with and distribution of curcumin into the cell. Using absorption and fluorescence spectroscopic methods the cellular distribution of curcumin was investigated in normal (spleen lymphocytes and NIH3T3 cells) and tumor (EL4, mouse T-cell lymphoma; MCF7, breast cancer cells, human origin) cell lines (17, 18). Several key findings were made, of which included, the distribution of curcumin throughout the cells with concentration of curcumin both in the cell membrane and nucleus, higher levels in the tumor cell lines compared to normal cell lines by ~2-fold and an increased cytotoxicity to curcumin in tumor compared to normal cells.

The distribution and metabolism of liposomal curcumin (Lipocurc™) following short exposure times (0.25 hr) has also been investigated in red blood cells and peripheral blood mononuclear cells (PBMC) from dog and human with detection of both curcumin and tetrahydrocurcumin (THC) in cell extracts by LC/MS-MS (19). Key findings from this study were that both the distribution and metabolism of curcumin to THC were found to occur in red blood cells in a manner that was highly correlated with the pharmacokinetics of curcumin and THC following intravenous infusion in the dog and human and that on a per cell basis, curcumin had a higher distribution and metabolism to THC in PBMCs compared to red blood cells. Together these lines of evidence suggest that gaining a better understanding of the distribution of curcumin into cells is relevant to both the potential anti-cancer benefits and pharmacokinetics of curcumin.

Given the important anti-cancer potential of curcumin in leukemia and the increased distribution of curcumin into cell lines of tumor origin, we have investigated the distribution and metabolism of curcumin following short incubation times in PBMC derived from healthy individuals and CLL patients. Both the time and temperature-dependence of curcumin distribution were evaluated using methods for determining the cell distribution of curcumin that have been previously applied (19). The utilization of a highly sensitive analytic method (20) also permitted investigation of the dihydrocurcumin reductase product of curcumin, THC, to assess the level of reductase activity in PBMC.

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 human plasma (obtained from whole blood using lithium or sodium heparin as the anticoagulant and stored frozen, BioreclamationIVT, Westbury, NY, USA) with 3.75 ml of KHIM. Plasma protein supplemented KHIM was maintained at 4-8˚C prior to use and frozen for subsequent experiments if not used.

Diagnosis of CLL and informed consent. Diagnosis and staging of CLL were performed according to the IWCLL criteria of 2009 (21). In brief, peripheral blood lymphocytes were characterized by flow cytometry and diagnosed as CLL in case the following criteria were fulfilled: monoclonal light chain expression, CD5 and CD23 co-expression, positivity for CD19 and weak staining for the BCR (*i.e.* CD79). Zap 70 and CD38 expression are well established risk factors and were also determined by flow cytometry. In addition, analyses for IGVH chain gene rearrangement and cytogenetic aberrations with prognostic and predictive power were carried out routinely (22, 23). Informed consent and Ethics committee approval were obtained prior to drawing blood samples (Ethics committee Salzburg: 415-E/1287/4–2011, 415-E/1287/8–2011, 415-E/1287/13-2016).

Preparation of cells for distribution studies. One lot of cryopreserved normal human peripheral blood mononuclear cells were isolated from a healthy individual ($PBMC_{HI}$) and prepared by BioreclamationIVT (Westbury, NY, USA). The cryopreserved

$PBMC_{HI}$ (Lot#BRH1221012, expiry date December 31, 2018) were obtained from a single individual black male from blood samples treated with K_2EDTA and upon analysis of the blood cell distribution by the vendor the sample contained 83.7% lymphocytes, 14.9% monocytes and 0.6% granulocytes. Cryopreservation was done using the cryopreservative Cryostar CS10 from Biolife. $PBMC_{HI}$ were also isolated in-house from freshly drawn blood treated with K_2EDTA from a healthy male Caucasian donor (with consent) using 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 vols 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.

Cryopreserved PBMCs were also isolated from patients with chronic lymphocytic leukemia (CLL) who had a low lymphocyte cell count (17-58×10^6 CLL cells/ml; $PBMC_{CLL,Grp\ 1}$) or a high lymphocyte cell count (>150×10^6 CLL cells/ml; $PBMC_{CLL,Grp\ 2}$). $PBMC_{CLL,Grp\ 1/Grp2}$ were obtained from Dr. Michaela Schachner in the laboratory of Dr. Richard Greil, University Medical Hematology Clinic, Internal Oncology (Salzburg, Austria). One experimental trial was conducted with a pool of cryopreserved $PBMC_{CLL}$, Grp 1 obtained from three CLL patients (#89, #208, and #733; sample ID#'s 10863, 10326 and 10890; lymphocyte cell count 17-58×10^6 cells/ml). Two experimental trials were conducted with cryopreserved $PBMC_{CLL,Grp\ 2}$, one with a pool from two patients (#903 and #904; sample ID#'s 10971 and 10976; lymphocyte cell count 250-370×10^6 cells per ml; cell preparation #1) and the other from a single patient (#664; sample ID# 11710; lymphocyte cell count >150×10^6 cells per ml; cell preparation #2).

Cryopreserved $PBMC_{HI}$, $PBMC_{CLL,Grp\ 1/Grp\ 2}$ were rapidly and partially thawed by placing in a 37˚C water bath for between 60-90 sec and then resuspended in 10 ml of ice-cold KHIM. Freshly isolated $PBMC_{HI}$ were resuspended in 10 ml of ice-cold KHIM. All PBMC suspensions were centrifuged at 2,000 rpm for 20 min and the supernatant discarded. The pellets were gently resuspended in a minimum volume of ice-cold KHIM at an average suspension concentration of 27.0×10^6 cells/ml. The PBMC suspensions were maintained at ~4˚C in a water bath chilled with ice-packs and supplemented with O_2 prior to use within 2 h following preparation.

Stock solutions and dilutions of Lipocurc™. 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 (Stock A). 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 either in replicates of 3, 4 or 6 and was based on the method of Duan *et al*., (24). 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 between ~2-3 μM. Initially, tubes containing protein supplemented KHIM and the cell suspensions were pre-warmed 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 either carried out at 37˚C for 15 min or for investigating the time and temperature-dependence of curcumin at 2, 4, 8 and 16 min of incubation with Lipocurc™ at either 37˚C or 4˚C. Following incubation, all tubes were placed in a chilled water bath at 4˚-8˚C for ~5 min. The tubes were then centrifuged at 6,000 × *g* for 3 min. A 100 μl aliquot of the supernatant 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 and 100 μl of the supernatant was added a 300 μl volume of ice-cold extraction medium consisting of 98% acetonitrile, 2% formic acid (v/v). The extracted material was mixed by vortexing followed by ~5 sec of sonication and was incubated in dark for ~30 min at 4-8˚C. Subsequently, the extracted material was centrifuged at 6,000 × *g* for 3 min. The supernatant isolated and 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. Cell pellet weights for PBMCs ranged from 15.7-19.5 mg.

Sample analysis. The levels of curcumin and, for THC using published methodology (20), 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 acetonitrile/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 Bonna-Agela Technologies, Wilmington DE, USA) 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/H_2O, 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 2.1 min. THC and its internal standard were eluted at 1.1 min. The quantitation was based analyte/IS peak area ratios using quadratic calibration model with a weighting factor of $1/x^2$.

Data analysis and statistics. Data were presented as the group mean±standard error of the mean (SE). For the purpose of statistics, all statistical tests were performed using SigmaStat (v 3.5, Systat Software Inc., San Jose, CA, USA). Two group comparisons were performed using a Student's *t*-test. Multiple group comparisons were performed using a One-Way analysis of variance with a Student Neuman Keul's *post-hoc* analysis. In either case group differences were deemed statistically significant if they attained the $p<0.05$ level of significance.

Results

Cell viability was assessed to determine the number of viable PBMCs prior to cell distribution studies. The viability of the PBMCs either freshly isolated or cryopreserved averaged 74%. The number of viable cells used in the assay was on average 2.7×10^6 cells per assay. At the time blood was taken from two healthy individuals there was no outward signs of illness. All six of the CLL patients were chemonaive at the time when peripheral blood samples were taken, two of them had Rai stage IV, showed unmutated IGVH chain status as a prognostic unfavorable parameter, excessively high peripheral blood lymphocyte counts, and progressive disease immediately requiring treatment with chemoimmunotherapy (Table I). The other 4 cases showed unmutated *IgVH* gene in one and the favorable constellation of mutated *IGHV* status in 3 cases and with one exception had by far lower numbers of peripheral blood lymphocytes. None of them required therapy at the time blood was drawn.

Curcumin and THC (Figure 1) were measured in $PBMC_{HI}$, $PBMC_{CLL,Grp\ 1/Grp\ 2}$. PBMC were either incubated with ~2-3 μM curcumin (as Lipocurc™) at 37˚C for 15 min or at 37˚ and 4˚C for 2, 4, 8 and 16 min (to establish a time course and temperature-dependence for distribution) in 50% human plasma supplemented medium. Curcumin and THC levels were expressed as ng/g w.w. of cell pellet and ng/ml of medium. The results are presented in Tables II to IV and Figures 2 to 4.

The incubation time used to investigate the distribution of curcumin into human PBMC (either 15 min or a time course of up to 16 min) was chosen based on previous cell distribution studies performed in this laboratory (19) and reflected *in vivo* pharmacokinetic factors demonstrating very short half-lives for curcumin following bolus intravenous dosing and the termination of intravenous infusion of curcumin in the form of Lipocurc™ (19 and references cited therein). Human PBMC were employed either fresh or following cryopreservation to investigate the cell distribution

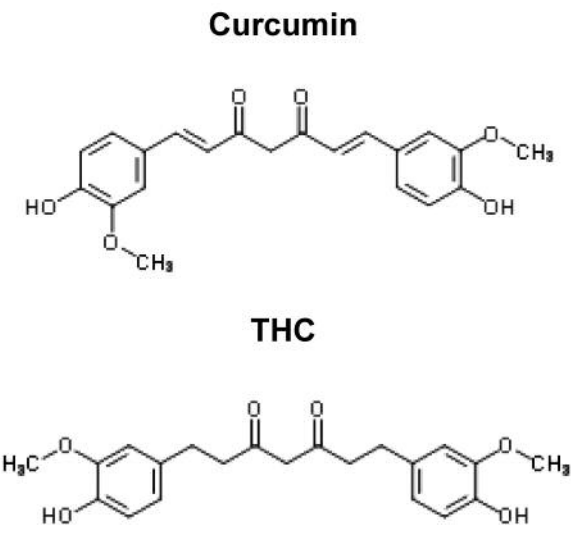

Figure 1. *Chemical structures of curcumin and tetrahydrocurcumin (THC).*

of curcumin (as Lipocurc™) and metabolism to THC. Compared to freshly isolated and cryopreserved $PBMC_{HI}$, $PBMC_{CLL,Grp\ 1/Grp\ 2}$ isolated from CLL patients had significantly and consistently higher levels of curcumin following 15 min of incubation at 37˚C (Table II). In contrast, the levels of THC ranged from 23.94-42.04 ng/g w.w. of cell pellet and from 23.08-48.22 ng/g w.w. of cell pellet for $PBMC_{HI}$ and $PBMC_{CLLGrp\ 1/Grp\ 2}$, similar ranges for each cell type and much lower than curcumin.

Compared to freshly isolated $PBMC_{HI}$, cryopreserved $PBMC_{HI}$ had higher levels of curcumin and THC, although the ratio of curcumin to THC was quite consistent at 5.1-5.2 for $PBMC_{HI}$ from both sources but different donors. In contrast the ratio of curcumin to THC in $PBMC_{CLL,Grp\ 1/Grp2}$ was higher with ratios ranging from 8.0-24.9.

Given the different extents of distribution of curcumin into $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 1/Grp\ 2}$, it was decided to examine both the time course and temperature-dependence for the distribution and metabolism of curcumin to THC. The time course for distribution of curcumin into $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$ displayed marked differences (Figure 2, Table III). Curcumin levels in $PBMC_{HI}$ clearly reached a maximum quickly with little change in cell pellet levels between 2 and 16 min of incubation at 37˚C. In contrast, there was a clear time-dependence for the distribution of curcumin into $PBMC_{CLL,Grp\ 2}$ with levels significantly increasing by 2.2-fold following 16 min of incubation compared to 2 min of incubation at 37˚C and appearing to reach a maximum. Partition coefficients for curcumin in $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$ based on ng/g w.w. of the cell pellet following 16 min of incubation at 37˚C were

Table I. *Patient characteristics*.

Patient ID/ sample ID	Age	Gender	RAI stage	IGVH[1]	Zap70/ CD38	Cytogenetics[2]	Treatment status	Lymphocyte count (10^6 cells/ml)
903/109717	60	M	IV	UM	High/high	Del 13q (83%)	Chemonaive/PD[3,4]	250
904/10976	65	F	IV	UM	Missing/high	Tris 12 (82%)	Chemonaive /PD[3,5]	370
733/10890	76	M	I	UM	High/low	Del 11q (51%)	Chemonaiv[6]	58
208/10326	86	M	0	M	High/high	No anomaly	Chemonaiv[6]	29
89/10863	90	M	0	M	Low/low	No anomaly	Chemonaiv[6]	17
664/11710	78	M	I	M	Low/high	Del 13q (85%)	Chemonaiv[6]	250

[1]IGVH: IgV heavy chain gene status UM (unmutated), M (mutated); [2]Cytogenetics: patients are routinely analyzed for del 17p, del 11q, deletion 13q and trisomy 12; [3]PD progressive disease; [4]At the time of freezing of cells, the patient was untreated but heavily progressive so that treatment was started immediately after blood was drawn; [5]Patient was recommended to start therapy but refused therapy; [6]No treatment indication according to the IWCLL criteria; [7]The patient also suffered from Non Small Cell Lung Cancer, requiring treatment shortly after three cycles of Chemoimmunotherapy with FCR (fludarabine, cyclophosphamide, Rituximab).

Table II. *Comparative levels of curcumin and THC in PBMCs derived from healthy individuals and CLL patients*.

Cells and number of determinations	Curcumin (ng/g.w.w.)	THC (ng/g.w.w.)	Curcumin/ THC	[Curcumin] Post incubation (μM)
$PBMC_{HI}$, n=4 (freshly isolated)[1]	122.27±28.53	23.94±1.44	5.1	1.92±0.03
$PBMC_{HI,}$ n=3 (cryopreserved)[2]	220.59±8.75	42.04±0.84	5.2	2.27±0.03
$PBMC_{CLL,Grp\ 1}$, n=6 (CLL cell count 17-58×10^6/mL, cryopreserved)[1]	574.50±39.24*	23.08±0.75*	24.9	1.96±0.03
$PBMC_{CLL,Grp\ 2}$, n=6 (CLL cell count >150×10^6/mL; cell preparation #1, cryopreserved)[1]	482.10±29.98*	26.38±0.85*	18.3	2.02±0.06
$PBMC_{CLL,Grp\ 2}$, n=3 (CLL cell count >150×10^6/mL; cell preparation #2, cryopreserved)[2]	384.75±3.06*	48.22±1.46*	8.0	3.18±0.11

$PBMC_{HI}$: PBMC from healthy individuals; PBMCCLL,ES: periperhal white blood cells isolated from early stage CLL patients with low CLL cell counts; $PBMC_{CLL,LS}$: PBMC isolated from late stage CLL patients with high CLL cell counts. [1]Incubation of PBMC with curcumin was for 15 min at 37˚C; [2]Incubation of PBMC with curcumin was for 16 min at 37˚C. Values are presented as the mean±SE. *Significantly different from $PBMC_{HI}$ fresh and cryopreserved, $p<0.05$, One-Way ANOVA.

0.264±0.012 and 0.328±0.003, respectively; the partition coefficient for $PBMC_{CLL,Grp\ 2}$ being significantly greater.

In contrast to curcumin, THC levels in $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$ (Figure 3, Table IV), did not display a marked dependence on time for development. However, THC levels were significantly higher (14% - 42%) dependent on incubation time point both at 37˚C and 4˚C in $PBMC_{CLL,Grp\ 2}$ compared to $PBMC_{HI}$ and were 19.3% and 7.4% of the curcumin levels in $PBMC_{HI}$ and $PBMC_{CLL,Grp2}$ following 16 minutes of incubation at 37˚C, respectively. A more detailed evaluation of the time and temperature-dependence of THC production (Figure 4) indicated that THC production displayed a greater time and temperature-dependence in $PBMC_{CLL,Grp\ 2}$ compared to $PBMC_{HI}$ based on the difference between the $AUC_{37°C}$ and $AUC_{4°C}$ (see legend of Figure 4).

Discussion

From the data presented in this report, it is clear that curcumin distributes to a higher degree in $PBMC_{CLL,Grp\ 1/Grp\ 2}$ compared to $PBMC_{HI}$. These findings are consistent with fluorescent microscopic determinations of curcumin's distribution into normal and tumor cell lines of lymphoid origin, where curcumin levels were, on average, 1.5-fold higher in tumor cells where there was an ~2-fold greater cytotoxicity (17, 18). In these studies, cryopreserved $PBMC_{CLL,ES/LS}$ displayed a 2.2-2.6-fold higher distribution of curcumin as Lipocurc™ compared to cyropreserved $PBMC_{HI}$. These results suggest that Lipocurc™ may be more effective at distributing curcumin into cells of lymphoid origin and strongly suggests that leukemic PBMCs will potentially be more susceptible to curcumin's anticancer and cytotoxic effects.

Table III. *Total free curcumin found in human normal and leukemic peripheral white blood cells and medium and partition coefficient following incubation with curcumin.*

Cell type and temperature	Incubation time (min)	Cell levels (ng/g w.w.)[1]	Medium levels post incubation (μM)	Kp[2]
$PBMC_{HI}$ 37˚C	2	218.90±5.38	2.27±0.03	0.264±0.012
	4	186.41±21.27		
	8	203.18±2.05		
	16	220.59±8.75		
$PBMC_{HI}$ 4˚C	2	226.96±21.12		
	4	211.47±16.86		
	8	204.03±2.12		
	16	209.13±6.15		
$PBMC_{CLL,Grp\ 2}$ 37˚C	2	171.81±7.18**	3.18±0.11 μM	0.328±0.003**
	4	254.26±27.11*		
	8	359.84±42.29*,**		
	16	384.75±3.06*,**		
$PBMC_{CLL,Grp\ 2}$ 4˚C	2	183.33±11.46		
	4	228.72±8.48**		
	8	356.74±18.78*,**		
	16	393.62±31.33*,**		

$PBMC_{HI}$: Cryopreserved PBMC isolated from a healthy individual; $PBMC_{CLL,Grp\ 2}$: cryopreserved PBMC isolated from late stage CLL patients with high CLL cell counts; cell preparation #2. Values are presented as the mean±SE of three determinations. The total amount of curcumin added to the assay tubes was 944.80 ng/ml and 1279.45 ng/ml for $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$ respectively (mean of 2 values). [1]Values are ng/g wet weight of cell pellet; [2]Kp is the ratio of the cell levels to the mean medium concentration assuming that 1 g of cell pellet has a volume of 1 ml. *Significantly different from 2 min incubation, $p<0.05$, One Way ANOVA. **Significantly different from $PBMC_{HI}$ $p<0.05$, Student's *t*-test.

Table IV. *Total free THC found in human normal and leukemic peripheral white blood cells and medium and ratio of THC to curcumin following incubation with curcumin.*

Cell type and temperature	Incubation time (min)	Cell levels (ng/g w.w.)[1]	Medium levels post incubation (μM)	Cell ratio THC/ Curcumin
$PBMC_{HI}$ 37˚C	2	42.04±0.74	0.020±0.001	0.192
	4	39.07±1.39		0.210
	8	40.34±0.42		0.199
	16	42.04±0.84		0.191
$PBMC_{HI}$ 4˚C	2	42.25±0.66		0.186
	4	38.22±1.12		0.181
	8	38.85±0.00		0.190
	16	39.91±0.49		0.191
$PBMC_{CLL,Grp\ 2}$ 37˚C	2	46.28±3.62	0.023±0.001	0.269
	4	48.58±3.66		0.191
	8	57.27±3.70*		0.159
	16	48.22±1.46*		0.125
$PBMC_{CLL,Grp\ 2}$ 4˚C	2	43.62±1.41		0.238
	4	44.86±1.93*		0.196
	8	49.65±0.94*		0.139
	16	45.39±1.47*		0.115

$PBMC_{HI}$: Cryopreserved PBMC isolated from a healthy individual; $PBMC_{CLL,Grp\ 2}$: Cryopreserved PBMC isolated from late stage CLL patients with high CLL cell counts; cell preparation #2. Values are presented as the mean±SE of three determinations. The total amount of curcumin added to the assay tubes was 944.80 ng/ml and 1279.45 ng/ml for $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$ respectively (mean of 2 values). [1]Values are ng/g wet weight of cell pellet; *Significantly different from $PBMC_{HI}$ $p<0.05$, Student's *t*-test.

Despite the higher curcumin levels in $PBMC_{CLL,Grp\ 1/Grp\ 2}$, comparatively, the metabolism of curcumin to THC was within a similar range for $PBMC_{CLL,Grp\ 1/Grp\ 2}$ compared to $PBMC_{HI}$. With respect to the levels of curcumin and THC measured in PBMC, the data were quite consistent amongst different lots and fresh versus cryopreserved $PBMC_{HI}$, $PBMC_{CLL,Grp\ 1}$ and $PBMC_{CLL,Grp\ 2}$ (greatest difference ~2-fold). Although the number of lots used was small, $PBMC_{CLL,Grp\ 1}$ and $PBMC_{CLL,Grp\ 2}$ were pooled from several CLL patients and thus it could be speculated that curcumin

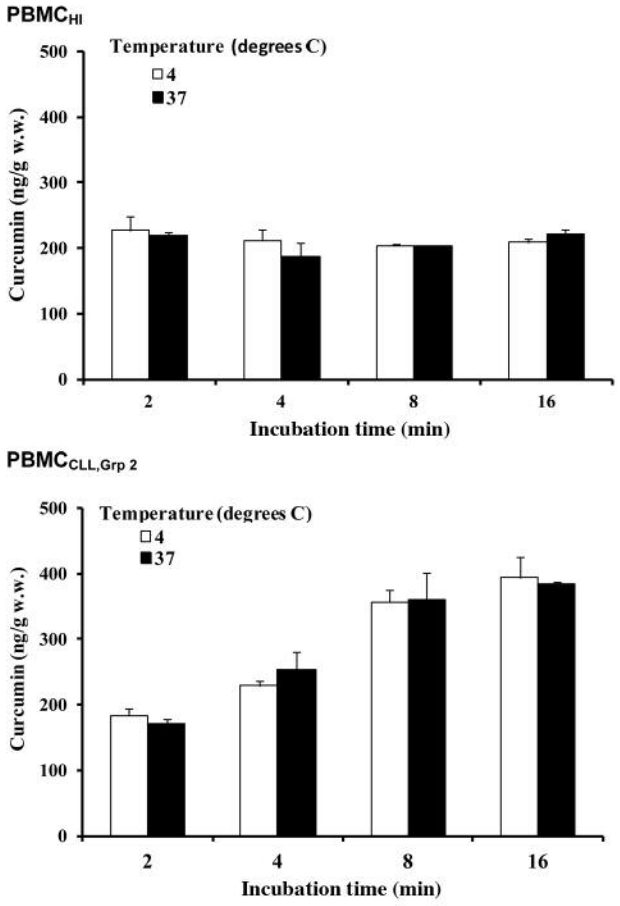

Figure 2. *Curcumin distribution into PBMC$_{HI}$ and PBMC$_{CLL,LP}$. The distribution of curcumin in PBMC$_{HI}$ and PBMC$_{CLL,LS}$ is illustrated above, the data are presented±S.E.M and the values along with their statistical significance are presented in Table II.*

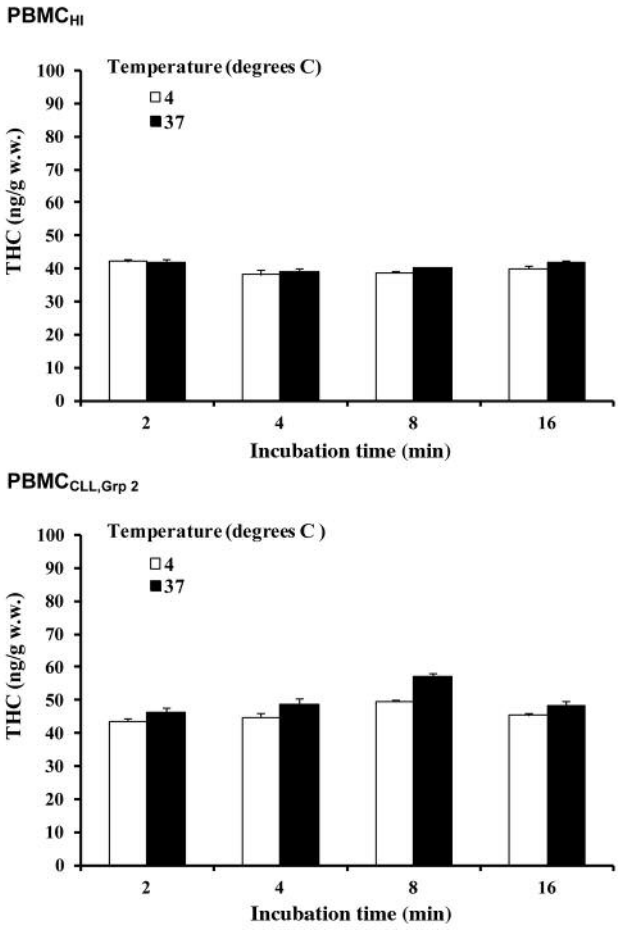

Figure 3. *THC levels in PBMC$_{HI}$ and PBMC$_{CLL,LP}$. The metabolism of THC to curcumin in PBMC$_{HI}$ and PBMC$_{CLL,LS}$ is illustrated above, the data are presented±S.E.M and the values along with their statistical significance are presented in Table III.*

distribution into and metabolism to THC may be quite consistent from patient to patient, although a greater number of patient samples would be needed to confirm this finding. Nonetheless, a greater and consistent distribution of curcumin into the PBMC of CLL patients may offer an advantage in the treatment of CLL with curcumin as a greater cytoxic effect of curcumin in tumor compared to normal cells has been observed (17).

Several other interesting aspects of the distribution of curcumin into PBMC and metabolism to THC were observed. The time dependence for distribution of curcumin into PBMC$_{HI}$ and PBMC$_{CLL,Grp\ 2}$ displayed marked differences. Curcumin levels in PBMC$_{HI}$ clearly reached a maximum quickly with little change in cell pellet levels between 2 and 16 min of incubation at 37˚C. In fluorescent microscopy studies of the distribution of curcumin in normal and tumor cell lines (17), while periods as long as 8 h were used for incubating cells with curcumin, distribution into cells was maximal by 0.5 h, consistent with a rapid distribution of curcumin into cells. In contrast, for PBMC$_{CLL,Grp\ 2}$, there was a clear time-dependence for the association of curcumin with levels significantly increasing by 2.2-fold at 16 min of incubation compared to 2 min of incubation at 37˚C and appearing to reach a maximum. This observation clearly suggest differences in the mechanisms involved in the distribution of curcumin between PBMC$_{HI}$ and PBMC$_{CLL,Grp\ 2}$. Consistent with these differences, studies on the fluidity of normal and leukemic white blood cell membranes using florescent probes indicated that there are compositional differences and a greater fluidity of the membrane for leukemic compared to normal PBMC (25-28), properties which may contribute to the difference between the distribution of nanocurcumin in the form of Lipocurc™ into PBMC$_{HI}$ and PBMC$_{CLL,Grp\ 2}$. Partition coefficients for curcumin in PBMC$_{HI}$ and PBMC$_{CLL,LS}$ based on ng/g w.w. of the cell pellet

following 16 min of incubation at 37˚C were low at 0.264 and 0.328, respectively, suggesting that it is unlikely there is a major active transport based mechanism to concentrate curcumin in either $PBMC_{HI}$ or $PBMC_{CLL,Grp\ 2}$. Nonetheless, compared to human red blood cells under similar incubation conditions (19) curcumin levels in $PBMC_{HI}$ were ~1.8-fold higher based on ng/g w.w. while in $PBMC_{CLL,Grp\ 2}$ they are 2.4-fold higher. In red blood cells, no major transport mechanisms have been characterized. Thus, $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$ may have non-active transport mechanisms that contribute to a greater distribution of curcumin as Lipocurc™ in PBMC compared to red blood cells.

Based on pmole/10^6 viable cells and normalized to a 1 μM incubation concentration of curcumin, the average levels of curcumin measured in $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$ were 4.3±1.5 and 7.8±3.6 pmole/10^6 cells, respectively. These levels are lower than those reported for normal and tumor cell lines which ranged from 22.6-23.2 pmles/106 cells and 34.5-44.2 pmoles/10^6 cells (17), respectively. The lower curcumin levels per cell reported in this study may be either due to the different cells used in this assay or that the assay contained much more protein in the form of plasma proteins compared to the study with normal and tumor cell lines as nanocurcumin has been shown to interact with plasma proteins (29), reducing its effective concentration for distribution into PBMC.

In contrast to curcumin, THC levels in $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 2}$, did not display a marked difference and were on average 19.4% and 7.3% of the curcumin levels in each cell type, respectively when incubated at 37˚C. However, the production of THC in $PBMC_{CLL,Grp\ 2}$ did show a slightly greater dependence on incubation time and temperature (both at 4˚C and 37˚C) compared to $PBMC_{HI}$ which may be related to the time-dependent increase of curcumin levels in $PBMC_{CLL,Grp\ 2}$. Given the finding that THC levels were only 7.3% of the curcumin levels in $PBMC_{CLL,Grp\ 2}$ compared to 19.3% of the curcumin levels in $PBMC_{HI}$, the possibility exists that metabolism of curcumin to THC is less robust in $PBMC_{CLL,Grp\ 2}$ compared to $PBMC_{HI}$, which may contribute to higher cell levels of curcumin. Nonetheless the production of THC is evidence that curcumin distributes across the cell membrane and into PBMC. This is consistent with fluorescent microscopy studies of the cellular distribution of curcumin after incubation with normal and tumor cells which demonstrated both a cell membrane and intracellular localization for curcumin (17).

Of interest, was the finding that $PBMC_{CLL,Grp\ 1}$ had a similar extent of curcumin distribution compared to $PBMC_{CLL,Grp\ 2}$, despite a higher number of lymphoctyes for $PBMC_{CLL,\ Grp\ 2}$. The independence of curcumin distribution from lymphocyte number in CLL may arise from the finding that in CLL patients the presence of CLL cells in the blood may alter the phenotype of the overall monocyte and macrophage population (30), in this case, potentially resulting in a greater ability to distribute curcumin in PBMC independent of the lymphocyte cell number in CLL.

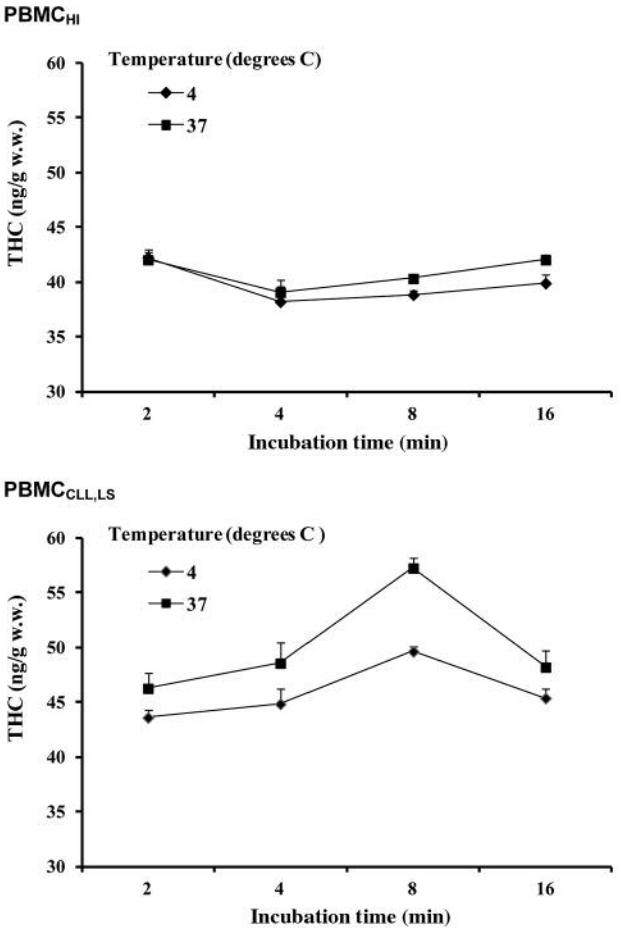

Figure 4. *THC levels in $PBMC_{HI}$ and $PBMC_{CLL,LP}$. The metabolism of THC to curcumin in $PBMC_{HI}$ and $PBMC_{CLL,LS}$ is illustrated above using a line graphs and an expanded scale, the data is presented±S.E.M and the values along with their statistical significance are presented in Table III. Area under the curves was calculated using the linear trapezoidal rule and the difference between 37˚C and 4˚C (in ng w.w.-min) for $PBMC_{HI}$ was 19 and for $PBMC_{CLL,LS}$ was 74.*

Distribution of curcumin into $PBMC_{HI}$ and $PBMC_{CLL,Grp2}$ and metabolism to THC did not display a marked dependence on temperature. This is to some extent consistent with the temperature independence of receptor ligand interactions and the cell type dependent interaction of molecules with cell membranes (31, 32), but also suggests, as previously discussed, that curcumin does not distribute into cells by a major active transport based mechanism. Furthermore, prior studies have shown that the greatest cell concentrations of curcumin exist in the plasma membrane and nucleus (17) consistent with the lipophilic nature of

curcumin and suggests that curcumin distributes into cells based on the simple diffusion of curcumin from a more aqueous environment (cell culture medium or plasma) to a more lipophilic (cell membrane) environment. Thus, Lipocurc™ may facilitate the concentration of curcumin in the PBMC cell membrane with curcumin subsequently diffusing into the cell.

In summary, we demonstrated that upon incubation of PBMC with Lipocurc™, the distribution of curcumin in $PBMC_{CLL,Grp\ 2}$ was time-dependent and greater for both $PBMC_{CLL}$ with low and high lymphocyte counts compared to distribution in $PBMC_{HI}$. The differences noted for the time-dependent distribution of curcumin into $PBMC_{HI}$ and $PBM_{CCLL,Grp2}$ are consistent with differences in the membrane fluidity of normal and tumor cells, suggesting that membrane fluidity may influence the membrane distribution of curcumin from Lipocurc™ in leukemic compared to normal PBMC. Curcumin metabolism to THC by curcumin reductase was observed both in $PBMC_{HI}$ and $PBMC_{CLL,Grp\ 1/Grp\ 2}$ consistent with the distribution of curcumin into PBMC. The potential greater and consistent distribution of curcumin as Lipocurc™ into PBMC from CLL patients suggests a potential for a treatment benefit for the utilization of Lipocurc™ in maintenance therapy for CLL. The PBMC used in this study clearly represent a heterogenous population of cells. Therefore, and quite importantly, a study on the comparative distribution of curcumin into plasma B cells from healthy individuals and CLL patients is now warranted based on the results of this study.

References

1 Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnet LJ, Gescher AJ and Steward WP: Pharmacodynamic and pharmacokinetic study of oral *Curcuma* extract in patients with colorectal cancer. Clin Cancer Res *7*: 1894-1900, 2001.

2 Chen AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen GA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC and Hsieh CY: Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res *21*: 2895-2900, 2001.

3 Dhillon D, Aggarwal BB, Newman RA, Wolfe RA, Kunnumakkara AB, Abbruzzese JL, Ng CS, Badmaev V and Kurzrock R: Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin Cancer Res *14*: 4491-4499, 2008.

4 Li M, Zhang Z, Hill DL, Wang H and Zhang R: Curcumin, a dietary component, has anticancer chemosensitization, and radiosensitization Effects by down-regulating the MDM2 oncogene through the PI3/mTOR/ETS2 pathway. Cancer Res *67*: 1988-1996, 2007.

5 Chang YF, Chuang HY, Hsu CH, Liu RS, Gambhir SS and Hwant JJ: Immunomodulation of curcumin on adoptive therapy with t cell functional imaging in mice. Cancer Prevention Res *5*: 444-452, 2012.

6 Bhattacharyya S, Hossain DMS, Mohanty S, Senj GS, Chattopadhyay S, Banerjee S. Chankraborty J, Das K, Dptendra S, Das T and Gaurisankar S: Curcumin reverses T cell-mediated adaptive immune dysfunctions in tumor-bearing hosts. Cellular and Mol Immunol *7*: 6306-6315, 2010.

7 Golombick T, Diamon TH, Manoharan A and Ramakrishna R: The effects of Curcumin (as Meriva) on absolute lymphocyte count (ALC), NK cells and T cell populations in patients with stage 0 / 1 Chronic Lymphocyteic Leukemia. J Cancer Ther *6*: 566-571, 2015.

8 Zhongfa L, Chiu M, Wang J, Chen W, Yen W, Fan-Havard P, Yee LD and Chan KK: Enhancement of curcumin oral absorption and pharmacokinetics of curcuminoids and curcumin metabolites in mice. Cancer Chemother Pharmacol *69*: 679-689, 2012.

9 Prasad S, Tyagi AK and Aggarwal BB: Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice Cancer Res Treat *46*: 2-18, 2014.

10 Rajan AP, Mukerjee A, Helson L, Gupta R and Vishwanatha JK: Efficacy of liposomal curcumin in a human pancreatic tumor xenograft model: inhibition of tumor growth and angiogenesis. Anticancer Res *33*: 3603-3609, 2013.

11 Tsai YM, Chien CF, Lin LC and Tsai TH: Curcumin and its nano- formulation: the kinetics of tissue distribution and blood-brain barrier penetration. Int J Pharmaceut *416*: 331-338, 2011.

12 Shanbhag VKL: Curcumin in chronic lymphocytic leukemia – a review. Asian Pac J Trop Biomed *7*: 505-512, 2017.

13 O'Brien S and Kay NE: Maintenance therapy for B-chronic lymphocytic leukemia. Clin Adv Hematol Oncol *9*: 22-31, 2011.

14 Gosh AK, Kay NE, Secreto CR and Shanafelt TD: Curcumin inhibits prosurvival patways in chronic lymphocytic leukemia b cells and may overcome their stromal protection in combination with ECGC. Clinical Cancer Res *15*: 1250-1258, 2009.

15 Everett PC, Meyers JA, Makkinje A, Rabbi M and Lerner A: Preclinical assessment of curcumin as a potential therapy for B-CLL. American J Hematol *82*: 23-30, 2007.

16 Pan MH, Sy LS and Lin JK: comparative studies on the suppresion of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IkappaB kinase and NFkappaB activation in macrophages. Biochem Pharmacol *60*: 1665-1676, 2000.

17 Kunwar A, Barik A, Mishra B, Rathinasamy K, Pandey R and Priyadarsini KI: Qualitative cellular uptake, localization and cytotoxicity of curcumin in normal and tumor cells. Biochimica Biophysica Acta *1780*: 673-679, 2008.

18 Kunwar A, Barik A, Mishra B and Priyadarsini KI: differential up-take and fluorescence of curcumin, a yellow pigment from turmeric, in normal *vs*. tumor cells. BARC Newsletter *285*: 202-207, 2007.

19 Bolger GT, Licollari A, Tan A, Griel R, Vcelar B, Majeed M and Helson L: Distribution and metabolism of Lipocurc™ (Liposmal Curcumin) in dog and human blood cells: Species Selectivity and Pharmacokinetic Relevance. Anticancer Res *37*: 3483-3492, 2017.

20 Tan A, Wu Y, Wong M, Licollari A, Bolger G, Fanaras J, Shopp G and Helson L: Use of basic mobile phase to improve chromatography and boost sensitivity for quantifying tetrahydrocurcumin in human plasma by LC–MS/MS. J Chromatograph B *1028*: 86-93, 2016.

21 Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, Hillmen P, Keating MJ, Montserrat E, Rai KR and Kipps TJ: International workshop on chronic lymphocytic leukemia. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International workshop on chronic lymphocytic leukemia updating the national cancer institute-working group 1996 guidelines. Blood *111*: 5446-5456, 2008; Erratum in: Blood *112*: 5259, 2008.

22 Pleyer L, Egle A, Hartmann TN and Greil R: Molecular and cellular mechanisms of CLL: Novel therapeutic approaches. Nat Rev Clin Oncol *6*: 405-418, 2009.

23 Huemer M, Rebhandl S, Zaborsky N, Gassner FJ, Hainzl S, Weiss L, Hebenstreit D, Greil R and Geisberger R: AID induces intraclonal diversity and genomic damage in CD86(+) chronic lymphocytic leukemia cells. Eur J Immunol *44*: 3747-3757, 2014.

24 Duan J, Bolger G, Garneau M, Amad M, Batonga J, Montpetit H, Otis F, Jutras M, Lapeyre N, Rheaume M, Kukolj G, White PW, Bethell RC and Cordingley MG: The liver partition coefficient-corrected inhibitory quotient and the pharmaco-kinetic-pharmacodynamic relationship of directly acting anti-hepatitis C virus agents in humans. Antimicrob Agents Chemother *56*: 5381-5386, 2012.

25 Inbar M, Ben-Bassat H, Fibach E and Sachs L: Mobility of carbohydrate-containing structures on the surface membrane and the normal differentiation of myeloid leukemic cells to macrophages and ganulocytes. Proc Natl Acad Sci *70*: 2577-2581, 1973.

26 Ben-Bassat H, Polliak A, Rosenbaum SM, Naparstek E, Shouval D and Inbar M: Fluidity of membrane lipids and lateral mobility of concanavalin A receptors in the cell surface of normal lymphocytes and lymphocytes from patients with malignant lymphomas and leukemias. Cancer Res *37*: 1307-1312, 1977.

27 Yanovich S, Harris K, Sallan SE, Schlossman SF and Inbar M: Dynamic parameters of membrane lipids in normal and leukemic human lymphocytes isolated from peripheral blood and bone marrow. Cancer Res *38*: 4654-4661, 1978.

28 Andoh Y, Okazaki S and Ueoka R: Molecular dynamics study of lipid bilayers modeling the plasma membranes of normal murine thymocytes and leukemic GRSL cells. Biochim Biophys Acta *1828*: 1259-1270, 2013.

29 Yallapu MM, Ebeling MC, Chauhan N, Jaggi M and Chauhan SC: Interaction of curcumin nanoformulations with human plasma proteins and erythrocytes. Int J Nanomed *6*: 2779-2790, 2011.

30 Maffei R, Bulgarelli J, Fiorcar S, Bertoncelli L, Marinelli S, Guarnotta C, Castelli I, Deaglio S, Debbia G, De Biasi S, Bonacorsi G, Zucchini P, Narni F, Tripodo C, Luppi M, Cossarizza A and Marasca R: The monocytic population in chronic lymphocytic leukemia shows altered composition and deregulation of genes involved in phagocytosis and inflammation. Haematologica *98*: 1115-11123, 2013.

31 Kawamura R, Shimizu K, Matsumoto Y Yamagishi A, Silberberg YR, Iijima M, Kuroda S, Fukazawa K, Ishihara K and Nakamura C: High efficiency penetration of antibody-immobilized nanoneedle through plasma membrane for in situ detection of cytoskeletal proteins in living cells. J Nanobiotechnol *14*: 74-83, 2016.

32 Man D and Olchawa R: Dynamics of surface of lipid membranes: theoretical considerations and the ESR experiment. Eur Biophys J *46*: 325-334, 2017.

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