Repurposing FDA-Approved Compounds for the Discovery of Glutaminyl Cyclase Inhibitors as Drugs Against Alzheimer's Disease

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Alzheimer's disease (AD) is one of the most common neurodegenerative causes of dementia, the pathology of which is still not much clear. It's challenging to discover the disease modifying agents for the prevention and treatment of AD over the years. Emerging evidence has been accumulated to reveal the crucial role of up-regulated glutaminyl cyclase (QC) in the initiation of AD. In the current study, the QC inhibitory potency of a library consisting of 1621 FDA-approved compounds was assessed. A total of 54 hits, 3.33% of the pool, exhibited QC inhibitory activities. The Ki of the top 5 compounds with the highest QC inhibitory activities were measured. Among these selected hits, compounds affecting neuronal signaling pathways and other mechanisms were recognized. Moreover, several polyphenol derivatives with QC inhibitory activities were also identified. Frameworks and subsets contained in these hits were analyzed. Taken together, our results may contribute to the discovery and development of novel QC inhibitors as potential anti-AD agents.

1. Introduction

Along with the aging of the population, Alzheimer's disease (AD) has become a huge economic and social burden all over the world. AD has been identified as a neurodegenerative disorder characterized by the progressive loss of neuronal function and substantial structural and functional damage of the healthy brain.[1–2] As the most prevalent form of dementia, AD accounts for more than 50 million cases currently, the number of which will be expected to triple to 152 million worldwide by 2050.[3] Despite considerable effort has been made to prevent and treat the disease in the past decades, AD is still incurable. So far, six prescription drugs have been approved by FDA to treat AD. However, they can only relieve AD symptoms temporarily, none of which exhibits the effect to cure or decelerate the progression of the disease.[4,5] Considering the fact that the number of individuals expecting to develop neurodegenerative conditions continues to increase, discovering and developing novel and effective agents against AD become extremely urgent.

One of the obstacles to discover anti-AD agents is attributed to the multifactorial nature of AD. Clinical evidence reveals that the main pathological characteristics of AD include the deposition of extracellular senile plaques composed of amyloid-β (Aβ) and the formation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau.[6,7] Aβ aggregates and plaques serve as the main causes of the neuron death. However, no anti-AD drug candidate directly targeting Aβ pathology has been identified.

Recently, a variety of N-truncated Aβs starting with Ala-2, pyroglutamylated Glu-3, Phe-4, Arg-5, pyroglutamylated Glu-11, etc, have been detected in AD brains, but not in normal age-matched control brains.[8] Undergoing truncation and cyclization of N-terminal glutamate (E) to pyroglutamate (pE), these pE-Aβs account for more than 50% of the total plaques in AD brains.[9,10] Compared with Aβs, pE-Aβs confer proteolytic resistance, exhibit increased hydrophobicity, aggregate more rapidly, and seed further Aβ aggregation. More importantly, pE-Aβs and oligomers drive the downstream toxicity cascade to destroy the plasticity of synapses and induce the death of neurons.[11–13] These findings indicate that pE-Aβs may act as an important inducer of AD before the Aβs aggregates are detected.

It has been well-confirmed that the cyclization of E to pE is mainly catalyzed by glutaminyl cyclase (QC, also known as QPCT) *in vitro* and *in vivo*. QC is widely distributed in mammalian brain with robust expression in the hippocampus and cortex.[14–16] The generation of pE-Aβs is directly associated with the up-regulation of QC, whose activity correlates with the accumulation of pE-Aβs and Aβs aggregates and the severity of AD. Furthermore, mRNA levels of QC are increased significantly only in AD brains. Therefore, the up-regulated mRNA level of QC in peripheral blood is suggested as an important biomarker for the diagnosis of AD.[17] On the other hand, inhibition of QC

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represents an alternative therapeutic strategy against AD by reducing the generation of pE-Aβs and blocking the activation of downstream cascades. In view of this, a number of QC inhibitors have been reported, which improve behavioral and cognitive deficits in AD mice by inhibiting QC activity.[18–22] PQ912, a competitive QC inhibitor, exhibited a beneficial effect on working memory and attention in the Phase II SAPHIR trial.[23]

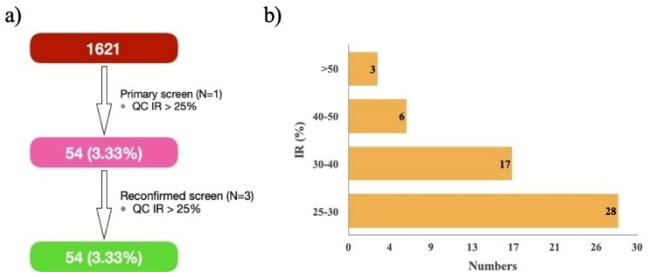


Figure 1. a) Experimental process for screening FDA-approved drug library and b) compounds exhibiting QC inhibitory potency in the screening.

2. Results and Discussion

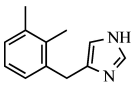
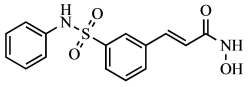
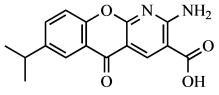
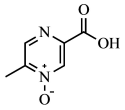
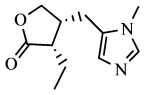
2.1. Identification of Potential QC Inhibitors in a FDA-Approved Compound Library

Increasing effort has been contributed for drug discovery over the years and a variety of potential compounds have been identified for targeting different diseases through structure-based screening. For example, selected furanocoumarins have been identified by structure-based multitargeted molecular docking analysis against breast cancer.[24] A natural product-like STAT3 dimerization inhibitor has been discovered through structure-based virtual screening.[25] Moreover, repurposing existing FDA-approved compounds for human therapy is proposed as an effective approach to improve the development of new drugs, such as the identification of Sorafenib as an anti-virus agent and Imipramine as a micropinocytosis inhibitor.[26–28] FDA-approved drugs are more likely to have lower risks of adverse drug reactions and much clearer mechanisms of action based on the fully pre-clinical and clinical evaluation for drug safety and efficacy.[29,30] In respect that most drugs have much broader ranges of action and exhibit the ability to regulate multiple targets in addition to the approved ones, the pool of FDA-approved compounds could be considered as a large reservoir of drug candidates.[31] Therefore, compared to *de novo* drug discovery and development, drug repurposing might possess a higher possibility and speed up drug development with a lower cost.

Considering the fact that inhibition of QC reduces the generation of pE-Aβs and blocks the activation of downstream cascades in anti-AD strategy, the substantial QC inhibitory potency of a pool of 1621 FDA-approved compounds was tested for the first time to improve the discovery and development of potential QC inhibitor in the current study. QC-glutamic acid dehydrogenase linked assay was used as described previously.[32] The rates of QC inhibition at 10 μM (IR, %) to DMSO control were recorded. Compounds were defined as candidates with inhibition rate higher than 25%. According to the flow chart shown in Figure 1a, a total of 54 compounds (3.33%) exhibited inhibition rate with 25% or higher, and the QC inhibitory activities were reconfirmed by following screening assay (Figure 1b and Table S1). The Ki of the top 5 compounds with the highest QC inhibitory activities were measured and shown in Table 1. Further analyzing the 54 FDA-approved hits may benefit the discovery of novel QC inhibitors more efficiently.

Up-regulated QC level has been shown as one of the most important signals in the development of AD, especially at the early stage. QC, distributed mainly in brain, is thus regarded as a critical target to prevent and treat AD. QC inhibitor is

Table 1. Top 5 FDA-approved drugs exhibiting QC inhibitory activities

Drugs	Target	Pathway	CAS No.	Formula (MW)	Structure	Ki (mM)
Detomidine HCl	Adrenergic Receptor	Neuronal Signaling	90038-01-0	$C_{12}H_{15}ClN_2$ (222.71)		2.58 ± 0.28
Belinostat (PXD101)	HDAC	Epigenetics	414864-00-9	$C_{15}H_{14}N_2O_4S$ (318.35)		2.98 ± 0.67
Amlexanox	Immunology & Inflammation related	Immunology & Inflammation	68302-57-8	$C_{16}H_{14}N_2O_4$ (298.29)		3.80 ± 0.61
Acipimox	Others	Others	51037-30-0	$C_6H_6N_2O_3$ (154.12)		6.23 ± 0.85
Pilocarpine HCl	AChR	Neuronal Signaling	54-71-7	$C_{11}H_{17}ClN_2O_2$ (244.72)		13.19 ± 7.27

therefore proposed to be involved in the regulation of neuronal signaling pathways by inhibiting the generation of neurotoxic pE-Aβs. Here, mechanism of actions of 16 FDA-approved compounds are related to neuronal signaling pathway, the targets of which include adrenergic receptor, AChR, 5-HT receptor, COX, histamine receptor, dopamine receptor, potassium channel and NMDAR (Figure 2, Table S2). Most of these 16 hits could be considered as drug candidates targeting central nervous system (CNS). In drug development, certain characteristics are required for the CNS drugs, e.g. proper blood-brain barrier (BBB) permeability of chemicals. Therefore, reviewing the development process of these hits may contribute to the discovery of QC inhibitors as potential anti-AD agents with lower risks. Moreover, these molecules could also be considered as candidates to develop multi-target compounds through structural modification based on the crystal structure of QC. In reverse, QC inhibitors could also be designed and developed as multi-target agents exhibiting inhibitory effect on targets showed in Table S2. This strategy would be helpful for the discovery of novel disease-modifying agents.

Meanwhile, mechanisms of other hits are related to DNA damage, microbiology, metabolism, epigenetics, transmembrane transporters, autophagy, protein tyrosine kinase, etc (Figure 2). And the targets of these compounds with QC inhibition activity include topoisomerase, autophagy, anti-infection, immunology & inflammation, PPAR, HDAC, potassium channel, carbonic anhydrase, casein kinase, CDK, etc (Table S1). Up-regulation of QC has also been demonstrated to play a role in the development of multiple diseases such as Huntington's disease, inflammation, septic arthritis, thyroid cancer, melanoma, osteoporosis, cerebral ischemia and rheumatoid arthritis by catalyzing the generation of pE modified mediators, e.g. pE-CCL2.[33–41] The FDA-approved chemicals selected in the current screening are suggested to be taken into account in QC inhibitor development, which might possess a much broader range of effect and serve as drug candidates for the therapies of many other diseases after further pharmacological evaluation.

2.2. Identification of Important Frameworks in Potential QC Inhibitors

The combination of molecular scaffolds and subsets contained in FDA-approved compounds presents as a novel approach to design new fragment-like libraries aiming at improving success rates and reducing risk in drug discovery.[42] In this research, scaffolds and/or subsets in 54-hit pool was analyzed to improve the success rates and druggability in design and discovery of novel QC inhibitors. Based on the crystal structure presented in compound PBD150 (PDB 3PBB), the active region of QC is composed of a hydrophobic entrance, a narrow active pocket, and a catalytic zinc ion located at the bottom of the pocket.[43] The development of series of QC inhibitors indicates that the chelation of zinc ion with N atom is the key for compounds to exhibit the QC inhibitory activity, and the π-π stacking between the aromatic motif and the Phe325 residue at the hydrophobic entrance is also important for the recognition and binding of the molecule to the protein.[18–23] The structure-activity relationship (SAR) is confirmed by the screening carried out in this research. Imidazole, aniline, pyrazine, piperazine, quinoline, indole, thiazole, pyridine, purine, triazine or their derivatives were identified in more than 55% of these hits (Figure 3, Table S1). Moreover, amide, hydroxyacetamide, amine, alkylamino, guanidine or imidamide were also detected in the side chains of more than 37% of hits. All of these motifs or subsets exhibit the potency to bind to the zinc ion. In accordance to the SAR reported previously, our results further supported the fact that the binding of catalytic zinc ion at the bottom of the pocket with N or other atoms can be considered as the main strategy to inhibit the QC activity. Considering that all of these chemicals have been approved by FDA, the scaffolds contained in the selected hits, e.g. Pilocarpine HCl, Sumatriplan Succinate, Belinostat (PXD101), etc., as well as the subsets showed in Figure 3, are suggested to be adopted into the inhibitor synthesis, which would contribute to the design and development of novel QC inhibitors.

Furthermore, polyphenols such as oleuropein, sulfolipids, and apigenin derivatives were also reported for the notable QC

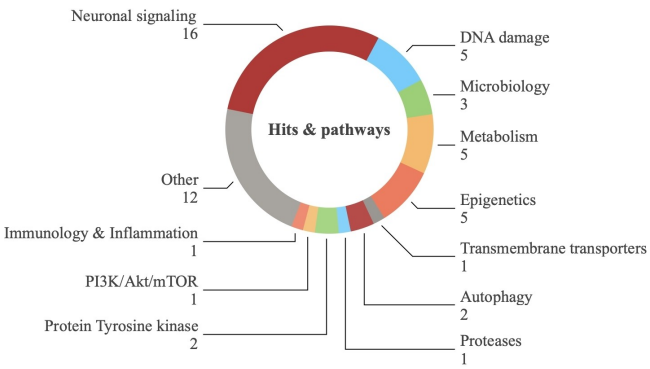


Figure 2. Groups of FDA-approved drugs affecting similar pathways.

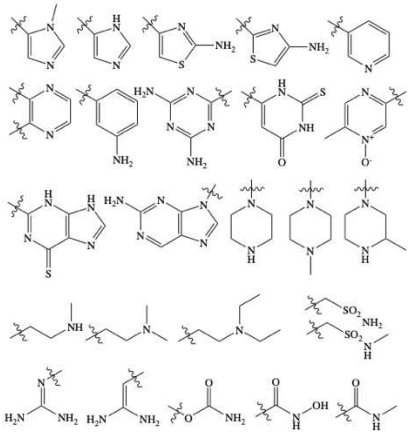


Figure 3. The motifs contained in FDA-approved drugs with QC inhibitory potency.

inhibitory activities.[44–46] Molecular docking illustrated the possible binding model of apigenin derivatives with QC. In the typical 'open sandwich' structure of active site, both the π-π stacking interaction between the phenolic skeleton and the aromatic residues in the hydrophobic pocket, and the chelation of zinc ion with -OH contributed to the binding of molecule to the protein. Hydrogen bond between C-OH and the carbonyl oxygen atom of residues inside was also important for the interaction. In our study, several polyphenols and carboxylic derivatives exhibited obvious QC inhibitory activities, e.g. Capsaicin (Vanilloid), Curcumin, Xylose, Suprofen, Genistein, Olsalazine Sodium, Resveratrol, Ketoprofen, ect (Table S2&S3). Therefore, the molecular frameworks from these hits could also be considered in generating QC inhibitor library. Meanwhile, the anti-oxidation effect of these compounds on their QC inhibitory activities needs to be further investigated to understand the underlying mechanisms. On the other hand, QC inhibitors derived from polyphenols might have the potential to prevent or treat diseases induced by oxidative stress.

3. Conclusions

Taken together, the QC inhibitory potency of a pool of 1621 FDA-approved compounds was investigated to improve the development of novel anti-AD agents, among which 3.33% of these compounds exhibited obvious QC inhibitory activities. Several groups of chemicals were identified, such as compounds affecting neuronal signaling pathway and polyphenol derivatives with QC inhibitory activities. The structures of these compounds can be considered as important references in design of new fragment like libraries targeting QC. This approach may be useful for evaluating the risk/benefit ratio in anti-AD drug discovery.

Experimental Section

A FDA-approved compound library (Cat No. L1300, pool: 1621, Order No. Z283685) was purchased from Selleckchem. Other materials were purchased from Alfa Aesar Co. All other reagents were of analytical grade.

Preparation of QC

Cloning, expression, and large scale preparation of QC were performed according to previous reports.[47–49] The gene for QC was inserted via the BamHI and XhoI restriction sites into Escherichia coli expression vector pET32a with additional introduction of an N-terminal His6-tag (primer pair Cs/Cas). Primers were as follows: 5-3 ACCTGGA- TCCGCTTCTGCTTGGCCGG, BamHI; 5-3 TATCCT- CGAGT-TACAGGTGCAGGTATTC, XhoI (Takara, China). E. coli strain DH5α was used for all cloning procedures. The cDNA was verified by sequencing (Samgon, China). Plasmid DNA was amplified, purified and linearized. QC was heterologously expressed in E. coli BL21 (DE3) using Fernbach flasks at room temperature overnight, and expression was induced by adding 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Cells were disrupted with 1 mg/mL lysozyme and a freeze-thaw cycle. The purification of QC protein followed two chromatographic steps: Ni2+-IMAC (immobilized metal affinity chromatography) and molecular sieve chromatography. QC-containing fractions were pooled, and purity was analyzed by SDS-PAGE (15%, sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Coomassie blue staining. The purified QC enzyme was stored at −80 °C without glycerol.

QC Inhibitory Activity Studies

The QC activity was assayed as described elsewhere.[50] Briefly, for spectrophotometric assessment, the assay reactions (200 μL) consisted of varying concentrations (0–4 mM) of freshly prepared H-gln-gln-H, 30 units/mL glutamic acid dehydrogenase, 0.5 mM $NADH/H^+$, and 15 mM α-ketoglutaric acid in 0.05 M Tris-HCl, pH 8.0. Reactions were started by the addition of QC. Activity was monitored by recording the decrease in absorbance at 340 nm for 15 min.

For inhibitory activity study, the assay reaction composition was the same as described above, except for the addition and pre-incubation of FDA-approved compounds with QC (5 min). The percentage of inhibition at 10 μM was calculated according to the formulation: inhibition $= (V_C - V_S)/V_C$, where V_C is the reaction velocity of control and V_S is the reaction velocity of samples. The IC_{50} values were determined graphically from log concentration versus % of inhibition curves. The K_i was calculated according to the formulation: $Ki = IC_{50}/(1 + [S]/K_m)$, where [S] is the concentration of substrate and K_m is the Michaelis constant of the substrate. All experiments were performed in triplicate.

The results were expressed as the mean ± SD of at least three independent experiments.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Alzheimer's disease · glutaminyl cyclase · FDA-approved compounds · oxidative stress · screening and repurposing

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