Biochemical and Biophysical Research Communications 532 (2020) 82–87

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A "keto-enol" plaque buster mechanism to diminish Alzheimer's β-Amyloid burden

Oláh Zoltán [a, b, c], Pecze László [d, *], Kocsis Éva [a, b, c], Viskolcz Béla [a]

[a] Bioinformatics and Molecular Surgery Unit, Institute of Chemistry, Faculty of Materials Science and Engineering, University of Miskolc, Miskolc, Hungary
[b] Acheuron Ltd, Szeged, Hungary
[c] Forget-Me-Not B2B Ltd, Szeged, Hungary
[d] University of Fribourg, Fribourg, Switzerland

ARTICLE INFO

Article history:
Received 26 June 2020
Accepted 17 July 2020
Available online 20 August 2020

Keywords:
Tautomer
Alzheimer's disease
Curcumin
Amyloid-β

ABSTRACT

Curcumin and related compounds have been validated to remove even well-developed human β-amyloid plaques from the brain of transgenic mice, *in vivo*. However, their molecular mechanism of the plaque buster activity is rather unknown. Computational chemistry was employed here to better understand the β-amyloid protein elimination. According to our docking studies, a tautomeric "keto-enol" flip-flop mechanism is proposed that may chop up β-amyloid plaques in Alzheimer's due to removing each hairpin-foldamers one by one from both ends of aggregated fibrils. According to the experimented models, other bi-stable "keto-enol" pharmacophores might be identified to break up amyloid plaques and enhance rapid clearance of toxic aggregates in Alzheimer's disease.

1. Introduction

Protein aggregates such as β-amyloids (Aβ) in Alzheimer's disease (AD), α-synuclein plaques in Parkinson's disease, islet amyloids in type 2 diabetes or prions in Creutzfeldt-Jakob's disease are potential drug targets [1,2]. These plaques can exist at least in two stable forms: i) in an asymptomatic state, ii) and in a life-threatening symptomatic state [3,4]. The numbers of AD-affected persons are forecasted to grow by 62% from 2015 to 2030, leading to 1 billion plaque-diseased patients worldwide [5,6].

Even the AD market alone justifies [7] the search for nature-made plaque-buster substances. These substances may ameliorate other plaque-diseases as well, but there is no validated plaque buster currently available on the market [8,9].

India-US cross-national epidemiological studies on the consumption of various curry foods have already suggested some pre-emptive role of curcumin (CURC) in AD [10–13].

Several in vitro structure-activity relationship studies concluded that CURC can obstruct protein miss-folding and it can prevent aggregation of the Aβ-peptides in the brain of transgenic mice serving as an AD model [12,13,15]. Asians ingest the highest amount of turmeric typically up to 1 g per day. This is equivalent to the consumption of 60–200 mg CURC per day. A study comparing rural populations in Northern India (n = 4450) and southwestern Pennsylvania (n = 886) showed a lower incidence and prevalence of AD in India [16,17]. Thus, the therapeutic use of CURC either to prevent or to treat AD has been started, first of all, with human bioavailability studies [14,18–20].

Initial randomized clinical trials, however, were suffered from the low bioavailability of oral CURC [21,22]. CURC did not have any beneficial effect on pro-inflammatory biomarkers such as serum Aβ and isoprostanes as well as failed to improve cognitive performance in mild-to-moderate AD patients [23]. This is in contrary to the strong *in vivo* plaque buster effect of CURC observed in transgenic mice [24–26].

Achiral Mannich-base CURC analogs have been validated to have better bioavailability and chaperon effect on miss-folded proteins [27–29], also a promise to AD patients, if the initial finding will be managed from bench-to-bedside [2,15,30,31].

Although, CURC and its homologs had undergone extensive preclinical development, showed remarkable efficacy in neuronal injury repair, and it is a validated anti-inflammatory agent, the selective mechanism of the plaque buster activity on misfolded Aβ, are rather obscure. A better understanding of the mechanism of plaque disintegration and removal of aggregation-prone proteins from the brain and other human tissues may accelerate bench-to-bedside processes of promising lead molecules. Another

* Corresponding author.
E-mail address: laszlo.pecze@unifr.ch (P. László).

https://doi.org/10.1016/j.bbrc.2020.07.086
0006-291X/

accelerated translation is to make available various spice-mixes as the epidemiologically proven curry, for the AD-vulnerable population [10–13]. Here, we suggest a flip-fop dissolution mechanism of how CURC can inhibit newly formed plaques or disaggregate plaques that have already been embedded in the brain tissue.

2. Methods

2.1. Screening plant extracts

Cold $A\beta_{1\text{-}42}$ peptide, radioactively labeled Leu-[^{3}H]Pro-Tyr-Phe-Asp-$CONH_2$ (L[^{3}H]PYFE) peptide (stock solution 35.3 Ci/mmol $8*10^{-8}$ M), and cold LPYFE were synthesized by in house core facility. The working solution of L[^{3}H]PYFE) was 1 μCi/μl in the binding assay. From synthesized $A\beta_{1\text{-}42}$ peptides, previously lyophilized twice, Aβ fibrils were grown with spontaneous aggregation. It was carried out in a standard assay buffer by vigorous stirring with a magnetic bar at room temperature (25 °C) for a week. Fibril formation was controlled by visual inspection. Fibrils were stained with Congo Red and samples were analyzed with an Olympus FV1000 confocal LSM microscope. The radiolabeled small peptide, L[^{3}H]PYFE binds to fibrils. Its binding can be inhibited by several test compounds. The assays were performed in duplicates in sterile flat bottom 96 well plates with components as follows: 70 μl buffer, 10 μl suspension of $A\beta_{1\text{-}42}$ stock (either 0.2 mM or the 0.04 mM), 20 μl LPYFE, 100 μl L[^{3}H]PYFE and the extract/compound of interest. After 30 min incubation at room temperature, the samples were transferred to a Multiscreen HTS (Millipore Corporation, Bedford, MA, USA) for filtration and extensive washing, to get rid of the excess of reporter peptide. After that 50 μl scintillation cocktail was added into each well and radioactivity was measured in a 96 well scintillation counter. Data were analyzed with Prism software (GraphPad™) and Microsoft Excel™.

2.2. Docking studies

The computational chemistry tasks were carried out using the AutoDock 3.05 software [32]. The initial structure of amyloid [5] was taken from the PDB database (accession number: 2BEG, the first model was chosen), and the input files were generated with AutoDockTools [32]. During the docking procedure, amyloid was kept rigid, and flexible (torsional) movements were allowed for the ligands at geometries obtained from the *ab initio* optimization.

Lennard-Jones potentials 12–10 and 12–6 were used to model H-bonds and van der Waals interactions, respectively. In calculations of the electrostatic grid map, the distance-dependent dielectric constant of Mehler and Solmajer was utilized [33]. Using the distance-dependent form in the grid calculation the screening effect of the solvent was taken into consideration. During the docking procedure, the Lamarckian Genetic Algorithm [34] with the pseudo-Solis and Wets method was used with 250 individuals in the population. The stopping criterion was defined by the total number of energy evaluations, which was set to 2.5×10^7. The translation step was set to 0.5 Å/step, and in both quaternation and torsional (only at flexible ligand docking) steps 5.0°/step was used. The docking box was centered on the macromolecule and the lattice point distance was set to 0.375 Å. The docking procedure was performed 256 times.

3. Results

Aβ fibrils were stained with Congo Red (Fig. 1A) to check the applicability in the binding assay. A typical binding assay can identify several compounds inhibiting the binding of the radioactive peptide, L[^{3}H]PYFE, to Aβ fibrils (Fig. 1B).

In silico the aggregation-prone polypeptide motif of the $A\beta_{1\text{-}42}$ folds in a hairpin molecular format and stack up on top of one another as a filament (Fig. 2).

Comparable to CURC, salicyl-curcuminoid, Bingo RS0406, and Congo Red have been shown to bind to $A\beta_{1\text{-}42}$ plaques and capable of tautomerism. Based on the type of tautomerism, Aβ-binding compounds form three groups. These functional groups were shown in Supplementary Fig. 1.

Group **A** is represented by curcumin and salicyl-curcuminoid. *Ab initio* molecular geometry optimizations have been performed on both compounds. The infrared spectra of the „keto" and „enol" forms of salicyl-curcuminoid have also been computed (Supplementary Fig. 2). *Group* **B** is represented by Congo Red and *Group* **C** is represented by Bingo-RS0406. The optimum geometries of the compounds were also determined by *ab initio* molecular geometry optimizations.

Congo Red occurs in two tautomeric forms. **B** is the completely planar form in which the terminal naphthalin rings are in ortho-quinoidal forms and the central biphenyl moiety is in para-quinoidal form. This is not the structure of the normal α-imino-phenylhydrazone. In contrast to that, the **B′** form which is the normal form of a phenylhydrazone, is a tautomer in which the proton is migrated from nitrogen atom, close to the naphthalin, to the adjacent nitrogen, close to the biphenyl. Consequently, only the ortho-quinoidal structure is retained while the para quinoidal structure disappeared. **B** and **B′,** two forms of Congo Red are shown in Supplementary Fig. 3. Note that the **B′** is not completely planar in comparison with the **B**-form. There is an about 10° twist in the C–C bond that connects the two benzene rings of the biphenyl groups (Fig. 3A).

Some of these molecules were subjected to docking computations, involving the amyloid peptide aggregate shown in Fig. 2. The **B′** tautomer of Congo Red is docked in this complex (Fig. 3B).

The thermodynamics of the docking of the two tautomer forms of Congo Red were represented by the Gibbs free energy (ΔG) profile (Fig. 3C). The total sum of the released Gibbs free energy is considered to be the arithmetical sum of the ΔG associated with the isomerization and the docking process

$$\Delta G_{\text{Total}} = \Delta G_{\text{Isom}} + \Delta G^{\text{ENOL}}_{\text{Docking}}$$

The Bingo-RS0406 total of Gibbs free energy (ΔG_{Total}) could perhaps range from −10 to −50 kcal/mol, which may be suitable to initiate the break off of the first polypeptide foldamer from the edge. These chains are hooked together in a parallel β-strand having 12 membered rings in their hydrogen-bonded network (Fig. 4). The calculated thermodynamic parameters are shown in Table 1.

4. Discussion

It is assumed that keto-enol tautomerization is involved in the mechanism of action of cyclohexanedione oxime herbicides. These herbicides act on acetyl-coenzyme A carboxylase, a key enzyme in the lipid biosynthesis pathway [35]. Besides that, it has been published that the antifungal activity of α-substituted acetophenones shows a linear correlation with the computed enolization energies [36]. Moreover, curcumin analogs have been tested for their potencies as inducers of detoxification enzymes in murine hepatoma cells depending on their keto-enol tautomerization [37]. However, to the best of our knowledge, no attempt has been made to connect keto-enol tautomerization with plaque buster potential.

AD is often called "proteinopathy" due to the presence of misfolded and aggregated proteins that lose their physiological roles and acquire neurotoxic properties [38,39]. CURC binds to and limit

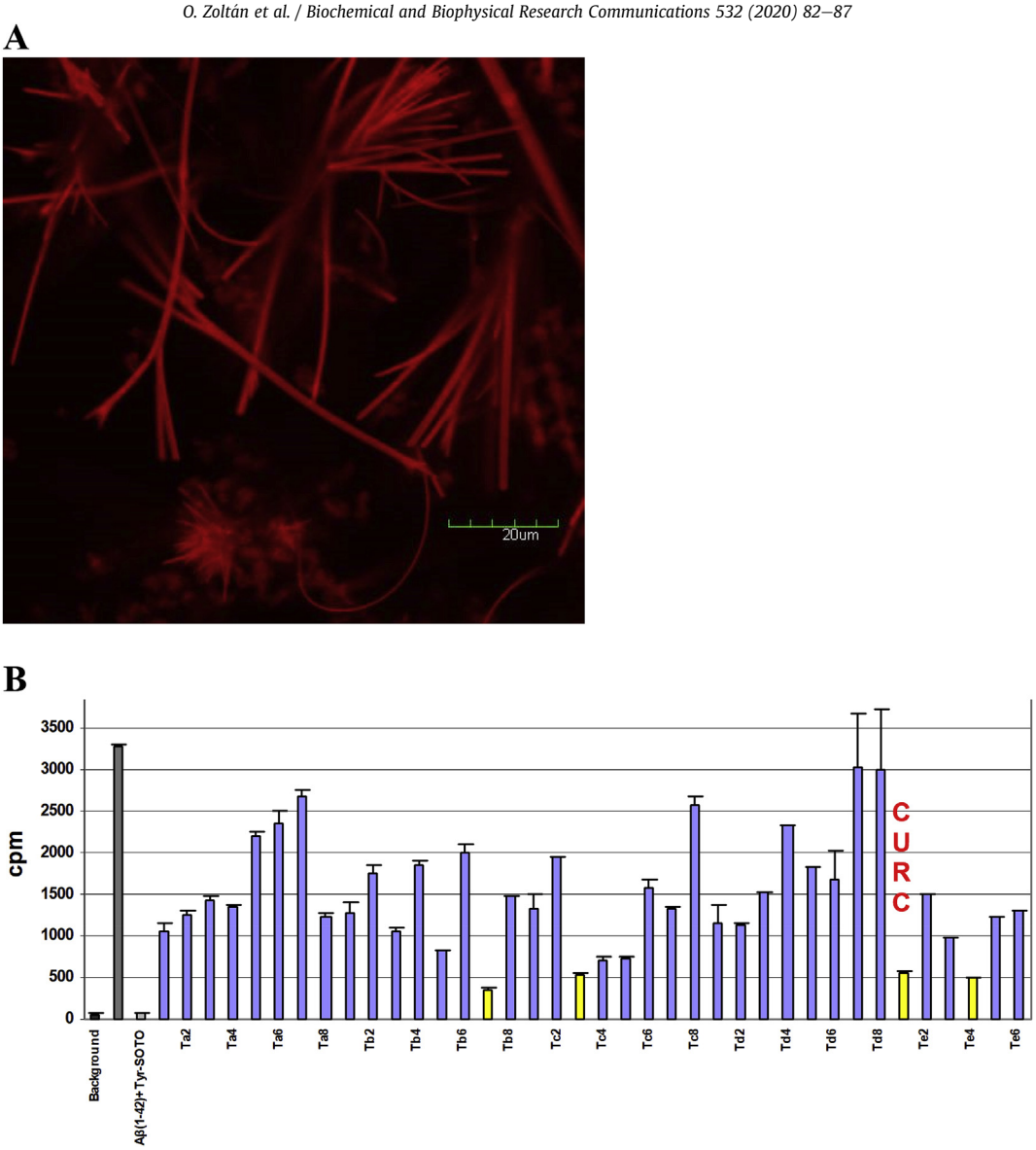

Fig. 1. Protein binding assay. A) Fibers were generated by in vitro aggregation at room temperature and depicted by confocal fluorescent microscopy. The branching Aβ fibers are shown their diameter is under 1 μm, determined by the calibration bar (20 μm). B**)** The result of a typical screening experiment illustrates the discovery of the biological effect of CURC among other hits (see Tb7, Tc3, Te4).

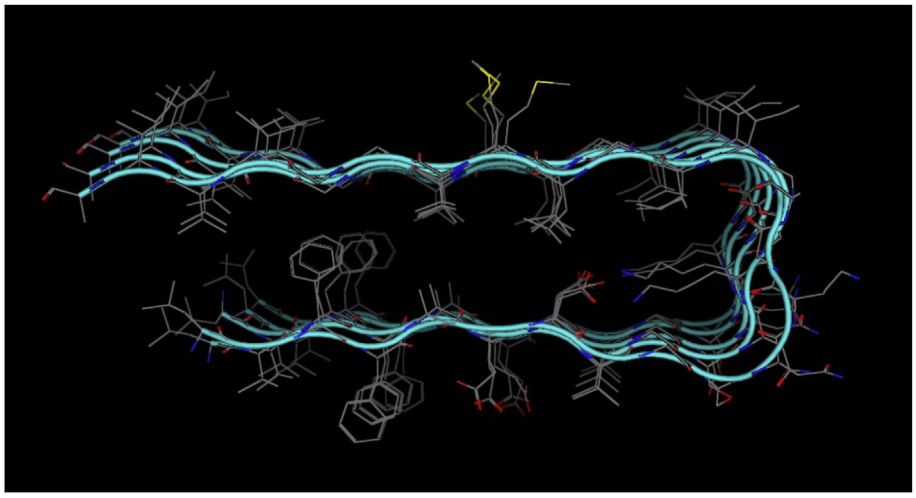

Fig. 2. *In silico* fibre of $A\beta_{1\text{-}42}$ peptides. $A\beta_{1\text{-}42}$ peptides fold in a hairpin molecular format and stack up on top of one another.

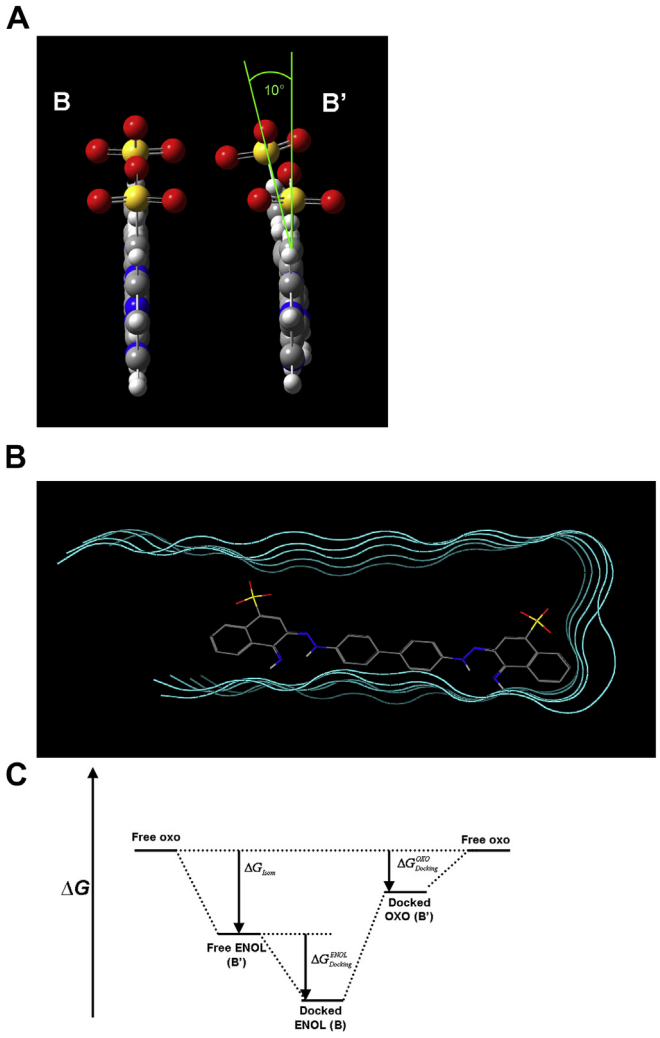

Fig. 3. Tautomeric forms and docking of Congo Red. **A**) Letters **B** and **B′** refer to the two tautomers of Congo Red. **B** is planar, and **B′** is three-dimensional tautomeric forms of Congo Red. There is a 10° twist about the C–C bond that connects the two benzene rings of the biphenyl. **B**) Congo Red docked to the amyloid peptide aggregate model. **C)** A schematic Gibbs free energy profile of tautomeric isomerization and docking. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the aggregation of Aβ oligomers into fibers and reduces Aβ plaques [15].

Our calculations by computation chemistry support a "keto-enol" tautomerization mechanism that may explain selectivity and efficacy of curcuminoids as plaque buster agents. Many of them, not only slows down or stop neurodegenerative diseases but also restores homeostasis of the inflammatory system [40]. CURC boosts certain heat shock proteins with chaperon activity [41–44] some of them helps to selectively refold even the miss-folded proteins involved in AD and other plaque diseases. In addition, CURC has generic anti-oxidant activity and induces response elements of scavengers that remove free radicals and chelates various heavy metals such as iron, and zinc in the liver [45].

Both CURC and Congo Red selectively bind to aggregated Aβ fibrils. We suppose that the "enol" form of CURC could nest in the hairpin of Aβ, while change over the "keto" might be suitable to dissociate and peel-off each foldamer, one by one, and eventually dissociate the aggregated structure fibrils. If this process repeats itself, the plaque may completely disintegrate.

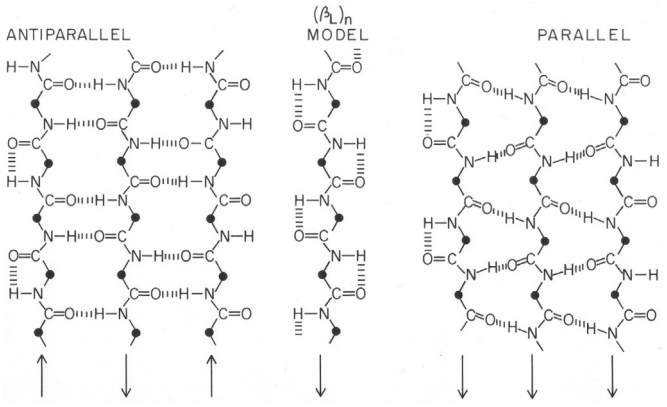

Fig. 4. Aβ disaggregation. The parallel β-strand (at the right) has relatively weak hydrogen bonds and each of the single strands (at the left) can be broken-off one at a time. As the parallel arrangement represents a weaker interaction than the antiparallel arrangement, the proposed break-off will become feasible at a certain critical ΔG_{Total} value. The heteroatoms

$$(=N:, :O:) \text{ and } -SO_3^{(-)})$$

in the plaque-busters will form hydrogen bonds with the $>N\text{-}H$ groups of the peptide chain just broken-off from the amyloid structure. This will also aid the process on energetic grounds.

Table 1
Thermodynamics of tautomerization and docking.

Compund	ΔG_{Isom}	$\Delta G^{ENOL}_{Docking}$	ΔG_{TOTAL}
Salicylcurcuminoid	−2.2	−5.4	−7.6
CongoRed	−13.5	−9.1	−22.6
Bingo RS0406	−20.8	−9.4	−30.2

CURC and various analogs induce a decrease in the plaque burden *in vivo*. Plaque busters as natural nutraceuticals, most likely directly involved in the prevention and delay the onset of AD [1,19,21,46,47]. Pharmaceutical development of tautomeric drug leads like CURC, and synthetic derivatives, such as the recently found curcumin-melatonin hybrid, have strong scientific rationale to increase drug specificity, stability, and bioavailability [48–50]. Moreover, this principle can be exploited in other protein-aggregation associated diseases such as Parkinson's disease, type 2 diabetes, or Creutzfeldt-Jakob's prion infection [1].

CRediT authorship contribution statement

Oláh Zoltán: Supervision, Writing - original draft, Writing - review & editing. **Pecze László:** Writing - original draft, Writing - review & editing. **Kocsis Éva:** Writing - original draft, Writing - review & editing. **Viskolcz Béla:** Writing - original draft, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgment

Professor emeritus Imre Csizmadia is highly acknowledged for critical reading of the manuscript. This research was supported by the European Union and the Hungarian State, co-financed by the European Regional Development Fund in the framework of the GINOP-2.3.4-15-2016-00004 project, aimed to promote the

cooperation between the higher education and the industry. ZO, EK, and BV were supported by grants from the Hungarian Government; EFOP-3.6.1-16-2016-00011, GINOP-2.2.1-15-2016-00005, and GINOP-2.3.2-15-2016-00058.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.07.086.

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