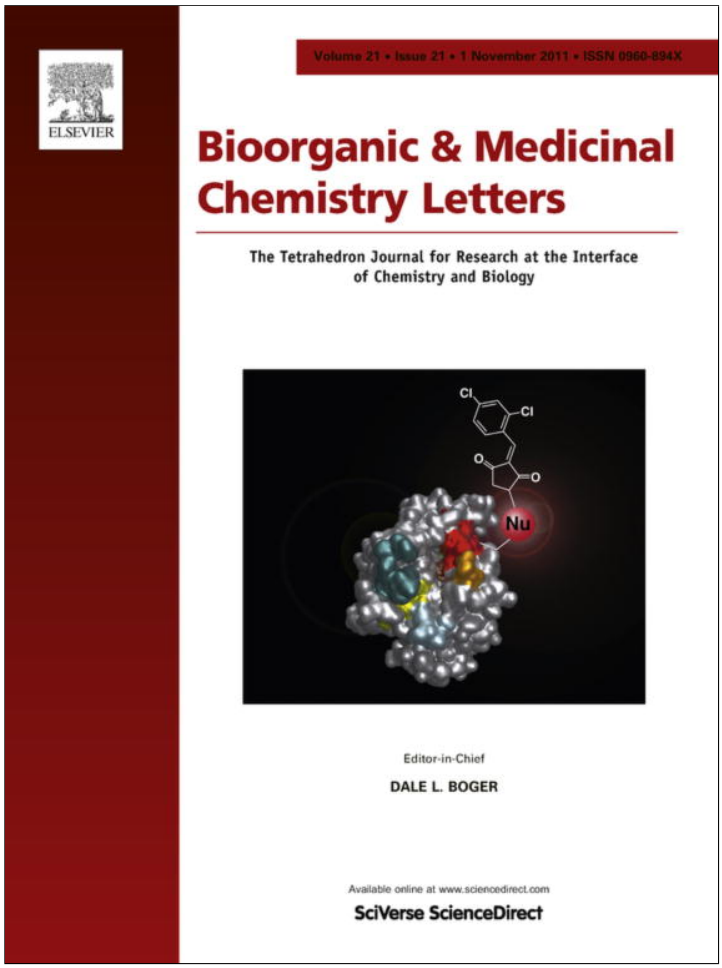

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Vitamin B6s inhibit oxidative stress caused by Alzheimer's disease-related Cu^{II}-β-amyloid complexes—cooperative action of phospho-moiety

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ABSTRACT

Cu^{II} complexes of Alzheimer's disease-related β-amyloid (Aβ) peptides exhibit metal-centered oxidation chemistry. The metallo-Aβ complexes are the hallmark of the disease and have been attributed to the generation of reactive oxygen species (ROS), causing oxidative stress. In this communication, the inhibitions of the oxidative activity of Cu^{II}-Aβ by vitamin B6 compounds pyridoxamine (PM), pyridoxine (PN), pyridoxal (PL), and pyridoxal-5'-phosphate (PLP) are presented. These B6's are competitive inhibitors toward dopamine oxidation by Cu^{II}-$A\beta_{1-20}$, with K_i values of 1.4, 8.3, 1.2, and 0.2 mM, respectively. The phospho-moiety in PLP seems to exhibit cooperative inhibition, affording a clue for future design of inhibitors.

Alzheimer's disease (AD), an irreversible progressive brain disease, is the most common cause of dementia in elderly. It is one of a series of neurodegenerative diseases that have been associated with misregulation of transition metal ions.[1,2] The disease is characterized by the formation of the insoluble Aβ plaques found in the brain with up to mM amounts of Cu^{2+}, Zn^{2+}, and Fe^{3+}. Full-length Aβ of 40–42 amino acids is generated by β- and γ-secretase cleavage of the amyloid precursor protein.[3] Aβ binds transition metal ions via His-6, His-13, and His-14 residues in its N-terminal domain (DAEFR **H**DSGY EV**HH**Q KLVFF; $A\beta_{1-20}$). The coagulate metallo-Aβ complexes with redox-active Cu^{+2} and $Fe^{2+/3+}$ ions are the hallmark of the disease and have been attributed to the generation of highly reactive oxygen species (ROS) that may induce oxidative stress in the brain of AD patients.[4–7]

Recent focus on AD chemistry has been directed toward correlating metal-centered pathways to the homeostasis of ROS.[1] With a good understanding, this could ultimately lead to ways of treating and preventing ROS-mediated damage in this disease. ROS can be generated in normal respiration in the brain and regulated by antioxidation systems. As the generation of ROS exceeds the capacity of the antioxidation, oxidative stress may happen. Chelating agents and antioxidants have been used for inhibition against ROS and oxidative stress.[8–11]

Studies have suggested that vitamin B6 compounds inhibit the production of radicals and serve as quenchers for singlet oxygen.[12] The three forms of vitamin B6, PM, PN, and PL, are eventually converted to the active form PLP by pyridoxal kinase, and utilized as a cofactor for over 140 enzymes.[13] Some of the enzymes are involved in amino acid and monoamine neurotransmitter synthesis.[14] Vitamin B6 is involved in the methionine/glutathione transsulfuration pathway to convert homocysteine to cysteine and ultimately to the natural antioxidant glutathione. Vitamin B6 deficiency can lead to insufficient insulin and altered hormone production,[14] which can be alleviated with recommended daily intake of 2 mg easily obtained from various vegetables, fish, and some fruits, whereas the tolerable/safe upper limit is generally considered high at 100 mg/day for adults set by the US FDA, 25 mg/day by the EU SCF, and 10 mg/day in the UK.[15] In addition to its regulatory roles, vitamin B6 has also been shown to serve as an antioxidant.[12] Moreover, dietary imbalance is considered an important risk factor of AD according to various clinical, transgenic mouse models of AD, and epidemiological studies.[16]

We previously showed that the Cu^{II}-Aβ complexes exhibit metal-centered redox chemistry consistent with the mechanism of the Type-3 copper enzyme catechol oxidase.[17–19] The studies presented herein explore the inhibitory effect of vitamin B6 compounds toward the oxidative activity of Cu^{II}-Aβ complexes.[20]

As redox-active transition metal ions are found to be involved in the formation of the plaques in AD brain, many studies have focused on the generation of ROS as well as the binding ability of metals to Aβ fragments to form the self-assembled metallo-Aβ complexes. Cu^{II}-Aβ complexes can catalyze the oxidation of

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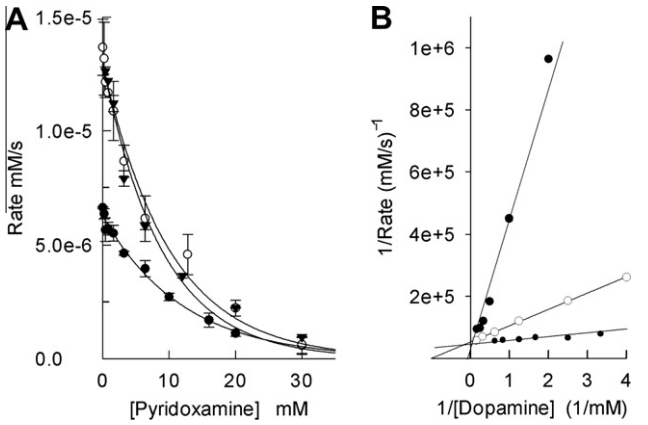

Figure 1. (A) PM inhibition plot of dopamine (1.0 mM) oxidation catalyzed by (●) 2.0 μM Cu^{II}-$A\beta_{1-40}$, (○) 3.0 μM Cu^{II}-$A\beta_{1-20}$, and (▼) 2.0 μM Cu^{II}-$A\beta_{1-16}$ in 0.1 M HEPES at pH 7.4 and 25 °C. (B) Lineweaver–Burk plot of 1/v versus 1/[dopamine] by Cu^{II}-$A\beta_{1-40}$ in the presence of PM (0.0, 2.0, and 4.0 mM from bottom to top trace).

catechol and derivatives to their corresponding *o*-quinone products following enzyme-like kinetics,[17,18] which was thus used as means for checking the effectiveness of vitamin B6 compounds as inhibitors against the oxidation.[21] PM can inhibit the oxidation of dopamine by the Cu^{II}-Aβ complexes of various lengths (Fig. 1A), albeit at relatively high IC_{50} values at around 7–8 mM. The inhibition of dopamine oxidation was further analyzed, which seems to follow a competitive inhibition pattern with K_i = 1.42 mM against the full-length Cu-$A\beta_{1-40}$ (Fig. 1B) following equation (1). Similarly, PM inhibits dopamine oxidation by Cu^{II}-$A\beta_{1-20}$ and Cu^{II}-$A\beta_{1-16}$, affording K_i of 1.40 and 1.03 mM, respectively (Fig. 1A, Table 1).

$$v = V_{max}[S]/\{Km(1 + [I]/Ki) + [S]\} \quad (1)$$

PN inhibits the oxidation of dopamine by Cu^{II} complexes of Aβ fragments with much less potency (Fig. 2). Fitting of the rate as a function of [PN] to eq. (1) affords K_i values of 8.29 and 5.56 mM toward dopamine oxidation by Cu^{II}-$A\beta_{1-20}$ and Cu^{II}-$A\beta_{1-16}$, respectively (Table 1). The use of the substrates THB and DTBC also confirms the relatively higher K_i values for PN inhibition (Table 1).

PL can react with the amino group of dopamine via its aldehyde group to form a Schiff base moiety. Thus THB and DTBC were used as the substrates to reveal PL inhibition capability. PL inhibits DTBC oxidation by Cu^{II}-$A\beta_{1-20}$ and Cu^{II}-$A\beta_{1-16}$ in a competitive manner, affording K_i of 1.12 and 0.67 mM, respectively, comparable to those of PM (Fig. 3, Table 1). The inhibition is confirmed with THB as the substrate, giving K_i = 2.8 mM.

Inhibition potencies of PM and PL are similar and higher than that of PN, whereas their only structural difference is at position-4 which may result in different H-bonding interactions. Further insight into such interactions awaits future exploration by the use of amyloid variants and chemically modified B6 derivatives.

PLP also inhibits the oxidation of DTBC by Cu^{II}-$A\beta_{1-16}$ and Cu^{II}-$A\beta_{1-20}$ in a competitive manner, affording much smaller K_i values of 0.13 and 0.20 mM, respectively (Fig. 4A and Table 1). The inhibition by PLP is much more pronounced as compared to PL which may be attributed to the phosphate group in PLP. Indeed, phosphate alone is observed to inhibit the oxidation of DTBC with K_i = 4.73 mM (Fig. 4B), similar to previously observed.[18] PLP thus behaves as a bifunctional inhibitor toward DTBC oxidation by Cu^{II}-$A\beta_{1-20}$, in which the phosphate group is also involved in inhibiting the oxidative activity. The inhibitory effect of PL and phosphate was further studied by determining the effect of PL:phosphate in 1:1 molar ratio toward the oxidation of DTBC catalyzed by Cu^{II}-AB_{1-20}, affording K_i = 0.80 mM which is significantly higher than that of PLP (Fig. 4B and Table 1). The decrease in K_i of PL in the presence of phosphate

Table 1
Inhibition constants (mM) for B6 compounds toward the oxidation of various catechol derivatives by Cu-Aβ complexes

Complex/B6	PM[a]	PN[a]	PL[b]	PLP[b]
Cu-$A\beta_{1-20}$	1.40	8.29	1.12 (2.8)	0.20
Cu-$A\beta_{1-16}$	1.03	5.56 (8.67) 5.05[b]	0.67	0.13

[a] Dopamine was used as substrate (or THB in parentheses).
[b] DTBC was used as substrate (or THB in parentheses).

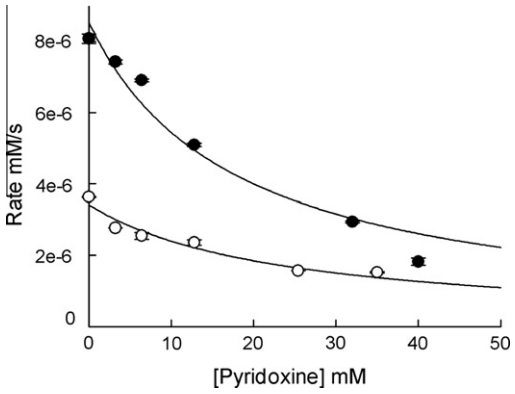

Figure 2. PN inhibition of dopamine oxidation (1.0 mM) catalyzed by (●) 17.0 μM Cu^{II}-$A\beta_{1-20}$ and (○) 3.0 μM Cu^{II}-$A\beta_{1-16}$ in 0.1 M HEPES pH 7.4 at 25 °C.

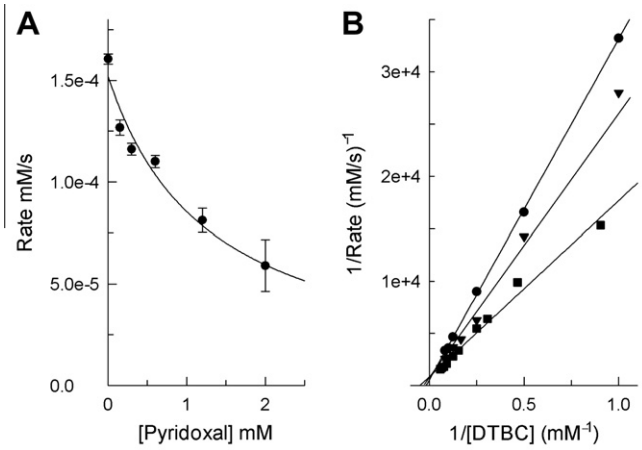

Figure 3. (A) PL inhibition toward DTBC (3.0 mM) oxidation by 1.4 μM Cu^{II}-$A\beta_{1-20}$ complex in 0.1 M HEPES at pH 7.4 and 25 °C. (B) Lineweaver–Burk plot of 1/rate versus 1/[DTBC] in the presence of fixed concentrations of PL at (■) 0.0, (▼) 0.4, and (●) 1.2 mM.

is an indication that PL and phosphate inhibit the oxidation reactions synergistically, which becomes more pronounced when they are covalently combined in PLP. The synergistic effect of phosphate is further supported by a previous study of the influence of redox-active agents $NAD(P)^+$/NAD(P)H toward the oxidation of dopamine by Cu-$A\beta_{1-20}$,[18] wherein NADP(H) exhibits a more pronounced inhibition than NAD(H).

Dietary deficiency of vitamins B6, B9 (folic acid), and B12 is known to increase the level of homocysteine in the blood, which was suggested to increase the risk of AD according to studies on transgenic mouse models of the disease.[16,22] Increase in homocysteine level was also shown to increase oxidative stress and alter DNA methylation.[23,24] Recent clinical trials show that daily doses of up to 95 mg total of vitamins B6, B9, and B12 can slow the onset of AD by decreasing the rate of brain shrinkage.[25] While another clinical trial concluded that there was insufficient evident that

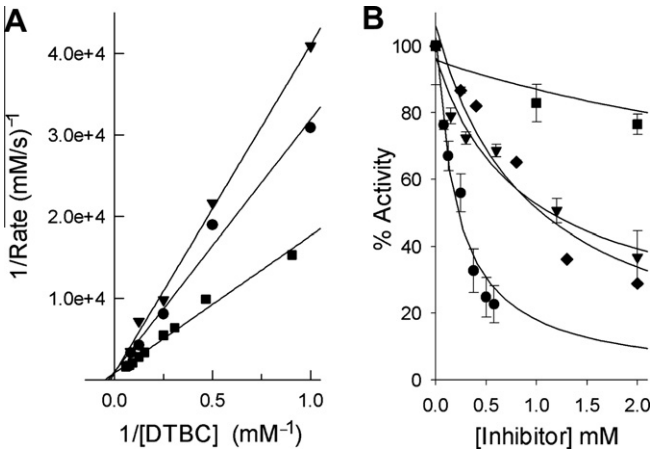

Figure 4. (A) Lineweaver–Burk plot of 1/rate versus 1/[DTBC] in the presence of 0.0, 0.13, and 0.30 mM (from bottom) of PLP toward DTBC oxidation catalyzed by 1.4 μM Cu^{II}-$A\beta_{1-20}$ in 0.1 M HEPES at pH 7.4 and 25 °C. (B) Percent activity of DTBC oxidation by Cu-$A\beta_{1-20}$ as a function of increasing concentration of PLP (●), PL (▼), phosphate (■, up to 50 mM), and 1:1 PL:phosphate (♦), all in 1:1 MeOH:HEPES buffer at pH 7.0 and 25 °C.

vitamin B6 alone has any benefit to AD patients.[26] The concern in terms of the clinical trials arises in terms of the time span, number of individuals, and the disease stage, which may affect the output of the results.

In conclusion, we presented herein that vitamin B6 compounds are competitive inhibitors toward the oxidation of catechol derivatives and the catecholamine neurotransmitter dopamine by Cu^{II}-Aβ complexes that may reflect the conditions under oxidative stress. A structure-activity relationship is evident based on the K_i values of the inhibitors. The functional group at C-4 position and the phosphoester moiety in PLP are significant in inhibiting the oxidative activity. Especially the more potent PLP may serve as a lead for future development of non-toxic compounds against the oxidative stress caused by redox-active metallo-Aβ complexes. Whether or not the Asp/Glu and Arg/Lys side chains in Aβ are involved in the interactions with the functional group at C-4 and/or the phosphoester moiety in PLP await future investigations. Since the oxidative activity of Cu-Aβ complexes and their oxidative damage toward neurotransmitters and biomolecules have been demonstrated, inhibition of the oxidative reactions may alleviate oxidative stress in the disease to certain extents and may provide a alternative for AD therapeutics and/or prevention.

Acknowledgments

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21. In a typical kinetic assay, several concentrations of the substrate (dopamine, 1,2,3-trihydroxylbenzene THB, or 3,5-di-*tert*-butylcatechol DTBC) were incubated with a constant concentration of Cu^{II}-Aβ in 0.1-M HEPES buffer at pH 7.4 and 25 °C. The formation of the corresponding *o*-quinone product was monitored for the oxidation of dopamine, DTBC, and THB (at ε_{480} = 3,300, ε_{414} = 1,910, and ε_{500} = 32,500 M^{-1} cm^{-1} with the last as an adduct of 3-methyl-2-benzothiazolinone hydrazone MBTH) on a Varian CARY50 spectrophotometer, and the rates determined by the change in absorbance over time. The effect of B6 on substrate oxidation was measured at various B6 concentrations following standard enzymological protocols to afford the inhibition pattern and inhibition constants K_i.
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