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# The effect of three polyphenols and some other antioxidant substances on amyloid fibril formation by Human cystatin C

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ABSTRACT

Human cystatin C (CysC) is an amyloid forming protein involved in the hereditary cerebral amyloid angiopathy (HCCAA) that affects arteries in the brain and the peripheral nervous system. In this study we measured the influence of several substances on human CysC aggregation and amyloid fibril formation, induced at pH 4 *in vitro*. The effect of three polyphenols: resveratrol, quercetin and curcumin and of two antioxidants: vitamin C (VitC) and N-acetyl-L-cysteine (NAC) was explored as well as the effect of sulphoraphane (SF) and α-lipoic acid (AL). The formation of amyloid fibrils was followed by Thioflavin T (ThT) fluorescence and by transmission electron microscopy (TEM). Effects on the length of the lag phase were revealed by following the increase of ThT fluorescence intensity with time. The amount and morphology of fibrils in comparison to prefibrillar aggregates and globular oligomers were evaluated by TEM at the plateau stage of the reaction. Thermal stabilization of the CysC monomer by the small compounds was measured by differential scanning fluorimetry (DSF). NAC, VitC and SF exhibited the largest inhibitory effect on amyloid fibril growth. The effects of polyphenols were not significant, apart from resveratrol, which partly inhibited the amyloid fibril growth.

## 1. Introduction

Protein misfolding and aggregation underlie the so called protein misfolding diseases, also known as proteinopathies. Proteinopathies are a group of disorders characterized by the accumulation of a specific protein within neurons or in the brain parenchyma (Bayer, 2015). A subtype of proteinopathies (or protein misfolding diseases) are amyloidoses, where the pathological hallmark is the deposition of amyloid fibrils (Nastou and Nasi, 2019). These are divided into systemic and local amyloidoses. Neurodegenerative diseases fall among the latter. **Neurodegenerative diseases** are the most prevalent chronic diseases of the aging population in the Western world. These include Alzheimer's (AD) and Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), polyglutamine expansion disorders, such as Huntington's, various other dementias and prion-related diseases. Protein misfolding and aggregation might also be involved in progressive myoclonus epilepsies (PMEs) (Polajnar and Zerovnik, 2011) and neuropsychiatric diseases, such as schizophrenia and major depression (Polajnar and Zerovnik, 2014).

**Amyloid fibril** formation represents a common property of misfolded proteins causing amyloidoses (Dobson, 2001, 2002). There is an agreement that prior to the formation of amyloid fibrils some form of the oligomeric intermediates likely interact with cellular membranes or even make amyloid pores, suggesting a common mechanism of down-stream signaling leading finally to cytotoxicity (Stefani and Dobson, 2003). Cerebral amyloid angiopathy (CAA) is a condition that can develop in aging individuals as well as in AD and Down's syndrome patients and is not directly related to vascular disease (Kovari et al., 2013). It presents with deposits of amyloid-beta (Aβ) peptide in the meningeal and intracerebral vessels. Human cystatin C (CysC) is co-deposited in amyloid plaques together with Aβ (Kaeser et al., 2007; Mi et al., 2007). Increased CysC expression leads to protection in AD by inhibiting cysteine proteases, inducing autophagy and cell division and preventing amyloidogenesis (Kaur and Levy, 2012). Supporting its role in disorders related to protein aggregation, Yamaguchi's group showed that CysC co-localizes with prosaposin in Bunina bodies of motor neurons from patients with ALS (Wada et al., 2018). In contrast to CAA, the

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hereditary CysC amyloid angiopathy (HCCAA) has only been found in a small portion of the population of Iceland. It is a rare autosomal dominant genetic disease presented in younger people who do not live past 35 years of age. Amyloid deposits of mutated CysC accumulate predominantly in the brain arteries but also in the peripheral nervous system and various other organs (Bjarnadottir et al., 1998). The deposited amyloid weakens arterial walls, which leads to repeated brain hemorrhages (mini-strokes) and results in dementia and finally in paralysis (Palsdottir et al., 2006). As for now no treatment to combat the deposition of amyloid and avoid early death of these patients is available (Ghiso et al., 1986). In order to contribute towards an efficient preventive therapy for HCCAA, EPM1 and possibly other neurodegenerative diseases, including AD, we have embarked on studies of the effect of antioxidant substances on protein aggregation of cystatins (Hasanbasic et al., 2018). In this study we have chosen vitamin C (VitC), N-acetyl-L-cysteine (NAC), sulphoraphane (SF) and α-lipoic acid (AL) and three polyphenols: curcumin, resveratrol and quercetin and measured their effect on the kinetics, yield and morphology of CysC amyloid fibril formation. This study has confirmed that some of the beneficial effects of the antioxidant and anti-inflammatory substances may arise from their anti-amyloid activity.

VitC acts as an enzyme cofactor and an antioxidant in many physiological processes including neuronal maturation and differentiation, myelin formation, modulation of neurotransmission, iron absorption, immune responses and antioxidant protection (Du et al., 2012). Furthermore, it has been shown that VitC can inhibit the formation of amyloid fibrils of polypeptides (Alam et al., 2017; Patel et al., 2018), which makes it a potential therapeutic additive for amyloid related diseases.

NAC is a derivative of L-cysteine, a precursor in the synthesis of the endogenous antioxidant glutathione (GSH). NAC penetrates the blood-brain barrier and causes an increase of GSH levels in the brain. NAC acts as a direct scavenger of free radicals (Kerksick and Willoughby, 2005). Furthermore, NAC shows anti-inflammatory properties, as it is able to limit the release of cytokines in the early stage of immune proliferation (Omara et al., 1997). Among its various properties is also the ability to inhibit and disrupt amyloid fibril formation (Hasanbasic et al., 2018). NAC was shown to minimize Tau expression and neuronal loss in animal model of AD (Joy et al., 2018).

SF is the product of glucoraphanin hydrolysis and is present in cruciferous vegetables such as broccoli (Cramer and Jeffery, 2011). It acts as an antioxidant, anti-inflammatory and anti-tumor agent via multiple targets and various mechanisms. It has been reported that neuroprotective effects of SF could be mainly ascribed to its ability to activate Nrf2 pathway (Ping et al., 2010). SF was shown to activate heat shock response and to enhance proteasome activity through up-regulation of Hsp27 (Gan et al., 2010). It is neuroprotective in AD disease models and it reduces Aβ cytotoxicity (Kim et al., 2013; Park et al., 2009).

AL is an organosulfur compound found in mitochondria. It acts as a co-factor of the multienzyme complexes such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Ghibu et al., 2009; Holmquist et al., 2007). AL is a strong antioxidant that improves and restores the intrinsic cellular antioxidant systems. It binds heavy metals in the blood stream and thus reduces oxidative stress (Caylak et al., 2008). AL also shows anti-inflammatory properties, and decreases neuronal cell death (Li et al., 2015). It has been proven to be protective in AD and PD disease models (Holmquist et al., 2007; Li et al., 2015).

Polyphenols are secondary plant metabolites that are composed of aromatic phenolic rings. Many epidemiological studies show a significantly reduced incidence of neurodegenerative diseases in populations with specific dietary regimens, where at least some of these compounds are present at high concentrations (Knight et al., 2016; Feart et al., 2010). Quercetin is a flavonol widely present in fruits, vegetables and herbs. Curcumin is the main coloring substance present in the spice turmeric (*Curcuma longa*), while resveratrol is a polyphenolic phytoalexin, abundant in grapes, berries and other plant species. Quercetin, curcumin and resveratrol have anti-inflammatory and antioxidant properties (Kim et al., 2016; Liu et al., 2016; Zhao et al., 2015a). Furthermore, they are capable of inhibition of the amyloid fibril formation (Yang et al., 2005). For example, resveratrol reshapes toxic aggregates into a non-toxic type of aggregates (Ladiwala et al., 2010) and prevents tau hyperphosphorylation (He et al., 2017). Moreover, it has been shown that *in vivo* studies quercetin and curcumin improve memory and cognitive impairment in AD models (Kim et al., 2016; Wang et al., 2014). Therefore, polyphenols may play an important role in the prevention of amyloid related neurodegenerative diseases (Andrade et al., 2019a, 2019b).

## 2. Materials and methods

### 2.1. Materials

Recombinant human CysC was prepared following the protocol developed at the Jožef Stefan Institute (PhD thesis by Nina Cimerman Uni. Ljubljana, 1993). The construct has a pI of 9.2 (with added 3 amino acid residues on the N-terminal – GSM). Thioflavin T (ThT) was purchased from Aldrich while antioxidants were from Sigma-Aldrich. Other chemicals were from Sigma and Merck.

### 2.2. Differential scanning fluorimetry (DSF)

Experiments were performed as described in Nature Protocols by Nielsen et al. (Niesen et al., 2007). All the samples were tested in triplicate. The final mixture contained 37.4 μM CysC, 10 mM sodium phosphate pH 7, 145 mM NaCl, 20x Sypro Orange and 250 μM ligand, with exception of quercetin, where the concentration used was 50 μM. The experiment was run for 90 cycles.

### 2.3. ThT fluorescence

Amyloid fibrils of the recombinant human CysC were grown at pH 4 (0.025 M acetate buffer, 0.1 M NaCl, pH 4) at 50 °C with constant shaking. Protein concentration was 18.7 μM. ThT was dissolved in a phosphate buffer (25 mM, 0.1 M NaCl, pH 7.5) to a final concentration of 15 μM ($A_{416} = 0.66$). This stock solution we term ThT buffer. At separate time points of the amyloid fibrillation reaction of CysC (alone or in presence of antioxidant substances), 150 μL of the reaction mixture were added to 1725 μL of the ThT buffer, just before the measurement. ThT fluorescence was measured using 1 cm cuvette at room temperature on Shimadzu model RF-5301 PC spectrofluorimeter. For ThT emission spectra, excitation was set at 440 nm and the spectra were recorded from 455 nm to 600 nm. Excitation slit was set at 10 nm, while the emission slit was 3 nm. Each measurement was performed four times.

### 2.4. Transmission electron microscopy (TEM)

15 μL of the reaction mixture in which fibrils were grown (protein concentration was 18.7 μM) was applied on a Formvar and carbon-coated grid. After 3 min the sample was soaked away and stained with 1% (w/v) uranyl acetate. Preparations were observed using the Philips CM 100 (FEI, The Netherlands) transmission electron microscope operating at 80 kV. Images were recorded by Bioscan CCD or ORIUS SC 200 camera (Gatan Inc., Washington, DC,USA), using Digital Micrograph software (Gatan Inc., Washington, DC, USA). Two parallel grids were prepared for each sample, and at least 10 grid squares were observed.

### 2.5. Circular dichroism (CD)

The samples used for the far-UV CD spectra experiments were prepared by mixing CysC and antioxidants dissolved in phosphate buffer

(0.05 M, 0.075 M NaCl, pH 7). Protein concentration was 0.2 mg/ml, while concentrations of antioxidants varied. The samples were incubated for 2 h at room temperature (r.t.). CD spectra were then measured also at r.t. with a Circular Dichroism Spectrometer Chirascan (Applied Photophysics, UK) using 1 mm quartz cuvette. Temperature was set at 20 °C. Data were recorded from 250 to 200 nm with 1 nm sampling interval. The final spectra were the average of three repeated measurements.

## 3. Results

### 3.1. TEM in comparison to ThT fluorescence

The amyloid fibril formation by CysC *in vitro* was induced by constant shaking at pH 4 and 50 °C. The effect of three polyphenols: resveratrol, quercetin and curcumin and of four antioxidants: VitC, NAC, SF and AL were examined by ThT fluorescence intensity measurement and by transmission electron microscopy (TEM). Both methods are necessary to better estimate the amount of amyloid fibrils in comparison to the prefibrillar oligomers.

Fig. 1A shows that at resveratrol concentrations from 50 to 200 μM

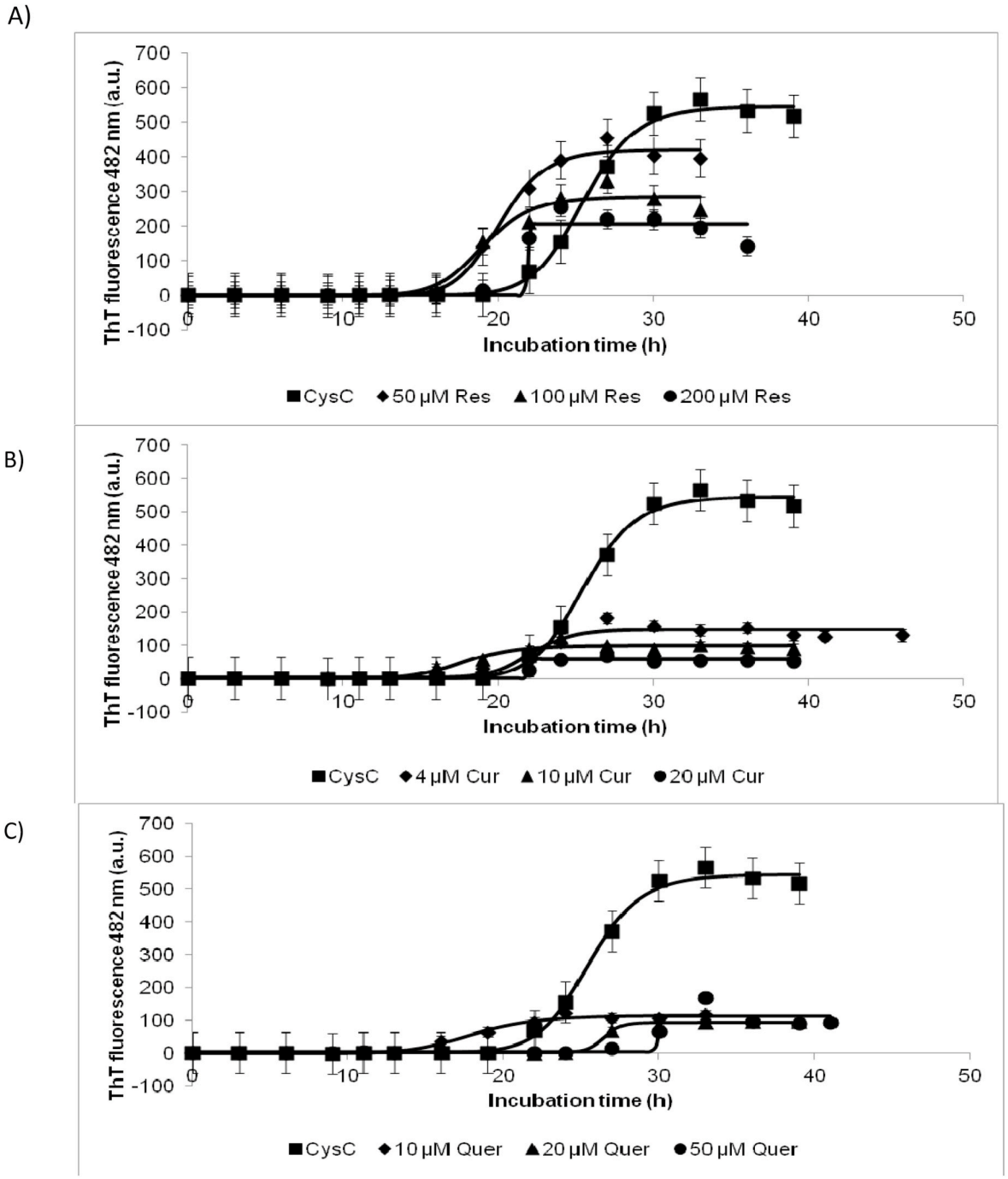


**Fig. 1.** Time dependence of ThT fluorescence intensity following the amyloid fibrillation reaction of CysC in presence of: A) resveratrol (Res) B) curcumin (Cur) and C) quercetin (Quer); at various concentrations.

the ThT fluorescence intensity slightly decreases but the lag phase does not increase, in fact it is shortened it by cca. 20%. Nevertheless, TEM reveals that the highest concentration of resveratrol, 200 μM (Fig. 2C), diminishes the amount of amyloid fibrils formed in comparison to the sample of CysC alone – as a control (Fig. 2A). This does not seem to occur at 100 μM concentration (Fig. 2B). TEM similarly does not show a decrease in the amount of amyloid fibril in the presence of the other 2 polyphenols (Suppl. Fig. 1), even though they reduce the intensity of ThT fluorescence Fig. 1B and C and quercetin even increases the lag phase (Fig. 1C).

In contrast to polyphenols, NAC and VitC inhibition of amyloid fibril growth was shown both by ThT fluorescence (Fig. 3A and B) and TEM (Figs. 4 and 5). NAC prolongs the lag phase the most out of all the probed substances and it reduces the ammount of fibrils detected by ThT fluorescence (Fig. 3A). It destabilizes CysC monomer (as it will be shown later with DSF measurements) and as confirmed by TEM it inhibits amyloid fibril growth in a concentration dependent manner. A 0.2 mM concentration of NAC already reduces the ammount and length of the fibrils (stage of protofibrils), whereas the oligomers are present only in traces (Fig. 4 A). At 1 mM of NAC a few shorter fibrils can bee seen, whereas the oligomers are prevalent (Fig. 4 B, C).

At 0.2 mM concentration VitC already diminishes ThT fluorescence intensity, while the maximum effect is observed at 0.5 mM concentration (Fig. 3B). TEM data confirm the ThT fluorescence results, showing reduced ammount and length of amyloid fibrils at 0.5 mM VitC, where one can hardly trace some short fibrils (Fig. 5A). At 1 mM VitC (Fig. 5B and C), however, the inhibition is diminished, as shown by more amorphous material attached to the fibrils (Fig. 5 B, C).

In Fig. 6A ThT fluorescence intensity of CysC under amyloid fibril forming conditions in the presence of SF is shown. It can be deduced, that the inhibition of amyloid fibril formation is the largest at 100 μM concentration, whereas at 200 μM the inhibition is lower and the lag phase gets reduced, thus, the effect is reversed. TEM results confirm the reduction of the amount and length of the fibrils, with changed morphology (Fig. 7A and B) in comparison to the control (Fig. 2A). Fig. 6B shows results of ThT fluorescence intensity for AL, which exhibits the highest inhibition at 100 μM, this, however, is not confirmed by TEM; where no reduction in the amount of amyloid fibrils can be observed (Suppl.Fig. 1).

### 3.2. Differential scanning fluorimetry (DSF) measurements

Melting points ($T_m$) of CysC alone and in the presence of the ligands were determined using DSF (Fig. 8). The results show that when NAC is present thermal stability of CysC monomer at pH 7 is significantly lowered (by 1.7 °C), while in the presence of all the other tested ligands it is stabilized (up to 2.2 °C for quercetin). This does not correlate with inhibitory effect of the ligands on amyloid fibrils formation by CysC at pH 4 and 50 °C. We discuss (see Discussion), how NAC in comparison to the stabilizing compounds might inhibit amyloid fibrillation reaction.

## 4. Discussion

In this study we investigated the inhibitory effects of several antioxidants on aggregation of human CysC (see Fig. 9 for 3D structure). CysC is a known amyloidogenic protein, that causes a hereditary amyloid angiopathy (HCCAA). This protein has 2 disulfide bonds and reduction of one of the bonds is not critical for protein's function and structure, because it has only local influence (Bjork and Ylinenjarvi, 1992). For chicken cystatin, the most stable homologue, it was shown that when both disulfide bonds get broken, the protein loses its structure and turns into molten globule state (Staniforth et al., 2000). Cystatins are prone to form domain-swapped dimers (Janowski et al., 2001; Staniforth et al., 2001), even if there are two disulfide bonds present in the monomer. Domain-swapping may lead directly to formation of amyloid fibrils, where the domain-swapped dimers associate into extended β-sheets (Janowski et al., 2005) or, by a less direct mechanism that involves monomers and domain-swapped dimers (Wahlbom et al., 2007) as the building blocks or, even off-pathway oligomers, as is the case with stefin B (Jenko Kokalj et al., 2007). In this latter case, proteolysis of the fibrils did not confirm domain-swapped dimers as building blocks (Davis et al., 2015), in contrast to previous H/D NMR exchange study (Paramore, 2012). However, this discrepancy might stem from different conditions and presence of fibril polymorphs.

The hypothesis, that some antioxidant substances may also have an anti-amyloid activity and act as inhibitors of amyloid fibril formation, is not new. This idea was first mentioned by Ono et al. (2004) and Porat et al. (2006), who studied anti-amyloid effects of curcumin and some other polyphenols on aggregation of Aβ peptide and islet amyloid polypeptide, respectively. In our present study of the effects of antioxidant substances on protein aggregation, we have studied three polyphenols: resveratrol, quercetin and curcumin and four antioxidants from other chemical classes: VitC, NAC, SF and AL. To probe the effect of the ligands on CysC structure and stability at neutral pH, we measured the far UV CD spectra. Secondary structure of CysC did not change upon binding of any of the measured substances (CD data not shown). In order to determine the stabilization of CysC monomer by the compounds, we used differential scanning fluorimetry (DSF), also known as ThermoFluor. This technique has become a common approach for detecting protein-ligand interactions (Bai et al., 2019; Simeonov, 2013). An increase in the temperature at which the protein unfolds in the presence of the ligand can serve as evidence of a direct interaction. At pH 7, all the substances stabilize CysC except NAC, which destabilizes it (Fig. 8). This might be due to possible partial reduction of one of the two disulfide bonds by NAC (Grubb et al., 1984).

To follow amyloid fibril growth, ThT fluorescence and TEM were measured. Their results were mostly in agreement, however, when ThT fluorescence intensity was low albeit TEM did not show reduction of

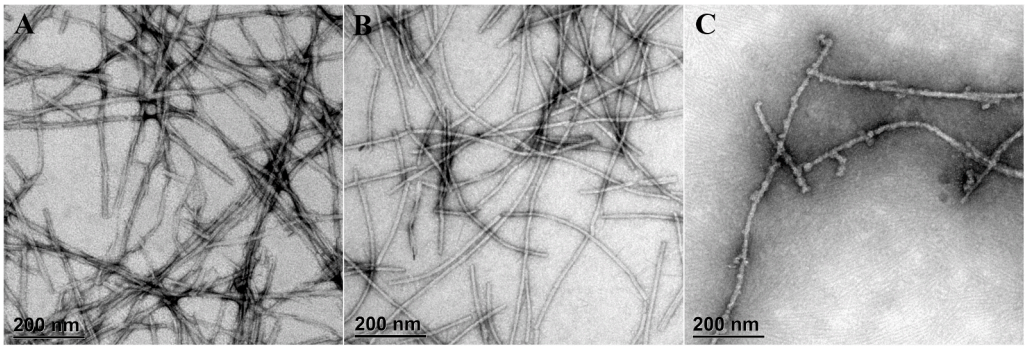


**Fig. 2. A –C** Representative TEM data of the plateau phase of cystatin C without treatment (A) and with resveratrol at 100 μM (B) and 200 μm (C) concentration. Protein concentration was 18.7 μM throughout.

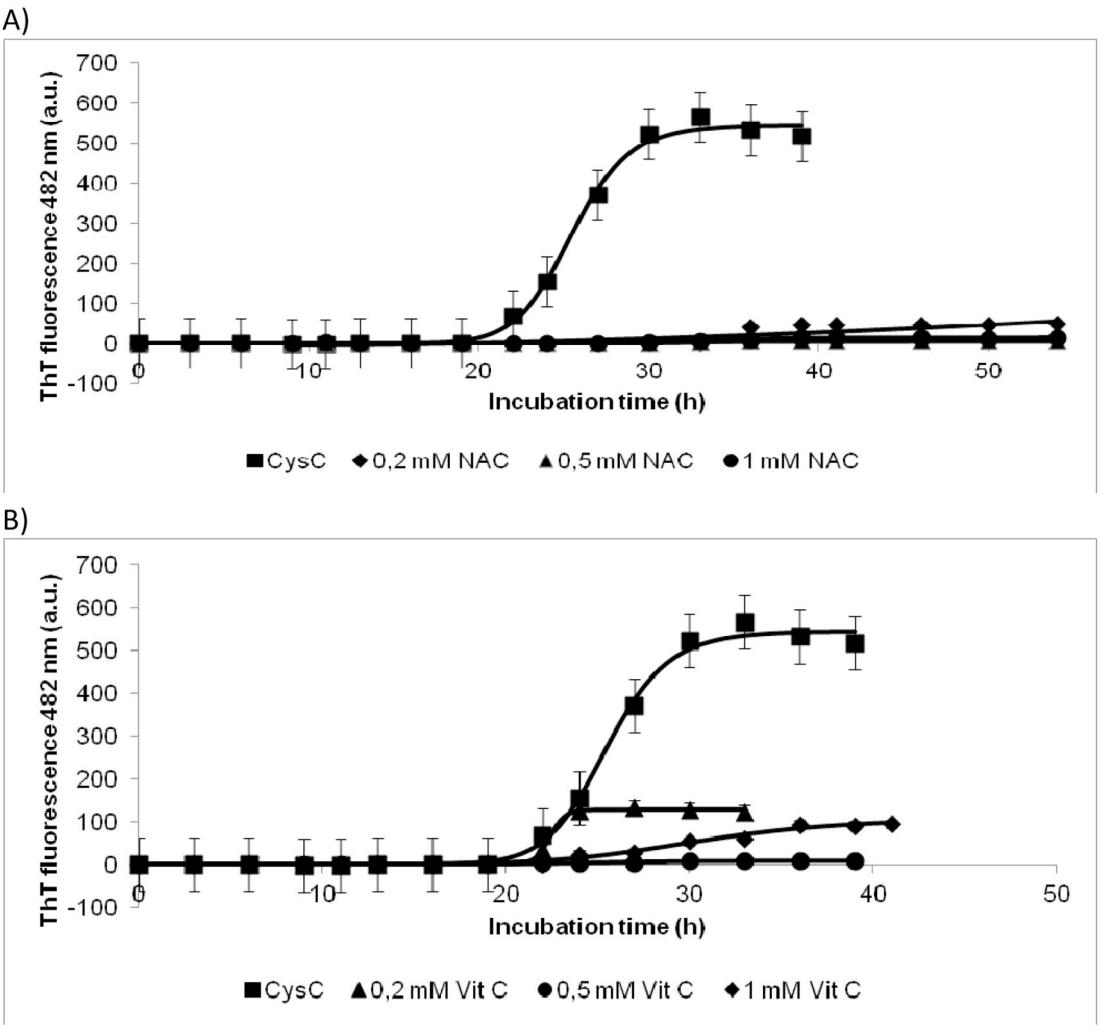


**Fig. 3. A, B:** Time dependence of ThT fluorescence intensity while following the amyloid fibrillation reaction of CysC in presence of: A) NAC and B) VitC; at various concentrations.

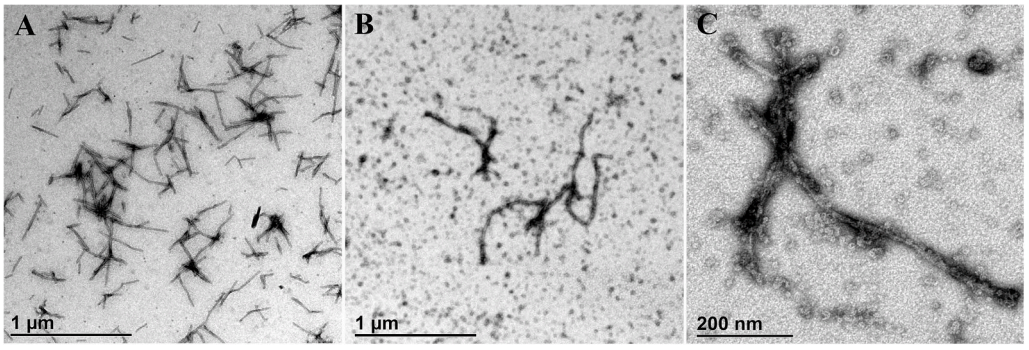


**Fig. 4.** TEM data of CysC with NAC at 0.2 mM (A) and 1 mM (B, C) concentration.

amyloid fibril formation, we took TEM results as more reliable. Possible differences could arise from the fact that ThT fluorescence can be quenched by aromatic compounds (Nedaei et al., 2018; Noormai et al., 2012; Hudson et al., 2009). For example, ThT fluorescence measurement alone indicated that curcumin and especially quercetin inhibited the amyloid fibril growth of CysC and that quercetin prolonged the lag phase (Fig. 1). TEM data did not confirm the reduction in the amount of amyloid fibrils (Suppl. Fig. 1) and we took this method as more reliable.

Previously we studied amyloidogenesis of human stefin B (Hasanbasic et al., 2018) in the presence of the same antioxidant and polyphenol compounds, apart from SF and AL. Among the polyphenols, curcumin showed the most prominent inhibition of stefin B amyloid aggregation (Hasanbasic et al., 2018); it prolonged the lag phase and diminished the amount of amyloid fibrils. Again, this effect was most prominent at lower concentrations (of 10 μM) and diminished with increasing concentrations (from 20 till 50 μM). Surprisingly, curcumin up to 20 μM did not show such a prominent effect on CysC aggregation as it did on stefin B aggregation. Actually, with CysC none of the

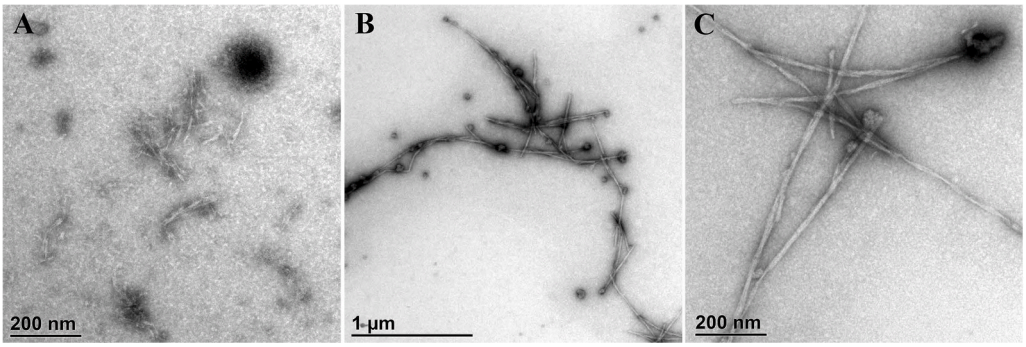


**Fig. 5.** TEM data of CysC with VitC at 0.5 mM (A) and 1 mM (B, C).

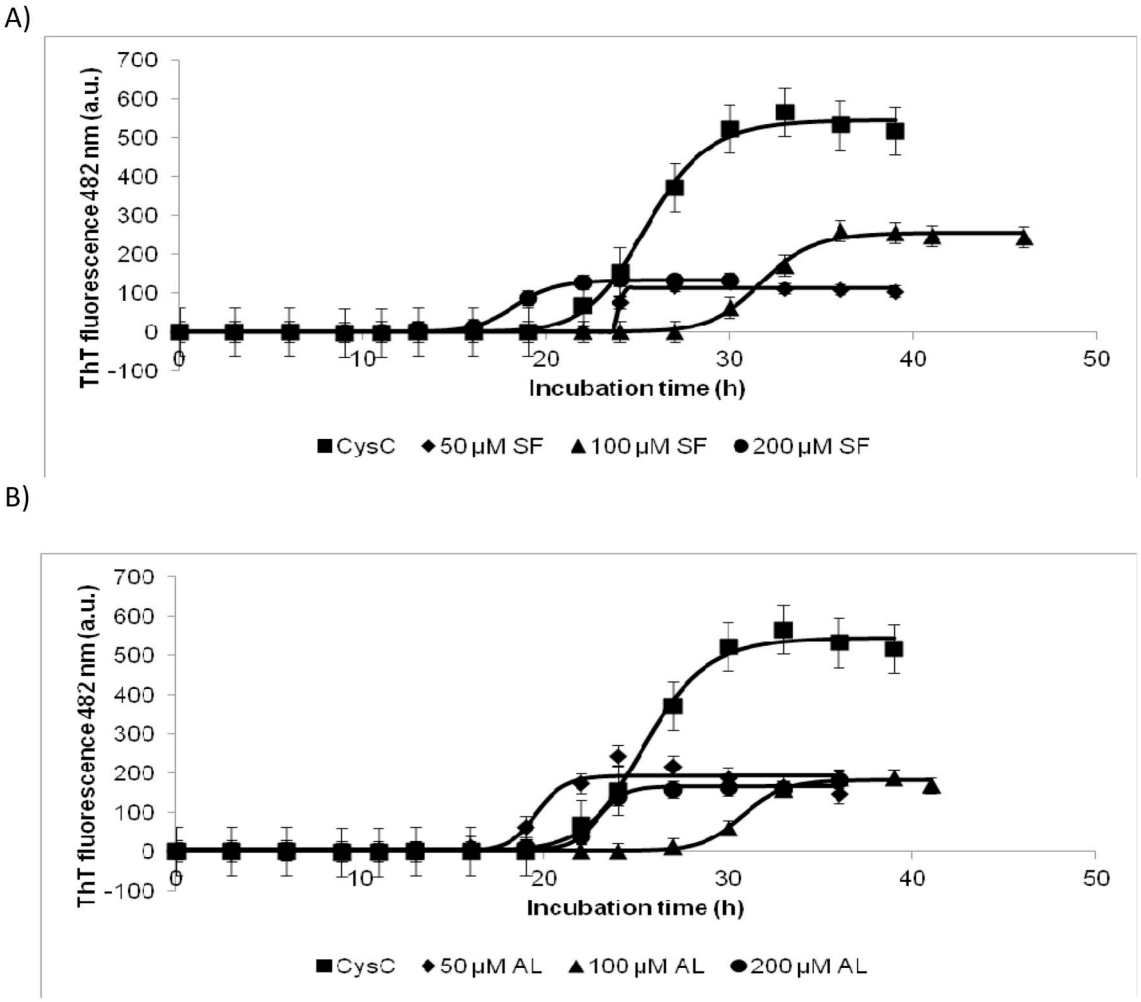


**Fig. 6. A, B:** Time dependence of ThT fluorescence intensity for the amyloid fibrillation reaction of CysC and: A) SF and B) AL; at various concentrations.

polyphenols apart from resveratrol exerted much inhibition of amyloid fibril growth as observed by TEM (Suppl. Fig. 1). Even though, curcumin and quercetin exhibited more inhibitory effect than resveratrol when measuring the intensity of ThT fluorescence (Fig. 1), TEM showed no difference (Suppl. Fig. 1). We have chosen slightly lower concentrations than previously based on the low physiological concentrations of the polyphenols reached in the blood (Nicholson et al., 2010). Porat et al. proposed that polyphenols display their inhibitory effect on amyloid formation by positioning aromatic rings in the amyloidogenic core (Porat et al., 2006), thus one could expect that polyphenols are able to inhibit the formation of amyloid fibrils by any protein. Therefore, the different effect on two different proteins (CysC and stefin B), which have a similarly structured amyloid core with cross-β structure, may be explained by an assumption that sequence details and precise arrangement of the subunits in the protofilaments influence aromatic-aromatic Π stacking interactions (Gazit, 2002).

In the present study we show NAC has the largest effect out of the probed substances on amyloid fibril formation by prolonging the lag phase and reducing the ammount of fibrils formed by CysC as judged by ThT fluorescence (Fig. 3). As confirmed by TEM, it inhibits amyloid fibril growth nearly completely at 1 mM and at 0.5 mM (Fig. 4B and C), whereas at 0.2 mM it also reduces their ammount and makes them much

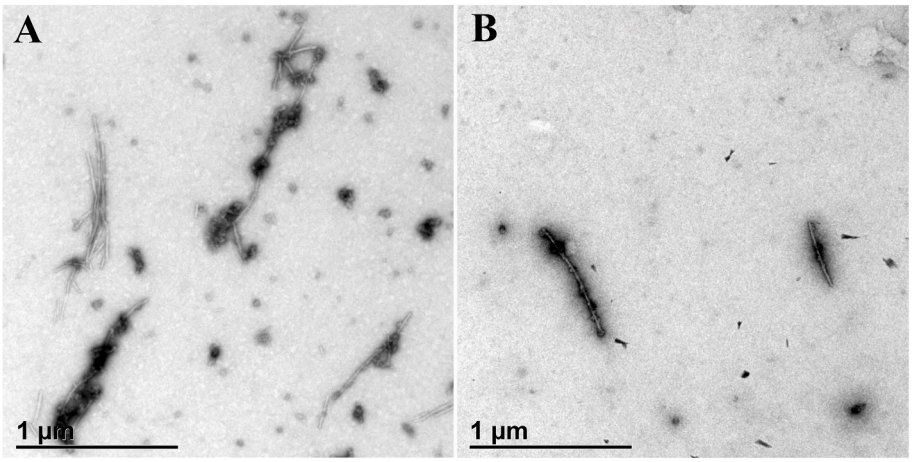


**Fig. 7. A,B:** TEM data of CysC with SF at two concentrations; 100 μM (A) and 200 μM (B).

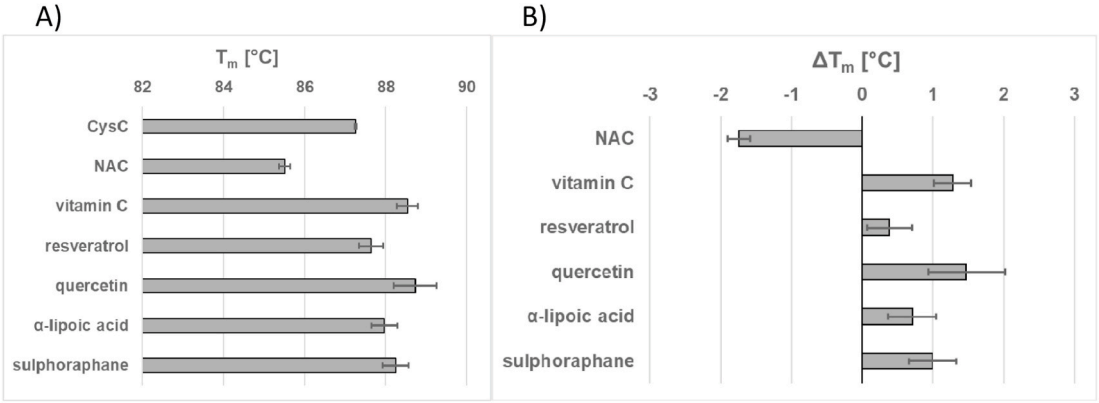


**Fig. 8.** Graphs representing A) the melting temperatures ($T_m$) and B) differences in the $T_m$ s between the experiments containing ligands and experiment with no ligand. Averages are depicted together with the standard deviation. The measured melting point of CysC without ligands was 87.2 ± 0.0287 °C.

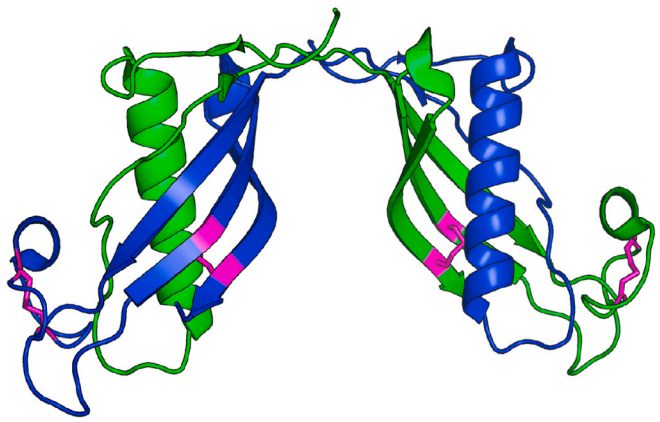

**Fig. 9. Structure of human CysC dimer (PDB code:** 1G96) (Janowski et al., 2001). Disulfides are presented as magenta sticks. Figure prepared using PyMol software (Janowski et al., 2001).

shorter (Fig. 4A). Using DSF we observed that NAC destabilizes CysC monomer at pH 7, which we explain by possible partial disulfide bonds reduction. Disulfide bonds remain intact during the fibrillation process. However, disulfide bonds appear to have an important role in the dimerization process. In an US patent application (*U.S. Provisional Application No. 62/555,496 filed Sep. 7, 2017*) it is suggested that specificity of NAC may be related to the reduction of disulfide bonds, thus changing the structure of monomers, which are then not able to open properly and to exchange the three-dimensional subdomains between the two subunits of the domain-swapped dimer (Fig. 9).

VitC inhibits the amyloid fibril formation of CysC at the concentrations 0.2 mM and 1 mM, by prolonging the lag phase (Fig. 3), while the detected fibrils are shorter and there are fewer in comparison to the control by TEM (Fig. 5A). Moreover, at 0.5 mM concentration, VitC completely inhibits the fibril formation of CysC (Fig. 5B and C). Similar effect was observed for stefin B in our previously study (Hasanbasic et al., 2018). Lee and coworkers (Lee et al., 2016) proposed the mechanism for the inhibitory effect of VitC might be due to its ability to interfere with exposed hydrogen atoms of the NH groups in the β-sheet backbone causing disruption of β-sheet stacking. Furthermore, Yang & Zeng, reported that the degradation products of VitC have more potent inhibitory impact on amyloid formation than the native molecule (Yang and Zeng, 2018).

In the previous work of stefin B aggregation (Hasanbasic et al., 2018) we also demonstrated that VitC and NAC inhibited amyloid fibril formation but only at low concentrations (of up to 1 and 0.5 mM, respectively), whereas high concentration (of 1.7 mM and 1 mM, respectively) increased fibrils amount as determined both, by TEM and ThT fluorescence. Even in the present study, we observe that different modes of action apply at low 0.2 mM medium 0.5 mM or high concentrations (1 mM) of VitC, which can act as antioxidant or pro-oxidant, depending on the conditions (Castaneda-Arriaga et al., 2018). There must be two opposing processes taking place. One could be direct binding of VitC to the protein, which would inhibit fibril growth, another could be the

change in the redox state and modification of susceptible amino-acids (pro-oxidant effect).

SF also inhibits amyloid aggregation of CysC. The present study shows that SF at 100 μM concentration prolongs the lag phase (Fig. 6A) and reduces the amount of fibrils that are short as shown by TEM (Fig. 7A). At 50 μM concentration SF doesn't impact the lag phase, while at 200 μM concentration it even decreases the lag phase. However, at both concentrations the intensity of ThT fluorescence (Fig. 6A) decreases, whereas TEM shows rarer, shorter fibrils (Fig. 7), not of smooth morphology as compared to the control.

There are some other studies that support the role of SF as an anti-amyloid agent. Nagaveni and coworkers (Nagaveni et al., 2014) studied the inhibitory effect of SF on amyloid fibrillation by Aβ peptide. They suggest that SF may bind covalently with Aβ. In the case of CysC we suggest that SF as an electrophile (Keum, 2011) may interfere with exposed neutrophil groups in the β-sheet backbone of CysC amyloid fibrils and thus disrupt the β-sheet stacking.

## 5. Conclusions

Based on the present study, it would seem reasonable to continue the studies of VitC, NAC and SF as food supplements with an aim to find means of preventing HCCA. However, even though we observe a strong inhibition of the CysC amyloid fibril formation by NAC and VitC, one has to be cautious with interpretation, as their use may not always be beneficial. If the reaction gets stuck at the level of prefibrillar aggregates this may be counterproductive. Cytotoxicity measurements would seem a necessary first step towards a safe preventive treatment. There are already some studies on animal models, that show that NAC reduces cerebral ischemia and reperfusion injuries (Turkmen et al., 2016; Wang et al., 2016). SF also displays neuroprotection against hemorrhagic strokes due to the activation of Nrf2 in microglia resulting in an increase of the anti-oxidative capacity, phagocytosis and hematoma clearance (Zhao et al., 2015b). Taken all the studies together, NAC and SF should be considered as potent candidates for preventive measures against HCCAA. As mentioned, the possible application of NAC and GHS for treatment of neurodegenerative diseases, including HCCAA, was protected by an US patent (*U.S. Provisional Application No. 62/555,496 filed Sep. 7, 2017*).

## Author contribution

Undersigned Eva Žerovnik, Jožef Stefan Institute, Ljubljana confirm that I am submitting the revised paper and that all the co-authors agree to this version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2020.104806.

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