Amyloid-Precursor-Protein-Lowering Small Molecules for Disease Modifying Therapy of Alzheimer’s Disease

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Abstract

Alzheimer’s disease (AD) is the most common form of dementia in the elderly with progressive cognitive decline and memory loss. According to the amyloid-hypothesis, AD is caused by generation and subsequent cerebral deposition of β-amyloid (Aβ). Aβ is generated through sequential cleavage of the transmembrane Amyloid-Precursor-Protein (APP) by two endoproteases termed beta- and gamma-secretase. Increased APP-expression caused by APP gene dosage effects is a risk factor for the development of AD. Here we carried out a large scale screen for novel compounds aimed at decreasing APP-expression. For this we developed a screening system employing a cell culture model of AD. A total of 10,000 substances selected for their ability of drug-likeness and chemical diversity were tested for their potential to decrease APP-expression resulting in reduced Aβ-levels. Positive compounds were further evaluated for their effect at lower concentrations, absence of cytotoxicity and specificity. The six most promising compounds were characterized and structure function relationships were established. The novel compounds presented here provide valuable information for the development of causal therapies for AD.

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Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disease [1]. Symptoms include cognitive dysfunction, behavioral disturbances and difficulties with performing activities of daily living [2]. Probable AD is diagnosed by presence of characteristic neurological and neuropsychological features and auxiliary tests such as neuroimaging and cerebrospinal fluid analyses, but the definite diagnosis of AD can only be made postmortem. The neuropathological hallmarks of the disease include brain atrophy, the presence of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau-protein and extracellular deposition of β-amyloid (Aβ) [3].

Aβ is generated by post-translational processing of the Amyloid-Precursor-Protein (APP), a transmembrane protein, implicated in synapse formation [4] and trophic support [5]. There are two cleavage pathways of APP, a nonamyloidogenic and an amyloidogenic pathway [6]. Nonamyloidogenic cleavage of APP by α-secretase and the γ-secretase complex releases an APP intracellular domain (AICD), the 23–25 amino-acid long p3 fragment and soluble APP (sAPP). Amyloidogenic cleavage of APP by β-site APP cleaving enzyme 1 (BACE1) and the γ-secretase complex generates Aβ-alleforms ranging from 38 to 43 ([Aβ]38–[Aβ]43) amino acid length [7]. The main alleforms of Aβ in amyloid deposits are 40 ([Aβ]40) and 42 ([Aβ]42) acids long. Besides this, a soluble N-terminal APP fragment (sAPPβ) and AICD is produced. According to the amyloid hypothesis, generation and tissue deposition of Aβ is causal for neurodegeneration with Aβ42 aggregating readily and possessing high neurotoxicity [8]. Current hypothesis to explain Aβ-induced neurodegeneration include direct toxicity via the mitochondrial apoptotic pathway [9] or through activation of caspases [10],[11] and receptor mediated toxicity involving the N-methyl-D-aspartate receptor (NMDAR) [12].

The majority of AD cases are sporadic and show an association to the apolipoprotein E (APOE) ε4 allele as a genetic risk factor [13],[14]. Familial AD (FAD) is an autosomal dominant disorder with early disease onset. FAD is associated with mutations in presenilin-1 (PS1), presenilin-2 (PS2) or APP genes [15],[16],[17]. Recently copy number variations of APP have been shown to be causative for AD [18],[19]. The importance of APP gene dosage effects for the development of AD has been studied extensively in trisomy 21 patients where triplication of chromosome 21 including the APP-gene locus invariably leads to early-onset AD [20],[21],[22],[23].

Presently, there are no validated and licensed Aβ-lowering therapeutics. Efforts to develop drugs which specifically target BACE1 or the γ-secretase complex are complicated due to the pleotropic effects of these proteases leading to dramatic side effects [24],[25],[26],[27].
Moderate decrease of APP expression seems to be an attractive target for AD therapy. Therefore we screened for APP-lowering compounds using a newly developed, cell-based screening method. Of 10,000 high diversity, quality drug-like and lead-like compounds, we identified five which were non-cytotoxic, were effective at lower concentrations and lead to a selective reduction of APP and its cleavage product Aβ. Our data opens a new therapeutic approach by targeting APP and may lead to development of novel drugs to treat AD.

Materials and Methods

Cell culture

Human embryonic kidney 293 (HEK) cells [28] were grown in Dulbecco’s modified Eagle’s medium high glucose with L-glutamine, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (PAA Laboratories, Paching, Austria) in a 5% CO2 incubator. The APPsw cells (gift from C.Haass), are stably transfected HEK cells overexpressing APP with a double mutation at codons 670/671 and 5–8 fold increase in Aβ production when compared to wild type HEK-cells [29]. They were grown as described above supplemented with 1% Gentamycin (G418; PAA Laboratories). N2a cells (mouse neuroblastoma cell line) were grown as described above without G418. N2a cells were stably transfected with APPsw using the FUGENE®HD transfection kit, cells were grown with G418. APPsw or N2asw cells were plated on 96-well plates (100 µl/well = 30,000 cells/well). After 1 day, compounds (one compound/well) were added to media, untreated controls received solvent only. Initial concentrations were 100 µM (1% DMSO) [30]. To check effects at lower concentrations, compounds were used at concentrations of 100 µM, 50 µM, 10 µM and 1 µM. Supernatants (for Dot blot, Western blot and ELISA) or cells (for Western blot and quantitative reverse transcription PCR) were collected after 3 days of incubation.

Immunofluorescence

To assess transfection-efficiency, cells were grown on coverslips, fixed in acetone (20 min at −20°C), rinsed with phosphate-buffered saline, pH 7.4 (PBS, PAA Laboratories) and blocked with 5% donkey serum (Dianova, Hamburg, Germany). As primary antibody, anti-APP/Aβ antibody 6E10 recognizing the first 16 amino acids of Aβ was used (Dianova, Hamburg, Germany) was rinsed in 10 ml of methanol for 3 sec, equilibrated in PBS for 1 min, and placed in a dot blot apparatus (TE70 ECL Semi-Dry Transfer Unit, Amersham Biosciences, Freiburg, Germany). The supernatant of each well was spotted with an Eppendorf Multipipette onto the PVDF membrane through the sample wells of the Dot blot apparatus to obtain reproducible 3-mm-diameter dots in the pattern of a 96-well microtiter plate. Proteins were blotted on the membrane by negative pressure using a vacuum pump. The membrane was dried at 37°C for 1 h, blocked in 5% (w/v) fat-free milk powder in PBS (PAA Laboratories), and incubated with the 6E10 antibody (1:10,000, 4°C over night, Covance). After washing with PBST (PBS containing 0.1% Tween-20), secondary anti-mouse antibody (Promega, Fitchburg, USA) was used at a dilution of 1:10,000. Blots were developed with ECL enhanced chemiluminescence (Sigma) in an Imager Gel Doc System (Biorad).

Western blot

For Western blot analysis of supernatants, 100 µl of media was centrifuged and supernatants were boiled with loading buffer and run on 8% SDS-PAGE, blotted and probed 6E10 (1:1000, Covance), 3F4 (1:100, Covance) and rabbit monoclonal β-actin antibody (1:5000, Sigma), as well as matching secondary antibodies (1:5000, Promega). For cell lysates procedures were identical but 200 µg of homogenate in RIPA buffer was used. For visualisation and quantification ECL enhanced chemiluminescence (Sigma), Imager Gel Doc System and Quantity One Software (Biorad) was used. For determination of glycosylation ratios between mature and immature APP, band intensities were compared and expressed as ratios.

Real-time RT-PCR

The RNA was isolated from cell lines using the RNA Miniprep Kit (Stratagene, Basel, Switzerland). The purification of RNA was carried out with RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) following the protocol provided by the manufacturer. During purification, samples were treated with RNase-free DNase Set (Qiagen, Germany) to avoid later amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with Nanodrop 1000. RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, US).

ELISA

For quantification of Aβ40 and Aβ42 we used Aβ40/Aβ42-specific sandwich Enzyme Linked-Immuono-Sorbet Assay according to manufacturer’s instructions. Briefly, media were centrifuged at 1000 rpm for 5 min (at 4°C), supernatants (50 µl) were added to antibody-coated wells, capture antibody was added and extinction was measured at 450 nm by spectrophotometer (μQuant, BioTec).

Statistical analysis

In all experiments, means +/- SD are reported. Statistical comparisons among groups were determined using Student’s t-test.
with statistical significance at p-values <0.05 (*), <0.01 (**), and <0.001 (***)

Identification of similar structures

To assess novelty and to identify similar activities a number of prominent databases were accessed. These included ChEMBL [33], ChemSpider [34], BindingDB [35], PubChem [36], Drug Bank [37], Espacenet [38] and Google Scholar [39] and were accessed between 29th May 2013 and 25th June 2013.

Results

A cell-based AD model

As a cell culture model for AD we used HEK cells stably translected with human APP harboring the Swedish mutation (APPsw). Expression of APP and Aβ was assessed by immunohistochemistry for APP/Aβ using the 6E10 antibody. This revealed strong expression of APP/Aβ on plasma membrane as well as intracellularly in nearly 100% of cells (Fig. 1A). Since compounds used for our screen were dissolved in DMSO we excluded unspecific effects of DMSO on expression of APP and generation of Aβ by exposing cells to ascending concentration of DMSO added to the media. There were no significant differences regarding APP expression or generation of Aβ up to a 1/100 dilution of DMSO in media (data not shown).

Screen for APP-lowering small molecules

The goal of this study was to identify compounds reducing expression of APP. To this end our initial screen using a compound library (ChemBridge DIVERSet™) with 10,000 small molecules designed for high chemical diversity and drug-likeness focused on identifying compounds that decrease the amount of sAPP in the supernatant.

For this, cells were incubated for 72 hours with above mentioned compounds and supernatants were analyzed by dot blot using the monoclonal antibody 6E10 recognizing sAPP. The validity of our approach is shown in Fig. 1B where strong uniform signal for sAPP is only observed in APPsw but not in control HEK cells. For the initial screen, compounds were added at a concentration of 100 µM to single wells in a 96-well format allowing for the simultaneous assessment of 80 compounds (Fig. 1C). Only compounds showing strong sAPP reduction in four independent experiments were evaluated as “positive”, thus eligible for further investigation. Of 10,000 compounds, 223 were “positive” and were assessed for effectiveness at lower concentrations by incubating cells for 72 hours with compounds at concentrations of 100 µM, 50 µM, 10 µM and 1 µM. As above, supernatants were analyzed by dot blot and compounds were evaluated as “positive” when effects were seen at lower concentrations in four independent experiments. Sixteen compounds were effective at 10 µM and two compounds showed a significant reduction of sAPP at 1 µM. In order to exclude unspecific effects on cell viability or cellular metabolism, we assessed cytotoxicity of compounds by Trypan Blue assay and influence on proliferation of compounds by MTT-assay. Of the sixteen compounds showing reduction of sAPP at lower concentrations, six did not show effects on proliferation and were non-cytotoxic (Fig. 2A). Two compounds which decreased sAPP signal up to 1 µM (Compound B and C) and four compounds that decreased sAPP signal up to 10 µM (Compound A, D–F) were chosen for further analysis (Fig. 2B). Cytotoxic compounds or compounds with effects only at 100 µM and 50 µM were excluded (Fig. S1A, B, C).

Reduction of full-length intracellular/plasma membrane bound APP and sAPP was confirmed by Western Blot analyses of cell lysates and supernatants 72 hours after incubation with the six selected compounds (at 10 µM). In this analysis, all of the six compounds led to a significant reduction of intracellular/plasma membrane bound APP and sAPP (Fig. 3A, n = 5, p***<0.001).

To determine if reduction of full-length intracellular/plasma membrane bound APP and sAPP also applies for neuronal cells, we treated stably transfected neuronal N2aSw cells with the six selected compounds (10 µM, 72 hours) and performed Western Blots of cell supernatants and cell lysates. We could not observe cytototoxic effects for these compounds when assessed by MTT-assay (Fig. S2A, n = 3, **p<0.01). Amounts of intracellular/plasma membrane bound APP and sAPP were decreased for all six compounds (Fig. S2B, n = 2).

Reduction of Aβ40 and Aβ42 by sAPP-lowering compounds

To confirm that selected compounds reduce the amount of Aβ40 and Aβ42 we analyzed supernatants following three-day compound treatment by Aβ40 and Aβ42 specific ELISA. HEK cells produce small amounts of Aβ40 whereas Aβ42-production is under the detection limit, whereas APPsw cells produce large amounts of Aβ40 and lower amounts of Aβ42. All six tested compounds significantly reduced amounts of Aβ40 and Aβ42 with relative reductions ranging from 36%–59% for Aβ40 and 21%–56% for Aβ42 (Fig. 3B, C, n = 3, **p<0.01; ***p<0.001).

Specificity of sAPP-lowering compounds

To exclude nonspecific reduction of neuronal membrane proteins we assessed expression of PrP^C as membrane-bound, highly abundant neuronal protein. PrP^C represents a good control protein since it localizes to similar membrane microdomains and its expression is not regulated by APP [40]. With the exception of compound B there was no significant regulation of PrP^C by tested compounds (Fig. S3).

Transcriptional and posttranslational effects of sAPP-lowering compounds

In order to investigate if decrease of sAPP is due to transcriptional regulation we determined mRNA levels of APP using quantitative RT-PCR using established methods [41]. For compound B we observed transcriptional effects on APP mRNA levels 72 hours after compound treatment whereas for compounds A, C, D, E and F no transcriptional effects were observed (Fig. 3D, n = 2).

APP is N-glycosylated in the ER and cis-Golgi followed by O-glycosylation in medial- and trans-Golgi [42]. The mature form of APP is fully glycosylated (mAPP) whereas the immature form is N-glycosylated (imAPP) [43]. mAPP undergoes cleavage by β- and γ-secretases in the secretory pathway or at the plasma membrane whereas imAPP localizes to endoplasmic reticulum or cis-Golgi and is not subject to cleavage [44]. To investigate if this posttranslational modification is changed upon treatment with compounds, we calculated the ratio between mature and immature APP 3 days after incubation with compounds at 10 µM. All of the tested compounds with the exception of compound A led to a shift of glycosylation pattern towards predominantly immature APP when compared to controls where the ratio between mAPP and imAPP is balanced (Fig. S4, n = 3, *p<0.05).
Identification of similar structures

We identified 6 compounds out of the 10,000 which decreased the amount of APP or Aβ at lower concentrations (10 μM) and which were not cytotoxic (Fig. 4A). These findings could be used as starting points for further investigations in developing therapeutic targets. All structures of the 6 compounds are presented in figure 4B. We performed structural comparisons to determine promising structures of the molecules (Fig. S5). The activities and the predicted physicochemical properties of these structures (e.g. cLogP), which are well within the 'lead-like' and 'Drug-like' space, make them very interesting starting points for Drug Discovery efforts. Two of these six structures, compounds A and D contain a 5-bromonicotinamide moiety. Within the screening set, there were other compounds with this moiety that showed reduced or no activity, indicating some SAR. Thus compounds M and N were weakly active at 100 μM, whilst O and P were inactive at this dose. Compound A has been widely screened within the NIH Molecular Libraries initiative and has only proved active nine times out of the five hundred and ninety one assays in which it has been screened, indicating that it is not a promiscuous structure. None of the assays run are similar to the one of this report. Compound A does appear in a patent containing diverse structures as ‘Ganglioside Biosynthesis Modulators’ [45]. This could be a mode of action by increasing the immature form of APP, even if there is no shift in the mature/immature APP ratio in our Western Blots, but further work would need to be done to confirm this. There is also some SAR around compound C within the 10,000 compounds screened. Compound Q shows weak activity at 100 μM whilst a compound that is very closely related to C, compound R, is inactive. Compound C has also been widely screened within the NIH Molecular Libraries programme and of the 652 assays run, it was only active in 10 – all of these were CYP450 assays. This cytochrome activity is unsurprising for a molecule with an exposed pyridine nitrogen atom and is not likely to be directly related to the activity of this current report. There is also some additional data from this screen for compound B, with compounds S and T both active without toxicity at 50 μM. This structural class has previously been reported to prevent Huntingtin protein aggregation which may have some relevance [46]. However, with the potentially nonspecific effects seen with B, interest in this compound/series is perhaps lower than the others. Few analogues of compound E were assayed, although compound U was inactive.
Figure 2. Identification of six compounds lowering APP levels at lower concentrations are non cytotoxic. A) Toxicity assays. Trypan Blue Assay was performed after 3 days of compound incubation. The percentage of surviving cells was calculated. 1% DMSO was used as a negative and 10% DMSO as a positive control. For the MTT assay absorbance of formazan was measured at 570 nm. All experiments were performed in triplets.
at 100 μM suggesting that pyridyl moiety is important for activity. Further investigation in this area of the molecule would also seem sensible, including CYP450 binding. There is again a hint of SAR around compound F – from the small set of related compounds screened, the data suggests that activity may require the benzimidazole and a second basic centre – compound V is inactive at 100 μM whereas compounds W and X show weak activity at this concentration.

**Discussion**

We have established a novel cell-based method to screen for compounds lowering APP. Using this approach we have indentified six promising compounds out of 10,000 which significantly reduce levels of APP, Aβ40 and Aβ42 at lower concentrations, are non-cytotoxic, do not change the metabolic activity and fulfill the terms fur drug-likeness, which is a key fact for developing AD drugs. APP-lowering effects could be seen in non-neuronal HEK cells and in neuronal N2a cells.

There are several possibilities how these compounds interfere with Aβ generation. One possibility is transcriptional downregulation of APP mRNA. APPsw cells mainly express APP 695 which is the APP isof orm with the highest propensity to be processed to Aβ [47]. The transcription of APP can be affected by negatively influencing transcription factors or regulatory sequences in promoter regions of APP [48],[49],[50]. Compound B reduces APP mRNA levels and may function on the transcriptional level,
Figure 4. Characterization of the six best compounds. A) Flowchart of the screening of the 10,000 compound library DIVER Set. 10,000 compounds were screened and hits were analyzed by serial dilutions (100 μM, 50 μM, 10 μM, 1 μM). Compounds effective at lower concentrations were checked for cytotoxicity and the non-cytotoxic ones were further analyzed by western blot, ELISA and RT-PCR. B) Structures of the highly potent 5 compounds (A, C–F) in the DIVER Set library which have a specific effect on APP/Ab-production at a concentration of a minimum of 10 μM and are not cytotoxic. The structure of compound B is added below.

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although this relative reduction is influenced by stable overexpression of APPsw in our cell culture model. Since this compound also decreased levels of PrPSc these actions may be due to unspecified effects making this compound the least attractive compound identified by us.

Another way of interfering with AB generation is by influencing the trafficking and proteolytic processing of APP [51],[52]. Once APP reaches the plasma membrane, it is rapidly internalized and subsequently trafficked through endocytic and recycling compartments back to the cell surface or degraded in the lysosome [43]. Disturbed trafficking to the plasma membrane or enhanced degradation in lysosomes, could explain reduced levels of intracellular/plasma membrane APP. A second possibility could be the inhibition of APP maturation by modification of the Golgi apparatus. Recently it could be shown that X11-like, a neural adaptor protein, regulates intracellular trafficking of APP by this process [44]. O-glycosylation is a prerequisite for γ-secretase cleavage [53], therefore interference with O-glycosylation may result in decreased AB production. Although we did not investigate this in detail, for compounds B–F we observed a shift to the immature form of APP.

Finally the sequential cleavage of APP by α-, β- and γ-secretases represents a putative target. Since we designed our compound screen looking at reductions of sAPP, which nicely correlates with APP-lowering, direct influence of our compounds on α-, β- and γ-secretases activity is unlikely.

Recently published studies identified lead compounds aimed at treating a wide range of conformational dementias based on their potential to inhibit protein aggregation [54],[51]. Our approach differs from this approach as we screened for compounds aimed at reducing the substrate subject to dementia causing misprocessing. Combining both approaches represents an attractive strategy to indentify highly potent compounds to treat dementia where protein aggregation is causally involved.

In conclusion, we have indentified six compounds which reduce the amount of AB10 and AB12 possibly by influencing APP expression (for instance compound B) or APP maturation (compound C, D, E, F). APP-lowering effects could be seen in non-neuronal and neuronal cells.

All compounds with the exception of compound B did not affect expression of other membrane bound neuronal proteins such as PrPSc. From a Medicinal Chemistry point of view, each of the screening hits constitutes an interesting starting point for Drug Discovery in this vitally important disease area. All of the compounds have excellent potency for lowering APP and predicted analogues may show enhanced profiles.

Future studies will focus on the in vivo relevance, optimization of lead structures and on assessing if these compounds have potential for treating other dementias.

Supporting Information

Figure S1 Some compounds reducing APP at lower concentrations are cytotoxic. A) Effect at lower concentrations was assessed using serial dilutions (100 μM, 50 μM, 10 μM, 1 μM) in four independent experiments. Untreated APPsw cells were used as controls. One example of a blot is presented. Compound G, H, J, K, L reduce the APP level in a dose of 50 μM like 33 other compounds. B) I is one of the 10 compounds, which reduce the APP level at 10 μM, but are cytotoxic. Results are shown as mean±S.D., n = 3, ***p<0.001. C) Structures of some compounds (G–I) which were cytotoxic or reduce the APP level only at a dose of 50 μM.

(TIF)

Figure S2 Effect of compounds on N2a cells. A) Toxicity assays. For MTT assay absorbance of formazan was measured at 570 nm. All experiments were performed in triplicates. 1%DMSO was used as a negative and 10% DMSO as a positive control. We exclude toxic effects on neuronal cells. Results are shown as mean±S.D., n = 3, **p<0.01. B) Western blot of the cell lysates and the supernatants of N2a and N2asw cells after 3 day incubation with compounds (10 μM). Arrows indicate fully glycosylated mature, incompletely glycosylated immature APP and sAPP. First Graph shows relative expression of mAPP and sAPP normalized to expression of actin, untreated N2asw were set to 1. Results show means of two experiments. Second Graph shows relative expression of sAPP normalized to expression of actin, untreated N2asw were set to 1. Results show means of two experiments.

(TIF)

Figure S3 Assessment of specificity. Western Blot of cells lysates and supernatants of APPsw cells after 3 day incubation with compounds (10 μM). Arrow indicates PrPSc (diglycosylated, monoglycosylated and unglycosylated). β-actin serves as a marker for equal loading. Histogram showing relative expression of PrPSc normalized to expression of actin, untreated APPsw controls were set to 1. Results are shown as mean±S.D., n = 3, *p<0.05.

(TIF)

Figure S4 Effect of compounds on APP-glycosylation. Analysis of Glycosylation ratio. The ratio of mature to immature APP was calculated. Compound B, C, D, E and F lead to a shift to immature APP. Results are shown as mean±S.D., n = 3, *p<0.05.

(TIF)

Figure S5 Structural comparison. We checked similarity to other compounds of the library (compound M–X) and their appearance in other databases to determine promising structures of the molecules.

(TIF)

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Author Contributions

Conceived and designed the experiments: SCR M. Geissen M. Glatzel. Performed the experiments: SCR KH BS JV IF. Analyzed the data: SCR KH SS M. Geissen M. Glatzel. Contributed reagents/materials/analysis tools: M. Glatzel SS. Wrote the paper: SCR M. Glatzel.

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