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INHALABLE CURCUMIN: OFFERING THE POTENTIAL FOR TRANSLATION TO IMAGING AND TREATMENT OF ALZHEIMER'S DISEASE

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Abstract

Curcumin is a promising compound that can be used as a theranostic agent to aid research in Alzheimer's disease. Beyond its ability to bind to amyloid plaques, the compound can also cross the blood brain barrier. Presently, curcumin can be applied only to animal models, as the formulation needed for iv injection renders it unfit for human use. Here, we describe a novel technique to aerosolize a curcumin derivative, FMeC1 and facilitate its safe delivery to the brain. Aside from the translational applicability of this approach, a study in the 5XFAD mouse model suggested that inhalation exposure to an aerosolized FMeC1 modestly improved the distribution of the compound in the brain. Additionally, immunohistochemistry data confirms that following aerosol delivery, FMeC1 binds amyloid plaques expressed in the hippocampal areas and cortex.

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Keywords

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INTRODUCTION

The pathology of Alzheimer's disease (AD) is characterized by the presence of extracellular deposits of misfolded and aggregated amyloid- β (A β) peptides. These form initially in the hippocampus and entorhinal cortex before being disseminated to other regions of the brain. Once propagated, these peptides contribute to the irreversible neuronal death that underlies clinically observed deficits in memory, logic and the ability to speak, all of which are characteristic of the disease [1]. Assuming that current amyloid-centric hypotheses of AD are correct relative to situating A β plaques at the core of AD pathogenesis, then preventing A β plaque formation or facilitating their demolition, particularly during the early disease process, represents a key therapeutic strategy. Toward this end, small organic molecules or antibodies that target A β plaques have been developed as therapeutic agents for the treatment of AD. However, due to the strenuous requirements that characterize efficacious AD therapeutics, including the ability to (i) cross the blood brain barrier (BBB), (ii) bind to A β plaques and (iii) inhibit A β plaque aggregation, the development of disease-modifying drugs for AD would certainly be counted among the most significant scientific discoveries with respect to world health. To gauge the potential impact of such a discovery, it must be borne in mind that AD represents 50–70% of all cases of senile dementia and impacts nearly 35 million people worldwide. To compound this crisis, as the baby-boom generation joins the geriatric clinical population, 20% of Americans, some 71 million individuals, will reach the typical age of AD onset by 2030 [2]. These projections illustrate clearly that the impetus to identify and characterize disease-modifying treatments for AD has never been greater. Unfortunately, despite the description of multiple treatment strategies capable of perturbing A β plaque formation, none have yet proven clinically efficacious. Particularly in the case of small molecule inhibitors of A β aggregation, drug distribution to the brain parenchyma represents a restricting factor that almost certainly contributes to the overall trend toward disappointing results in clinical trials [3]. In contrast to peripheral capillaries that have open endothelial junctions and exhibit constitutive pinocytosis which facilitate paracellular and transcellular routes of molecular transport from the blood to brain, the distribution of molecules from the blood to the brain's interstitial space is facilitated predominately via lipid-mediated free diffusion of small molecules [3]. We demonstrated previously that lipidization of drug molecules represents a promising approach to achieving trans-BBB delivery [4, 5]. However, modifying amyloid-binding compounds to enable BBB penetration remains a daunting task that to date, has yielded no clinically implemented drugs. At present, only a handful of diagnostic amyloid imaging probes are available for clinical study, including Pittsburgh compound B (PIB), Florbetapir F18 and Florbetaben. Therefore, as is the case among many other investigators motivated to identify a more practical approach to AD therapy, we focused recently on the naturally occurring turmeric extract curcumin as a potential theranostic agent for AD [6–8]. Notably, more than 100 clinical studies have credited curcumin with antioxidant, anti-inflammatory, anticancer, antiviral and antibacterial

properties, as well as the ability to bind and disrupt A β plaques. As a result, this compound is widely used by non-allopathic practitioners of medicine for a variety of diseases, and is particularly unique in its ability to bind A β plaques with high affinity [9–12]. Besides being used as a therapeutic agent, curcumin possesses an intrinsic fluorescence signal that can be used to image its association optically with A β plaques in the brain. For instance, Bacsikai et al. and others recently used two-photon microscopy to confirm the findings of other reports that curcumin can cross the BBB, bind to A β deposits and clear existing plaques in a transgenic mouse model of AD [13, 14]. Other groups have also successfully radiolabeled curcumin derivatives for A β imaging [15]. Similarly, we recently developed a perfluoro curcumin analog (FMeC1) for ^{19}F NMR imaging (Fig. 1) to facilitate in vivo visualization of the mechanism that enables curcumin to bind to A β [16]. As with many A β aggregation inhibitors, however, the clinical utility of curcumin therapy has been severely limited due to its poor aqueous solubility and relatively low penetration across the BBB [11, 17, 18]. In fact, curcumin's amphiphilic properties require the compound be dissolved in a combination of buffer solutions, which contain excessively high concentrations of detergent. As the resultant formula is exceptionally viscous, bolus administration by intravenous (iv) injection has proven lethal in testing subjects and is therefore unsuitable for human trials [19]. One way to circumvent the problem is by using a controlled pump to infuse the substance over an extended period; however, enormous uncertainty persists regarding how these methodological adaptations can be implemented in translational studies without major unforeseen complications. To alleviate the problem of trans-BBB delivery, we describe herein a novel approach for delivering curcumin to the brain via inhalation. In our approach, FMeC1 compound was prepared in the same suspension formulation used for iv injection; however, rather than bolus administration, the suspension was aerosolized using a center-flow atomizer, diluted with air and subsequently delivered by nose-only inhalation. Preliminary data demonstrated that drug delivery using our atomization approach alleviated toxicity concerns and efficiently deposited FMeC1 compound in the brain slightly better than iv injection. Notably, delivery of the FMeC1 compound reached concentrations in the brain detectable by ^{19}F NMR. Additionally, plaque-like punctate fluorescence attributable to complexes of the inhaled FMeC1 analog were colocalized to immuno-stained A β plaques in the cortex and hippocampal regions of the 5XFAD mouse brain under fluorescence microscopy.

MATERIALS AND METHODS

Animals

The C57BL/6 and 5XFAD mice (8–12 months old) were maintained at Vanderbilt University under standard conditions, in a 12-hour light/dark cycle and with free access to food and water. The 5XFAD mice overexpress both mutant human APP and PS1 express high APP levels correlating with high burden and accelerated accumulation of the A β as described by Vassar et al. [20]. A colony of 5XFAD transgenic mice obtained from Jackson Laboratories was maintained by crossing 5XFAD mice with a wild-type C57BL/6J strain. The mice were genotyped by a standard polymerase chain reaction using DNA isolated from tail tips with the following primers: PSEN1 forward, 5'-TCATGACTATCCTCCTGGTGG3' and reverse, 5'-CGTTATAGGTTTAAACACTTCCCC-3'. For APP, forward, 5'-

AGGACTGACCACTCGACCAG-3' and reverse, 5'-CGGGGGTCTAGTTCTGCAT-3'. We also genotyped mice for the presence of retinal degeneration Pde6b^{rd1} mutation using forward, 5'-AAGCTAGCTGCAGTAACGCCATTT-3' and reverse, 5'-ACCTGCATGTGAACCCAGTATTCTATC-3'. After polymerase chain reaction amplification, the DNA product of each reaction was analyzed by size fractionation through a 1% agarose gel; with Pde6b mutant = 560 bp, APP transgene = 377 bp and PSEN1 transgene = 608 bp. The 5XFAD mice were maintained as homozygous. Animal experiments were conducted per the guidelines established by Vanderbilt University's Institutional Animal Care and Use Committee.

Inhalation Exposure and Compound Atomization

The atomizer system is comprised of a PVDF fluorinated polymer cross-flow atomizer (Single Pass Atomizer, SPA), an L-shaped conveyer, three inhalation ports, inlets for pressurized gas and liquid and a control unit to regulate aerosol generator air (Fig. 2). In a typical experiment, an aqueous solution containing FMeC1 was pumped through a capillary the SPA. Because of the rapid flow of air across the capillary tip, the airflow shears FMeC1-containing solution into micron sized that is diluted with a stream of air delivered by an adjacent source of clean, dry air. Together, these processes generate the FMeC1-containing aerosol, the concentration of which and in some instances its aerodynamic size being controlled by a flow of dilution air through the atomizer at ambient temperature (23–25°C) [21]. Delivery of the generated aerosol to cohorts of experimental animals is facilitated via a delivery trumpet assembled within each inhalation chamber. Similarly, exhaled aerosol is conveyed to an exhaust outlet via escape channels just below the connector cone. In its current configuration, the stainless steel inhalation chamber is fitted with multiple ports and protected septum seals that enable the simultaneous respiratory exposure of three animals. The system can be expanded to accommodate up to five animals.

Prior to atomization of the test compound airflow was calibrated as a function of pressure to ensure that every experiment ran at a consistent airflow rate of 3 L/min and an operating pressure of 20 psi. The solution to be sprayed consisted of 15 mg of FMeC1 in 30 mL of 1:6 PBS:Tween 20 mixture that was delivered to the SPA atomization via syringe pump (Harvard Apparatus) at a rate of 50 mL/hour (833 µL/min).

The atomization process commenced when compressed air was introduced into the aerosol generator inlet via a pressure regulator. The pressurized air interacted with fluid within the SPA. The resulting aerosol particles of respirable size were then emitted into the exposure apparatus. To reduce aerosol size by evaporation, the aerosol was diluted with additional clean dry air. The resulting mixture was then directed to three nose ports within the reduced volume inhalation chamber via a connector that directs the aerosol flow to each chamber inlet. During animal trials, non-anesthetized mice were placed into a restraining tube which was inserted into the exposure chamber. The animal's snout was secured within the restraint tube by a stainless steel nose cone within the tube to focus delivery of the aerosol to the breathing zone of the animal. The tapered nose cone inside the restraint tube enables the animal to inhale the presented aerosol. As noted above, exhaled air is redirected to a second channel that leads to the system's exhaust. Rebreathing of exhaled air does not occur.

Prior to commencing the inhalation exposure of animals, an analysis of each port's aerosol flow was conducted to ensure the amount of aerosol delivered to each inhalation chamber was identical. In this work, 50-mL condensing tubes were inserted into each outlet port and sealed with parafilm. This configuration ensured that all aerosolized FMeC1 delivered through the outlet port was deposited on the surface of the condensing tube. To assess atomization efficiency, 1 mL of the 0.9 mM FMeC1 solution was aerosolized by the atomizer system. The condensing tubes for each port were then repeatedly rinsed with 1 mL of PBS and assessed for FMeC1 concentration by employing absorbance spectroscopy in combination with Beer's law (Figure S1). Over the course of three atomization trials, no significant difference in aerosol delivery efficiency was detected between outlet ports (mean variance = 0.006 micrograms per 500 micrograms aerosolized), confirming that each inhalation chamber, and thus each animal, would receive identical amounts of the aerosolized product. Additionally, we determined from this data that 7.8% of the administered dose was successfully aerosolized and conveyed to the inhalation chambers. This quantitative data enables us to calculate and normalize equivalent doses to compare probe distribution between respiratory exposed and iv-injected animal groups (Figure S2).

Biodistribution analysis

Intravenous administration of the FMeC1 solution was accomplished through the caudal tail vein. Aerosol administration of FMeC1 was performed as described above. A 5 mg/kg dose of FMeC1 was administered to each animal cohort. One hour after administration of FMeC1 to the 5XFAD and wild-type mice cohorts, all major organs including brain, lungs, heart, liver and kidneys were harvested and processed for FMeC1 extraction using methods previously described [22]. Briefly, each tissue was weighed before homogenization in 1 mL of PBS, after which 100 μ L of acetonitrile was added. The solution was vortexed for 5 minutes, heated to 50°C for 10 minutes, then vortexed again before being centrifuged at 12,000 g for 1 hour. The supernatant was collected and transferred to a conical tube before removing all of the PBS/acetonitrile solvent via lyophilization. The residue was reconstituted in a 1:1 solution of acetonitrile and PBS to a total volume of 30 μ L for concentration analysis using a UV-Vis spectrophotometer (Agilent). Fortunately, FMeC1 is a fluorophore that has a reliable molar extinction coefficient of 68,000 $M^{-1}cm^{-1}$. This enabled us to quantify the compound's bioavailability using Beer's law in conjunction with a preestablished calibration curve of concentration versus absorbance intensity.

Image analysis of ex vivo fluorescence brain imaging

Monochromatic images of slide-mounted coronal brain slices (n=40 per cohort) were manually segmented to create individual ROIs that encompassed the hippocampus and isocortex using ImageJ software. The Allen mouse brain atlas was used as a reference for the segmentation of the ROIs, and manual tracing was performed by a blinded collaborator. Mean signal intensity at $\lambda = 557$ nm was calculated for each ROI across three cohorts of animals; (i) iv-injected 5XFAD mice, (ii) respiratory exposed 5XFAD mice and (iii) respiratory exposed wild-type mice (n=4 per cohort).

Brain perfusion

Following exposure or treatment, deeply anesthetized animals were laid onto ice after which the thoracic cavity was accessed via a sharp transverse incision into the abdomen. This was followed by a series of longitudinal cuts with a scalpel to open the thoracic cavity, which then was stabilized with a retractor. Perfusion was performed with a 25-gauge syringe containing ice-cold PBS (30 mL, pH 7.4) inserted through the left ventricle and injected slowly into the ascending aorta. Upon initiation of perfusion, the right atrium was snipped to facilitate drainage of the systemic venous return. Immediately following PBS perfusion, 30 mL of paraformaldehyde (PFA) (pH 7.4) was perfused. With perfusion completed, the animals were decapitated and their brains quickly harvested and fixed in 4% PFA overnight at 4°C, which was followed by 10% sucrose precipitation overnight at 4°C. The fixed brains were then embedded in Cryo-OCT compound before cryosectioning.

Ex vivo imaging of the brain slides

Excised brain sections were imaged using a fluorescence tomographic imaging system (Visen FMT, PerkinElmer, MA, USA). Using a laser diode, the system scanned the surface of the FMeC1-containing brain tissues in a user-adjustable grid pattern and generated a two-dimensional map of FMeC1 deposition. Laser power and exposure time were kept constant between imaging sessions and were automatically optimized by the system to provide a maximum signal-to-noise ratio while avoiding pixel saturation. Configuration of the system to image FMeC1 fluorescence was accomplished via manual insertion of excitation (450–490 nm) and emission (560–600 nm) optical filters. Using this imaging system coupled with Maestro 3.1 software (PerkinElmer, MA, USA), monochromatic images of three slides were acquired simultaneously at FMeC1 emission $\lambda_{\max}$ of 557 nm, at an exposure time of 1 msec.

Immunohistochemistry

Midbrain coronal sections (12 μ m) were mounted onto charged glass slides, placed in 4% PFA for 10 minutes, transferred to tris buffer for 5 minutes and then allowed to dry before being subjected to a citrate buffer antigen retrieval protocol. Briefly, slide mounted sections were incubated for 40 minutes in a 100°C bath of sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and allowed to cool for 20 minutes before PBS washing and blocking. Treated sections were then incubated overnight at room temperature with primary antibodies: rabbit anti-A β (1:1000 dilution, Thermo, Pittsburgh, PA, USA). Following PBS washes, the sections were subsequently incubated with secondary biotinylated goat anti-rabbit (1:2000, Thermo, Pittsburgh, PA, USA) for 2 hours at room temperature. The ABC Elite Kit (Vector Laboratories) was used with DAB as a chromogen to visualize the reaction product. The sections were then counterstained with hematoxylin, dehydrated in a series of alcohols, cleared in xylene and then cover-slipped.

Fluorescence microscopy

To localize the distribution of FMeC1 in the brains of treated mice and corroborate its intrinsic fluorescence signal with that of the fluorescently labeled A β antibodies, dual channel fluorescence microscopy was employed. For all fluorescence imaging, a GFP-BP filter (excitation filter 450–490 nm; dichroic mirror 495 nm; emission filter 510–560 nm)

was used to image FMeC1 fluorescence. A Texas Red filter (excitation filter 540–580 nm; dichroic mirror 595 nm; emission filter 600–660 nm) was utilized to visualize the anti-A β antibodies. All images were acquired using a CCD camera and data were analyzed using Nuance 2.6 software.

Colocalization analysis of A β -antibody and FMeC1 dual-channel fluorescence microscopy

To demonstrate that the A β -specificity of FMeC1 is retained when administered via atomization, coronal sections (n=30) isolated from respiratory exposed 5XFAD mice (n=5) were co-stained with anti-A β antibodies. Fluorescence images of the co-stained sections were acquired in hippocampal and cortical brain regions in the Cy5 channel, thus reflecting A β plaque expression as reported via anti-A β antibodies. Without changing the position of the sample, a second set of images that captured FMeC1 fluorescence were acquired in the GFP channel. To qualitatively demonstrate the colocalization between anti-A β antibody fluorescence and that of FMeC1, line plots of mean pixel intensity as a function of pixel coordinates were generated using ImageJ software for both channels and overlaid. To demonstrate colocalization quantitatively between the FMeC1 and antibody signals, the red and green channels for Cy5 and GFP images were isolated, respectively. The pixels in each monochromatic image set were then segmented into two groups using the k-means clustering algorithm to generate binary images with pixel values that represented signal versus no signal. Quantitative assessment of the degree of colocalization between each processed image set (n=20) was then completed using the JACoP plugin for ImageJ following well-established protocols [23].

Detection and quantification of FMeC1 in the brain by NMR spectroscopy

A 5 mg/kg dose of FMeC1 was administered to 5XFAD mice (n=4) via atomization. One hour after administration, the brain of each animal was removed and processed for FMeC1 extraction using methods previously described [22]. Briefly, each tissue was weighed before homogenization in 2 mL of PBS, after which 200 μ L of acetonitrile was added to the tissue lysate. The solution was then vortexed for 5 minutes, heated to 50°C for 10 minutes, and vortexed again before being centrifuged at 12,000 g for 1 hour. The supernatant was collected and all PBS/acetonitrile solvent removed via lyophilization. The resulting FMeC1 residue was reconstituted in deuterated chloroform to a total volume of 200 μ L for ^{19}F NMR analysis using a 300 MHz Bruker NMR spectrometer.

The concentration of FMeC1 was calculated using ^{19}F NMR spectroscopy. To enhance the detection threshold, 200 μ L of the extracted FMeC1 solution was placed in a 3-mm NMR tube. The ^{19}F NMR experiment for this sample acquired over a period of 15 hours (31,000 scans), revealed a single peak at –75.96 ppm. To provide accurate and reproducible quantitative results, 0.1 M trifluoroacetic acid (TFA) (–76.55 ppm) served as an external reference. Since sample material was not a consideration, 600 μ L of the TFA sample was placed in a 5-mm NMR tube and analyzed using the same experimental NMR parameters as the FMeC1 sample; however, it required only a 20-minute scan time. The calculation of FMeC1 concentration involved first adding the processed NMR spectra for both samples. The NMR spectrum for the TFA sample was offset to avoid spectral overlap. The spectra were then integrated by fitting the NMR peaks using either Gaussian and/or Lorentzian line

shapes. Once the values were calculated using line fitting techniques, the integral ratios were scaled to compensate for the differences in the number of scans and sample volume.

Statistical analysis

All values were measured by independent experiments, conducted in triplicate, and represented as the mean $\pm$ standard deviation. Statistical analysis was performed using a 2-tailed Student's t-test conducted with StatView for Windows Ver. 4.5 (Abacus Software) with a $p < 0.05$, which is considered to represent a statistically significant difference.

RESULTS

FMeC1 aerosol crosses the blood brain barrier and binds to A β

Two cohorts of awake and alert 8-month-old mice, one comprised of C57BL/6 wild-type controls and a second of 5XFAD mice ($n=5$, each), were exposed by inhalation to a solution containing FMeC1 at a dose equivalent to a 5 mg/kg injection. One hour after respiratory exposure, transcardiac perfusion was performed prior to removal of the animal's brain, which was then processed for histological analysis. FMeC1 accumulation in the brain was assessed via wide-field fluorescence microscopy of FMeC1's intrinsic fluorescence at $\lambda_{\text{max}}=557$ nm. Critically, one hour following exposure via the inhalational route, no fluorescence signals, either from FMeC1 or anti-A β antibodies, were detected in the brains of wild-type mice (Fig. 3A, E). In contrast, we observed remarkable punctate fluorescence emitted by FMeC1 in the frontotemporal cortex and hippocampus, brain regions known to harbor robust A β plaque deposition in the 5XFAD mouse model [20] (Fig. 3B, F). Importantly, the signal detected in the 5XFAD mouse brain is distinct from background as autofluorescence measured in untreated 5XFAD cohorts is orders of magnitude below the fluorescence observed in the respiratory exposed 5XFAD brain sections. Impressively, fine details of the morphology of individual A β plaques are recognizable in higher magnification images (Fig. 3C, G). Having demonstrated that respiratory exposed FMeC1 penetrates the BBB efficiently and is retained in the brain of A β -burdened mice, we then worked to confirm the binding specificity of FMeC1 for A β plaques. In this effort, immunohistochemical staining of A β plaques using fluorescent anti-A β antibodies was employed to colocalize the punctate fluorescence of FMeC1 with A β plaques. Here, we employed dual-fluorescence microscopy to image both FMeC1 deposition and A β plaques in the same section, relying on the GFP channel to image FMeC1 deposition and the Cy5 channel to image monoclonal anti-A β antibodies labeled with a near-infrared dye that provided a distinct absorption/emission profile. Of note, a high degree of colocalization was observed between the clusters of FMeC1 fluorescence and the signal emitted from the distinctly labeled anti-A β antibodies in both the hippocampus (Pearson's coefficient = 0.9, $n=25$) and frontotemporal cortex (Pearson's coefficient = 0.9, $n=25$) (Fig. 3D, H). In addition to this quantitative analysis of FMeC1's A β -specificity, a representative line plot through image sets acquired in the GFP and Cy5 channels, for FMeC1 and A β antibodies, respectively, qualitatively, demonstrates quantitatively the high degree of corroboration between FMeC1 deposits and the location of A β plaques (Fig. 3I). Overall, this data confirms the ability of atomization technology to deliver A β -targeted diagnostics or therapeutics, such as FMeC1, to the brain. Furthermore, this work confirms that FMeC1

delivered to the brain via the respiratory route penetrates midbrain structures, such as the hippocampus and retains its binding specificity for A β plaques.

Bioavailability of FMeC1 in the brain by respiratory delivery atomization versus iv injection

To compare the biodistribution of FMeC1 in the brain by inhalation versus iv injection and assess whether FMeC1 deposition significantly increased in regions that harbor A β plaques, high-throughput ex vivo brain imaging was employed. Briefly, respiratory exposed 5XFAD and wild-type, and iv injected 5XFAD cohorts (n=5 per group) were similarly dosed with FMeC1 (5 mg/kg) via their respective administration method. One hour after treatment, all mice were subjected to cardiac perfusion and their major organs promptly removed and processed for sectioning using the protocols we reported in the past [5, 24]. In this work, we employed the Visen 3D imaging system (PerkinElmer) to perform high-throughput imaging of multiple slide-mounted brain slices. Using this experimental configuration, the global FMeC1 distribution in each cohort was assessed via fluorescence imaging (λ =557 nm) of brain slices containing midbrain structures of interest such as the hippocampus. Then, employing NIH ImageJ software, isocortical and hippocampal regions of interest were manually selected by a blinded collaborator and quantitatively evaluated for mean fluorescence intensity. As illustrated in Fig. 4A, a higher FMeC1-derived fluorescence signal is readily appreciable in the isocortex of 5XFAD mice as compared wild type mice following the administration of FMeC1 either by iv or atomization (p =0.02 & p <0.001, respectively). Similarly, the A β -plaque dependency of FMeC1 retention holds in the hippocampus, where both iv and respiratory exposed 5XFAD mice demonstrate greater FMeC1 fluorescence signals as compared to respiratory exposed wild-type controls (p =0.002, p =0.002, respectively) (Fig. 4B). Interestingly, the brains of both iv injected and respiratory exposed 5XFAD mice exhibit a similar topographic retention of FMeC1 in A β plaque-laden regions, which alludes strongly to an amyloid dependency with respect to FMeC1 retention in the brain. In contrast, no differences in the FMeC1 signal were detected in non-amyloid-burdened brain regions such as the hypothalamus. As perhaps the best illustration of the amyloid dependency of FMeC1 retention, FMeC1 fluorescence measured from the hippocampus or cortex of respiratory exposed 5XFAD mice was significantly greater than that of the thalamus (n=60, p <0.001) (Fig. 4B). Notably, the result of interregional analysis is markedly different within the wild-type cohort, with no statistically significant differences were noted between any of the analyzed regions. Again, taken as a whole, these results confirm the specificity of respiratory exposed FMeC1 for A β plaque as well as the ability of FMeC1 to report A β plaque load in the brain with regional specificity. Lastly, when comparing the FMeC1 signal measured in the isocortex of iv-injected and respiratory exposed animals, it is interesting to note that a modest, yet statistically significant improvement in FMeC1 delivery is achieved by implementing inhalation technology (p <0.05).

Biodistribution studies reveal that inhalable FMeC1 was distributed in the lungs and other major organs

To corroborate our ex vivo imaging data, which demonstrated that atomization of an FMeC1-containing aerosol can deliver diagnostic or therapeutic compounds to the brain with an efficacy similar to that of iv injection but without associated toxicity, we

quantitatively compared the biodistribution of FMeC1 in all major organs following the administration of a 5 mg/kg dose by either administration method. As in previous atomization administration experiments, the awake and alert mice were kept in the inhalation chamber, which was attached to the atomizer via the nosecone. By comparison, iv administration of the FMeC1 solution was accomplished via the caudal vein. One hour after iv injection or the completion of respiratory, animals were sacrificed and their major organs surgically isolated and processed for FMeC1 extraction using methods previously described [22]. Briefly, liquid-liquid extraction was employed to isolate FMeC1 into an acetonitrile organic layer from individual tissues homogenized in 1.0 mL of PBS. Following additional processing, the acetonitrile-PBS solvent was removed from the FMeC1 via lyophilization. This resulted in an FMeC1 residue that could be reconstituted in 30 μ L of 1:1 acetonitrile:PBS for concentration analysis using a spectrophotometer. While this protocol facilitates the isolation of FMeC1 from each tissue, it also allows the total tissue FMeC1 to be concentrated into a minimal volume, which ensures the sample absorption falls into the linear range of the Beer-Lambert plot for FMeC1. To avoid overestimating the mass of FMeC1 deposited in each organ, secondary to absorption by proteins intrinsic to the tissue, each measurement was normalized to similarly processed control samples isolated from untreated 5XFAD mice. Using these techniques, in a comparison of two age-matched 5XFAD mice cohorts (8 months, n=3, each), quantitative analysis of the mass of FMeC1 in the brains of mice injected with a 5 mg/kg bolus (mean = 630 ng FMeC1/g tissue) did not differ significantly from the amount retained in the brains of the respiratory exposed cohort (mean= 567 ng FMeC1/g tissue) (p=0.6) (Fig. 5A). Interestingly, comparison of the FMeC1 mass retained in the brains of the respiratory exposed 5XFAD cohort with that of a similarly treated cohort of age-matched wild-type mice revealed a greater deposition and retention of FMeC1 in the brain of the 5XFAD cohort (2 ng versus 567 ng FMeC1/brain). Again, this result corroborates our ex vivo imaging studies and suggests an amyloid-dependent retention of FMeC1 in the brains of A β -plaque-expressing 5XFAD animals (p=0.005). Cumulatively, this analysis confirms that respiratory exposed FMeC1 is delivered and preferentially retained in the brains of 5XFAD mice in an A β -dependent manner, and with an efficacy similar to that achieved via iv injection.

In addition to confirming that respiratory efficaciously delivers FMeC1 to the brain, our tissue-specific analysis of FMeC1 deposition provided additional insight into the biodistribution pathways employed during atomization. As expected, FMeC1 deposition in the lungs of respiratory exposed cohorts, both 5XFAD (p=0.009) and wild-type (p=0.003), was significantly greater than that of iv-injected animals (Fig. 5A). Cumulatively, the data suggest that two major pathways are utilized for the delivery of FMeC1 during atomization. First, aerosolized FMeC1 is likely directly transported to the brain following deposition onto the nasal mucosa and is subsequently transported in a retrograde manner along trigeminal and olfactory neurons [25–28]. Secondly, a fraction of respiratory exposed FMeC1 likely deposits in the lungs and nasal mucosa, and is subsequently conveyed to the blood stream, where it avoids the deleterious effects of first-pass metabolism associated with enteric administration methods [29, 30]. Combined, these biodistribution pathways result in a cumulative deposition of FMeC1 in the brain via atomization, which is at least as efficacious as that of iv injection. As approximately 0.25% of the original 5 mg/kg dose reaches the

brain, a figure similar to that reported in previous studies [31]. However, the major advantage of drug administration via atomization is the robust reduction in toxicity, since the system shock associated with bolus injection is avoided. In fact, during the course of this work, the iv injection of high doses of curcumin and related analogs produced mortal toxicity in approximately 50% of the mice. Moreover, that systemic toxicity is well reflected in the significantly elevated FMeC1 load measured in the liver ($p=0.01$) and kidneys ($p=0.004$) of iv-dosed mice as compared to the respiratory exposed cohorts (Fig. 5A). By comparison, no toxicity was observed in any mouse dosed using the atomization protocol described in this study; this observation is in line with the previous study reported by Minko et al. [32].

To corroborate our biodistribution studies and demonstrate the potential of capitalizing on FMeC1's perfluoro labeling for the detection of amyloid plaques, the concentration of FMeC1 in the brain was calculated using ^{19}F NMR spectroscopy. In that effort, 5XFAD mice ($n=4$) were respiratory exposed with a 5mg/kg dose of FMeC1 and perfused prior to removal of the brain tissues for homogenization. The FMeC1 was then extracted from the homogenized lysate samples and reconstituted in deuterated chloroform to facilitate detection by ^{19}F NMR. The acquisition of a quantitative NMR experiment typically does not require any special pulse sequences or new parameter settings. Nevertheless, attention must be paid to standard parameter settings such as the excitation pulse length and recycle delay. The ^{19}F NMR experiment for this sample was acquired for 15 hours (31,000 scans) and revealed a single peak at -68.33 ppm (Fig. 5B).

DISCUSSION

Drug delivery to the brain for imaging or treatment of AD requires a perfect balance of the chemical bioisosteres that enable the compound to be sufficiently hydrophilic enough to traverse blood circulation yet maintain the lipophilicity that underlies its ability to cross the BBB. Curcumin is one such compound among a mere handful of known $\text{A}\beta$ -binding agents. However, its strength as an amphiphilic molecule is also its shortcoming. As noted in the literature, polyphenols, such as curcumin present a unique challenge with respect to their formulation for in vivo application. With a log p value of 2.9, curcumin is insoluble in any physiological aqueous condition suitable for in vivo application [33]. Therefore, it must be dissolved in saline with a high proportion of methanol [33] or Tween [16]. In congruence with significant mortality associated with iv injection of curcumin noted our study, bolus administration of a viscous solution like that used to administer curcumin can be fatal. Highlighting the inherent difficulties and most profound limitations associated with the in vivo use of amphiphilic molecules, all past and ongoing clinical trials that seek to evaluate the therapeutic efficacy of curcumin in AD patients have been forced to employ oral dosing regimens despite the compound's abysmal bioavailability via that method of administration. In addition to its inherent weak nature, this dependence on oral dosing is cited frequently as the principal reason human studies have failed to replicate the robust improvements in memory observed in preclinical animals studies that employ curcumin to treat AD [34, 35]. In support of this hypothesis, investigators have reported that even following oral doses of 4 grams, the highest curcumin dose utilized in a completed AD-specific human study to date, curcumin does not reach detectable levels in the blood [36]. Furthermore, despite more than

40 reports that have attempted to increase the bioavailability of curcumin through a variety of methods such as nanotechnology, encapsulation in liposomes/cyclodextrin/polylactic-co-glycolic acid or administration with metabolic inhibitors, traditional methods employed to overcome curcumin's poor biodistribution have proven insufficient [37–41]. Thus, the development of administration techniques that better facilitate the delivery of amphiphilic molecules to the brain is of critical importance, as reliance on non-optimized dosing regimens may lead to the premature dismissal of potentially clinically impactful molecules such as curcumin. As emphasized earlier, the clinical utility of curcumin extends beyond AD, thus given the large number of studies that characterize the disease-modifying effects of curcumin's wide spectrum of biological and pharmacological activities, it is indeed in our interest to develop innovative, practical and translatable administration methodologies that can be used to deliver curcumin to the brain [42]. Toward that end, we have demonstrated the utility of a novel atomization-based drug administration technology that rivals iv injection with respect to delivery of the compound to the brain.

The advantages of curcumin delivery by inhalation include: (i) effective delivery of the compound to pathologically relevant regions of the brain such as the hippocampus; (ii) avoidance of the glucuronidation and biotransformation attributable to first-pass liver metabolism by employing retrograde transport along cranial nerves [27, 28, 36, 43]; (iii) circumvention of the low bioavailability of curcumin associated with its poor absorption in the gut [44] and (iv) negligible toxicity. Notably, none of the animals exhibited any signs of toxicity or discomfort during the atomization procedure, a finding that is consistent with studies of intranasal drug administration [45]. In contrast, severe respiratory distress and tachycardia were apparent in all animals following bolus iv injection. Further, while both delivery techniques resulted in distribution and A β -dependent retention of curcumin in the brains of 5XFAD mice, atomization facilitated improved delivery in some brain regions compared to iv injection. Nevertheless, there remains a few important caveats need to be confirmed such as therapeutic efficacy of FMeC1 versus curcumin. Assuming they have similar inhibitory effect, does inhibition of plaques aggregation will lead to cognitive improvement in AD. Addressing those issues necessitate the confirmation of the usefulness of FMeC1 compound would enhance its role for theranostic of AD. Currently, work is in preparation in our laboratory to test the cognitive function of FMeC1-treated animals. Given FMeC1 is a perfluoro compound, another parallel approach is pursued, that is to detect the distribution of the compound non-invasively in targeted region such as hippocampus and cortex of 5XFAD mice using ¹⁹F-NMR imaging.

In conclusion, the work described herein provides sufficient evidence to support the conclusion that generating aerosolized curcumin for inhalation-assisted delivery to the brain is a promising alternative to the field-dominant iv injection administration method. Recently, there is a large body of literature show optimistic results of curcumin in reversing the pathogenesis of AD in small animal models [46–49], we hope our work will eventually help to facilitate the translation of those knowledge into human applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Curcumin (A) and its perfluoro analogs, FMeC1 (B).

Figure 2. Atomizer design and aerosol delivery system

(A) Atomizer schematic. (B) Magnified view of inhalation chamber and nosecone assembly. FMeC1-containing aerosol (red arrows) is conveyed through the inlet while air exhaled by the animal (blue arrows) is directed toward the system exhaust port via outlet channels.

Figure 3. Internalization and amyloid-dependent retention of aerosolized FMeC1 in the brain
 Cohorts of wild-type and 5XFAD mice (n=5, each) were respiratory exposed with a 5 mg/kg dose of FMeC1 and allowed to recover for one hour prior to cardiac perfusion and removal of the brain tissue. Note the absence of a GFP signal associated with deposits of FMeC1 associated with A β plaques in both the hippocampus (A) and isocortex (E) of respiratory exposed wild-type animals. In contrast, the hippocampus (B) and isocortex (F) of respiratory exposed 5XFAD mice show a robust deposition of FMeC1. At 20 $\times$ magnification, the clusters of FMeC1 fluorescence (GFP channel) in the hippocampus (C) of respiratory exposed 5XFAD mice colocalizes profoundly (mean Pearson's coefficient = 0.9, n=25) with anti-A β antibody staining (Cy5 channel) within the same coronal section (D). Similarly impressive colocalization of FMeC1 (G) and anti-A β antibody (H) generated fluorescence was observed in the isocortex (mean Pearson's coefficient = 0.9, n=25). (I) Line plot of pixel intensity as a function of image coordinates along the white line overlaid on subfigures (G) and (H). Peaks in signal intensity in the Cy5 channel (anti-A β antibodies) reflect A β plaque deposits and are highlighted in blue. For all A β plaque deposits, FMeC1 fluorescence intensity is increased in magnitude almost identically to fluorescence changes in the anti-A β antibody channel. Overall, these data confirm that respiratory exposed FMeC1 is delivered to the brain across the BBB, associates with A β plaques and is retained in the brain in an A β -dependent manner.

Figure 4. Ex vivo imaging of amyloid-dependent FMeC1 retention

(A) Representative monochromatic ($\lambda=557$ nm) images of respiratory exposed wild-type (left), respiratory exposed 5XFAD (center), and iv-injected 5XFAD (right) mice dosed with 5 mg/kg of FMeC1. Note the robust FMeC1-derived fluorescence observed in cortical and hippocampal regions of the respiratory exposed and iv injected 5XFAD cohorts. In comparison, no fluorescence above background was noted in the respiratory exposed wild-type cohort. This suggests that in the absence of A β plaques FMeC1 is washed out of the brain within 1 hour. (B) Regional quantification of FMeC1 fluorescence in the brains of three described cohorts (n=60, 5 animals per group). Measured fluorescence intensity emanating from the isocortex of respiratory exposed and iv-injected 5XFAD mice was significantly greater than that of respiratory exposed wild-type mice ($p=0.00001$ and $p=0.02$, respectively). No significant difference in fluorescence was observed between iv and respiratory exposed 5XFAD cohorts. For the hippocampus area, FMeC1 fluorescence increased significantly in respiratory exposed and iv-injected 5XFAD mice compared to respiratory exposed wild-type animals ($p=0.002$ and $p=0.002$, respectively). As in the isocortex, administration method did not produce statistically significant differences in hippocampal FMeC1 retention between 5XFAD cohorts. For the thalamus region, no significant difference in FMeC1 fluorescence was measured between cohorts in this brain region. However, among the iv and respiratory exposed cohorts, FMeC1 emission was significantly higher in the A β -burdened regions of the brain, such as the isocortex and hippocampus, compared to the amyloid plaque-free hypothalamus ($p<0.001$).

Figure 5. Biodistribution of FMeC1 by iv and atomization

(A) Equivalent doses of FMeC1 (5 mg/kg) were administered by iv injection or atomization. One hour after dosing, FMeC1 was isolated from each homogenized tissue using liquid-liquid extraction. Total FMeC1 mass was calculated using standard spectrophotometric methods. (A) FMeC1 mass retained in the brain. Respiratory exposed and iv-dosed 5XFAD mice retained significantly greater amounts of FMeC1 compared to untreated 5XFAD controls ($p=0.005$, $p=0.01$, respectively). Correspondingly, respiratory exposed 5XFAD mice retained greater FMeC1 in the brain compared to similarly treated wild-type mice ($p=0.005$). These results confirm the A β plaque dependency of FMeC1 retention. (B) FMeC1 mass retained in the liver. The livers of respiratory 5XFAD and wild-type cohorts exhibited no difference in FMeC1 mass compared to untreated controls. The iv-injected mice demonstrated elevated levels of FMeC1 in the liver compared to untreated animals ($p=0.01$). (C) FMeC1 mass retained in the kidney. No significant difference in FMeC1 retention was measured in the kidneys of respiratory exposed and untreated mice. The livers of iv- injected mice showed enhanced FMeC1 retention as compared to untreated cohorts ($p=0.04$). (D). FMeC1 mass retained in the lung. Respiratory exposed 5XFAD and wild-type cohorts retained greater masses of FMeC1 compared to untreated controls ($p=0.009$, $p=0.03$, respectively). (B) Fluorine NMR spectroscopy was used to detect FMeC1 compound extracted from the brains of respiratory route exposed 5XFAD mice.