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1           **Curcumin derivatives and A $\beta$ -fibrillar aggregates: an interactions' study for**  
2           **diagnostic/therapeutic purposes in neurodegenerative diseases.**

3  
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1 **ABSTRACT**

2 Several neurodegenerative diseases, like Alzheimer's (AD), are characterized by amyloid  
3 fibrillar deposition of misfolded proteins, and this feature can be exploited for both diagnosis and  
4 therapy design. In this paper, structural modifications of curcumin scaffold were examined in order  
5 to improve its bioavailability and stability in physiological conditions, as well as its ability to interfere  
6 with  $\beta$ -amyloid fibrils and aggregates. The acid-base behaviour of curcumin derivatives, their  
7 pharmacokinetic stability in physiological conditions, and *in vitro* ability to interfere with A $\beta$  fibrils  
8 at different incubation time were investigated. The mechanisms governing these phenomena have  
9 been studied at atomic level by means of molecular docking and dynamic simulations. Finally,  
10 biological activity of selected curcuminoids has been investigated *in vitro* to evaluate their safety and  
11 efficiency in oxidative stress protection on hippocampal HT-22 mouse cells.

12 Two aromatic rings,  $\pi$ -conjugated structure and H-donor/acceptor substituents on the aromatic  
13 rings showed to be the *sine qua non* structural features to provide interaction and disaggregation  
14 activity even at very low incubation time (2 h). Computational simulations proved that upon binding  
15 the ligands modify the conformational dynamics and/or interact with the amyloidogenic region of the  
16 protofibril facilitating disaggregation. Significantly, *in vitro* results on hippocampal cells pointed out  
17 protection against glutamate toxicity and safety when administered at low concentrations (1  $\mu$ M). On  
18 the overall, in view of its higher stability in physiological conditions with respect to curcumin, of his  
19 rapid binding to fibrillar aggregates and strong depolymerizing activity, phtalimide derivative  
20 **K2F21** appeared a good candidate for both AD diagnostic and therapeutic purposes.

21

22 **KEYWORDS:** Amyloid  $\beta$  fibrillar aggregates; Alzheimer's Disease; curcumin-derivatives;  
23 Molecular Dynamics simulations; hippocampal HT-22 mouse cells.

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25

## 1 1. INTRODUCTION

2 The amyloid deposition of misfolded proteins, such as hyperphosphorylated tau, amyloid  
3  $\beta$  ( $A\beta$ ) peptide, or  $\alpha$ -synuclein, has been recognized as a feature of many neurodegenerative diseases  
4 (NDD) [1]. In fact, the degree of abnormal protein deposition commonly correlates with the clinical  
5 progression and severity of the pathology [1]. In addition to the accepted role of amyloid plaques in  
6 Alzheimer's disease (AD) pathogenesis, amyloid deposition plays a key contribution in cognitive  
7 impairments associated to synucleinopathies, such as Parkinson's disease (PD), related Parkinson's  
8 disease with dementia (hPDD) and dementia with Lewy bodies (DLB) [2]. Monomeric  $A\beta$  peptides  
9 normally present in the human brain have no deleterious effects on neurons. However, they have a  
10 tendency to self-assembly into amyloid aggregates from which the oligomeric species are most likely  
11 responsible for the pathogenesis of AD and cerebral amyloid angiopathy (CAA) [3].

12 The amyloid accumulation of  $A\beta$  peptides has a crucial role in neuronal dysfunction,  
13 eventually leading to cell death. Indeed, apoptosis occurs and contributes to AD onset and progression  
14 [4]. Several stimuli trigger the apoptotic events, i.e. oxidative stress, endoplasmic reticulum stress,  
15 metabolic dysfunction, DNA damage and reduced levels of anti-apoptotic genes [4–6]. In particular,  
16 oxidative stress has a pivotal role in the AD pathophysiology: reactive oxygen species (ROS) in  
17 association with nitrogen species (RNS), precede the formation of senile plaques [7]. The vicious  
18 circle, existing between ROS produced by damaged mitochondria during oxidative stress and  $A\beta$   
19 peptides' accumulation, accelerates AD progression [8]. Moreover, the oxidative stress, together with  
20 the deposition of  $A\beta$  in senile plaques and chronically increased concentrations of glutamate, trigger  
21 an increase in the activity of the glutamatergic system, which finally leads to neuronal dysfunction  
22 and cell death in AD [9].

23 Shining light on the mechanisms of amyloid fibril formation and disaggregation paves the  
24 way to several strategies to mitigate AD that remains an extremely challenging ailment to defeat, as  
25 recently pinpointed by Doig *et al.* [10]. Drugs under investigation comprise antibody-based  
26 immunotherapeutics such as aducanumab, peptidomimetics, glutamatergic system targeting  
27 molecules (memantine), and natural occurring small-molecules [11,12]. Antioxidants based on  
28 polyphenols, among them epigallo catechin gallate (EGCG), resveratrol and curcumin, have a  
29 demonstrated protecting activity against  $A\beta$ -induced neurotoxicity [13] with the advantage of being  
30 naturally occurring and non-toxic at reasonable concentration [14,15].

31 Planar molecules, such as Congo Red, chrysamine G and curcumin [16,17], demonstrated to  
32 have high affinity for  $A\beta$  amyloid aggregates, hence they could be used as diagnostic tools for  
33 detection of amyloid aggregation at early stages of the disease [18,19]. Whereas the importance of  
34 specific amino acid residues in the recognition process has been recently highlighted [20], the

1 structures of fibrils and oligomers, the precise location of ligand binding sites and the mechanisms of  
2 fibril destabilization upon ligand interaction with small molecules still remain largely obscure and  
3 few studies are reported [21]. Computational simulations have been recently used to complement  
4 experimental studies in the elucidation of curcumin binding modes and functionalities [22–24].

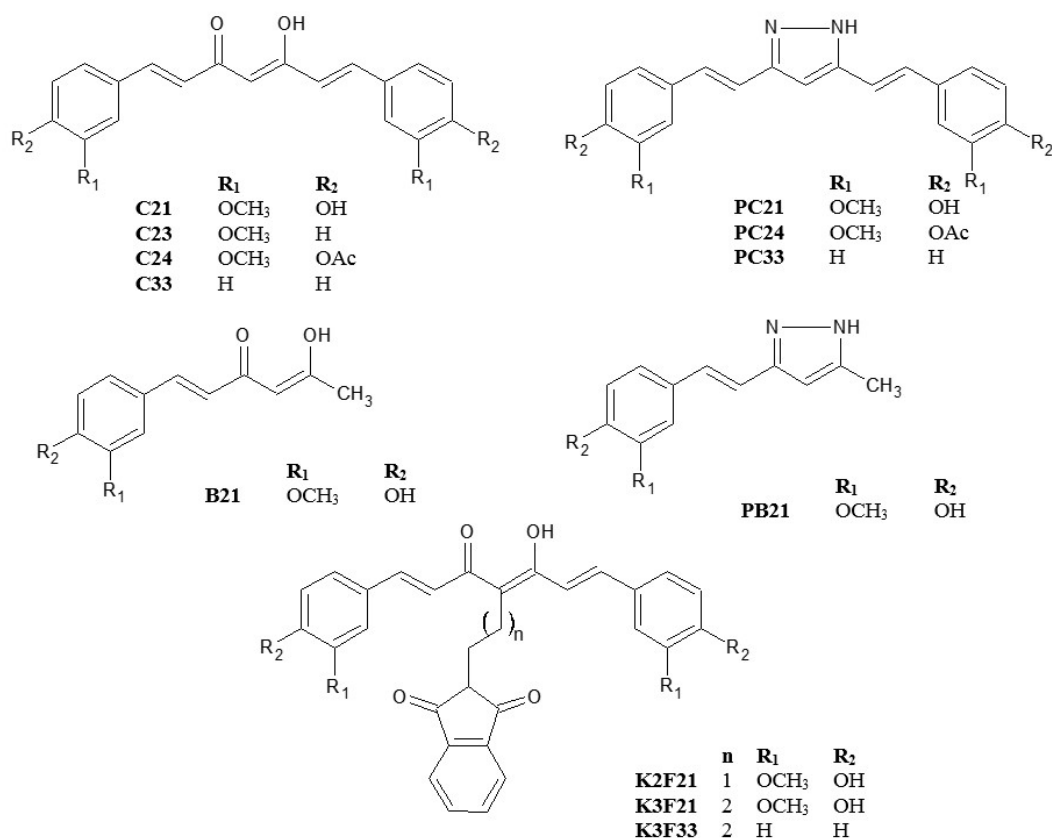
5 In particular, *in silico* studies highlighted the existence of several putative non-overlapping  
6 binding sites of the full-length A $\beta$  models for curcumin [25–29]. Moreover, based on molecular  
7 dynamics (MD) simulation studies, protofibril distortion upon curcumin interaction has been  
8 identified as the main cause for prevention of oligomerization by perturbation of A $\beta$  aggregation  
9 pathway and the formation of nontoxic aggregates [25,30].

10 Despite Curcumin (C21) possesses pleiotropic activities of considerable benefit, its use in  
11 clinical applications is limited by low bioavailability, instability and poor water solubility, as recently  
12 pinpointed by Nelson *et al.* [31]. Several articles about effect of modified curcumin on A $\beta$  amyloid  
13 aggregation has been published recently [32,33], however new scaffold modifications that may turn  
14 curcumin into stable, safe, and potent ligand for fibrillar aggregates still need to be explored.

15 In the present study, structural modifications of curcumin scaffold were examined in order to  
16 improve its bioavailability and possibly the ability to interfere with  $\beta$ -amyloid fibrils and aggregates.  
17 In particular, since tautomeric equilibrium is supposed to play a key role in curcumin instability, the  
18 replacement of the 1,3-dicarbonylic portion with isosteres is a promising strategy. The  
19 functionalization of  $\beta$ -diketo moiety was carried out by the insertion of phthalimide-functionalized  
20 chain in  $\alpha$  position to the two carbonyl groups (K series), consistently to previously synthesized KT  
21 series that demonstrated improved stability with respect to the lead compound [34]. Finally, the  
22 removal of keto-enol moiety in favor of a pyrazole ring (P series) allows to stiffen the structure and  
23 favor  $\pi$  conjugation, features that might improve both stability and interaction with A $\beta$  aggregates,  
24 as previously reported for other heterocyclic curcumin analogs [33].

25 The new derivatives (**Figure 1**) have been characterized in depth in relation to acid-base  
26 behaviour and pharmacokinetic stability in physiological conditions, and tested for their *in vitro*  
27 ability to interfere with A $\beta$  fibrils at different incubation time so to inspect their potential applications  
28 for therapy as well as for AD early diagnosis. The mechanisms governing these phenomena have  
29 been studied at atomic level by means of molecular docking and dynamic simulations. Finally,  
30 biological activity of selected curcuminoids has been investigated *in vitro* to evaluate their safety and  
31 efficiency in oxidative stress protection on neuronal cells.

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**Figure 1.** Chemical structure of investigated curcuminoids.

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## 2. RESULTS AND DISCUSSION

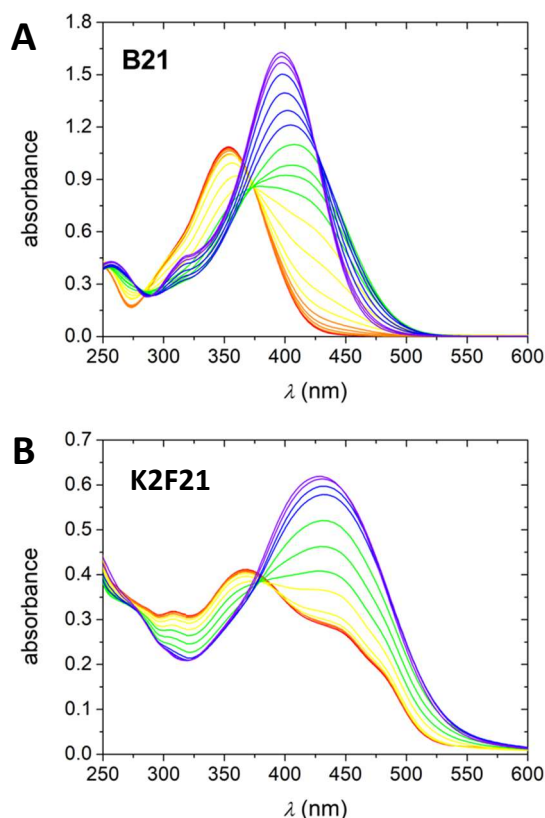
### 2.1 Chemistry.

7 All curcumin-like compounds were synthesized according to one-pot “Pabon reaction” [35] or its  
 8 modifications [36], in order to gain derivatives reported in **Figure 1**. In solution, all the compounds  
 9 gifted with the  $\beta$ -diketo moiety, namely C and K serie, show tautomerism between diketo (DK) and  
 10 keto-enol (KE) forms, that typically display two main UV-vis absorption bands at 350–370 nm and  
 11 400–430 nm, respectively [37,38]. The P series, in which the addition of pyrazole ring is aimed to  
 12 rise molecular planarity and stability, is characterized by only one main absorption band around 340  
 13 nm (**Figure 1 SI**).

14 Since acid/base behaviour may strongly affect drug uptake, distribution and interaction with  
 15 biological targets, the evaluation of overall protonation constants and species distribution curves have  
 16 a key role in the development of pharmaceuticals. The overall protonation constants (**Table 1 SI**)  
 17 were refined from spectrophotometric data with HypSpec Software [39,40]. For **B21** and **K2F21**  
 18 (**Figure 2**), as pH is increased from 5 to 11, an isosbestic point appears at about 375 nm, hinting the  
 19 equilibrium between neutral and dissociated forms around pH 8.5/9. All C and K compounds display

1 a keto-enol dissociation constant around 8.5, followed by the deprotonation of phenols at pH 9-11.  
2 As previously observed for curcumin analogs [34,41], the length of alkyl spacer in K series does not  
3 impact significantly the acidity of keto-enol moiety, hence we may predict that acid-base properties  
4 of K2F21 and K3F21 should overlap.

5 For PC21, a first deprotonation is observed at very acid pH (~2), due to the dissociation of  
6 pyrazolidinium cation to give the neutral form of pyrazole, followed by the deprotonation of the two  
7 phenolic groups at pH 8.5-9 (Figure 1 SI).



8  
9 **Figure 2.** pH-metric spectrophotometric titration of **B21** (A) and **K2F21** (B) in aqueous medium at 298 K in  
10 the 250–600 nm spectral range, pH starting from 5 (red) to 11.0 (violet).  
11

12 Lipophilicity is of utmost importance in view of uptake and distribution in biological systems.  
13 Particularly when non-specific mechanisms take place, neutral species with low molecular weight (<  
14 600 Da) are commonly better internalized than charged molecules. All the investigated compounds  
15 are mainly in the neutral form in physiological conditions (pH 7.4, Figure 2 SI), especially, **K2F21**  
16 is almost completely undissociated, suggesting a shift in favour of the diketo form and a decrease in  
17 keto-enol acidity compared to C compounds, driven by the addition of the alkyl chain to the keto-  
18 enol moiety.

19 With respect to the lead curcumin, K and P type of compounds showed an improved stability  
20 in physiological conditions (Figure 3 SI), with a residual percentage (%) within the first 2 hours close

1 to 60 % and 25 % for P series and K series, respectively. The residual % was estimated as  $A_t \cdot 100/A_0$ ,  
2 where  $A_t$  and  $A_0$  stand for absorbance at  $\lambda_{\max}$  at *time t* and *time zero*, respectively.

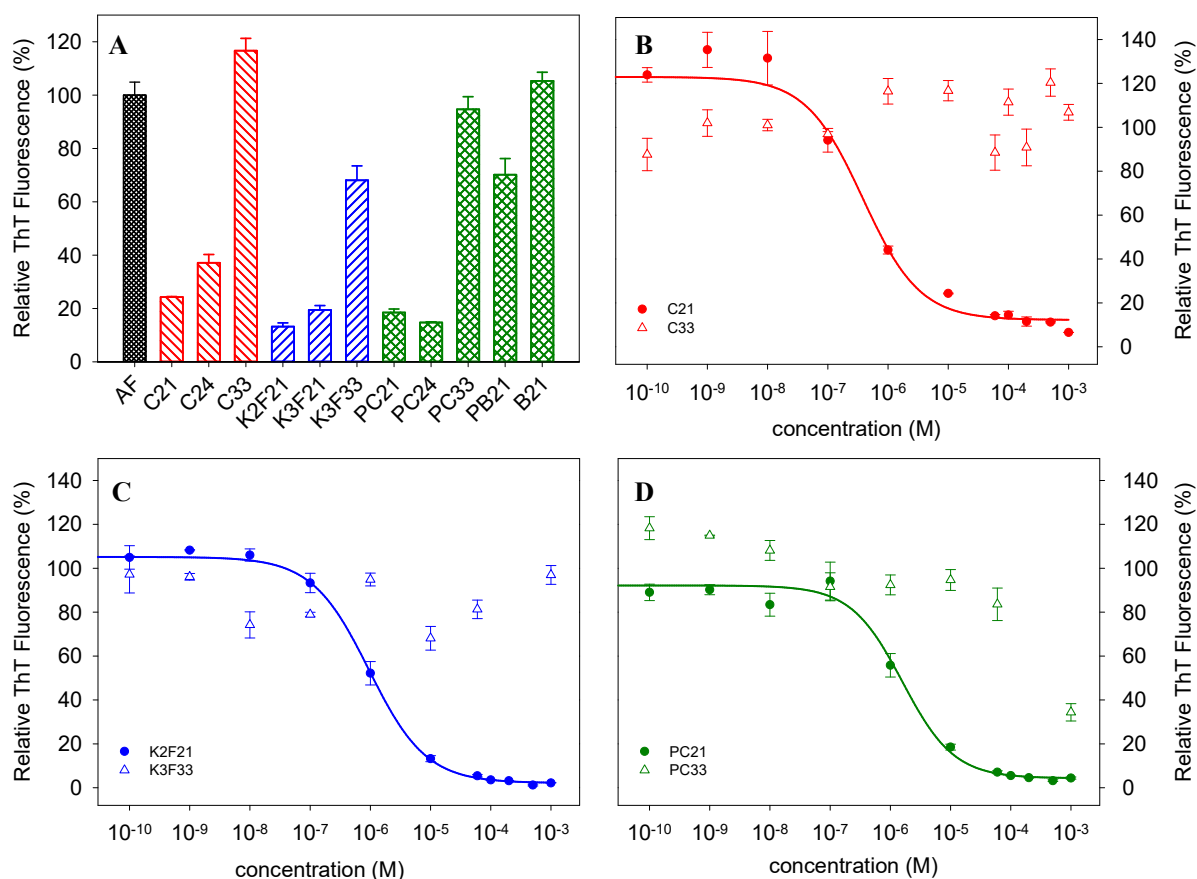
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#### 4 **2.2 Interference of curcumin derivatives with $A\beta_{1-40}$ amyloid fibrils in vitro.**

5 All derivatives were screened for their ability to interfere with  $A\beta_{1-40}$  amyloid fibrils. Initially we  
6 investigated the interaction of 10  $\mu\text{M}$  solution of each derivative with equimolar amounts of  $A\beta_{1-40}$   
7 fibrils using the Thioflavin T (ThT) fluorescence assay. The fluorescence intensities observed for  
8 fibrils after 24 h incubation with all derivatives (normalized to the fluorescence signal observed for  
9 fibrils alone (AF)) are shown in **Figure 3A**. The decrease in fluorescence intensity corresponds to the  
10 efficiency of derivatives to destroy the fibrils.

11 Addition of derivatives to  $A\beta_{1-40}$  amyloid fibrils led to different effects depending on their  
12 structure. Significant fluorescence decrease, corresponding to binding of compounds to fibrils and  
13 subsequent fibril disruption, was observed for derivatives with vanillin-like aromatic structure (**C21**,  
14 **K2F21**, **K3F21**, **PC21** and **PC24**). The fluorescence intensities are lower than 25% of fluorescence  
15 signal observed for untreated fibrils, indicating higher than 75% destroying efficacy. On the other  
16 hand, weak destroying activity was observed for derivatives from each series that have no substituent  
17 on the aromatic rings ( $R_1 = R_2 = \text{H}$ ), i.e. **C33**, **K3F33** and **PC33**. The fluorescence intensities observed  
18 for fibrils in presence of these derivatives were comparable or only slightly lower than signal detected  
19 for fibrils alone. A similar negligible destroying effect was determined for derivatives **PB21** and **B21**.  
20 In order to compare the ability to destroy  $A\beta_{1-40}$  amyloid fibrils, the  $\text{DC}_{50}$  values (compound  
21 concentration at which 50 % of fibrils are destroyed) were determined using ThT assay. ThT  
22 fluorescence intensities of 10  $\mu\text{M}$  solution of fibrils after 24 h incubation with curcumins at  
23 concentrations ranging from 100 pM to 1 mM were measured and data for selected derivatives are  
24 shown in **Figure 3B-D**. The  $\text{DC}_{50}$  values calculated for all studied compounds are reported in **Table**  
25 **1**.

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**Figure 3.** A) ThT fluorescence intensities of 10  $\mu\text{M}$   $\text{A}\beta_{1-40}$  fibrils alone (AF) and after 24 h treatment with 10  $\mu\text{M}$  curcuminoid derivatives. **B - D)** The effect of increasing curcumin derivative concentration on 10  $\mu\text{M}$   $\text{A}\beta_{1-40}$  fibrils detected using ThT assay: **B)** Curcumin (red circles), **C33** (empty red triangles); **C)** **K2F21** (blue circles), **K3F33** (empty blue triangles); **D)** **PC21** (green circles), **PC33** (empty green triangles). The average fluorescence values were fitted by a nonlinear least-square methods using SigmaPlot software and used for calculation of  $\text{DC}_{50}$  values. The fluorescence intensities were normalized to the fluorescence intensities observed for fibrils in the absence of derivatives (taken as 100%). The error bars represent the average deviation for measurements of fluorescence intensities of three replicates.

It is evident that the studied compounds have a different impact on  $\text{A}\beta_{1-40}$  amyloid fibrils, in particular the aromatic substituents rather than the central moiety ( $\beta$ -diketo/pyrazole) seems to drive the interaction with fibrils. Indeed, derivatives with no substituents on aromatic ring (i.e. **C33**, **K3F33**, **PC33**) have not noteworthy effect on amyloid fibrils (**Figure 3**, empty triangles), while the substituted ones favor disaggregation as shown by the fluorescence decrease that is dose-dependent (**Figure 3**, full circles) and allows to calculate  $\text{DC}_{50}$  values (**Table 1**).

**Table 1.** DC<sub>50</sub> values observed for studied curcumin derivatives after 24 h (\*) and 2 h (\*\*) incubation by Thioflavin T assay. (N/A - not available due to very weak depolymerizing activity.)

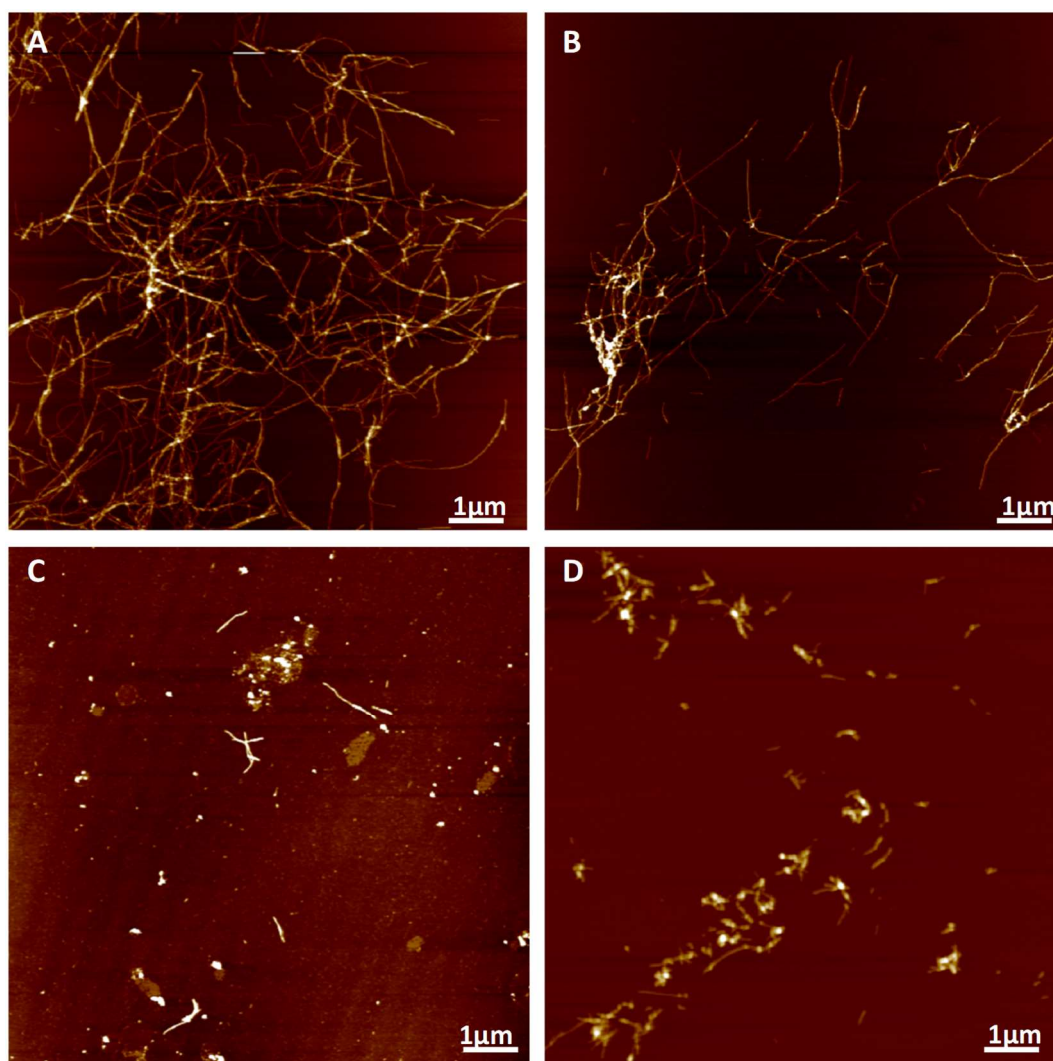
Compound	DC <sub>50</sub> (μM)*	DC <sub>50</sub> (μM)**
C21	0.78 ± 0.04	0.73 ± 0.07
C24	1.95 ± 0.54	1.0 ± 0.05
C23	N/A	
C33	N/A	
K2F21	1.11 ± 0.23	0.14 ± 0.02
K3F21	1.16 ± 0.18	
K3F33	N/A	
PC21	1.47 ± 0.37	
PC24	0.31 ± 0.01	0.65 ± 0.03
PC33	N/A	
PB21	38.8 ± 5.67	
B21	N/A	

Derivatives with the aromatic structure of vanillin ( $R_1 = \text{OCH}_3$  and  $R_2 = \text{OH/AcO}$ ), especially **PC24** and **C21**, showed the highest destroying effect, consistently with their lowest DC<sub>50</sub> values. Comparing **PC21** (1.47 μM) and **PB21** (38.8 μM), it is clear the need of a highly conjugated structure with π delocalized electrons. For **K** series, there is no major effect given by the length of the spacer in K series, and in general, all K derivatives showed a slightly lower activity compared to **C21**. These results are in great agreement with comprehensive study about structure-activity relationship of different curcumin derivatives done by Reinke and co-workers [16]. They have shown that the important features for activity of curcumin-based Aβ fibril ligands are: i) presence of both aromatic rings; ii) connection by rigid linker with a length of 8 - 16 Å; iii) hydroxyl or other polar substituents on the peripheral aromatic groups. Accordingly, top lead derivatives (**C21**, **C24**, **K2F21**, and **PC24**) satisfy all these criteria.

Furthermore, it was recently demonstrated that gallium-68 curcuminoids are able to bind Aβ<sub>1-40</sub> aggregates after 3 h incubation suggesting their potential applications as diagnostic tools for fibrils targeting using PET imaging technique [42,43]. Consequently, in order to evaluate the possibility to employ these derivatives for diagnostic purposes, we tested the activity of top lead compounds after 2 h incubation with Aβ<sub>1-40</sub> fibrils. As it is evident from **Table 1**, DC<sub>50</sub> values after 2h incubation are similar or even one order of magnitude lower (**K2F21**) than DC<sub>50</sub> values after 24 h incubation, hinting

1 a rapid binding to fibrillar aggregates and strong depolymerizing activity, with the strongest activity  
2 provided by **K2F21**.

3 Atomic force microscopy (AFM) can be exploited to confirm ThT results and visualize the drug  
4 effect on amyloid fibrils, Indeed,  $A\beta_{1-40}$  fibrils (control - untreated sample) have a typical unbranched,  
5 linear amyloid morphology (**Figure 4A**) that becomes less dense and shortens upon 24 h incubation  
6 with **K2F21** and **C21** (**Figure 4C and D**), confirming their strong interaction and destroying  
7 propensity. Unsubstituted compounds, like **C33**, don't spoil fibrillar aggregates (**Figure 4B**),  
8 confirming ThT assay inactivity.



9  
10 **Figure 4.** AFM images of 10  $\mu\text{M}$   $A\beta_{1-40}$  fibrils alone (**A**) and after 24 h treatment with 10  $\mu\text{M}$  curcumin  
11 derivatives with different extent of destroying activities: **C33** (**B**), **K2F21** (**C**) and **C21** (**D**).  
12

### 13 2.3. Atomic description of the binding to the $A\beta_{1-40}$ protofibrils by selected **curcuminoids**.

14 In order to investigate at atomic details the perturbations induced by curcuminoids binding to  
15 the  $A\beta_{1-40}$  preformed fibrils, MD simulations have been carried out on representative compounds of  
16 the three investigated series (C, P, K), that exhibited high affinity for  $A\beta_{1-40}$  fibril aggregates by ThT

1 assay, namely **C21**, **PC24**, and **K2F21**. The ligand putative binding sites were identified by means of  
2 docking experiments to a protofibril formed by ten A $\beta$ <sub>1-40</sub> monomer units replicated along the  
3 principal axis in order to obtain a continuous structure 6.5 nm long. The best poses obtained from the  
4 docking analysis of each compound on each binding site were then used as starting configuration for  
5 MD simulation runs, and the molecular mechanics-Poisson-Boltzmann surface area (MM-PBSA)  
6 method [44] has been used to obtain an accurate analysis of the stability of the ligand-A $\beta$  protofibril  
7 complexes.

8 The docking poses obtained for the ligands can be clustered into four binding sites, labelled  
9 as  **$\beta$ -1 side**,  **$\beta$ -2 side**, **in** and **top**, as shown in **Figure 5**. Moreover, **Table 2** lists the MM-PBSA  
10 interaction energy and the probability of the occupancy of each protofibril site, obtained by the  
11 docking exercises. In agreement with the previous studies [28,29], the major component of the total  
12 free binding energies is given by the van der Waals (vdW) interactions.

13 The  **$\beta$ -1** binding site comprises the amino acid residues 16-22. The interaction of **C21** with  
14 this region was first hypothesized on the basis of the results obtained by solid-state NMR, using  
15 dipolar assisted rotational resonance [45]. Successively this site was investigated by computational  
16 studies on models of A $\beta$  hexapeptide <sup>16</sup>KLVFFA<sup>21</sup> and full length A $\beta$  fibril [25,27]. The results  
17 obtained in the present study indicate a negligible probability for interaction of the ligands with the  
18  **$\beta$ -1** site locate at the external surface of the protofibril. Moreover, **only moderate free binding energy**  
19 **was obtained for** the ligands at this site (**Table 2**).

20 However, the <sup>16</sup>KLVFFA<sup>21</sup> stretch of amino acid can also be approached by the ligands after  
21 interaction with the **in** binding site (**Figure 5**), which is located inside the upper patch of the  $\beta$ -hairpins  
22 (19-34 amino acid residues), and in the **top** binding site, an extended region that involves amino acid  
23 residues 15-40, depending on the ligand (**Figure 5**). The **in** binding site was also found in the  
24 computational studies by Ngo *et al.* [28,29], and Battisti *et al.*[27]; whereas the **top** binding site has  
25 been previously investigated by Kundaikar *et al.* [26] by means of site map analysis.

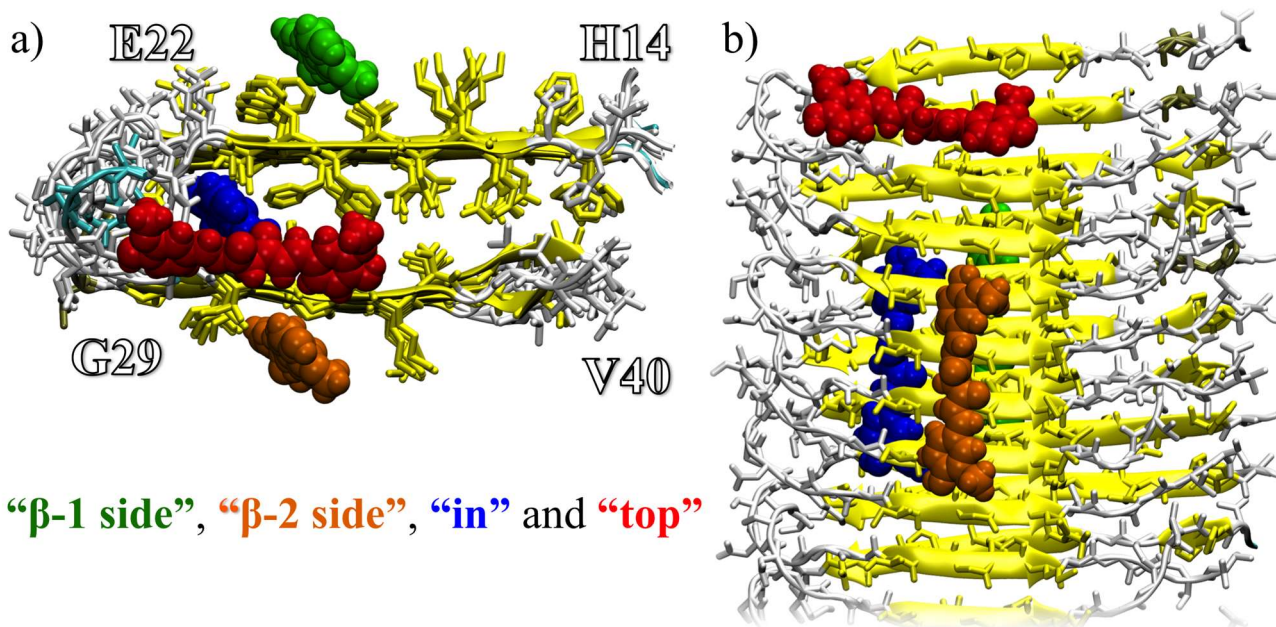
26 Finally, the  **$\beta$ -2** binding site is located in the 31-40 region, which is known to modulate fibril  
27 aggregation by means of methionine at the position 35 (M35) [46]. Therefore, this binding site is of  
28 potential interest to prevent aggregation in this zone [20], and it has been very recently targeted by  
29 Battisti *et al.* [27] in their combined computational and experimental study to design curcumin  
30 derivatives able to modify the aggregation pattern of A $\beta$  peptides.

31 Interestingly, all the ligands show a high probability of docking the **top site**, where they realizes loose  
32 interaction due to the high mobility during dynamics, whereas better interactions are observed at  
33 the  **$\beta$ -2** site (**Table 2**). The  **$\beta$ -1** and **in** binding sites give only a limited contribution to the overall  
34 landscape.

1 Perturbation in the fluctuations due to A $\beta$ <sub>1-40</sub> fibrils binding can be quantified by the Root  
 2 Mean Square Fluctuation (RMSF), and values of the fibril-C $\alpha$  atoms in the absence and presence of  
 3 the ligands are reported in **Table 2**. In the unbound fibril, the structured  $\beta$ -sheets regions  
 4 (corresponding to amino acid residues 13-22 and 31-40) show low flexibility with RMSF values of  
 5 around 0.9 Å, whereas the turn region connecting them (amino acid residues 23-30) shows RMSF  
 6 values of around 2.5 Å. These observations are in agreement with the recent ssNMR experimental  
 7 study of A $\beta$  peptide [47] and with the results of structural models of double-layer A $\beta$  segmental  
 8 polymorphism [48].

9  
 10 **Table 2.** MM-PBSA binding free energies (kcal mol<sup>-1</sup>) and probability of ligand occupancy of each binding  
 11 site ( **$\beta$ -1 side**,  **$\beta$ -2 side**, **in** and **top**). The variation of the RMSF values (Å) of the fibril-C $\alpha$  atoms (region aa10-  
 12 40), in the absence and presence of the ligands, are listed for the most significant sites ( **$\beta$ -2 side** and **top**).  
 13

Ligand	P%	E <sub>kcal/mol</sub>	P%	E <sub>kcal/mol</sub>	P%	E <sub>kcal/mol</sub>	P%	E <sub>kcal/mol</sub>	$\Delta$ RMF <sub>top</sub> (Å)	$\Delta$ RMF <sub><math>\beta</math>-2</sub> (Å)
	$\beta$ -1		$\beta$ -2		in		top			
C21	9	-26,3 ± 4,6	14	-22,4 ± 3,9	5	-28,5 ± 5,2	62	-16,7 ± 3,2	1.5	0.02
K2F21	9	-28,3 ± 5,4	19	-39,7 ± 4,7	5	-46,4 ± 5,3	77	-21,2 ± 9,5	0.5	0.11
PC24	7	-25,8 ± 5,1	30	-36,9 ± 3,8	2	-45,3 ± 4,3	71	-31,4 ± 4,5	1.0	0.03



15  
 16 **Figure 5.** The four main binding sites for the curcuminoid ligands as detected from AutoDock<sup>8</sup>; a) top view,  
 17 and b) side view. The fibril regions involved in the interactions are labelled as  **$\beta$ -1 side**,  **$\beta$ -2 side**, **in** and **top**.  
 18 The fibril is coloured accordingly to its secondary structure and the ligands with different colours for each  
 19 docking region.  
 20

1 The analysis of the evolution of the trajectories of the ligand-fibril complexes as a function of  
2 time reveals that in the **top** pose ligands are rather mobile and travel on the surface of the fibril  
3 contacting several residues (**Figure 4 SI**). As a result, curcumin leads to large fluctuations in the  
4 natural “dynamic breath” with respect to the isolated fiber; this effect is observed, to a minor extent,  
5 also for **K2F21** and **PC24**. On the contrary, the effect at **β-2** site is smaller, stiffening the protofibril.

6 Previous computational studies also found an increase of flexibility upon curcumin binding to  
7 the Aβ peptide [25,26,49]. In particular, Rao *et al.* [25] hypothesized that by increasing the fibril  
8 flexibility and promoting conformational changes, curcumin favours the formation of non-neurotoxic  
9 intermediate Aβ aggregates including dimers, oligomers, protofibrils, and fibrils in the Aβ-  
10 aggregation pathway.

11 In summary, we can conclude that curcumin shows a significant tendency to destabilize the  
12 protofibril by binding to the **top** site. The effect of distortion of the peripheral chain by curcumin may  
13 inhibit the process of elongation of the fibril along the principal axes, and/or catalyse the disruption  
14 of the β secondary structure. This perturbation is particularly strong at the peptide C-terminals, where  
15 M35 lies and can exert an indirect detrimental effect on the conformation/dynamics of this zone  
16 responsible for the hierarchical assembly of amyloid fibrils, as also pointed out by Kundaikar *et al.*  
17 [26]. Besides the interaction with the **top** site, **K2F21** and **PC24** show also a preference to bind at  
18 **β-2** site with moderate perturbative effect on the protofibril dynamics.

19 Therefore, in order to explain the perturbation induced by ligand binding and the consequent  
20 destruction of amyloid aggregates a concurrence of several effects should be invoked. These can be  
21 summarized as: a) alteration of the protofibril natural “dynamic breath” upon binding, and b) hiding  
22 of the amyloidogenic region by direct binding to the saddle near M35 (**β-2**) or indirectly affecting  
23 the M35 conformation and dynamics by binding to other sites.

#### 24 25 **2.4 Biological activity of curcuminoids on neuronal cells in vitro.**

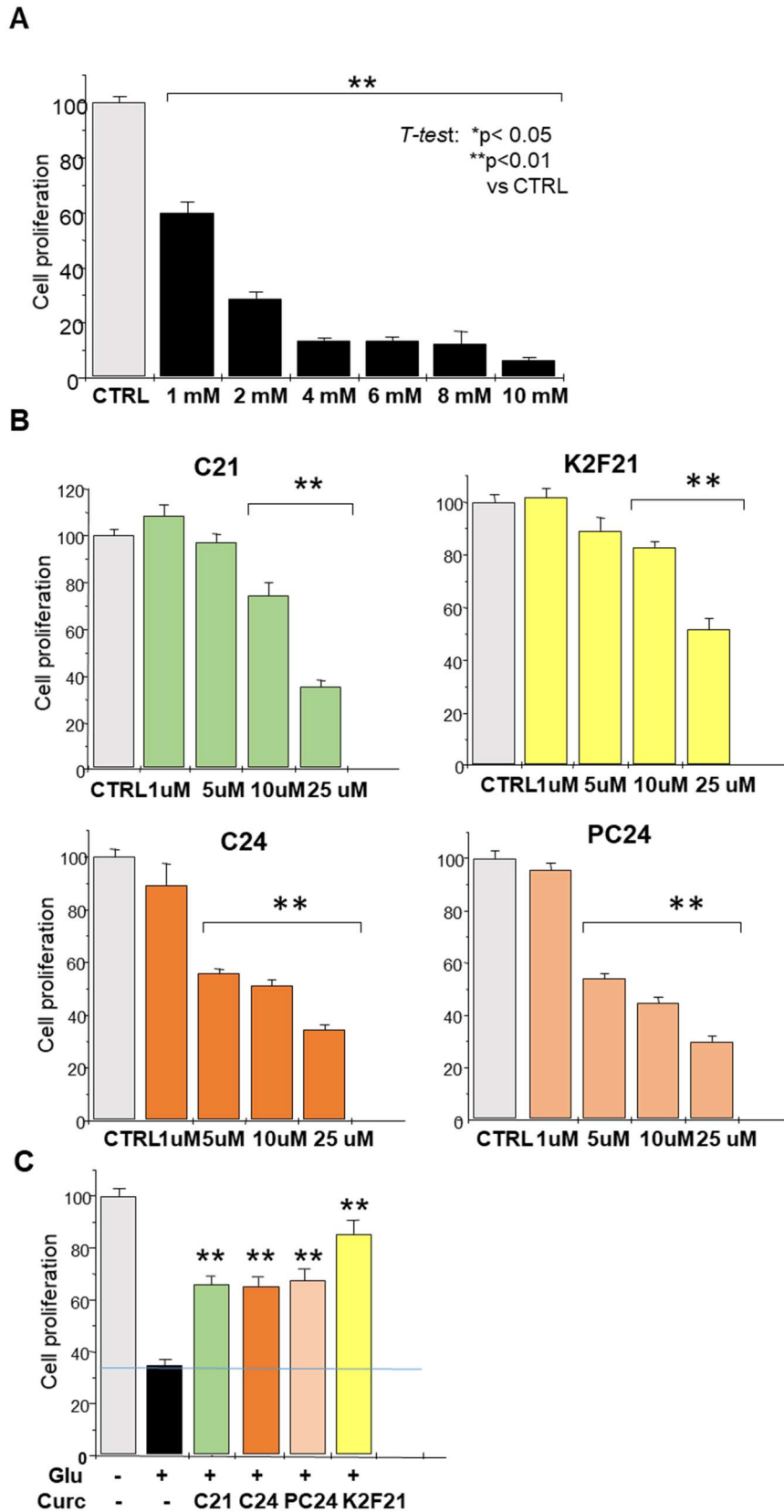
26 The glutamatergic system plays a key role in synaptic dysfunction and neuronal death induced by Aβ  
27 peptide in AD. Excessive extracellular glutamate concentration, due to disrupted glutamate  
28 uptake/recycling mechanisms and impaired glutamate neurotransmission, can induce excitotoxicity,  
29 a pathological process leading to neuronal cell death [50]. Therefore, we studied whether the  
30 curcuminoids with demonstrated disaggregating activity towards Aβ aggregates could exert a  
31 protective function in non-receptor mediated oxidative glutamate toxicity in mouse hippocampal HT-  
32 22 cells [50]. Through MTT assay, we first evaluated the concentration of glutamate able to halve  
33 cell proliferation and we selected 2 mM as IC<sub>50</sub> dose (**Figure 6 A**). Similarly, we identified the

1 concentrations at which the selected curcuminoids could have toxic effects on HT-22 cells (**Figure 6**  
2 **B**).

3 While curcumin (**C21**) and **K2F21** showed effects on cell viability at 10  $\mu$ M, **C24** and **PC24** resulted  
4 toxic already at 5  $\mu$ M. We thus selected 1  $\mu$ M as non toxic dose of curcuminoids that were further  
5 investigated for protective effect towards glutamate excitotoxicity. Co-administration of curcuminoids  
6 to glutamate was able to partially recover cell proliferation and limit glutamate toxicity (**Figure 6 C**).  
7 In particular, **C24**, **PC24** and **K2F21** showed similar or higher cytoprotective activity compared to  
8 **C21**.

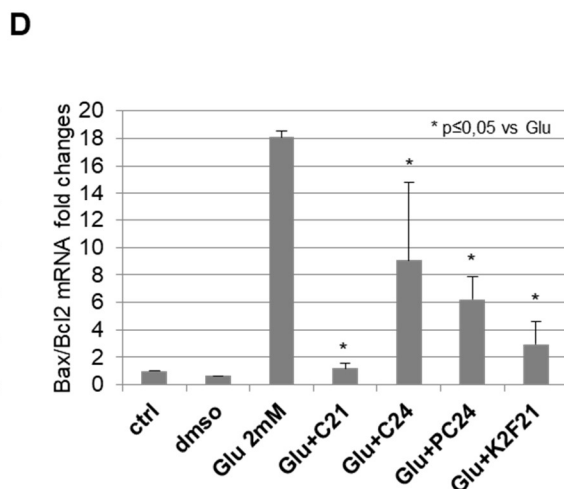
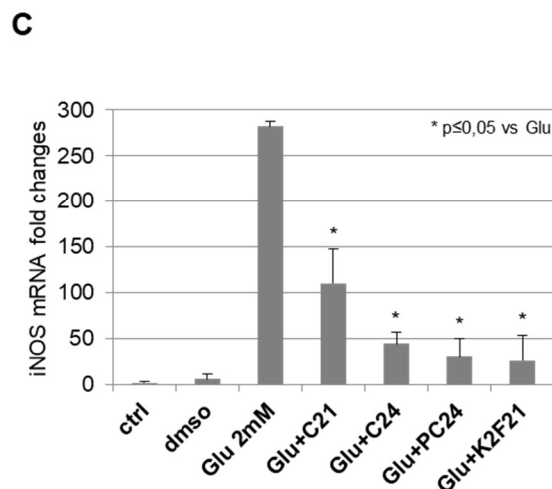
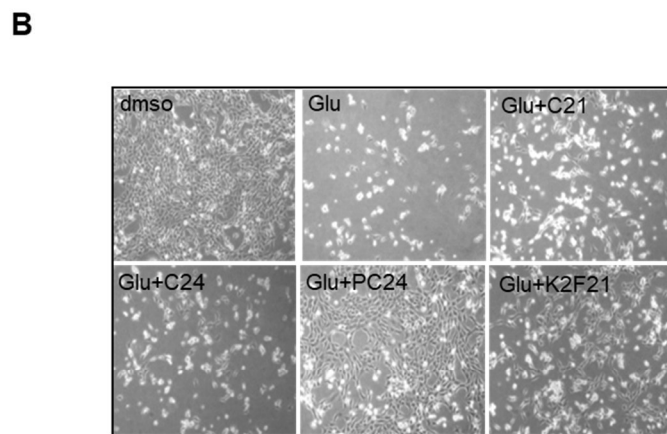
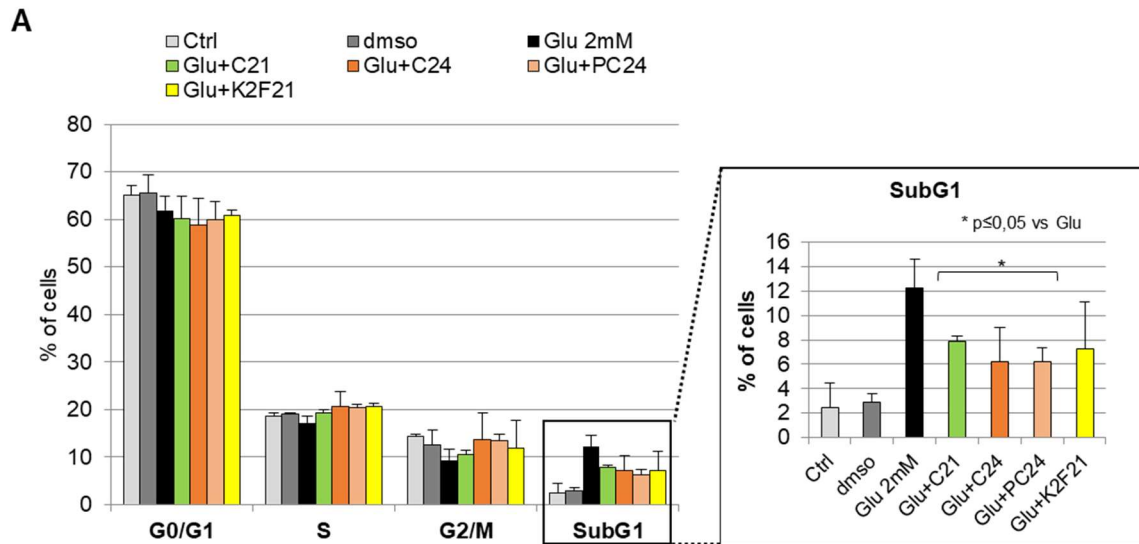
9 By means of Propidium-Iodide staining, we analyzed the effect of glutamate and  
10 curcuminoids co-treatment on cell cycle progression (**Figure 7A**). As expected, glutamate  
11 administration increased the number of cells in SubG1 phase, representing apoptotic or necrotic cells,  
12 from about 3% in DMSO to 12% in glutamate treated cells (**Figure 7A**). The co-administration with  
13 **C21**, **C24**, **PC24** and **K2F21** significantly reduced cell death induced by glutamate. Microscopic  
14 analysis of HT-22 cells corroborated the increase in viable cells following co-treatments compared to  
15 glutamate alone (**Figure 7 B**).

16 Since glutamate evokes oxidative stress in HT-22 and consequently activates apoptosis [51],  
17 we decided to better characterize the activity of **C24**, **PC24** and **K2F21** *versus* **C21**, through the  
18 analysis of the expression levels of key genes involved in oxidative stress and apoptotic cell death.  
19 qRT-PCRs showed an increase in the expression levels of inducible nitric oxide synthase (iNOS)  
20 following glutamate administration, corroborating the activation of oxidative stress response (**Figure**  
21 **7 C**). All the tested molecules reduced iNOS transcript levels, with **C24**, **PC24** and **K2F21** working  
22 even better than **C21**. To evaluate the anti-apoptotic activity of curcuminoids, we analyzed the *ratio*  
23 between the pro-apoptotic Bax and the anti-apoptotic Bcl-2 genes (**Figure 7 D**). The Bax/Bcl-2 *ratio*  
24 was arbitrarily set at 1 in control cells. The *ratio*, strongly increased by glutamate administration, was  
25 significantly decreased by all tested curcuminoids, consistently with cell cycle data (**Figure 7 A**).



1

2 **Figure 6.** Cytoprotective activity of curcuminoids against glutamate-induced toxicity in hippocampal HT-22  
3 mouse cells. A) Anti-proliferative activity of different concentrations of glutamate compared to control cells  
4 (CTRL, its proliferation arbitrarily set at 100%). B) Dose-response effect (1, 5, 10, 25  $\mu$ M) of the indicated  
5 ligands on HT-22 cell proliferation. C) Effect of the co-administration of glutamate (Glu = 2  $\mu$ M) with  
6 curcuminoids (1  $\mu$ M) on cell proliferation. Data are the mean of at least three independent experiments +/-  
7 standard deviation. \*\* pvalue < 0.01.



1  
 2 **Figure 7.** Anti-apoptotic activity of curcuminoids in hippocampal HT-22 mouse cells. **A)** Cytofluorimetric  
 3 cell cycle analysis of HT-22 cells treated with the indicated molecules (left panel). Right panel: enlargement  
 4 of the percentages of SubG1 events. Data are the mean of three independent experiments  $\pm$  SD. **B)**  
 5 Representative optical microscope images of HT-22 cells treated with DMSO, glutamate or glutamate together  
 6 with curcuminoids. **C)** qRT-PCR analysis of iNOS transcript in treated or untreated HT-22 cells. Expression  
 7 levels have been reported as fold change vs control cells (ctrl) (arbitrarily set at 1). **D)** Fold changes of the *ratio*  
 8 between Bax and Bcl2 transcripts, evaluated by qRT-PCR, in control (ctrl), DMSO or glutamate and  
 9 curcuminoids HT-22 treated cells. The *ratio* in control cells has been arbitrarily set at 1.  
 10 \* indicates pvalue < 0.05.

### 1 3. CONCLUSIONS

2 On the overall, the presented results suggest that the studied curcumin-based derivatives are  
3 able to interfere with A $\beta$  fibrils, and the entity of the exerted effect is modulated by their structure. In  
4 particular, the presence of a  $\pi$ -conjugated structure and both peripheral aromatic rings is confirmed  
5 to be of utmost importance in A $\beta$  fibrils' interaction, since **B21** and **PB21** showed very weak activity.  
6 Moreover, H-donor/acceptor substituents on the aromatic rings are the *sine qua non* structural feature  
7 for interaction and disaggregation activity even at very low incubation time. When comparing **C21**,  
8 **PC21** and **K2F21**, only minor differences are observed in the interaction with A $\beta$  fibrillar aggregates.  
9 Upon binding, the ligands modify the conformational dynamic and/or interact with the amyloidogenic  
10 region of the protofibril facilitating disaggregation, as demonstrated by computational simulations.  
11 However, since the ligand binding is non-specific, the A $\beta$  fibrils display several partially or not  
12 overlapping binding sites, in which dispersion interactions (vdW) act as the driving force.

13 Significantly, *in vitro* results on hippocampal cells demonstrate that the molecules are safe if  
14 administered at low concentrations, and can protect from glutamate toxicity.

15 To wrap up, this study pinpointed the importance of vanillin aromatic structure of  
16 curcuminoids to accomplish both interaction with A $\beta$  fibrils and cytoprotection against oxidative  
17 stress, while substitution on  $\beta$ -diketo moiety (K series) or the presence of pyrazole ring (P series)  
18 have effect on increasing stability in physiological conditions, and hopefully bioavailability in further  
19 *in vitro* and *in vivo* investigations.

20 On the whole, among all compounds, **K2F21** stands out as the best candidate for both  
21 diagnostic/therapeutic purposes, due to its high stability in physiological conditions, its rapid binding  
22 to fibrillar aggregates and strong depolymerizing activity, high cytoprotection against oxidative stress  
23 and low cytotoxicity.

24

### 25 4. EXPERIMENTAL SECTION

#### 26 4.1 Chemistry

27 All chemicals were reagent grade and used without further purification unless otherwise specified.  
28 Elemental analyses were performed on ThermoScientific<sup>TM</sup> FLASH 2000 organic elemental analyzer.  
29 UV-visible spectra were recorded with a Jasco V-570 UV/Vis/NIR spectrophotometer at 298 K in the  
30 250–600 nm spectral range employing quartz cells (1 cm optical path).

31 Liquid chromatography – mass spectrometry (LC-MS) experiments were performed on an Agilent  
32 6300 Ion Trap LC-MS system equipped with an electrospray ionisation (ESI) interface. The  
33 compounds were separated using Agilent Zorbax SB C18 30 $\times$ 2.1 mm, 3.5  $\mu$ m. Samples were  
34 prepared in MeOH and diluted to 10 ppm in MilliQ water; blank was MilliQ water. Eluent phase:

1 pump A H<sub>2</sub>O (formic acid 1%), pump B CH<sub>3</sub>CN (formic acid 1%), gradient: 10 % of B for 1 minute,  
2 10% –100% of B for 5 minutes, then 100 % of B for 4 minutes, flux 0.3 mL min<sup>-1</sup>, injection volume  
3 10 μL. The ion spectra were obtained in positive mode, using a scan range between *m/z* 100 and 1500.  
4 High-purity nitrogen was used as the nebuliser and the drying gas. The nitrogen drying gas was at a  
5 constant flow rate of 10 L min<sup>-1</sup>, heated to 350 °C. The nebuliser gas pressure was 32 psi and the  
6 capillary voltage was 3.5 kV.

7 NMR spectra were recorded on a Bruker FT-NMR AVANCE III HD 600 MHz spectrometer with  
8 5mm CryoProbe BBO H&F at 298 K. Nominal frequencies were 600.13 MHz for <sup>1</sup>H and 150.9 MHz  
9 for <sup>13</sup>C. For each sample, ~5 mg were weighed and diluted up to 0.6 mL with the proper deuterated  
10 solvent into 5 mm NMR tube. 90° pulse was calibrated for each sample and standard NMR parameters  
11 were used to achieve quantitative results (relaxation delay 10 s). Proton and carbon chemical shifts  
12 are given in parts per million (ppm) *versus* external TMS, and were determined by reference to the  
13 solvent residual signals (7.26 for CHCl<sub>3</sub> and 2.05 ppm for CD<sub>2</sub>HCOCD<sub>3</sub> for proton, and 77.2 for  
14 CDCl<sub>3</sub> and 29.8 ppm for (CD<sub>3</sub>)<sub>2</sub>CO for carbon). Typical 2D homo- and hetero-nuclear techniques  
15 were used for assignment, i.e. <sup>1</sup>H, <sup>1</sup>H-COSY, <sup>1</sup>H, <sup>13</sup>C-HSQC, <sup>1</sup>H, <sup>13</sup>C-HMBC. <sup>1</sup>H and <sup>13</sup>C NMR spectra  
16 for all compounds are reported in Supplementary Information. The purity of final compounds was  
17 determined to be at least 95% pure by a combination of HR-MS, NMR, and elemental analysis.

#### 18 **4.1.1 Synthesis.**

19 Compounds **C23**, **C24**, **C33** and **PC21** were synthesized as reported in the literature [36,52–54].  
20 *4-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-(3Z,5E)-3,5-esadien-2-one* (**B21**). This compound was  
21 synthesized by a modification of a procedure reported in the literature [55]. A suspension of boric  
22 anhydride (10 mmol) and 2,4-pentandione (10 mmol) in DMF (12 mL) was stirred for 30 min at 80  
23 °C, then tributylborate (4 mmol) was added, and the mixture was kept under stirring at 80 °C  
24 overnight. Vanillin (2 mmol) was added and followed by slow addition of *n*-butylamine (0.4 mmol  
25 in 0.5 mL of DMF). After stirring at 80 °C for 6 h, the solution was acidified with HCl 0.5 M (30 mL)  
26 under heating at 80 °C, then cooled down to room temperature. The aqueous phase was extracted  
27 with ethyl acetate, then the organic phases were washed with NaHCO<sub>3</sub> 5% and brine, before  
28 anhydrification under MgSO<sub>4</sub>. The crude product was purified by flash-column chromatography  
29 (silica gel, gradient: petroleum ether/ethyl acetate from 90/10 v/v to 30/70 v/v). Yellow powder, 40%  
30 yield. Elemental analysis calc. (%) for C<sub>13</sub>H<sub>14</sub>O<sub>4</sub> (234.25 g/mol): C, 66.66; H, 6.02. Found: C, 67.05;  
31 H, 6.17. LC-MS (ESI): *m/z* 235.1 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ (ppm) 5.79 (H-1, s, 1H), 6.51 (H-3,  
32 d, 1H), 2.15 (H-3', s, 3H), 7.54 (H-4, d, 1H), 7.21 (H-6, d, 1H), 6.83 (H-9, d, 1H), 7.09 (H-10, dd,  
33 1H), 3.92 (OCH<sub>3</sub>, s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ (ppm) 100.0 (C-1), 178.1 (C-2), 197.4 (C-2'), 119.2

1 (C-3), 25.3 (C-3'), 140.3 (C-4), 127.2 (C-5), 110.4 (C-6), 148.1 (C-7), 149.1 (C-8), 115.2 (C-9), 122.7  
2 (C-10), 55.2 (OCH<sub>3</sub>). Atom numbering refers to **Scheme 1 SI**.

3 *3,5-bis[(E)-2-(3-methoxyphenyl)ethenyl]-1H-pyrazole (PB21)*. This compound was synthesized by a  
4 modification of a procedure reported in the literature [56]. **B21** (2 mmol) was dissolved in glacial  
5 acetic acid (20 mL) and kept under stirring at 70 °C for 30 min, then hydrazine monohydrate (8 mmol)  
6 was added dropwise. The reaction was stopped after 8 h by removal of solvent under reduced pressure.  
7 The crude product was suspended in water, filtered and purified by flash-column chromatography  
8 (ethyl acetate/*n*-hexane 9/1 v/v). Pale yellow powder, 80% yield. Elemental analysis calc. (%) for  
9 C<sub>13</sub>H<sub>14</sub>N<sub>2</sub>O<sub>2</sub> (230.26 g/mol): C, 67.81; H, 6.13; N, 12.17. Found: C, 67.75; H, 6.18; N, 12.10. LC-MS  
10 (ESI): *m/z* 231.3 [M+H]<sup>+</sup>. <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (*ppm*) 9.06 (NH, s, 1H), 6.24 (H-1, s, 1H), 6.96  
11 (H-3, d, 2H), 2.27 (H-3', s, 3H), 7.03 (H-4, d, 2H), 7.19 (H-6, d, 2H), 6.82 (H-9, d, 2H), 6.98 (H-10,  
12 dd, 2H), 3.91 (OCH<sub>3</sub>, s, 6H). <sup>13</sup>C NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (*ppm*) 101.2 (C-1), 147.8 (C-2), 143.2 (C-2'),  
13 117.1 (C-3), 10.0 (C-3'), 129.1 (C-4), 129.5 (C-5), 108.9 (C-6), 147.8 (C-7), 146.7 (C-8), 115.1 (C-  
14 9), 120.2 (C-10), 55.4 (OCH<sub>3</sub>). Atom numbering refers to **Scheme 1 SI**.

15 *4,4'-[1H-pyrazole-3,5-diyl di(E)ethene-2,1-diyl]bis(2-methoxyphenol) (PC21)*. Synthesis of PC21  
16 was performed as previously reported [56]. Orange powder, 80% yield. Elemental analysis calc. (%)  
17 for C<sub>21</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub> (364.39 g/mol) C 69.22%, H 5.53%, N 7.69%. Found: C 69.13%, H 5.62 %, N 7.75  
18 %. LC-MS-IT *m/z* 365.4 (M + H)<sup>+</sup>. <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (*ppm*) 9.17 (NH, s, 1H), 6.68 (H-1, s,  
19 1H), 7.02 (H-3, d, 2H), 7.14 (H-4, d, 2H), 7.22 (H-6, d, 2H), 6.85 (H-9, dd, 2H), 7.01 (H-10, dd, 2H),  
20 3.92 (OCH<sub>3</sub>, s, 6H). <sup>13</sup>C NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (*ppm*) 98.94 (C-1), 146.94 (C-2), 120.83 (C-3), 129.85  
21 (C-4), 129.45 (C-5), 108.89 (C-6), 147.75 (C-7), 146.94 (C-8), 115.19 (C-9), 120.4 (C-10), 54.77  
22 (OCH<sub>3</sub>). Atom numbering refers to **Scheme 1 SI**.

23 *1H-pyrazole-3,5-diyl bis[(E)ethene-2,1-diyl(2-methoxyphenyl-4,1-diyl) diacetate (PC24)*. This  
24 compound was obtained by direct acetylation of **PC21** [52]. Light brown powder, 20% yield.  
25 Elemental analysis calc. (%) for C<sub>25</sub>H<sub>24</sub>N<sub>2</sub>O<sub>6</sub> (448.47 g/mol): C, 66.95; H, 5.39; N, 6.2. Found: C,  
26 66.83; H, 5.42; N, 6.18. LC-MS (ESI): *m/z* 449.4 [M+H]<sup>+</sup>. <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (*ppm*) 5.64 (H-1,  
27 s, 1H), 7.27 (H-3, d, 2H), 7.78 (H-4, d, 2H), 7.34 (H-6, d, 2H), 6.89 (H-9, d, 2H), 7.08 (H-10, dd,  
28 2H), 3.97 (OCH<sub>3</sub>, s, 6H), 2.70 (OCOCH<sub>3</sub>). <sup>13</sup>C NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (*ppm*) 55.4 (C-1), 146.5 (C-2),  
29 134.0 (C-3), 114.8 (C-4), 129.5 (C-5), 109.1 (C-6), 147.8 (C-7), 146.9 (C-8), 115.2 (C-9), 120.9 (C-  
30 10), 55.4 (OCH<sub>3</sub>), 22.2 (OCOCH<sub>3</sub>), 170.7 (OCOCH<sub>3</sub>). Atom numbering refers to **Scheme 1 SI**.

31 *3,5-bis[(E)-2-phenylethenyl]-1H-pyrazole (PC33)*. Synthesis of **PC33** was performed as previously  
32 reported, starting from **C33** [56]. Light yellow powder, 80% yield. Elemental analysis calc. (%) for  
33 C<sub>19</sub>H<sub>16</sub>N<sub>2</sub> (272.34 g/mol): C, 83.79; H, 5.92; N, 10.29. Found: C, 83.72; H, 5.98; N, 10.02. LC-MS  
34 (ESI): *m/z* 273.3 [M+H]<sup>+</sup>. <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (*ppm*) 9.06 (NH, s, 1H), 6.82 (H-1, s, 1H), 7.20

1 (H-3, d, 2H), 7.25 (H-4, d, 2H), 7.58 (H-6/H-10, d, 4H), 6.39 (H-7/H-9, d, 2H), 7.29 (H-8, d, 2H).  
2 <sup>13</sup>C NMR ((CD<sub>3</sub>)<sub>2</sub>CO): δ (ppm) 100.0 (C-1), 146.5 (C-2), 118.5 (C-3), 129.7 (C-4), 137.2 (C-5), 126.3  
3 (C-6/C-10), 128.8 (C-7/C-9), 127.7 (C-8). Atom numbering refers to **Scheme 1 SI**.

4 *2-(4-acetyl-5-oxohexyl)-1H-isoindole-1,3(2H)-dione (K3F)*. 2,4-pentandione (25 mmol) is added to  
5 a suspension of K<sub>2</sub>CO<sub>3</sub>/KI (50/3 mmol) in dry acetone (15 ml) at 80 °C and kept under stirring for 1  
6 h. Then, a solution of 2-(3-bromopropyl)-1H-isoindole-1,3(2H)-dione (25 mmol) in dry acetone (5  
7 mL) is added dropwise. After stirring overnight at 80°C, the solution was diluted with aqueous NH<sub>4</sub>Cl  
8 and extracted twice with CH<sub>2</sub>Cl<sub>2</sub>. The organic phase was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>.  
9 Removal of the solvent afforded an oily residue, which was purified through distillation under  
10 reduced pressure to give the desired product as yellow oil (yield 45%). Elemental analysis calc. (%)  
11 for C<sub>16</sub>H<sub>17</sub>NO<sub>4</sub> (287.31 g/mol): C, 66.89; H, 5.96; N, 4.88. Found: C, 67.00; H, 6.01; N, 4.95. LC-  
12 MS (ESI): *m/z* 288.3 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ (ppm) 2.15 (H-3/H-3', s, 6H), 2.32 (H-11, t, 2H)  
13 1.83 (H-12, m, 2H), 3.77 (H-13, t, 2H), 7.88 (H-16, dd, 2H), 7.77 (H-17, dd, 2H); <sup>13</sup>C NMR (CD<sub>3</sub>OD):  
14 δ (ppm) 109.1 (C-1), 190.0 (C-2), 26.0 (C-3), 25.1 (C-11), 29.1 (C-12), 37.8 (C-13), 168.5 (C-14),  
15 123.3 (C-15), 132.5 (C-16), 134.0 (C-17). Atom numbering refers to **Scheme 2 SI**.

16 *2-(3-acetyl-4-oxopentyl)-1H-isoindole-1,3(2H)-dione (K2F)*. This compound was obtained as **K3F**  
17 using 2-(2-bromoethyl)-1H-isoindole-1,3(2H)-dione. Yellow oil, 40% yield. Elemental analysis calc.  
18 (%) for C<sub>15</sub>H<sub>15</sub>NO<sub>4</sub> (273.28 g/mol): C, 65.92; H, 5.53; N, 5.13. Found: C, 65.88; H, 5.59; N, 5.10.  
19 LC-MS (ESI): *m/z* 274.3 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ (ppm) 2.63 (H-3/H-3', s, 6H), 3.56 (H-11, t,  
20 2H), 4.04 (H-12, t, 2H), 7.80 (H-16, m, 2H), 7.69 (H-17, m, 2H); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ (ppm) 108.1  
21 (C-1), 189.0 (C-2), 36.8 (C-3), 28.3 (C-11), 39.1 (C-12), 167.7 (C-14), 131.9 (C-15), 123.4 (C-16),  
22 134.1 (C-17). Atom numbering refers to **Scheme 2 SI**.

23 *(2-((4Z,6E)-5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-4-((E)-3-(4-hydroxy-3-  
24 ethoxyphenyl)acryloyl) hepta-4,6-dien-1-yl)isoindoline-1,3-dione (K3F21)*. This compound was  
25 synthesized as other similar curcuminoids, using K3F and vanillin as reagents [34]. The crude  
26 product was recrystallized from EtOH to give the title compound as red powder (yield: 15%).  
27 Elemental analysis calc. (%) for C<sub>32</sub>H<sub>29</sub>NO<sub>8</sub> (555.58 g/mol): C, 69.18; H, 5.26; N, 2.52. Found: C,  
28 68.97; H, 5.32; N, 2.60. LC-MS (ESI): *m/z* 556.3 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ (ppm) 6.72 (H-3, d,  
29 2H), 7.63 (H-4, d, 2H), 7.06 (H-6, d, 2H), 6.94 (H-9, d, 2H), 7.12 (H-10, dd, 2H), 1.93 (H-11, t, 2H),  
30 1.49 (H-12, m, 2H), 3.70 (H-13, t broad, 2H), 7.85 (H-16, m, 2H), 7.73 (H-17, m, 2H). <sup>13</sup>C NMR  
31 (CDCl<sub>3</sub>): δ (ppm) 109.0 (C-1), 182.8 (C-2), 121.3 (C-3), 145.1 (C-4), 128.3 (C-5), 109.6 (C-6), 147.2  
32 (C-7), 148.3 (C-8), 114.8 (C-9), 124.3 (C-10), 27.8 (C-11), 23.2 (C-12), 38.0 (C-13), 168.0 (C-14),  
33 132.3 (C-15), 123.1 (C-16), 133.9 (C-17). Atom numbering refers to **Scheme 2 SI**.

1 2-((4Z,6E)-4-cinnamoyl-5-hydroxy-7-phenylhepta-4,6-dien-1-yl)isoindoline-1,3-dione (**K3F33**).  
2 This compound was obtained as **K3F21** from **K3F** and benzaldehyde, yielding a yellow powder  
3 (yield: 35%). Elemental analysis calc. (%) for C<sub>30</sub>H<sub>25</sub>NO<sub>4</sub> (463.52 g/mol): C, 77.74; H, 5.44; N, 3.02.  
4 Found: C, 77.63; H, 5.52; N, 3.09. LC-MS (ESI): *m/z* 464.2 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ (ppm) 7.07  
5 (H-3, d, 2H), 7.80 (H-4, d, 2H), 7.60 (H-6/H-10, m, 4H), 7.42 (H-7/H-8/H-9, m, 6H), 2.67 (H-11, t,  
6 2H), 2.04 (H-12, m, 2H), 3.91 (H-13, t broad, 2H), 7.92 (H-16, m, 2H), 7.79 (H-17, m, 2H). <sup>13</sup>C NMR  
7 (CDCl<sub>3</sub>): δ (ppm) 110.4 (C-1), 182.7 (C-2), 120.1 (C-3), 142.1 (C-4), 135.2 (C-5), 128.5 (C-6/C-10),  
8 129.0 (C-7/C-9), 130.1 (C-8), 23.7 (C-11), 30.8 (C-12), 37.7 (C-13), 168.5 (C-14), 134.3 (C-15),  
9 123.3 (C-16), 134.1 (C-17). Atom numbering refers to **Scheme 2 SI**.

10 2-((3Z,5E)-4-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-3-((E)-3-(4-hydroxy-3-methoxyphenyl)  
11 acryloyl) hexa-3,5-dien-1-yl) isoindoline-1,3-dione (**K2F21**). This compound was obtained as **K3F21**  
12 from **K2F** and vanillin, yielding a red powder (yield: 20%). Elemental analysis calc. (%) for  
13 C<sub>31</sub>H<sub>27</sub>NO<sub>8</sub> (541.55 g/mol): C, 68.75; H, 5.03; N, 2.59. Found: C, 68.63; H, 5.12; N, 2.55. LC-MS  
14 (ESI): *m/z* 542.2 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ (ppm) 7.29 (H-3, d, 2H), 7.75 (H-4, d, 2H), 7.47 (H-  
15 6, dd, 2H), 6.99 (H-9, dd, 2H), 7.20 (H-10, dd, 2H), 2.90 (H-11, t, 2H), 3.89 (H-12, t (broad), 2H),  
16 2H), 7.88 (H-16, m, 2H), 7.83 (H-17, m, 2H), 4.15 (OCH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ (ppm) 106.7 (C-1),  
17 183.1 (C-2), 117.6 (C-3), 142.4 (C-4), 128.1 (C-5), 109.1 (C-6), 147.1 (C-7), 148.1 (C-8), 114.6 (C-  
18 9), 124.1 (C-10), 25.7 (C-11), 38.6 (C-12), 168.0 (C-14), 132.2 (C-15), 133.9 (C-16), 123.0 (C-17),  
19 56.2 (OCH<sub>3</sub>). Atom numbering refers to **Scheme 2 SI**.

20 **4.1.2 Kinetics stability at physiological conditions.** The chemical stability of the selected  
21 curcuminoids **C23**, **C24**, **PC21**, **PC24** and **K2F21** at 37 °C in darkness was evaluated by UV-vis  
22 spectroscopy. The change in absorbance in the 250–600 nm range over an overall period of 24 h was  
23 recorded for 50 μM solutions of the compounds in phosphate buffered solution (PBS) 0.01 M at pH  
24 7.4. A constant ionic strength of 0.1 M (NaCl) was maintained in all experiments.

25 **4.1.3 Spectrophotometric pH-dependent titrations.** 50 μM aqueous solution of selected compounds  
26 (**B21**, **K3F21** and **PC21**), obtained by dilution of 2.50 × 10<sup>-3</sup> M methanol solution, was investigated  
27 in the 250–600 nm range on varying pH by negligible additions of concentrated NaOH or HCl (4 M)  
28 in the 2–11 (**PC21**) or 5–11 (**B21**, **K3F21**) pH range. The overall protonation constants (logβ<sub>qr</sub>) were  
29 evaluated from spectrophotometric data using computer program HypSpec [40].

## 30 **4.2 Studies on Aβ<sub>1-40</sub> peptide *in vitro*.**

31 Recombinant human Aβ<sub>1-40</sub> peptide (Cat #A-1001) was purchased from rPeptide Company (Georgia,  
32 USA). Aβ<sub>1-40</sub> was dissolved in aqueous NaOH 10 mM to reach 665 μM stock concentration defined  
33 by UV-Vis spectroscopy using extinction coefficient 2300 M<sup>-1</sup> cm<sup>-1</sup> at λ = 292 nm. The solution was  
34

1 sonicated for 1 min in bath sonicator, and centrifuged for 10 min at 4 °C and 12000 rpm. The  
2 concentration of solution was checked spectrophotometrically. For amyloid fibril preparation, the  
3 stock solution was incubated for 5 days at 37 °C without agitation.

4 The stock solution of A $\beta$ <sub>1-40</sub> amyloid fibrils was diluted with 150 mM 3-(N-morpholino)  
5 propanesulfonic acid (MOPS) buffer (0.035% NaN<sub>3</sub>, pH = 6.9) to a final concentration of 10  $\mu$ M.  
6 Curcuminoid derivative at 10  $\mu$ M concentration was added to solution of 10  $\mu$ M amyloid fibrils and  
7 incubated for 24 h at 37 °C. The interference of derivatives with A $\beta$ <sub>1-40</sub> fibrils was evaluated using  
8 Thioflavin T (ThT) fluorescence assay and atomic force microscopy. For quantification of the  
9 destroying ability the interference of the studied curcumin derivatives were examined for samples  
10 containing 10  $\mu$ M A $\beta$ <sub>1-40</sub> fibrils and derivative in the concentration range 100 pM – 1 mM, using ThT  
11 assay. Briefly, ThT was added to the samples containing 10  $\mu$ M A $\beta$ <sub>1-40</sub> fibrils alone and after treatment  
12 with curcuminoid to 20  $\mu$ M final concentration. The samples were incubated at 37 °C for 1 h in the  
13 dark. Then, the samples were excited at 440 nm (slit width 9 nm) and the subsequent emission was  
14 measured between 465 and 600 nm (slit width 9 nm) with maximal fluorescence intensity peak at 485  
15 nm. ThT fluorescence intensities were detected in black 96-well plates using a Synergy Mx (BioTek  
16 Company, USA) well plate reader. To measure the intrinsic fluorescence of the tested compounds the  
17 peptide was replaced with buffer solution. The volume of DMSO in measuring samples was lower  
18 than 2% and has no effect on the stability of A $\beta$ <sub>1-40</sub>. All ThT experiments were performed in triplicate  
19 and the final value is the average of measured values with standard deviation ( $\pm$  SD).

20 Samples for Atomic Force Microscopy (AFM) were prepared by spreading solutions of 10  
21  $\mu$ M fibrils and 10  $\mu$ M derivatives on a freshly cleaved mica surface and leaving them for 5 min to  
22 adsorb on the surface. After 5 min adsorption, the samples were washed with ultrapure water (18.2  
23 M $\Omega$  cm) and left to dry under nitrogen. AFM images were taken using a Scanning Probe Microscope  
24 (Veeco di Innova, Bruker AXS Inc., Madison, USA) in a tapping mode using an NCHV cantilever  
25 with specific resistance of 0.01 – 0.025  $\Omega$  cm, antimony (n) doped Si, radius of the tip curvature of  
26 10 nm. The resolution of image was 512 pixels per line (512  $\times$  512 pixels per image) and scan rate  
27 0.5 kHz. No smoothing or noise reduction was applied.

28 Interaction of curcumin derivatives with A $\beta$ <sub>1-40</sub> fibrils leading to their destroying was  
29 characterized using DC<sub>50</sub> values (half-maximal concentration with 50% destroying activity). The  
30 destruction of A $\beta$ <sub>1-40</sub> (concentration was fixed at 10  $\mu$ M) induced by increasing concentration of  
31 curcuminoid derivatives ranging from 100 pM to 1 mM was detected using ThT assay. ThT  
32 fluorescence intensities measured in the presence of derivatives were normalized to the fluorescence  
33 signal of amyloid fibrils alone. The final DC<sub>50</sub> value represents the average value obtained by fitting  
34 three independent concentration dependencies with the non-linear least-squares method (SigmaPlot:

1 sigmoidal, 3-parameters logistic:  $y = a / [1 + \exp(x - x_0)/b]$  where  $x_0$  corresponds to  $DC_{50}$  value). To  
2 measure the intrinsic fluorescence of the tested compounds the peptide was replaced with buffer  
3 solution. The volume of DMSO in measuring samples was lower than 2% and has no effect on the  
4 stability of fibrils and  $A\beta_{1-40}$  fibrillization.

5

### 6 **4.3 Computational details.**

7 The structure of **C21**, **K2F21** and **PC24** each curcumin derivative was built in its DK form,  
8 except for **C21**, for which both the DK and KE forms were considered. The simulation results are  
9 essentially the same, therefore we reported the results for the KE form. The force field for each  
10 derivative was built in the Gromacs format [57] by using the Automated Topology Builder[58,59]  
11 (ATB) web server.

12 The structural model of  $A\beta$  fibrils formed by the 40-residue peptide based on numerous  
13 constraints from solid state NMR and electron microscopy was retrieved from the Protein Data Bank  
14 (PDB ID: 2LMN [60]). The missing N-terminal peptide region of  $A\beta$  peptide was built using the  
15 Molefacture plugin in the VMD package [61] as random coils as predicted by both the Jpred web  
16 server [62] and by the Modeller package [63] for protein secondary structure assignments.

#### 17 **4.3.1 Molecular Docking**

18 The binding modalities of the compounds studied with the  $A\beta_{1-40}$  fibril were investigated by  
19 means of the AutoDock [64] program. The  $A\beta$  fibril was built by replicating the monomeric unit 10  
20 times along its principal axis obtaining a continuous structure 6.5 nm long. The region of the fibril  
21 used to perform the docking comprises the  $\beta_1$ ,  $\beta_2$   $\beta$ -sheets and the coil connecting them (amino acid  
22 residues from 13 to 40). The N-terminal region was not considered because of its high mobility. The  
23 fibril was kept rigid, whereas flexibility was allowed to the compounds in order to obtain a good  
24 accuracy and affordable computational costs.

25 A grid of  $126 \times 126 \times 126$  points, with a grid spacing of  $0.375 \text{ \AA}$ , was selected. It was centred  
26 at centre of mass of the first five monomers, with the aim of covering half of the whole the fibril  
27 surface. The docking of the ligands was performed using the Lamarckian Genetic Algorithm with a  
28 population of 150 individuals, during 200 runs for 27000 generations with 25 million energy  
29 evaluations. The docking results were afterward clustered setting an RMS tolerance value of 2.0, to  
30 identify the principal orientations of the ligands. The most representative structure of each of the  
31 principal clusters for every ligand/docking system was selected for further analysis.

#### 32 **4.3.2 Molecular Dynamics Simulations.**

33 Molecular dynamics simulations were performed with GROMOS 54a7 force field [65]. **This**  
34 **force fields has been shown to improve the stability of secondary structure elements, while retaining**

1 the agreement with observed data such as NOE intensities and 3J-couplings [65]. Very recently, the  
2 a99SB-disp, a force field of the Amber family, has been developed to achieve excellent agreement  
3 with experiment for disordered proteins, while maintaining state-of-the-art accuracy for folded  
4 proteins [66]. However, a recent work on the interaction of natural compounds on full-length A $\beta$ <sub>40</sub>  
5 fibrils showed that the Gromos54a7 force field is able to grasp the destabilizing effects on the rotein  
6 structure upon binding [67].

7 Simple point charge (SPC) water [68] was added to the simulation box (7.5 × 9.7 × 8.0 nm)  
8 and a salt concentration of 150 mM NaCl was used to neutralize the system. The particle-mesh Ewald  
9 (PME) algorithm was used to calculate long-range electrostatics [69,70] with a fourth-order cubic  
10 interpolation, a grid spacing of 0.16 nm, and a real-space cutoff of 1 nm. Both van der Waals and  
11 neighbour list cutoffs describing short-range interactions were set to 1.0 nm. The temperature in all  
12 simulations was kept constant at 310 K and the pressure was set to 1 bar in order to mimic  
13 physiological conditions. The temperature was controlled using a velocity-rescaling thermostat with  
14 a coupling time of 0.1 ps. During the equilibration run the pressure was controlled by the Berendsen  
15 barostat, while during the production run by the Parrinello-Rhman barostat with coupling time of 2  
16 ps and an isothermal compressibility of 4.5 × 10<sup>-5</sup> bar<sup>-1</sup>. All computational simulations (production  
17 runs) were 100ns long and the time step used was 2.0 fs. Data analysis were performed using the  
18 Gromcas-5.0.4 package[71].

19 MM\_PBSA [44,72] is based on the single-trajectory approach, thus 250 snapshots (25 ns)  
20 sampled over the course of the 100 ns simulation were used, starting the calculation when the system  
21 reached an equilibrium configuration (approximately after 10 ns of the beginning of the production  
22 run).

#### 24 4.4 Cell culture and treatments.

25 The mouse clonal hippocampal neuronal cell line HT-22 was a generous gift from Dr. Pamela Maher  
26 (The Salk Institute for Biological Studies, La Jolla, San Diego, CA). Cells were maintained at 37 °C  
27 and 5% CO<sub>2</sub> in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%  
28 heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS-Gibco), 50 U/mL penicillin, 50 mg/mL  
29 streptomycin, 5 mM glutamine (Biowest). Cells were harvested and cultured in 96- or 6-well plates  
30 according to the requirement of the experiment.

31 To examine the effect of C21, C24, PC24 and K2F21, cells were treated the day after plating  
32 with different concentrations (1, 2, 5, 10, 25 μM in complete medium) for 24 h. A stock solution of  
33 the drugs (10 mM) was prepared in DMSO and diluted in culture medium keeping the final DMSO  
34 concentration at 0.025%. The same concentration of DMSO was used as negative control. To examine

1 the effect of glutamate (#G8415-Sigma Aldrich) cells were exposed to different concentrations (1, 2,  
2 4, 6, 8, 10 mM) in complete medium for 24 h. **C21**, **C24**, **PC24** and **K2F21** (1  $\mu$ M) were added to  
3 the media containing glutamate 2 mM for the co-treatment. The selection of concentrations was based  
4 on the results of MTT assays.

#### 5 **4.4.1 Cell viability assay (MTT assay)**

6 Cell viability was determined through the analysis of the conversion of MTT to MTT-formazan by  
7 mitochondrial enzymes as follows. HT-22 cells ( $5 \times 10^3$ ) plated in 96-well plates were treated for 24  
8 h with **C21**, **C24**, **PC24**, **K2F21** and glutamate, or co-treated with curcuminoids and glutamate at the  
9 selected concentrations. After 24 h at 37 °C, 150  $\mu$ L of MTT solution (5 mg/mL Thiazolyl Blue  
10 Tetrazolium Bromide in PBS) was added to cells at a final concentration of 0.5 mg/ml per well and  
11 incubated for 2 h at 37 °C. The medium was then removed carefully and 150  $\mu$ L of MTT solvent (4  
12 mM HCl, 0.1% NP40 in isopropyl alcohol) was added to resolve the blue formazan in living cells.  
13 After 15 min of incubation with a gently shaking in the dark, the absorbance at 570 nm was read with  
14 an ELISA reader (Multiskan FC, ThermoScientific).

#### 15 **4.4.2 Flow cytometric cell cycle analysis**

16 HT-22 cells ( $1.5 \times 10^5$ ) plated in 6-well plates were co-treated with glutamate and curcuminoids at  
17 the selected concentrations, for 24 hours. Cell pellets were then collected by centrifugation, washed  
18 with PBS and resuspended in 400  $\mu$ l of PI solution (0.1%, Triton, 3.4 M Na citrate, 50  $\mu$ g/mL  
19 Propidium Iodide). After 30 min of incubation, cells were analyzed for DNA content using  
20 cytofluorimeter (Beckman coulter).

#### 21 **4.4.3 mRNA expression analysis (RT-PCR)**

22 RNA was extracted from cells by using RNeasy mini kit (Qiagen, Hilden, Germany), according to  
23 the manufacturer's protocol. For cDNA synthesis, 1  $\mu$ g of RNA was retrotranscribed with a Moloney  
24 murine leukemia virus reverse transcriptase (RT) (Promega). Quantitative Real-Time PCR was  
25 performed with SsoAdvanced Universal SYBR Green Supermix (Biorad) in a Roche LC480 Cycler.  
26 mRNA amplification was performed with the following oligonucleotides: Rplp0 forward: 5'-  
27 GAGCCAGCGAGGCCACACTG -3'; Rplp0 reverse: 5'- CCACGTTGCGGACACCCTCC -3'; Bax  
28 forward: 5'- AGGGTTTCATCCAGGATCGAGCAG -3'; Bax reverse: 5'-  
29 ATCTTCTTCCAGATGGTGAGCGAG -3'; Bcl2 forward: 5'- CACAGAGGGGCTACGAGTG-3';  
30 Bcl-2 reverse: 5'- CAAAGGCATCCCAGCCTCC-3'; iNOS forward: 5'-  
31 ACGAGACGGATAGGCAGAGA- 3'; iNOS reverse: 5'- GAGTAGTAGCGGGGCTTCAA-3'.

32 Expression levels of target genes were normalized to the levels of the housekeeping gene Rplp0. The  
33 relative fold change expression of sample was calculated with the comparative  $\Delta\Delta$ Ct method [73].

34

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