Glutaminyl Cyclase Inhibitor PQ912 Improves Cognition in Mouse Models of Alzheimer’s Disease—Studies on Relation to Effective Target Occupancy

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ABSTRACT

Numerous studies suggest that the majority of amyloid-β (Aβ) peptides deposited in Alzheimer’s disease (AD) are truncated and post-translationally modified at the N terminus. Among these modified species, pyroglutamyl-Aβ (pE-Aβ, including N3pE-Aβ40/42 and N11pE-Aβ40/42) has been identified as particularly neurotoxic. The N-terminal modification renders the peptide hydrophobic, accelerates formation of oligomers, and reduces degradation by peptidases, leading ultimately to the accumulation of the peptide and progression of AD. It has been shown that the formation of pyroglutamyl residues is catalyzed by glutaminyl cyclase (QC). Here, we present data about the pharmacological in vitro and in vivo efficacy of the QC inhibitor (S)-1-(1H-benzo[d]imidazol-5-yl)-5-(4-propoxyphenyl)imidazolidin-2-one (PQ912), the first-in-class compound that is in clinical development. PQ912 inhibits human, rat, and mouse QC activity, with Kₐ values ranging between 20 and 65 nM. Chronic oral treatment of hAPP₆₉₅xQC double-transgenic mice with approximately 200 mg/kg/day via chow shows a significant reduction of pE-Aβ levels and concomitant improvement of spatial learning in a Morris water maze test paradigm. This dose results in a brain and cerebrospinal fluid concentration of PQ912 which relates to a QC target occupancy of about 60%. Thus, we conclude that >50% inhibition of QC activity in the brain leads to robust treatment effects. Secondary pharmacology experiments in mice indicate a fairly large potency difference for Aβ cyclization compared with cyclization of physiologic substrates, suggesting a robust therapeutic window in humans. This information constitutes an important translational guidance for predicting the therapeutic dose range in clinical studies with PQ912.

Introduction

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disorder. Pathogenic hallmarks of AD are mainly extracellular aggregates of amyloid-β (Aβ) and intracellular neurofibrillary tangles, which are composed of the hyperphosphorylated protein tau (Hardy and Higgins, 1992; Mudher and Lovestone, 2002). Aβ oligomers have been shown to poorly predict the cognitive status of patients. Rather, nonfibrillary soluble Aβ oligomers appear to correlate with the development of the disease and to induce tau pathology (Lambert et al., 1998; Selkoe, 2005; Shankar et al., 2008; Ittner and Götz, 2011). These oligomers contain truncated and modified forms of Aβ at a significant extent, as recently shown by Esparza et al. (2016) and discussed at Alzforum (http://www.alzforum.org/print-series/620566).

A substantial degree of Aβ heterogeneity is attributed to the N terminus (Bayer and Wirths, 2014). Among these species, truncated Aβ variants starting at positions 3 or 11 with an N-terminal glutamyl residue are post-translationally modified by pyroglutamyl (pE) formation. Pyroglutamyl-Aβ peptides have been shown to be major constituents of Aβ deposits in sporadic and familial AD (Saido et al., 1995; Miravalle et al., 2005; Piccini et al., 2005). In postmortem tissue, the pE-Aβ content of deposits varies between 10 and 25% or even higher, depending on the methods of extraction (Näslund et al., 1994; Lemere et al., 1996; Saido et al., 1996; Kuo et al., 1997; Portelius et al., 2010; Wu et al., 2014). The N-terminal formation of pE renders the Aβ peptide more hydrophobic (Schlenzig et al., 2009, 2012). Furthermore, the pE formation triggers rapid oligomerization, which negatively interferes with synaptic and neuronal physiology as...
captured by, e.g., impairments in long-term potentiation (Nussbaum et al., 2012; Schlenzig et al., 2012). The data suggest that Aβ oligomers formed from N3pE-Aβ structurally differ from those of Aβ(1-42), and it is assumed that these structural modifications constitute the basis for the increased toxicity (Nussbaum et al., 2012; Gillman et al., 2014; Matos et al., 2014). Moreover, the toxic oligomeric structure induced by pE-Aβ might be transmitted to full-length Aβ in a mechanism of molecular priming (Nussbaum et al., 2012). Recent studies also suggest that the abundance of pE-Aβ correlates with the appearance of tau-paired helical filaments (Mandler et al., 2014), and that the concentration of N3pE-Aβ in cortical tissue of postmortem human AD brain samples inversely correlates with the cognitive status of the patients (Morawski et al., 2014). In contrast to the content of unmodified Aβ in plaques, the level of pE-Aβ increases and correlates with disease stages. The modified pE-Aβ is first measurable on the brink from the preclinical to clinical stage (Rijal Upadhaya et al., 2014; Thal et al., 2015). These results link the formation and accumulation of N3pE-Aβ to the cognitive status and disease progression of AD. The size and structure of native Aβ oligomers is currently being intensively investigated.

The formation of pE-Aβ is catalyzed by the metal-dependent enzyme glutaminyl cyclase (QC) (Schilling et al., 2004). QC is highly expressed in the human brain and has been shown to be upregulated in AD (Schilling et al., 2008), thereby causing an increase in pE-Aβ formation. Likewise, the concomitant accumulation of Aβ also favors formation of pE-Aβ due to increased QC substrate levels. Previous studies showed that expression of human QC in amyloid precursor protein (APP) transgenic mice increases pE-Aβ formation and induces a behavioral deficit (Jawhar et al., 2011; Nussbaum et al., 2012), whereas a depletion of murine QC prevents the development of the AD-like phenotype in 5xFAD transgenic mice (Jawhar et al., 2011). A pharmacological proof of principle has been shown previously in two different AD mouse models using the QC inhibitor PBD150 [1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea] as a tool compound. The compound prevents the generation of pE-Aβ(3-42) and improves spatial learning and memory (Schilling et al., 2008).

Within a comprehensive drug discovery program (Buchholz et al., 2006, 2009; Ramsbeck et al., 2013), PQ912 ((S)-1-(1H-benzo[d]imidazol-5-yl)-5-(4-proxyphenyl)imidazolidin-2-one) (Fig. 1) has been selected as a development candidate based on its excellent overall drug-like profile. PQ912 is a first-in-class inhibitor of glutaminyl cyclases currently in clinical development (Lues et al., 2015).

In this paper, we summarize key primary and secondary pharmacological data relevant to evaluating the compound’s in vivo efficacy and target occupancy as well as the in vivo substrate selectivity of PQ912 as a basis for the translational assessment of the therapeutic window. The data support a favorable profile of the compound for QC engagement coupled to a reduction of pE-Aβ and behavioral improvements, setting the cornerstones for translation of the approach to clinical trials.

**Materials and Methods**

**Materials**

Human and murine QCs were heterologously expressed in *Pichia pastoris* and purified as described previously (Schilling et al., 2002a, 2005). PQ912-HCl was synthesized and purchased from Carbogen Amcis AG (Aarau, Switzerland). For in vitro studies, the drug was dissolved in dimethylsulfoxide (10 mM) and further diluted in the appropriate buffer. For in vivo studies, PQ912 was applied in pellet standard rodent chow (R/M 10 mm, Ssniff Spezialdiäten, Soest, Germany).

**Animals**

hAPPSLxhQC (mice double transgenic for the human APP gene containing Swedish and London mutation and human QC, Nussbaum et al., 2012) and 5xFADxhQC transgenic mice (mice transgenic for the human APP gene containing Swedish, Florida and London mutation, the human PS1 gene variant (M146L, L286V) and human QC, Jawhar et al., 2011) were used to assess the efficacy of PQ912. Animals were housed in individually ventilated cages on standardized rodent bedding supplied by Rettenmaier Austria GmbH & Co. KG (Viena, Austria). Mice were kept in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility of QPS Austria GmbH (previously JSW Lifesciences, GmbH, Grambach, Austria). Animal studies conformed to the Austrian guidelines for the care and use of laboratory animals and were approved by the Styrian government, Austria (Approval numbers: FA10A-78-Jo45-2009; FA10A-78-Jo58-2010; FA10A-78-Jo68-2011; FA10A-78-Jo69-2011). The room temperature during the study was maintained at approximately 24°C, and the relative humidity was maintained between 40 and 70%. Animals were housed under a constant light cycle (12 hours light/dark). Dried pelleted standard rodent chow (Ssniff R/M 10 mm) with or without PQ912 and normal tap water were available to the animals ad libitum. Each animal was checked regularly for any clinical symptoms, and body weight and food consumption of the animals were measured once a week.

Mice of both genders were used in all studies using hAPPSLxhQC. Wild-type controls were used only in experiments with behavioral assessment to ensure that the transgenic animals show a behavioral phenotype. For the longitudinal characterization of the double-transgenic APPSLxhQC mouse model, only the respective single-transgenic models were used as adequate controls. With regard to 5xFADxhQC, our earlier studies were done in female mice only.
In Vivo Pharmacology

The in vivo efficacy of PQ912 was assessed by analyzing its effects on lowering the burden of β-amyloid Aβ in reporter mice (5xFADxhQC). The 5xFADxhQC mice were used here again. The in vitro efficacy of PQ912 was assessed using a cellular model of Alzheimer's disease (AD). Male and female hAPP-ΔEaQC double-transgenic mice received PQ912 food pellets (0.8 g/kg) for 5 weeks, starting at 7.5 months of age. Animals of the Tg control group received regular food pellets. The effect of the test compound on learning and memory (MWM) was evaluated at approximately 8.5 months of age. Mice were sacrificed at the age of 9 months and the brain amyloid burden was quantified, applying ELISA for N3pE-Aβ as described later.

Therapeutic Short-Term Treatment Regimen. Male and female hAPP-ΔEaQC double-transgenic mice received PQ912 food pellets (0.8 g/kg) for 4 months, starting at 8 months of age. Tg controls received normal food pellets. Tg animals of the control group and non-Tg littermates received drug-free food pellets. Mice were sacrificed at the age of 12 months. The brain amyloid burden was quantified, applying ELISA for N3pE-Aβ.

Female 5xFADxhQC mice (Jawhar et al., 2011) received PQ912 orally at doses of 0.8- and 2.4-g/kg food pellets. Treatment was started at 3 months of age. Animals were sacrificed at 6 months of age.

MWM. The Morris water maze consisted of a white circular pool (diameter: 100 cm) filled with tap water at a temperature of 21 ± 2°C. The pool was virtually divided into four quadrants. A transparent platform (8-cm diameter) was placed about 0.5 cm beneath the water surface. During all test sessions, the platform was located in the southwest quadrant of the pool. Each mouse had to perform three trials with a time lag of 10 minutes in between (intertrial time) on each of 4 consecutive days. A single trial lasted for a maximum of 1 minute. During this time, the mouse had to find the hidden, diaphanous platform. After each trial, mice were allowed to rest on the platform for 10–15 seconds to orientate in the surroundings. At least 1 hour after the last trial on day 4, mice had to fulfill a probe trial (PT). During the PT, the platform was removed from the pool, and the number of crossings over the former target position and the abscissa in this quadrant were recorded.

A computerized tracking system was used for the quantification of the escape latency (time in seconds the mouse needs to find the hidden platform and, therefore, escape from the water) and of the
ELISAs detecting Aβ SDS and FA fractions was considered as the insoluble pool of Aβ no. 37571; ThermoFisher Scientific). The sum of Aβ dilutions to a final volume of 10 ml using ELISA blocking buffer (catalog acid extract was neutralized by addition of 3.5 M Tris solution and (SDS fraction), and 0.5 ml of 70% formic acid (FA fraction). The formic Triton X-100 (TBS/Triton fraction), 2.5 ml of 2% SDS in distilled water homogenized in Tris-buffered saline [TBS, 20 mM Tris, 137 mM NaCl [pH 7.6], 2 volumes of buffer per brain weight, Dounce homogenizer] brains were removed, and the cerebellum was cut off and CSF of hAPPSLxhQC mice treated for 6 months with chow (ad libitum) treated for 1 week with chow (ad libitum) containing 0.8 g/kg PQ912 (equivalent to approximately 200 mg/kg/day). On the last day, animals were sacrificed at 5 a.m., 9 a.m., 1 p.m., 7 p.m., and 11 p.m. CSF, brain, and cerebellum were analyzed for PQ912 using LC-MS/MS as described later. Mean ± standard deviation.

Statistics

Descriptive statistical analysis was performed on all evaluated parameters. Data were averaged and (if not stated otherwise) represented as the mean ± S.E.M. Differences compared with respective control groups were analyzed by t test or analysis of variance (ANOVA). In the case of non-normally distributed data, Kruskal-Wallis test was used. Post-hoc comparisons with the respective control group were done by Dunnett’s or Dunn’s test. For the MWM, outliers detected with Grubb’s test were excluded from data analysis. Differences in MWM learning curves were evaluated by a two-way ANOVA followed by Dunnett’s post-test.

Pharmacokinetics

Pharmacokinetics Day Profile in CSF and Brain after 1 Week of Treatment. PQ912 exposure was determined in a satellite experiment using 10-month-old hAPPSLxhQC mice (n = 5 per time point) treated for 1 week with chow (ad libitum) containing 0.8 g/kg PQ912. Animals were sacrificed in the morning after 6 weeks (0.8 g/kg only) or 6 months of treatment, and CSF and cerebellum samples were analyzed for PQ912 using LC-MS/MS as described later.

PQ912 Concentrations in Brain and CSF after Long-Term Treatment. PQ912 exposure was determined in the cerebellum and CSF of hAPPSLxhQC mice treated for 6 months with chow (ad libitum) containing 0.24, 0.8, and 2.4 g/kg PQ912. Animals were sacrificed in the morning after 6 weeks (0.8 g/kg only) or 6 months of treatment, and CSF and cerebellum samples were analyzed for PQ912 using LC-MS/MS.

Determination of Free Brain Concentration. The free concentration of PQ912 in brain was determined using equilibrium dialysis in vitro. PQ912 (1 µM final concentration) was spiked into buffer-diluted mouse brain homogenate and buffer and added to either side of the membrane of the rapid equilibrium device (RED; target-zone crossings and the abidance in the target quadrant in the PT. All animals had to perform a visual test after the PT to rule out visual impairments that may influence the results of the MWM test.

Tissue Sampling. Blood (plasma), cerebrospinal fluid (CSF), and brain samples were collected from Tg mice. Mice were sedated by standard inhalation anesthesia (Isoba, Essex Tierarznei, Munich, Germany). Cerebrospinal fluid was obtained by blunt dissection and exposure of the foramen magnum. Upon exposure, a Pasteur pipette was inserted to an approximate depth of 0.3–1 mm into the cisterna magna. CSF was collected by suction and capillary action until flow fully ceased. CSF samples were immediately frozen on dry ice and stored at −80°C. After CSF sampling, each mouse was placed in dorsal position, the thorax was opened, and a 26-gauge needle attached to a 1-cc syringe was inserted into the right cardiace ventricular chamber. Blood was collected into EDTA-coated vials and used to obtain plasma.

To obtain plasma, blood samples from each mouse were centrifuged (1000 × g, 10 minutes, room temperature). Following blood sampling, mice were transcardially perfused with physiologic (0.9%) saline. Thereafter, brains were removed, and the cerebellum was cut off and stored at −80°C. Brains were hemisected and immediately frozen on dry ice. One brain hemisphere was used for determination of Aβ level by ELISA, and cerebellum and CSF were used for measurement of compound exposure using liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Analysis of N3pE-Aβ42. Brain tissue without cerebellum was homogenized in Tris-buffered saline (TBS, 20 mM Tris, 137 mM NaCl (pH 7.6), 2 volumes of buffer per brain weight, Dounce homogenizer) containing protease inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland) and 0.1 mM 4-(2-Aminoethyl)benzensulfonylfluoride (Carl Roth, Karlsruhe, Germany), sonicated, and centrifuged at 75,500 × g for 1 hour at 4°C. The supernatant was stored at −80°C, 25 ± 3 17 1.3e6 2.3e−2

| Species     | pH 6.0     | pH 8.0     | Kd    | koff   | koff   |
|-------------|------------|------------|-------|--------|--------|
| Human QC    | 19 ± 3     | 25 ± 3     | 17    | 1.3e6  | 2.3e−2 |
| Mouse QC    | 41 ± 3     | 62 ± 7     | nd    | nd     | nd     |

nd, not determined; SPR, surface plasmon resonance.

For enzyme kinetic measurements of QC, two different batches of PQ912 free base were analyzed. For each batch and each pH value, three separate weighings were performed and analyzed as described in Materials and Methods; Kd determination with SPR for human QC were done once.

TABLE 2

Human APP constructs used in cell culture experiments

Conjugate | APP Sequence* at BACE1 Cleavage Site | Aβ1 Peptide Released after BACE1 Cleavage | Used in Cell Culture Experiments |
|----------|--------------------------------------|-------------------------------------------|----------------------------------|
| hAPPwt   | 593 …EVKM/DAEFRH DSGYEVHQQKL         | Aβ(1-40) DAEFRHDSGY EVHHQQKLFF            |                                   |
| APP-NLE  | 593 …EVNL/ERFRH DSYEYHQQKL           | Aβ(3-40) EFRHDSGY EVHHQQKLFF             |                                   |
| APP-NLQ  | 593 …EVNL/QFRHD SGEYVHQQKL          | Aβ(3-40) QFRHDGSY EVHHQQKLFF             |                                   |

*Numbering refers to human APP695 wt variant, Frame and slash: BACE1 cleavage site; bold: amino acid exchange compared with wild-type (wt) sequence.
Inhibition of QC-catalyzed N3pE-Aβ40 formation by PQ912 in cell culture (means ± S.E.M. and four-parameter fits) with the following constraints for all curves: top = 100%, bottom = 6%, hilleslope = 1. HEK293 cells were transfected to express APP-NLE or APP-NLQ constructs alone or together with human QC. The cells generate E3-Aβ40 (NLE) or Q3-Aβ40, respectively. Hence, QC catalyzes either cyclization of N-terminal glutamate (E-) or glutamine (Q-) residues. We determined EC50 of 200 and 800 nM for PQ912-mediated reduction of N3pE-Aβ40 from APP-NLE/hQC and APP-NLQ, respectively. Inhibition of Q-cyclization in the QC-overexpressing model (APP-NLQ/hQC) was negligible (EC50 > 10 μM). The results support a higher potency of PQ912 to inhibit cyclization of glutamic acid residues.

ThermoFisher Scientific, Darmstadt, Germany). After equilibration at 37°C for 4 hours, the compound concentration on both sides of the membrane was analyzed by LC-MS/MS, and the unbound fraction was calculated following correction for the dilution factor.

Bioanalysis with LC-MS/MS. Brain hemispheres or cerebellum samples were homogenized in 2-volume equivalents of 90% acetonitrile using a Precellys 24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) containing 1.4-mm ceramic beads. After centrifugation, the supernatant was further diluted with 90% acetonitrile containing the stable isotope-labeled internal standard.

CSF and in vitro samples were extracted with 3-volume equivalents of acetonitrile containing the stable isotope-labeled internal standard.

Quantification of PQ912 in CSF, brain homogenate, and buffer extracts was done using a specific and sensitive LC-MS/MS method. After protein precipitation and centrifugation, an aliquot of extracts (2 μl) was injected into a Synergi Polar RP column (50 × 2 mm, 2.5 μm; Phenomenex, Torrance, CA) and separated using a linear gradient of water/acetonitrile from 5 to 95% organic within 4 minutes at 0.3 ml/min (HP1200; Agilent, Santa Clara, CA). PQ912 was quantified in the selected reaction-monitoring mode (mass transition 337.4 > 160.1 Da) using either an API3200 or API4000 (Sciex, Framingham, MA) with heated electrospray ionization in positive ion mode. The lower limit of quantification was defined with 0.25 ng/ml, and all six QC samples (low, medium, high), analyzed within each analytical batch, were valid according to bioanalytical guidelines (U.S. Department of Health and Human Services et al., 2001).

Calculation of Target Occupancy. Mean target occupancy was calculated according to the following formula: target occupancy as a percentage = 100°C/Ki + C, where C is the free concentration of PQ912. The free (unbound) concentration in brain was determined by equilibrium dialysis. K represents the inhibitory constant of PQ912 for human QC. The transgenic mice used in the studies were transgenic for human QC, which is specifically expressed in and mainly responsible for pE-Aβ formation in these mice.

Secondary Pharmacology

To assess an influence of treatment on pE-hormone maturation, we determined the concentration of the hormones testosterone and thyrotropin (TSH) and thyroxine (T4) in plasma. The production of the thyroid and the gonadal hormones is regulated by the hypothalamic-pituitary-thyroid (HPT) and the hypothalamic-pituitary-gonadal (HPG) axes, respectively. Mice (C57/B16) were treated orally for 2 weeks, receiving food pellets containing PQ912 at doses of 2.4 and 4.8 g/kg. Afterward, animals were sacrificed and blood plasma was prepared. Specific ELISAs were used according to the manufacturer’s instructions for determination of TSH (#SY45021; Cusabio, College Park, MD), T4 (#RE55261), and testosterone (#RE53151; IBL).

In Vitro Binding Kinetics

Mammalian glutaminyl cyclases are metalloenzymes that contain a typical architecture of a catalytic zinc-binding motif. PQ912 showed competitive inhibition of human QC activity (Fig. 1) with a Ki value of about 25 nM (pH 8.0). Similar Ki values were found for inhibition of recombinant murine QC (Table 1). The binding constant of PQ912 for human QC was also determined using surface plasmon resonance (SPR) technology. PQ912 showed fast association and dissociation kinetics with a Kd value of 17 nM. The koff value of 0.023 second⁻¹ corresponds to a half-life of the QC-inhibitor complex of about 30 seconds.

Cellular Assays

The effect of human QC inhibition in formation of N3pE-Aβ40 was determined using a cellular assay, which is based on expression of human APP695 variants (Table 2) together with QC (Cynis et al., 2008). HEK293 cells expressing human APP-NLE and human QC were used to determine the potency of PQ912 (Fig. 2). The APP-NLE construct leads to formation of E3-Aβ40/42 by BACE1 and γ-secretase cleavage of APP. Human QC converts the N-terminal glutamate residue to form N3pE-Aβ40/42. With overexpression of APP-NLE alone (without QC overexpression), there was no pE-Aβ detectable within an acceptable time frame for the cell-culture experiment. Therefore, the APP-NLE/hQC model was used to investigate the effect of QC inhibitors on Glu cyclization. The EC50 values for PQ912 to inhibit N3pE-Aβ40/42 formation in the APP-NLE/hQC model were determined to be in the range of 0.14–0.25 μM. Figure 2 shows an experiment in which the APP-NLE/hQC model was directly compared with the APP-NLQ and APP-NLQ/hQC models. In the case of an N-terminal glutamine residue, the inhibitory potency of PQ912 on pE-Aβ formation in the comparable APP-NLQ/hQC model was very low (no noteworthy inhibition up to 10 μM). Also, for inhibition of glutamyl cyclization in the APP-NLQ model without QC overexpression (only intrinsic cellular QC activity), about 4-fold higher inhibitor concentrations are needed (EC50 = 0.8 μM) compared with the APP-NLE/hQC model. These observations point toward a higher potency to inhibit cyclization of glutamate compared with glutamine residues in cell culture. This Glu-Gln potency difference provides an important basis for the target selectivity of the approach.

PQ912 Exposure and Target Occupancy in hAPP695xhQC Mice

The PQ912 exposure in the CSF and brain of hAPP695xhQC mice after oral application of PQ912 (0.8 g/kg chow, ad libitum) is shown in Fig. 3. At this dose, the mean free PQ912 concentration was about 2 times Ki (47 nM for CSF and...
and 62 nM for brain and cerebellum homogenate, respectively), resulting in a mean target occupancy of more than 60% (65, 69, and 71% for CSF, brain, and cerebellum, respectively).

Previous studies in 5xFAD mice cross-bred with QC knockout mice (Jawhar et al., 2011) implied that more than 50% QC inhibition is required to achieve an effect on pE-Aβ formation and a concomitant behavioral improvement (unpublished observations). Hence, doses of 0.24, 0.8, and 2.4 g were selected for further long-term treatment.

The treatment of hAPPSLxhQC mice for 6 months resulted in brain concentrations of about 50, 230, and 700 ng/g brain, respectively (Fig. 4). Considering an unbound fraction of 0.06 ± 0.02 (n = 3), the free brain concentrations corresponded well with the PQ912 concentrations in CSF. Thus, these data suggest that an oral dose of 0.8 g/kg is sufficient to achieve more than 60% inhibition of QC in brain over 24 hours.

Pharmacodynamic Effects of PQ912 in Transgenic Mice

Two AD-like mouse models were used to assess the in vivo effect of PQ912: the double-transgenic hAPPSLxhQC mice and the 5xFADxhQC mice. The 5xFADxhQC mice were characterized in a previous study (Jawhar et al., 2011). These mice start to develop pE-Aβ-containing deposits at an age of 3–4 months. Also, hAPPSLxhQC mice have been briefly described previously (Nussbaum et al., 2012), but a detailed characterization of pE-Aβ deposition and behavioral changes at different ages for these mice was lacking. The development of AD pathology reflected by pE-Aβ increase in the brain and deficits in behavioral tests was evaluated in a longitudinal study as the basis for the definition of prophylactic treatment versus therapeutic treatment paradigms (Fig. 5). PQ912 was then tested for reduction of pE-Aβ formation in hAPPSLxhQC mice.

![Fig. 3. Time-dependent concentration of free PQ912 in CSF (A) and brain (C) (median ± range, n = 5 per time point; data point in gray was extrapolated) and calculated QC target occupancy in CSF (B) and brain (D) after 1 week of PQ912 treatment (0.8 g/kg chow, ad libitum) in 10-month-old hAPPSL/hQC mice. The Kᵢ (PQ912) for human QC of 25 nM corresponds to a PQ912 concentration of 8.4 ng/ml. The mean exposure over 24 hours is about 2 × Kᵢ, resulting in a mean target occupancy (TO% = 100*C/(Ki + C)) of about 60%.

![Fig. 4. PQ912 concentrations in brain (circles) and CSF (squares) after 6 months of treatment of hAPPSL/hQC mice with 0.24, 0.8, and 2.4 g PQ912/kg chow (ad libitum, n = 11–15 per group, mean ± S.D.). Black-filled circles represent calculated free brain concentrations (fu = 0.06). Mean compound concentrations in CSF can be translated to approximately 0.45*Kᵢ, 1.47*Kᵢ, and 4.36*Kᵢ, respectively. The red symbols refer to 6-week PQ912 treatment, which results in nearly same brain and CSF levels compared with the 6-month treatment.](https://jpet.aspetjournals.org/jpet/10.1124/jpet.112.196625/f3.png)
or 5xFADxhQC mice in both treatment paradigms. Furthermore, the effect of PQ912 on learning and memory was assessed in the hAPP SLxhQC mice.

The deposition of Ab in hAPP SLxhQC transgenic mice starts at an age of about 6 months and increases continuously with age. There was a 2- to 4-fold increase of total Ab between 9 and 12 months, reaching similar levels in hAPP SL single-transgenic and hAPP SLxhQC double-transgenic mice (Fig. 5A). N3pE-Ab is detectable in some animals above the lower level of quantification for the first time at an age of about 7.5 months and is present in all animals at 9 months of age (Fig. 5B). pE-Ab progressively accumulates during aging in single-transgenic hAPP SL (~15-fold increase between 9 and 12 months) and especially in the double-transgenic hAPP SLxhQC mice (~30-fold increase), resulting in significantly more N3pE-Ab in the brains of the double-transgenic mice.

First behavioral changes of the double-transgenic mice in the Morris water maze were already detected at 4 months of age (data not shown), thus slightly preceding the presence of pE-Ab at quantifiable concentrations. Double-transgenic mice perform clearly worse in the Morris water maze between 6 and 9 months of age (Fig. 5, C–F). Therefore, preventive treatment was initiated before the onset of pathophysiological changes, e.g., at an age of 3 months, and therapeutic paradigms began after detection of behavioral or pathologic changes, at 7–8 months of age.

Preventive Long-Term Treatment. In this set of experiments, transgenic mice were treated orally beginning at 3 months of age. PQ912 was applied via food pellets containing 0.24, 0.8, or 2.4 g of compound per kilogram of chow. Behavioral assessment in the Morris water maze test was performed at 8.5 months of age. The animals were sacrificed 2 weeks later, and brain tissue was collected for analysis of compound concentration and Ab content.

After sacrifice, Ab was sequentially extracted from brain hemispheres, as described earlier, using TBS, SDS, and formic acid. The concentration of N3pE-Ab42 within the TBS fraction is depicted in Fig. 6A. The treatment with PQ912 resulted in a significant reduction of N3pE-Ab42 in the TBS extracts at doses of 0.8 and 2.4 g PQ912 per kilogram of chow. The reduction of N3pE-Ab42 within the fractions of the insoluble pool (SDS and formic acid summed up) did not reach significance (data not shown).

For the behavioral assessment, wild-type littermates were used as naive (non-Tg) controls. The effect of PQ912 was compared with vehicle-treated transgenic animals (Tg control). At age 8.5 months, wild-type animals were able to learn to find the target position, whereas vehicle-treated hAPP SLxhQC double-transgenic mice showed significant spatial learning impairment measured as longer escape latencies on days 1–4. Treatment with the lowest dose of PQ912 (0.24 g/kg chow) did not show a beneficial effect on learning capabilities. The two higher doses of PQ912 (0.8 and 2.4 g/kg chow) caused a significant amelioration of spatial learning abilities compared with hAPP SLxhQC double-transgenic controls, reflected by shorter escape latencies on days 3 and 4 (Fig. 6B, P < 0.05 for 0.8-g/kg dose at day 3). In the probe trial, non-Tg controls tended to show better retention abilities reflected by higher abidance in the target quadrant than their vehicle-treated hAPP SLxhQC littermates. Treatment with PQ912 showed a dose-dependent trend to enhance the time hAPP SLxhQC animals spent in the target zone (ANOVA P value = 0.122) (Fig. 6C).

Therapeutic Short-Term Treatment. In an additional arm of the same study, hAPP SLxhQC mice were treated with a PQ912 dose of 0.8 g/kg chow beginning at about 7.5 months of age. The study was performed to assess whether a short treatment period might already result in biochemical changes and a behavioral improvement of the mice. The Morris water maze test was performed after 3 weeks of treatment, thus animal age matched the previous analysis of the preventive long-term treatment. Subsequently, animals (9 months of age) were sacrificed for biochemical analysis. This short-term treatment with the QC inhibitor PQ912 did not affect the N3pE-Ab42 concentration in brain (Fig. 7A). However, the treatment caused an improvement of spatial learning abilities compared with vehicle-treated hAPP SLxhQC double-transgenic controls, shown by significantly shorter escape latencies on day 3 and day 4 (Fig. 7B). With regard to spatial learning, short-term-treated animals showed an abidance in the target quadrant comparable to long-term-treated animals. The effect on spatial memory as assessed in the probe trial did not reach significance (Fig. 7C).

Therapeutic Long-Term Treatment. In an additional set of experiments, we assessed the effect of PQ912 at a dose of 0.8-g/kg food pellets for 4 months, starting at 8 months of age. The treatment resulted in a clear reduction of N3pE-Ab42 in soluble (P = 0.052, t test) and insoluble (P = 0.022) Ab fractions at the 12-month endpoint (Fig. 8, A and B).

We also investigated the effect of PQ912 in 5xFADxhQC mice, which has also been used in a genetic proof-of-concept study (Jawhar et al., 2011). Because these mice start to develop plaques at 2–3 months of age, i.e., earlier than hAPP SLxhQC, we treated these animals from 3 to 6 months. The total pE-Ab load in the brain of vehicle-treated animals was similar to 12-month-old hAPP SLxhQC mice (Fig. 8, C and D). Treatment with PQ912 at a dose of 0.8 g of PQ912 per kg chow (~200 mg/kg/day) resulted in a significant reduction of pE-Ab by about 30% in the TBS (soluble Ab) as well as the SDS/FA (insoluble Ab) fraction. Thus, the results obtained with PQ912 in 5xFADxhQC correspond to the results observed in the hAPP SLxhQC model.

In Vivo Secondary Pharmacology Related to Substrate Specificity—HPT and HPG Axis. As indicated in the Introduction, physiologic substrates of QC carry an N-terminal glutamine (Gln) residue without exception, being cyclized by QC to produce pE at the N terminus. The conversion of N-terminal glutamate residues (Glu), however, seems to be restricted to pathologic situations, such as accumulation of Ab in AD. To assess an in vivo therapeutic window between pathologic Glu and physiologic Glu cyclization, the effect of PQ912 on testoster-
on and T4 was measured in male C57/B16 mice after 2 weeks of treatment. These hormones function as indicators for the maturation of hypothalamic pE hormones gonadoliberin (GnRH) and thyroliberin (TRH), regulating the HPG or HPT axis, respectively.

Because the pharmacological experiments pointed toward an efficient reduction of pE-Ab formation and an accompanying behavioral improvement at a dose of 0.8 g PQ912/kg food pellet, 3- and 6-times higher doses of PQ912 were used (2.4 and 4.8 g/kg food pellet). Afterward, animals were sacrificed, and the hormone concentrations in plasma were assessed. With these doses, the downstream hormones of the HPT and...
HPG axes, testosterone and T4, were not affected by the treatment (Fig. 9).

**Discussion**

Compelling evidence suggests a crucial role of N-terminally truncated and pE-modified Aβ in Alzheimer’s disease (Russo et al., 2002; Gunn et al., 2010; Bayer and Wirths, 2014). These modified peptides have been shown to correlate with progression of AD and tau pathology (Güntert et al., 2006; Mandler et al., 2014; Morawski et al., 2014; Thal et al., 2015). The N-terminal blockage by pE stabilizes against degradation (Saido et al., 1995; Russo et al., 2002) and increases the surface hydrophobicity of oligomeric aggregates, which is most probably linked to toxicity (Schlenzig et al., 2012). It was also shown that pE-Aβ facilitates the formation of
hetero-oligomers, inducing toxicity in a tau-dependent manner (Nussbaum et al., 2012). The pE modification is catalyzed by glutaminyl cyclases, enzymes that are present in brain and upregulated in AD (Schilling et al., 2008; De Kimpe et al., 2012). Overexpression of QC and Aβ accumulation in transgenic mice has been shown to induce pE-Aβ formation and...
behavioral impairment, and a knockout of QC rescued the observed phenotype (Jawhar et al., 2011; Nussbaum et al., 2012). Hence, inhibitors of QC represent potential therapeutics to treat AD. PQ912 is the first inhibitor of QC that entered clinical development. The results of a comprehensive phase 1 study have been recently published (Lues et al., 2015).

The aim of the present study was 2-fold. First, we aimed to determine an effective dose of PQ912, which results in reduction of pE-Aβ formation and concomitant behavioral improvement of transgenic mice. These data provide a key translational finding for the clinical assessment of PQ912 in humans. Second, we addressed a potential functional selectivity for inhibition of pE formation from N-terminal glutamic acid over glutamine. Glutamate3-Aβ represents the precursor of N3pE-Aβ, whereas glutamine is the precursor of N-terminal pE in all physiologic substrates, including TRH and GnRH. Therefore, the results should provide evidence for a reasonable therapeutic window.

To assess the efficacy in vivo, we used the hAPPslxhQC and 5xFADxhQC mouse models. These mice generate pE-Aβ at higher levels than other mouse models, and the appearance of pE-Aβ is linked to behavioral changes in spatial learning and memory, beginning at age 4–6 months (Fig. 5) (Jawhar et al., 2011; Nussbaum et al., 2012). The preventive treatment of hAPPslxhQC mice with an oral PQ912 dose of 0.8 g/kg chow (≈200 mg/kg/day) for 6 months starting at 3 months of age resulted in a significant reduction of pE-Aβ formation. The reduction of pE-Aβ was accompanied by an improvement of spatial learning, assessed using a Morris water maze paradigm (Fig. 6). Suppression of pE-Aβ was corroborated in the therapeutic treatment of hAPPslxhQC and 5xFADxhQC mice where 0.8 g PQ912/kg in food pellets caused a significant reduction of pE-Aβ after 4 months of treatment (Fig. 8). The CSF concentration of about 47 nM at the end of the experiment predicted a QC inhibition of about 65%. An effective dose in this range, resulting in >50% target occupancy, is in good agreement with previous results on genetic ablation of QC activity in 5xFAD mice (Jawhar et al., 2011). A 50% reduction of QC activity by heterozygous ablation of QC did not affect pE-Aβ formation and had only weak effects on behavior (unpublished results). In contrast, homozygous depletion of QC resulted in a rescue of the behavioral impairment and a significant reduction of pE-Aβ (Jawhar et al., 2011). This indicates that the average QC inhibition necessary to obtain a robust therapeutic effect should be higher than 50%. Thus, our studies in transgenic mice highlight an effective brain exposure that can be used for translation to human trials. Results of a phase 1 study with PQ912 in healthy volunteers suggested...
that, with well tolerated doses, an average QC inhibition in CSF of 90% could be achieved (Lues et al., 2015).

QC has a physiologic substrates; therefore, it is important to evaluate not only the primary pharmacological effect of the cyclization of the N-terminal glutamic acid residue in N3pE-Aβ, but also the effect of PQ912 on those peptides which carry an N-terminal glutamic residue. Reduction of the pE hormones TRH or GnRH, which are generated from Gln precursors, results in hypothyroidism or hypogonadism, respectively (Mason et al., 1986; Yamada et al., 1997).

Therefore, the preference of PQ912 to inhibit Glu over Gln cyclization was addressed in an HEK cell model overexpressing different APP constructs as precursors of E3- or Q3-Aβ. PQ912 effectively inhibited formation of N3pE-Aβ40 from E3-Aβ40 (APP-NLE/hQC), with an EC50 of about 200 nM. However, the cyclization of the N-terminal glutamic residue, which is generated by a mutated APP construct leading to Q3-Aβ instead of E3-Aβ (APP-NLQ), was not inhibited, with at least 50-fold higher PQ912 concentrations and otherwise identical conditions (overexpression of QC).

Different potential reasons could be considered for this apparent selectivity of PQ912 for inhibition of cyclization of glutamic acid residues. First, the difference in the specificity constants of the Glu versus Gln substrates likely play a role. The apparent dissociation constant (Kd) of Glu substrates was shown to be about 3 orders of magnitude higher compared with the respective Gln substrates (Schilling et al., 2004; Seifert et al., 2009). This, in turn, results in enforced competitive replacement of the Glu substrate by the inhibitor, which might account for the apparent specificity of PQ912 to more strongly suppress cyclization of glutamic acid residues. Second, the cyclization of glutamic residues occurs mainly intracellularly, i.e., under conditions of high substrate and enzyme concentrations. Evidence for this was provided by studies on the maturation of peptide hormones (Nillni and Sevarino, 1999; Keire et al., 2003). These peptides are matured within secretory vesicles and are secreted as the modified pE species. Under these high-enzyme/high-substrate conditions, the excess substrate “protects” the enzyme from binding the inhibitor so that much higher inhibitor concentrations are needed for considerable inhibition of the Glu cyclization. Finally, we have previously shown that N-terminal glutamine is prone to spontaneous cyclization with a half-life of several hours. Thus, spontaneous cyclization of N-terminal glutamine residues might also contribute to pE formation in physiologic substrates. In contrast, N-terminal glutamic acid residues cyclize at negligible rates spontaneously (Seifert et al., 2009). All of the aforementioned mechanisms could play a role in vivo, causing a higher potency of QC inhibitors to prevent Glu cyclization.

To translate these findings to physiologic substrates in vivo, the effect of PQ912 on plasma levels of gonadal and thyroid hormones was assessed in mice treated with PQ912. The secretion of T3 and T4 is regulated by the HPT axis, which also consists of the hypothalamic hormone TRH (pyroGlu-His-Pro-amide) and the pituitary hormone TSH. A reduction in mature TRH may occur due to reduction in formation of N-terminal pE in response to QC inhibition. A pronounced reduction of TRH would result in hypothyroidism, as is observed in TRH knockout mice (Yamada et al., 1997). These mice show a 50% reduction of the thyroxine concentration, increased TSH concentration, and hypoglycemia. Homozygous QC knockout mice show a very mild hypothyroidism as suggested by a 20% reduction of thyroxine virtually no effect on TSH, and no hypoglycemia. The effect is likely caused by a reduction of mature pE-TRH (Schilling et al., 2011).

To estimate the therapeutic window based on the differences in inhibition of pE formation in Gln and Glu substrates, we assessed TSH, thyroxine, and testosterone in plasma of mice treated with high doses of PQ912. Importantly, we did not observe an effect on testosterone, nor on the TSH and thyroxine concentration, even after treatment with a dose 6-fold higher than an efficacious pharmacological dose (Fig. 9) necessary for inhibition of pE-Aβ formation. This corresponds with results in the multiple ascending dose phase 1 study, where T3/T4 levels were not affected at a dose, which leads to 90% QC inhibition, on average, in the spinal fluid. Thus, the apparent specificity of PQ912 on cyclization of Glu residues opens a therapeutic window for effectively reducing the pE-Aβ formation without effect on hormonal regulation cascades.

To summarize, our results suggest a robust therapeutic effect of PQ912 in transgenic mouse models of AD. These data further strengthen the hypothesis that the formation of pE-Aβ can be effectively reduced by inhibition of glutaminyl cyclase, and that brain QC is a druggable target. The therapeutic effect of PQ912 is observed at an oral dose of about 200 mg/kg/day, which translates to about 60–70% brain target occupancy. Notably, these observations match very well with a pharmacokinetic/pharmacodynamic relationship in human phase 1 studies, which revealed an EC50 of 30 nM in human CSF (Lues et al., 2015). Moreover, the results suggest a comfortable therapeutic window for the compound’s primary pharmacological effect on pE-Aβ and behavior in AD animal models versus its effects on hormonal regulation cascades driven by glutamine cyclization.

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Authorship Contributions

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