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Anti-amyloidogenic activity of tetracyclines: studies in vitro

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Abstract Cerebral deposition of β-amyloid is a major neuropathological feature in Alzheimer's disease. Here we show that tetracyclines, tetracycline and doxycycline, classical antibiotics, exhibit anti-amyloidogenic activity. This capacity was determined by the exposure of β 1-42 amyloid peptide to the drugs followed by the electron microscopy examination of the amyloid fibrils spontaneously formed and quantified with thioflavine T binding assay. The drugs reduced also the resistance of β 1-42 amyloid fibrils to trypsin digestion. Tetracyclines not only inhibited the β-amyloid aggregates formation but also disassembled the pre-formed fibrils. The results indicate that drugs with a well-known clinical profile, including activity in the central nervous system, are potentially useful for Alzheimer's therapy.

Key words: Alzheimer's disease; β-Amyloid; Fibrillogenesis; Antibiotic; Neuroprotection

1. Introduction

Alzheimer's disease (AD) is the main cause of cognitive decline and the third cause of death in the elderly. Despite recent progress in the symptomatic therapy with cholinergic drugs, we are still awaiting an effective therapeutic approach that interferes directly with the neurodegenerative process in AD. AD is neuropathologically characterized by neurofibrillary tangles, an intracellular cytoskeletal alteration, and deposits of β-amyloid (Aβ) in the brain parenchyma and on the wall of cerebral blood vessels.

The formation of Aβ deposits, which are insoluble fibrils of Aβ peptide 1-40 and 1-42, is an early event in AD and there are now numerous genetic, biochemical and neuropathological studies pointing to a causal role of Aβ in the pathogenesis of AD [1]. The recent identification of an aspartyl-protease responsible for Aβ synthesis might further help in clarifying the essential role of Aβ [2]. According to the 'amyloid cascade' hypothesis in AD [3] recently confirmed with neuropathological examinations [4], prevention of the formation of Aβ aggregates or their elimination once formed is a potential therapeutic approach to the disease. We have found that iododoxorubicin, an anthracycline with anti-tumoral activity, inhibited the formation of amyloid aggregates in vitro and in vivo [5–7]. The intrinsic toxicity and low blood–brain barrier passage have inhibited the possibility of a direct use of this drug in AD therapy. Based on some chemical analogies with anthracyclines, we tested the anti-amyloidogenic capacity of the antibiotic tetracycline, a molecule with a safety toxicological profile, whose derivatives (i.e. doxycycline) have a favorable distribution in the central nervous system (CNS) [8]. We investigated the anti-amyloidogenic capacity of tetracycline and doxycycline using β 1-42 synthetic peptide, this peptide is highly represented in AD amyloid deposits and has been proposed as a seed molecule for amyloid formation. β 1-42 amyloid fibrils spontaneously formed in vitro were analyzed by electron microscopy (EM). The anti-amyloidogenic effect was quantified by fluorescent thioflavine T binding to amyloid structures. Since the self-aggregation capacity of β 1-42 amyloid peptide is associated with a resistance to protease digestion, we also tested whether tetracyclines facilitated the hydrolysis of β 1-42 peptide by the proteolytic enzyme trypsin [9].

2. Materials and methods

2.1. 'Binding' thioflavine T

The synthetic peptide βA 1-42 (Tecnogen, Caserta, Italy) was dissolved at a concentration of 0.22 mM in water:acetonitrile (1:1) and aliquots of 30 μg were lyophilized. The samples were dissolved in 30 μl of Tris–HCl 100 mM, pH 7.4 and incubated for 5 days at 37°C. After a centrifugation for 10 min at $13\,000\times g$, the supernatant was discharged and the pellet resuspended in 300 μl of 50 mM glycine-NaOH buffer, pH 9.6 including thioflavine T, 2 μM. The samples were incubated for 5 min and the fluorescence was determined by a spectrofluorimetric apparatus (LS 5OB Perkin Elmer Instr., Bekomfield, UK) at the excitation and emission wavelengths of 420 and 480 nm, respectively. The fluorescence values of the samples containing only tested drug were determined and subtracted from the values of the samples containing βA 1-42 fibrils.

2.2. Protease digestion

After 5 days of incubation at 37°C the solution of βA 1-42 in Tris–HCl 100 mM, pH 7.4 was digested with trypsin (Merck, Darmstadt, Germany) at 37°C for 30 min at 1:120 (w/w) enzyme to substrate ratio. After centrifugation at $13\,000\times g$ for 10 min at 4°C, the pellets were dissolved in 80 μl of 10% formic acid containing 0.1% trifluoroacetic acid and analyzed by reverse-phase high performance liquid chromatography (HPLC). Parallel samples were run in the absence of tetracycline and analyzed as specified above with or without trypsin digestion. The extent of proteolysis was calculated as the percentage of peptide present in the pellet compared to the total amount originally present.

2.3. EM

The synthetic peptide βA 1-42 was suspended in 100 mM Tris–HCl, pH 7.4, at a concentration of 0.22 mM either in the presence or in the absence of an equimolar concentration of tetracycline. Following 5 days incubation at 37°C, 5 μl aliquots of peptide suspension were applied to Formvar-coated nickel grids, negatively stained with 5%

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(w/v) uranyl acetate, and observed in an electron microscope (EM 109, Zeiss, Germany) at 80 kV.

3. Results

As shown in Fig. 1A, co-incubation of β 1-42 with tetracycline for 5 days (Fig. 1B) markedly reduced the amyloid fibrils formation as detected by EM in comparison with the control (Fig. 1A). This was confirmed by the measurement of specific binding of thioflavine T to amyloid fibrils formed by β 1-42, tetracyclines increasing ratios progressively reduced amyloid formation (Fig. 1C). In similar conditions gentamicin and 4-epianhydrotetracycline did not affect thioflavine T binding (data not shown). The inhibition of β 1-42 amyloid fibrils formation was associated with an increase of trypsin digestion of the peptide, as shown in Fig. 2. The enzymatic degradation of β 1-42 by trypsin was facilitated by the co-incubation with tetracycline (Fig. 2A) or doxycycline (Fig. 2B) as determined by HPLC analysis of residual peptide dissolved in formic acid/trifluoroacetic acid (9:1) solution. The HPLC determinations showed an anti-amyloidogenic effect of tetracyclines, without trypsin, slightly inferior compared to the measure of thioflavine T binding (Fig. 1C). This is likely due to the fact that HPLC analysis identified the monomeric form of the peptide deriving not only from fibers but also from oligomeric structures or non-amyloidogenic aggregates.

The tetracyclines not only inhibited the β 1-42 amyloid aggregation but also exerted a de-fibrillogenic effect against pre-formed β 1-42 amyloid fibrils. β 1-42 peptide was incubated alone at 37°C for 48 h and the tetracyclines were successively added for an other 48 h, thioflavine T binding and the resistance to trypsin were both reduced in relation to the amount of drugs added (Fig. 3). At the maximal concentration the tetracyclines induce a complete degradation of β 1-42 exposed to trypsin (Fig. 2B). We also used fluorescent microscopy to analyze the specific binding of tetracycline to β 1-42

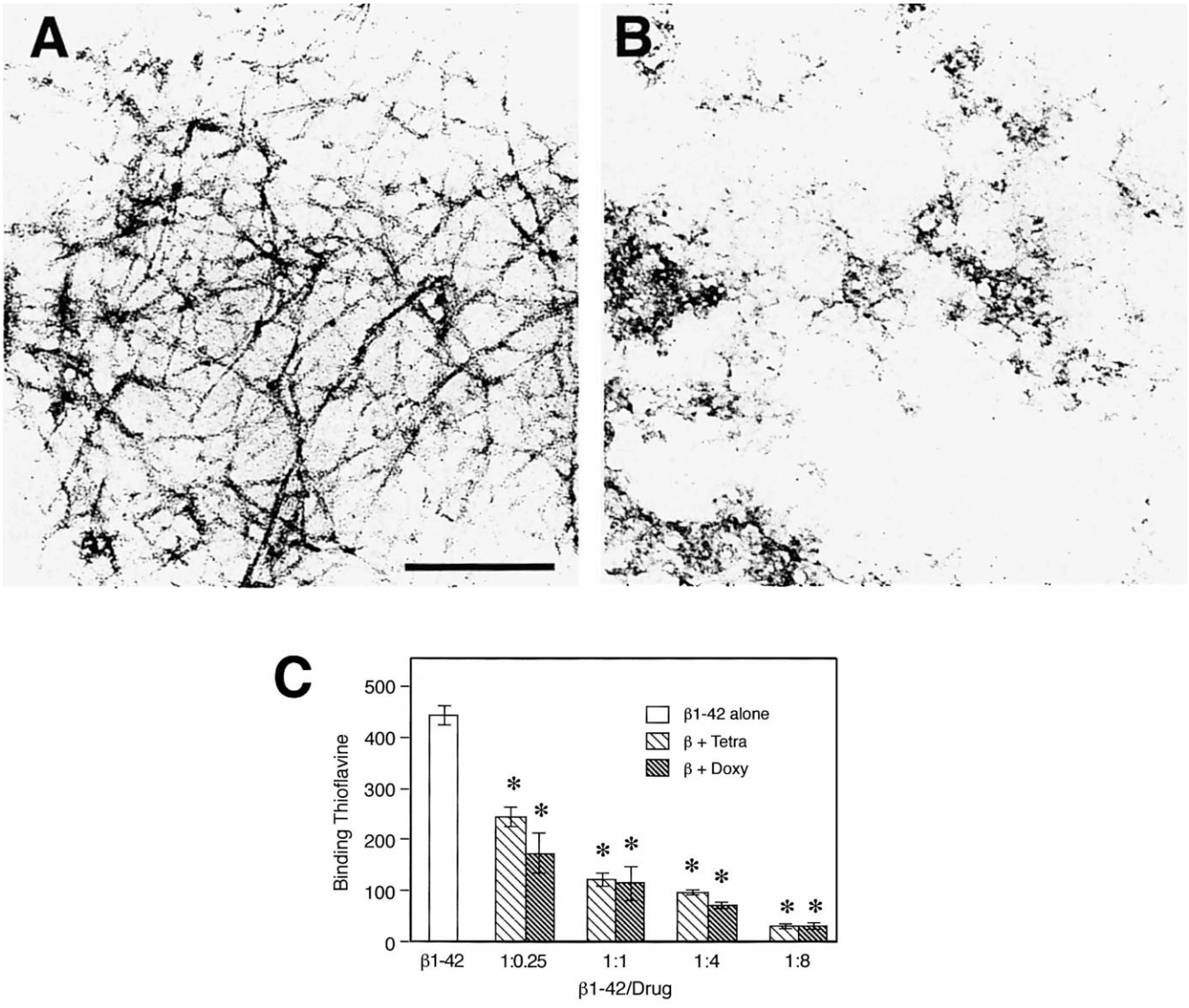

Fig. 1. Effect of tetracyclines on the self-aggregation capacity of β 1-42 incubated for 5 days at 37°C. Electron micrographies of β 1-42 (220 μM) fibrils with (A) or without (B) equimolar concentration of tetracycline; in B the fibrils disruption is evident. (C) Dose–response effect of tetracycline and doxycycline on thioflavine T binding to β 1-42 fibrils spontaneously formed. The data are expressed as the percentage of β 1-42 assayed without digestion. Mean ± S.E.M. of 10–12 replications. $*P < 0.01$ vs. respective control condition (Dunnett's test). Bar = 100 nm.

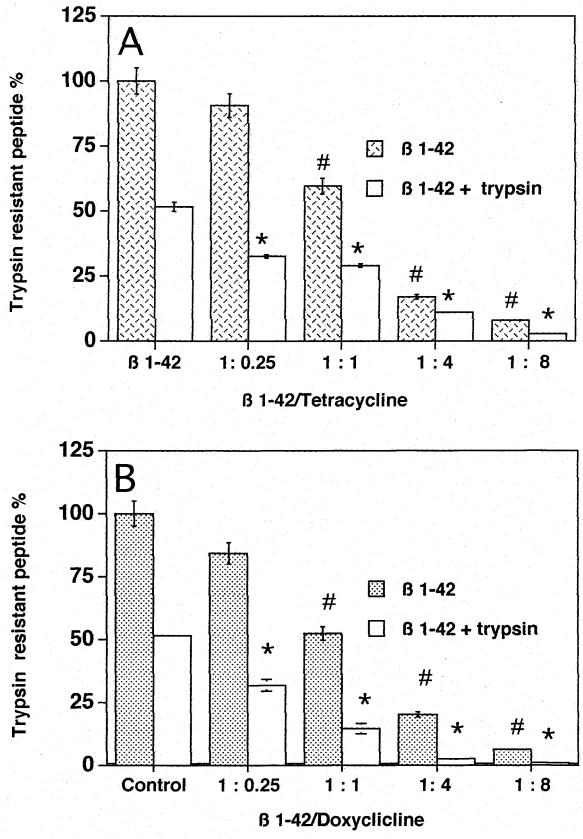

Fig. 2. Effect of tetracyclines on the trypsin resistance of β 1-42 fibrils. The presence of tetracycline (A) or doxycycline (B) progressively reduced the partial resistance of β 1-42 digestion to trypsin. The data are expressed as the percentage of β 1-42 assayed without digestion. Mean ± S.E.M. of 10–12 replications. $*P < 0.01$ vs. respective control condition with trypsin. $\#P < 0.01$ vs. control condition without trypsin (Dunnett's test).

amyloid fibrils in comparison with thioflavine T and the results confirmed the affinity of tetracycline for amyloid structures (data not shown).

4. Discussion

Several substances, including endogenous compounds like melatonin and laminin, have been found to inhibit the amyloid formation [10,11]. These compounds inhibit the fibrillogenesis but their ability to dissolve pre-formed fibrils has never been shown. On the other hand short synthetic peptides, designed as β-sheet breakers, inhibited amyloid formation and disassemble pre-formed β 1-42 fibrils [12] but several chemical modifications of the peptides structure will be necessary before obtaining derivatives suitable for clinical use. On the contrary, tetracyclines are a well-know category of antibiotics that include doxycycline and minocycline, used in clinical practice for CNS injuries [13].

The selection of tetracycline as test compound was based on structural analogies with Congo red and iododoxorubicin. Although the molecular basis of tetracyclines anti-amyloidogenic activity is not understood, it may be related to the propensity of these drugs to bind the fibrillogenic structure. Like the anthracyclines, tetracyclines contain an extended hydrophobic core formed by aromatic moieties which permits interactions with lipophilic residues of Aβ. A second characteristic of these compounds is the presence of a variety of polar substituents that can form hydrogen bonds with specific residues of Aβ, strengthening the drug to protein interaction. Furthermore, the anti-amyloidogenic capacity of tetracyclines is not exclusively against Aβ but it can also prevent and even reverse the prion protein conformation changes associated with spongiform encephalopathies that became sensitive to protease degradation [14]. Tetracycline's capacity to attenuate the resistance of amyloid fibrils to proteolysis is relevant for the clearance of Aβ and to prevent the accumulation of the peptide. As recently shown in experimental conditions [15] the role of physiological catabolism of Aβ is essential for the formation of cerebral amyloid deposition and a treatment, like tetracyclines, that can facilitate the proteolytic degradation of Aβ is extremely useful. In terms of potential curative intervention in AD, the capacity to disrupt pre-formed Aβ aggregates exhibited by tetracyclines is also promising of efficacy. It has recently been shown that an other derivative of tetracycline, minocycline, exerted a neuroprotective effect in a transgenic model of Huntington disease [16]. This finding confirms the blood–brain barrier passage and the curative effect of a tetracycline, independent from the antibiotic activity. Furthermore, since the inhibition of caspase-1 and -3 expression has been proposed as potential mechanism responsible of the minocycline effect, this element could be favorable also in

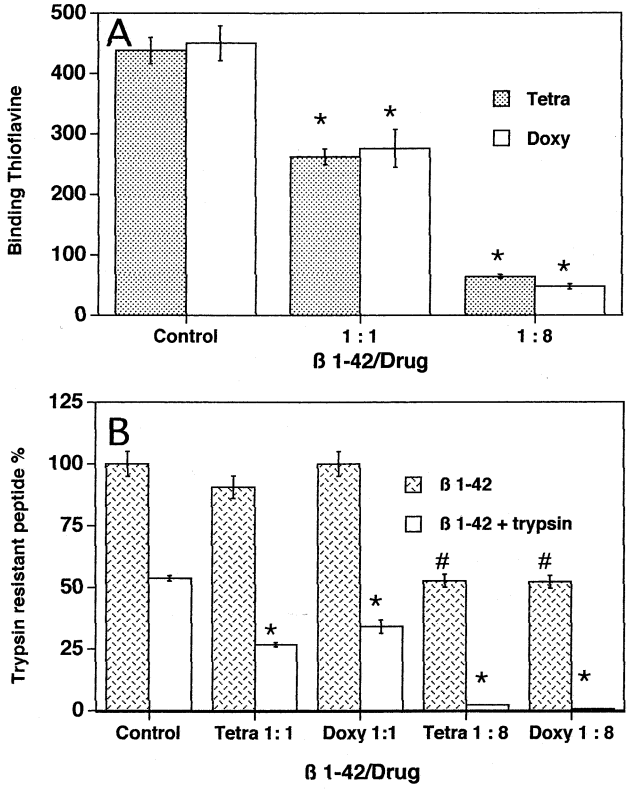

Fig. 3. Disassembled effect of tetracyclines on pre-formed β 1-42 amyloid fibrils. β 1-42 (220 μM) was incubated at 37°C for 48 h alone (thioflavine T binding = 404.00 ± 12.25). Subsequently tetracycline or doxycycline were added to the samples and the incubation was continued for further 48 h, β 1-42 fibrils were identified by thioflavine T binding (A) or digested with trypsin (B) as explained in the legend to Fig. 1. Mean ± S.E.M. of 10 replications. Ten–12 determinations, $*P < 0.01$ vs. respective control condition in A; $*P < 0.01$ vs. respective control condition with trypsin, $\#P < 0.01$ vs. control condition without trypsin in B (Dunnett's test).

AD therapy. Thus, although in vivo studies will be necessary to establish the efficacy of the anti-amyloidogenic capacity of tetracyclines, our data strongly suggest that these drugs might offer a useful therapeutic approach to AD and other central amyloidosis.

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References

[1] Selkoe, D.J. (1999) Nature 399, A23–A31.
[2] Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M.A., Biere, A.L., Curran, E., Burgess, T., Louis, J.C., Collins, F., Treanor, J., Rogers, G. and Citron, M. (1999) Science 286, 735–741.
[3] Hardy, J.A. and Higgins, G.A. (1992) Science 256, 184–185.
[4] Naslund, J., Haroutunian, V., Mohs, R., Davis, K.L., Davies, P., Greengard, P. and Buxbaum, J.D. (2000) J. Am. Med. Assoc. 283, 1571–1577.
[5] Tagliavini, F., McArthur, R.A., Canciani, B., Giaccone, G., Porro, M., Bugiani, M., Lievens, P.M.-J., Bugiani, O., Peri, E., Dall'Ara, P., Rocchi, M., Poli, G., Forloni, G., Bandiera, T., Varasi, M., Suarato, A., Cassutti, P., Cervini, M.A., Lansen, J., Salmona, M. and Post, C. (1997) Science 276, 1119–1122.
[6] Merlini, G., Ascari, E., Amboldi, N., Bellotti, V., Arbustini, E., Perfetti, V., Ferrari, M., Zorzoli, I., Marinone, G., Diegoli, M., Trizio, D. and Ballinari, D. (1995) Proc. Natl. Acad. Sci. USA 92, 2959–2963.
[7] Forloni, G., Salmona, M., De Gioia, L., Canciani, B., Jansen, J., Della Vedova, F., Molinari, A., Suarato, A., Varasi, M., Post, C. and Tagliavini, F. (1996) 26° Ann. Meet. Soc. Neurosci 26, 651.8.
[8] Yrjanheikki, J., Keinanen, R., Pellikka, M., Hokfelt, T. and Koistinaho, J. (1998) Proc. Natl. Acad. Sci. USA 95, 15769–15774.
[9] Nordstedt, J., Naslund, J., Tjernberg, L.O., Thyberg, J. and Therenius, L. (1994) J. Biol. Chem. 269, 30773–30776.
[10] Pappolla, M., Bozner, P., Soto, C., Shao, H., Robakis, N.K., Zagorsky, M., Frangione, B. and Ghiso, J. (1998) J. Biol. Chem. 273, 7185–7188.
[11] Monji, A., Tashiro, K., Yoshida, I. and Tashiro, N. (1998) Brain Res. 788, 187–190.
[12] Soto, C., Sigurdsson, E.M., Morelli, L., Kumar, R.A., Castano, E. and Frangione, B. (1998) Nat. Med. 4, 822–826.
[13] Yrjanheikki, J., Tikka, T., Keinanen, R., Goldsteins, G., Chan, P.H. and Koistinaho, J. (1999) Proc. Natl. Acad. Sci. USA 96, 13496–13500.
[14] Tagliavini, F., Forloni, G., Colombo, L., Rossi, G., Girola, G., Canciani, B., Angeretti, N., Giampaolo, L., Peressini, E., Awan, T., De Gioia, L., Ragg, E., Bugiani, O. and Salmona, M. (2000) J. Mol. Biol. 300, 1309–1322.
[15] Iwata, N., Tsubuki, S., Takaki, Y., Watanabe, K., Sekiguchi, M., Hosoki, E., Kawashama-Morishima, M., Lee, H.-J., Hama, E., Sekine-Aizawa, Y. and Saido, T.C. (2000) Nat. Med. 6, 143–150.
[16] Chen, M., Ona, V.O., Li, M., Ferrante, R.J., Fink, K.B., Zhu, S., Bian, J., Guo, L., Farrell, L.A., Hersch, S.M., Hobbs, W., Vonsattel, J.P., Cha, J.H. and Friedlander, R.M. (2000) Nat. Med. 6, 797–801.