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Inhibition of amyloid fibril formation and cytotoxicity by a chemical analog of Curcumin as a stable inhibitor

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ABSTRACT

Clinical application of curcumin for Alzheimer's disease treatment is severely limited with regard to its poor bioavailability, high rate of metabolism, and instability under neutral condition. In the current study, we designed three compounds in which the diketone moiety of curcumin was replaced by cyclohexanone. In these compounds, the linker length of the molecules was optimal; and substitution of dioxolane for hydroxyl groups on compound 3 should prevent metabolic inactivation. The inhibitory effect of the compounds was investigated against hen egg white lysozyme (HEWL) fibrillation using AFM (atomic force microscope), ThT (thioflavin T) and MTT assay. We found that all three compounds were able to inhibit HEWL aggregation in a dose-dependent manner and inhibit the cytotoxic activity of aggregated HEWL. Docking results demonstrated that the compounds could bind into lysozyme and occupy the whole active site groove. In conclusion, we present chemical analogs of curcumin with various modifications in the spacer and the phenolic rings as improved inhibitors of amyloid aggregation.

1. Introduction

Progressive neurodegenerative disorders (e.g. Alzheimer's and Parkinson's disease) are responsible for significant, increasing incidence of morbidity and mortality in the world [1]. Deposition of amyloid-like plaques is an early observed histological modification in the brain of the patients [2]. These plaques are related to the symptoms of cognitive impairment and suggested to have their role in the events resulting into the clinical presentation of dementia [3,4]. Aβ oligomers have been demonstrated to induce cognitive dysfunction and synaptic impairment during AD progression [5]. Insoluble and large fibrillar aggregates of amyloid polypeptides were previously supposed to be cytotoxic [6,7] but more recent studies suggest the soluble oligomeric forms of amyloid proteins to have higher cytotoxicity [8–10]. With regard to the role played by the progressive accumulation of Aβ aggregates in the development of neurodegenerative pathology, proposed therapeutic approaches toward AD have focused on decreasing the concentration of cerebral Aβ. On the other hand, amyloid formation is not an exclusive property of the disease-related proteins. In fact formation of *in vitro* fibrillar aggregates has been demonstrated for several other proteins that have no link to pathologies [11]. It has recently been found that a protein has no direct link to AD or other human diseases can induce memory impairment, resulting in an neurological effect similar to that caused by Aβ oligomers [12]. These findings suggest the existence of a common molecular mechanism in amyloid formation, including amyloid diseases [13]. As a consequence, studies on the inhibition or reversal of amyloid formation in disease – unrelated proteins could provide general information about the aggregation processes and finally result in finding potential preventive means related to amyloid diseases [14,15]. Several *in vitro* studies have reported amyloid aggregation of hen egg white lysozyme (HEWL) and wild- type human one under acidic pH and high temperature [16,17]. Lysozyme is considered a useful model in such studies, with regard to the fact that its structure and folding mechanisms are well characterized. Classical suggested approaches to treat amyloidogenic diseases target the amyloidogenic formation of proteins (reduction), or the clearance rate of

Abbreviations: AD, Alzheimer's disease; HEWL, hen egg white lysozyme; ThT, thioflavin T; CR, Congo red; AFM, atomic force microscope; MTT, 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetraazolium bromide; ANS, 8-anilinonaphthalene-sulfonate; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's Medium.

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misfolded/aggregated proteins (increase); accordingly, the stability of proteins native state could be increased, or direct inhibition of the self-assembly process may be aimed [18]. Multiple studies have reported that small organic molecules containing single or multiple (hetero) aromatic residues, possess *in vitro* anti aggregation activity [19,20]. This inhibitory effect may be exerted through several mechanism: stopping formation of mature fibrils from monomers [19], preventing both oligomers and fibril formation [21], affecting only oligomers without effect on mature fibrils formation, or block mature fibrils while allowing oligomer formation [22]. However, the practical use of these inhibitors is limited due to potential toxicity or possible inability to cross the blood–brain barrier. Clinical studies have demonstrated the chemotherapeutic activity of curcumin in AD and its lack of toxicity even at very high doses (500–8000 mg/day for 3 months) [23]. However, curcumin has not yet been approved as a therapeutic agent due to poor bioavailability and high rate of metabolism as well as instability under neutral-basic condition. Considering these facts, modifications in the core structure of curcumin may result in the generation of improved AD therapeutics. It has been suggested that the chemical scaffold of curcumin, with two aromatic groups separated by a planar backbone, is needed to fulfill its inhibitory role [2,24]. On the other hand, the hydroxyl groups of curcumin are modified in the kidneys, liver, and mucosal intestine, leading into production of curcumin glucuronide and curcumin sulfate, resulting into low bioactivity [25–27]. Moreover, both *in vitro* and *in vivo* experiments have shown that the enolic moiety in curcumin is responsible for its instability [28,29].

The present study aimed at presenting new curcumin derivatives which would possess the anti-amyloidogenic potential of the natural compound, and concurrently, be improved with regard to stability and bioavailibity. Designed compounds (Table 1) have the following characteristics: first, all three have curcumin scaffold with two aromatic ends and a rigid linker. Second, we found that all three are more or less active in amyloid disaggregation processes. Third, investigators have reported that the optimal length of curcumin derivatives linker lies within a 6–19 Å distance [24] and linkers of the selected compounds fall in that range (8.84 Å).

2. Materials and methods

2.1. Materials

HEWL (EC 3.2.1.17), Curcumin, Congo red, Thioflavin T (ThT), 2, 6 Bis (3, 4-methylenedioxy benzylidene) 1-cyclohexanone, 2, 6-divanillylidene cyclohexanone, 2, 6 Bis (3,4-dimethoxybenzylidene)-1-cyclohexanone were purchased from Sigma (St Louis, MO, USA). 8-anilinonaphthalene-sulfonate (ANS), all salts and organic solvents were obtained from Merck (Darmstadt, Germany). Protein concentration was determined spectrophotometrically at 280 nm, using an extinction coefficient (ε_{280}) of 2.65 L g^{-1} cm^{-1} [30].

2.2. Preparation of compounds solution

All the compounds were dissolved in DMSO to a concentration of 30 mM and then diluted with 50 mM glycine buffer, pH 2.5 to a specific final concentration (containing 2% (v/v) DMSO).

2.3. Defining linker length and flexibility

Linker length was quantified according to Reinke and Gestwick [24] by summing the bond lengths according to the following values: C–C, 1.54 Å; C=C, 1.34 Å; C–O, 1.43 Å. Linker flexibility was quantified based on simple quantification of sp^3 carbons and not based on dynamic simulations. This was described by Reinke and Gestwick [24].

2.4. UV–visible absorption spectra study

Absorption spectra measurements were made in the range of 250–600 nm using a Cecil 7200 UV–visible spectrophotometer (England). A stock solution of 20 mg/mL of all three compounds and curcumin in DMSO were prepared. Each stock underwent dilution with phosphate buffer 50 mM pH 7.4 to achieve a final concentration of 40 μM in the cuvette, then the UV–vis spectra of compounds were separately recorded at 25 °C immediately and after 15 min [31].

2.5. Amyloid preparation

Lysozyme was dissolved at 2 mg/mL in 50 mM glycine buffer (pH 2.5), and then incubated at 57 °C for the specified durations while stirred gently by Teflon magnetic bars [32].

2.6. Fluorescence spectroscopy

Fluorescence experiments were performed on a Cary Eclipse VARIAN fluorescence spectrophotometer. The intrinsic emission spectra were obtained at protein concentration of 0.05 mg/mL. The excitation wavelength was 280 nm and the emission spectra were collected between 290 and 450 nm. The excitation and emission slit widths were both set at 5 nm. ANS fluorescence studies were measured using a final ANS concentration of 250 μM, and the molar ratio of protein to ANS was 1:50. ANS fluorescence emission was scanned between 400 and 700 nm with an excitation wavelength of 380 nm.

2.7. ThT assay

All fluorescence experiments were carried out on a Cary Eclipse VARIAN fluorescence spectrophotometer (Mulgrave, Australia) at room temperature. Stock solution of ThT was prepared in phosphate buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7) at a concentration of 2.5 mM, passed through a 0.45 μm filter paper and stored at 4 °C. At different time intervals, an aliquot (10 μL) of incubated solution was mixed with 490 μL of ThT solution in a quartz cuvette with 1 cm path length. Fluorescence emission spectra were then taken using excitation at 440 nm. The excitation and emission slit widths were set as 5 nm and 10 nm, respectively [33].

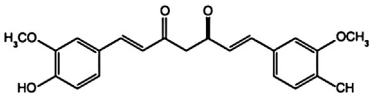
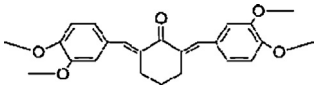
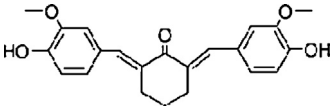
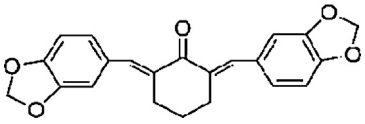
2.8. CR assay

Samples were prepared as described above. At different time intervals, 60 μL aliquots of each sample were mixed with 440 μL of a solution containing 20 mM CR, 5 mM sodium phosphate buffer, and 150 mM NaCl at pH 7.4. The Congo red solution was filtered using a center-glass N4 filter. Optical absorption spectra were acquired from 400 to 700 nm, after a 2–3 min equilibration at 25 °C, using a 1 cm path-length cell and a Cecil 7200 UV–visible spectrophotometer (England).

2.9. Atomic force microscopy (AFM)

In order to visualize HEWL amyloid fibrils, atomic force microscopy was used. For this purpose, an aliquot (10 μL) from the incubated solution (with or without the compounds) was placed on freshly cleaved mica at room temperature. After a few minutes mica was slowly washed with 100 μL of deionized water, followed by drying with nitrogen gas. Each image was acquired in a tapping mode at a scan speed of 30 μm/s, loop filter of 3 Hz and force of

Table 1
Structures of Curcumin and three its analog compounds.

Compound	Sigma code	Name	Structure	Linker length
		Curcumin		11.92 Å
1	S878863	2,6-bis(3,4-methylenedioxybenzylidene)1-cyclohexanone		8.84 Å
2	S432474	2,6-divanillylidenecyclohexanone		8.84 Å
3	S878901	2,6-bis(3,4-dimethoxybenzylidene)-1-cyclohexanone		8.84 Å

200 nN with a Dual Scope Probe Scanner (Solver next, model NT-MDT, DME, Russia) with an area of $5 \times 5\ \mu m^2$. Conical shape silicon tips (mikromasch NSC16) with a resonance frequency of 150 kHz and a nominal constant of 40 N/m were used.

2.10. 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetraazolium bromide (MTT) reduction test

MCF7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) F-12 Ham with 25 mM HEPES and $NaHCO_3$ (1:1) supplemented with 10% fetal calf serum (Sigma–Aldrich), 1.0 mM glutamine and antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin). The cells were maintained in a 5.0% CO_2 humidified atmosphere at 37 °C and grown until 80% confluence for a maximum of 20 passages. HEWL amyloid samples (in presence or absence of the compounds) were prepared as described above. The amyloid samples were centrifuged and dried under N_2 to remove any compounds and DMSO, dissolved in DMEM at a final lysozyme concentration of 2 μM. Aggregate toxicity was assessed in 96-well plates by the MTT assay. Briefly, the cells were seeded at 5000 cells per well in 96-well cell culture plates. After 24 h incubation, growth media was replaced with fresh growth medium containing HEWL amyloid samples (2 μM), pre incubated with or without the compounds. After the exposure to 2 μM HEWL aggregates for 48 h at 37 °C, the medium was removed and MTT solution (5 mg/mL in PBS) was added to the cells. The cells were incubated at 37 °C for 4 h, then the medium was aspirated, and the formazan product was solubilized with DMSO. The absorption was measured at 570 nm (620 nm

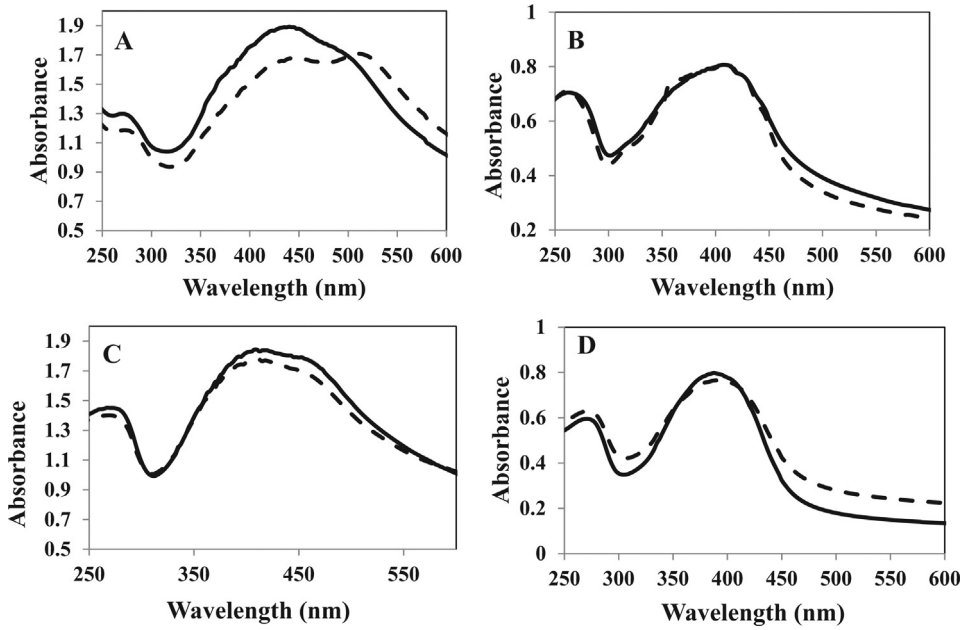


Fig. 1. UV–vis absorption spectra of curcumin and the three tested compounds. A) Curcumin spectrum immediately (□), and 15 min after incubation (—). B) Compound 1 spectrum immediately (□), and 15 min after incubation (—). C) Compound 2 spectrum immediately (□), and 15 min after incubation (—). D) Compound 3 spectrum immediately (□), and 15 min after incubation (—). For more detail please see Experimental section 2.4.

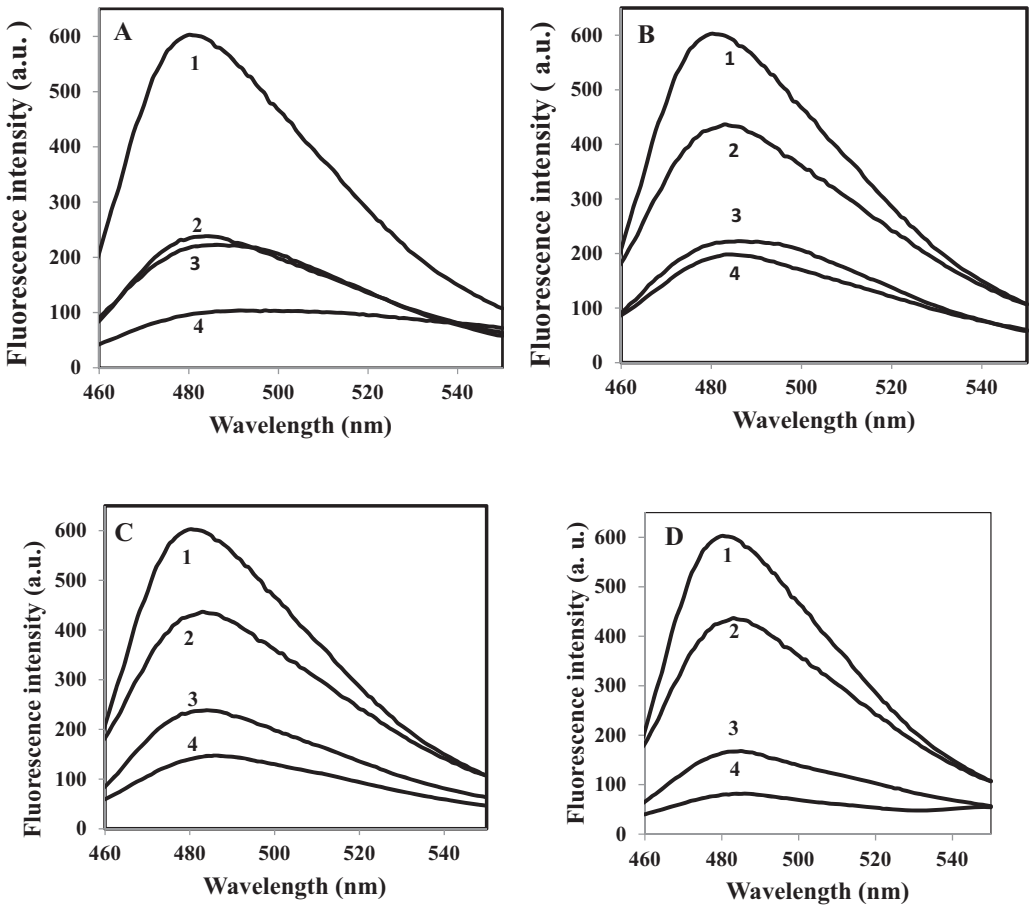


Fig. 2. Concentration-dependent prevention of HEWL aggregation. The compounds were added at different concentrations, from 0.2 (2), 0.4 (3) to 0.8 (4) mM. Spectra were obtained at pH 2.5 and 57 °C by monitoring change in ThT emission enhancement after 48 h. The tested compounds were curcumin (A), compound 1 (B), compound 2 (C), and compound 3 (D). Protein concentration was 2 mg/mL. ThT enhancement of HEWL without inhibitors (1) is also shown for comparison.

as a reference) in an ELISA reader. Cell viability was expressed as percent of MTT reduction in treated cells as compared to cognate untreated cells.

2.11. Docking studies

Auto dock vina was used to do the docking experiment [34]. The 2VB1.pdb file was used as receptor whose protonation state was adjusted at pH 2.5 with the use of the corresponding module of MOE 2012.10 (*Molecular Operating Environment* (*MOE*), 2012.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2012). Grid box of $40 \times 40 \times 58$ points was used with a spacing 1.0 Å, encompassing the whole molecule. The ligands were prepared using MOE 2012.10 and converted with MGLtools to perform docking. Gasteiger charges were assigned to protein and ligand molecules. Exhaustiveness was set on 20 and a computer with four processors was utilized for the computation. 100 poses were generated for the ligand, computing ligand interactions and preparation of the image representing the best poses were performed with MOE 2012.10.

2.12. Statistical analysis

All data are presented as Mean ± SD. The mean values were calculated based on the data from at least three independent experiments. The statistical significances were achieved when $p < 0.05$.

3. Results and discussion

Several studies have suggested that interactions between aromatic small molecules may play an important role in the inhibition of amyloid aggregate formation [35,36]. Moreover; recent studies on Aβ amyloid formation indicate that these small aromatic compounds would interfere with aggregation pathways, and may "remodel" the amyloid intermediates via different mechanisms or interactions [37]. Also, a wide range of studies have demonstrated that curcumin (as a natural aromatic small molecule) as well as curcumin derivatives, could inhibit β-amyloid fibril formation from Aβ [38]. The structural particularity of curcumin, which include the presence of two aromatic end groups and a suitable length of linker between the aromatic ends, is suggested to be necessary for its optimal activity [24]. Modifications of the native structure of curcumin can result in the identification of improved inhibitors of amyloid aggregation, three compounds have been chosen that may useful in this regard. It has been suggested that curcumin would be rapidly degraded at physiological pH, in presence of light and reducing agents [29]. As described in the Section 1 we anticipated that our suggested compounds could be more stable in comparison with curcumin at physiological pH. To validate this theory, the UV–visible absorption spectrum of curcumin and our three suggested compounds were obtained in phosphate buffer (50 mM, pH 7.4). The method presented by Chakraborti et al. [31] was used as a guideline. As depicted in Fig. 1A, the intensity of curcumin spectrum was significantly decreased and a red shift occurred after 15 min of its incubation in phosphate buffer pH 7.4 while both the

intensity and shape of the compounds spectra taken in the same condition were unchanged (Fig. 1B–D). This observation suggests that all three compounds are stable at physiological pH in contrast with curcumin.

To test the activity of these compounds on amyloid aggregation processes, the well-known ThT assay was employed. First, HEWL was driven toward amyloid formation by the use of acidic pH (pH 2.5) and high temperature (57 °C), and then, Thioflavin T and Congo red were used as markers for detection of amyloid fibrillation [30]. ThT fluorescence emission recorded for 48 h showed a significant increase (from 28.4 ± 5.2 to 646.6 ± 27.3 a.u.). The kinetics of amyloid formation was nucleation-dependent, and included the phases of initial nucleation, elongation and equilibration. Furthermore, congo red absorbance increase was detected coupled to gradual higher red shift during the incubation time. Both data are in accordance with previous reports on HEWL amyloid formation [32]. Finally, atomic force microscopy (AFM) image of HEWL samples incubated in this condition confirmed the formation of long, unbranched fibrils that are found in amyloid structures.

3.1. Effect of curcumin and compounds 1, 2 and 3 on fibrillation of HEWL

To determine whether these compounds affect the process of HEWL amyloid formation, curcumin and curcumin analogs were separately added to the incubation medium in various concentrations ranging from 0.2 to 0.8 mM. As depicted in Fig. 2, all compounds clearly showed a pattern of dose-dependent inhibition as suggested by the observed decrease of ThT fluorescence intensity but compound 3 and curcumin were most effective in this regard.

We also designed a control experiment to rule out the possibility that the low ThT intensity in the fluorescence assay might result from a quenching effect of the inhibitors. To this end, the most effective concentration of inhibitors (0.8 mM) was added to HEWL amyloid sample and fluorescence was immediately measured. No significant quenching effect was detected (data not shown). DMSO is commonly used as a co-solvent to improve the aqueous solubility of small organic compounds. Throughout the study, we used DMSO for dissolving our compound and thus a first control was conducted to determine whether DMSO itself had an effect on the HEWL aggregation. It has been reported that at low concentration, organic solvents including dimethyl sulfoxide (DMSO) would have little effect on protein structure and function. However, higher concentrations of the reagent could result into partial unfolding and inactivation of enzymes [39]. However, 2% (v/v) DMSO used here has a minimal effect on the aggregation of HEWL, and as so, all compounds were dissolved in DMSO prior to use. The effect of these compounds on the kinetics of HEWL amyloid formation was also investigated through monitoring maximal ThT emission intensity over the course of 48 h. As depicted in Fig. 3, the ThT fluorescence curve exhibited a characteristic sigmoid shape representing three phases: the nucleation phase, polymerization phase and equilibrium phase. Based on Fig. 3, all reagents were found to be inhibitory in the polymerization phase, changed the slope of the curve, but with different intensity (curcumin ≥ compound 3 > compound 2 > compound 1).

To determine which phase is inhibited in presence of these inhibitors, the compounds were added to HEWL medium after the polymerization phase reached completion and the fibrillogenesis had already ended. All compounds were active against HEWL amyloid formation even when they were added after the polymerization phase but curcumin was a much better inhibitor for disaggregating pre-formed HEWL fibrils (Fig. 4).

All compounds were inhibitory in the polymerization and equilibrium phases, and all changed the slope of the curve, but with different magnitudes. A study has shown that the most effective

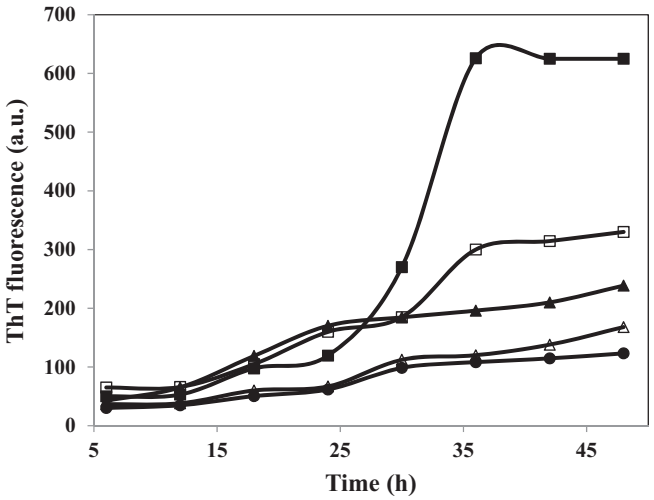


Fig. 3. Kinetics of HEWL fibrillation in the presence of 0.8 mM of the different compounds. This was followed by monitoring changes in ThT fluorescence intensity after 48 h of incubation at pH 2.5 and 57 °C. The studied compounds were curcumin (●), compound 1 (□), compound 2 (▲), and compound 3 (△). HEWL in the absence of any inhibitors indicated by (■). The protein concentration was kept at 2 mg/mL.

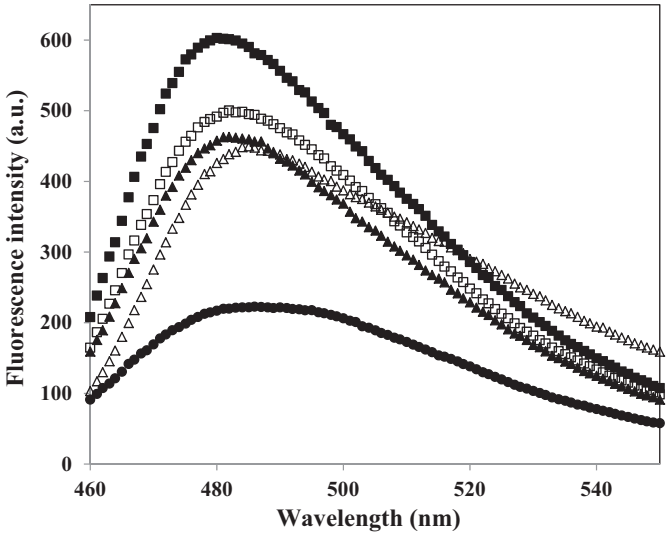


Fig. 4. Effects of curcumin and the compounds on HEWL amyloid fibrils when added after the polymerization Step. Curcumin and compounds were added at 0.8 mM final concentration to HEWL samples incubated for 48 h, at pH 2.5 and 57 °C. ThT fluorescence changes were then monitored over a 24 h incubation in the same conditions. HEWL spectrum in the absence of any compounds is indicated by (■) and samples containing HEWL in the presence of 0.8 mM are compound 1 (□), compound 2 (▲), compound 3 (△) and curcumin (●). Protein concentration was 2 mg/mL.

curcumin-like inhibitors identified within a set of 20 compounds were the ones possessing 7-carbon linker length with meta- and para-substituted methoxy and hydroxyl groups which are similar to curcumin substitutions [2]. Reinke and Gestwick [24] have reported the efficacy of some compounds similar with what we have used in our study but with 7-carbon linker length. They have shown that upon OH substitution with o-methoxy groups, no activity would be observed even in high concentration of the inhibitor. In contrast, our results showed that compound 1 which has 2 o-methoxy groups per ring has still a significant effect in comparison with other compounds (Figs. 2–4). A similar observation has been made about a compound that has

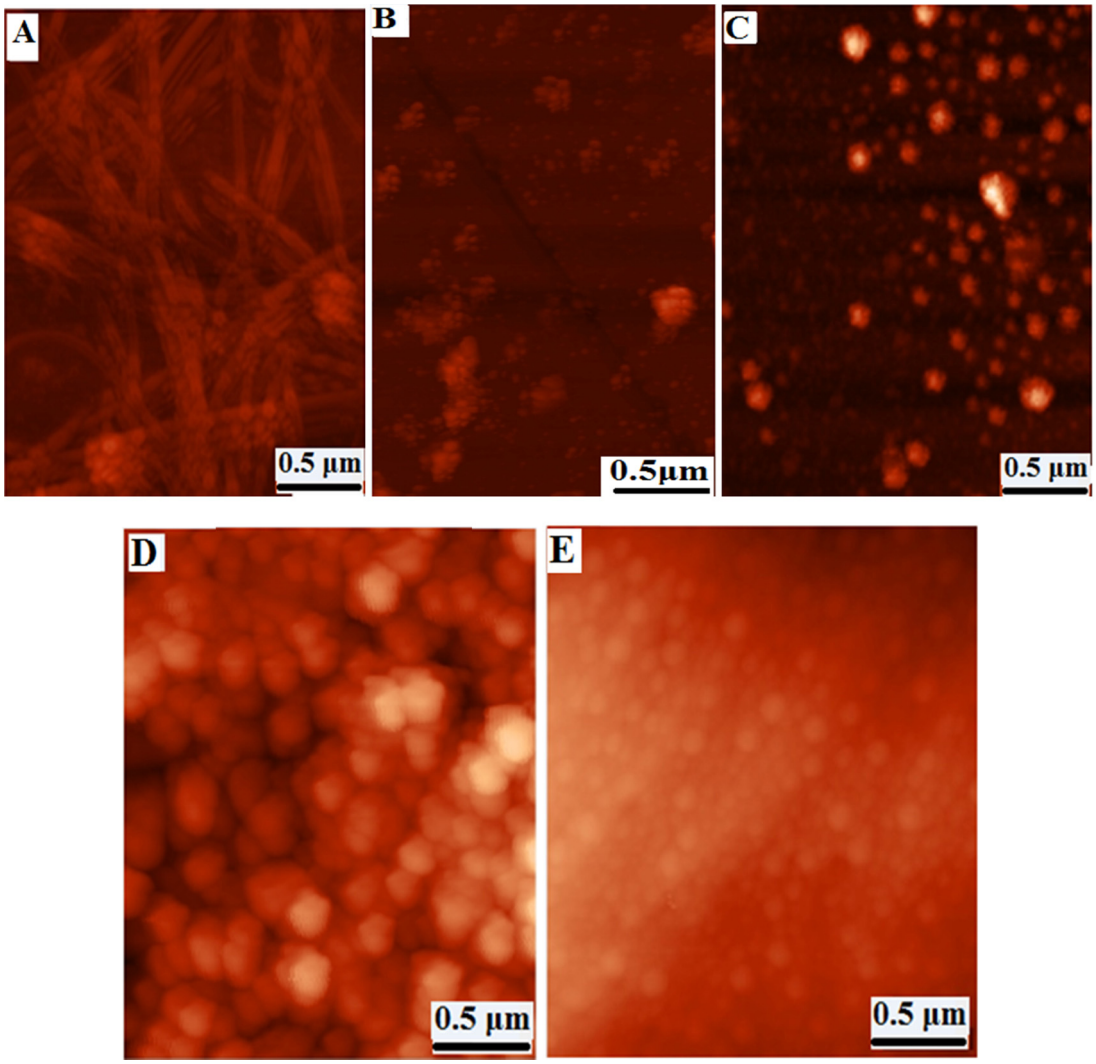


Fig. 5. AFM images of HEWL aggregates deposited on mica flowing inhibition by different inhibitors. HEWL was incubated under fibrillation conditions alone (after 48 h of incubation at pH 2.5 and 57 °C) (A) or in the presence of 0.8 mM curcumin (B), compound 1 (C), compound 2 (D) and compound 3 (E).

methoxy groups placed in both position but yet was active in amyloid disaggregation (compound 2 from their library) [2]. Overall, compound 3, which does not have curcumin instability at physiological pH, could be considered as a suitable curcumin analog that may find potential application as anti-aggregation therapeutic.

3.2. Morphology analysis of HEWL fibrillation

Morphological characteristics of HEWL incubated under amyloidogenic conditions in absence or presence of curcumin and curcumin analogs were analyzed by AFM. In the AFM micrographs of HEWL without inhibitors, single strands and unbranched fibrils were observed. In the samples containing HEWL and inhibitors, no fibrils were detected (Fig. 5). We hypothesize that these compounds may bind to on-pathway intermediates and effectively inhibit lateral association of oligomers to form larger structures.

3.3. Effect of curcumin and curcumin analogs on HEWL amyloid induced cytotoxicity

Cytotoxicity studies were performed using both fibrils and the non-fibrillar structures formed in presence of the compounds and curcumin according to the protocol described in the Section 2. To test whether these compounds can inhibit the formation of cytotoxic structures, aggregated formed in presence/absence of compounds were transferred to a physiological medium and the resulting suspensions were added to the cell culture media of MCF7 cells at a concentration of 2 μM (Final concentration of aggregates in cell culture). The ability of the resulting aggregates to cause cellular dysfunction was assayed by performing the MTT reduction Test [40]. As demonstrated in Fig. 6, native HEWL did not cause any detectable decrease in the ability of cells to reduce MTT relative to untreated cells. By contrast, the cell viability of MCF7 cells was significantly decreased to 38 ± 8.3% after a 48 h exposure to HEWL aggregates. In presence of compounds 1, 2, 3 and curcumin the ability of cells to reduce MTT were increased to 69.6 ± 10%, 65.3 ± 7.5%, 81.5 ± 9.2% and 92.5 ± 3.028 respectively. Thus these compounds could significantly protect MCF7 ($p < 0.05$) from the cytotoxicity of lysozyme oligomers and improved cell viability. Based on these results and AFM micrographs presented in Fig. 5, it is suggested that these aromatic compounds and also curcumin are capable to remodel soluble oligomers into large, off-pathway aggregates that are non-toxic. Moreover, they also remodel mature amyloid fibrils into non-toxic structures [37]. These findings clearly show that curcumin and compound 3 are more efficient in the reduction of HEWL induced cytotoxicity in comparison with the other tested compounds.

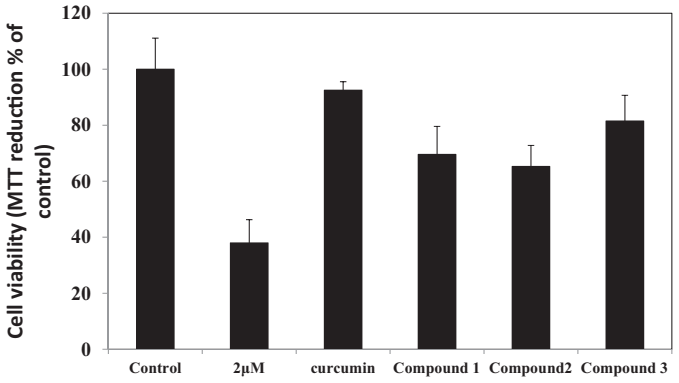


Fig. 6. Cytotoxicity of HEWL aggregates. Cell viability was determined by the MTT reduction test in MCF7cells exposed to 2 μM native HEWL aggregates incubated for 48 h at 57.5 °C, pH 2.5 (bar 2). HEWL aggregates incubated for 48 h at 57.5 °C, pH 2.5 and in presence of 0.8 mM, curcumin (bar 3), compound 1 (bar 4), compound 2 (bar 5) and compound 3 (bar 6). The reported values are representative of two independent experiments, each carried out four times. Untreated cells were assumed to be viable as a control (bar 1).

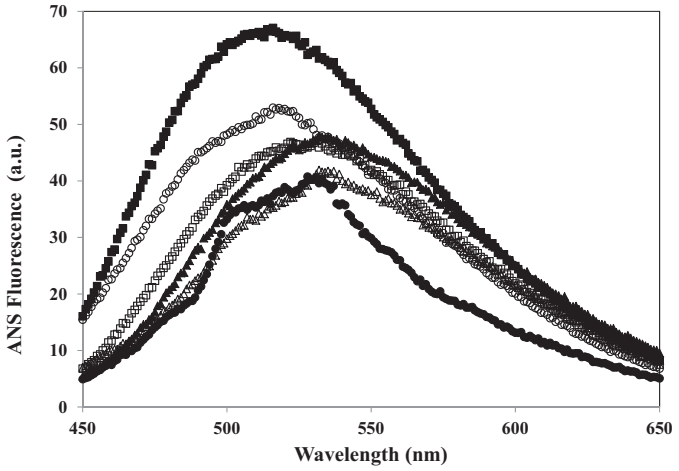


Fig. 7. Effect of 0.8 mM of curcumin and the compounds on the surface hydrophobicity of HEWL. Changes in ANS emission spectra obtained in the presence of HEWL in glycine buffer 50 mM, pH 2.5 without compounds (■), and or with curcumin (○), compound 1 (●), compound 2 (△) and compound 3 (▲). Background ANS fluorescence recorded in the absence of the protein is also presented as (□). Further details are given in experimental section.

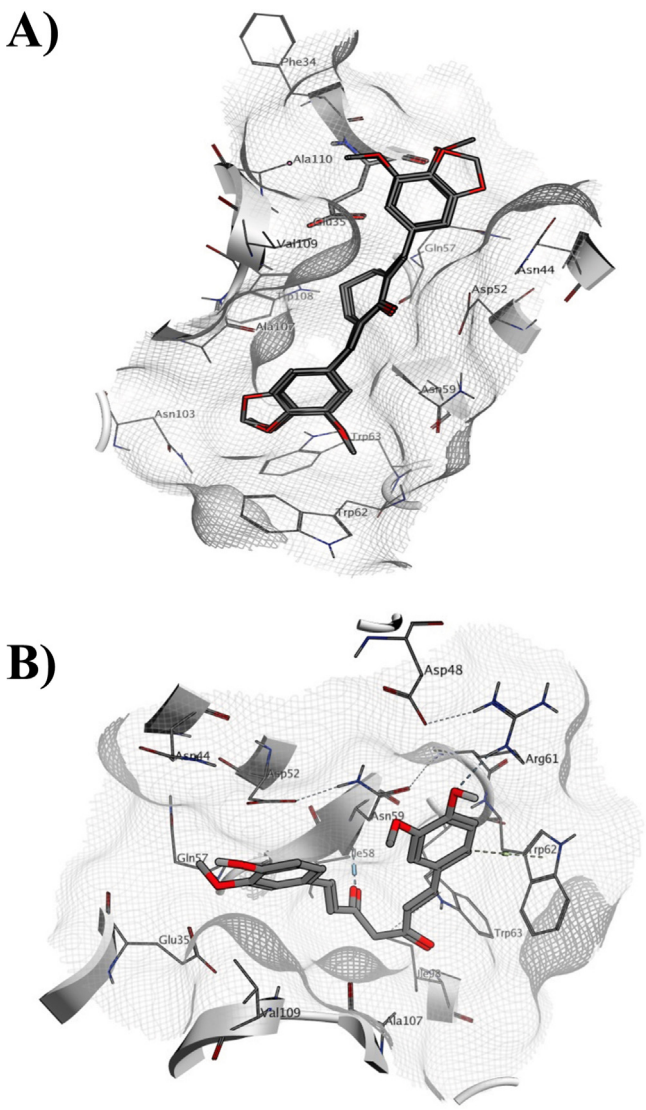


Fig. 8. Binding mode of the compounds into HEWL, obtained by a docking experiment. The three compounds (shown with sticks) are superimposed (A), and the docking pose of curcumin is indicated separately (B). The binding cavity volume is represented by surface computation. HEWL residues present in the vicinity of the ligands (4.5 Å) have been labeled.

3.4. Compounds potentially binds to HEWL and stabilize native state conformation

Small molecules can inhibit amyloid aggregation with various mechanisms. Some small molecules may prevent the process with one or more than one of the mechanisms [41]. Aggregation events generally require the presence of partially unfolded proteins state, which makes the stabilization of native state making one of the widely sought after strategies of aggregation inhibition [42]. Several studies have shown that some indole derivatives can bind directly into the protein and stabilize its native state and also inhibit its aggregation. In contrast, our recent study showed that cinnamon extract whose effective compound is cinnamaldehyde could bind to the protein and destabilize HEWL but is also capable to inhibit fibrillation processes (unpublished data). An experiment was thus designed to test the theory that the compounds can stabilize native state of the protein. Fluorescence measurements involving 8-anilinonaphthalene-sulfonate (ANS) a hydrophobic reporter probe, revealed exposure of large hydrophobic patches on the surface of the protein molecule in the amyloid aggregation-prone state (Fig. 7). After addition of curcumin and curcumin analogs, significant decrease of intensity and red shift took place. Thus, the presence of all compounds prevents exposure of hydrophobic patches and result into packed native state. Taking these findings together, it seems that these reagents may also inhibit HEWL amyloid fibril formation by protecting the native protein from structural changes.

3.5. Docking studies

Based on the previous section results, that show the ability of the tested compound to preserve HEWL native structure, a modeling experiment was performed to assess the possible interaction site of the compounds and curcumin. In order to investigate the binding site of the ligands, blind docking was done with the use of Autodock Vina. Compounds 1, 2, 3 were found to bind HEWL in a similar location (Fig. 8A). Docking scores of the best poses were −7.2 kcal/mol, −7.3 kcal/mol, and −8.0 kcal/mol for compounds 1, 2, and 3 respectively, indicating a slightly better score for compound 3. All compounds were found to adopt an extended

conformation, and to interact with HEWL in its active site. Interestingly, the docked pose of curcumin is different in this regard (Fig. 8B), possibly due to a higher flexibility of the structure; the overall conformation was found to be non-extended in the first 10 poses (docking scores of −7.1 to −6.8 kcal/mol). In the case of the curcumin derivatives, the carbonyl component of the cyclohexanone group is pointing toward the more hydrophilic residues of the active site. Catalytic residues E35 and D52 are also observed in the interacting site of the ligands, and a hydroxyl group of compound 2 could make a hydrogen bond with E35 backbone. Weak π–H interactions may also occur between the ligands and W62. Curcumin, on the other hand, may interact mainly with R61 side chain (hydrogen bond), as well as with N59 (backbone hydrogen bond). π–H interaction may also occur with W62, and there is a possibility to make a hydrogen bond with E35 side chain. However, D52 is not located close enough to curcumin. Due to the flexibility of curcumin structure, other patterns of interaction may be detected in the other poses; as an example, in pose #2, the R61 interaction is still present, but a hydrogen bond may be formed between D52 and the ligand, instead of E35. The overall result is that curcumin may not be able to interact with both catalytic residues, and has the potential to form various interactions with the active side residues, due to the potential of making multiple hydrogen bonds, and the curved conformation that it takes. On the contrary, the curcumin derivatives are positioned into HEWL with an extended conformation, thus occupying the whole active site groove. The obtained docking scores of derivatives are more or less similar, with a slightly better result obtained by compound #3, which may be due to its more constrained conformation: this structure lacks the methoxy and hydroxyl groups of compounds 1 and 2. The overall result is in accordance with the experimental data. Ligands binding location spans from HEWL catalytic residues to the tryptophan residues 62 and 63, from which W62 was previously found to interact with cinnamaldehyde (unpublished data) and indole derivatives [32]. The size of these compounds and presence of aromatic/cyclic components may have a role in their effectiveness. These compounds are able to affect amyloid formation at various stages: not only they are able to interact with HEWL native structure, but their structural characteristics may confer them the potential to be inserted between formed fibrils.

4. Conclusion

In spite of proven efficacy and relative safety of curcumin; however, its clinical application is severely limited because of its poor bioavailibity under physiological conditions. The main goal of the present study was to identify more effective aggregation inhibitors based on curcumin structure by avoiding the weak stability and bioactivity of curcumin. To find improved inhibitor, three compounds were chosen with suitable similar properties to curcumin including rigid and suitable linker length, in which the diketone moiety of curcumin was replaced by benzene ring and various substituted groups were placed on aromatic moiety in the molecules. It was previously reported that presence of the diketone part may be essential for the biological activity of curcumin [43]. However, our study showed that that compounds that lack this group are still more or less active and other studies confirm our results [44,45]. On the other hand, at physiological conditions, curcumin exists in equilibrium between a keto and enol form and the studies have shown that the enol form of curcumin is responsible for its degradation [46]. However, compounds tested here that are devoid of this enol group should be more stable in comparison to curcumin. It was recently reported that a series of derivatives with substitution in the diketone region of curcumin show dramatic improvement in curcumin stability while possessing efficacy that is comparable with that of curcumin [31]. Moreover, flexibility of the linker is also other parameter may influence on curcumin derivatives activities [24]. Relative flexibility was approximated by Reinkel model based on number of freely rotating sp^3-hybridized carbons within the backbone [24]. Compounds tested here have not any rotating sp^3-hybridized carbon within their backbone and are therefore more rigid than curcumin. It is concluded that with regard to both length and flexibility of the linker region, the compounds are promising curcumin analogues that may be considered as amyloid aggregation inhibitor. Moreover, the methoxy substitution of hydroxyl groups on compound 1 and absence of hydroxyl groups on compound 3 should prevent these enzyme modifications and may improve their bioactivity [2]. Overall, we found new compounds and evaluated their inhibitory ability and protective effect on HEWL aggregates. It should be noted that our results presented here showed that curcumin is most effective in preventing HEWL aggregation and toxicity but as mentioned previously, its low stability and poor bioavailibity severely limit its clinical application. We showed that although our suggested compounds showed not significant improvement as HEWL aggregation inhibitors in comparison with native curcumin, these analogs are extremely stable at physiological pH, thus strongly suggested as good replacement for curcumin in future drug development. Especially, compound number 3 is most effective (in comparison with compound 1 and 2). The results of the present study may be considered as a preliminary report of the beneficial effect of these curcumin derivatives as inhibitors of amyloid fibrillation. Though in-depth biochemical and biophysical studies are necessary to clarify the effect of our compounds on Aβ fibrillization and establish their anti-aggregation mechanism, this study highlights new curcumin derivatives eliciting high inhibitory activity against amyloid aggregation.

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