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Evaluation *in Vitro* of Synthetic Curcumins As Agents Promoting Monocytic Gene Expression Related to β-Amyloid Clearance

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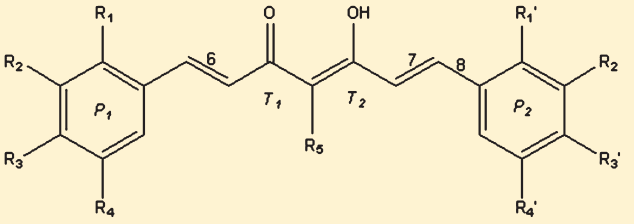
ABSTRACT: Accumulation of amyloid-beta (Aβ) is one of the hallmarks of Alzheimer's disease (AD), and efficient clearance of Aβ by cells of the innate immune system may be an important mechanism for controlling or preventing disease onset. It was reported that peripheral blood mononuclear cells (PBMCs) of most AD patients are defective in the phagocytosis of soluble Aβ. Natural curcumins were shown to restore Aβ phagocytosis by AD PBMCs and to up-regulate the expression of key genes including *MGAT3* and those encoding Toll-like receptors (*TLR*s). Bisdemethoxycurcumin (BDC), a minor component of natural curcumin, was shown to have the greatest potency for stimulating AD PBMCs. Because natural curcumins have inherent limitations with regard to physicochemical properties, synthetic curcumin analogues were developed that showed improved solubility, stability, and bioavailability. An *in vitro* system using human monocytic cell lines (U-937, THP-1) was used to evaluate analogues for the potency of innate immune cell stimulation. These cell lines showed responses to curcuminoids and to 1α,25-dihydroxyvitamin D3 (VD3) resembling those seen in human PBMCs. From more than 45 curcuminoids analyzed, the most potent compounds possessing enhanced pharmaceutical properties were identified. The most promising candidates included prodrug versions containing water solubility-enhancing amino acids and stability-increasing modifications near the central diketone. *In vivo* studies showed compound (**5**) substantially increased bioavailability by combining several promising structural modifications. Studies examining *ex vivo* phagocytosis of Aβ and bead particles in mouse microglia showed that BDC and several water-soluble analogues were quite effective compared to curcumin or an unnatural analogue. *In vitro* studies using monocytic cell lines reported herein complement those using human PBMCs and represent a routinely accessible and uniform cellular resource allowing direct comparisons between compounds.



INTRODUCTION

The mechanisms of neurodegeneration associated with abnormally folded proteins, including beta-amyloid (Aβ) and phosphorylated tau in Alzheimer's disease (AD), remain unclear. Considerable interest exists in the role of the innate immune system in neuropathology and defective innate immunity in AD patients has been suggested to play a significant role in amyloidosis leading to inflammation and neurodegeneration.[1,2] One approach to addressing the neurodegenerative diseases described previously [2] has focused on stimulating innate immune cells, including blood-borne macrophages and brain-based microglia to promote Aβ clearance. Peripheral blood mononuclear cells (PBMCs) of AD patients have been shown to cross the blood−brain barrier into the brain, but generally are defective in Aβ phagocytosis and in clearing Aβ plaques from AD brain sections.[1,3] Defective Aβ phagocytosis was associated with dramatically decreased expression of certain target genes,[2,4] including those encoding the glycosylation-modifying enzyme MGAT3 (GnT-III), and the Toll-like receptors (TLRs) that are crucial for macrophage function.[2,3] A direct correlation between MGAT3 function and efficient phagocytosis was shown by experimental knockdown of gene expression in PBMCs from healthy control subjects using siRNA, resulting in a significant (>85%) inhibition of Aβ phagocytosis.[2] It was further shown that the commonly used natural product curcumin isolated from the curry spice turmeric restored these deficiencies associated with gene down-regulation in PBMCs from AD patients.[2,3] A minor curcuminoid constituent, bisdemethoxycurcumin (BDC), was isolated from the natural mix and shown to possess the greatest potency for enhancing Aβ phagocytosis and clearance.[2] Restored uptake of Aβ by AD patients' macrophages was associated with significant up-regulation in the levels of *MGAT3* and *TLR* mRNAs.[5]

The hormone 1α,25-dihydroxyvitamin D3 (VD3) regulates the transcription of important human genes including genes for innate immune antimicrobial defenses, such as cathelicidin[6] and has important extranuclear immunostimulating effects.[7] Administration of VD3 improves phagocytosis and degradation of Aβ by monocyte/macrophages[8] and protects neurons against Aβ toxicities related to increased intracellular calcium and decreased vitamin D receptor (VDR) and nerve growth factor.[9] Recently, the intracellular receptor for both VD3 and BDC was identified as the VDR, suggesting that VDR is associated with VD3- and BDC-mediated improvement in Aβ phagocytosis by monocytes of AD patients.[8] Although the precise molecular mechanisms are

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not fully understood, *MGAT3*, *TLRs*, and *VD3* genes are involved with innate immune cell functional activity for Aβ degradation and cellular immune capacity in response to curcumin treatment of relevance to AD.

Given that natural curcumins have been consumed safely by humans for thousands of years,[10,11] the observed potency of these compounds to enhance Aβ phagocytosis suggests the potential for a novel and safe therapeutic approach to stimulate uptake and degradation of Aβ peptide by AD patients' innate immune cells. Natural curcumins have also been reported to have strong anti-inflammatory and antioxidant properties, and therefore may have additional benefits for treating other diseases beyond clearance of abnormal protein accumulation in AD.[12,13] Antioxidant function may be distinct from immune stimulation. Support for the potential of curcumins as anti-AD agents come from both epidemiological and animal studies. In populations in Asia where curcumins are widely consumed, the prevalence of AD is less than 4-fold that in the United States.[10,14] Enhanced cognitive performance among elderly curry-consuming Asian individuals, compared to individuals that regularly do not consume curry, has also been reported.[15] More direct support comes from studies showing that curcumin enhanced the clearance of brain Aβ in mouse transgenic models of AD.[16,17]

Natural curcumins have inherent limitations with regard to bioavailability that restrict their potential as drug candidates. Most curcumins possess poor aqueous solubility and stability, thus prompting their use in clinical trials at multigram/day quantities in an effort to reach effective physiological levels.[18] In an effort to overcome the pharmaceutical limitations of natural curcumins, novel analogues were synthesized with improved pharmaceutical properties. We previously reported on the development of synthetic curcuminoids that were tested to stimulate Aβ clearance and expression of key genes associated with improved innate immune function using PBMCs from AD patients.[2,3,8] Certain analogues were found to have greater potency for stimulating Aβ clearance with corresponding gene expression changes, and a preliminary description of certain physicochemical properties associated with these analogues was presented.[8]

Herein, we report on the development of an *in vitro* approach for evaluating curcuminoids using human monocytic cell lines, offering the potential of a uniform and accessible cellular resource that can be tested in higher throughput. We show that established monocyte cell lines mimic the responses observed in *ex vivo* human PBMCs and possess functional hormonal signaling that likely mediates innate immune activation by curcuminoids. We have expanded our prior analyses to include a larger library of synthetic curcumin analogues, have characterized the analogues for the potency of stimulating innate immune gene expression relevant to Aβ clearance, and have identified chemical features associated with improved pharmaceutical properties. We have verified the potency of certain curcumin analogues in *ex vivo* particle bead phagocytosis and Aβ clearance studies in mouse microglia. The *in vitro* system was used in structure−activity relationship (SAR) studies to guide the iterative synthesis of novel curcuminoids with superior potency and drug-like characteristics.

MATERIALS AND METHODS

Synthesis and Characterization of Curcuminoids. Natural curcumins, synthetic analogues of BDC, amino acid-BDC prodrugs, and cyclohexyl-containing BDC analogues **1**−**42** were synthesized and analyzed using previously published methods.[2,8] Compound **43** was synthesized according to a previously reported method,[19] and the spectral data was consistent with the published data. The synthesis of compounds **44** and **45** is reported below. Characterization of physicochemical properties including aqueous stability and solubility, metabolic stability in the presence of human liver microsomes and S9 fraction, and permeability were also as described.[8,18] Values for compound lipophilicity (log *P*), topological surface area (tPSA), and Gibbs free energy (ΔG) were calculated using ChemDraw software (v 11.0, CambridgeSoft). Three fragmentation methods for estimating log *P* were used.[20−22]

*Preparation of 1-(4-(Dimethylamino)phenyl)-5-hydroxyhexa-1,4-dien-3-one (**44**).* 2,4-Pentanedione (2 mL, 20 mmol) was dissolved in EtOAc (4 mL), and boric anhydride (0.9 g, 11 mmol) was added to the solution. The mixture was stirred at 70 °C for 40 min. To this solution, 4-dimethylaminobenzaldehyde (995 mg, 6.7 mmol) and tributylborate (1.8 mL, 6.7 mmol) were added, and the mixture was stirred at 70 °C for 30 min. The temperature was raised to 85 °C, and *n*-butylamine (0.66 mL, 6.7 mmol) was added dropwise over a period of 20 min. The organic layer was separated, and the aqueous layer was extracted with EtOAc. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography (hexane/EtOAc, 2:1, v/v) to give 291 mg, 19% yield, of the desired product as a red powder, R_f = 0.27. ESI/MS: m/z = 232 (MH^+). ^{1}HNMR ($CDCl_3$) δ 7.38 (s, 1H), 7.30 (d, J = 9.3 Hz, 2H), 6.64 (d, J = 9.3 Hz, 2H), 3.04 (s, 6H), 1.9 (d, J = 5.7 Hz, 6H).

*Preparation of 2-(3-(4-Hydroxyphenyl)acryloyl)cyclohexanone (**45**).* 2-Acetyl cyclohexanone (1.6 mL, 12.3 mmol) was dissolved in EtOAc (2 mL) and boric anhydride (571 mg, 8.2 mmol) was added. The mixture was stirred at 70 °C for 40 min. 4-Hydroxybenzaldehyde (0.5 g, 4.1 mmol) and tributylborate (1.3 mL, 4.1 mmol) were added to this solution. The resulting mixture was stirred at 70 °C for 30 min. *n*-Butylamine (0.2 mL) was added dropwise, and the solution was stirred at 100 °C for 1 h and then cooled to 56 °C. Then, 0.4 N HCl (10 mL) was added, and the mixture was stirred at 56 °C for 30 min. The resulting mixture was cooled to room temperature and then extracted with EtOAc. The organic extracts were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography (100% hexane followed by a gradient increase to 50% EtOAc/hexane) to give 300 mg, 10% of the product as a yellow powder, R_f = 0.83; ESI/MS m/z = 243 (MH^-), ^{1}H NMR ($CD_3OD/CDCl_3$, 4/1, v/v) δ 7.6 (d, J = 15.4 Hz, 1H), 7.46 (m, 2H), 6.85 (d, J = 15.4 Hz, 1H), 6.79 (m, 2H), 2.54 (m, 2H), 2.37 (m, 2H), 1.74 (m, 4H).

Cell Culture and Compound Treatments. Two human monocytic cell lines, U-937[23] and THP-1,[24] were used for *in vitro* characterization of gene expression in response to treatments with BDC analogues and Aβ. Cell lines were obtained from ATCC (www.atcc.org). Both cell lines were maintained in RPMI-1640 medium plus glutamine (ThermoScientific/HyClone, Waltham MA), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For cell treatments with compounds, replicate 0.5−1 mL cultures containing 1×10^6 cells/mL were plated in 24- or 48-well tissue culture plates. Curcumin analogues (Figure 1) were resuspended from dried aliquots just prior to addition to cell cultures, initially in DMSO and then diluted to varying test concentrations in culture medium. Oligomeric amyloid-beta (1−42) peptide (Aβ) was obtained from California Peptide Research (Napa, CA). Dried aliquots of peptide were resuspended in water just prior to use in cultures at a concentration of 5 μg/mL. Tests typically involved overnight treatment with curcuminoid (24 h), followed by a second overnight treatment with or without added Aβ, as previously reported for human PBMCs.[2,8]

Gene Expression Analysis. Assays were designed for characterizing mRNA levels using Sybr Green real-time, quantitative PCR

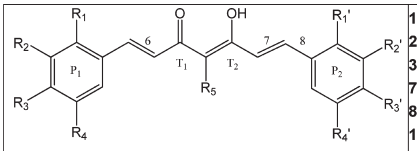
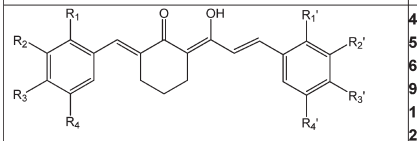
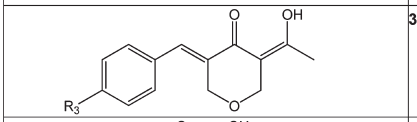
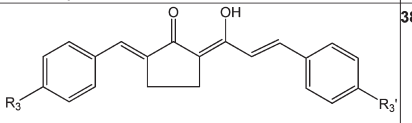
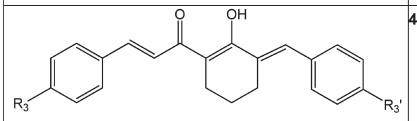
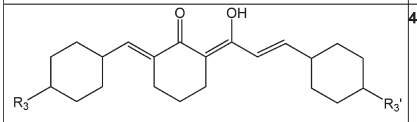
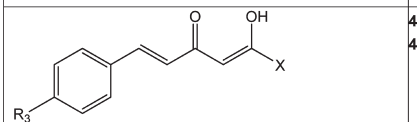
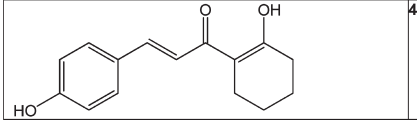
	**1***: R_1,R_2,R_4=H, R_3=OH (**BDC**) **2***: R_1,R_2,R_4=H, R_3=Val **3***: R_1,R_4=H, R_2=OCH_3, R_3=Lys **7**: R_1=OH, R_2,R_4=H, R_3=OCH_3 **8**: R_1=OCH_3, R_2,R_4=H, R_3=$N(CH_3)_2$, R_5=CH_3CH_2 **10**: R_1,R_4=H, R_2=Cl, R_3=OH **12**: R_1,R_2,R_4=H, R_3=OH, 6,7=sat.bonds **13**: R_1,R_2,R_4=H, R_3=OCH_3 **14**: R_1,R_2,R_4=H, R_3=$OCOCH_3$ **15**: R_1,R_2,R_4=H, R_3=OCO-tBu **16***: R_1,R_3,R_4=H, R_2=OH **17**: R_1R_4=H, R_2=OH, R_3=OCH_3 **18***: R_1,R_4=H, R_2=OCH_3, R_3=OH (**Curcumin**) **19**: R_1=OCH_3, R_2,R_4=H, R_3=OH **20**: R_1=OCH_3, R_2, R_3,R_4=H **21**: R_1,R_2,R_4=H, R_3=SCH_3 **22**: R_1,R_2,R_3,R_4=H **23**: R_1,R_2,R_4=H, R_3=CH_3 **24**: R_1,R_2,R_4=H, R_3=tBu **25**: R_1,R_4=H, R_2= OCH_3, R_3=OH **26**: R_1=OCH_3, R_2,R_4=H, R_3=$N(CH_3)_2$ **27**: R_1,R_2,R_4=H, R_3=OH, R_5= CH_3CH_2 **28**: R_1,R_2,R_4=H, R_1'=OCH_3, R_3=$N(CH_3)_2$, R_4'=F **30***: R_1,R_2,R_4=H, R_3=$N(CH_3)_2$ **31**: R_1,R_2,R_4=H, R_3=Lys **32***: R_1,R_4=H, R_2= OCH_3, R_3=Val **33***: R_1=OCH_3, R_2,R_3=H, R_4=F **34**: R_1,R_2,R_4=H, R_3=$N(CH_3)_2$, R_5= CH_3CH_2 **36**: R_1=OCH_3, R_2,R_3=H, R_4=F, R_5= CH_3CH_2
	**4***: R_1,R_2,R_4=H, R_3=$N(CH_3)_2$ **5**: R_1,R_2,R_4=H, R_3=Val **6**: R_1,R_2,R_4=H, R_3=Lys **9**: R_1,R_4=H, R_2=OCH_3, R_3=Val **11**: R_1=OCH_3, R_2,R_4=H, R_3=$N(CH_3)_2$ **29***: R_1,R_2,R_4=H, R_3=OH **35**: R_1=OCH_3, R_2,R_3=H, R_4=F **39**: R_1,R_4=H, R_2=OCH_3, R_3=OH **40**: R_1,R_4=H, R_2=OCH_3, R_3=Lys
	**37**: R_3=OH
	**38**: R_3, R_3'=OH
	**41**: R_3=Val, R_3'=$N(CH_3)_2$
	**42**: R_3=H,R_3'=OH
	**43**: R_3=OH, X=CH_3 **44**: R_3=$N(CH_3)_2$, X=CH_3
	**45**:

Figure 1. Natural curcumins and synthetic analogues. Generally, the corresponding R sites on both phenolic rings (i.e., P_1, P_2) were the same. R′ indicates differences between the rings at the corresponding site. Compound **1** is natural bisdemethoxycurcumin (BDC), and **18** is the predominant curcumin in the natural mixture. The following terms are abbreviated: amino acids, Val = valine, Lys = lysine, OCH_3 = methoxy; SCH_3 = S-methyl; *t*Bu = tertiary-butyl, Sat. = saturated bond. The asterisk indicates compounds that are also in ref 8. Compounds **1**−**12** were identified to be overall the most potent; however, numbering does not imply potency rankings.

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(qPCR). Using human gene sequences available from NCBI (www.ncbi.nlm.nih.gov/nucleotide), PCR oligonucleotide pairs were selected spanning introns to optimize amplification from mRNA templates and avoiding nonspecific amplification products, using NCBI's Primer-BLAST or online Primer 3.0.[24,25] Oligonucleotide sequences for qPCR analysis of the test genes were as follows:

MGAT3, (F3) CGGACGCCAGCATCTCC, (R4) AGGGCTGAGGGAGGCCAGTTCTC;

TLR2, (2F) GCCCATTGCTCTTTCACTGC, (3R) TGATGATGACCCCCAAGACC;

TLR3, (1F) AAAGGAAAGGCTAGCAGTCATCC, (2R) CAGTGCACTTGGTGGTGGAG;

TLR4, (2F) ATCCCCTGAGGCATTTAGGC, (3R) TGCCCCATCTTCAATTGTCTG;

VDR, (7F) CAGCATCCAAAAGGTCATTGG, (8R) CAATGGCACTTGACTTCAGCAG;

and the control gene for normalizing expression of the test genes, *HPRT*, (F407) GACCAGTCAACAGGGGACAT, (R588) GTCCTTTTCACCAGCAAGCT.

Following compound treatments, cultured cells were harvested by brief centrifugation and were lysed, and total cellular RNAs were extracted using RNeasy Minikits (QIAGEN, Valencia, CA). RNAs were quantified using a Nanodrop spectrophotometer (ThermoScientific, Waltham, MA), and total cDNAs were prepared from 1 to 2 μg of total RNA using SuperScript III reverse transcriptase (LifeTechnologies, San Diego, CA). qPCR reactions included 200 nM of each oligonucleotide, 1× SYBR Green SuperMix (BioRad, Richmond, CA), and 1 μL of cDNA template (or water control). Cycling conditions using a BioRad iQ5 real-time thermocycler were 5 min denaturing at 95 °C, followed by 40 cycles of 95 °C (10 s) and 58 °C annealing (30 s).

Data Analysis. Cycle threshold (*Ct*) values were automatically recorded for each replicate qPCR reaction, and mean *Ct* values were normalized against those determined for *HPRT*. Fold-expression differences relative to untreated control cells were determined using the $2^{-\Delta\Delta Ct}$ method.[26] Significance of gene expression changes relative to controls was analyzed using one-way ANOVA (Kruskal–Wallis) and the Dunns post-test for all possible test pairings using Prism GraphPad 3.03 software (GraphPad Software, San Diego, CA). *P*-values (two-tailed) with 95% confidence intervals were computed, and $P < 0.05$ was considered statistically significant.

Correlation studies involving chemical properties of curcumin analogues and gene expression responses were done using both nonparametric (Spearman) and Gaussian data (Pearson) methods, both yielding similar results. The analyses provided *r*-values and significance of observed correlations.

Murine Microglial Cell Isolation. Microglial cells were obtained from cerebral cortices of neonate DBA/2N mice (1–3 days old). Cortices were mechanically dissociated in Hanks' Balanced Salt Solution with 100 μg/mL DNase I (Sigma, St. Louis, MO). Dissociated cells were filtered through a 100 μm cell strainer, (Falcon, Heidelberg, Germany) and centrifuged at 200*g* for 5 min. Pellets were resuspended in growth medium consisting of high glucose DMEM (Life Technologies), 10% FBS (Atlanta Biologicals, Lawrenceville, GA), and 25 ng/mL recombinant mouse granulocyte–monocyte colony stimulating factor, (rmGM-CSF, R&D Systems, Minneapolis, MN), and cells were plated at a density of two brains per T-75 plastic culture flask. After seven days, the flasks were shaken at 200 rpm using a Lab-line orbital shaker for 2 h at 37 °C. Cells suspensions were centrifuged at 200*g* and resuspended in the appropriate assay buffer.

Bead Phagocytosis Assay. Mouse microglia were treated with 100 nM BDC or BDC analogues overnight. FluorSpheres particles (10 μm, Life Technologies) (5×10^6) were washed with 1 mL of PBS three times to remove azide. Bead particles were added (1:10) to mouse microglia in assay buffer (DMEM, 10% FBS) and incubated for 90 min at 37 °C. Unbound particles were washed with PBS, and cells were stained with Hema 3 reagent (Fisher Diagnostics, Pittsburgh, PA) for 30 s, and phagocytized beads were visualized by light microscopy. Phagocytotic index was calculated by dividing the number of cells that ingested beads by the total number of cells counted × 100.

Ex Vivo Phagocytosis Assay. The *ex vivo* assay was conducted with PDAPP frozen sections in the presence of 3 μg of an anti-Aβ IgG2a antibody 3D6 as a positive control, an irrelevant Ms IgG2a antibody as a negative control, and BDC and BDC analogues. Sections were treated with antibodies for 30 min prior to the addition of mouse microglia, at 5% CO_2 at 37 °C. The cocultures were then extracted the next day, and the remaining Aβ was measured by ELISA (21F12 capture, 266-B reporter) to assess Aβ clearance.

Amyloid-β ELISA Assay. For quantification of Aβ, either mAb 266 (made at Elan Pharmaceuticals) at 10 μg/mL or mAb 21F12 (made at Elan Pharmaceuticals) at 5 μg/mL was diluted into well coating buffer (100 μL per well, 0.1 M sodium phosphate, pH 8.5, and 0.1% sodium azide) and added into immunoassay plates (Immulon 4HBX, Thermo LabSystems) and incubated overnight at room temperature. The solution was removed by aspiration, and wells were blocked by the addition of 200 μL of 0.25% human serum albumin in PBS buffer for at least 1 h at room temperature. Blocking solution was removed, and the plates were stored desiccated at 4 °C until used. Plates were rehydrated with TTBS wash buffer just prior to use. Samples, standards, and controls were added (100 μL per well) in triplicate to coated plates and incubated overnight at 4 °C. The plates were washed at least three times with TTBS. Biotinylated mAb 3D6, diluted to 0.5 μg/mL in casein assay buffer (0.25% casein, PBS, 0.05% Tween 20, pH 7.4), was added at 100 μL per well and incubated for 1 h at RT. Avidin–horseradish peroxidase conjugate, (Avidin-HRP, Vector Laboratories, Burlingame, CA), was diluted 1:4000 in casein assay buffer, then added at 100 μL per well to the plates, and incubated for 1 h at RT. One hundred microliters per well of colorimetric substrate, One-Step Slow TMB-ELISA, (Pierce Chemical Company, Rockford, IL) was added and allowed to react for 15 min at RT, after which the enzymatic reaction was stopped by the addition of 25 μL of 2 N H_2SO_4. The reaction product was detected using a Molecular Devices Spectramax measuring the difference in absorbance at 450 and 650 nm. Absorption data was quantified using Softmax Pro software (Molecular Devices, Sunnyvale, CA) by extrapolation from a quadratic fit equation of the standard curve.

Bioavailability. Plasma profiles of certain compounds were determined by validated HPLC or LCMS analysis of organic extracts of plasma from mice treated with the test compound. Male BALB/c mice ($n = 3$/group) were administered **1**, **2**, or **5** at a dose of 25 mg/kg by the oral route of administration (p.o.) or 100 mg/kg by the intraperitoneal (i.p.) route. At designated time points, blood was obtained from the animal, rapidly acidified to pH 3, and centrifuged to provide serum. Acidic serum samples were treated with CH_3CN and NaCl to precipitate protein and extracted with CH_2CI_2/IPA (4:1, v:v). The extraction efficiency of >85% was superior to a previously reported study.[27] The organic fraction was evaporated to dryness and resuspended in MeOH and injected onto an HPLC-MS. *In vivo* bioavailability was determined by solving the equation $F = AUC_{po}/AUC_{ip}$ after the administration of a curcumin analogue by both oral and intraperitoneal (i.p.) routes. Area under the concentration time profile (AUC) was calculated by a plot of plasma concentration (nmol/L) versus time (h). The HPLC-MS system efficiently separated the parent compound from material extracted from the plasma or the solvent front, as well as metabolites. On the basis of the ion intensity of each material, the parent compound was compared and quantified with peak areas of the chromatogram. Standard curves were run before and after each series of chromatographic runs. The amount of material injected into the HPLC or LCMS was plotted against the peak area to determine the correlation. Linear regression analysis of

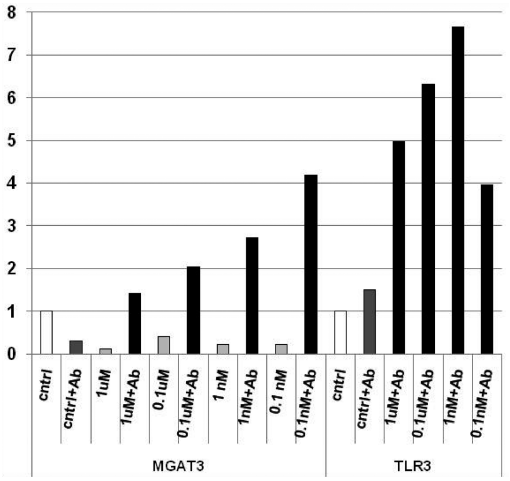

Figure 2. Fold mRNA changes in THP-1 monocytes. Duplicate THP-1 monocyte cultures were treated overnight with BDC (compound **1**, Figure 1) in a range of 1 μM−0.1 nM, and a second overnight treatment with ± Aβ (5 μg/mL). Following mRNA isolation and cDNA synthesis, *MGAT3* and *TLR3* mRNA levels were determined by qPCR and normalized to *HPRT* mRNA levels. Gene expression is shown as fold-increases (*y*-axis) relative to untreated control cells.

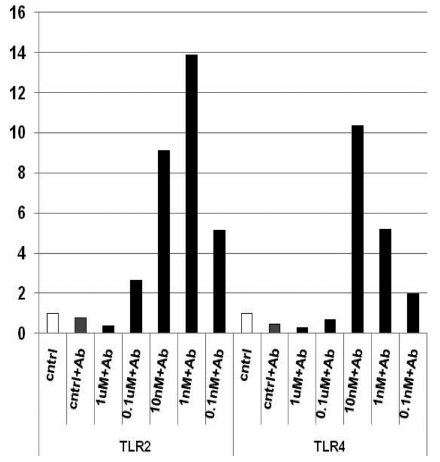

Figure 3. Fold mRNA changes in U-937 monocytes. Duplicate U-937 monocyte cultures were treated overnight with **4** in a range of 1 μM−0.1 nM and a second overnight treatment +Aβ (5 μg/mL). Mean *TLR2* and *TLR4* mRNA levels are shown as fold increases (*y*-axis) relative to untreated control cells.

such plots typically gave *r*-values of at least 0.99, and interexperiment variation for external standard curves was typically less than 5%.

RESULTS

Synthesis of Curcumin Analogues. Following observations that natural product curcumins enhanced Aβ phagocytosis by macrophages from AD patients,[28] the most potent immunostimulatory component was isolated from the natural mix and identified by LCMS as bisdemethoxycurcumin (BDC).[2,3] Medicinal chemistry approaches were applied to develop analogues of BDC to overcome limitations of the pharmaceutical properties of natural curcumins. As described previously for a subset of compounds,[8] several promising analogues were shown to enhance phagocytosis and clearance of Aβ by PBMCs from AD patients. However, the small set of compounds tested did not constitute a rigorous structure−activity relationship study. We report here the synthesis of a larger set of curcumin analogues (Figure 1). They were characterized for potency in stimulating innate immune gene expression *in vitro*. Using established synthetic schemes as reported previously,[8,18,19] the synthesis of most curcuminoids (**1**−**42**) was based on modifications of one core structure (Figure 1). Some analogues were distinguished by possessing novel compositions and placement of substituents on either or both of the phenolic groups. Modification of the *para*-phenolic hydroxyl groups included introduction of the amino acids valine or lysine that afforded prodrugs with greater aqueous solubility. Other analogues included modifications of the central bridge region, including substituents and ring structures near the tautomeric position of the diketone moiety. Several asymmetric analogues lacking one of the phenyl rings (**43**−**45**) were represented by a second core structure (Figure 1). These compounds were tested for their ability to stimulate the expression of genes in monocytes reported to be relevant to efficient Aβ uptake, and a subset of compounds were investigated for the phagocytosis of particle beads and Aβ in the presence of mouse microglia.

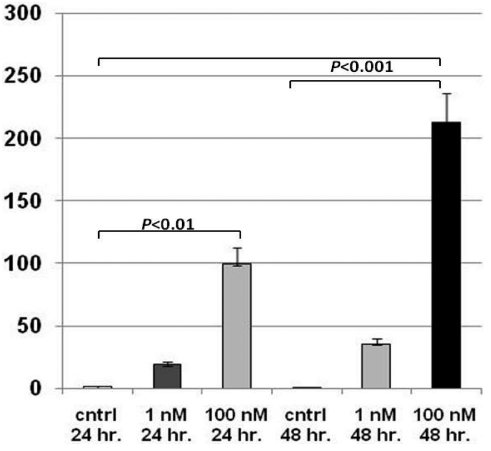

Figure 4. U-937 monocytes are responsive to VD3. Triplicate U-937 cultures were treated with VD3 at 1 nM and 100 nM for either 24 or 48 h. Mean *CAMP* mRNA levels from replicate qPCR reactions (n = 6) are shown as fold-increases (*y*-axis) relative to untreated control cells.

Curcumin Analogues Stimulate Gene Expression in Innate Immune Cells. The analysis of the effects of compounds on PBMCs obtained from human subjects can be complicated by access to AD patient samples and by variability among samples.[2,8,28] To complement those studies, an *in vitro* system for testing curcuminoids was developed using well-established human monocytic cell lines that represented readily accessible and uniform alternatives to cells from AD patients. Two monocytic cell lines, U-937[23] and THP-1,[24] were tested and shown to retain components of the Aβ phagocytosis pathways present in PBMCs from human subjects including expression of the gene *MGAT3* and those encoding *TLR*s, previously shown to be up-regulated by curcumins in the presence of Aβ.[2,8] Further, curcuminoid-treated monocytic cell lines showed up-regulated expression of *MGAT3* and *TLR*s, and the pattern resembled those of *ex vivo* human PBMCs treated with curcumin compounds. As shown for BDC-stimulated *MGAT3* expression in THP-1 cells (Figure 2), *MGAT3* and other genes typically

showed little response to curcuminoids administered alone. BDC caused an inverse dose−response where greater gene expression was observed at lower doses (Figure 2). This was likely due to cell toxicity at greater concentrations of BDC. In general, administration of curcumin compounds in the 0.1−10 nM range potentiated gene expression response after subsequent Aβ treatment. In a similar fashion, compound **4** induced TLR2 and TLR4 gene expression in a dose-dependent fashion in U-937 monocytes (Figure 3).

It has been reported that both THP-1 and U-937 cell lines undergo differentiation in response to treatment with 1,25-dihydroxyvitamin D3 (VD3).[29−31] Given that VD3 was also shown to stimulate Aβ phagocytosis in PBMCs from AD patients and that curcumin effects are likely mediated through binding to the VD3 receptor (VDR),[8] the monocyte cell lines were also tested for response to VD3 after the administration of curcumin analogues in the presence of Aβ. Figure 4 shows that VD3-treated U-937 cells showed functional VD3 signaling and significantly increased the expression of the gene encoding cathelicidin antimicrobial peptide (*CAMP*), reported to be highly responsive to VD3[32] (Figure 4). *CAMP* up-regulation was dependent on the concentration of VD3 and the amount of time cells were treated (Figure 4). Further, it was observed that treatment of cells with curcuminoid **2** plus Aβ induced the expression of *VDR* mRNA (Figure 5). The expression of VDR mRNA followed an inverse dose−response where 0.1 nM curcumin **2** was an optimal concentration (Figure 5).

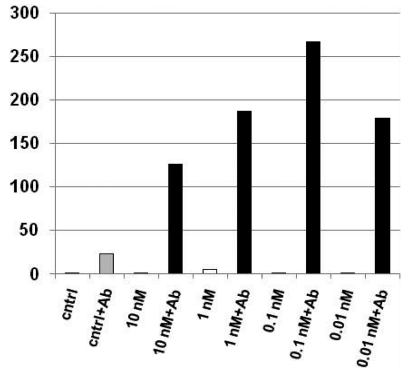

Figure 5. *VDR* mRNA levels in U-937 monocytes are responsive to curcuminoids. Duplicate U-937 cultures were treated with the water-soluble prodrug Val-BDC (**2**) in a range of 10 nM−0.01 nM and a second overnight treatment ± Aβ (5 μg/mL). Mean *VDR* mRNA levels are shown as fold-increases (*y*-axis) relative to untreated control cells.

For the curcuminoids tested, gene expression was generally more responsive to low curcuminoid concentrations, and gene expression increased in a dose-dependent manner, but this may be related to the cell toxicity observed for certain compounds at higher concentrations (i.e., ≥1 μM, data not shown). Maximum *MGAT*, *TLR*, and *VDR* gene expression in response to curcuminoids was typically seen in the range of 0.1−10 nM, but some compounds significantly increased gene expression *in vitro* at levels as low as 1 pM. While some compounds were tested at additional concentrations, for the purpose of comparing potency, most curcuminoids were tested in U-937 cells at two nominal concentrations (i.e., 0.1 nM and 0.001 nM). Compounds were evaluated for relative potency based on observed fold-expression increases at tested concentrations at or below 10 nM and on the number of genes showing >2-fold expression increases, relative to untreated controls. Table 1 lists the curcuminoids with the greatest overall potency for stimulating *MGAT3*, *TLR*, and *VDR* gene expression in the presence of Aβ (i.e., compounds **1**-**12**, Figure 1). Of the 45 compounds evaluated, 12 induced the expression of at least 3 of the 5 tested genes, and 8 induced significant expression of all 5 genes. The curcumin analogues showing the greatest overall potency for stimulating gene expression in monocytes included prodrug versions containing solubility-enhancing amino acids and modification of the central portion of the molecule. Introduction of a cyclohexyl group into the central portion of curcumin markedly increased the aqueous stability of the molecule at neutral pH. As we reported previously,[2] natural BDC (**1**) was among the most potent curcuminoids as an immune stimulant. On the basis of the data of Table 1, the most potent immune-stimulating compound overall appeared to be **2**, the valine-containing prodrug of BDC, although relatively modest induction of *TLR*s-3 and *TLR-4* by this compound was observed. *MGAT3* was the most responsive of the genes tested, with an average increase of nearly 50-fold among the most potent curcuminoids examined. *TLR4* appeared to be the least responsive gene

Table 1. Curcumins with Greatest Potency for Inducing Gene Expression Relevant to Aβ Phagocytosis[a]

compd #	(fc) *MGAT3*	(fc) *TLR2*	(fc) *TLR3*	(fc) *TLR4*	(fc) *VDR*	av (fc)	log *P*	tPSA (Å^2)	Δ*G* (kJ/mol)	HLM $t_{1/2}$(min)
1	14.0	8.5	8.5	8.5	51.0	18.1	3.93	77.8	−8.95	16.4
2	300	19.0	4.7	4.8	260.0	117.7	2.81	141.9	−132.37	≥300
3	55.0	9.0	18.0	18.0	17.0	23.4	1.97	212.4	−195.17	19.9
4	52.0	14.0	10.2	10.2	2.1	17.7	6.45	43.8	530.5	126.6
5	11.8	4.0	3.6	7.7	38.0	13.0	2.89	141.9	−138.14	≥300
6	28.6	2.4	3.0	3.2	7.5	8.9	1.78	194.0	16.5	≥300
7	6.1	6.4	4.3	4.1	22.6	8.7	4.19	96.2	−221.37	n.d.
8	9.7	2.7	5.5	2.5	3.6	4.8	7.08	62.2	332.1	n.d.
9	2.8			6.2	18.3	9.1	1.97	157.2	−387.28	≥300
10		3.7		2.1	12.0	5.9	5.17	77.8	−52.07	n.d.
11	6.9	2.7	8.0			5.9	6.71	62.2	318.1	126.7
12		3.2		2.2	2.4	2.6	3.74	77.8	−169.39	n.d.

[a] Shown are the maximal fold-changes (fc) in expression at tested concentrations (10 nM or lower), in the presence of Aβ at 5 μg/mL. Average fc was determined across all five genes. Shown also are calculated values for certain physicochemical properties included in correlation studies with gene expression. Topological polar surface area (tPSA), is presented as Å^2. HLM refers to metabolic stability in the presence of human liver microsomes, with compound half-lives presented in minutes (measured up to a maximum of 300 min).

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to curcuminoids, averaging approximately 6-fold increased expression compared to that of the vehicle control for the most potent curcumins tested.

Gene response was also related to the time of exposure of cells to compounds. U-937 monocytes were tested for gene expression responses during various times of exposure to curcumins (followed by a fixed Aβ exposure time). The length of time of administration of Aβ to cells (following fixed curcumin exposure times) was also varied. For example, as shown for both *MGAT3* and *TLR3* (Figure 6), up-regulation of gene expression was observed in response to 8 h of BDC pretreatment followed by 24 h treatment with Aβ. Similarly, mRNA levels increased with increasing Aβ exposure times up to at least 24 h.

Physicochemical Properties of Curcumins Affecting Gene Expression. Knowledge of the chemical properties of curcumin analogues that most likely influenced the potency of innate immune cell function helped guide drug candidate development. Both predicted and experimentally determined values for various physicochemical properties of curcuminoid compounds were analyzed for correlation with the potency of gene expression (i.e., *MGAT3*, *TLRs* 2−4, and *VDR*), determined *in vitro* using U-937 monocytes (Table 1). Calculated values for certain properties were available for 45 curcuminoids, including log *P*, topological polar surface area (tPSA), Gibbs free energy (ΔG), and molecular critical volume. For subsets of the compounds, experimentally determined data included measures of chemical stability in either water or buffer (pH 7.4), metabolic stability in the presence of human liver microsomes (HLM) and human S9 fraction (i.e., cytosol plus microsomes), and average solubility and permeability, as described previously for the evaluation of curcuminoids.[8,18] It is notable that the amino acid derivatives of curcumins had water solubility in excess of 5 mg/mL, whereas the parent curcumins were virtually insoluble in water. Undoubtedly, increased water solubility increases absorption into cells and aided in overall biological response. Gene expression data were determined for the 45 curcuminoids tested in U-937 cells at 0.1 nM and 0.001 nM, followed by 24 h treatment with Aβ. Absolute and log values of the fold-expression changes were analyzed for correlation with their chemical properties. Certain properties showed significant correlation with increased expression of the tested genes. In particular, calculated values for both log *P* (calculated as in ref 22) and free energy (ΔG) showed strong correlation with the expression of all five tested genes ($P < 0.01$). tPSA[33] and metabolic stability (in HLM) also showed significant correlation with the expression of all tested genes ($P < 0.05$). The remaining properties analyzed showed little evidence for correlation with gene expression. For the most potent curcuminoids, values of the physicochemical properties correlated with gene expression and were included in Table 1.

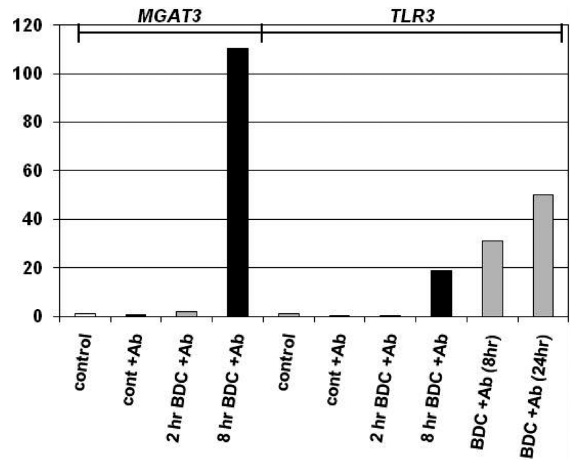

Figure 6. Time dependence of monocyte response to compound treatments. Left 8 columns: duplicate U-937 cultures were treated with BDC (**1**) for varying times, followed by 24 h treatment with Aβ (5 μg/mL). Shown for *MGAT3* and *TLR3*, gene expression response was seen with BDC treatments between 2 and 8 h. Right 2 columns: following 24 h BDC treatment, duplicate U-937 cultures were treated with Aβ for varying times. Shown for TLR3, gene expression increased with increased Aβ exposure time up to at least 24 h. Mean mRNA levels are shown as fold-increases (*y*-axis) relative to untreated control cells.

Optimized Curcumin Analogues Have Improved Bioavailability. The *in vitro* gene expression results suggested that enhanced innate immune function was associated with greater solubility and stability of curcuminoids. In order to validate the compounds with regard to improved *in vivo* bioavailability, we administered prodrug analogues **2** (Val-BDC) and **5** (cyclohexyl-Val-BDC) to adult mice, and after four hours, *in vivo* plasma concentrations were determined and compared with those for the parent compound **1** (BDC). Table 2 shows that **5** attained 55-fold greater plasma concentration on a molar basis by oral administration, and 73-fold greater concentration by i.p. administration, than did BDC. In contrast, increased *in vivo* plasma concentrations were not observed for the valine-containing prodrug, compound **2**, although the plasma concentration of **2** was not statistically significantly different than BDC. The data suggested that the cyclohexyl-stabilized, water-soluble prodrug was stable in the gut and facilitated effective transport into the blood, substantially increasing the *in vivo* concentrations of BDC available for innate immune stimulation.

***Ex Vivo* Phagocytosis.** To examine a functional ex vivo correlate of the cell-based gene expression studies described above for phagocytosis, we examined the effect of curcumin and selected curcumin analogues on Aβ removal and bead phagocytosis in mouse microglial cells. Cultured microglial cells obtained from cerebral cortices of neonate DBA/2N mice were treated with 100 nM curcumin or curcumin analogues overnight and

Table 2. Plasma Levels of Curcuminoids after Oral or i.p. Administration to Mice[a]

	oral administration (25 mg/kg)		i.p. administration (100 mg/kg)	
compd	μg/mL of plasma	nmol/mL of plasma	μg/mL of plasma	nmol/mL of plasma
BDC (**1**)	0.01 ± 0.01	0.03 ± 0.02	0.42 ± 0.15	1.36 ± 0.47
Val-BDC (**2**)[b]	0.00	0.00	0.27 ± 0.16	0.47 ± 0.15
Cyc-Val-BDC (**5**)[c]	1.02 ± 0.29	1.65 ± 0.26	61.57 ± 1.48	99.37 ± 1.34

[a] Values are the mean of 3 animals. [b] Product quantified in the plasma was de-esterified BDC. [c] Product quantified in plasma was the de-esterified cyclohexyl version.

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Table 3. Effect of Curcumins on Phagocytosis

compd	percent amyloid-β removal[b]	bead phagocytosis index[c]
none[a]	24.2 ± 3.1	9 ± 3
1, BDC	68.3 ± 3.6	33.5 ± 4
18, curcumin	39 ± 5.5	15.5 ± 3
17	25 ± 5	12 ± 2.8
32	65 ± 9.9	22 ± 1.9
26	65.5 ± 4.7	21.1 ± 0.9
30	60.2 ± 7.9	16.3 ± 1.2
33	60.5 ± 5.2	18.6 ± 2.1
20	45 ± 4.3	17.4 ± 2.2
2	63.3 ± 4.2	16.1 ± 2.9
24	52.3 ± 8.8	28 ± 0.8

[a] Vehicle-treated cells. [b] Mean of three determinations ± standard deviation. [c] Mean of three determinations ± standard deviation.

examined for phagocytosis of FluorSphere bead particles. Phagocytized beads were visualized by light microscopy. As shown in Table 3, BDC **1** was more effective at phagocytizing particle beads than curcumin **2**. In a parallel experiment, BDC was more effective than dexamethasone or curcumin (i.e., phagocytosis indexes of 48, 35, and 28, respectively, at 100 nM compared with a control phagocytosis index equal to 17). On the basis of the bead phagocytosis index, (Table 3), compounds **24** and Val-curcumin **32** were effective at phagocytizing beads followed in potency by **26** and **33**. The remaining compounds examined (i.e., **2**, **18**, **20**, and **30**) showed only modest bead phagocytosis potency compared to that of the unnatural curcumin analogue **17**.

Removal of Aβ in an *ex vivo* assay with tissue sections from PDAPP mice was conducted to determine the potency of curcumin and curcumin analogues. On the day of the experiments, an antibody previously known to remove Aβ and an irrelevant antibody worked fine as positive and negative controls, respectively. Tissue sections were treated with curcumin or curcumin analogues prior to the addition of mouse microglia, the cocultures were incubated overnight, and the remaining Aβ was measured by ELISA to quantify Aβ clearance. BDC **1** induced Aβ clearance in a dose-dependent manner (i.e., 10 nM to 1000 nM). Maximal functional activation of Aβ removal was linearly dependent on time, and maximal phagocytosis occurred at 6 h. On the basis of immunocytochemical detection, BDC appears to enhance removal of Aβ via enhanced phagocytosis (data not shown). As shown in Table 3, BDC and several analogues (i.e., **2**, **26**, **30**, **32**, and **33**) were considerably more potent than curcumin **18** and the unnatural curcumin isomer **17** at removing Aβ. However, the SAR results for Aβ removal did not exactly parallel the results for bead phagocytosis probably due to the distinct biological mechanisms involved.

DISCUSSION

An *in vitro* system was developed for evaluating analogues of natural product curcumins for potency in stimulating innate immune gene expression for Aβ phagocytosis of relevance to AD. This permitted the rapid screening of many novel synthetic curcumin compounds to identify the most promising candidates in an experimental system that mimicked human PBMCs. This system was useful for directed development of novel curcuminoids with therapeutic potential as amyloid-clearing anti-AD drug candidates.

In addition to their capability for promoting Aβ phagocytosis in the context of AD, curcumins have also been intensively investigated as potential therapeutics against cancer and inflammatory diseases because of their well-characterized antiproliferative and anti-inflammatory properties, respectively.[34,35] Given their multivaried therapeutic potential, considerable interest therefore exists in the characterization of curcumins and the relative impact of modifications made in curcumin analogues on a variety of biological processes. However, the inherent limitations of natural curcumins with regard to solubility, stability, protein binding, and extensive metabolic transformations[8,18] contribute to poor overall bioavailability and restrict their potential as drug candidates. Many clinical trials have been done or are underway testing safety, tolerance, drug formulation, and efficacy of curcumins for treating an array of diseases including AD, cancer, and others. The low bioavailability of curcumins has necessitated their testing in humans of multigram quantities per day. Limited reports thus far indicate safety and tolerance, even up to oral administration of 8−12 g per day,[36] although only low physiological levels have been attained. In one phase II study,[37] plasma levels of curcumin quickly reached (3 days) steady-state levels of 22−41 ng/mL (detected upon release from glucuronide and sulfate conjugated forms), despite 8 g daily oral doses over a 4 week period. Further, the most potent component BDC, representing only 10−15 percent of the administered natural curcumin mix, would be expected to be bioavailable at much lower levels.

Prior to the current studies, our evaluations of curcuminoids as pro-Aβ phagocytic agents employed monocyte and tissue samples obtained from AD patients and healthy control subjects. While the *ex vivo* PBMC analyses identified several promising leads, only a subset of the potential compounds were analyzed, thus limiting the structure−activity relationship (SAR) studies. Also, variability among PBMCs from different AD patients based on responses to curcuminoids and VD3 (i.e., Types I and II macrophages[5]) introduced additional complexity to the analysis. We therefore developed a complementary *in vitro* approach, using established human monocytic cell lines for evaluating novel analogues. Representing uniform, renewable, and accessible resources, these cell lines permitted more direct comparisons between test compounds.

The cell lines U-937 and THP-1 were selected based on their description as human monocytes and because of reported responsiveness and capacity for differentiation mediated by VD3.[38] It was therefore anticipated that U-937 and THP-1 cell lines would show response to curcumins in the presence of Aβ that resembled those observed for human PBMCs. Strong upregulation of the gene *CAMP*, known to contain several promoter VDE response elements,[39] showed these lines possessed functional VD3 signaling pathways affecting transcription. Further, we observed that these cells responded to VD3 treatment by increased expression of the gene encoding its receptor, VDR. Having shown that monocyte cells possessed the machinery proposed to mediate curcumin signaling, natural and synthetic curcuminoids were tested and were shown to induce expression patterns of genes (i.e., *MGAT3*, *TLRs*, and *VDR*) similar to those associated with efficient Aβ phagocytosis in PBMCs from control humans. Providing additional support, we observed similar gene responses to curcumins plus Aβ in mouse macrophage and microglial cell lines (i.e., J77A.1 and BV-2, respectively; data not shown), indicating conserved innate immune responses.

The cell lines generally responded more efficiently at lower curcuminoid levels, and toxicity *in vitro* was observed for some compounds at higher concentrations ($\geq$10 μM, data not shown). Certain structural features may also be important, as one analogue tested in human PBMCs was uniquely associated with toxicity.[8] Gene expression response *in vitro* was typically greater for compounds in the range of 0.1−10 nM, a range consistent with the lower end of physiological VD3 levels, and the same as that reported for VD3-induced differentiation in U-937 cells.[29] The low curcumin concentrations that are effective in the study herein are also in agreement with those reported for stimulating human PBMCs.[2,8] For some curcuminoid compounds, significantly increased gene expression *in vitro* was observed at concentrations as low as 1 pM. Time-dependent gene expression changes were also observed, with increased mRNA levels becoming evident by 8 h of curcuminoid treatment (followed by fixed Aβ treatment times). The reported interaction of curcumins with the alternative binding pocket of VDR led to the hypothesis that curcuminoid signaling may involve modulation of calcium or chloride channel activity.[7,8,40] The observed temporal effects of curcuminoid action *in vitro* are consistent with rapid, nongenomic pathways that ultimately influence transcriptional changes.

Interestingly, curcuminoids did not significantly affect gene expression directly but appeared to potentiate the cells to subsequent Aβ exposure. This may be similar to the reported priming of U-937 monocytes by VD3 to differentiate in response to subsequent treatment with other agents that independently showed no differentiation capability.[29] Cell priming was achieved in less than 20 h (and as little as 1 h for certain differentiating agents) and was shown to be independent of RNA and protein synthesis and thus may provide further support for the existence of functional, nongenomic signaling paths in these cells.

The relative potency for promoting monocyte gene expression *in vitro* was examined for most of the synthetic curcuminoids at similar concentrations prior to the administration of Aβ. The most potent compounds for stimulating expression of the genes examined included BDC (**1**) and prodrug versions of BDC (**2**,**3**) containing solubility-enhancing amino acids (valine, lysine) at the phenolic R_3 positions. The water-soluble valine-BDC prodrug (**2**) appeared to be the most potent agent overall from among all the curcuminoids examined. Curcumins are insoluble in water, but attachment of amino acids dramatically improves solubility. For example, Val-containing curcumins (i.e., **2**, **5**, **9**, **32**, and **41**) had water solubililty of 8−10 mg/mL, Lys-containing curcumins (i.e., **3**, **6**, **31**, and **40**) had water solubility of 5−8 mg/mL. Incorporation of a cyclohexyl moiety into amino-acid containing curcumins increased the water solubility further. Improved water solubility appears to be a major component of curcuminoid potency, possibly facilitating interaction with receptors or transporters to promote cellular uptake. As observed using human PBMCs, BDC was observed to be more potent than the predominant natural curcumin (**18**).[2] Twelve compounds (**1**−**12**) were identified as having the greatest overall potency based on the induction of the expression of at least three of the tested genes >2-fold compared to controls, and eight of those curcuminoids induced all five genes.

The fold-expression results showed that *MGAT3* was the most responsive gene to these compounds, followed by *VDR*, and then roughly equivalent responses among the three tested *TLR*s. Elevated *MGAT3* levels have been associated with the process of Aβ uptake and clearance, but the detailed mechanism has not been clearly established. MGAT3-mediated *N*-glycosylation may be important for efficient phagocytic ability, and altered *N*-glycosylation resulting from dysregulated *MGAT3* expression may underlie observed defects of Aβ clearance by AD PBMCs. TLRs have crucial and well-established roles as innate immune cell receptors and initiators of intracellular signaling in response to pathogen markers and other stimuli.[41] Together with VDR, these gene targets of curcuminoid and VD3 signaling appear to be important components of the response of monocytes to Aβ, and potentially represent important biomarkers for AD onset or progression or response to therapeutics.[8] The ability to characterize responses of these genes *in vitro* may help clarify their roles in promoting Aβ phagocytosis and to understand the generalized dysfunction in Aβ clearance by AD patients' monocytes.

Certain chemical structural features were found to be prevalent among the most potent curcuminoid gene inducers. Among the top 12 compounds, 5 were amino acid prodrugs (i.e., 3 contained Val, and 2 contained Lys). The top 12 curcuminoids identified also included 5 compounds containing a cyclohexyl group at the T_1 position in the bridge region, associated with greater chemical and metabolic stability. As further support for the relevance of the *in vitro* studies, several of these same compounds were also identified as potent for stimulating Aβ phagocytosis by AD macrophages.[2,8,28] With regard to substitutions on the phenyl rings, with a few exceptions the most potent compounds showed a preference for no substitution at the R_1, R_2, and especially the R_4 positions (Figure 1). As observed previously, there was a strong preference for the most favorable type of substituents at the phenyl R_3 (*para*) position.[8] Only one curcuminoid among the most potent 12 compounds, (i.e., compound **12**), contained saturated bonds within the bridge region. Despite the anticipation that the saturated analogue was to have a stabilizing effect, this modification was associated with decreased potency relative to BDC, possibly due to decreased solubility.

To gain insight into other important chemical features underlying biological potency, various physicochemical properties were determined for each curcuminoid tested and correlated with *MGAT3*, *TLR*, and *VDR* gene expression results. Significant correlations were observed between gene expression and calculated values for log *P*, free energy (ΔG), and topological polar surface area (tPSA). The log *P* value is frequently used as an estimate of relative lipophilicity and distribution to biological targets. The most potent compound (**2**) had a log *P* value of 2.81, compared to an average for all compounds of 3.93. Possibly, for highly lipophilic curcuminoids, nonspecific protein binding may decrease potency. tPSA is a commonly used metric for estimating cell permeability, allowing the prediction of drug transport properties.[33] Among the most potent compounds, **2**, **3**, **5**, **6**, and **9** had the highest calculated polar surface areas, suggesting that they might have been poor at permeating cell membranes. However, it is likely that the amino acid substituents present in each of these prodrugs improved their solubility and provided recognition to cellular transporters to more efficiently take up the curcumin prodrugs. After uptake into the blood, the amino acid prodrugs are likely spontaneously and/or enzymatically de-esterified in the gut or bloodstream to the parent compounds (i.e., **1** and **4**), and **1** and **4** have PSA values more consistent with cellular entry. Results of gene expression studies also showed significant correlation with experimentally determined metabolic stability in the presence of human liver microsomes (HLM)

suggesting that metabolic stability is also an important parameter that contributes to potency. Other analyzed physicochemical properties showed little evidence of correlation with gene expression, although limited data for experimentally derived average solubility and permeability (i.e., $n = 8$ and 7 data points, respectively) likely precluded the determination of significant correlations.

Various reports describe the synthesis and evaluation of curcuminoids, providing opportunities for comparison with our results focused on innate immune phagocytosis. In support of our prior and current results, it was recently reported that BDC was also the most potent natural curcumin in antiproliferation and anti-inflammation assays.[34,35] Relative potencies varied among other curcuminoids examined in those assays, suggesting that specific structural features may affect different processes to differing extents. At least one asymmetric curcuminoid lacking one of the aromatic groups was reported to retain anti-inflammatory and antiproliferative activities.[35] It remains to be shown whether the curcuminoid analogues characterized herein have similar activities in other processes and other cell types or if the structural features highlighted in this report uniquely promote phagocytosis in innate immune cells.

Preliminary pharmacokinetic studies were done to test the hypothesis that water-soluble and chemically stable analogues would have greater bioavailability than natural BDC (**1**). In support of this, we observed substantially increased mean *in vivo* serum concentrations in adult mice for the cyclohexyl- and valine-containing **5**, relative to BDC, by both oral and i.p. administration (i.e., 55-fold and 73-fold, respectively). The results suggested that **5** was effectively transported into the blood and that the valine was spontaneously or enzymatically cleaved to the active parent compound. Such efficient delivery could substantially increase *in vivo* concentrations available for innate immune stimulation. In contrast, the valine-only analogue **2** did not show increased levels *in vivo* by either route of administration, suggesting perhaps metabolic lability in the gut and/or lack of efficient uptake into the bloodstream. Thus, **5** and related compounds represent excellent candidates for further preclinical lead development.

Treatment of microglia from mice with BDC and several BDC analogues enhanced phagocytosis capability as measured by bead engulfment and removal of Aβ. In good agreement with the gene expression results, of the analogues examined, BDC and water-soluble BDC prodrugs **2** and analogue **32** were the most potent at stimulating Aβ clearance and particle bead phagocytosis. Curcumin and unnatural curcumin **17** were not potent at enhancing Aβ clearance or particle bead phagocytosis. For the compounds examined, with the exception of BDC, the SAR for bead phagocytosis did not exactly parallel the SAR for Aβ phagocytosis. This is not surprising in view of the fact that phagocytosis of Aβ by normal monocytes is very robust compared to phagocytosis by monocytes derived from AD patients.[2] However, both types of monocytes phagocytize bacteria with similar ability.[2] That a distinct BDC analogue SAR is in operation for Aβ removal and a different SAR is apparent for bead phagocytosis suggests that, similar to bacteria engulfment, different mechanisms may be in operation for plastic bead engulfment and Aβ phagocytosis. On the basis of the results of phagocytosis studies (above) and image analysis experiments of cells labeled with fluorescent Aβ (data not shown), BDC and the most potent analogues tested induced Aβ removal via enhanced uptake and phagocytosis and not increased degradation of Aβ.

In summary, an *in vitro* system using human monocytic cell lines was developed for evaluating curcuminoids as drug candidates for treating AD. This system complements the use of cells from human patients, with potential advantages in throughput, accessibility, and comparability between tested compounds. The cell lines used herein were shown to reproduce patterns of gene expression resembling those seen with human PBMCs and allowed a more comprehensive evaluation of structure−activity relationships among multiple curcuminoid analogues. Several compounds were identified as the most potent at stimulating innate immune cell expression of genes associated with efficient Aβ phagocytosis, and this was verified for several curcumin analogues by *ex vivo* phagocytosis studies. At least one of these compounds showed substantially increased bioavailability over natural BDC. Knowledge of the structural features and chemical properties associated with superior pharmaceutical attributes will guide further development of curcumin-based drug candidates with potential for treating AD and possibly other diseases.

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