Research paper

Antioxidants from the class of polyphenols, NAC and Vitamin C act differently on amyloid fibril formation by two human cystatins: stefin B and cystatin C.

Alma Jahić1, Samra Hasanbašić2, Magda Tušek Žnidarič3, Selma Berbić1 and Eva Žerovnik2,4*

[1] *Faculty of Pharmacy, Department of Biochemistry, University of Tuzla, Univerzitetska 1, 75000 Tuzla, Bosnia and Herzegovina.*

[2] *Jožef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia.*

[3] *Department of Biotechnology and Systems Biology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia.*

[4] *Department of Biochemistry and Molecular and Structural Biology, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia.*

Corresponding author: Eva Žerovnik e-mail: eva.zerovnik@ijs.si

Abstract

We compare the effect on amyloid fibril formation by two homologous proteins from the family of cystatins, human stefin B (stB) and cystatin C (cysC) in presence of 3 polyphenols: curcumin, resveratrol and quercetin and 2 non-phenolic anti-oxidants: vitamin C (VitC) and N-acetyl cystein (NAC). Some of the experimental data have already been presented, here we compare, further discuss and highlight the results. The amyloid fibril formation was followed by ThT fluorescence and transmission electron microscopy. Inhibitory effects on amyloid fibrillation reaction depended on anti-oxidant class and concentration. The fact that different effect of polyphenols was observed with the two cystatins; Cur acted inhibitory on stB but not on cysC fibril formation, could be explained if the 3 polyphenols would not bind to the same binding site in the fibrils core. Other differences are pointed out and discussed. Synergistic effects of VitC and chosen polyphenols on amyloid fibrilllation of human stB have been explored and are reported here for the first time.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

INTRODUCTION

Neurodegenerative diseases, among them most prevalent Alzheimer's and Parkinson's disease, stem from protein misfolding and aggregation. Misfolded proteins form deposits extracellularly and inclusions inside cells, including neurons. The soluble fractions of these aggregates are more toxic to the cells than fibrils and it is believed that they interact with membranes and even perforate them. Many indirect membrane oligomers interacton studies confirm this notion, among them in silico and structural studies[1] as well as biophysical lipid protein interaction studies[2]. However, it remains to see amyloid pores in living cells.

As a model for folding and later mis-folding and aggregation to amyloid fibrils we have studied human stefin B in comparison to a more stable stefin A and numerous of its mutants and chimeric variants. Solving 3D structure of the tetramer of stefin B by both X-rays and by NMR in solution, we have shown that the oligomers are domain-swapped and that the tetramer is composed of two domain-swapped dimers formed in a process of loops exchange[3]. Cystatin C also forms domain swapped dimers and their structure has been determined by X-ray and NMR in solution, respectively[4, 5]. In the mechanism of stefin B amyloid fibril formation, we have studied the role of oligomers in the process, wher the lower oligomers might be off-pathway[6, 7]. Interaction of stefin B oligomers with membranes was demonstrated *in vitro*. It was shown that the size, morphology and interaction of the oligomers with lipid membranes correlate with toxicity[8-10], resembling pore forming toxins[11].

Under normal physiological conditions, human stefin B (alternative name is cystatin B), as a housekeeping gene, acts as intracellular cathepsins inhibitor. It is localized in the cytosol but also in the nucleus[12]. Stefin B protein was found multimeric in cells[13, 14], which suggests it may have alternative functions than cysteine proteases inhibition. Its oligomers were shown to interact with cytoskeletal proteins[13] and to bind Aβ[15], suggesting a chaperone-like function[16, 17]. Recently, the Italian group has demonstrated that stefin B (cystatin B) is important in physiology of the synapse[18] and is essential for proliferation and interneuron migration[19]. When cystatin B gene is mutated and either loses activity or aggregates or both, it causes a rare progressive myoclonus epilepsy – EPM1[20, 21], spred in the Baltics and some parts of the Mediteranian.

Human cystatin C mutation L68Q also causes a severe disease, hereditary cystatin C amyloid angiopathy (HCCAA). HCCAA is a rare autosomal dominant genetic disease observed in a small part of population of Iceland, only. The amyloid deposits of the L68Q mutated CysC, which accumulate in the brain arteries, weaken arterial walls, which leads to repeated brain hemorrhages (mini-strokes) and may result in dementia and paralysis[22]. Cystatin C also binds Aβ[23-25] and was found neuroprotective, loaded in extracellular vesicles[26]. Not at least, cystatins are constituents of amyloid plaques[19].

As we are studying antioxidant effects on protein aggregation it is worth to mention that stefin B was shown to bind Cu2+ in contrast to stefin A[27]. The binding of the metal influenced protein aggregation, it inhibited amyloid fibrils but increased granular and amorphous aggregates[27]. It is known that deposits of amyloid fibrils interact with metals, such as Cu2+ and Fe3+ and raise free radicals in the Fenton's reaction, generating H2O2. Externally applied antioxidant substances can help the body to fight free radicals. Sometimes antioxidants can become pro-oxidant under certain conditions, such as

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

transition metals presence, generating by themselves H2O2 as is the case with vitamin C (ascorbic acid) at higher concentrations [28]. Indirectly, some substances could also act as antioxidants if they would reduce protein aggregation. The idea that polyphenolic compounds reduce protein aggregation by direct binding to amyloid core structure is not new[29].

Here, we highlight and discuss our own studies of the influence of chosen anti-oxidants on protein aggregation to amyloid fibrils, comparing 2 different human proteins. We studied amyloid fibril formation in presence of polyphenolic compounds, vitC and NAC of human stefin B (stB) and cystatin C (cysC) from the family of cystatins and found different behavior. We try to explain these observations by subtle differences in protein sequence and by several binding sites to different anti-oxidant substances.

RESULTS AND DISCUSSION:

1. Effect of curcumin on stefin B fibrillation; kinetics and TEM measurements.

We followed the kinetics of amyloid fibril formation of stefin B in presence of various concentrations of curcumin (Cur) and without this substance - as a control, by using ThT fluorescence. Figure 1 shows the fibrillation reaction of stefin B as a function of time for various concentrations of Cur. Stefin B alone was made to fibrillate at 25°C, pH 4.8, adding 10% 2,2,2, trifluoro-ethanol (TFE) . as described before[30] and is given in more details under Methods.

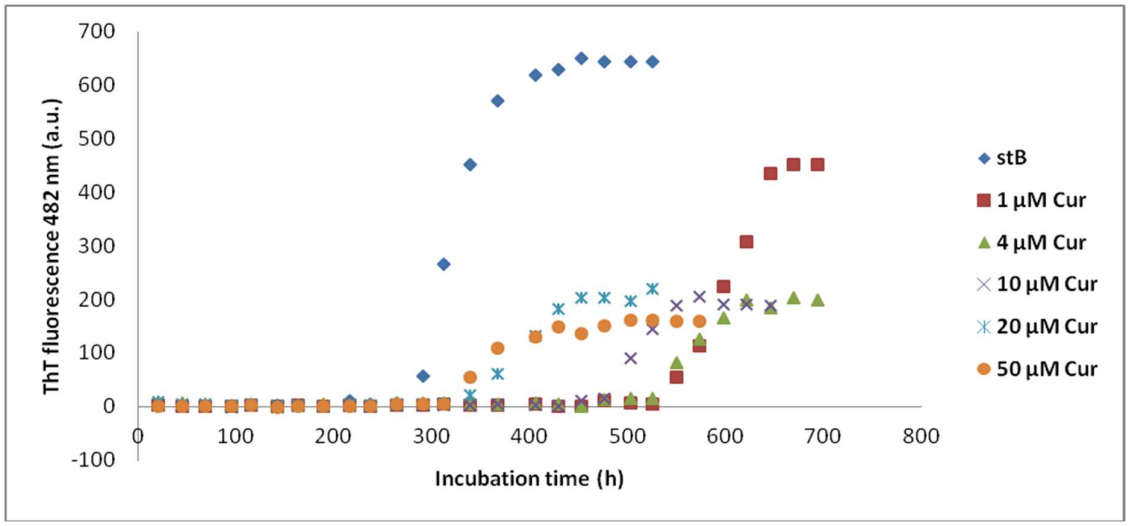

Figure 1: Time dependence of ThT fluorescence intensity following the aggregation kinetics of stefin B in the absence and presence of different concentrations of curcumin (Cur). ThT fluorescence emission at 482 nm was monitored upon excitation at 440 nm. Protein concentration was 34 µM. Each sample was incubated at room temperature in 0.015 M acetate buffer, 0.15 M NaCl at pH 4.8, in presence of 10% TFE [31].

The results of the kinetics of amyloid fibril formation (Figure 1) show that stB in presence of Cur forms less amyloid fibrils at the plateau phase of the reaction as revealed by final ThT fluorescence

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

intensity and that the effect on the lag phase is significant and dependent on Cur concentration. Inhibitory action on amyloid fibrils formation is most expressed at lower concentrations (1 µM Cur and 4 µM Cur), where the lag phase as compared to stB alone is prolonged to 250 hours. The lag phase prolongation at presence of 10 µM Cur is 150 hours, while at higher Cur concentrations (20 µM Cur and 50 µM Cur) it is even less, only 50 hours.

The intensity of ThT fluorescence is substantially lower at all Cur concentrations apart from the lowest (1 µM Cur), which might indicate inhibition of fibril growth, i.e., that the amount of the amyloid fibrils by stB is less in presence of Cur as compared to stB alone. However, one should be aware that quenching effects might be present on ThT fluorescence by aromatic and polyphenolic compounds [32], therefore, another method is needed to check the inhibitory effect.

To check the morphology and to some degree also amount of the fibrils we conducted transmission electron microscopy (TEM) experiments. The samples of stB with Cur, which showed the biggest decrease in ThT intensity (50 µM Cur) as compared to stB alone in Figure 2A are shown in Figure 2B. It can be seen that there are less fibrils and that they are shorter yet thicker. At the lowest Cur concentration (1 µM Cur) fibrils are very scarce, instead, granular aggregates are abundant (Figure 2C), which agrees with the longest lag phase and the strongest inhibition. Intensity of ThT fluorescence of stB amyloid fibrils at the plateau (Figure 1), therefore, does not reflect their true amount (Figure 2B); at the 2 highest Cur concentration fluorescence intensity gets lowered due to quenching phenomenon.

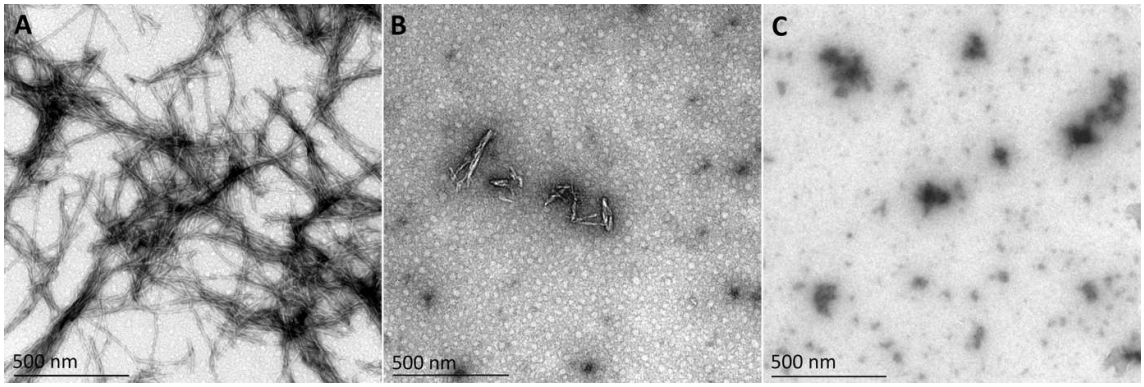

Figure 2: Representative TEM data of the plateau phase of the fibrillation reaction by stefin B without curcumin in water, as a control (A) and with curcumin at 50 µM (B) and 1µm (C) concentration. Protein concentration was 34 µM throughout.

2. The effect of resveratrol on stefin B fibrillation; kinetics and TEM measurements.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

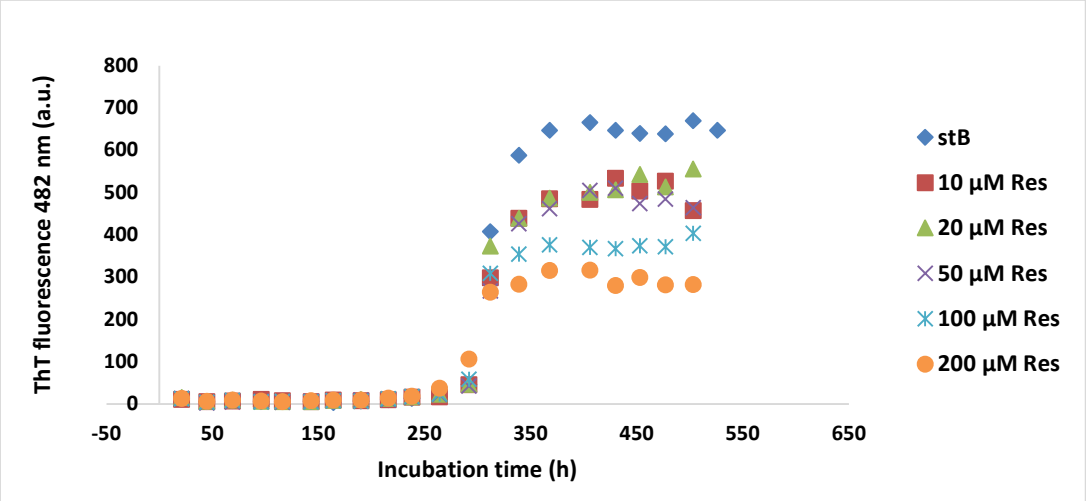

Figure 3: Time dependence of ThT fluorescence intensity following the aggregation kinetics of stefin B in the absence and presence of different concentrations of resveratrol (Res). ThT fluorescence emission at 482 nm was monitored upon excitation at 440 nm. Protein concentration was 34 µM. Each sample was incubated at room temperature in 0.015 M acetate buffer, 0.15 M NaCl at pH 4.8, in presence of 10% TFE.

As shown in Figure 3, Res also diminishes ThT fluorescence of stB fibrils, depending on Res concentration. ThT fluorescence intensity gets lowered at higher concentrations of 100 and 200 µM Res, while it does not influence the lag phase of the fibrillation reaction at any concentration.

To check the amount and morphology of stB fibrils in presence of Res, TEM was performed. The results are shown in Figure 4. TEM of samples from the plateau phase at the 2 highest concentrations of (200 µM Res and 100 µM Res) and those at the 2 lower concentrations (10 µM Res and 50 µM Res) were measured. At 10 µM Res (Figure 4A) no major difference can be observed as compared to the control (Figure 2A). At 50 µM Res and at 100 µM Res the fibrils are thicker and their amount is less (Figures 4 B,C). At 200 µM Res aggregates are more abundant, whereas the amyloid fibrils are thicker (Figure 4D). Alltogether, there was no marked inhibition observed concominant with unchanged lag phase as in Figure 3.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

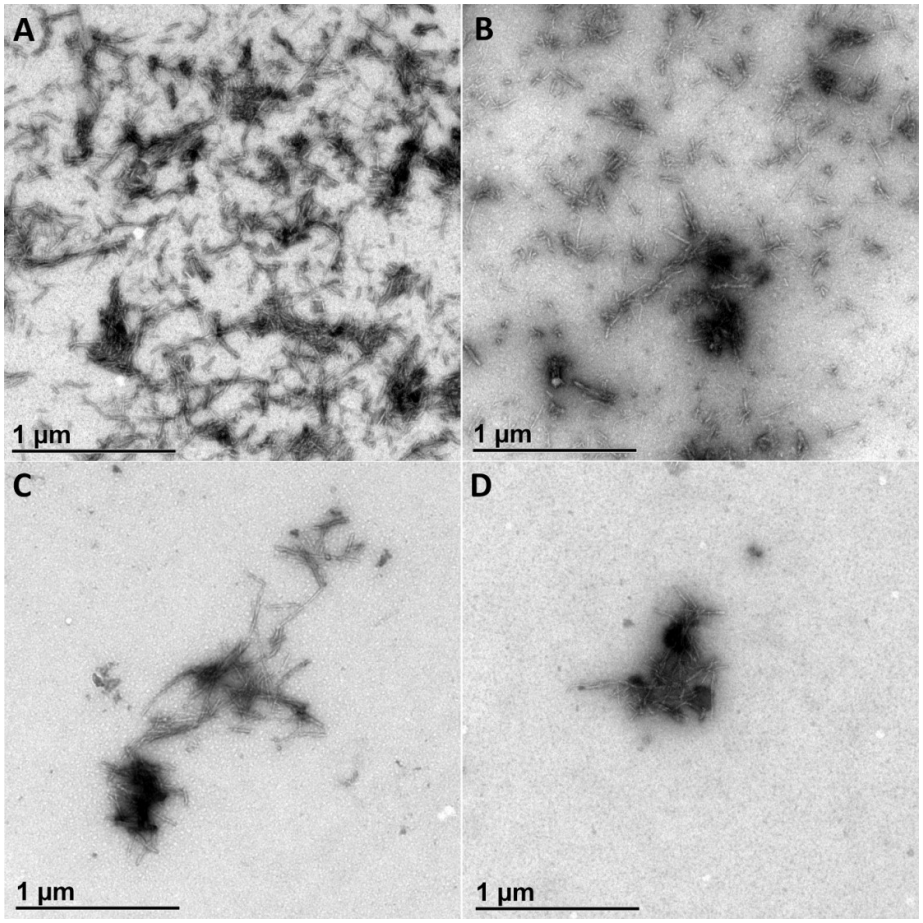

Figure 4: Representative TEM data of the plateau phase of the fibrillation reaction by stefin B with resveratrol at 10 µM (A) and 50 µm (B) and 100 µM (C) and 200 µM (D) concentration. Protein concentration was 34 µM throughout.

3. Effect of quercetin on stefin Bfibrillation

As studied by Hasanbasić et al.[31] in presence of Quer ThT fluorescence intensity of stB amyloid fibrils got lowered and the lag phase prolonged by 50 hours. The effect on ThT fluorescence intensity was stronger at the higher concentrations. Only, 1 µM Quer did not show any effect as compared to stB alone.

TEM of the final amyloid fibrils was also measured after the plateau phase at 50 µM Quer and 1 µM Quer. At both concentrations there were more amorphous aggregates while amyloid fibrils were more scarce and of changed morphology[31].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

4. Comparison of the effect of 3 polyphenols to stefin B and cystatin C amyloid fibril formation

As shown by Hasanbasic et al.[31], representative data are presented again in Figures 1 and 3 (ThT fluorescence) and Figures 2 and 4 (TEM), stB amyloid fibril formation is strongly inhibited by Cur and modestly by Res and Quer.

On the contrary, cysC is not much affected by the 3 polyphenols, as shown by Jahić et al., 2020[33]. For that study, cysC amyloid fibril formation was induced at 50°C and pH 4 at constant shaking (mixing). CysC amyloid fibril formation in presence of Res was studied first. 200 μM Res produced a significant decrease in ThT fluorescence, however, no change in the lag phase was observed as is the case with stB. At the two lower concentrations of 100 and 50 μM Res, the lag phase got slightly shorter, however, this may be within the error of measurements. No reduction in the amount of amyloid fibrils at the plateau phase of the reactions could be observed by TEM for any of Res concentrations. As well curcumin and quercetin did not demonstrate inhibition of amyloid fibril formation of cysC[33]. TEM measurements thus confirm that the 3 studied polyphenols do not change amyloid fibrils morphology and qualitatively also the amount of cysC fibrils in comparison to the control.

We are trying to explain these open questions: 1. Why do the 3 polyphenols affect amyloid fibril formation of stB and cysC differently? and, 2. Why does Cur inhibit stB fibrillation and not as much cysC? It seems that Res acts similarly on cysC and stB, while Cur and Querc have different effects on the two homologous proteins. This would be possible if Res and Cur would bind to different sites on the amyloid core. Previously Porat, Abramovitz and Gazit[29] thought that all polyphenols position their aromatic rings to the cross-β structure of amyloid core and therefore would act similarly. In line with this assumption the effect of all polyphenols on cysC and stB would be expected similar. However, one observes different action of Res, Cur and Quer on amyloid fibril formation of cysC and stB. Regardless of the similarly structured amyloid core with the cross-β structure, this may be explained by an assumption that sequence details and precise arrangement of the subunits in the protofilaments influence aromatic-aromatic π stacking interactions[34]. Indeed, precise arrangement of protofilaments can influence π stacking[35, 36] and the same protein can form polymorphic amyloid fibrils, which have specific chemical and biological properties[37, 38].

5. The effect of of N-acetylcystein (NAC) on stefin B fibrillation; kinetics and TEM measurements.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

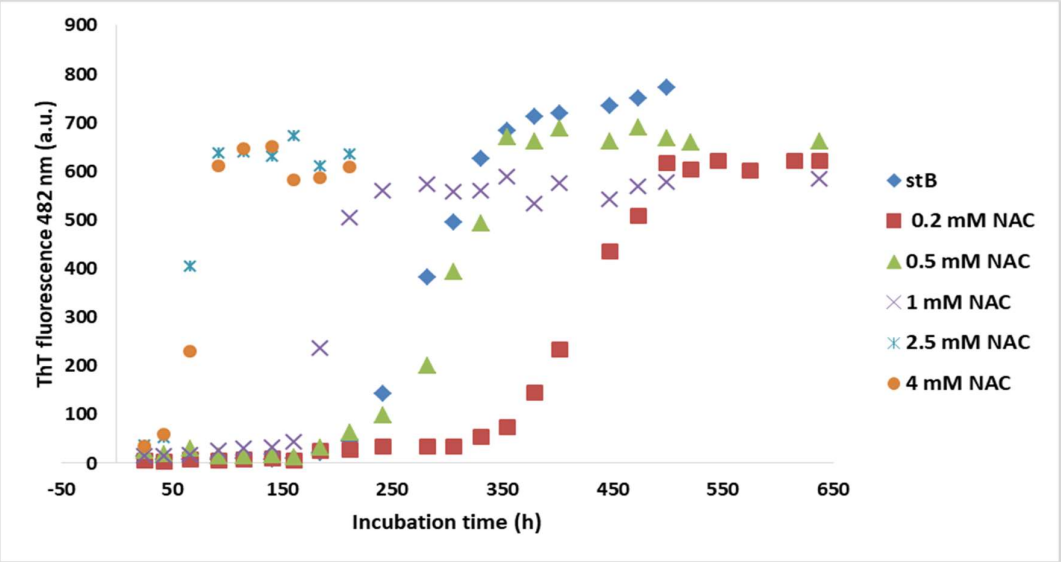

Figure 5: Time dependence of ThT fluorescence intensity following the aggregation kinetics of stefin B in the absence and presence of different concentrations of N-acetyl cystein (NAC). ThT fluorescence emission at 482 nm was monitored upon excitation at 440 nm. Protein concentration was 34 µM. Each sample was incubated at room temperature in 0.015 M acetate buffer, 0.15 M NaCl at pH 4.8, in presence of 10% TFE.

As shown in Figure 5 the time course of ThT fluorescence, as of interest, the highest concentrations of NAC (2,5 mM NAC and 4 mM NAC) decrease the lag phase of stB fibrillation reaction substantially. In contrast at the lowest concentration of 0.2.mM NAC the lag phase gets prolonged. No effect on the lag phase can be observed at 1 mM NAC. At all concentrations of NAC the ThT fluorescence intensity remains more or less the same.

That NAC does not change the amount of amyloid fibrils, it even seems to increase the rate of their formation at higher than 0.2 mM concentration, was confirmed by TEM. Samples of stB at the plateau of the reaction in presence of 4mM NAC were measured[33]. The fibrils were abundant and did not differ from the control (not shown).

.

6. The effect of vitamin C on stefin B fibrillation; kinetics and TEM measurements.

From the results of the kinetics by ThT fluorescence (Figure 6) one can deduce similar concentration effect of vitamin C (VitC) on the lag phase as was the case with NAC. At the highest VitC concentration of 2.5 and 4 mM VitC this substance accelerates fibrils growth. In contrast, at the lowest concentrations measured (0.2 and 0.5 mM) VitC prolongs the lag phase by nearly 250 hours. Inhibitory effect is also observed at 1 mM VitC concentration where the lag phase is prolonged by nearly 150 hours. However, in distinction to NAC, VitC at all concentrations decreases the intensity of

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

ThT fluorescence. TEM results (not shown) confirmed that less amyloid fibrils and more aggregates were presentat 0.2 mM VitC. Highest concentrations, especially 4mM VitC accelerated the fibrillation reaction. At the plateau there were more fibrils seen[33].

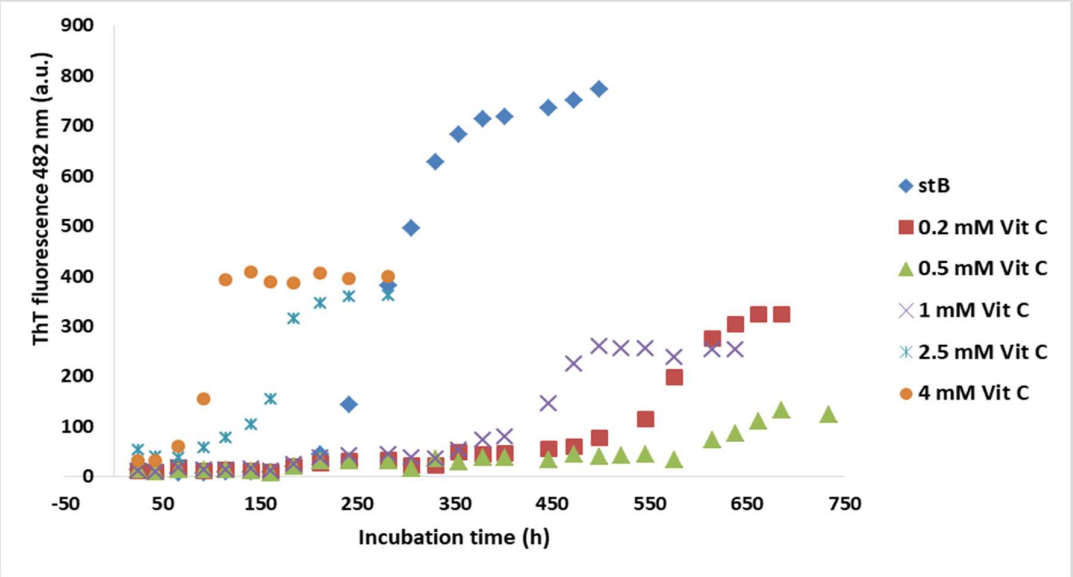

Figure 6: Time dependence of ThT fluorescence intensity following the aggregation kinetics of stefin B in the absence and presence of different concentrations of vitamine C (Vit C). ThT fluorescence emission at 482 nm was monitored upon excitation at 440 nm. Protein concentration was 34 µM. Each sample was incubated at room temperature in 0.015 M acetate buffer, 0.15 M NaCl at pH 4.8, in presence of 10% TFE.

7. Comparison of the effects of VitC and NAC on kinetics of stB and cysC fibril formation

ThT measuremnts of cysC in presence of vit C show that at the concentration of 0.5 mM VitC completely inhibits amyloid fibril formation, while it does not prolong the lag phase (not shown). This was confirmed by TEM measurements where nearly no fibrils of cysC were seen at 0.5 mM VitC concentration. At 1 mM concentration also much less fibrils could be detected by TEM in comparison to cysC alone but there was more amorphous material seen[33]. The effect of vitC on CysC is thus similar than the effect on stB, namely, it acts inhibitory at lower concentrations while at higher the effect gets reversed. We explain different effects at lower and higher concentrations of vitC by dual, contrasting effects. The first mechanism is interference with cross-β structure of amyloid, the second is pro-oxidative effect, due to change in the redox state of the redox sensitive amino-acids, such as cysteine and methionine.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

In distinction to stB, NAC at the higher concentrations, such as 0.5 mM and 1 mMalso completely inhibits amyloid fibril formation of cysC (Fig.7), while at 0.2 mM concentration it significantly prolongs the lag phase.

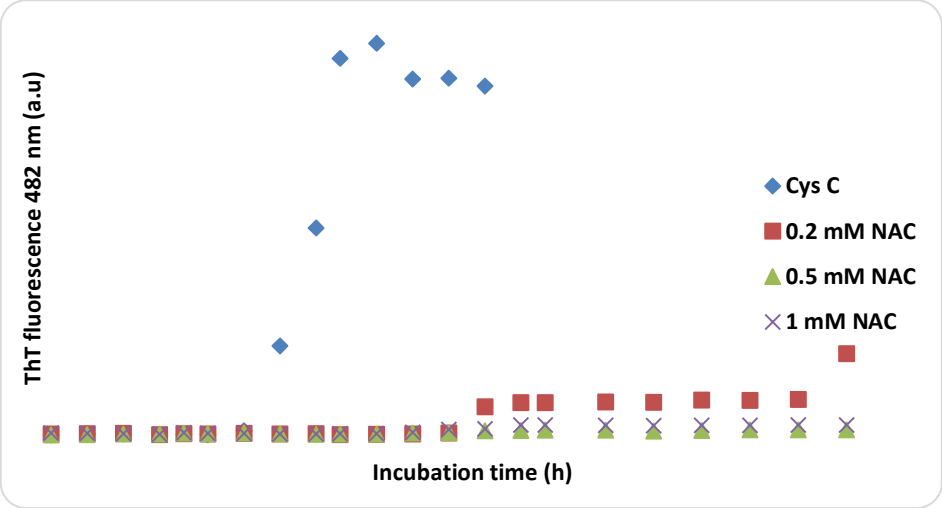

Figure 7: Time dependence of ThT fluorescence intensity following the aggregation kinetics of cystatin C in the absence and presence of different concentrations of N acetyl cystein (NAC). ThT fluorescence emission at 482 nm was monitored upon excitation at 440 nm. Protein concentration was 18.73 µM. Each sample was incubated at 50°C with constant stirring at 250 rpm in 0.025 M acetate buffer, 0.1 M NaCl at pH 4,

TEM results (Fig.8) confirm ThT fluorescence (Fig.7). Namely, at 1 mM NAC nearly no fibrils appear and at 0.2 mM NAC their number is less in comparison to the control of cysC alone.

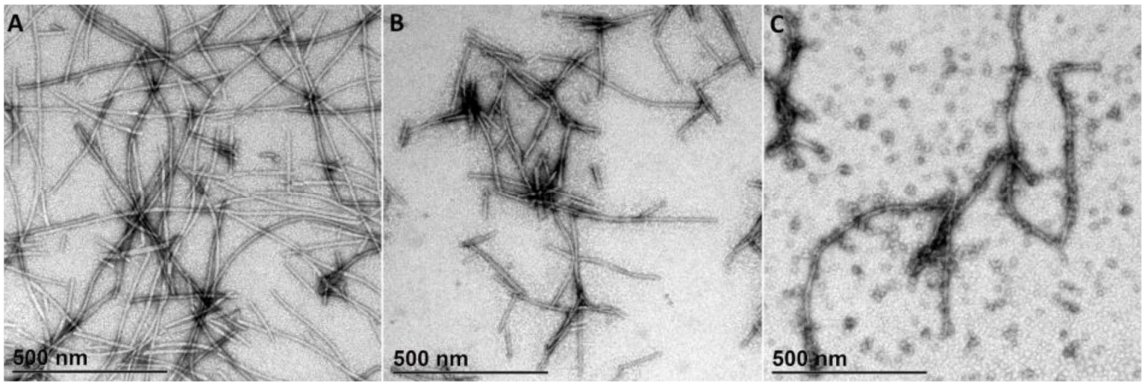

Figure 8: Representative TEM data of the plateau phase of the fibrillation reaction by cystatin C without NAC in water - as a control (A) and with NAC at 0.2 mM (B) and 1 mM (C) concentration. Protein concentration was 18,7 µM throughout.

In conclusion, cysC amyloid fibrils formation is inhibited both by vit C and NAC. Especially significant is the effect of NAC. This was not observed for stB and is explained by possible reduction by NAC of at least 1 disupfide bond in cysC. Using scanning fluorimetry - DSF[33], have shown that NAC destabilizes CysC monomer at pH 7, which can be explained by partial disulfide bonds reduction. Disulfide bonds

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

remain intact during the fibrillation process, however, they may have have an important role in the dimerization process. An US patent application (U.S. Provisional Application No. 62/555,496 filed Sep. 7, 2017) suggests that specificity of NAC may be related to the reduction of disulfide bonds, influencing the structure of monomers and affecting domain-swapping necessary for dimerization.

8. Synergistic effects of VitC and polyphenols on stB fibrillation

It can be disputed whereas VitC is beneficial or not in protecting cells against ROS and protein aggregation. At higher concentrations > 1 mM it can act pro-oxidant[39] and as shown with stB also pro-amyloid. In order to see, how VitC acts in presence of Cur, which sometimes are added as supplements together, new data were collected (here presented for the first time) of synergistic effects of Cur and VitC on stB amyloid fibril formation. We have chosen the most inhibitory 0.5 mM VitC concentration and varied Cur concentration as shown in Figure 8

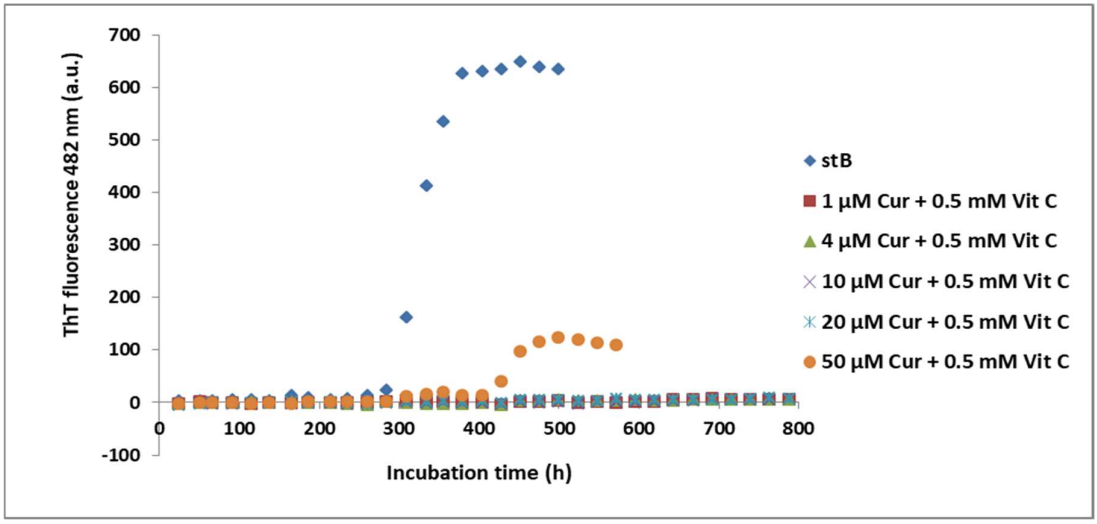

Figure 9: Kinetics of amyloid fibril formation of human stefin B at different concentrations f curcumin (Cur) and parallel concentration of 0,5 mM VitC as measured by ThT fluorescence. Intensity of ThT emission was measured against time at 482 nm, while excitation wavelength was 440 nm. Protein concentration was 34 µM. The amyloid fibrils of stB were grown under the set conditions: 0,015 M, 0,15M NaCl, pH 4,8 at room temperature in presence of 10% TFE.

Results of the time course of ThT fluorescence along stB fibrillation reaction show that a complete inhibition is observed at all Cur concentrations (1 µM Cur + 0,5 mM VitC, 4 µM Cur + 0,5 mM VitC, 10 µM Cur + 0,5 mM VitC, 20 µM Cur + 0,5 mM VitC) apart the highest 50 µM Cur + 0,5 mM VitC. Even here the lag phase is significantly prolonged by cca 100 hours than stB with 50 µM Cur. One can judge (comparing Figs 6 and 9) that Vit C and Cur act synergistically and strongly inhibit stB amyloid fibril formation. TEM analysis (Fig. 10B) shows that even at 50 µM Cur + 0,5 mM VitC the amount of

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2020 doi:10.20944/preprints202010.0274.v1

amyloid fibrils is low in comparison to stB alone (Fig. 10A). Detailed viewing of the fibrils showed that they are of different morphology (shorter and thicker).

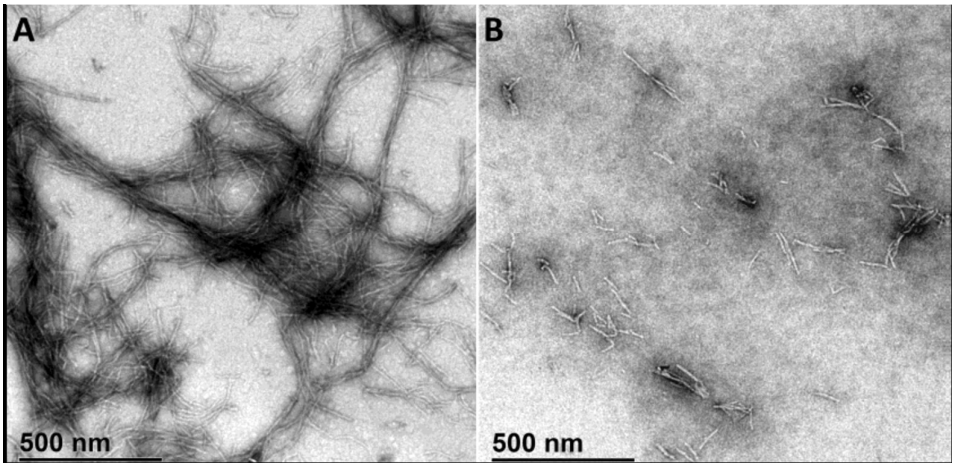

Figure 10: TEM analysis of the parallel effect Cur and VitC (50 µM Cur + 0,5 mM Vit C) on amyloid fibril formation of stB. Samples of stB fibrils were taken at the plateau phase of the fibrillation reaction. A) stB fibrils alone in water B) stB in presence of 50 µM Cur + 0,5 mM Vit C. .

Synergistic effects of 0.5 mM VitC and different concentrations of resveratrol and quercetin on stB amyloid fibril formation were also probed, both by ThT fluorescence and TEM (Supplementary). Quer and vitC act synergistically, whereas Res and vit C do not. The lag phase gets much prolonged in case of Quer plus vit C (Supplementary Figure 1), more so than adding 0.5 mM VitC alone, and the amount of fibrils is much reduced as detected by TEM (Supplementary Figure 2). In contrast, when Res plus vitC are added the lag phase stays the same as with 0.5 mM vit C alone (Supplementary Fig.3). This is concominant with similar behaviour of curcumin and quercetin on stB fibril formation in comparison to resveratrol, probably binding to a different site, whereas the binding site of resveratrol and vitC may be the same.

METHODS n.a. New experiments (synergistic effect of vitC and polyphenols) were studied by the same methods as described before. The details have been described in papers by Jahic et al [33] for cysC and Hasanbasic, Jahic et al.[31] for stB.

Acknowledgements:

This work was funded by ARRS – the Slovenian research agency, programme Proteolysis and its regulation P1-0140 (led by B. Turk).

 doi:10.20944/preprints202010.0274.v1

REFERENCES:

1. Ciudad, S., Puig, E., Botzanowski, T., Meigooni, M., Arango, A. S., Do, J., Mayzel, M., Bayoumi, M., Chaignepain, S., Maglia, G., Cianferani, S., Orekhov, V., Tajkhorshid, E., Bardiaux, B., and Carulla, N. (2020) Abeta(1-42) tetramer and octamer structures reveal edge conductivity pores as a mechanism for membrane damage, *Nature communications 11*, 3014.
2. Younger, S., Jang, H., Davies, H. A., Niemiec, M. J., Garcia, J. G. N., Nussinov, R., Migrino, R. Q., Madine, J., and Arce, F. T. (2020) Medin Oligomer Membrane Pore Formation: A Potential Mechanism of Vascular Dysfunction, *Biophysical journal 118*, 2769-2782.
3. Jenko Kokalj, S., Guncar, G., Stern, I., Morgan, G., Rabzelj, S., Kenig, M., Staniforth, R. A., Waltho, J. P., Zerovnik, E., and Turk, D. (2007) Essential role of proline isomerization in stefin B tetramer formation, *Journal of molecular biology 366*, 1569-1579.
4. Janowski, R., Kozak, M., Jankowska, E., Grzonka, Z., Grubb, A., Abrahamson, M., and Jaskolski, M. (2001) Human cystatin C, an amyloidogenic protein, dimerizes through three-dimensional domain swapping, *Nat Struct Biol 8*, 316-320.
5. Staniforth, R. A., Giannini, S., Higgins, L. D., Conroy, M. J., Hounslow, A. M., Jerala, R., Craven, C. J., and Waltho, J. P. (2001) Three-dimensional domain swapping in the folded and molten-globule states of cystatins, an amyloid-forming structural superfamily, *EMBO J 20*, 4774-4781.
6. Skerget, K., Vilfan, A., Pompe-Novak, M., Turk, V., Waltho, J. P., Turk, D., and Zerovnik, E. (2009) The mechanism of amyloid-fibril formation by stefin B: temperature and protein concentration dependence of the rates, *Proteins 74*, 425-436.
7. Taler-Vercic, A., Kirsipuu, T., Friedemann, M., Noormagi, A., Polajnar, M., Smirnova, J., Znidaric, M. T., Zganec, M., Skarabot, M., Vilfan, A., Staniforth, R. A., Palumaa, P., and Zerovnik, E. (2013) The role of initial oligomers in amyloid fibril formation by human stefin B, *International journal of molecular sciences 14*, 18362-18384.
8. Anderluh, G., Gutierrez-Aguirre, I., Rabzelj, S., Ceru, S., Kopitar-Jerala, N., Macek, P., Turk, V., and Zerovnik, E. (2005) Interaction of human stefin B in the prefibrillar oligomeric form with membranes. Correlation with cellular toxicity, *Febs Journal 272*, 3042-3051.
9. Ceru, S., and Zerovnik, E. (2008) Similar toxicity of the oligomeric molten globule state and the prefibrillar oligomers, *FEBS letters 582*, 203-209.
10. Rabzelj, S., Viero, G., Gutierrez-Aguirre, I., Turk, V., Dalla Serra, M., Anderluh, G., and Zerovnik, E. (2008) Interaction with model membranes and pore formation by human stefin B: studying the native and prefibrillar states, *The FEBS journal 275*, 2455-2466.
11. Anderluh, G., and Zerovnik, E. (2012) Pore formation by human stefin B in its native and oligomeric states and the consequent amyloid induced toxicity, *Front Mol Neurosci 5*, 85.
12. Ceru, S., Konjar, S., Maher, K., Repnik, U., Krizaj, I., Bencina, M., Renko, M., Nepveu, A., Zerovnik, E., Turk, B., and Kopitar-Jerala, N. (2010) Stefin B interacts with histones and cathepsin L in the nucleus, *The Journal of biological chemistry 285*, 10078-10086.
13. Cipollini, E., Riccio, M., Di Giaimo, R., Dal Piaz, F., Pulice, G., Catania, S., Caldarelli, I., Dembic, M., Santi, S., and Melli, M. (2008) Cystatin B and its EPM1 mutants are polymeric and aggregate prone in vivo, *Biochim Biophys Acta 1783*, 312-322.
14. Di Giaimo, R., Riccio, M., Santi, S., Galeotti, C., Ambrosetti, D. C., and Melli, M. (2002) New insights into the molecular basis of progressive myoclonus epilepsy: a multiprotein complex with cystatin B, *Hum Mol Genet 11*, 2941-2950.
15. Skerget, K., Taler-Vercic, A., Bavdek, A., Hodnik, V., Ceru, S., Tusek-Znidaric, M., Kumm, T., Pitsi, D., Pompe-Novak, M., Palumaa, P., Soriano, S., Kopitar-Jerala, N., Turk, V., Anderluh, G., and Zerovnik, E. (2010) Interaction between oligomers of stefin B and amyloid-beta in vitro and in cells, *Journal of Biological Chemistry 285*, 3201-3210.
16. Zerovnik, E. (2017) Putative alternative functions of human stefin B (cystatin B): binding to amyloid-beta, membranes, and copper, *Journal of Molecular Recognition 30*.

17. Zerovnik, E. (2017) Co-chaperoning by amyloid-forming proteins: cystatins vs. crystallins, *European biophysics journal : EBJ 46*, 789-793.
18. Penna, E., Cerciello, A., Chambery, A., Russo, R., Cernilogar, F. M., Pedone, E. M., Perrone-Capano, C., Cappello, S., Di Giaimo, R., and Crispino, M. (2019) Cystatin B Involvement in Synapse Physiology of Rodent Brains and Human Cerebral Organoids, *Front Mol Neurosci 12*, 195.
19. Di Matteo, F., Pipicelli, F., Kyrousi, C., Tovecci, I., Penna, E., Crispino, M., Chambery, A., Russo, R., Ayo-Martin, A. C., Giordano, M., Hoffmann, A., Ciusani, E., Canafoglia, L., Gotz, M., Di Giaimo, R., and Cappello, S. (2020) Cystatin B is essential for proliferation and interneuron migration in individuals with EPM1 epilepsy, *EMBO molecular medicine 12*, e11419.
20. Lalioti, M. D., Mirotsou, M., Buresi, C., Peitsch, M. C., Rossier, C., Ouazzani, R., Baldy-Moulinier, M., Bottani, A., Malafosse, A., and Antonarakis, S. E. (1997) Identification of mutations in cystatin B, the gene responsible for the Unverricht-Lundborg type of progressive myoclonus epilepsy (EPM1), *Am J Hum Genet 60*, 342-351.
21. Pennacchio, L. A., Lehesjoki, A. E., Stone, N. E., Willour, V. L., Virtaneva, K., Miao, J., D'Amato, E., Ramirez, L., Faham, M., Koskiniemi, M., Warrington, J. A., Norio, R., de la Chapelle, A., Cox, D. R., and Myers, R. M. (1996) Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1), *Science 271*, 1731-1734.
22. Palsdottir, A., Snorradottir, A. O., and Thorsteinsson, L. (2006) Hereditary cystatin C amyloid angiopathy: Genetic, clinical, and pathological aspects, *Brain Pathol 16*, 55-59.
23. Mi, W., Jung, S. S., Yu, H., Schmidt, S. D., Nixon, R. A., Mathews, P. M., Tagliavini, F., and Levy, E. (2009) Complexes of amyloid-beta and cystatin C in the human central nervous system, *Journal of Alzheimer's disease : JAD 18*, 273-280.
24. Mi, W., Pawlik, M., Sastre, M., Jung, S. S., Radvinsky, D. S., Klein, A. M., Sommer, J., Schmidt, S. D., Nixon, R. A., Mathews, P. M., and Levy, E. (2007) Cystatin C inhibits amyloid-beta deposition in Alzheimer's disease mouse models, *Nat Genet 39*, 1440-1442.
25. Sastre, M., Calero, M., Pawlik, M., Mathews, P. M., Kumar, A., Danilov, V., Schmidt, S. D., Nixon, R. A., Frangione, B., and Levy, E. (2004) Binding of cystatin C to Alzheimer's amyloid beta inhibits in vitro amyloid fibril formation, *Neurobiology of aging 25*, 1033-1043.
26. Perez-Gonzalez, R., Sahoo, S., Gauthier, S. A., Kim, Y., Li, M., Kumar, A., Pawlik, M., Benussi, L., Ghidoni, R., and Levy, E. (2019) Neuroprotection mediated by cystatin C-loaded extracellular vesicles, *Scientific reports 9*, 11104.
27. Zerovnik, E., Skerget, K., Tusek-Znidaric, M., Loeschner, C., Brazier, M. W., and Brown, D. R. (2006) High affinity copper binding by stefin B (cystatin B) and its role in the inhibition of amyloid fibrillation, *The FEBS journal 273*, 4250-4263.
28. Podmore, I. D., Griffiths, H. R., Herbert, K. E., Mistry, N., Mistry, P., and Lunec, J. (1998) Vitamin C exhibits pro-oxidant properties, *Nature 392*, 559.
29. Porat, Y., Abramowitz, A., and Gazit, E. (2006) Inhibition of amyloid fibril formation by polyphenols: Structural similarity and aromatic interactions as a common inhibition mechanism, *Chemical biology & drug design 67*, 27-37.
30. Hasanbasic, S., Taler-Vercic, A., Puizdar, V., Stoka, V., Tusek Znidaric, M., Vilfan, A., Berbic, S., and Zerovnik, E. (2019) Prolines Affect the Nucleation Phase of Amyloid Fibrillation Reaction; Mutational Analysis of Human Stefin B, *ACS chemical neuroscience 10*, 2730-2740.
31. Hasanbasic, S., Jahic, A., Berbic, S., Znidaric, M. T., and Zerovnik, E. (2018) Inhibition of Protein Aggregation by Several Antioxidants, *Oxidative medicine and cellular longevity 2018*, 8613209.
32. Hudson, S. A., Ecroyd, H., Kee, T. W., and Carver, J. A. (2009) The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds, *Febs Journal 276*, 5960-5972.
33. Jahić, A., Tušek Žnidarič, M., Pintar, S., Berbić, S., and Žerovnik, E. (2020) The effect of three polyphenols and some other antioxidant substances on amyloid fibril formation by Human cystatin C, *Neurochemistry international 140*, 104806.

34. Gazit, E. (2002) Global analysis of tandem aromatic octapeptide repeats: The significance of the aromatic-glycine motif, *Bioinformatics 18*, 880-883.
35. Knowles, T. P. J., Vendruscolo, M., and Dobson, C. M. (2014) The amyloid state and its association with protein misfolding diseases (vol 15, pg 384, 2014), *Nat Rev Mol Cell Bio 15*, 496-496.
36. Chiti, F., and Dobson, C. M. (2017) Protein Misfolding, Amyloid Formation, and Human Disease: A Summary of Progress Over the Last Decade, *Annu Rev Biochem 86*, 27-68.
37. Petkova, A. T., Leapman, R. D., Guo, Z., Yau, W. M., Mattson, M. P., and Tycko, R. (2005) Self-propagating, molecular-level polymorphism in Alzheimer's beta-amyloid fibrils, *Science 307*, 262-265.
38. Qiang, W., Yau, W. M., Lu, J. X., Collinge, J., and Tycko, R. (2017) Structural variation in amyloid-beta fibrils from Alzheimer's disease clinical subtypes, *Nature 541*, 217-+.
39. Garry, A., Edwards, D. H., Fallis, I. F., Jenkins, R. L., and Griffith, T. M. (2009) Ascorbic acid and tetrahydrobiopterin potentiate the EDHF phenomenon by generating hydrogen peroxide, *Cardiovasc Res 84*, 218-226.