CURCUMIN STRUCTURE-FUNCTION, BIOAVAILABILITY AND EFFICACY IN MODELS OF NEUROINFLAMMATION AND ALZHEIMER'S

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a) Running Title (47 char, <60 characters): Curcumin structure-function and bioavailability

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c) Manuscript Information: The number of text pages: 43
Number of tables: 2
# Figures: 10
# References: 40
# words Abstract: 243
# words Introduction: 749
# words Discussion: 1500

d) Nonstandard abbreviations: Curc, curcumin; TC, tetrahydrocurcumin; i.p., intraperitoneal, i.m., intramuscular, NFkB, nuclear factor kappa B, Aβ, beta-amyloid peptide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; JNK, c-jun n-terminal kinase; NT, nitrotyrosine; glial fibrillary acidic protein, GFAP; IL-1 β, interleukin-1β; AD, Alzheimer's disease; ROS, reactive oxygen
species; RNS, reactive nitrogen species; CNS, central nervous system; COX, cyclooxygenase; HAT, histone acetyltransferase; NSAIDs, non-steroidal anti-inflammatory drugs.

e) Section Assignment:

Neuropharmacology

(alternative: Inflammation, Immunopharmacology, and Asthma)
Abstract

Curcumin can reduce inflammation and neurodegeneration, but its chemical instability and metabolism raises concerns, including whether the more stable metabolite tetrahydrocurcumin (TC) may mediate efficacy. We examined the anti-oxidant, anti-inflammatory or anti-amyloidogenic effects of dietary curcumin and TC, either administered chronically to aged Tg2576 APPsw mice or acutely to LPS-injected wild-type mice. Despite dramatically higher drug plasma levels after TC compared to curcumin gavage, resulting brain levels of parent compounds were similar, correlating with reduction in LPS-stimulated iNOS, nitrotyrosine, F2 isoprostanes and carbonyls. In both the acute (LPS) as well as chronic inflammation (Tg2576), TC and curcumin similarly reduced IL-1beta. Despite these similarities, only curcumin was effective in reducing amyloid plaque burden, insoluble Abeta and carbonyls. TC had no impact on plaques or insoluble Abeta, but both reduced TBS-soluble Abeta and pJNK. Curcumin but not TC prevented Abeta aggregation. The TC metabolite was detected in brain and plasma from mice chronically fed the parent compound. These data indicate that the dienone bridge present in curcumin, but not in TC, is necessary to reduce plaque deposition and protein oxidation in an Alzheimer's model. Nevertheless, TC did reduce neuroinflammation and soluble Abeta, effects, which may be attributable to limiting JNK-mediated transcription. Because of its favorable safety profile and the involvement of misfolded proteins, oxidative damage and inflammation in multiple chronic degenerative diseases, these data relating curcumin dosing to the blood and tissue levels required for efficacy should help translation efforts from multiple successful preclinical models.
**Introduction**

Neuroinflammation is implicated in the pathogenesis of many neurodegenerative disorders including Alzheimer’s disease (AD). In AD, several mediators in the inflammation cascade contribute both to neurodegeneration and to the production and accumulation of the beta-amyloid peptide, including interleukin-1β (IL-1β), phospho-c-Jun N-terminal kinase (pJNK), reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) mediated production of reactive nitric oxide species (RNS) and lipid peroxidation products (e.g. 8-iso-PGF2α) (reviewed in Akiyama et al., 2000). For AD therapeutics, there is rationale to develop drugs that attenuate inflammatory cascades contributing to neurodegeneration and amyloid production or accumulation. However, there is concern that some anti-inflammatory drugs may interfere with phagocytic clearance of amyloid. Curcumin, a compound known to inhibit inflammation while reducing plaque deposition in AD models is the yellow pigment of the widely used spice, turmeric. Curcumin antagonizes many steps in the inflammatory cascade, including AP-1 transcription, activation of NF-κB, iNOS and JNK (Pendurthi et al., 1997; Weber et al., 2006). Unlike α-tocopherol (vitamin E), which is a poor scavenger for NO-related oxidation, curcumin exerts potent antioxidant activity for NO-related radical generation (Chan et al., 1998). In contrast to NSAIDs whose adverse side-effects include gastrointestinal ulceration and liver or kidney toxicity, curcumin appears to be relatively safe, even in clinical trials for prevention of relapse of ulcerative colitis (Hanai et al., 2006). Curcumin administration has been reported to attenuate cognitive deficits, neuroinflammation and plaque pathology in AD models (Frautschy et al., 2001; Yang et al., 2005; Garcia-Alloza et al., 2007). Beyond AD, oral curcumin efficacy *in vivo* has been shown in models for many conditions with oxidative damage and inflammation, including many types of cancer, diabetes, atherosclerosis, arthritis, stroke, peripheral neuropathy, inflammatory bowel and brain trauma (Aggarwal et al., 2007).
Curcumin's poor oral absorption in both humans and animals has raised several concerns that this may limit its clinical impact (Kelloff et al., 1996). Curcumin, is a biphenolic compound with hydroxyl groups at the ortho-position on the two aromatic rings that are connected by a β-diketone bridge, containing two double bonds (dienone), which can undergo Michael addition, critical for some of curcumin's effects (Weber et al., 2006), but contributing to chemical instability in aqueous solution (Pan et al., 1999). Pharmacokinetic studies of curcumin demonstrate extensive intestinal sulfation and glucuronidation (Kelloff et al., 1996; Pan et al., 1999; Ireson et al., 2001; Sharma et al., 2004). Typically, clinical trials show negligible unconjugated curcumin plasma levels with oral dosing (Lao et al., 2006; Baum et al., 2008), leading to the suggestion that in vivo efficacy may come from a more bioavailable and/or potent metabolite (Kelloff et al., 1996).

Potential metabolites, some of which may be active, include tetrahydrocurcumin (TC), hexahydrocurcumin, hexahydrocurcuminol, vanillin, vanillic acid, and ferulic acid (Ireson et al., 2002). However, detectable levels of these metabolites in active unconjugated forms after administration of the parent compound have not been reported, presumably due to their low concentrations, or vulnerability to sulfation, glucuronidation or hydrolysis (Baum et al., 2008). TC, produced by reduction of dienone double bonds in curcumin, primarily in the intestinal cells (Okada et al., 2001; Ireson et al., 2002), is resistant to hydrolysis (Pan et al., 1999) and a candidate active metabolite. TC has potent antioxidant and anti-inflammatory properties in vitro (Sugiyama et al., 1996; Pan et al., 2000) and in vivo (kidney) (Okada et al., 2001). Because of its activity and putative increased bioavailability, TC has been chronically fed to aging mice resulting in significantly increased mean and maximum lifespans (Kitani and Osawa, 2002). However, since detection methods have not revealed unconjugated TC in plasma (Pan et al., 1999; Okada et al., 2001), TC’s role as a candidate active curcumin metabolite is unclear and it has not been tested in AD models. Experiments comparing TC and curcumin are also useful to evaluate the functional importance of curcumin’s dienone bridge. In our studies, the purpose was to determine efficacious blood and tissue TC and curcumin levels for in vivo antioxidant, anti-inflammatory and anti-amyloid activity and the importance of the dienone bridge in an acute inflammatory model as well as a chronic AD model.
In the acute paradigm, we investigated the differential impacts of curcumin versus TC on the drug levels in plasma and brain achieved and on the neuroinflammatory responses to an acute inflammatory stimulus (LPS) after different routes of administration. In the chronic model, we investigated curcumin metabolism and how chronic dietary administration of curcumin or TC modified Aβ and inflammatory parameters in an aging APPsw transgenic mouse model of AD.
Methods

Animals and treatments — Use of animals was approved by the Greater Los Angeles Veterans Administration Institutional Animal Care and Use Committee. In the acute dosing paradigm C57Bl6/J male and female mice (3–4 months of age, n=4) were administered curcumin, TC, or vehicle by gavage (gav), i.p. or i.m., following periodic fasting for two consecutive days as shown in Fig. 1, A. One hour following the second day's dosing, mice were administered LPS (0.5 µg/g of body weight) by i.p. and sacrificed 3 hours later. The gav and i.p. doses were each 148 µg (0.4 µmoles) of curcumin (Cayman Chemicals, Michigan, MI) or TC (Sabinsa Corp. Piscataway, NJ) and the i.m. dose was 74 µg (0.2 µmoles). Briefly, curcumin for gavage or injection was dissolved in 0.5 N NaOH, diluted to desired concentration, then neutralized in PBS as described previously (Chan et al., 1998). For acute gavage studies we dissolved curcumin in NaOH to ensure delivery and efficacy at low doses (Chan et al., 1998). However, this method has the disadvantage of exposing curcumin to hydrolysis, which occurs rapidly at neutral pH (Wang et al., 1997). Therefore, to minimize the impact of the major and minor metabolites of curcumin, we neutralized curcumin less than 5 minutes prior to gavage. Although it is possible that some effects observed after gavage were in part due to the metabolites such as ferulic acid, 6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal or vanillin, it seems unlikely since absorbance levels at 426 nm indicated greater than 90% added curcumin was still present at time of administration, and ferulic acid has poor blood brain barrier permeability, so would be unlikely to have a significant impact on brain responses. A similar acute gavage experiment was performed to investigate the impact of fasting on tissue levels of curcumin and TC and responses to LPS (n=4).

Lyophilized TC or curcumin was mixed in chow for the chronic oral dosing paradigm 500 ppm chow (which is equivalent to ~1.25 mg/day or 83 mg/kg body weight) using male and female APPsw Tg2576 mice (Fig. 1, B). Mice were fed these diets ad libitum for 4 months with TC (n=5-7) or curcumin (n=5-11) during the accelerated period of plaque deposition (12-16 months of age). We also fed transgene
negative animals 2000 ppm chow (~10 mg/day or 333 mg/kg body weight) to obtain more tissue to study curcumin metabolism. At sacrifice, mice were first anesthetized with pentobarbital (40 mg/kg body weight, i.p.) for blood sample collection. Blood was drawn from the abdominal aorta into EDTA-containing tubes, and centrifuged at 1,600 x g for 20 minutes at 4°C. Collected plasma was aliquoted and snap frozen, using liquid nitrogen. Mice were subsequently perfused with PBS (pH 7.4) containing protease inhibitors (20 µg/mL each pepstatin A, aprotinin, phosphoramidon, and leupeptin, 0.5 mM PMSF, and 1 mM EGTA). Brains were removed and dissected, then snap-frozen using liquid nitrogen and stored at -80°C for further analysis.

In vitro studies. Aβ oligomer treatment of neurons — Non-fibrillar high molecular weight Aβ42 oligomer was prepared as previously described (Yang et al., 2005). Briefly double-lyophilized synthetic Aβ (1-42) peptide was monomerized by solubilization in (1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). Fibril formation was minimized during subsequent HFIP evaporation by adding sterilized water (to achieve stock solution of 315 µg/ml or 63 µM). Fibrils were formed in the solution by continuous stirring (600 rpm) for 48 h at room temperature (25°C). 10 mM NaHCO₃ buffer (pH 10-11) was added to optimize high molecular weight (HMW) Aβ oligomer stability. Oligomers (100 nM) were added to cells for 48 h at 37 °C with curcumin (2.5 µM), TC (2.5 µM), curcumin+TC (1.25 µM each), or vehicle. After 48h, a LDH toxicity assay was performed on culture media using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), and absorption was read at 490 nm. Each data point was determined in triplicate.

To evaluate the impact of curcumin and/or TC on Aβ oligomer toxicity, Aβ oligomers were added to SH-SY5Y human neuroblastoma cells plated at 10,000 cells/well in 96-well plates and differentiated in low serum Dulbecco's modified Eagle's medium (DMEM) with N₂ supplement and 10 µM all-trans-retinoic
acid for 7 days. The medium was removed and replaced with fresh maintenance medium containing 0.1% BSA.

**In vitro studies. LPS Treatment of microglia and primary hippocampal neurons** — LPS is commonly known to induce iNOS in the CNS in microglia cells, and there is one report that it induces iNOS in neurons, so we investigated the effect of TC and/or curcumin in both primary neurons and a microglial cell line in vitro.

Cultures of cortical neurons from embryonic day 18 (E18) Sprague Dawley rat fetuses were prepared yielding ~97% neurons and 3% glia. Cells were first grown in B27 medium (containing cytosine arabinoside to minimize glial cell proliferation) supplemented with 25 µM glutamate, 0.5 µM glutamine, 5U/ml penicillin, and 5 mg/ml streptomycin, and 10% fetal bovine serum (FBS). Three hours before treatment, neurons were incubated in Neurobasal Medium (NBM), but without B27 and glutamate. Then cells were preincubated with 2.5 µM of curcumin, TC, curcumin+TC, or vehicle for 1 hour before adding LPS (1 µg/ml or 100 nM) before harvesting 24 hr later. Cells were washed with cold PBS, scraped into 1 ml of PBS, and then centrifuged at 5000 rpm for 5 min. The cell pellet was sonicated in lysis buffer (SDS, deoxycholate, triton X100, NaCl, NaH₂PO₄, 0.5%, 0.5%, 1%, 150 mM, 10 mM, respectively) with a protease and phosphatase inhibitor cocktail (fenvalerate, cantharidin, sodium vanadate, sodium pyrophosphate, sodium fluoride, phosphoramidon, EGTA, EDTA, leupeptin, aprotinin, pepstatin A, phenylmethylsulphonyl fluoride (PMSF), 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.05, 0.05, 1, 1, 50, 0.02, 1, 0.1, 0.02, 0.0015, 0.0145, 0.250, 0.5 mM, respectively). After incubation at 4°C for 30 min, the samples were again centrifuged (12,000 rpm for 10 min), and the supernatants were collected and used for determining protein concentration (Bio-Rad DC protein assay kit, Hercules, CA) and iNOS levels by Western blot.
The BV-2 microglial cell line (a gift from Prof. Jean deVellis, UCLA), was used to evaluate the impact on iNOS in the brain, since microglia are primary source of iNOS in the brain. BV-2 cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS (Gibco, Invitrogen Corporation, Carlsbad, CA) at 37 °C in 5% CO₂ in a humidified cell incubator. One day before treatment, serum was removed and cells incubated in 0.1% BSA and DMEM. Then cells were pretreated with curcumin, TC, curcumin+TC, or vehicle one hour prior to LPS, and cells harvested 24 hours later, as described above for cortical neuron cultures.

**MC65 neuroblastoma cells** We also investigated the neuroprotective effects of TC or curcumin on inducible intraneuronal expression of APP C99. MC65 cells were kindly provided LW Jin (University Washington, Seattle, WA). Briefly, for cell viability experiments, cells were plated onto 96 well plates at 25000 cells/well in Dulbecco’s Modified Eagle’s medium, DME (GIBCO #11995-065, Carlsbad, CA) plus 10% FCS and 1 µg/ml tetracycline. 24 hours later, cells medium was replaced with tetracycline free Opti-MEM. After that cells were treated for three days with and without 2 µM of curcumin and TC in 0.1% ethanol. After 72 hr, MTT reduction viability assay was performed at 550 nm.

**Plasma and tissue sample preparation for HPLC** — Mouse plasma and brain samples were extracted in 95% ethyl acetate/5% methanol and analyzed by HPLC with detection at 262 (curcumin) and 282 (TC) nm, using 17β-estradiol as an internal standard as described previously except with the following modifications (Heath et al., 2003)(Heath et al., 2005). For brain tissue sample preparation, whole brains were first homogenized in 10 vol of 1 M ammonium acetate (pH 4.6). The extraction efficiency for curcumin and TC was 88-97% and 82-90% from plasma and brain homogenate, respectively. Using the HPLC method, the lower limits of detection for Curc were 35 ng/ml (95.1 nM) in plasma and 100 ng/g wet tissue (271 nM) in brain, and for TC were 8 ng/ml (21.5 nM) in plasma and 250 ng/g wet tissue (672 nM) in brain.
Curc and TC LC/MS/MS analysis of brain from chronically fed mice — Methods for assessing efficacy of drugs for anti-oxidant, anti-inflammatory and anti-amyloid activity utilized considerable brain tissue, so there was limited tissue available to assess brain concentrations of parent drug and metabolites. Therefore we developed a more sensitive, efficient and specific method to evaluate curcumin metabolism and levels in the brain after chronic feeding.

The curcumin-related compounds monitored by LC/MS/MS were curcumin, tetrahydrocurcumin, and tetramethoxycurcumin (the internal standard). All gave strong signals for the (M-H)⁻ ion in negative ion electrospray mass spectrometry when infused in solutions of water/acetonitrile containing 10 mM ammonium acetate. The tandem mass spectra (MSMS) of these ions produced a number of fragment ions, the most intense of which were selected for quantitation by MS/MS-multiple reaction monitoring (MRM).

Dried extracts were redissolved in acetonitrile/methanol/water/acetic acid (41/23/36/1, all by volume), and an aliquot of the solution (typically 100 µl) was injected onto a reverse phase HPLC column (Supelco Ascentis Express C18, 150 x 2.1 mm) equilibrated in 10 mM ammonium acetate (buffer A) and eluted (100 µl/min) with an increasing concentration of acetonitrile/isopropanol (1/1, v/v, buffer B: min/%B; 0/10, 5/10, 30/100, 33/100). The effluent from the column was passed directly to an Ionspray™ ion source connected to a triple quadrupole mass spectrometer (PerkinElmer Life Sciences Sciex API III⁺) operating in the negative ion MS/MSMRM mode in which the intensity of parent to fragment ion transitions were recorded with an orifice voltage of 65 and argon collision gas (instrumental setting CGT of 120). The m/z values of the specific transitions were: curcumin, 367.1→271.1, 367.1→173.1, 367.1→149.0; tetrahydrocurcumin, 371.1→235.1; and tetramethoxycurcumin, 395.2→380.2, 395.2→365.1, (the internal standard). To enhance signal intensity for low abundant samples, both Q1 and Q3 were detuned so that the isotope clusters for the polypropylene calibrant ions between m/z 200-
1000 were not separated from one another. This strategy resulted in a signal enhancement for the curcumin-related compounds between 3.4-8-fold. Instrument manufacturer-supplied software was used to calculate peak heights (PE Sciex MacSpec version 3.3). For quantitation the most intense of the parent→fragment transitions for each compound were used. These were 367.1→149.0 for curcumin and 395.2→365.1 for tetramethoxycurcumin (the internal standard).

MRM sensitivity was routinely checked with injections of and 0.01, 0.1, and 1 pmol of each of the curcuminoid compounds. At maximal sensitivity (detuned mass spectrometric conditions), the limit of detection corresponded to 100 pg/g wet tissue of extracted material or 272 pM for curcumin and 1 ng/g wet tissue or 2.68 nM for tetrahydrocurcumin. Injection of 0.1 pmol for each compound gave strong signals with peak height/baseline noise ratios in excess of 50/1. The retention time of Curc, TC and internal standard was 28.24, 26.36 and 30.27 minutes respectively.

**Quantitative PCR (QPCR)** — Brain levels of iNOS mRNA were measured by reverse transcription followed by quantitative PCR (QPCR). Briefly, total RNA was isolated from brain using the RNAqueous kit (Ambion) as per the manufacturer’s instructions and was treated with DNase. RNA (1 µg) was then reverse-transcribed with dT primers, using the Retroscript kit and aliquoted for single uses. The analysis of mRNA levels was performed using QPCR with the SYBR Green PCR Master Mix (Applied Biosystems) in an SDS7700 thermocycler (Applied Biosystems), followed by a dissociation curve analysis, that verified the amplification of a single PCR species. Thermocycling parameters were standard for the SDS7700 (Applied Biosystems) instruments: 95°C for 10 min, and 40 cycles of 95°C for 15s and 60°C for 1 min. Each sample was tested in triplicate. A standard curve was run to allow relative comparisons of the sample values. The PCR primers for iNOS were TGAGACAGGAAAGTCGGAAG (forward) and TCCCATGTTGCCTTGAA (reverse) and normalized to GADPH mRNA levels (an
internal housekeeping gene control) for each cDNA sample, using the primers from the TaqMan Rodent GADPH Control Reagents (Applied Biosystems).

**Tissue Extraction and ELISAs for Aβ, 8-iso-PGF₂α (F2 isoprostanes), and interleukin1β (IL-1β)** — For Aβ and IL-1β ELISAs, an aliquot of frozen brain tissue (powderized on dry ice with a mortar and pestle) was homogenized in 10 vol of TBS containing the protease inhibitor cocktail (first without detergents). Samples were sonicated briefly and centrifuged at 100,000xg for 20 min at 4°C. The TBS-extracted supernatant was analyzed by ELISA for interleukin 1β and Aβ to measure an inflammatory endpoint and levels of ‘soluble Aβ’, respectively. To evaluate ‘insoluble Aβ’, the pellet was dissolved in guanidine and analyzed by Aβ ELISA as described previously (Lim et al., 2000). For F2 isoprostane ELISA, a separate aliquot of powderized tissue was used, and total lipids were extracted as described previously and measured using, the 8EPI-F2 Immunoassay Kit (EA84; Oxford Biomedical Research, Oxford, MI) (Frautschy et al., 2001).

**Western blot for brain iNOS, pJNK, nitrotyrosine, and carbonyls** — To detect iNOS, nitrotyrosine and pJNK, the TBS fraction was used. Samples (20 µg) were electrophoresed onto a 10% Tris–glycine gel. After transfer of proteins to PVDF membrane, blot was blocked for 1 h at room temperature in 5% milk with 0.05% Tween20 in PBS. The membrane was incubated with primary mouse monoclonal antibodies at 1:1,000 dilution overnight at 4°C: anti-iNOS antibody (Transduction laboratories, BD Biosciences, Bedford, MA), anti-nitrotyrosine (Cayman Chemical, Michigan, MI) or anti-pJNK (Cell signaling, Beverly, MA). The secondary antibody was horseradish peroxidase conjugated anti-mouse (1:30,000) incubated for 1 h at room temperature, and developed with ECL.

For measurement of carbonyls, we used the “Oxyblot” kit (Intergen, Purchase, NY) as previously described (Lim et al., 2001). The TBS-insoluble pellet was sonicated in lysis buffer (see above) with
protease inhibitor cocktails and then centrifuged at 100,000 × g for 20 min at 4°C. The resultant supernatant was collected as the “lysis fraction”. 10 µg of protein from this fraction were reacted with 1 X dinitrophenylhydrazine (DNPH) for 15~30 min, followed by neutralization with a solution containing glycerol and β-mercaptoethanol. These samples were electrophoresed on a 10% Tris–glycine gel, transferred, and blocked. The blot was incubated overnight with a rabbit anti-DNPH antibody (1:150) at 4°C, followed by incubation in goat anti-rabbit (1:300) for 1 hr at room temperature. Bands were visualized using chemiluminescent techniques with nonsaturating exposures, and the resulting bands were scanned and quantified using a densitometer and Molecular Analyst software (Model GS-700, Bio-Rad Laboratories, Hercules, CA).

**Immunostaining and image analysis** — In addition to analyzing Aβ and GFAP by Western of dissected brain homogenates, brains were also analyzed immunohistochemically. Hemibrain cryosections (8 µM thick) of Tg2576 mice on curcumin or TC diet were analyzed immunohistochemically from the anterior middle and posterior hippocampal Bregma regions for Aβ (rabbit polyclonal antibody; 1:500 anti-Aβ1–13, DAE) or GFAP (1:10000, monoclonal antibody, Sigma, St Louis, MO) as described previously (Lim et al., 2000). Briefly, antigen retrieval was accomplished by steaming sections in an unmasking solution (Vector Laboratories, Burlingame, CA). After quenching with 0.6% hydrogen peroxide and blocking with normal serum, sections were incubated with primary antibodies overnight at 4°C. After incubating with the peroxidase-containing ABC reagent (Vector Lab), sections were developed with metal-enhanced diaminobenzidine (DAB, Pierce, Rockford, IL).

Acquire images of anti-Aβ (DAE) or GFAP stained sections were analyzed using NIH Image public domain software to calculate number of plaques, plaque burden and percent GFAP-area stained in cortical and hippocampal layers. Statistical analysis of variables was by 2x2 ANOVA (treatment x region).
Aβ42 aggregation assays by Western blot and oligomer disaggregation by A11 dot blot assay — We investigated impact of TC and curcumin on aggregation using two different preparations of oligomer starting with lyophilized Aβ42, monomerized with HFIP. The first oligomer was prepared by incubating 5 µM Aβ for 4 hr at 37 °C in F12 media and the second prepared by incubating 67 µM Aβ in water at 48 hr at room temperature, and then running the preparations that had been coincubated with different concentrations of TC or curcumin on a Western immunoblotted with 6E10 anti-Aβ antibody as described previously (Yang et al., 2005). We also examined the impact of TC and curcumin on Aβ42 disaggregation in a cell-free system, using a dot blot with an oligomer-specific antibody as described previously (Yang et al., 2005) except oligomers were pre-formed. Freshly prepared Aβ42 oligomers (11 µM, Dr. Charles C. Glabe, UC Irvine) were co-incubated with curcumin (16 µM), TC (16 µM), curcumin+TC (curcumin and TC together, 8 µM each), or vehicle for 72h at 25°C, and then 500 ng per well applied to the nitrocellulose membrane in a Bio-Dot apparatus (Bio-Rad). The membrane was blocked with 10% nonfat milk in TBS-T at room temperature for 1 h, washed with TBS-T, and probed with either the anti-Aβ (6E10) or the anti-oligomer A11 antibody (Yang et al., 2005) (1:10,000) in 3% BSA-TBS-T overnight at 4 °C. After washing, it was probed with anti-rabbit horseradish peroxidase-conjugated antibody (Pierce) solution (1:12,000) for 1h at room temperature. The blot was developed with SuperSignal (Pierce) for 2–5 min. Dots were scanned and analyzed with a model GS-700 densitometer using Molecular Analyst software (Bio-Rad).

Statistical analysis — The comparative effects of curcumin and TC treatment were analyzed by one-way ANOVA analysis, followed by post-hoc Bonferroni/Dunn test. All Western blot densitometry was analyzed by one-way ANOVA analysis (Graph Pad InStat v.3.05, San Diego, CA) and image analysis by 2 x 2 ANOVA (Treatment x Region) at anterior, middle and posterior hippocampus, 4 consecutive sections per Bregma. Coefficient of correlation and significance of the degree of linear relationship between parameters were determined with a simple regression model using StatView (Abacus, Berkeley,
discontinued) or JMP (SAS Institute Inc., Cary, NC) software. All experiments were performed in triplicate or quadruplicate. GraphPad Prism software (Version 5.00, GraphPad Software Inc.) was used to evaluate the dose response curves and EC50 for responses in iNOS, IL-1beta, and isoprostane. The drug response curves were determined by using nonlinear regression. For Figures 3 and 4, the concentration of drug is shown in Log 10 scale in X-axis and curve fit determined using a variable slope model. The response variable was normalized, according to the percent maximal response. The curves for the effect of Curc or TC on iNOS, IL-1beta and isoprostane were fitted using the equation:

\[ Y = \frac{100}{1 + 10^{((\log_{10}\text{EC50} - X) \times \text{HillSlope})}} \]
Results

Previous measurements of curcumin or TC using our published HPLC methods showed detection of TC or curcumin in tissue samples spiked with TC or curcumin. However, it was not known if these methods were sufficiently sensitive or specific to detect unconjugated TC or curcumin in animals treated with curcumin or TC prior to sacrifice. We delivered curcumin or TC using three routes of administration to investigate impact of GI tract on tissue levels and drug efficacy. The structures of curcumin and TC are shown in Fig. 2, A and B, respectively, illustrating that curcumin's double bonds flanking the β-diketone bridge are lost upon reduction to TC. Data demonstrated successful detection of these compounds in treated mice (Fig. 2 and Table 1). Representative chromatograms from brains or plasma of vehicle-treated mice, showed the internal standard peak, without signal for curcumin or TC, respectively (Fig. 2, C and D). However, when mice were injected i.m. with either drug, their respective peaks were present in both brain (Fig. 2, E and F) and plasma fractions (Fig. 2, G and H).

We also examined metabolism of curcumin. Although in the acute study we did not detect metabolites of curcumin in the brain or plasma, measureable levels were obtained in the chronic study. HPLC analysis revealed that plasma containing the parent compound curcumin (Fig. 2, I) also contained the metabolite TC (Fig. 2, J, Table 2). The plasma values for the metabolite of mice fed 500 ppm curcumin were close to the lower limit of detection, but higher in animals fed a higher dose (2000 ppm). Similarly the metabolite was also detected by LC/MS/MS in the brains of mice chronically fed the parent compound curcumin, using LC/MS/MS (Fig. 2, K and Table 2). Brain levels of the metabolite were approximately 6-fold less than that of the parent compound (Table 2).

Table I (acute study) shows that plasma levels of drug are higher with TC than with curcumin, regardless of route of administration. Similar results are observed in chronic study, Table 2.
Brain to plasma ratios of parent compounds were calculated. In the acute gavage study (Table 1), curcumin ratios were approximately 5 fold higher in brain than plasma, while TC brain to plasma ratios were much lower (Table 1), particularly in chronically fed mice (where brain TC levels were ~0.5-fold that of plasma, Table 2).

Brain levels of the curcuminoids ranged from 0.7 µM to 6 µM for TC and 1.4 µM to 3 µM for curcumin, depending on route of administration. We then assessed the association of these brain levels with anti-inflammatory effects, using iNOS as an index of inflammation. We induced inflammation by injecting LPS (i.p.), and then analyzed brains of TC or curcumin-treated mice for iNOS protein (Fig. 3, A and B) and mRNA (Fig. 3, C). LPS caused robust increases in iNOS protein and mRNA. Although i.m. injection, which produced the highest brain drug levels, tended to be slightly more effective in attenuating these LPS responses, all routes of administering curcumin and TC caused large decreases in iNOS protein and mRNA. Regression analysis showed a close correlation between inhibition of iNOS mRNA and increasing concentrations of the administered curcuminoids (Fig. 3, D). The EC50 for iNOS mRNA inhibition in vivo was 1.186 and 0.701 µM for curcumin and TC, respectively.

Since LPS induces CNS expression of IL-1β (Hsu and Wen, 2002), an inflammatory cytokine known to be elevated in neuroinflammatory diseases including AD (Akiyama et al., 2000), we evaluated the relative impact of curcumin or TC on IL-1β induction. While LPS effectively induced robust elevations in IL-1β brain levels compared to vehicle, all routes of administration of either curcumin or TC equally inhibited this response (Fig. 4, A). The EC50 for IL-1β inhibition in vivo was 1.722 and 1.286 µM for curcumin and TC, respectively (Fig. 4, B).

LPS can induce brain lipid peroxidation, as measured by oxidation of arachidonic acid to F2 isoprostanes, which are elevated in AD and correlate with synaptic loss and (Montine et al., 2004) and AD models.
(Frautschy et al., 2001). Therefore, we investigated the impact of curcumin or TC on induction of F2 isoprostanes. Results showed that LPS increased F2 isoprostanes. Although acute oral delivery of curcumin or TC failed to produce brain levels sufficient to modify isoprostanes, i.p. delivery slightly reduced, and i.m. delivery greatly reduced LPS-induced F2 isoprostanes (Fig. 4, C). The EC50 for F2 isoprostane inhibition in vivo was 1.067 and 0.501 µM for curcumin and TC, respectively (Fig. 4, D).

Superoxide reaction with nitric oxide, resulting in increased peroxynitrite, typically occurs in neuroinflammatory diseases such as AD (Castegna et al., 2003). An indirect index of peroxynitrite can be estimated by measuring anti-nitrotyrosine (NT) reactive proteins, formed by peroxynitrite-mediated nitration of protein tyrosine residues. Results demonstrated that LPS induced prominent 75 kDa and 90 kDa NT-reactive bands, but gavage with either compound was not effective in modifying NT (Fig 5, A), similar to the failure of gavage to suppress F2 isoprostane induction. In contrast, curcumin was effective in suppressing LPS-induced protein oxidation, as measured by carbonyls, regardless of route of administration (Fig. 5, B), unlike TC which was only effective in reducing carbonyl induction if injected by i.m. (Fig. 5, C).

Because it has been reported that fasting may enhance absorption or utilization of curcumin (Chan et al., 1998), and because chronic treatment is likely necessary for therapeutic intervention of diseases, we evaluated the impact of food ad libitum on curcumin and TC plasma and brain levels. We increased the gavage dose to 480 µg, which is closer to the daily dietary consumption of curcumin used to reduce AD pathogenesis in the Tg2576 mouse (Lim et al., 2001). Compared to fasted mice, mice previously fed ad libitum showed a large reduction in absorption of both curcumin and TC (by gavage) as measured by their contents in the brain (Fig. 6, A). TC was not detectable in brain if given with food, and curcumin was detectable, but reduced. The diminished plasma levels associated with food-intake correlated with impaired (curcumin) or abolished (TC) efficacy in inhibition of LPS-induced brain iNOS (Fig. 6, B).
Since curcumin was not detectable with acute gavage dosing with food (non-fasted), we then investigated whether it could be detected after chronic administration in chow. We chose doses previously shown to reduce oxidative damage, neuroinflammation and plaque pathology in the APPsw Tg2576 mouse (Lim et al., 2001). Table 2 shows that in contrast to acute gavage, chronic oral dosing in diet of curcumin or TC resulted in detectable plasma levels of 0.095 µM and 0.73 µM, respectively. Like acute dosing, chronic dosing resulted in plasma levels of TC that were several-fold higher than those of curcumin.

We then evaluated the relative efficacies of curcumin- and TC-fed mice on plaque pathology in the Tg2576 APPsw mouse during plaque deposition (from 12 to 16 months of age). Immunostaining for anti-Aβ revealed that, compared to control-fed mice, curcumin-fed mice showed a marked reduction in plaques (Fig. 7, A-D), similar to that previously described (Lim et al., 2001). But this was not observed with TC-fed mice (Fig. 7, E and F). Quantitation of plaque size (Fig. 7, G) and plaque burden (Fig. 7, H) confirmed that curcumin, but not TC, reduced Aβ pathology. We then analyzed the dissected cortex for Aβ levels and showed that curcumin-fed, but not TC-fed animals had reduced Aβ in the insoluble fraction (Fig. 7, I). The only significant impact of TC on Aβ variables was on soluble Aβ, which it reduced more than curcumin (Fig. 7, J).

GFAP was analyzed immunohistochemically to evaluate the impact of curcumin and TC on the elevated gliosis in this transgenic model of AD (Fig. 8, A-H). Quantitation of GFAP confirmed the known induction of GFAP in this transgenic model. Both curcumin and TC significantly attenuated the gliosis associated with the transgene (Fig. 8, I). IL-1β is an index of neuroinflammation, thought to contribute to AD pathogenesis and elevated in this model. Like gliosis, IL-1β was effectively reduced by both curcumin and TC compared to Tg+ mice on the control diet (Fig. 9, A). However, although carbonyls known to be induced by this transgene (Lim et al., 2001) were reduced by curcumin as previously
described, TC showed only a non-significant trend for reducing carbonyls (Fig. 9, B). Since the stress-activated (phosphorylated) c-Jun N-terminal kinase (pJNK) has been reported to be elevated in cortical homogenates of this model (Puig et al., 2004), and JNK is a putative target of curcumin, we also examined the relative impacts of curcumin and TC on pJNK. Western analysis showed two bands at 46 and 56 kDa, which when quantified demonstrated that both curcumin and TC effectively reduced pJNK, with TC being particularly potent (Fig. 9, C and D). Because of data suggesting that pJNK may influence Aβ, we evaluated the association by a regression analysis, and showed that the degree of TBS soluble Aβ reduction was proportional to that of pJNK reduction (Fig 9, E).

*In vitro* data confirmed that both TC and curcumin were potent suppressors of iNOS in primary neurons (Fig. 10, A) and in BV2 microglia (not shown). Both protected from oligomeric Aβ toxicity in a neuroblastoma model as measured by LDH (Fig. 10, B). In contrast, using a model of intraneuronal APP C99 fragment accumulation (M65 cells, Jin L-W, APP mutation lacking leader sequence), only TC protected from intraneuronal Aβ toxicity, possibly indicating differing neuroprotective mechanisms of TC and curcumin (Fig. 10, C). We then investigated the impact of TC and curcumin on Aβ oligomer formation (Fig. 10, D-F). Aggregating Aβ at low concentrations was robustly attenuated by curcumin, but not by TC (Fig. 10, D), similar to previous data (Yang et al., 2005). Although TC had no impact on aggregation of Aβ at the lower concentration, at the higher Aβ concentration, TC appeared to selectively reduce aggregates of the intermediate MW oligomers (30-200 KDa, Fig. 10, E) and also the level of pre-aggregated oligomers (11 µM) in a cell-free dot blot assay using oligomer-specific A11 (Fig. 10, F). Unlike curcumin, TC appeared to increase (rather than reduce) very high aggregates associated with the stack (Fig. 10, E). When curcumin (but not TC) was added to pre-aggregated oligomers, intense monomeric bands appeared, suggesting de-aggregation (not shown).
Discussion

We demonstrate target plasma and brain levels needed for \textit{in vivo} antioxidant, anti-inflammatory and anti-A\(\beta\) activities of TC and curcumin, which is relevant to clinical utility and the functional aspects of the molecular structure. Evidence supports similar mechanisms of TC versus curcumin on neuroinflammatory cascades but a differential mechanisms on amyloid pathogenesis.

\textit{Curcumin metabolism to TC} — We demonstrate for the first time that mice chronically fed the parent compound curcumin, showed detectable levels of the unconjugated metabolite TC in both the plasma and brain. Curcumin is reduced to TC by the intestinal cells as the major metabolite (Ireson et al., 2002), but only the unglucuronidated metabolite was detected in mice (Pan et al., 1999; Okada et al., 2001; Liu et al., 2006) or humans (Baum et al., 2008) administered curcumin. One explanation for the discrepancy might be our improved sensitivity of the HPLC (Heath et al., 2005) and LC/MS/MS assays. Brain concentrations of the parent compound were approximately six to ten fold higher than levels of the metabolite, depending on curcumin dose.

\textit{Differential brain plasma ratios for TC versus Curc}—Plasma TC levels were nearly 8-fold higher than curcumin levels after chronic dosing (with food), consistent with either TC having better GI absorption or greater stability than curcumin (Okada et al., 2001). However, fasting increased plasma curcumin levels, consistent with a previous report (Chan et al., 1998). Fasting also increased TC plasma levels. Fasting slows stomach emptying and gastric motility, so it could result in an increase in absorption from the stomach, as opposed to the small intestine where glucuronidation is thought to occur. However, the relatively high plasma TC levels cannot be attributed solely to improved GI absorption, since injections of TC also resulted in comparatively high plasma levels. Thus these observations are more likely due to TC’s resistance to hydrolysis in plasma, consistent with \textit{in vitro} stability data and similarly high glucuronidation rates (Pan et al., 1999; Okada et al., 2001).
Brain to plasma ratios from acute studies were highest for curcumin, suggesting its stability in lipid-rich compartments, where hydrolysis is limited. In the chronic study with 500 ppm the brain to plasma ratio dropped for TC and increased for curcumin. This may reflect differences in blood-brain-barrier transport, brain or plasma clearance. For example TC may be more readily exported from brain or less stable in the brain than curcumin. Although curcuminoid entry into the brain is limited by glucuronidation, both TC and curcumin are readily glucuronidated. Thus glucuronidation cannot explain the different brain to plasma ratios.

**Effects of TC and curcumin on brain inflammation** — Curcumin and TC similarly inhibited IL-1β in the acute inflammation model (1.722 and 1.286 µM) and IL-1β and the astrocytic marker GFAP in the chronic AD model (at 1.3 and 0.7 µM, respectively). It was previously reported that curcumin reduced CNS IL-1β (Lim et al., 2001; Chainani-Wu, 2003), but this is the first demonstration that TC reduced brain IL-1β. Similarly, both compounds strongly inhibited LPS induction of iNOS protein and mRNA. Curcumin's anti-inflammatory activity is mediated by limiting the activity of transcription factors (Weber et al., 2006), or co-activators of AP-1, including p300 histone acetyltransferase (HAT, (Marcu et al., 2006)). Our data support the hypothesis that the phenolic ring methoxy groups, but not the C=C double bonds of curcumin’s dienone, mediate anti-inflammatory effects *in vivo*.

**Anti-oxidant effects of TC and Curcumin** — Curcumin inhibited protein oxidation better than TC in the AD model. Previously curcumin's brain antioxidant effects have been well-documented in models of cerebral ischemia (Thiyagarajan and Sharma, 2004), AD (Lim et al., 2001) and brain trauma (Wu et al., 2006). Although the 4-hydroxyl-3-methoxy phenolic groups, which are common to both TC and curcumin are critical for some anti-oxidant effects, metal chelation can also reduce oxidative damage (Cherny et al., 2001), which along with amyloid reduction might contribute to the slightly better...
antioxidant effects of curcumin in the Tg2576 model. The present report is the first showing TC efficacy on CNS oxidative damage.

**TC and curcumin effects on Aβ and JNK in the Tg2576 mouse** — Curcumin was more effective than TC in attenuating plaque pathogenesis, demonstrating an important difference between the two compounds. The inhibitory effect of curcumin on fibril and oligomer formation and disaggregation was consistent with previous reports (Kim et al., 2005; Yang et al., 2005). It is also consistent with *in vitro* data showing that, while addition or removal of the phenolic ring methoxy groups did not attenuate potency of curcuminoids in reducing fibril formation, loss of the dienone double bonds resulted in loss of fibril inhibition (Kim et al., 2005). Curcumin readily metabolize to ferulic acid, which also impacts fibril formation; however, the EC50 concentrations for disaggregation of fibrillar Aβ is about ten-fold higher for ferulic than for curcumin (Ono et al., 2005), arguing against a substantial role for this charged and likely poorly brain permeant metabolite. Factors such as metal chelation, which is known to reduce plaque formation may contribute to the more potent plaque-reducing effects of curcumin *in vivo* (Cherny et al., 2001). Curcumin but not TC, readily binds Cu²⁺ and Fe²⁺ and may remove metal from Aβ (Baum et al., 2008).

Our data showing curcumin reduction in soluble Aβ levels confirmed our previous report showing a similar 50% reduction (Lim et al., 2001). Our soluble (TBS) Aβ levels in aged Tg2576 were well above baseline Aβ monomer production levels in Tg2576 and clearly included soluble aggregates (oligomers) as shown on Westerns. It might be presumed that, if insoluble and soluble Aβ compartments were in equilibrium, drugs reducing Aβ aggregation or increasing Aβ clearance would similarly reduce both amyloid fibrillar plaques and soluble oligomers, as we observed for curcumin. However this was not the case with TC. The surprising finding was that despite TC having no statistically significant impact on plaque burden, it reduced soluble Aβ by 75% (Fig. 7, J). One possibility is that, despite curcumin’s greater affinity for fibrils (Kim et al., 2005), TC and curcumin have similar affinity for oligomers as
suggested by our cell-free aggregation studies. Another possibility is that TC’s reduction of soluble Aβ related to the observed stronger inhibition of pJNK. Aβ levels can be lowered by pJNK inhibitors (Borsello and Forloni, 2007) or in JNK knockout mice crossed with APP Tg mice in vivo (unpublished observations, Prof. Reinhard Schleibs, University of Leipzig, Germany). Soluble Aβ appears to more closely correlate with synaptic loss and neurodegeneration in AD (Frautschy et al., 2001). Curcumin does not interact directly with JNK, but via an unknown mediators upstream to SEK1, MEKK1 or HPK1 (Chen and Tan, 1998). JNK is a tau kinase hypothesized to mediate end stage neurodegeneration and LTP deficits in AD models (Puig et al., 2004; Wang et al., 2004). In summary, although TC was not as effective in reducing brain oxidation and plaque burden, its slightly better impact on reducing soluble Aβ and pJNK merits its further consideration potential clinical use.

Conclusions — Curcumin, but not TC reduced plaque burden and protein oxidation in the AD model, demonstrating the importance of the hydrolysis-vulnerable dienone bridge in curcumin's anti-amyloid action. In contrast, both drugs had similar potent effects on neuroinflammation and CNS pJNK, showing that the phenolic rings, but not the dienone bridge are required for suppression of these targets which is not simply secondary to amyloid reduction. Overall, our data support the concept of pleiotropic anti-amyloid, antioxidant and anti-inflammatory treatment for AD. Effacious tissue levels can be achieved with chronic oral 500 ppm curcumin which is well within the safety range established in chronic animal experiments by the National Toxicology Program (Kelloff et al., 1996). Similar levels are likely needed to control inflammation, oxidative damage and protein aggregation in other diseases. However, in humans high oral dosing fails to achieve detectable plasma levels (Lao et al., 2006). The reported failure to achieve these modest target levels in humans with oral supplements predicts limited success in translating to the clinic. In our studies, increasing curcumin solubility with phosphatidyl choline, olive oil or stearic acid increases plasma and brain levels compared to administering unformulated curcumin
powder. For example, oral gavage of an optimized lipidated curcumin formulation (Verdure Sciences, Noblesville, Indiana) resulted in 11-fold higher levels of curcumin in plasma and 4-fold higher levels in brain compared to equal doses of curcumin powder or curcumin-piperine extracts. A 5 mg curcumin dose delivered by acute gavage in this lipid rich formulation (n=5) resulted in 2.15 ± 0.744 µM mouse brain curcumin levels after 3 hrs. After 2 weeks lipidated formulation at 500 ppm curcumin in chow (n=5) we observed 5.79 ± 1.22 µM mouse brain curcumin, well above the 1-2 µM range of EC50’s for the inhibition of iNOS, IL-1β, PGE2 and isoprostanes. This suggests oral delivery can achieve our target tissue levels. Finally, the traditional method of dissolving turmeric in fat during cooking is likely an effective method to improve absorption, and could play a role in India’s low incidence and prevalence of Alzheimer’s (references in (Lim et al., 2001)).
Acknowledgments

We thank Walter Beech for preparation of primary neuron cultures, M65 neuroblastoma and BV-2 cells. We are grateful to Drs. C. Glabe and Rakez Kayed (UC Irvine) for oligomeric-specific polyclonal antibody, to Prof. Jean deVellis, Univ. California, Los Angeles, for BV-2 cells and Prof. Lee Way Jin, for M65 cells (University of Washington, Seattle). Lipidated curcumin was provided by Verdure Sciences (Noblesville, Indiana, www.vs-corp.com).
References


a) U01028583 (SAF), VA Merit (SAF, GMC), AG021975 (SAF), AG016570 (Cummings. Project 1. GMC). We are grateful for the original support of Ms. K.K. Siegel on curcumin in 1998 on a UCLA Pilot Center on Aging Grant.

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Legends for Figures

Fig. 1. Schematic paradigms of curcumin and TC treatments. A, mice were treated with curcumin (curc) or TC by gavage (gav), intraperitoneal injection (i.p.), or intramuscular injection (i.m.) using doses of 0.4, 0.4, or 0.2 μmoles (148, 148, 73.4 μg), respectively. B, Aged Tg2576 APPsw transgenic mice were fed diets ad libitum with or without curcumin and TC for four months at 500 ppm (500 mg/kg chow).

Fig. 2. Detection of curcumin or TC in plasma and brain after acute injection or chronic feeding. Structural differences between curcumin (A) and TC (B) are indicated by circles, highlighting the presence (curcumin) or absence (TC) of diketone bridge. HPLC chromatograms with UV detection for plasma (C-D, G-J) and brain (E,F) are shown for curcumin (left) and TC (right). Control samples only showed the ISD peak, with no TC or curcumin peaks (C and D). At 262 nm, curcumin was detected at peak retention time 5.56 min, and ISD at 10.898 minutes (E, G and I), while TC was detected at 10.6 min and ISD at 19.9 min (F,H and J). mAU indicates milli absorbance unit. I and J, Chromatograms of plasma collected from mice fed for 4 months ad libitum feeding with curcumin in chow (2000 ppm, n=5) revealed predominantly curcumin (I), but also significant bioconversion to TC (J). LC/MS/MS-MRM chromatograms of a brain extract from mice chronically fed the parent compound curcumin (K) show clear peaks for Curc (m/z 371→149 transition) and TMC (m/z 395.2→365.1 transition), and in addition a peak for TC (m/z 371.1→ 235 transition), which elutes as an earlier and broader peak on the chromatographic system employed.

Fig. 3. Curcumin and TC suppressed LPS-induced iNOS protein and mRNA. Mice injected with LPS or vehicle, were sacrificed 4 hr after administration of curcumin (A) or TC (B), and
the supernatant of TBS-extracted brains was electrophoresed on Western blot and immunostained with anti-iNOS and β-actin. Representative lanes and their densitometric quantitation are shown. C, RNA was extracted from brain and measured for iNOS mRNA using quantitative RT-PCR. D, Percent iNOS inhibition was regressed on curcumin or TC concentrations. Closed circles, Curcumin; open circles, TC. Values shown are the amount of iNOS mRNA as the mean ± SD. * p < 0.05, ** p < 0.01 and *** p < 0.001 represents a significant difference compared to positive controls (LPS treatment alone; n=4).

**Fig. 4.** Acute injection of TC or curcumin similarly attenuated LPS induced-IL-1β or F2 isoprostane. A, Quantitation of IL-1β levels in mouse brain homogenates was determined by sandwich ELISA. LPS injection (i.p) increased IL-1β levels over 6-fold, an effect that was partially (>50%) suppressed by acute administration of either curcumin or TC, regardless of route of administration. B, Either curcumin or TC levels correlated positively with percent IL-1β inhibition. C, Lipid extracts of brain were measured for 8-iso-PGF2α by ELISA. The 40% increase in 8-iso-PGF2α caused by LPS was partially reduced by i.p. injection and completely suppressed by i.m injection of either compound. D, Brain curcumin or TC correlated positively with brain F2 isoprostane inhibition. Values shown are the mean ± SD. * p < 0.05, and ** p < 0.01 and *** p < 0.001 represents significant difference compared to positive controls (LPS treatment alone; n=4).

**Fig. 5.** Acute curcumin or TC similarly suppresses LPS induction of brain nitrotyrosine (NT), but curcumin more effective at suppressing carbonyls. A, NT proteins increased over 7-fold after LPS from mouse brain homogenates measured by Western blot with anti-nitrotyrosine antibody and normalized to β-actin. Injection (i.m.) of either compound suppressed NT induction. Injection (i.p.) of curcumin partially suppressed NT induction,
while i.p. injection of TC did not impact NT induction. Oxidized protein levels of brain were determined in the lysis-extracted supernatant of the TBS-insoluble pellet using Oxyblot analysis with an anti-DNP antibody. Representative lanes are shown and quantified for curcumin- (B) or TC- (C) fed mice. Values shown are the mean ± SD. * p < 0.05 and ** p < 0.01 represents a significant difference compared to positive controls (LPS treated alone; n=4).

**Fig. 6.** Brain levels and efficacy of curcumin and TC administered by gavage are increased by fasting. 480 µg of curcumin or TC were administered to mice by gavage with fasting (FAST) or non-fasting (nFAST). After four hours, brains were removed, and prepared for the analysis of curcumin and TC levels by (A) HPLC and (B) iNOS protein measured by Western blot of iNOS and normalized with β-actin (endogenous control). When administered with food, brain curcumin levels were not detectable, and TC levels were reduced 50% compared to if mice had fasted. Curcumin and TC reduced iNOS less if administered with food. Values shown are the mean ± SD. * p < 0.05 and ** p < 0.01 represents a significant difference compared with control (con, untreated, n=4) curcumin or TC by gav n=4, each treatment (ND, not detectable).

**Fig. 7.** Dietary curcumin appears more effective than TC in reducing plaque pathology in the Tg2576 APPsw mouse, but both reduce soluble Aβ levels. Curcumin or TC was administered to aged Tg2576 mice for four months (12-16 month-old) in the chow (500 ppm) during accelerated plaque deposition. Representative micrographs stained with antibodies to Aβ (DAE, anti Aβ 1-13). Compared to Tg+ mice on control diet (A and B), mice on curcumin diet showed a noticeable reduction in plaque size and number (C and D). However sections from mice on dietary TC (E and F) showed plaque distribution similar to...
those from mice on control diet. Although image analysis quantification confirmed that dietary curcumin could reduce plaque size \((G)\) and plaque burden \((H)\), dietary TC appeared to have no impact on plaque pathology. \(I\), Insoluble (guanidine-insoluble) and soluble A\(\beta\) (TBS-soluble) in cortical homogenates were also evaluated by A\(\beta\) sandwich ELISA. Curcumin, but not TC, reduced A\(\beta\) levels in detergent insoluble fraction. \(J\), In contrast, both drugs suppressed soluble A\(\beta\) levels (in TBS-extracted brain homogenates), with a trend for TC being more potent. Magnification bar=75 \(\mu\)m. Statistical analysis of Western data was performed by one way ANOVA and immunohistochemistry data by 2 x 2 ANOVA (Treatment x Brain region) and values shown are the mean ± SD. * \(p < 0.05\), ** \(p < 0.01\) and *** \(p < 0.001\) represent significant difference of control (n=5) and standard diet (n=5) compared to curcumin (n=9) and TC (n=7) treatment of Tg+ mice respectively.

**Fig. 8.** Curcumin or TC diets ameliorated Tg2576-dependent glial activation. Micrographs demonstrated that, compared to brains of Tg- mice (A and B), brains of Tg+ mice (C and D) showed increased staining for GFAP. Compared to Tg+ mice fed control diet, Tg+ mice fed either curcumin (E and F) or TC (G and H) in chow showed reduced GFAP (glial activation). Quantification of percentage GFAP staining demonstrated significant attenuation of transgene-dependent gliosis by both TC and curcumin (I). Values shown are the mean ± SD. **\(p < 0.01\) represents significant difference of curcumin (n=9) or TC (n=7) treatment of mice compared to control diet (n=5). Magnification bar=75 \(\mu\)m.

**Fig. 9.** In APP\(\text{sw}\) Tg2576 mice, both dietary curcumin or TC similarly reduced IL-1\(\beta\) and phospho N-terminal c-Jun kinase (pJNK), while curcumin, but not TC, reduced carbonyls. A, Compared to Tg+ mice on control diet, mice fed curcumin or TC showed 25% reduction in IL-1\(\beta\) levels as measured by sandwich ELISA of TBS-extracted...
supernatant fraction of brain homogenate. B, Compared to Tg+ mice on control diet brain curcumin reduced carbonyls measured on Western with anti-DNP antibody from the detergent buffer -extracts of brain homogenates. Although TC-fed mice showed a trend for reduction, it was not statistically significant. C, Western analysis of main pJNK bands of 46 and 56 KDa, showed that compared to mice fed control diet, mice fed TC or curcumin showed reduced pJNK. D, Densitometric quantification showed that compared to control fed Tg+ mice, mice fed curcumin or TC showed reductions in pJNK, with TC showing the greater reduction. E, pJNK was positively correlated with soluble Aβ. Values shown are the mean ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001 represents significant difference of curcumin (n=9) or TC (n=7) treatment of Tg+ mice with control diet (n=5).

**Fig. 10.** Differential impact of TC versus curcumin on iNOS, Aβ toxicity and Aβ aggregation

**in vitro.** Primary cortical neuron cultures (A) or the microglial cell line BV-2 (not shown), were treated with LPS (1 µg/ml), and iNOS protein levels in the detergent soluble fraction were measured by Western blot with or without curcumin (2.5 µM), TC (2.5 µM), curcumin+TC (1.25 µM each), using β-actin as an endogenous control to ensure equal protein loading. LPS induction of iNOS was attenuated by curcumin (50%), TC (80%) or combined Curcumin + TC (95%). B, Attenuation of Aβ42 oligomer (500 nM)-induced toxicity (% maximum LDH) in SH-SY5Y neuroblastoma by pretreatment with curcumin (40%), TC (20%), or curcumin + TC (70%). C, TC, but not curcumin minimized the reduction in viability (MTT reduction) in MC65 neuroblastoma cells caused by intraneuronal expression of C99, 3 days after tetracycline withdrawal. D-E, Western blot for Aβ-ir bands (6E10) to assess drug effect on aggregation of specific MW oligomers. Impact of co-treatment of TC and curcumin on aggregation of low dose (5 µM) Aβ (D) or high dose 67 µM Aβ (E), initially monomerized with HFIP. F, Both TC and curcumin reduced levels of
oligomeric-specific antibody A11 during oligomerization of 11 μM Aβ, using starting curcuminoid:Aβ molar ratio of 1.45 to 1. Equal loading is demonstrated by a second dot blot with 6E10. Values shown are the mean ± SD; * p < 0.05, ** p < 0.01, and *** p < 0.001 represents significant differences compared to positive control (n=4) and treatments (n=4).
TABLE 1

Detection of curcumin and tetrahydrocurcumin (TC) in plasma and brain after multiple dosing routes.

These data showed brain and plasma levels of curcumin or TC 4h after administration (by gavage, i.p., i.m.). The lower limits of detection for curcumin were 35 ng/ml in plasma and 100 ng/g t in brain, and for TC were 8 ng/ml in plasma and 250 ng/g t in brain. Data are presented as mean ± SD. Different superscript letters represent statistical differences of means from each other between treatments within route of administration or between routes within treatments. p <0.05 SD; standard deviation, ND; not detectable, g t; grams wet tissue; gav, gavage; Curc, curcumin; TC, tetrahydrocurcumin

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TABLE 2

Parent and metabolite compounds in plasma and brain after chronic dietary administration.

Curcumin or tetrahydrocurcumin (TC) in chow was fed to mice for 4 months duration at 500 ppm (~2.5 mg/day) or 2000 ppm (~10 mg/day). After sacrifice, plasma was collected, animals perfused and brains removed. Plasma values were detected using HPLC where the lower limits of detection (LLQ) for curcumin and TC were 35 and 8 ng/ml, respectively. Brain levels were measured using LC/MS/MS where the LLQ was 100 pg/g t for curcumin and 1 ng/g t for TC. Data represent mean ± SD, standard deviation; ND; not detectable, NA; not applicable, t; wet tissue, Curc, curcumin; TC, tetrahydrocurcumin.

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<th>Brain Curc (µg/g t or µM)</th>
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<td>0.052 ± 0.054 (0.143 µM)</td>
<td>3.07</td>
</tr>
<tr>
<td>500</td>
<td>TC</td>
<td>ND</td>
<td>0.270 ± 0.003 (0.734 µM)</td>
<td>ND</td>
<td>0.128 ± 0.019 (0.344 µM)</td>
<td>0.47</td>
</tr>
</tbody>
</table>
Figure 1

A
Acute dosing of Curc or TC

Day 1
9:00

1st dose

Overnight fasting

5 h Absorption

7 h food ad libitum

Overnight fasting

Day 2
9:00

2nd dose

4 h Absorption

10:00 LPS

Sacrifice

B
Chronic dosing of Curc or TC

14.5 month old

18.5 month old

APPsw Tg2576 mice, food ad libitum, 500 ppm dosing in chow

Sacrifice
Figure 3

A

kDa

130

42

iNOS

β-actin

p < 0.001

Curc

Route LPS

LPS +

gav

i.p.

i.m.

B

kDa

130

42

iNOS

β-actin

p < 0.001

TC

Route LPS

LPS +

gav

i.p.

i.m.

C

p < 0.0001

<table>
<thead>
<tr>
<th>Curc</th>
<th>TC</th>
</tr>
</thead>
</table>

D

p < 0.05

Curc, R²=0.993, p<0.0001
EC⁵₀ = 1.186 μM (log -5.926)

TC, R²=0.968, p<0.0001
EC⁵₀ = 0.701 μM (log -6.154)
Figure 7

(A) Con Diet
(B) Con Diet
(C) Curc Diet
(D) Curc Diet
(E) TC Diet
(F) TC Diet

(G) % control plaque size μm²
(H) % control plaque burden

(I) % control insol Aβ μg/mg
(J) % control sol Aβ fg/μg
Figure 8

(A) Tg- Control
(B) Gfap- Tg-
(C) Tg+ Control
(D) Gfap+ Tg+
(E) Tg+ Curc
(F) Gfap+ Tg+ Curc
(G) Tg+ TC
(H) Gfap+ Tg+ TC

(I) Graph showing the percentage of Tg+ Control (% area GFAP) across different diet conditions:
- **Tg-**
- **Tg+ Control**
- **Tg+ Curc**
- **Tg+ TC**

Bars indicate significance levels with **indicating a p-value < 0.01 for comparison of Tg+ Control to other conditions.
Figure 10

A. LPS induction of iNOS in 1′ neuron

B. Aβ oligomer-induced SH-SY5Y toxicity

C. Intraneuronal C99 toxicity (M65 cells)

D. Impact on aggregation: low [Aβ]

E. Impact on aggregation: high [Aβ]

F. Anti-oligomer (A11) rel OD

(Charts and graphs showing data with statistical comparisons and experimental conditions)