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RESEARCH ARTICLE

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Proton pump inhibitors act with unprecedented potencies as inhibitors of the acetylcholine biosynthesizing enzyme—A plausible missing link for their association with incidence of dementia

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Abstract

Introduction: Several pharmacoepidemiological studies indicate that proton pump inhibitors (PPIs) significantly increase the risk of dementia. Yet, the underlying mechanism is not known. Here, we report the discovery of an unprecedented mode of action of PPIs that explains how PPIs may increase the risk of dementia.

Methods: Advanced *in silico* docking analyses and detailed enzymological assessments were performed on PPIs against the core-cholinergic enzyme, choline-acetyltransferase (ChAT), responsible for biosynthesis of acetylcholine (ACh).

Results: This report shows compelling evidence that PPIs act as inhibitors of ChAT, with high selectivity and unprecedented potencies that lie far below their *in vivo* plasma and brain concentrations.

Discussion: Given that accumulating evidence points at cholinergic dysfunction as a driving force of major dementia disorders, our findings mechanistically explain how prolonged use of PPIs may increase incidence of dementia. This call for restrictions for prolonged use of PPIs in elderly, and in patients with dementia or amyotrophic lateral sclerosis.

KEYWORDS

acetylcholine, Alzheimer's disease, amyotrophic lateral sclerosis, choline-acetyltransferase, cholinergic system, dementia, Down's syndrome, esomeprazole, *in silico* analyses, Lewy body dementia, omeprazole, lansoprazole, pantoprazole, proton pump inhibitors, rabeprazole, tenatoprazole

1 | INTRODUCTION

The cholinergic system is one of the oldest and the most widely spread neuronal and non-neuronal signaling systems in the body, and in an evolutionary perspective has acquired regulatory functions in diverse biological processes and organs. The cholinergic neurons and their projections are identified by intracellular presence of the acetylcholine (ACh) bio-synthesizing enzyme, choline-acetyltransferase (ChAT). All

other downstream neurons and non-excitable cells which express ACh-receptors are cholinoceptive cells. The central cholinergic system consists of four "ChAT-containing" neuronal nuclei (Ch1–Ch4) located in the basal forebrain.[1] Ch1 and Ch2 innervate the hippocampal complex, Ch3 the olfactory bulb. Ch4-neurons are located in the nucleus basalis of Meynert (nbM), which innervate the rest of cerebral cortex and amygdala.[1] These cholinergic nuclei and their widespread projections throughout the brain have recently been mapped in detail as a 3D whole-brain atlas together with the morphology and the connectivity of individual neurons from the basal forebrain.[2] In addition, extensive cholinergic neuronal projections, originated from 13 cranial nerves (CN0 and I-XII, constituting the parasympathetic system[3]), reach throughout the body, thereby controlling autonomic function of diverse organs, muscles, and glands.[4,5] Another major neuronal system with extensive cholinergic circuitry is the enteric nervous system (ENS), in which about 64% of the neurons are cholinergic.[6]

Regardless of the underlying causes or molecular mechanisms, an indisputable and paramount feature of a spectrum of neurodegenerative diseases (commonly leading to manifestation of cognitive impairment and movement disorders) is the degeneration of cholinergic neuronal networks. For instance, one of the key features of Alzheimer's disease (AD), that is also shared by dementia with Lewy bodies (DLB), Parkinson's disease dementia (PDD), and Down syndrome (DS), is an early and severe degeneration of these central cholinergic projections throughout the brain.[7-13] There are also cholinergic interneurons in the striatum, which target the nigrostriatal system, which are involved in Parkinson's disease,[14] in the corticobasal degeneration syndrome (CBD) and progressive supranuclear palsy (PSP).[15-19] Another particularly devastating neurodegenerative disease is amyotrophic lateral sclerosis (ALS), a deadly motor neuron disease, in which a highly selective degeneration of the cholinergic motor neurons is one of the key pathophysiological findings. Postmortem ALS studies indicate severe decline both in the number of large motor neurons and their positivity for ChAT.[20,21]

Another well-established characteristic that is shared by AD, LBD, and DS is accumulation of amyloid-β (Aβ) peptides in the brain.[22,23] Intriguingly, one report shows accumulation of intracellular Aβ in spinal cord motor neurons of patients with ALS.[24] Several lines of evidence implicate an inter-loop between Aβ peptides and the cholinergic machinery and neuronal integrity, in which Aβ peptides play a modulatory role through a direct allosteric interaction with several components of the cholinergic machinery, namely acetylcholinesterase (AChE); butyrylcholinesterase (BChE); the high affinity choline transporter; and the core ACh biosynthesizing enzyme, ChAT.[22,25-28]

Accumulated evidence further indicates that cholinergic dysfunction may occur long before the manifestation of clinical symptoms. For example, it is reported that early changes in the basal forebrain predict atrophic changes in the entorhinal cortex, one of the first brain regions that becomes affected by AD.[29] Similarly, earliest symptoms of ALS usually include muscle weakness, indicating that the cholinergic motor neurons become affected early and progressively in the course of the disease. In addition, both neuronal and non-neuronal cholinergic activities decline with advancing age, that is, one of the strongest risk factors of AD.[30,31] These together with the apparent close inter-loop between the Aβ peptides and cholinergic function suggest that the cholinergic dysfunction may be a driving force rather than a consequence of AD.[32] Aligned with this hypothesis are the findings from numerous pharmacoepidemiological studies, reporting that prolonged and accumulative exposure to drugs with anti-cholinergic burden does not merely produce clinical symptoms resembling dementia but actually increases the incidence of dementia.[33-40]

Nonetheless, similar pharmacoepidemiological association is reported between incidence of dementia and use of other drugs with, to date, no known anti-cholinergic burden. A prominent example is the proton pump inhibitors (PPIs) as several pharmacoepidemiological studies indicate that PPIs significantly increase the risk of dementia[41-44] (albeit not without controversies[45]). PPIs belong to one of the most common classes of drugs that are used for management

RESEARCH IN CONTEXT

1. Systematic review: Literatures were reviewed using a PubMed search. Reference lists of the assessed literature were screened further for related papers. Several pharmacoepidemiological studies indicate that prolonged exposure to proton pump inhibitors (PPIs) significantly increases the risk of dementia. Yet, the underlying mechanism for this association is not known. Given that accumulating evidence points at cholinergic dysfunction as a driving force of the major dementia disorders, we examined whether PPIs alter the function of the core-cholinergic enzymes, in particular choline-acetyltransferase (ChAT).
2. Interpretation: *In silico* molecular docking analyses on the human ChAT structure revealed that PPIs interact with ChAT. Initial *in vitro* analyses on ChAT protein confirmed that the interaction of PPIs with ChAT results in a significant inhibition of ChAT activity. Further systematic enzymological analyses showed that PPIs act as highly selective and reversible ChAT inhibitors with unprecedented potencies. A comparison of the inhibition constant in relation to the well-known concentrations of these drugs in humans suggested with high probability that PPIs might exert significant anticholinergic activity, consistent with several adverse events recorded for PPIs in human. Due to the compelling nature of the findings, PPIs should be prescribed for the shortest period of time possible with special care in the elderly, and in patients suffering from dementia.
3. Future directions: Future pharmacoepidemiological studies combined with measurement of PPI concentration in the plasma and cerebrospinal fluid are highly warranted to ascertain the clinical significance of identified anticholinergic activity of these drugs.

of hyperacidity disorders such as gastroesophageal reflux and peptic ulcers, and many of them are available over the counter.

Currently, there are no credible underlying mechanisms for the association between the exposure to PPIs and the increased risk of dementia.[41-43] Here, we report the discovery of an unprecedented mode of action for PPIs, namely that all the tested members of this class of drugs exhibit a potent inhibitory action on the activity of the core cholinergic enzyme ChAT. This provides not only the highly plausible mechanism that PPIs increase the risk of dementia by reducing the biosynthesis of ACh through the identified anti-ChAT activity but also reinforces the hypothesis that malfunctioning of the cholinergic system should be considered a driving force of AD-type dementia.

2 | MATERIAL AND METHODS

2.1 | *In silico* analyses

In silico docking analyses was performed on all U.S. Food and Drug Administration (FDA)-approved PPI drugs identified after an initial virtual screening step to elucidate their mode of binding in the active sites of ChAT protein. The crystal structure of ChAT (PDB ID: 2FY3)[46] was downloaded from the Protein Data Bank (PDB) database and a 3D structure of ChAT was prepared by addition of hydrogens, repairing side chain, treating termini, fixing atom type, setting protonation state, fixing bond order, adding charge, and fixing side chain amide. The prepared structure was minimized to remove the strain produced during the earlier protein preparation steps. The defined binding pocket, that is, a "Protomol" was generated using the co-crystallized ligand in the active site of ChAT. The chemical structure of the drugs was sketched covering both R and S stereoisomers and converted into 3D conformation. To ascertain selection of compounds with potential blood-brain barrier (BBB) permeability, the library was screened against modified Lipinski parameters for central nervous system (CNS) drugs with potential to penetrate BBB[47] as described in detail previously.[48] Finally, the prepared dataset of compounds were docked into the active site of ChAT using Surflex-Dock GeomX (SFXC) module interfaced in SYBYL-X 2.1.1[49] and the compounds were ranked using Total_Score (-logK_d).

The theoretical lipophilicity of the selected compounds (as a measure of BBB permeability) was further estimated by *in silico* calculation of logD values, which represent the octanol-water coefficient of compounds at a given pH value. The calculation was done using ChemAxon's Instant JChem 18.8.0 software for pH range of 6.5 to 8.0.

2.2 | *In vitro* enzyme-kinetic analyses

2.2.1 | Purification of recombinant human ChAT

Recombinant human ChAT was produced in *Escherichia coli* using pProEXHTa-ChAT plasmid as previously described.[26,27,48,50] Briefly, DYT media was inoculated with a preculture of *E. coli* BL21 Rosetta2 cells transformed with pProEXHTa-ChAT (a generous gift from Brian Shilton[50]). The bacteria were grown at 37°C and induced with 0.5 mM IPTG at 0.5 OD. His_6-ChAT was allowed to express for circa 16 hours at 18°C. Afterward, cells were harvested and His_6-ChAT was purified with "Ni-NTA fast start Kit" (Qiagen) following the manufacturer's instructions. The elution buffer was exchanged to storage buffer (10 mM Tris pH 7.4, 500 mM NaCl, 10% [v/v] glycerol) using 30 kDa cut-off Amicon Ultra concentrators (Merck Millipore). ChAT protein was produced and purified by the Protein Science Facility (PSF) at Karolinska Institute/SciLifeLab (http://ki.se/psf). The protein preparation was aliquoted, frozen on dry ice, and stored at -80°C. The purity of protein was determined using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) stained with Coomassie blue dye. The total protein concentration was measured using BioRad DC protein Assay (BioRad).

2.3 | *In vitro* high-throughput enzyme kinetic assays

2.3.1 | AChE and BChE activity inhibition assays

A modified version of Ellman's colorimetric assay was designed and adapted for high-throughput assay to monitor activity of the *in silico* hits against the enzymatic activity of BuChE and AChE in real time, as described previously.[48] The reagent, butyrylthiocholine iodide (BTC), acetylthiocholine iodide (ATC), 5,5′-dithiobis (2-nitrobenzoic acid; DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The assays were run on Nunc 384-well plates. Briefly, 25 μL/well of a 1:1600 diluted solution of a pooled human plasma and 1:750 diluted (133 ng/mL final concentration) purified recombinant human AChE protein (Sigma, cat no. C1682) was used for measurement of BuChE and AChE activity, respectively. The wells were pre-incubated with 25 μL/wells of 100 μM concentrations of the *in silico* hits for 30 minutes at room temperature. To the control wells (no hits) just 25 μL/well of buffer was added. After 30 minutes, 25 μL of a cocktail mix prepared in Na/K phosphate buffer, containing DTNB (final concentration 0.4 mM) and BTC (final concentration 5 mM) or ATC (final concentration 0.5 mM) was added to each well. The plates were read using a microplate spectrophotometer reader (Infinite M1000, Tecan) and changes in the absorbance was monitored at 412 nm wavelength for 15 to 20 minutes with 30 second interval. Negative controls were wells without enzyme and each hit was run in octuplicate.

2.3.2 | Fluorometric ChAT inhibition assay

ChAT activity was measured using our newly developed fluorometric assay, using human recombinant ChAT (rChAT) protein.[26,27,48] The reagents, choline chloride, acetyl coenzyme-A (ACoA, A2181) and 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) were purchased from Sigma-Aldrich. The ChAT assay could be run in either 96-well or 384-well plates in real time, as described previously.[26,27,48]

Initial activity inhibition screening was done using 96-well plates. Briefly, 50 μL/well of 0.212 μg/mL (final concentration) of the

recombinant ChAT was incubated with 100 μM of different *in silico* hits (50 μL/well) for 10–30 minutes at room temperature in dilution buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0 mM etyhlenediaminetetraacetic acid [EDTA], 0.05% [v/v]) Triton X-100). Then, 50 μL of a cocktail-A (dilution buffer containing choline chloride [final concentration 150 μM], ACoA [final concentration 13.3 μM], and CPM [final concentration 15 μM]) was added to each well. Immediately after adding the cocktail-A, the changes in fluorescence were monitored kinetically at 479 nm after exciting at 390 nm at 1- to 2-minute intervals for 15 to 20 minutes using a microplate spectrophotometer reader (Infinite M1000, Tecan).

Each *in silico* hit was run in at least triplicate. On each 96-well plate, several enzyme wells without inhibitor were also included during measurements as control and for estimating the inhibition level. Negative controls were wells without enzyme. The percentage inhibition for each hit was calculated based on the enzyme control value as a reference (100% activity). The fluorometric ChAT assay was used when real-time kinetics of the enzyme was assessed. The top hits showing more than 98% inhibition of enzyme activity were selected for further kinetic studies.

2.4 | Enzyme kinetic parameters (K_i, IC_{50}, and mode of action) of top hits

For enzyme kinetic studies, a similar protocol as inhibition assay was followed; a dilution series of five different concentrations ranging from μM to nM were prepared for each selected top hit. For the enzyme kinetics, the concentration of choline chloride was varied between 320 and 10 μM, but the ACoA was kept constant at 10 μM (final). Each PPI compound concentration was measured in duplicate. The rate of enzyme activity (as ΔFU/hour kinetic data) was calculated and analyzed using the GraphPad Prism 8 analysis software.[15] The inhibitory constant (K_i) values were determined from the dose-response curve and the half-maximal inhibitory concentration (IC_{50}) values were calculated by plotting the percentage enzyme activity versus the log of the compound concentrations and fitting the data using the nonlinear regression enzyme kinetics-inhibition function.

The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) values were calculated from substrate-velocity curve after fitting the data with non-linear regression Michaelis-Menten kinetic function. The data were further used to plot the Lineweaver-Burk plots; the plots were fitted using linear regression function.

3 | RESULTS

3.1 | PPIs exhibit high *in silico* scores against human ChAT

In the context of an ongoing research project for the development of a new *in vivo* positron emission tomography (PET) imaging tracer for mapping the cholinergic neurons and their widespread projections throughput the brain, we initiated *in silico* screening on a subset of FDA approved drugs (Figure 1A). This *in silico* screening analysis identified all PPIs (viz, omeprazole, lansoprazole, pantoprazole, rabeprazole, tenatoprazole, and ilaprazole) as potential ligands of human ChAT. Then we performed advanced *in silico* molecular docking analyses on all PPIs, all of which exhibited high *in silico* binding scores for the core cholinergic enzyme, ChAT (Table 1). The 3D and 2D representations for the most common PPIs, omeprazole, lansoprazole, and pantoprazole, are shown in Figure 1B. Initially, these *in silico* high-scoring PPIs were then screened *in vitro* by an in-house real-time kinetic ChAT assay[26,48] at a single concentration of 100 μM. At this screening condition, all the tested PPIs exhibited an almost complete inhibition of the activity of the ChAT enzyme (Figure 1C). Of note, we also tested the S-enantiomer of omeprazole, that is, esomeprazole, which is also an FDA approved drug, on its own. Esomeprazole also passed this initial *in vitro* screening.

In addition, we tested omeprazole sulfone, which is one of the major metabolites of omeprazole or esomeprazole.[51] At a concentration of 100 μM, omeprazole-sulfone caused 31 $\pm$ 2% ChAT inhibition, while omeprazole completely inhibited ChAT at this concentration. Thus, this major omeprazole metabolite is expected to exhibit very weak, if any, *in vivo* activity on ChAT in the brain.

3.2 | The IC_{50} values of PPIs for human ChAT are in low micromolar ranges

We then determined IC_{50} values for the most commonly used PPIs, lansoprazole, omeprazole (and esomeprazole), and pantoprazole. These analyses were run at the substrate concentrations of 150 μM for choline and 10 μM for acetyl-coenzyme A (acetyl-CoA), a co-factor that is required by ChAT for the biosynthesis of ACh. These analyses revealed that omeprazole, lansoprazole, and pantoprazole inhibited ChAT with remarkable potencies as can be deduced from IC_{50} values of 0.1, 1.5, and 5.3 μM, respectively (Figure 1D). This indicates that omeprazole as a ChAT inhibitor is about 15 to 50 fold stronger than lansoprazole or pantoprazole. Esomeprazole, the S-enantiomer of omeprazole, exhibited an IC_{50} between 0.05 and 0.07 μM. This IC_{50} range is about half of the IC_{50} of omeprazole as a ChAT inhibitor. Omeprazole is a racemic mixture, meaning that at a given concentration it contains 50% esomeprazole. Thus, we may conclude that esomeprazole is fully responsible for ChAT inhibition by racemic omeprazole.

3.3 | The inhibition constants (K_i) of PPIs for human ChAT are in nanomolar ranges

IC_{50} values are however prone to vary depending for instance on the concentration of the enzyme substrates, which under certain conditions make interpretation of IC_{50} somewhat problematic. We therefore performed systematic enzyme-inhibition kinetics analyses, including multiple concentrations of the substrate and the drug. This way we

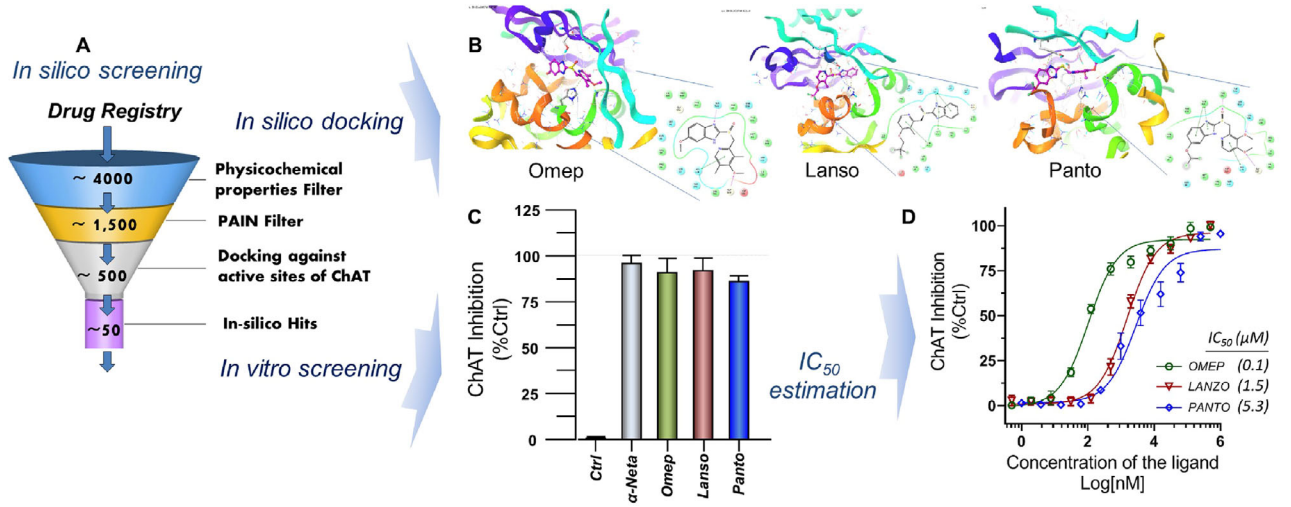

FIGURE 1 *In silico* and *in vitro* screening workflow for the identification of novel choline-acetyltransferase (ChAT) inhibitors from U.S. Food and Drug Administration drug registries. A, The *in silico* screening procedure and the most important selection filters. For more details please see the Material and Methods section. These *in silico* analyses identified proton pump inhibitors (PPI)s as top scoring compounds. B, Results of docking analyses of omeprazole, lansoprazole, and pantoprazole in the binding pocket of the catalytic domain of ChAT, respectively. The insets are 2D presentation of the binding of the compounds. C, Result of the initial *in vitro* screening of the high *in silico* scoring PPIs at a single concentration of 100 μM of the drugs. The substrate concentrations were 150 μM for choline and 10 μM for acetyl-coenzyme A. The inhibitory effect of PPIs and α-NETA (a known commercially available ChAT inhibitor) were compared with the activity of the enzyme in presence of buffered control (Ctrl, no inhibitors; 100% activity). D, Half maximal concentration (IC_{50}) estimation curves for the most commonly used PPIs, that is, omeprazole, lansoprazole, and pantoprazole. The IC_{50} value was calculated after fitting the dose-response curves using nonlinear regression function of GraphPad Prism 8. The values are presented as mean ± standard error of the mean. Omep = omeprazole, Lanso = lansoprazole, Panto = pantoprazole

TABLE 1 *In silico* molecular docking scores for the representative identified ligands of choline-acetyltransferase

Compound name	[a]Total_score (-logK_d)	[b]Crash	[c]Polar
(R)-Omeprazole	9.457	−4.158	3.373
(S)-Omeprazole	9.374	−2.706	1.653
(R)-Lansoprazole	7.803	−0.766	0.547
(R)-Pantoprazole	8.457	−1.253	3.297
(S)-Pantoprazole	9.151	−2.409	2.248
(R)-Tenatoprazole	8.433	−2.257	1.262
(S)-Tenatoprazole	8.782	−1.236	1.586
(R)-Rabeprazole	9.902	−2.659	3.855
(S)-Rabeprazole	9.013	−3.44	1.912
(R)-Ilaprazole	8.774	−2.376	2.378
(S)-Ilaprazole	7.55	−1.714	2.478

[a]Total score is the total Surflex-Dock score expressed as -log(K_d).
[b]Crash is the degree of inappropriate penetration by the ligand into the protein and of interpenetration (self-clash) between ligand atoms that are separated by rotatable bonds. Crash scores close to 0 are favorable.
[c]Polar is the contribution of the polar interactions to the total score.

determined the inhibition constants (K_i) that are most reliable as well as the mode of action of PPIs as inhibitors of ChAT.

The estimated K_i for omeprazole ranged between 70 and 140 nM and for esomeprazole between 50 and 70 nM (Figure 2A). Non-linear enzyme-ligand kinetic analyses indicated with high probability (>99%) that this PPI behaves with regard to choline concentration as a mixed-competitive reversible inhibitor of ChAT. This mode of action is also graphically illustrated by the classical Lineweaver-Burk plot analyses in Figure 3A. An important implication of this mode of action is that omeprazole (and/or esomeprazole) will bind with high affinity to the enzyme, regardless of the state of the enzyme (ie, being in free state or bound with the substrate choline). It also means that the endogenous *in vivo* concentration of choline may minimally prevent inhibition of ChAT by this compound, as expected when a compound behaves as a full competitive inhibitor.

We further found that two other PPIs exhibited even higher potency as ChAT inhibitor than esomeprazole. Tenatoprazole exhibited a K_i value of 18 nM (Figure 2B), and rabeprazole a K_i of 17.5 nM for ChAT (Figure 2C). In addition, both behaved as non-competitive reversible ChAT inhibitors (Figure 3B and C). A non-competitive mode of action implies that both raberprazole and tenatoprazole have equal high affinity for both free and choline-bound enzyme, and that they can inhibit ChAT regardless of *in vivo* concentration of the substrate, choline.

ChAT is an enzyme that simultaneously uses two substrates, choline and acetyl-CoA (or ACh and –CoA). Thus, we examined the K_i value and the mode of action of PPIs with regard to acetyl-CoA. The enzyme kinetic analyses for omeprazole and esomeprazole was performed at 0 to 100 μM concentration range of acetyl-CoA. The result is shown in Figure 4. Non-linear regression analyses estimated a K_i of 6.5 μM for omeprazole (ranging between 3.2 and 9.7 μM, Figure 4A). The corresponding K_i for esomeprazole was 4.2 μM (ranging between 2.2 and 16.0 μM, Figure 4B). These analyses further showed with 99%

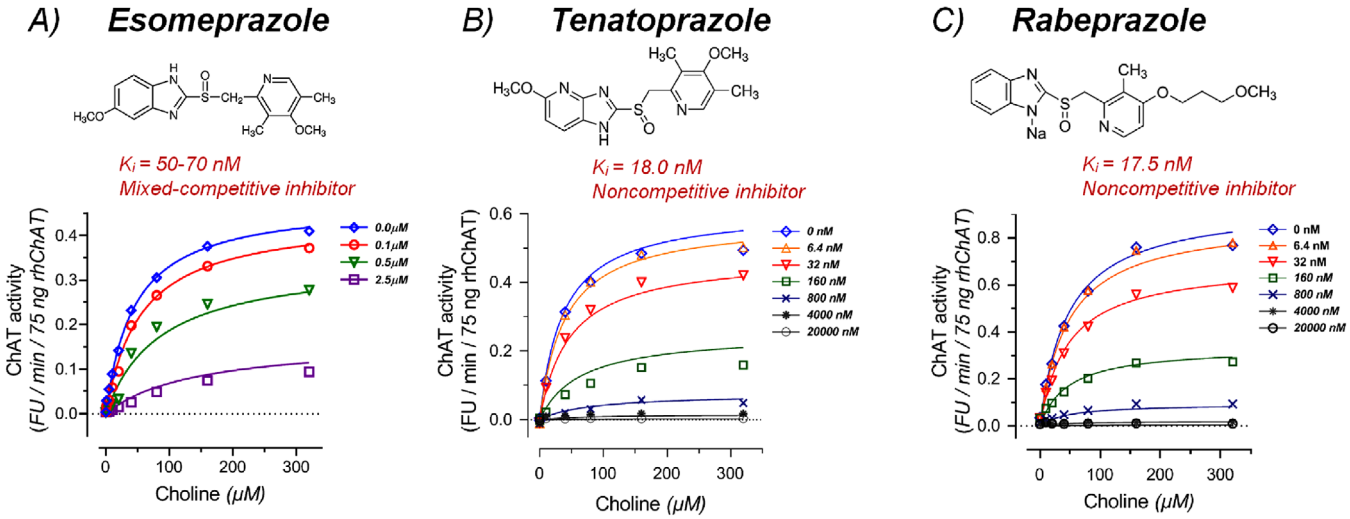

FIGURE 2 Enzyme-inhibition kinetic analyses for the most potent proton pump inhibitors. Graphs (A), (B), and (C) show the substrate-velocity curves of the choline-acetyltransferase (ChAT) enzyme activity at different concentrations of the substrate, choline (ranging from 10 to 320 μM) in the presence of specified concentrations of esomeprazole, tenatoprazole, and rabeprazole, respectively. The 2D structure of each compound is also provided. Nonlinear regression analyses were used to estimate inhibition constant (K_i) value and their mode of inhibition, which are also given in (A)—(C). These were determined using GraphPad Prism 8. The values are presented as mean ± standard error of the mean (SEM). It should be noted that due to small SEM values the error bars are not visible

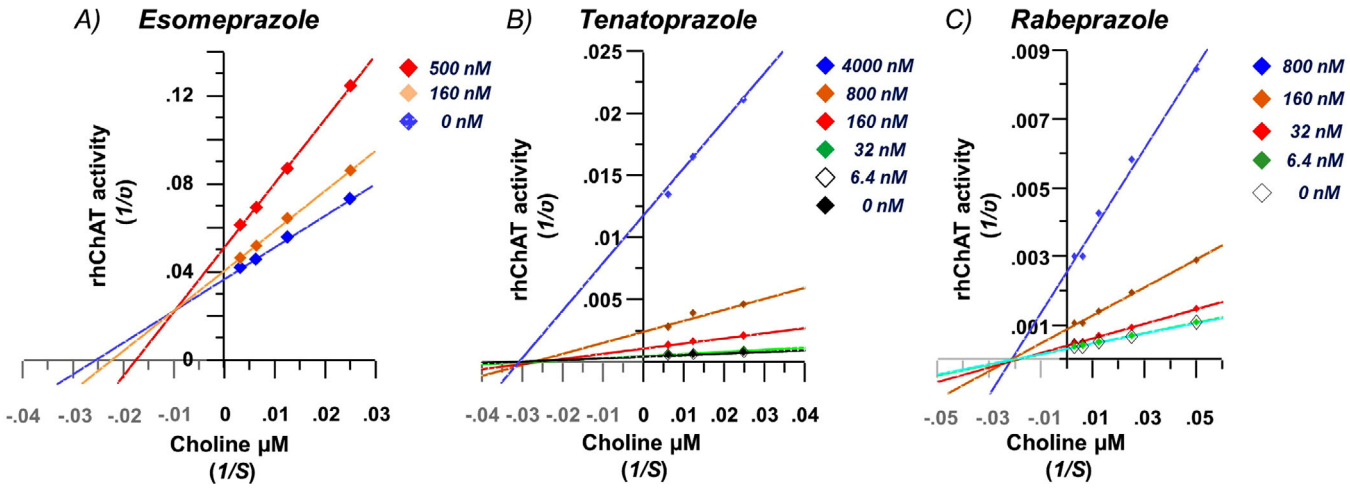

FIGURE 3 Lineweaver-Burk plot illustration of the mode of action of proton pump inhibitors as choline-acetyltransferase (ChAT) inhibitors. A, Esomeprazole behaves as a mixed-competitive reversible ligand because the lines are crossing each other over the *x*-axes. B and C, Tenatoprazole and rabeprazole behave as non-competitive reversible ligands of ChAT, respectively, as the lines are crossing each other on the *x*-axes

probability that omeprazole behaved like a fully competitive inhibitor while esomeprazole behaved as a mixed-competitive inhibitor in relation to acetyl-CoA (Figure 4).

3.4 | PPIs inhibit human ChAT with higher potencies than α-NETA a known potent inhibitor of ChAT

To fully appreciate the potency of these compounds we also tested one of the strongest known inhibitors of ChAT, a commercially available compound known as α-NETA, which exhibited a potency (IC_{50}) of ~90 nM in our laboratory.[27] Thus, the racemic mixture of omeprazole has similar potency as α-NETA, while esomeprazole is about twice as strong, and tenatoprazole or rabeprazole are about five times stronger than α-NETA as ChAT inhibitors.

3.5 | PPIs exhibit higher selectivity for human ChAT than the related enzymes

The enzymatic pocket of ChAT enzyme may share similarities with other enzymes that use ACh or choline as substrates. We hence tested

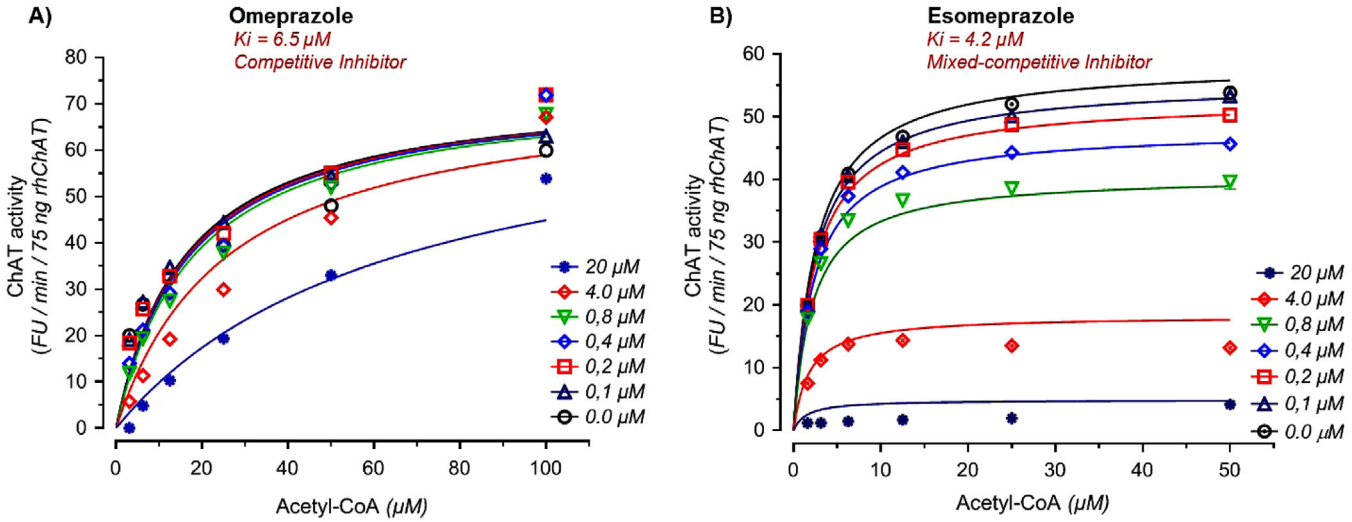

FIGURE 4 Enzyme-inhibition kinetic analyses for the most potent proton pump inhibitors (PPIs) with regard to acetyl-CoA as substrate. A and B, Substrate-velocity curves of the choline-acetyltransferase (ChAT) enzyme activity at different concentrations of the substrate, acetyl-Co enzyme A (ACoA) in the presence of various concentrations of omeprazole and esomeprazole, respectively. The values are presented as mean ± standard error of the mean (SEM). It should be noted that due to small SEM values the error bars are not visible. The half maximum inhibition constants (K_i) values and the mode of action of the ligands were determined by nonlinear regression analyses with regard to ACoA binding site, using GraphPad Prism 8

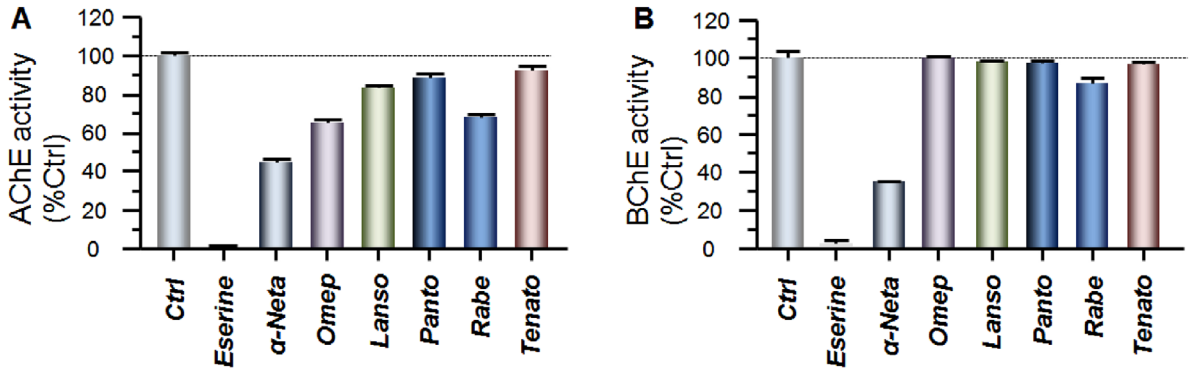

FIGURE 5 Inhibitory effects of proton pump inhibitors (PPIs) on choline-acetyltransferase (ChAT) related cholinergic enzymes. A, Result of the activity of PPIs on the acetylcholine-degrading enzyme, acetylcholinesterase (AChE) at a single concentration of 100 μM of each PPI. B, Corresponding results for butyrylcholinesterase (BChE). Eserine, a known inhibitor of AChE and BChE served as a positive control. α-NETA, a known ChAT inhibitor, was used for a comparison with PPIs. The activity of the enzymes in the absence of the drugs was used as control (Ctrl: no inhibition, 100% activity). Each compound was run in octuplicate and data are shown as mean ± standard error of the mean. Omep = omeprazole, Lanso = lansoprazole, Panto = pantoprazole, Rabe = rabeprazole, Tenato = tenatoprazole

how PPIs affected the activity of choline-oxidase (oxidizing choline to betaine), and AChE and BChE, which hydrolyze ACh when released into synapses to terminate ACh signaling.

At the high screening concentration of 100 μM, four of the five PPIs showed less than 30% inhibition of human AChE (Figure 5A). These PPIs inhibited less than 10% of human plasma BChE activity (Figure 5B). None of the PPIs affected the activity of choline oxidase (data not shown).

Considering that the PPIs' IC_{50} or K_i for ChAT is between 0.017 and 5.3 μM, these PPIs exhibited at minimum between 20 (100 μM/5.3 μM) and 5800 fold (100 μM/0.017 μM) higher affinity for ChAT than AChE or BChE. As a comparison, α-NETA (the known ChAT inhibitor) showed IC_{50} values of 88 nM for ChAT versus 34 μM for both AChE and BuChE, indicating merely 386 fold selectivity toward ChAT.[27] Thus, we may conclude that PPIs act with an unprecedentedly high selectivity and potency as inhibitors of ChAT compared to these related cholinergic enzymes and one of the known strongest ChAT inhibitors.

Various acyltransferases, similar to ChAT, also use acetyl-CoA as one of their substrates, raising the question whether PPIs may also inhibit these enzymes. As depicted in Figure 4, the estimated K_i values for omeprazole and esomeprazole were 6.5 and 4.2 μM, respectively. Given that omeprazole's K_i value for ChAT with regard to choline as substrate is ~0.1 μM, the result indicates that omeprazole has at least

65 fold (6.5/0.1 = 65) less affinity/selectivity toward the acetyl-CoA binding sites on ChAT. The comparison of the corresponding K_i values for esomeprazole with regard to acetyl-CoA (4.2 μM) versus choline (0.05–0.07 μM) suggest 60 to 84 fold (4.2/0.07 = 60 or 4.2/0.05 = 84) lesser selectivity of esomeprazole for acetyl-CoA site. These analyses clearly indicate that PPIs have most likely at least 60 to 80 times lesser activity toward other acyltransferases that use acetyl-CoA but not choline as their substrates.

3.6 | Plasma and the expected brain concentrations of PPIs are several fold higher than their K_i values for human ChAT

The peak concentrations of PPIs in human blood/plasma varies depending, for example, on the dosage of the drug but it generally ranges between 1 and 20 μM with a plasma elimination half-life (t½) of about 1 hour.[53] For instance, the human plasma concentration range for omeprazole following a single standard 20 mg oral dose is between 0.23 and 23.2 μM.[53] This plasma concentration range represents up to 23 fold higher concentration than the estimated K_i of 70 to 100 nM for omeprazole. Although the brain permeability for PPIs is not well documented we had included certain *in silico* screening filters to ascertain selection of compound with potential BBB permeability.[47] In addition, the logD (a measure of lipophilicity) values were also calculated and were 2.4, 2.4, 2.1, and 2.2 for omeprazole, esomeprazole, rabeprazole, and tenatoprazole, respectively. Overall, these theoretical measures indicate that the PPIs are potentially able to pass the BBB. Studies in rats are in agreement with this assessment and have shown a brain/blood concentration ratio of 0.1 to 0.15, suggestive of ~10% to 15% brain permeability for omeprazole.[52,54] In humans, it is expected to be about the same ratio, that is, 10% to 15%.[55,56] Finally, preliminary data on radio-labeled omeprazole in non-human primates indicated a standard uptake value (SUV) of 1.5, which together with *in vivo* PET images clearly showed that omeprazole as radio tracer reached the brain and was distributed quite evenly to all the brain regions (preliminary unpublished data). Given that all PPIs share high similarity in their molecular structure, at least same level of brain permeability could be expected for all the other PPIs, as well. Thus, a conservative brain concentration range is between 150 and 3000 nM, a range that represents a brain penetration level of at least 1.5 to 30 fold higher than the K_i values of omeprazole and esomeprazole. This is, however, 8 to 170 fold higher than the *Ki* values for rabeprazole and tenatoprazole (Figure 2).

4 | DISCUSSION

This report provides compelling evidence that PPIs are able to inhibit the key cholinergic enzyme responsible for the biosynthesis of the cholinergic signal substance, ACh. We also showed that in this aspect PPIs are more potent and more selective inhibitors of ChAT than one of the strongest known inhibitors of this enzyme, that is, α-NETA. We also found that the potency of PPIs as determined by the inhibition constant lies several fold below the known plasma concentrations for these drugs in human patients even following standard daily dosages of 10 to 20 mg of the drugs. However, higher dosages for these drugs are not uncommon, and several of the PPIs are purchasable over the counter in many countries. Overall, these findings together with the results of pharmacoepidemiological studies, linking exposure to PPIs with incidence of dementia,[41-44] are pointing at an alarming secondary mode of action for PPIs in terms of probability of exerting a clinically relevant anti-cholinergic burden.

Given that the best strategy against AD is the detection and when possible elimination of the risk factors, the current findings are important and suggest that the use of PPIs should be considered a legitimate risk factor for both incidence of dementia and its further progression, in particular in the elderly and in patients already suffering from dementia.

Furthermore, the PPIs' anti-cholinergic burden is most likely also relevant for several other diseases, in which a cholinergic dysfunction may be involved, such as ALS and the related motor neuron disorders. Thus, the findings warrant specific pharmacoepidemiological studies on PPI use as risk factor (or risk modifier) for ALS and the related motor neurons disorders.

To the best of our knowledge, all drugs with known anti-cholinergic burden do so by acting as antagonists of the cholinergic receptors, in particular the muscarinic subtypes, while it is the first time that the identified target is the key ACh synthesizing enzyme. Thereby studies on biological consequences of inhibition of ChAT are scarce. There is a large diversity among the cholinergic receptors in peripheral organs as well as in the CNS. This diversity may be in some degree able to biologically mitigate the anti-cholinergic burden of a certain receptor antagonist via other receptor subtypes. This possibility is lacking for ChAT, because this is the single known cytoplasmic enzyme that is capable of biosynthesizing ACh within the cytoplasm of all cholinergic cells and neurons. Given that PPIs have access to the cholinergic interfaces in CNS, peripheral nervous system (PNS), and enteric nervous system (ENS), the mode of action of PPIs in inhibiting ChAT is expected to mediate broader long-term consequences than antagonists of cholinergic receptors, thereby warranting careful investigation in future studies.

We also examined whether PPIs are able to simultaneously inhibit the activities of the ACh-degrading enzymes, AChE and BChE. The results indicated that PPIs have negligible activity on these two enzymes. This finding has an important implication, appreciated as follows. If PPIs inhibited both ChAT and the ACh-degrading enzymes, then their anti-cholinergic action (ie, reduced production of ACh) could have been negated by their cholinergic-enhancing action (inhibition of ACh degradation). Thereby, the net expected biological implication would be low. However, the above analyses indicated that this is very unlikely because the potency of PPIs concerning their inhibition of ChAT is in absolute dominance, even with a highly conservative estimation of 20 to 5800 fold selectivity of PPIs for ChAT versus the cholinesterases. Thus, it is unlikely that the apparent anti-cholinergic burden of PPIs and the possible consequences could be moderated by their much weaker activity on the cholinesterases.

Similarly, a comparison of Ki values for omeprazole and esomeprazole for acetyl-CoA versus choline binding site on ChAT predicts that PPIs most likely exhibit 60 to 84 fold lesser affinity or selectivity toward other enzymes that use acetyl-CoA but not choline as one of their substrates. An important exception is however the mitochondrial enzyme carnitine acetyl-transferase (CART), which has been shown to be able to biosynthesize ACh, albeit at much lesser degree than ChAT. Nonetheless, further enzyme kinetic analyses on other acyl-transferases, particularly on CART, are warranted.

In the context of the biological consequences, it could be argued that PPIs have well-documented safety profiles based on data spanning a period of 20 years and their use in millions of people around the world. However, with the mechanistic hindsight implied by the findings in this report and the aforementioned pharmacoepidemiological studies concerning the association between PPIs and incidence of dementia, it becomes evident the biological consequences of PPIs' anti-cholinergic burden cannot be rejected. Rather it merely indicates that the pathophysiological consequences of the anti-cholinergic burden of PPIs are difficult to detect or to assert clinically, most likely because it requires long build-up time for at least two main reasons. One is that PPIs have very short plasma half-life of about 1 hour.[53] Theoretically, following the most common dosage regimen of PPIs (ie, once daily), the plasma concentration of the drug will rapidly decline to <3% of its initial concentration within 5 hours (5 t½ of the drug). Thus, the duration of the anti-cholinergic activity of the drug may be too short to allow immediate clinical manifestation, particularly because the cholinergic system is known to be highly adaptable and resistant to short term insults. Second, evidence indicates that cholinergic neurons contain an intracellular ACh depot,[57] making it possible for neurons to release ACh after limited short-term insult on ChAT activity. Nonetheless, under certain conditions the cholinergic system may be highly vulnerable to even such a short insult. For instance, it is well established that stress stimuli result in a prolonged hyper-excitation state of cholinergic circuitries,[58] which may greatly deplete the ACh depot, thereby increasing immediate demand on ACh biosynthesis by ChAT. The same may occur after longer-term usage, more frequent daily intake of PPIs, and/or higher dosage regimens as well as other conditions resulting in heavy workload on cholinergic neuronal activity. Under these conditions even short insults on this core cholinergic enzyme may be manifested as symptoms that are normally considered rare side effects or as overdose symptoms of PPIs. These known symptoms are confusion, agitation, hallucinations, depression, dizziness, blurred vision, muscle weakness, fall and risk for fracture, constipation, etc,[59] all of which are known to be associated with anti-cholinergic activity.[60] The anti-cholinergic burden of PPIs is also expected to be more relevant in the elderly as cholinergic activity declines with advancing age.[61,62] The same is also true for patients already suffering cholinergic related disorders, such as AD, LBD, DS, and ALS and the related motor neuron disorders.[7-12,14-21]

Subgroup analyses have been done for the three most prescribed and often used PPIs (omeprazole, pantoprazole, and esomeprazole) in one of the referenced pharmacoepidemiological studies.[41] Among these three PPIs, the estimated risk ratios for incidence of dementia is reported highest for esomeprazole (hazard ratio of 2.12).[41] This is in line with our finding here that esomeprazole was the most potent inhibitor of ChAT compared to the other two. In fact, we found that the overall anti-ChAT activity of omeprazole was attributed to its S-enantiomer, esomeprazole.

However, there is a study on the effect of short-term exposure to PPIs in young healthy human volunteers.[63] The study had six treatment groups composed of 10 young healthy individuals, who were treated for 7 days with omeprazole (40 mg/d), lansoprazole (30 mg/d), rabeprazole (20 mg/d), pantoprazole (40 mg/d), esomeprazole (40 mg/d), or a placebo capsule (as control group). Various cognitive measures were assessed by Cambridge Neuropsychological Test Automated Battery software. The results clearly indicated that even such a short-term exposure to PPIs significantly affected the majority of the assessed cognitive domains. In contrast, no significant changes were observed among the placebo group.[63] The investigators also assessed the effect size of the exposure to PPI using Cohen's d test value, which indicated that the observed outcomes had practical and clinical significance. The investigators concluded that PPIs influence different cognitive domains and have associations with AD. However, they did not find any significant difference among the PPI-exposed groups.[63] This in the context of the current report may suggest that other pharmacokinetic properties of each PPI may be as relevant as their potencies as ChAT inhibitors (ie, K_i values). Such properties could be the relative BBB permeability, protein binding, and enantiomeric specific activity toward ChAT as well as activity of their metabolites toward ChAT, all of which merits further investigations. Nonetheless, we tested one of the major metabolites of omeprazole/esomeprazole that was commercially available, namely omeprazole sulfone. We found that this metabolite inhibited ChAT merely by about 30% at a concentration of 100 μM, while the mother compound, omeprazole, completely inhibited the enzyme at this concentration. Thus, in the case of omeprazole or esomeprazole, the activity of this metabolite is unlikely to be related to the lack of difference between these two treatment arms with regard to the observed cognitive impairment in the aforementioned study.

One of the most important questions in the AD research field concerns identifying the cause or the driving force of the disease. Numerous pharmacoepidemiological studies indicate that prolonged and/or accumulative exposure to drugs with strong anti-cholinergic burden is associated with incidence of dementia.[33-40] This means that such drugs are not merely inducing symptoms of dementia but actually causing dementia. The current consensus in the AD field is however that the observed early cholinergic deficit in AD is merely a consequence of the pathological events in AD. The findings in this report together with the results of numerous pharmacoepidemiological studies[33-40] strongly rejects this consensus, and rather dictate that early cholinergic deficit should be considered a causative force of AD-like dementias. This is also in line with the facts that advanced age is the strongest risk factor of AD and that both neuronal and non-neuronal cholinergic systems are subject to an age-dependent decline.[61,62]

The question is then how cholinergic deficit may act as a driving force. A compelling body of evidence points at complex, multilayered mechanisms. ChAT enzyme kinetic analyses have shown that Aβ,

particularly Aβ42 peptides, act as allosteric potentiator of ChAT activity, by increasing the enzyme efficiency by about 30% at physiological Aβ concentrations.[26] Another group has shown that PPIs (lansoprazole, omeprazole, esomeprazole, and pantoprazole) augment APP metabolism and thereby Aβ production.[64] Given that Aβ is hypothesized to play a pivotal role in the overall ACh homeostasis in the brain,[26,32] and the hindsight provided by the current data we hypothesize that the inhibition of ChAT by PPIs initiates a compensatory feedback loop to restore ChAT activity by increasing the level of the allosteric potentiator of ChAT, that is, Aβ42 peptides. In line with this hypothesis are reports indicating that Aβ induces translocation of a ChAT variant protein to nucleus, which ultimately alters APP metabolism and reduces Aβ production.[65,66] Thus, inhibition of ChAT by PPIs may have various more or less simultaneous outcomes—first it may cause a reduction in ACh biosynthesis that undermines the proper cholinergic signaling, and second it may increase production of Aβ peptides, that is, the positive ChAT modulator to restore the enzyme's activity. However, whether these phenomena work in synergy, discretely, or in opposition requires further investigation.

Additional mechanisms may be appreciated through compelling evidence for presence of a cross-talk between intracellular ACh and mitochondrial integrity.[25,67-71] Indeed, one of the yet to be fully recognized functions of ACh seems to be related to the prevention of the intrinsic mitochondrial apoptotic pathway.[72,73] Accumulating evidence in this context suggests the presence of an inter-loop between ACh biosynthesis and the mitochondrial integrity and bioenergetics function. On one hand, the biosynthesis of ACh is directly linked to the bioenergetics function of mitochondria because its biosynthesis by ChAT requires equimolar amount of acetyl-CoA, a cofactor that is mainly produced within mitochondria during glycolysis. On the other hand, ACh controls the mitochondria-derived cellular apoptotic pathway, through activation of mitochondrial nicotinic ACh receptors present on the outer membrane of mitochondria.[69] Reports indicate that nicotine (as the agonist) inhibits the release of mitochondrial cytochrome C.[67,71] Vice versa, inhibition of cholinergic signaling is shown to cause apoptosis.[72,73] Thereby conditions or drugs that can directly or indirectly reduce the cellular ACh production or level could trigger neurodegeneration.

Another equally relevant consequence of the discovered secondary mode of action of PPIs as inhibitors of ChAT is a possible disturbance in the cholinergic anti-inflammatory pathway.[74] Lymphocytes possess an almost complete cholinergic machinery, including ChAT, and use ACh as an autocrine immune modulator.[75] Given that unchecked neuroinflammatory cascades may be an important feature in various neurodegenerative disorders, ChAT blockage may act as a predisposing factor, particularly among the elderly population, rendering them susceptible to a diverse spectrum of neurodegenerative and autoimmune disorders.

Overall, these apparently complex multilevel mechanisms offer an explanation for some of the controversies observed among the reports concerning the association between PPI exposure and the incidence of AD.

In conclusion, we report for the first time compelling evidence that PPIs reversibly inhibit the activity of the core-cholinergic enzyme ChAT, with unprecedented selectivity and potencies that lie far below their *in vivo* plasma and CSF concentration in humans even at lowest dosages used by millions of people worldwide. This unexpected mode of action together with pharmacoepidemiological observation warrant further mechanistic studies on PPIs in relation to dysfunction of the cholinergic system.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

The concept and design of the study were done by Taher Darreh-Shori and Rajnish Kumar. The enzyme kinetics was done by Rajnish Kumar and Amit Kumar. *In silico* analysis was done by Rajnish Kumar. The first draft of the manuscript was written by Taher Darreh-Shori. All other authors provided critical input and suggestions in finalizing the manuscript. All the authors read and approved the final draft of the manuscript.

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