Yeast growth selection system for the identification of cell-active inhibitors of β-secretase

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1. Summary

The production and deposition of the cytotoxic Aβ peptide is a central event in the pathogenesis of Alzheimer's disease (AD). Aβ is excised from the amyloid precursor protein (APP) through sequential actions of the β-secretase, which cleaves at the so-called β-site, and the γ-secretase, which cleaves at the so-called γ-site of APP. Inhibition the β-secretase BACE1 is a promising approach for AD therapy, but the search for small molecule inhibitors has proven to be challenging. In this thesis I describe a novel screening assay to identify cell-active BACE1 inhibitors by a positive yeast growth selection system, which combines the practicability of *in vitro* assays with the advantages of a cell-based assay. Analogue to the situation in mammalian cells, the β-site cleavage reaction was reconstituted in the secretory pathway of yeast cells using membrane-bound BACE1 and a membrane-bound APP-derived substrate. In contrast to conventional mammalian cell-based assays, false positive compounds can be rapidly excluded by the use of simple specificity controls that mimic the readout in the absence of BACE1. Furthermore, there is counter-selection for toxic compounds due to the positive growth readout upon inhibition of BACE1. The system was initially validated with two bona fide BACE1 inhibitors that stimulated the growth of BACE1-expressing cultures in a concentration-dependent manner, whereas the growth of control cultures remained unaffected in the presence of these inhibitors. In order to identify novel BACE1 inhibitors and to further validate the system, we screened a library of 15'000 small molecules. This screening revealed six compounds, which significantly reduced the secretion of Aβ from a human cell line overexpressing APP.

1. Zusammenfassung

In der Entstehung der Alzheimer'schen Krankheit spielt das zytotoxische Peptid Aβ eine zentrale Rolle. Zwei aufeinanderfogende Schnitte, welche durch die sogenannte β- und γ-Sekretase ausgeführt werden, setzen das Aβ Peptid aus dem Amyloid Precursor Protein (APP) frei. Die Inhibierung der β-Sekretase BACE1 ist ein vielversprechender Ansatz für die Therapierung der Alzheimer'schen Krankheit. Leider hat sich die Identifizierung von BACE1 Inhibitoren mittels bestehender Screening-Methoden als schwierig erwiesen. In der vorliegenden Arbeit beschreibe ich eine neuartige Methode für die Identifizierung von BACE1 Inhibitoren mittels einer positiven Wachstumsselektion von Hefezellen. Diese Methode kombiniert die einfache Durchführbarkeit von *in vitro* Methoden mit den Vorteilen von Zell-basierten Methoden. Für die Rekonstituierung der proteolytischen Reaktion der β-Sekretase wurden BACE1 und ein APP-basiertes Substrat als membranständige Proteine in sekretorischen Kompartimenten der Hefe exprimiert. Dies ist analog zu der natürlichen Situation in menschlichen Zellen. Im Gegensatz zu herkömmlichen Screening-Methoden mit kultivierten Säugetierzellen, bietet das Hefesystem Möglichkeiten, die es erlauben falsch-positive Substanzen mittels einfacher Spezifizitätskontrollen auszuschliessen. Die positve Wachstumsantwort, welche auf die Inhibierung von BACE1 erfolgt, verhindert zudem die Selektion von toxischen Substanzen. Das Hefesystem wurde mit Hilfe von zwei bestehenden BACE1-Inhibitoren validiert. Beide Inhibitoren ergaben eine konzentrationsabhängige Wachstumsantwort in BACE1-exprimierenden Hefezellen, während das Wachstumsverhalten von Hefezellen, die eine BACE1-unabhängige Kontrolle exprimierten, durch die Inhibitoren nicht beeinflusst wurde. Mit der Absicht, neuartige BACE1-Inhibitoren zu identifizieren und das Hefesystem weiter zu validieren, wurde ein Screening von 15'000 chemischen Verbindungen durchgeführt. In diesem Screening wurden sechs chemische Verbindung identifiziert, welche die Aβ-Sekretion einer APP-überexprimierenden humanen Zelllinie signifikant reduzierten.

2. Introduction

2.1. Alzheimer's disease: A short introduction

Alzheimer's disease (AD) is a progressive disease of the brain leading to loss of cognitive functions and ultimately to death. The major risk factor for the disease is age. The incidence of AD rises exponentially with age (Kawas et al., 2000) and therefore the increased life expectancy of human beings has made AD one of the predominant medical problems for elderly people. The disease is named after Alois Alzheimer (1864-1915). In 1906 he described a female patient in her early 50s, who suffered from progressive problems with memory, language and behavior. After the patient died, Alzheimer found two major changes in her brain, which he called neurofibrillary tangles and amyloid plaques.

Neurofibrillary tangles and amyloid plaques are hallmarks of the disease and the elucidations of their molecular compositions were milestones towards a profound understanding of the mechanism underlying the disease. The neurofibrillary tangles (NFT) are intracellular aggregates of hyperphosphorylated versions of the microtubule-associated protein tau (Wood et al., 1986; Wischik et al., 1988; Lee et al., 1991). The major component of the extracellular amyloid plaques is the β-amyloid peptide (Aβ) (Masters et al., 1985), which is excised from the so-called amyloid precursor protein (APP) (Kang et al., 1987) through sequential cleavage by the β- and the γ-secretases. Cleavage at the so-called α-site in the middle of the Aβ sequence precludes β-secretase cleavage and gives rise to the shorter non-amyloidogenic p3 peptide (Figure 1, for review see Selkoe, 2001).

Soon the question came up, which of the two hallmarks occurs first in the course of the disease and hence, which is the causative agent. The "tauists" believed that hyperphosphorylated tau occurs first and that Aβ-deposition is a consequence of it, whereas the "βabtists" claimed that it is the other way round.

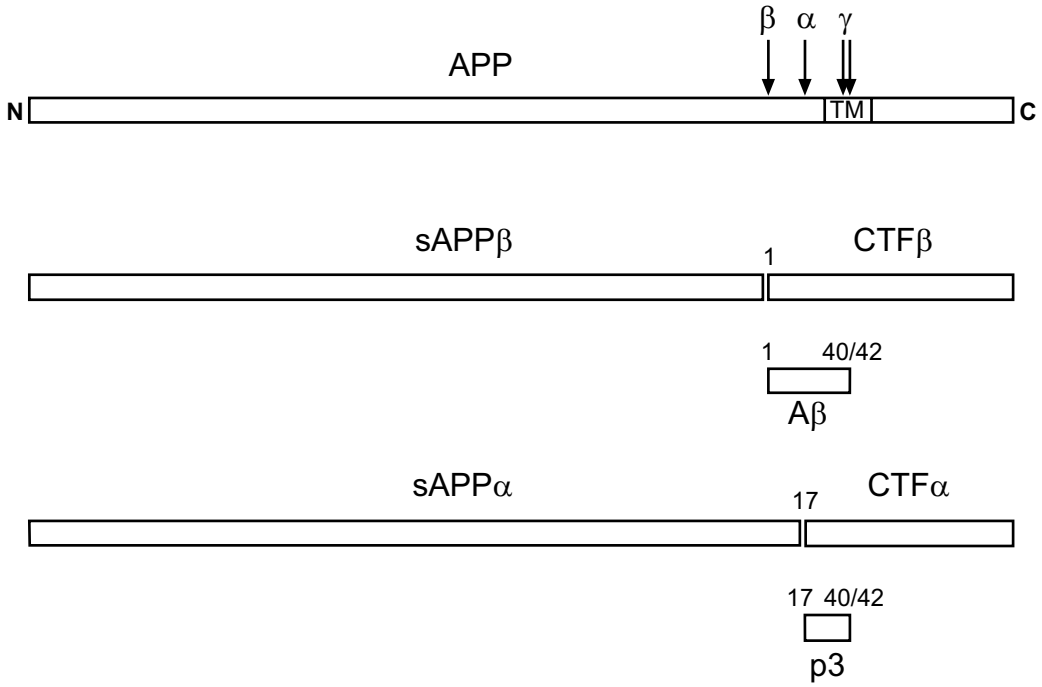

Figure 1 Proteolytic processing of the type-I transmembrane protein APP. The cleavage sites of the α-, β- and γ-secretase are indicated by α, β and γ. The amyloidogenic pathway is initiated by cleavage of APP at the β-site, which gives rise to two proteolytic fragments: The soluble APP ectodomain sAPPβ and the membrane-bound C-terminal fragment CTFβ. Subsequent cleavage of the γ-secretase at position 40 or 42 within the transmembrane domain (TM) of APP releases Aβ40 or Aβ42. The non-amyloidogenic pathway is initiated by α-secretase cleavage of APP giving rise to sAPPα and CTFα. Subsequent γ-secretase cleavage releases the non-amyloidogenic p3 peptide. The indicated numbers refer to Aβ numbering.

The importance of neurofibrillary tangles was emphasized by the recognition, that there exists a whole group of neuropathological disorders with filamentous intracellular inclusions composed of hyperphosphorylated tau. A prominent member of these so-called "tauopathies" is the autosomal dominant fronto temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), for which mutations could be mapped to the tau gene on chromosome 17 (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). The most common mutation in FTDP-17, the tau P301L was expressed in transgenic mice, which exhibited motor and behavioral deficits and developed NFTs in an age and gene-dose-dependent manner (Lewis et al., 2000; Götz et al., 2001a). These findings demonstrate that aberrant tau processing can be sufficient to cause dementia. In the case of AD however, this is not yet the whole story. If the mice expressing tau P301L are crossed with mice expressing mutant APP (Tg2576), or if Aβ peptide is injected into their brains, the number of NFTs becomes several fold enhanced (Götz et al., 2001b; Lewis et al., 2001), which are strong arguments for Aβ playing a central role in the development of AD.

2.2. Putative mechanism of Aβ toxicity

It is known for a long time that Aβ is neurotoxic *in vitro* (Yankner et al., 1990) and *in vivo* (Kowall et al., 1992). However the exact mechanism of this toxicity remains unclear. The literature is often contradictive in the details, but the following minimal consensus seems to be widely accepted: Aβ toxicity is associated with oxidative stress, which causes oxidative damage to macromolecules such as proteins, nucleic acids and lipids, and which then leads to apoptosis. (Signs of necrotic cell death have been observed as well in the AD brain, but most publications describe prevalence for apoptotic cell death implicated in AD.)

How is Aβ associated with enhanced occurrence of reactive oxygen species (ROS) that cause oxidative stress? Does it generate the harmful ROS directly or indirectly? Many studies describe a disturbance of the intracellular Ca^{2+} homeostasis that goes along with Aβ toxicity (for review see Canevari et al., 2004). Since Ca^{2+} signals play a fundamental role as coordinators and integrators of cellular signaling pathways, they represent a good candidate as mediator for the manifestation of pathological processes. However, the

causal relationship of oxidative stress and the disturbance of Ca^{2+} homeostasis is contradictive. Are the observed changes in intracellular Ca^{2+} concentration directly caused by Aβ, and oxidative stress results as a consequence of it, or is the disturbance of the Ca^{2+} homeostasis a consequence of oxidative stress? Models supporting a direct role for Aβ in disturbing Ca^{2+} homeostasis suggest that Aβ alters the current through Ca^{2+} channels or that it directly alters the leakiness of the membrane for Ca^{2+}, e.g. by the formation of a pore.

A direct role for the generation of oxidative stress by Aβ is supported by the observation that Aβ binds Cu(II) with very high affinity and that this complex catalyzes the production of cytotoxic hydrogen peroxide (H_2O_2) via the reduction of Cu(II) to Cu(I) (Huang et al., 1999a; Huang et al., 1999b). Furthermore, it was shown that this reaction depends on the presence of a reducing agent like dopamine, cholesterol or vitamin C (Opazo et al., 2002). The cytotoxic effect of the Aβ·Cu(II) complex could be attenuated by the addition of catalase, which catalyzes the reaction of 2 H_2O_2 to yield 2 H_20 and O_2. Beside neurotoxic effects there is also a number of publications describing neurotrophic effects of Aβ (for review see Atwood et al., 2003). Atwood et al. summarize evidence that Aβ can protect neurons from Fe(II) induced toxicity by binding to it and that it can function as antioxidant with superoxide dismutase (SOD)-like activity that catalyzes the reduction of superoxide (O_2^-) to H_2O_2. In this view, the transition of Aβ from a neuroprotective to a neurotoxic factor is concentration and age dependent. At a certain concentration of active Aβ complexes the generation of H_2O_2 would exceed the removal capacity of catalase and glutathione peroxidase resulting in cell damage. An analogous scenario is known for the Cu,Zn-SOD, which reduces oxidative stress when its activity is in balance with catalase activity, but when it is overexpressed relative to catalase activity, oxidative stress is increased (Amstad et al., 1991).

2.3. Therapeutic approaches to reduce Aβ

Interference with the generation or deposition of Aβ is considered as a promising approach for AD therapy. An obvious method to interfere with the production of Aβ is inhibiting the secretases, which excise it from APP. The identities of these secretases have been revealed in recent years. Five groups published almost simultaneously the

identification of the β-secretase BACE1 (β-site APP cleaving enzyme 1; alternative names are memapsin 2 or Asp 2), which is a single type-I transmembrane aspartic protease (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000). BACE1 as a drug target and the search for inhibitors thereof are discussed in a separate chapter of this introduction.

The elucidation of the composition of the γ-secretase, which is a multimeric transmembrane complex comprised of presenilin, nicastrin, aph-1 and pen-2 was achieved recently (Edbauer et al., 2003; Kimberly et al., 2003). In the absence of presenilin 1 (PS1), γ-secretase cleavage of APP is prevented (De Strooper et al., 1998), but the gene knockout mice exhibit severe defects of the skeleton and of the CNS, and die shortly after birth (Shen et al., 1997; Wong et al., 1997). Indeed, their phenotype is strongly reminiscent of the one exhibited by mice deficient for Notch (Wong et al., 1997), which is a substrate for the γ-secretase as well (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). An induced knockout of Notch function in mice after birth caused a severe deficiency in thymocyte development (Radtke et al., 1999). Beside Notch and APP, there are several other targets of the γ-secretase known (for review see Medina and Dotti, 2003). For these reasons, inhibition of γ-secretase is associated with the risk of adverse side effects. Indeed, *ex vivo* and *in vivo* studies with γ-secretase inhibitors revealed severe interference with lymphopoiesis (Hadland et al., 2001; Wong et al., 2004). These side effects could be circumvented by developing γ-secretase inhibitors that specifically affect APP processing, or by finding a range of inhibition, which sufficiently impairs Aβ production, but still allows for the biological function of the other targets of the γ-secretase. In fact, there are already potent γ-secretase inhibitors tested in clinical trials (see for example: http://www.myriad.com/pharma/clinicaltrials/alzheimers.htm or http://www.centerwatch.com/bookstore/nmt/nmtb_alzheimers.pdf).

Another possibility to reduce Aβ would be to shift APP processing from the amyloidogenic to the non-amyloidogenic pathway. This approach is especially attractive, because it has been shown that the soluble APP ectodomain released by the α-secretase (sAPPα) has neuroprotective effects (Furukawa et al., 1996). There are three members of

the ADAMs (a disintegrin and metalloproteinase) family (ADAM9, ADAM10 and ADAM17), which fulfill the criteria required of α-secretase (for review see Allinson et al., 2003).

At the moment, the only FDA-approved AD drugs are acetylcholine esterase inhibitors, which stabilize acetylcholine levels in the brain. Treatment with these drugs does not stop, but slows down the progression of the disease and thereby the decline of cognitive impairment. There is evidence that the beneficial effect of these drugs is not only due to enhanced cholinergic neurotransmission, but also that acetylcholine upregulates the production of sAPPα, possibly via an agonistic effect on cholinergic muscarinic receptors. Agonists of muscarinic receptors are known to stimulate the production of sAPPα (for review see Allinson et al., 2003).

Retrospective clinical studies on cholesterol-lowering drugs and on NSAIDs (non-steroidal anti-inflammatory drugs) revealed a reduced risk for developing AD upon chronic treatment with these drugs (Rich et al., 1995; Jick et al., 2000; Wolozin et al., 2000). Increased cholesterol levels are a known risk factor for AD (Kuo et al., 1998) and there is evidence that high cholesterol levels shift the balance of amyloidogenic and non-amyloidogenic APP processing to the amyloidogenic pathway (for review see Allinson et al., 2003). For a subset of NSAIDs it was shown that they directly inhibit the γ-secretase (Eriksen et al., 2003), however for others it has been demonstrated that they upregulate the non-amyloidogenic pathway via activation of the Erk MAP kinase signaling cascade (Avramovich et al., 2002).

The role of a forth secretase, the BACE1 homologue BACE2 (71% sequence homology; Acquati et al., 2000), in the amyloidogenic pathway is less clear at the moment. Upon overexpression, BACE2 cleaves at the β-site and more efficiently at alternative sites near the α-site, after Phe-19 and Phe-20 of the Aβ sequence (Farzan et al., 2000; Hussain et al., 2000). The Flemish mutation at residue 21 increases the proportion of β-site cleavage by BACE2 (Farzan et al., 2000), which is an indicator that BACE2 can be involved in amyloidogenic processing of APP. However, overexpression of BACE2 caused an increased release of β-secretase derived soluble APP ectodomain (sAPPβ), but this was not accompanied by increased secretion of Aβ (Hussain et al., 2000). Selective inactivation of BACE1 and BACE2 transcripts using RNA interference

(RNAi) revealed antagonistic effects of BACE1 and BACE2 (Basi et al., 2003). Interference with BACE1 expression caused a decrease of Aβ secretion (as expected), whereas interference with BACE2 expression caused an increase of Aβ secretion suggesting an analogous role for BACE2 in the amyloidogenic process as for the α-secretase. Concomitant reduction of BACE1 and BACE2 transcripts did not have any effect on Aβ secretion (Basi et al., 2003). These results point out the potential importance of developing inhibitors, which are highly specific for BACE1, as they suggest that inhibitors, which inhibit both, BACE1 and BACE2 could compromise the desired lowering of Aβ.

An alternative therapeutic approach for reducing adverse effects of Aβ does not involve altering the proteolytic processing of APP, but is based on active or passive immunization against Aβ. Since the first discovery of beneficial effects of active Aβ-immunization in an APP-transgenic mouse model of AD (Schenk et al., 1999), there have followed many more promising studies in mice and humans (for review see Dodel et al., 2003). In 2002 a phase IIa clinical study for active immunization was suspended due to signs of meningoencephalitis in several patients (Senior, 2002). The exact cause for this adverse reaction of the vaccination is not yet clear, but is topic of intense investigations. Not clear as well are the mechanism by which the beneficial effects of the immunization approach are caused. There exist at least three hypotheses (Dodel et al., 2003). The first one predicts that the antibodies have to be present in the brain, where they directly bind to aggregated Aβ and thereby trigger Fc-mediated phagocytosis of aggregated Aβ. The so-called peripheral sink hypothesis is based on the assumption that there exists a dynamic equilibrium between Aβ in the brain, in the cerebral spinal fluid (CSF) and in the plasma. Aβ·antibody complex formation in the plasma would therefore cause an Aβ efflux out of the brain. The third predicted mechanism, involves direct inhibition of Aβ-aggregation and neurotoxicity by the antibodies.

Recent findings suggest that inhibition of kinases could be an approach to reduce the production of Aβ. It was found that the kinase inhibitor gleevec (STI571) reduced Aβ production in rat primary neuronal cultures and *in vivo* in guinea pig brain (Netzer et al., 2003). The exact mode of action for this has not yet been determined. Although it was

shown that the effect is dependent on the presence of ATP, it can still be that it does not involve the inhibition of a kinase. Remarkably, it was shown that gleevec interferes with γ-secretase processing of APP, but not with γ-secretase processing of Notch (Netzer et al., 2003). Similarly, it was found that therapeutic concentrations of lithium, a known inhibitor of glycogen synthase kinase-3 (GSK-3), interfere with γ-secretase processing of APP, but not of Notch (Phiel et al., 2003). The authors of this study furthermore demonstrated that overexpression of GSK-3α causes an increased secretion of Aβ. GSK-3 is also one of the kinases found to hyperphosphorylate tau (Hanger et al., 1992) and therefore its inhibition is especially interesting for AD therapy, since it could affect both, amyloid deposition and neurofibrillary pathology.

2.4. BACE1 as a drug target for AD therapy

As discussed above, inhibition of the γ-secretase can be problematic due to interference with the Notch (and eventually other targets of the γ-secretase) signaling pathway. For BACE1 inhibition, the issue of side effects could not yet be addressed, since there are no publications about BACE1 inhibitors being active in an animal model. The data obtained from BACE1 knockout mice, however, are promising. Gene knockout mice for BACE1 do not produce any detectable amounts of Aβ. Furthermore, these mice do not exhibit any obvious developmental defects indicating that inhibition of this enzyme would probably reduce Aβ production, without causing any severe side effects (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001; Luo et al., 2003). A minor behavioral phenotype of BACE1 knockout mice, however, was detected. Harrison et al. found that their knockout mice were more timid and less exploratory than their wildtype littermates (Harrison et al., 2003). Apart from APP, there are two other targets of BACE1 described in the literature, the Golgi resident sialyltransferase ST6Gal I and the P-selectin glycoprotein ligand-1 (PSGL-1), which are both involved in the regulation of the immune system (Kitazume et al., 2001; Lichtenthaler et al., 2003). The gene knockout mice for ST6Gal I or PSGL-1 are both viable (Hennet et al., 1998; Xia et al., 2002), but unlike the BACE1 knockout mice they exhibited immune system related phenotypes, which indicates that the functions of these two proteins are not fully dependent on BACE1 activity.

From a biological point of view BACE1 is widely considered as potentially ideal therapeutic target for the treatment of AD (see for example: Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001; Hong et al., 2002a; John et al., 2003; Citron, 2004)). Hook and Reisine however challenge this view and provide evidence that β-site processing of APP could in addition to BACE1 also be accomplished by as yet unidentified cysteine proteases. According to their model, Aβ produced by BACE1 would be secreted via the (minor) constitutive secretion pathway, whereas Aβ produced by these cysteine proteases would be secreted via the (major) regulated secretion pathway of neurons, which can be stimulated by neurotransmitters or changes in electrical firing activity (Hook et al., 2002; Hook and Reisine, 2003).

From a pharmacological point of view BACE1 has proven to be a tough target. The requirement of therapeutic BACE1 inhibitors to penetrate the blood-brain barrier would be best met by nonpeptidic compounds of low molecular weight. However, extensive campaigns of big pharma companies to identify small molecule inhibitors of BACE1 by random screenings of huge compound collections have had limited success so far (see also *part 2.6.* of this thesis, Middendorp et al. 2004). There are patents from Takeda, Vertex and Neurologic that disclose structures of nonpeptidomimetic compounds (for review see John et al., 2003). The most potent inhibitor claimed by Takeda reveals an *in vitro* IC_{50} of 0.35 μM, the most potent compound from Vertex has a K_i of 3 μM. The compounds disclosed by Neurologic were shown to shift β-site processing of APP to the non-amyloidogenic α-secretase pathway, which is – although a promising result for a potential AD therapy anyway – not a clear proof that these compounds target BACE1.

The best progress towards therapeutic inhibitors of BACE1 has been achieved using rational approaches. The elucidations of the structures of the BACE1 ectodomain in complex with two different inhibitors (Hong et al., 2000; Hong et al., 2002b) and most recently of free BACE1 ectodomain (Hong and Tang, 2004) provided important information for these approaches. A second essential achievement to aid rational approaches was the determination of the subsite specificity of BACE1 using a combinatorial peptide library (Turner et al., 2001). Exploiting the subsite specificity information yielded a very potent inhibitor consisting of an eight amino acid peptide, in which the peptidic bond between the p1 and the p1' residue was replaced by an

uncleavable hydroxyethylene transition state isostere. The K_i of this compound is 0.31 nM (Turner et al., 2001). It has not been reported to have an effect in cell culture, but it can be used as a lead structure for designing smaller and potentially cell-permeable, peptidomimetic inhibitors.

Elan Pharmaceuticals successfully developed a peptide inhibitor with an uncleavable statine group into a cell-permeable statine-based peptidomimetic inhibitor, which reduced the production of Aβ in HEK 293 cells by 50% at a concentration of 4 μM (EC_{50} = 4 μM) (Hom et al., 2003). A more recent publication from Elan Pharmaceuticals reports an even improved statine-based compound displaying an IC_{50} of 1 nM and an EC_{50} of 1000 nM (Hom et al., 2004). Furthermore, they produced a series, in which the statine groups were replaced by hydroxyethylene isosteres. The best compound of this series revealed IC_{50} and EC_{50} values of 30 nM and 3000 nM, respectively (Hom et al., 2004). The authors pointed out that the EC_{50} to IC_{50} ratio was a factor of 1000 for the statine-based compound, whereas this factor was only 100 for the hydroxyethylene-based compound, suggesting that the hydroxyethylene-based compound displays a better cell-permeability.

In summary, considerable progress has been achieved with peptidomimetic inhibitors of BACE1, whereas there is limited information about non-peptidomimetic compounds. However, the identification of a non-peptidomimetic lead structure is still desirable. The last part of this introduction summarizes existing screening methods and previews the work of this thesis, a novel screening system in yeast to identify inhibitors of BACE1.

2.5. References

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2.6. Methods to identify β-secretase inhibitors

(Searching for the most effective screening system to identify cell-active inhibitors of β-secretase)

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Minireview

Searching for the most effective screening system to identify cell-active inhibitors of β-secretase

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Abstract

The β-secretase BACE1 is an attractive drug target for reducing the level of the Alzheimer's disease-promoting Aβ peptide in the brain. Whereas potent peptidomimetic *in vitro* inhibitors of BACE1 have been designed, screening approaches to identify cell-permeable small molecule inhibitors have had limited success so far. In the present minireview we summarize existing screening methods, discuss their scope of application in the drug discovery process and compare them to a novel cell-based screening system to identify BACE1 inhibitors by a positive yeast growth selection.

Keywords: Aβ; Alzheimer's disease; BACE1; β-secretase; β-secretase inhibitor; screening system.

Introduction

Alzheimer's disease (AD), the most common cause for senile dementia, is characterized by two neuropathological lesions in the brain: Neurofibrillary tangles and amyloid plaques. The major component of the amyloid plaques is aggregated amyloid β (Aβ) peptide, which is excised from the type-I transmembrane protein amyloid precursor protein (APP) through sequential cleavage by the β- and the γ-secretase. An alternative cleavage at the so-called α-site precludes β-site cleavage and gives rise to the shorter, non-toxic p3 peptide. The amyloid cascade hypothesis states that the Aβ peptide is the central trigger in the pathogenesis of AD (Citron, 2004). Indeed, most of the current approaches towards an AD therapy aim to inhibit the production or deposition of the cytotoxic Aβ peptide (Michaelis, 2003). Inhibition of the β- or γ-secretase is a straightforward strategy to achieve this goal and consequently, extensive endeavors have been undertaken for their identifications. The search for the γ-secretase revealed a transmembrane protein complex composed of nicastrin, Pen-2, Aph-1 and presenilin-1 (PS1), the latter being the suspected enzymatic subunit (Edbauer et al., 2003). For the γ-secretase there exist already potent inhibitors, which reduce secreted Aβ peptide in AD mouse models (Dovey et al., 2001; Wong et al., 2004). However, chronic treatment of mice with one of these inhibitors caused severe side effects, most probably by interference with the Notch signaling pathway, which depends on γ-secretase activity (Wong et al., 2004). It remains to be seen whether a therapeutic window can be found for γ-secretase inhibition, in which the production of Aβ peptides is sufficiently reduced to be therapeutically beneficial, but Notch signaling still can occur.

The search for the β-secretase revealed a type-I transmembrane aspartic protease termed β-site APP cleaving enzyme (BACE) 1 (Vassar and Citron, 2000). Furthermore, recent studies published by Hook and Reisine suggest that, in addition to BACE1, there are cysteine proteases involved in the β-site processing of APP. These yet unidentified cysteine proteases are relevant for the production of Aβ peptide in the regulated secretion pathway of chromaffin cells, whereas the Aβ peptide produced by BACE1 is mainly secreted through the constitutive secretion pathway (Hook and Reisine, 2003). Since there are no publications about specific β-secretase inhibitors being active in mouse models, the therapeutic potential of β-secretase inhibitors can so far only be estimated from results obtained with BACE1 gene knockout mice. In support of BACE1 as an important target for developing β-secretase inhibitors is the observation that BACE1 knockout mice do not produce any detectable amounts of Aβ peptide. In addition, these mice are viable, fertile, and behave like their wild-type littermates (Cai et al., 2001; Luo et al., 2001) indicating that treatment with a BACE1 inhibitor would not cause any severe side effects. The best progress towards efficient and cell-permeable inhibitors of BACE1 has been achieved so far by the use of peptidic transition state mimetic compounds (Hom et al., 2003), whereas there are no publications describing the successful screening of a collection of small compounds to identify BACE1 inhibitors. In the present minireview we describe current methods to monitor β-secretase activity and discuss their scope of application in the drug discovery process. We compare existing screening methods and discuss a novel cell-based screening system to identify BACE1 inhibitors by a positive yeast growth selection. A link of current methods is provided in Table 1.

Biochemical methods to monitor BACE1 activity

The identification of BACE1 as β-secretase allowed the establishment of biochemical methods to investigate β-secretase activity of recombinant BACE1 *in vitro*. Naturally, this reaction involves at least two membrane-bound proteins that interact with each other: BACE1 and its substrate APP. Since purification of large amounts of

Table 1 Overview of current methods to monitor β-secretase activity.

	β-secretase	Substrate	Readout	Purpose
Biochemical methods				
FRET	soluble BACE1	modified peptide	continuous, direct	HTS, kinetics, hit validation
HPLC/MS	soluble BACE1	(modified) peptide	discontinuous, direct	hit validation, kinetics
ELISA	soluble BACE1	soluble fusion protein	discontinuous, indirect	hit validation, kinetics (MTS)
Cell-based assays				
Aβ ELISA	endogenous β-secretase	full-length APP	discontinuous, indirect	MTS, hit validation (standard test for cell activity)
Cleavage-dependent secretion of AP				
mammalian cells	endogenous β-secretase	membrane-bound AP-APP fusion protein	discontinuous, indirect	MTS
insect cells	transgenic BACE1	membrane-bound β-site-AP fusion protein	discontinuous, indirect	MTS
Growth readout in yeast cells	transgenic BACE1	membrane-bound invertase-APP fusion protein	continuous, indirect	MTS-HTS

Abbreviations: HTS, high-throughput screening; MTS, medium-throughput screening; AP, alkaline phosphatase

membrane-bound proteins is intricate, BACE1 activity was reconstituted *in vitro* using soluble BACE1 fragments and soluble substrates. Soluble BACE1 fragments comprising the prodomain (which is removed after folding of the protein in certain expression systems) and the catalytic domain, but lacking the transmembrane domain and the cytosolic part were purified from various expression systems, including 293T cells, *E. coli* and *Drosophila* S2 cells. In a recently published study the enzymatic activities of these soluble BACE1 versions were compared to the β-secretase activity of membrane preparations from HEK 293 cells that over-expressed full-length BACE1 (Kopcho et al., 2003). The authors did not find significant differences between the soluble and the membrane-bound BACE1 versions regarding enzymatic activity and inhibition by peptidic transition state mimetic inhibitors. However, the substrates used for this study were three peptides of 9 to 27 amino acids of length, which is still crucially different from the natural situation, in which both BACE1 and its substrate APP are membrane-bound. It remains to be seen, whether inhibition of BACE1 cleavage measured with soluble substrates is comparable to inhibition determined with membrane-bound substrates, or whether the membrane-anchoring properties of the substrate could be important for the activity of certain inhibitors. Indeed, all of the most common *in vitro* methods to detect BACE1 activity involve soluble substrates.

The majority of the *in vitro* high-throughput screening (HTS) systems to identify inhibitors of BACE1 is based on fluorescence detection methodologies. Fluorescence-based readouts allow for rigorous assay miniaturization; especially emerging confocal detection systems for HTS applications enable to use assay volumes in the submicroliter range without loss of signal quality (Jäger et al., 2003). The most common fluorescence-based methods involve fluorescent resonance energy transfer (FRET) substrates. These substrates are peptides with a fluorescent group and a quenching group attached to it. Upon cleavage of the peptide the fluorescent group is separated from the quenching group, which results in an increase of fluorescence (Ermolieff et al., 2000). This kind of assay is homogenous, direct and continuous, and therefore allows for online recording of the enzymatic reaction. The most frequent problem of FRET readouts is optical interference of the test substances, which can lead to both, false negative and false positive signals. This problem can be down-sized by the use of a time-resolved fluorescence (TRF) readout. Since most fluorescence signals from small molecules found in compound collections have short emission lifetimes and are emitted at near-UV wavelengths, they can be distinguished from TRF signals, which have long emission lifetimes and are emitted at higher wavelengths (Kennedy et al., 2003).

Other frequently used *in vitro* methods to measure BACE1 activity involve the quantification of cleavage products by high-performance liquid chromatography (HPLC; Toulokhonova et al., 2003), mass spectroscopy (MS; Turner et al., 2001) or ELISA (Sinha et al., 1999). These methods are discontinuous and because of their limited throughputs they are not the first choice for a HTS. In return, these assays are generally quite robust and consequently they are used to validate and characterize the hits obtained from the primary screening.

Cellular assays to monitor APP cleavage

Biochemical assays have some evident advantages over cellular assays. The most obvious of them are higher-throughput and, in comparison to cellular assays, less unknown parameters resulting in better controllability and reproducibility of the assay. On the other hand, cellular assays cover issues that cannot be addressed by biochemical assays. One important advantage of cell-based assays is that the target protein and its interaction partners are produced by the cells and presented under physiological conditions in their native conformation to the test substance. The potential relevance of this issue is demonstrated for example by the 'magic cancer bullet', the kinase inhibitor gleevec (STI-571). Gleevec binds to the inactive form of kinases (Schindler et al., 2000), which is in equilibrium with the active form *in vivo*. However, most *in vitro* kinase assays employ the active form, for which gleevec is a poor inhibitor (Bohmer et al., 2003).

The second advantage of cell-based HTS is that they directly select for compounds that are active in the appropriate cellular compartment. This suggests that respective compounds are not only able to permeate biological membranes, but that they are also resistant against inactivation by enzymes of the cellular metabolism. However, it has to be stated that in the case of *in vitro* HTS for BACE1 inhibitors, membrane permeability is not yet an issue, since BACE1 proved to be a tough target and it first seems to be a problem to identify any potent small molecule inhibitor by *in vitro* screenings.

A well-established method to monitor APP cleavage in whole cells is provided by a sandwich ELISA that allows quantifying the amount of Aβ present in biological fluids or released to the medium of cultured cells. This assay, which is not target-specific, was extensively used to screen for substances that lower Aβ secretion, even before the identities of β- and γ-secretase were revealed. These screening campaigns resulted in the identification of several compounds that reduced the amount of Aβ in the supernatant of cultured cells, but none of them was developed into approved AD therapy drugs and none of them targeted the β-secretase. However, a recent ELISA-based screening of 25 000 compounds yielded a lead compound for γ-secretase inhibition, which could be further developed to be effective in an animal model (Dovey et al., 2001).

The ELISA approach enables to differentiate between Aβ40 and the two amino acids longer Aβ42 peptide by the use of specific antibodies. This provides an opportunity to screen for substances that selectively inhibit the production of the longer and more aggregation-prone Aβ42, which is considered to be critical for the development of the disease (Citron, 2004). A HTS system based on this concept was established and the screening of a selection of natural product extracts revealed an extract that selectively reduced secretion of Aβ42 (Yager et al., 2002).

An alternative concept to monitor β-secretase activity of entire cells involves the cleavage-dependent release of a reporter enzyme, generally alkaline phosphatase (AP), from a chimeric transmembrane protein. The AP moiety, which is oriented to the topologically extracellular space, is connected to the membrane via a portion of APP harboring the β-site. Cleavage at the β-site liberates the AP moiety, which is then secreted to the cell culture medium. The activity of the secreted AP molecules is an indicator for the activity of the β-secretase and can be quantified by a colorimetric reaction. For a β-secretase inhibitor screening, this method has the advantage that it allows to exclude false positives by the use of a cell line that expresses constitutively secreted AP instead of membrane-bound AP. If a compound produces a positive signal (a reduction of the secreted AP activity) with both cell lines, it does obviously not affect β-secretase, but interferes somewhere else with the readout, e.g. with the secretion or catalytic function of AP. An application of this system is described in a patent from Schering (Dyrks et al., 1998). We do not have information whether this assay has been successfully applied in a HTS format. However, for other purposes, this concept of cleavage-dependent secretion of AP has been successfully used, e.g. to clone the SREBP-cleaving protease S1P (Sakai et al., 1998) or to identify an inhibitor of L-selectin ectodomain shedding (Feehan et al., 1996). Recently, this concept was also exploited to establish a screening system for BACE1 inhibitors in *Drosophila* S2 cells (Oh et al., 2003).

The identification of BACE1 as β-secretase was a prerequisite to establish target-specific assays in heterologous systems like *Drosophila* or yeast cells, which do not express endogenous β-secretase activity. In the last section of this minireview we describe a novel method to monitor a reduction of BACE1 activity in the yeast *Saccharomyces cerevisiae* by a positive growth selection.

Positive growth selection system to identify BACE1 inhibitors in yeast

Heterologous expression of disease-relevant proteins in yeast allows setting up target-specific screening platforms in an isolated but eukaryotic *in vivo* system, which can be suitable for the identification of small molecule inhibitors against a broad spectrum of proteins (Young et al., 1998; Grozinger et al., 2001; Perkins et al., 2001; Zaks-Makhina et al., 2004). BACE1 activity was reconstituted in yeast with the aim to establish a target-specific cell-based HTS system with minimal liquid handling requirements to identify inhibitors of BACE1. Functionality and specificity of an engineered BACE1 variant in yeast was demonstrated by the use of a type-I transmembrane invertase-APP fusion protein as a substrate (Lüthi et al., 2003). In this fusion protein, the invertase moiety is fused N-terminal to the APP part, which comprises a short lumenal stump harboring the β- and the α-site, the transmembrane domain and the cytosolic domain. Natural invertase is a secreted enzyme that catalyzes the hydrolysis of sucrose into fructose and glucose and thereby enables yeast to grow on medium with sucrose as the sole carbon source. The invertase-APP fusion construct, however, is retained in intracellular compartments of the secretory pathway via the transmembrane domain of APP. Consequently, recombinant yeast cells, which express invertase-APP instead of the natural invertase, can not grow on medium with sucrose as the sole carbon source, unless they express a secretase activity that cleaves within the lumenal APP portion and thereby liberates the invertase moiety, which is subsequently secreted (Figure 1A). Mkc7p and Yps1p, two endogenous yeast proteases, were shown to cleave APP at the α-site (Zhang et al., 1997). Since they both restored growth of the recombinant yeast cells on sucrose plates, their genes had to be deleted in order to demonstrate activity of the engineered BACE1 variant in yeast cells.

The system described above confers growth to yeast cells in the presence of active BACE1. This setup is not ideal for a screening for BACE1 inhibitors, since every toxic compound would reduce the growth of yeast cells and thus would produce a false positive signal. To circumvent this problem, the original readout was reverted in a way that yeast cells only grow if BACE1 activity is inhibited. To achieve this, the natural regulation of the yeast GAL gene network was exploited. A reporter con-

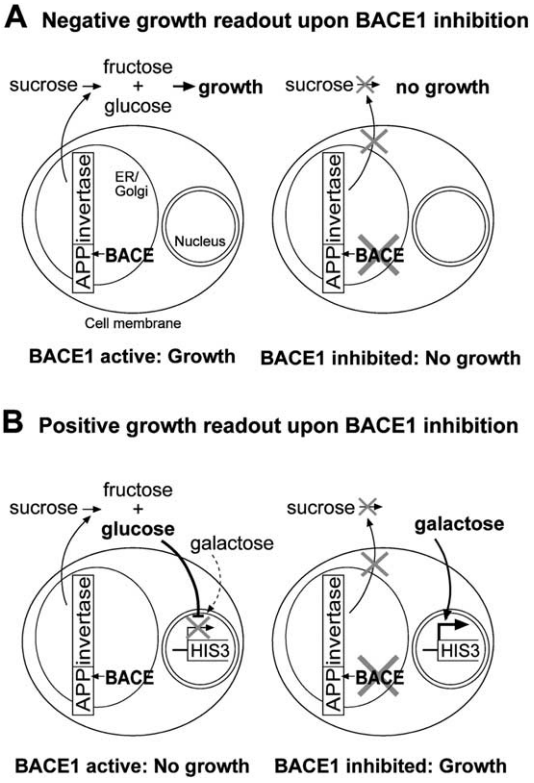

Figure 1 Yeast assays to monitor BACE1 inhibition: principles of the negative and the positive growth readout.
(A) Negative growth readout upon BACE1 inhibition: Invertase activity that is retained in the ER to Golgi compartment via the transmembrane domain of APP becomes liberated and secreted upon BACE1 cleavage at the β-site within the APP portion. Secretion of invertase activity, which hydrolyzes sucrose into fructose and glucose, is a prerequisite for growth on sucrose medium. If BACE1 is inhibited, secretion of invertase activity is prevented and growth on sucrose medium cannot take place. (B) Positive growth readout upon BACE1 inhibition: BACE1 dependent secretion of invertase activity occurs as described in A. The medium contains, in addition to sucrose, galactose and is depleted of histidine. The invertase-dependent hydrolysis of sucrose produces glucose that dominantly represses the transcription of the *HIS3* gene under the control of the *GAL1/10* promoter. Since the product of the *HIS3* gene is required for growth in histidine-depleted medium, the yeast cells cannot grow in the presence of active BACE1. If BACE1 is inhibited, secretion of invertase activity is prevented and consequently no glucose is produced. This allows for the activation of the *HIS3* gene by galactose and finally for growth on histidine-depleted medium.

struct, which expresses the *HIS3* gene as a growth selection marker and the *lacZ* gene as a colorimetric marker under the control of the divergent *GAL1/10* promoter, was integrated into the genome of yeast cells. The *GAL1/10* promoter is activated by galactose, but dominantly repressed by glucose. Thus the two reporter genes are actively transcribed if the yeast cells are grown on galactose medium or on medium containing galactose and sucrose, as sucrose is inert to the *GAL1/10* promoter. However, if glucose is added to the medium, the transcription of the reporter genes is repressed. If yeast cells grown on galactose and sucrose secrete invertase, the sucrose is hydrolyzed into fructose and glucose and the freshly generated glucose dominantly represses the expression of the two reporter genes. A combination of the glucose-repressible reporter gene expression with the secretase dependent secretion of invertase as described above couples the activity of the secretase to a transcriptional readout. In the presence of an active secretase, the invertase moiety is secreted and the reporter genes are repressed. Inhibition of the secretase impedes secretion of the invertase moiety and consequent transcriptional repression (Figure 1B).

Together with the BACE1 variant that was engineered to be active in yeast, this system allows for a positive growth selection of samples with impaired BACE1 activity. In a proof-of-concept study two validated BACE1 inhibitors from Novartis Pharma (Middendorp et al., 2004) stimulated growth of BACE-expressing cultures in a dose-dependent manner. Importantly, the two tested BACE1 inhibitors did not stimulate growth of cultures expressing the yeast α-secretase Yps1p, thus demonstrating the specificity of the system. On the other hand, the non-specific compound MG132 stimulated the growth rate of both BACE1- and Yps1p-expressing cultures. These results emphasize the importance of having a control like Yps1p that allows a rapid distinction between compounds that impair BACE1 function directly and compounds that interfere with the system in an unspecific manner.

The main difference of the yeast system compared to the other cell-based systems described above is the unique positive growth readout, which prevents the selection of toxic compounds as false positives. With the other systems, inhibition of BACE1 is associated with a decrease of readout signal, which requires monitoring of cell viability in order to exclude toxic false positives. The other advantage of the yeast growth readout described here is provided by the simplicity of the technique with minimal liquid handling steps and relatively low costs. Once the yeast suspension has been added to wells containing the test substances, the automated reader simply measures the cell density after defined time intervals.

The potential of this new technology is demonstrated by a pilot screening experiment with a chemical compound library of 15 000 small molecules that revealed a number of compounds that significantly reduced Aβ secretion from HEK 293 APP(Sw) cells.

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3. Results

Part 3.1.

Yeast growth selection system for the identification of cell-active inhibitors of β-secretase

Part 3.2.

Identification of potential small molecule inhibitors of the β-secretase

Part 3.3.

Selection of ankyrin repeat protein-based inhibitors of β-site cleavage

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Yeast growth selection system for the identification of cell-active inhibitors of β-secretase

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Abstract

Aβ peptides, which are believed to be at the origin of Alzheimer's disease (AD), are produced through the sequential processing of the transmembrane amyloid precursor protein (APP) by the β- and γ-secretase. The identification of small molecules that penetrate the brain and inhibit these secretases is of great therapeutic potential. Here, we describe a cellular selection system in yeast for the identification of inhibitors of the human β-secretase BACE-1. Similar to the natural situation in mammalian cells, BACE-1 and its substrate APP are bound to membranes in secretory pathway compartments. Yeast cells expressing these human proteins have been engineered so as to grow under selective conditions only upon reduction of BACE-1 activity, thus allowing identification of compounds that, in addition to inhibiting BACE-1, must permeate cellular membranes and present no cytotoxic effects. Our results show that gradual reduction of BACE-1 expression in the engineered yeast strain resulted in gradual increase of cell growth rate. Moreover, two validated BACE-1 inhibitors, which have IC_{50} values between 7 and 8 μM in mammalian cell assays, stimulated yeast growth in a concentration-dependent manner. This effect was specific for BACE-1 since these compounds had no effect on yeast cells expressing a different secretase cleaving the APP substrate at the α-site. The target-specific cellular assay presented here is applicable in high-throughput screens for selecting inhibitors of defined secretases acting on natural substrates in a membrane-bound protein configuration.

Keywords: Alzheimer; β-Secretase; BACE; β-Secretase inhibitor; Amyloid precursor protein; APP; Cell-based assay

1. Introduction

Alzheimer's disease (AD) is the most common cause of senile dementia. The prevalence of AD within the human population rises considerably with age [1]. By the age of 65 years, about 3% of the human population is affected, whereas by the age of 85 years the prevalence reaches almost 50% [2]. A characteristic of the disease is the presence of extracellular senile plaques, the major component of which is the β-amyloid peptide (Aβ). The hydrophobic, 39–43-amino-acid-long Aβ peptide is excised from the amyloid precursor protein (APP) by sequential cleavage by the so-called β- and γ-secretase. An alternative cleavage can occur at the α-site that precludes subsequent β-site cleavage and, upon proteolysis at the γ-site, gives rise to the shorter, nonpathogenic p3 peptide [3].

Known genetic predispositions for AD mostly affect genes involved in Aβ generation or Aβ deposition. Mutations that lead to increased Aβ generation are found in the APP as well as in the γ-secretase subunits presenilin 1 (PS1) and presenilin 2 (PS2). All disease-linked mutations of APP are clustered around the three cleavage sites (for review, see Ref. [4]). Transgenic mice expressing mutant human APP develop many of the neuropathological hallmarks of AD in an age-dependent manner [5,6]. Since the Aβ peptide seems to play an important role in the pathogenesis of AD, current therapeutic strategies often focus on inhibition of Aβ deposition and generation [7]. Inhibition of β-secretase activity represents an attractive option to achieve this goal. The identification of the aspartic protease BACE-1 as β-

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secretase [8–11] allowed the generation of gene knockout mice [12–14]. Analysis of these mutated mice revealed no detectable endogenous Aβ production, demonstrating that BACE-1 is the major β-secretase. Furthermore, these BACE-1 knockout mice showed no additional phenotype, thus supporting the notion that β-secretase is potentially an ideal therapeutic target for treating AD.

Despite major efforts to identify novel β-secretase inhibitors by applying in vitro high-throughput screening (HTS) assays with purified soluble BACE-1 fragments and fluorogenic peptide substrates [15], the best progress towards efficient BACE-1 inhibition has been achieved so far by the use of peptidic transition-state mimetic compounds [10,16–18]. These molecules are in general highly specific and potent inhibitors of BACE-1 proteolytic activity in biochemical, in vitro assays. However, for efficient inhibition of β-secretase in cells, their molecular weight must be reduced and their structure modified so as to allow for permeation of cellular membranes, the blood–brain barrier and for activity in the natural cellular environment [19]. Some of these crucial issues regarding biological properties of specific inhibitors could be addressed already during the early phase of the drug discovery process by the use of cell-based assays instead of in vitro assays for the HTS. In addition, there is a need to establish novel cell-based assays for identifying low molecular weight inhibitors of secretases that can block these membrane-bound enzymes at the natural location within intracellular compartments [20–22]. However, cell-based HTS assays are generally faced with the problem that selection signals, as expected if the function of the defined target is directly affected by specific inhibitors, are often caused by compounds that interfere with cellular processes or pathways that are redundant with that of the target. Indeed, most putative secretase inhibitors identified by mammalian cell-based screening assays for compounds reducing the generation of soluble Aβ did not specifically affect β- or γ-secretase activity. For example, bafilomycin A1 or acidotrophic amines impair the production of Aβ through the increase of the pH in intracellular compartments [23–25]; compounds like phorbol 12,13-dibutyrate or okadaic acid function through protein phosphorylation [26], and compounds like RO-47-1816/001 simply catalyze polymerization of Aβ, thus reducing the percentage of soluble peptide [27].

With mammalian cell systems, the distinction between specific and so-called false-positive signals is especially challenging due to the high complexity of the redundant pathways and the laborious genetic manipulation of mammalian cells. Nonspecific or indirect effects of compounds can be much more readily controlled by functionally reconstituting target activity in yeast, which provides a heterologous, yet eukaryotic cellular environment. One major advantage of yeast systems over mammalian cells is given by the versatile genetic malleability of this organism, which has made it the model of choice for pioneering research in molecular and cell biology over the last three decades [28]. Moreover, in contrast to mammalian cells, in which pathway redundancies can often obscure putative specific effects of compounds on a given target, a heterologous yeast system permits a clean readout in a null-background environment for the expression of many human proteins and the action of inhibitors [29]. Notably, reconstitution of the multimeric γ-secretase complex has been achieved recently in yeast [30]. The use of yeast for cell-based HTS should allow not only for identification of compounds that can permeate cellular membranes and be active in an intracellular environment but also for rapid exclusion of molecules that are not specific for the chosen target.

We recently published a yeast growth selection system for detection of secretase activity that cleaves APP in a cellular context [31]. Yeast cells do not contain endogenous β- and γ-secretase [32]. In this system, a fragment of APP bearing the β- and the α-site, the transmembrane domain with the γ-site, and the cytosolic tail was fused to the C-terminus of the yeast enzyme invertase (Suc2p), which is normally secreted to enable cell growth in the presence of sucrose as the sole carbon source. Expression of such invertase–APP fusion protein as a type-I transmembrane protein in intracellular compartments (ER and Golgi) of yeast cells lacking endogenous invertase (*suc2Δ*) did not allow growth on sucrose because the invertase moiety was retained within these compartments. However, co-expression of an active version of the human β-secretase BACE-1 or of the endogenous α-secretase Yps1p restored cell growth on selective sucrose plates upon specific cleavage of the invertase–APP fusion protein and subsequent liberation and secretion of the invertase moiety [31].

We have exploited the system described above as the basis for developing a cellular, target-specific HTS system for identifying secretase inhibitors that are active in eukaryotic cells. In order to establish a positive selection assay for inhibitory compounds, and thus automatically eliminate general cytotoxic compounds, we have reversed the original cellular readout to confer on yeast cells the ability to grow only upon inhibition of BACE-1. We show that the system is sensitive towards various levels of inhibition of BACE-1 and we provide specificity control for rapid exclusion of false-positive compounds.

2. Materials and methods

2.1. Recombinant plasmids

All BACE-1 constructs are carried by ARS1-CEN4 plasmids bearing the URA3 auxotrophic marker and are derived from the previously described $BACE_{APP}$ plasmid [31]. For constructs expressed from the *ADH1* full-length promoter and the *ADH1* truncated promoter (deletion upstream of the natural *Sph*I site), the $BACE_{APP}$ *Xba*I/*Sal*I

fragment from the *ACT1* version [31] was subcloned on plasmids pMH26 and pMH28, respectively. For the BACE-1 version expressed from the $tetO_7$ promoter, a *Bam*HI site was introduced into the *Xba*I site, and an *Hpa*I site was introduced into the *Sal*I site of $BACE_{APP}$. The resulting $BACE_{APP}$ fragment was isolated by *Hpa*I/*Bam*HI digestion and cloned into pCM189 [33]. Plasmid vectors for the expression of the invertase–APP fusion protein Suc2-$pAPP^{Sw}$ (referred to as Suc2$pAPP_{KKXX}^{Sw}$ by Lüthi et al. [31]), which bears the so-called Swedish mutation at the β-site, and of Yps1p have been described [31]. The integrative reporter gene plasmid expresses a Gal10p(aa1–47)–His3p fusion under the control of the *GAL10* promoter and the LacI–LacZ fusion under the control of the divergently oriented *GAL1* promoter.

2.2. Yeast media and transformation

All media were prepared according to Burke et al. [34]. Transformation of yeast cells was performed following the lithium acetate method [35].

2.3. Yeast strains

The parental strain of Biscre1 is derived from JPY9 and has been described by Lüthi et al. [31] (MATα *ura3-52 his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385 gal4Δ11 suc2::URA3 yps1::*kanMX4 *mkc7::HIS3*). For constructing Biscre1 (MATα *ura3-52 his3Δ200::lacZ-HIS3 leu2Δ1 trp1Δ63 lys2Δ385 suc2Δ yps1::*kanMX4 *mkc7::LYS2*), uracil-auxotrophy was reconstituted by cultivating the parental strain on 5-fluoro orotic acid (5-FOA). *GAL4* was reintroduced by transformation of a PCR-fragment bearing this gene and subsequent selection on 2% galactose plates (YPAG). *HIS3* was substituted by *LYS2* with the use of homologues recombination. Finally, the *lacZ-HIS3* reporter gene plasmid was integrated at the *his3Δ200* locus. The strain Biscre11 is a derivative of Biscre1 carrying the deletion of the three major ABC transporter genes, *pdr5Δ*, *snq2Δ*, *yor1Δ*.

2.4. Western blot analysis

Whole cell extracts from transformed yeast were prepared as described by Burke et al. [34]. Proteins were separated by SDS PAGE and Western blot analysis was performed according to standard procedures. A mouse monoclonal antibody from Sigma (A8717) that recognizes residues 676–695 of human APP695 was used at a final concentration of 25 ng/ml to detect expression of $BACE_{APP}$ and the CTFβ fragment.

2.5. β-Galactosidase assay

Cultures of transformed cells (2 ml) were grown overnight in 2% glucose −trp −ura medium at 30 °C. These cells were collected by centrifugation, washed with 5-ml H_2O and resuspended in 2-ml assay medium (5% sucrose 2% galactose −trp −ura). Aliquots of these processed pre-cultures were used to inoculate further 2-ml assay medium to obtain a starting OD_{600nm} of 0.25. These assay cultures were grown at 30 °C to $OD_{600nm} \cong 1$. One hundred microliters of each sample was subjected to β-galactosidase assays using the method as described by Burke et al. [34]. Activity was normalized to the number of cells assayed. The experiments were performed in triplicates.

2.6. Growth assays

Growth assays were performed either in conventional test tubes with manual handling and cell density measurement at OD_{600nm}, or in microtiter plates for automated handling and cell density measurement at OD_{595nm} by a microtiter plate reader. For growth assays in conventional test tubes, 2-ml pre-cultures were inoculated with transformed cells and grown overnight in 2% glucose −trp −ura medium at 30 °C. After about 14-h incubation, they were diluted with 2% glucose −trp −ura medium to obtain a cell density corresponding to $OD_{600nm} = 0.25$ and further incubated at 30 °C to reach $OD_{600nm} \cong 1$. These exponentially growing cultures were washed with 5-ml H_2O and resuspended in 2-ml 5% sucrose 2% galactose −his −trp −ura medium (=growth selection medium). At time zero of the growth selection experiment, 2-ml cultures of growth selection medium were inoculated with the processed pre-cultures to reach a starting OD_{600nm} of 0.05. At the time points indicated on the relevant figures, OD_{600nm} was determined in a Shimadzu UV mini-1240 spectrometer. For the OD measurement, samples were diluted 1:10 in plastic cuvettes (l=1 cm). For the experiment with the doxycycline-repressible BACE-1 expression construct, doxycycline (Sigma D9891) was added as water solution to the growth selection medium at time zero.

For growth assays in microtiter plates, the experimental conditions were adjusted in order to optimise them for automated handling and cell density measurement in a microtiter plate reader. The differences from the growth assays described above are the following: The overnight pre-cultures as well as subsequent dilutions were performed with the same drop-out medium containing 2% galactose instead of 2% glucose. The assay cultures had a volume of 150 μl and were incubated in 96-well microtiter plates (Greiner 655101) without shaking at 30 °C in a 100% humidity incubator. The BACE-1 expression construct for these experiments was BACE-ACT1. The BACE-1-specific inhibitors 1.2. and 4.1. (Novartis Pharma), as well as the control compound MG132 (Sigma C2211), were dissolved in DMSO and added to the assay cultures at time zero of the growth assays; the final DMSO concentration was always kept at 1%. Prior to light scattering measurement at 595 nm in a Tecan Genios reader for determining cell density without diluting the cultures, the microtiter plates were shaken on a plate shaker for 2 min at 1100 rpm.

3. Results

3.1. Principle of the growth selection system for the identification of β-secretase inhibitors

In order to establish a cell-based HTS system for the positive selection of β-secretase inhibitors, we modified our previously published system for detection of BACE-1 activity in yeast [31] so as to confer on yeast cells the ability to grow only upon inhibition or reduction of BACE-1 activity. The readout of the original system, in which BACE-1 activity correlated with cell growth, is indeed not suitable for a BACE-1 inhibitor screening system, since toxic compounds, which are generally not rare in chemical libraries, would significantly increase the number of false-positive hits. The new readout exploits the natural regulatory network that controls activation and repression of the divergent *GAL1–10* promoters by galactose and glucose, respectively. In a typical experiment, addition of galactose to yeast cultures grown on glycerol, a neutral carbon source with respect to *GAL1–10* promoter regulation, induces expression of the *GAL* genes at least 1000-fold. If glucose is added to the galactose-containing media, expression of the *GAL* genes is repressed to a level below 1% of that obtained with galactose alone. This phenomenon is known as glucose repression [36]. For the BACE-1 inhibitor screening system, a reporter construct was cloned which carries the *lacZ* gene under the control of the *GAL1* promoter and the divergently oriented *HIS3* gene under the control of the *GAL10* promoter. This reporter construct was integrated in the yeast genome to generate the strain Biscre1 (BACE inhibitor screen 1) (see Materials and methods). Since the natural regulation mode of the *GAL1–10* promoters is preserved in the context of the new reporter construct, the *lacZ* and the *HIS3* genes of the strain Biscre1 are fully induced in the presence of galactose and dominantly repressed upon addition of glucose to the medium. The product of the *HIS3* gene is needed for growth on histidine-depleted (− his) medium. Consequently, Biscre1 can only grow on − his media that contain galactose but no glucose. The *HIS3* reporter gene of the strain Biscre1, which lacks the endogenous invertase activity (*suc2Δ*), is also induced when these cells grow in the presence of sucrose together with galactose (Fig. 1A). However, if Biscre1 is allowed to secrete the invertase enzyme, glucose is generated by the hydrolysis of sucrose. The newly generated glucose dominantly represses the *HIS3* gene expressed from the *GAL10* promoter and growth on − his medium is inhibited (Fig. 1B).

The properties of Biscre1 combined with the BACE-1-dependent release of invertase from the invertase–APP fusion protein (Suc2pAPP^{Sw}) provide a useful tool to select for reduced or inhibited BACE-1 activity. Indeed, in the presence of active BACE-1, the fusion protein Suc2pAPP^{Sw} undergoes cleavage at the β-site; as a consequence, the invertase moiety is liberated and secreted into the periplasm,

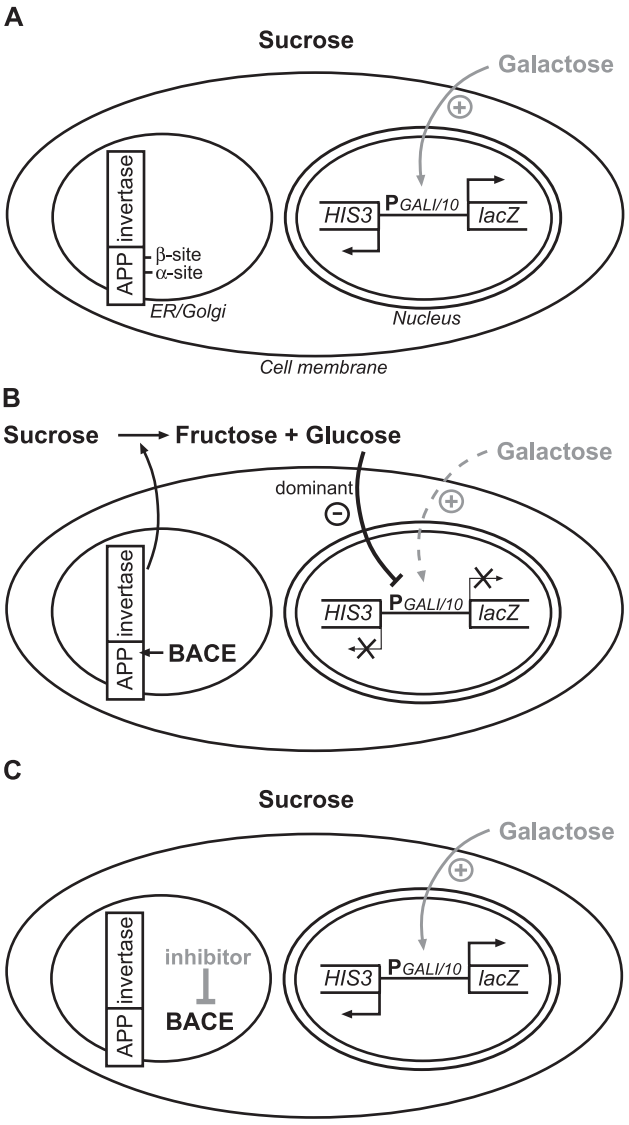

Fig. 1. Principle of the system to select BACE-1 inhibitors. Each drawing depicts a schematic yeast cell that is grown in medium containing sucrose and galactose. Two cell organelles are indicated: The nucleus containing the reporter construct with the *HIS3* gene and the *lacZ* gene under the control of the bidirectional *GAL1–10* promoter, and the ER/Golgi compartments harbouring the membrane-anchored invertase–APP fusion protein. (A) State of the system in the absence of an active secretase: The invertase moiety is retained in the ER/Golgi compartments, sucrose remains inert to the system, and galactose induces transcriptional activation of the reporter genes. (B) An active BACE-1 enzyme form expressed in the yeast cell cleaves invertase–APP at the β-site of APP; the invertase moiety is liberated and secreted to the periplasmic space of the cell, where it hydrolyses sucrose to generate fructose and glucose. The freshly generated glucose dominantly represses transcription of the reporter genes. (C) Addition of a BACE-1 inhibitor restores induction of reporter gene expression by galactose, thus allowing cell proliferation under selective conditions.

where it hydrolyses sucrose into fructose and glucose, the latter of which represses the *HIS3* and the *lacZ* reporter genes (Fig. 1B). If BACE-1 is inhibited, either by mutations or by an inhibiting compound, the invertase moiety remains anchored to intracellular membranes via the APP domain,

sucrose in the medium is no longer hydrolyzed, and expression of the *HIS3* and the *lacZ* gene is induced by galactose (Fig. 1C).

3.2. Expression of active secretases causes transcriptional repression

In the system outlined above, expression of Suc2-pAPP^{Sw} alone in Biscre1 cells cultivated in the presence of sucrose and galactose should allow transcription of the *lacZ* and *HIS3* reporter genes. Conversely, co-expression of an active and APP-specific secretase together with Suc2pAPP^{Sw} should cause transcriptional repression of the Biscre1 reporter genes under these conditions. The GPI-anchored aspartic proteases Yps1p and Mkc7p of *Saccharomyces cerevisiae* have been shown to cleave APP at the α-site [32]. Since the α-site is also present and accessible in Suc2pAPP^{Sw} [31], the endogenous genes encoding these two yeast α-secretases were deleted in Biscre1 (*yps1Δ mkc7Δ*). For proof-of-concept experiments we engaged Yps1p and the BACE-1 construct that has been shown to be active and specific towards the APP^{Sw} sequence in yeast [31].

Fig. 2 shows that expression of active secretases together with Suc2pAPP^{Sw} in Biscre1 caused repression of the reporter genes in the presence of 5% sucrose and 2% galactose in the liquid medium. Transcription of the *lacZ* gene was indirectly measured by the β-galactosidase assay. Co-expression of Suc2pAPP^{Sw} with either Yps1p or active BACE-1 caused a significant reduction of relative β-galactosidase activity down to 5.7% and 16% of the activity measured with cells expressing Suc2pAPP^{Sw} alone, respectively (Fig. 2A). Co-expression of Suc2-pAPP^{Sw} with a mutated form of Yps1p lacking its pro-domain sequence (Fig. 2A, column 3) or with a BACE-1 mutant carrying an amino acid substitution as its active site (D93N) (Fig. 2A, column 5) resulted in full activation of the *lacZ* reporter gene.

The same transformed Biscre1 cells that were used for the β-galactosidase assay shown in Fig. 2A were examined for growth in histidine-depleted liquid medium containing 5% sucrose 2% galactose (growth selection medium). Cultures of differentially transformed Biscre1 cells carrying the five pairs of vector plasmids were inoculated in quadruplets with equal amounts of exponentially growing cells from nonselective medium. Cell density was determined after 26-h incubation by measuring light scattering at 600 nm (Fig. 2B). Since the results of these growth selection experiments correlate very closely with the results of the β-galactosidase assay presented above (compare Fig. 2A with 2B), we conclude that the observed growth differences reflect different expression levels of the *HIS3* reporter gene. Taken together, the results of both experiments fulfil the prediction that the presence of active secretases causes repression of the reporter genes and subsequent growth inhibition of the Biscre1 strain under

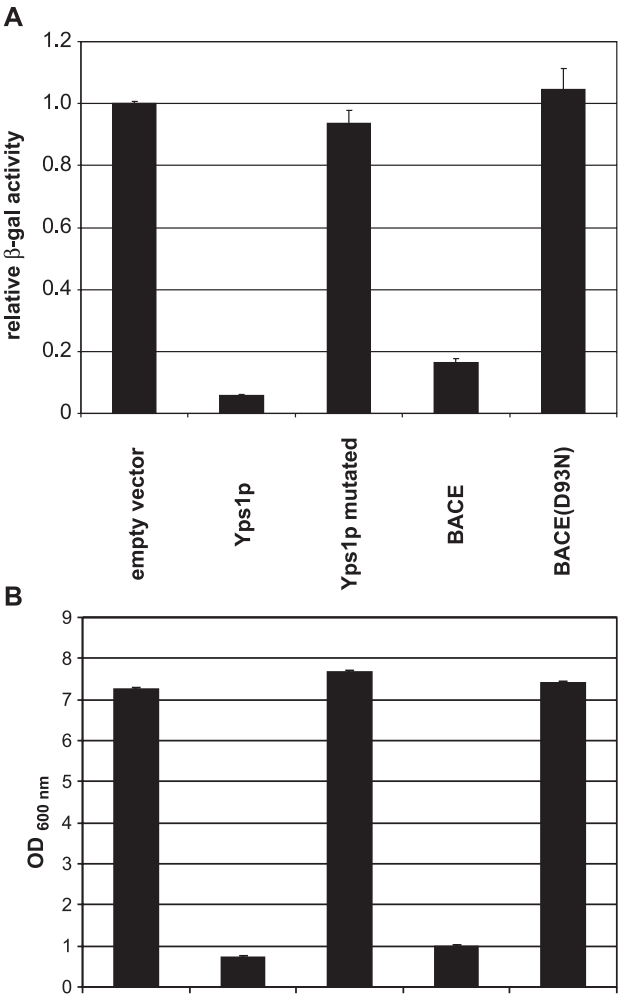

Fig. 2. APP-cleaving secretases cause transcriptional repression of the reporter genes. (A) β-Galactosidase assay with Biscre1 cells co-transformed with Suc2pAPP^{Sw} (invertase–APP) and an empty vector (column 1), Yps1p (column 2), mutated Yps1p (column 3), BACE-1 (column 4) or mutated BACE-1(D93N) (column 5). Each culture was grown in triplicates in medium containing 5% sucrose and 2% galactose before performing β-galactosidase assays. The β-galactosidase activity resulting from cultures harbouring an empty vector (column 1) was arbitrarily set to 1. (B) Growth assays with aliquots of the same transformed cells described above. Equal amounts of exponentially growing cultures were used to inoculate 2 ml of −his 5% sucrose 2% galactose medium. Cell density was determined after 26 h by measuring OD_{600nm}. The assay was performed in quadruplets.

selective conditions, while inactivation of the secretases allows induction of reporter gene expression and cell proliferation.

3.3. Discrete reduction of BACE-1 expression level results in discrete increase of cell growth rate

For the proof-of-concept experiments described above, BACE-1 had been expressed from the strong *ACT1* promoter (BACE-ACT1). In order to examine the sensitivity of the system towards variations of active BACE-1 concentrations within the cell, two additional BACE-1 expression vectors were constructed by substituting the *ACT1*

promoter with increasingly weaker promoters, namely the full-length *ADH1* promoter (BACE-ADH1) and a truncated version of this *ADH1* promoter (BACE-ADH1tr). The relative strength of these promoters had been previously tested by comparing β-galactosidase activity from cells expressing the *lacZ* gene under the control of one of the three promoters. In these experiments, the full *ADH* promoter gave an activity that was about 40% compared to that obtained with the *ACT1* promoter, whereas the β-galactosidase activity from the truncated *ADH* sequence barely reached 10% of that from the *ACT1* promoter (data not shown). The three different BACE-1 expression vectors were introduced into the Biscre1 strain expressing the invertase–APP fusion protein Suc2pAPP^{Sw}, and their potentially different impact on cell growth under selective conditions was tested as described above and in Materials and methods. Fig. 3A shows that cultures of Biscre1 cells harbouring the BACE-ACT1 construct grew to a maximum of about 12% of the cell densities reached by Biscre1 cells expressing either the inactive BACE-1 mutant (BACE(D93N)-ACT1) or no BACE-1 protein (empty vector). The BACE-ADH1 cultures reached about 50% of this level, whereas the cultures expressing BACE-1 from the *ADH1* truncated promoter grew to about 90% of the maximum level.

Expression levels of BACE-1 from the three different promoters were analysed by Western blotting assay (Fig. 3B). The strongest signals on the immunoblot film were observed as expected in the case of BACE-1 expression from the strong *ACT1* promoter, which also controls expression of the inactive BACE(D93N) mutant (Fig. 3B). BACE-1 expressed from the full-length *ADH1* promoter yielded a less intense signal, whereas expression from the truncated *ADH1* promoter was below detection limits in this experiment (Fig. 3B). We then examined whether different intracellular concentrations of active BACE-1 correlated with differences in the efficiency of cleavage of the Suc2pAPP^{Sw} substrate, as we would expect from the results of the growth assays shown in Fig. 3A. Aliquots of the cell extracts were analysed by Western blotting using a monoclonal antibody directed against the C-terminus of APP, which allows detection of the APP cleavage product named CTFβ. Fig. 3C shows that the intensity of the CTFβ signal on the immunoblot film decreased in correlation with the reduced expression level of active BACE-1 shown in Fig. 3B. The reduced levels of the APP cleavage product CTFβ were inversely proportional to the levels of cell growth shown in Fig. 3A. As internal control for the Western blotting assays, we used detection of the yeast endogenous protein calmodulin (Cmd) by a specific antibody (Fig. 3C).

These results taken together show that intracellular concentration of active BACE-1 can be limiting for cleavage of APP in our cell growth selection system, and it may be equated with β-secretase activity. Thus, this system is expected to allow detection of gradual reduction of β-secretase activity by potential BACE-1 inhibitors.

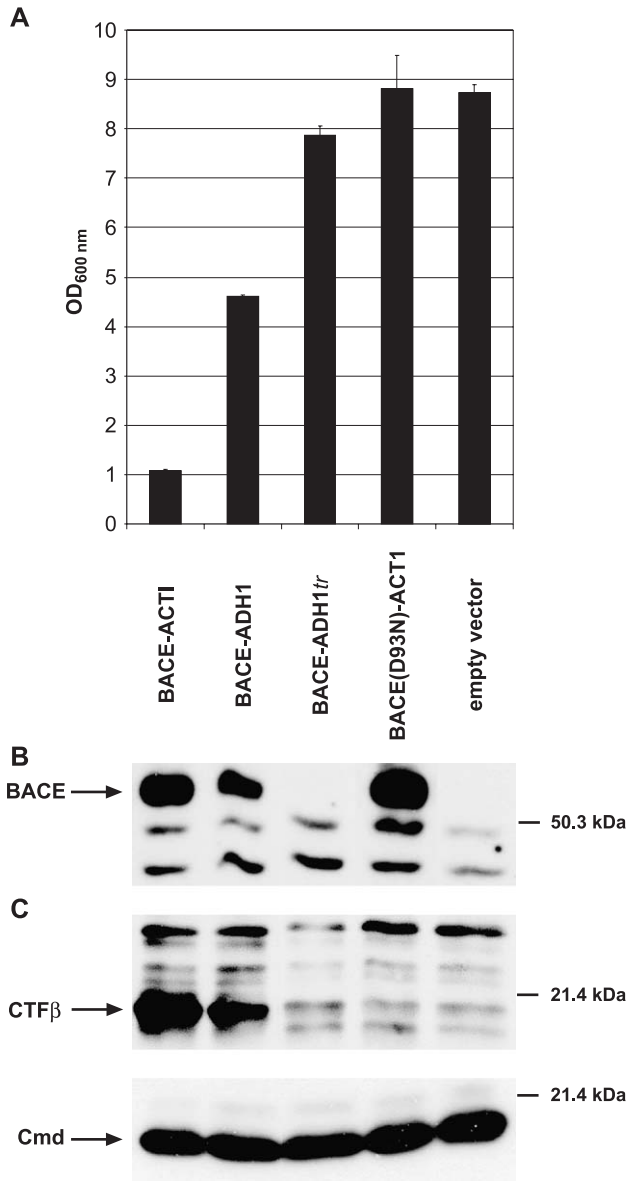

Fig. 3. The growth readout is sensitive towards variations of intracellular β-secretase levels. (A) Growth assay with Biscre1 cells co-transformed with Suc2pAPP^{Sw} and with BACE-1 expressed from different promoters (see text for detailed information). Equal amounts of exponentially growing cultures were used to inoculate 2 ml of −his 5% sucrose 2% galactose medium. Cell densities were monitored after 37 h by measuring OD_{600nm}. The assays were performed in triplicates. (B) Different intracellular concentrations of BACE-1 were detected by Western blotting. The signal corresponding to BACE-1 expressed in yeast cells is indicated by an arrow. (C) Western blotting analysis of the C-terminal cleavage fragments of Suc2pAPP^{Sw} (CTFβ) produced upon cleavage by various BACE-1 concentrations (upper panel). The CTFβ band partly overlaps with a much weaker background band that is also visible in the absence of BACE-1 (empty vector lane). The lower panel shows the results of a Western blotting analysis of the same whole cell extracts using an antibody directed against calmodulin (Cmd) as control for general protein concentration.

3.4. Modulation of BACE-1 expression by variable doses of doxycycline correlates with distinct changes in cell growth rate

In the experiments described above, BACE-1 was expressed at different levels from constitutive promoters of different strengths. From the results of these experiments, one can conclude that the described growth selection system is sensitive towards differences in total BACE-1 activity within a cell. Nevertheless, because of the constitutive nature of BACE-1 expression at various levels, these results do not indicate how the system would perform in a screening for BACE-1 inhibitors, in which BACE-1 activity is relatively high at the onset of the experiment (in the overnight pre-culture) and is then reduced to various extents upon addition of potential BACE-1 inhibitors to the yeast cell culture. To address this question, and to test conditions that would mimic more closely a screening experiment, the BACE-1 gene construct described above was put under the control of the doxycycline-repressible promoter *tetO*$_7$ [33], which allows modulation of gene expression in yeast by the addition of different concentrations of doxycycline to the cell culture.

Biscre1 cells co-transformed with BACE-tetO$_7$ and Suc2pAPP^{Sw} were subjected to growth selection assays as described above with the only difference that various concentrations of doxycycline were added to the yeast cell culture at time zero (onset of the growth assay). According to published results [33], the *tetO*$_7$ promoter is fully active in the absence of doxycycline and completely silenced in the presence of 1 μg/ml doxycycline. In addition to this concentration, we used 0.1 and 0.05 μg/ml doxycycline in order to obtain different levels of partial repression of the promoter. Fig. 4 shows that growth rate and maximal level of cell density increased along with increasing doxycycline concentrations. The maximal OD_{600nm} value measured in this experiment is very close to the OD_{600nm} values that were obtained in the presence of inactivated BACE(D93N)-ACT1 and in the absence of any BACE-1 derivative (see Fig. 3A). In the absence of doxycycline, yeast cultures grew to a level that is in the range of that reached upon constitutive expression of BACE-1 from the *ADH1* promoter, thus indicating that *tetO*$_7$ and *ADH1* most likely show similar strength.

These results show that the growth selection system can discriminate variations of active β-secretase levels caused by a small compound in a concentration-dependent manner.

3.5. Two validated BACE-1 inhibitors specifically stimulate growth in a screening assay format

The cellular system described in this paper was designed with the aim of applying it in screening experiments to identify specific inhibitors of β-secretase activity. Thus, in addition to testing the system for selectivity towards modulation of intracellular expression levels of BACE-1 as

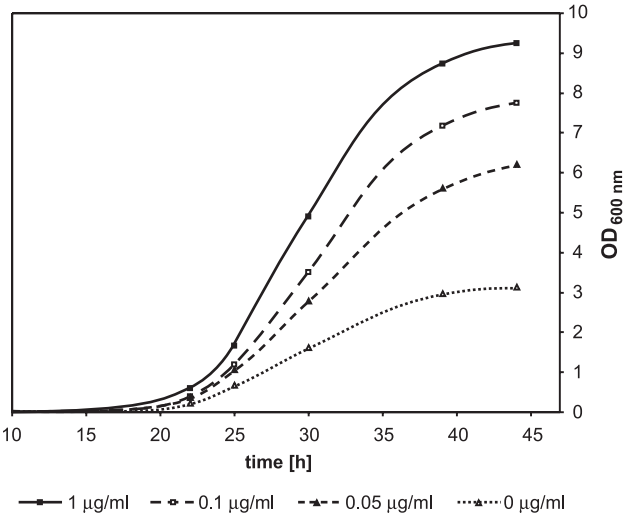

Fig. 4. The growth readout detects modulation of BACE-1 expression by addition of doxycycline to yeast cells. Biscre1 cells were co-transformed with Suc2pAPP^{Sw} and with BACE-1 expressed from the doxycycline-repressible *tetO*$_7$ promoter. The cultures were grown in −his 5% sucrose 2% galactose medium with variable concentrations of doxycycline (as indicated) at time zero. Cell growth rate was monitored by measuring OD_{600nm} at the indicated time points. Each point represents the average measurement of three different cultures.

described above, we wanted to test whether this system could also be applied for detecting small molecules that directly inhibit BACE-1 activity by binding to this enzyme. To this purpose, we challenged the growth selection system with the validated BACE-1-specific inhibitors 1.2. and 4.1. of Novartis Pharma. The in vitro IC_{50} and the cell culture IC_{50} values of compound 1.2. are 16 nM and 8 μM, respectively. The corresponding values for compound 4.1. are 140 nM and 7 μM, respectively. A more detailed characterisation of these compounds will be published elsewhere (U.N. and P.P., manuscript in preparation).

For these experiments, we used the yeast strain Biscre11, a derivative of Biscre1 in which the genes for the three major ATP-binding cassette (ABC) transporters Snq2p, Pdr5p and Yor1p have been deleted. These ABC transporters, also known as efflux pumps, are important components of the yeast pleiotropic drug resistance network, which counteracts accumulation of unwanted or potentially toxic compounds within the yeast cell [37]. For efficiently screening small molecule libraries in yeast with manageable compound concentrations, it is much preferable to use strains lacking the major ABC transporters [38]. Biscre11 cells co-expressing either BACE-1 and Suc2pAPP^{Sw} or Yps1p and Suc2pAPP^{Sw} were incubated with various concentrations of the BACE-1 inhibitors 1.2. and 4.1. in microtiter plates and cultivated under selective conditions for 24 h as described in Materials and methods. Fig. 5A shows that increasing concentrations of the BACE-1 inhibitors 1.2. and 4.1. correlated with increasing growth of the BACE-1-expressing Biscre11 cultures. At the highest tested concentration, growth stimulation was about 30% for compound

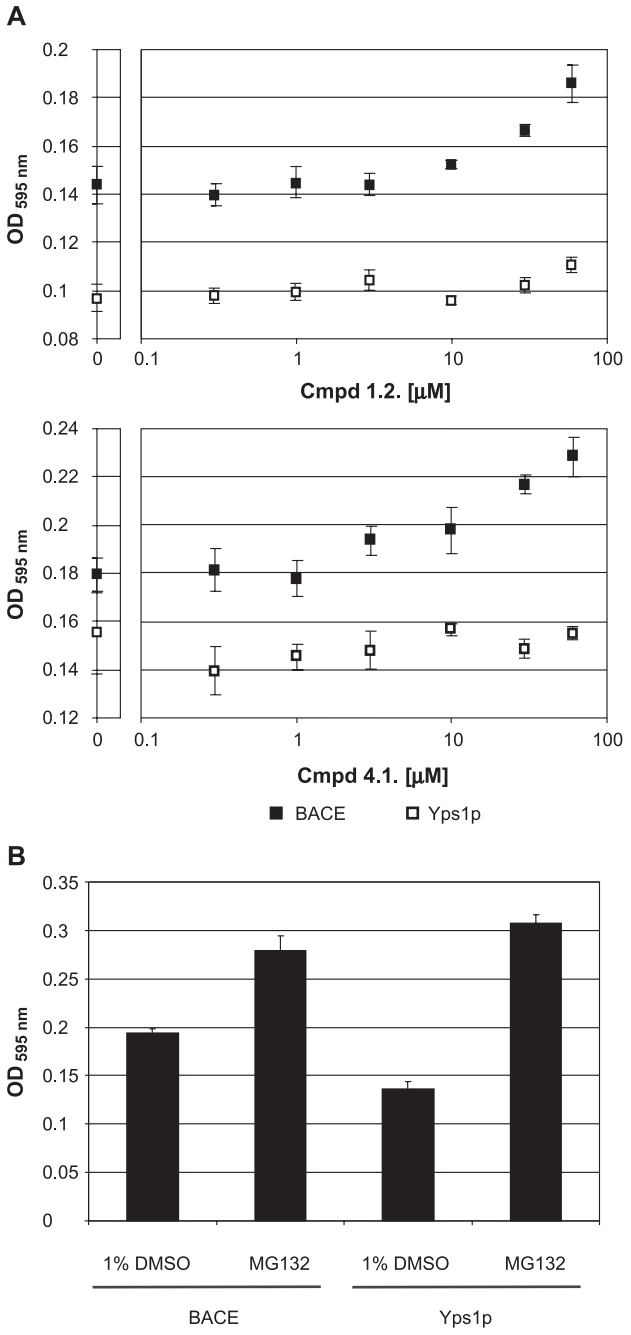

Fig. 5. The validated BACE-1 inhibitors 1.2. and 4.1. stimulate growth in a specific manner under selective conditions. (A) Increasing concentrations of compound 1.2. (upper panel) and 4.1. (lower panel) increasingly stimulate cell growth. 150 μl cultures of Biscre11 cells expressing Suc2pAPP^{Sw} and BACE-1, or Suc2pAPP^{Sw} and Yps1p, were grown either in the absence (0) or in the presence of increasing concentrations of compounds 1.2. and 4.1. (indicated in micromolar). The histidine-lacking medium contained 1% DMSO 5% sucrose 2% galactose. The experiment was performed in quadruplets. Growth was monitored with a microtiter plate reader at OD_{595nm} after 24 h. (B) Effect of 10 μM MG132 on growth of Biscre11 cells expressing Suc2pAPP^{Sw} and BACE-1, or Suc2pAPP^{Sw} and Yps1p. The applied growth conditions were identical as described above.

1.2. and 27% for compound 4.1. In contrast to the growth rescue effect in the presence of BACE-1, the growth of cultures expressing the yeast α-secretase Yps1p could not be rescued by neither of the two compounds at the tested concentrations. The slight differences in cell densities between BACE-1- and Yps1p-expressing cultures observed in the absence of these inhibitors are consistent with the results of previous experiments under different conditions (see, e.g. Fig. 2), and are most likely the consequence of differences in substrate cleavage efficiency by the two secretases and subsequent invertase secretion [31]. These results show that the BACE-1 inhibitors 1.2. and 4.1. are able to rescue growth of engineered Biscre11 cells in a concentration-dependent manner that is specific for cells expressing BACE-1.

To further control our growth selection system and to establish optimal conditions for application in screening assays, we tested the proteasome inhibitor MG132, which causes reduction of Aβ secretion from mammalian cells by preventing β-secretase cleavage in an indirect manner, most likely by impeding progression of APP through the secretory pathway [39]. Addition of 10 μM MG132 to Biscre11 cultures co-expressing either BACE-1 and Suc2pAPP^{Sw} or Yps1p and Suc2pAPP^{Sw} resulted in both cases in significant growth stimulation when compared to control cultures incubated with DMSO alone (Fig. 5B). Since the same effect was observed in the absence of BACE-1 when natural invertase (Suc2p) was expressed instead of Suc2pAPP^{Sw} (data not shown), we conclude that, similarly to what is believed to happen in mammalian cells, MG132 probably acts by generally inhibiting progression of proteins through the secretory pathway. These results, taken together, emphasize the importance of having a specificity control for compounds identified by cellular screenings, a condition that can be readily and inexpensively achieved with the genetically malleable yeast cells.

In summary, the cellular growth selection system presented here allows detection of the effect of the validated BACE-1 inhibitors 1.2. and 4.1. in an efficient manner. Moreover, components of the system can be readily changed for establishing important specificity controls for the assay, as it has been shown with the use of the Yps1p secretase and the nonspecific compound MG132. In a screening for novel inhibitors of BACE-1, compounds that could block the residual endogenous α-secretase activities in *yps1Δ mkc7Δ* strains (which have been reported to be responsible for about 14% APP cleavage [32]) might also cause growth stimulation in the presence of BACE-1. If such compounds would not inhibit the control α-secretase Yps1p, and if they would show their effect only in the presence of BACE-1 but not when Yps1p is expressed, they could appear as BACE-1-specific inhibitors in our system. Since characterization of so-called primary hits selected by a compound library screen would in any case require further experiments like test of activity in mammalian cells and protein binding assays, such expectedly low number of yeast residual secretase inhibitors

would be discarded as false-positive at this stage of the drug discovery process.

4. Discussion

The present work describes a readily applicable cellular screening system for the identification of in vivo β-secretase inhibitors. The system, which is based on positive growth selection, combines the previously described BACE-1-dependent release of the yeast enzyme invertase [31] with the properties of the newly engineered strain named Biscre1. Biscre1 harbours the reporter genes *lacZ* and *HIS3* under the control of the *GAL1–10* promoter, which is repressed in the presence of active BACE-1 (see Fig. 1). Reduction of BACE-1 expression or inhibition of its activity by a specific inhibitor relieves the reporter genes from repression and allows transcriptional activation. Reporter gene expression endows these cells with readily detectable properties such as the ability to grow under selective conditions and to be sorted by colorimetric assays, thus providing a means for a cell-based HTS of compound libraries.

We have performed proof-of-concept experiments to show that changes in the expression level of BACE-1 inversely correlate with changes in the growth rate of the engineered yeast cells. Results supporting such correlation have been reproducibly obtained both in the case of constitutive expression of different levels of BACE-1 and in the case of reduction of BACE-1 expression by the small molecule doxycycline (see Figs. 3 and 4). The latter experimental setup more closely mimics the situation in which a BACE-1 inhibitor added to the yeast culture can be identified because it reduces activity of β-secretase to a given percentage of the originally fully active enzyme population.

Although the results of such BACE-1 expression experiments provided good evidence that the system should allow detection of a BACE-1 inhibitor, the final proof came by testing the effect of the validated BACE-1 inhibitors 1.2. and 4.1. on cell growth stimulation in the selection system. The small molecules 1.2. and 4.1. can indeed stimulate growth of yeast cells expressing BACE-1 in a concentration-dependent manner (see Fig. 5A). It is noteworthy that, although the in vitro IC_{50} values of the compounds 1.2. and 4.1. differ by about a factor of 10 (16 and 140 nM), the levels of growth stimulation exerted by increasing concentrations of these two compounds in our yeast system are comparable between each other. Such similar behaviour in the yeast selection system is in better agreement with the properties of the compounds observed in cell culture experiments, in which these compounds showed similar IC_{50} values for inhibition of BACE-1 in vivo (U.N. and P.P., unpublished results). Importantly, neither compound 1.2. nor compound 4.1. did rescue growth of yeast cultures expressing Yps1p, whereas 10 μM of the peptide aldehyde MG132 rescued both Yps1p and BACE-1-expressing cultures. These results demonstrate that our system is able to detect BACE-1 inhibition and, furthermore, that false-positive compounds such as MG132, which would be detected in a screening assay, can be rapidly excluded by use of a control secretase like Yps1p. The proteasome inhibitor MG132 has been known for a long time to reduce Aβ secretion from mammalian cells [40], but the precise mode of action remains unclear. To our knowledge, MG132 has not been shown to directly inhibit BACE-1 activity towards APP. It has been suggested that MG132 inhibits γ-secretase activity [40]; other results have rather indicated that this effect of MG132 might be due to a direct or indirect impairment of APP progression through the secretory pathway [39]. Our results support the second explanation. Indeed, in addition to the fact that yeast cells do not exhibit any γ-secretase activity, MG132 has an effect on the readout of our invertase secretion system even when plain invertase is expressed in the absence of BACE-1.

Our yeast selection system combines advantages of in vitro assays with those of mammalian cellular systems. Cell-based assays allow early selection of compounds that show membrane permeability and the ability to function in the reactive environment of a living cell. Addressing these issues already in the primary screening might crucially accelerate the drug discovery process and thus lower the costs. Moreover, the activity of the target protein and its substrate, as well as the readout to monitor the effect of compounds, are examined in a cellular context that should recapitulate more closely than in vitro assays the natural physiological state in which the specific compound should ultimately function. In vitro assays to identify BACE-1 inhibitors are usually established with a soluble fragment of this enzyme together with a short peptide substrate. A recent publication described a novel in vitro assay with a membrane-bound form of BACE-1 incubated with the short peptide substrate. The authors of that paper observed no significant difference between their novel assay and the classical one with soluble BACE-1 regarding inhibition by two peptidomimetic competitive inhibitors [41]. However, while these results revealed that membrane anchoring was not relevant for BACE-1 activity (and inhibition) on the short peptide substrate, they did not address the potential importance of presenting the natural β-secretase substrate APP as transmembrane protein. It is plausible that there are small molecule inhibitors of β-secretase that efficiently function only on the cleavage of the natural substrate, for example by causing allosteric changes that interfere with the BACE-APP interaction.

The yeast cell growth selection system described here overcomes one major disadvantage of conventional screening assays with mammalian cells, which is the difficulty to distinguish direct inhibitory effects on the defined target (β-secretase) from other effects on redundant pathways, cell metabolism and cell viability [42]. The versatile genetic malleability of yeast has allowed us to readily address this problem by engineering cells in which inhibition of human

BACE-1 is associated with cell growth, and by including simple control tests such as those with alternative APP-cleaving enzyme (see Fig. 5). These controls allow for rapid exclusion of molecules that are not specific for the chosen target. Thus, similar to in vitro assays, the heterologous yeast system allows for a clean readout for the expression of human BACE-1 and the effects of inhibitors. At the same time, the use of yeast for cell-based HTS also allows selection of compounds that can permeate cellular membranes and that are active in the intracellular environment without causing toxicity.

In summary, we have developed a novel cellular system for the identification of BACE-1 inhibitors, which comprises a convenient method for rapid exclusion of so-called false-positive compounds and allows concomitant selection for cell permeability and against general cytotoxicity. We recently performed a screening experiment with a chemical compound library of 15,000 small molecules. This screening assay delivered 13 compounds that stimulated growth of yeast cells in a BACE-1-dependent manner. Initial characterization of these compounds revealed significant inhibition of BACE-1 activity in vitro by some of the molecules and reduction of Aβ secretion from HEK 293 APP(Sw) cells. Results of these experiments will be published elsewhere after further analysis of the identified compounds and optimised derivatives of them.

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Part 3.2.

Identification of potential small molecule inhibitors of the β-secretase

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3.2. Identification of potential small molecule inhibitors of the β-secretase

3.2.1. Results

Primary screening for BACE1 inhibitors

The "Yeast growth selection system for the identification of cell-active inhibitors of β-secretase" (Middendorp et al., 2004) as described in *part 3.1.* of this thesis was applied for the screening of a chemical compound collection of 15'000 small molecules.

The yeast strain that was used for this screening is a Biscre1 derivative (Biscre11), which was genetically modified with the aim to increase the intracellular concentration of the library compounds during the screening. It is known that intracellular accumulation of small molecules is generally much more limited in yeast cells compared to cultured mammalian cells. This is primarly due to the very efficient yeast pleiotropic drug resistance network, which pumps potentially toxic substances out of the cell, and, to some extent also, due to a different permeability of yeast cell membranes compared to mammalian cell membranes. A crucial difference between mammalian and yeast cell membranes is that cholesterol is replaced by ergosterol. A strain, which is deleted for the *ERG6* gene, the product of which is involved in the ergosterol synthesis, displays an increased rate of passive diffusion through the cell membrane (Emter et al., 2002). Our screening strain Biscre11 carries a wildtype allele for *ERG6* (We were able to delete *ERG6*, but any further attempts to genetically manipulate the strain failed, despite the usage of a special "*erg6Δ* transformation protocol" as described by Gaber et al. (Gaber et al., 1989).), but is deleted for the genes *PDR5*, *SNQ2* and *YOR1*, which encode components of the yeast pleiotropic drug resistance network. A combination of *pdr5Δ*, *snq2Δ* and *yor1Δ* produces a strain, which is sensitive towards a broad spectrum of different compound classes (Kolaczkowski et al., 1998). For the screening we used Biscre11 cells that were pre-transformed with plasmids encoding BACE1 (BACE-ACT1) and the invertase-APP (Suc2pAPP^{Sw}) fusion protein (herein after referred to as BACE strain).

The screening was performed in a 96 well plate format. Beside 80 wells containing different test substances dissolved in dimethyl sulfoxide (DMSO), on each plate there were eight wells containing the negative control (DMSO) and four wells containing the positive control being MG132. Addition of 150 µl of BACE strain suspension, resuspended in histidine-depleted medium with 5% sucrose and 2% galactose (growth selection medium), resulted in a final DMSO concentration of 1% in every well. The final concentration of the test substances was approximately 50 µM; the final concentration of MG132 was 10 µM, which stimulates the growth of BACE cultures by about a factor of two (see *part 3.1.* figure 5c). The variability of the signals for the negative and the positive controls as well as the width of the signal window is taken into account by the so-called Z' factor (see materials and methods for formula), which is calculated for each plate. The Z' factor is a widely accepted indicator for the statistical performance of a screening assay (Janzen, 2002). It is a dimensionless parameter that ranges from 1 to values < 0. If the Z' factor is < 0, the signal population of the positive control overlaps with the signal population of the negative control (a Z' factor of 1 would indicate infinite separation of the two populations). For a cellular screening, a Z' factor > 0.3 is acceptable. This was achieve with the BACE1 inhibitor screening in yeast, for which the Z' factors usually ranged from 0.3 to 0.8.

The chemical library was screened twice with the BACE strain. The cell densities were determined after 20, 26 and 32 hours incubation by measuring light scattering at 595 nm ($OD_{595\ nm}$). The relative effects of the test substances on cell growth were calculated in relation to the growth of the negative control. A primary hit was defined as a compound, which stimulated growth of BACE cultures repeatedly ≥ 5% at one (or several) given time point(s). This yielded 662 primary hits. (For an overview of the screening and the further proceedings see figure 1.)

BACE dependence of primary hits

Interference with BACE1 function is one of many possibilities for a compound to stimulate growth of BACE cultures. Since BACE activity is coupled to the growth readout via secretion of invertase activity and the transcriptional regulation of the *GAL1-10* promoter (see part 3.1. figures 1 and 2), every compound that interferes with the

function of invertase (secretion or enzymatic activity) or with the glucose repression of the *GAL1-10* promoter produces a false positive signal. These kinds of false positive compounds can be excluded by testing them in a strain that secretes invertase activity in a BACE1-independent way. This can be achieved by replacing BACE1 by another secretase, e.g. Yps1p that liberates the invertase moiety from the invertase-APP fusion protein by cleaving at the α-site, or by using a strain, which constitutively secretes natural invertase. If a compound stimulates not only the growth of the BACE strain, but also the growth of one of these two control strains, its mode of action is obviously not BACE-dependent.

Three growth assays were performed to confirm the growth stimulation observed in the screening and to investigate the BACE dependence of the 662 primary hits. The growth assays were performed twice with the BACE strain (reproduction of the screening results) and once with Biscre11 cells transformed with a plasmid encoding natural invertase (herein after referred to as invertase strain; BACE dependence test). For these growth assays each compound was tested at concentrations of approximately 50 µM, 25 µM and 12.5 µM. Cell densities were determined after 20, 26 and 32 hours incubation. This produces a dataset of nine measure points per compound and growth assay. A primary hit was considered as BACE-dependent if the following requirements were met at one measure point for all growth assays: The growth stimulation of the BACE strain must be at least 5% in both growth assays, whereas the growth stimulation of the invertase strain must be less than 0%. These criteria were met by 28 compounds. However, since a computer produced this list by considering isolated measuring points, the datasets of these 28 compounds were manually reviewed to get a more complete picture of the performance of the compounds. After this procedure there were 14 compounds left, which showed concentration-dependent growth stimulation of the BACE strain, but not of the invertase strain.

For the final test in yeast, the 14 compounds were reordered and freshly dissolve in DMSO. The setup of the experiment was the same as for the BACE dependence test of the 662 primary hits except that the compounds were tested additionally in Biscre11 cells transformed with plasmids encoding Yps1p and the invertase-APP fusion protein (herein after referred to as Yps1p strain). As shown in table 1, after this test there were eight

compounds still considered as BACE-dependent, four were rejected and two were proceeded because of convincing previous results although in this test they were no more BACE-dependent.

Analysis of candidate compounds by measuring Aβ secretion from HEK 293 cells

The final goal of therapeutic BACE1 inhibitors would be to reduce the deposition of Aβ peptide in the brain. It is therefore an early and important milestone for candidate compounds to reduce Aβ secretion in cell cultures. To investigate this issue, HEK 293 cells overexpressing the Swedish mutation of APP (Citron et al., 1992) were incubated with 20 µM of the test candidates for two days before the secretion of Aβ40 peptide was quantified by the use of a sandwich ELISA. In parallel to the Aβ ELISAs there were also XTT reduction assays performed to monitor cell viability. The results normalized to the negative control (0.1% DMSO) are shown in figure 2A. The γ-secretase inhibitors VII (Durkin et al., 1999) and IX (Dovey et al., 2001), which served as positive controls, were added at concentrations of 5 µM and 0.1 µM causing a reduction of Aβ40 secretion of about 40% and 80%, respectively. None of the ten tested compounds exhibited significant toxicity to the cells and six of them caused a significant reduction of Aβ40 peptide in the conditioned cell culture medium. Five compounds caused a comparable reduction of about 20%. The biggest reduction was observed for the compound 102H6, for which a dose dependency is shown in figure 2B. However, the initially observed reduction by 20 µM 102H6 was not reproduced to the extent of 60%. For the dose dependence experiment the maximal reduction was 40% at a concentration of 25 µM.

In vitro analysis of putative BACE1 inhibitors

In order to confirm the mode of action of the compounds that were selected by the yeast screening system they were tested with an *in vitro* assay for inhibition of BACE1. This is necessary, since both, the yeast readout and the measurement of secreted Aβ from HEK 293 cells involve the secretory pathway, which leaves the possibility that selected compounds do not act on BACE1, but interfere with the secretory pathway instead.

In vitro BACE1 activity was assessed by the use of soluble BACE1 ectodomain and two different fluorescence resonance energy transfer (FRET) substrates. The two

substrates referred to as FS-IV and FS-VI essentially differ in their fluorescence donor:acceptor pairs and the adjacent amino acids, whereas the p4-p4' residues are identical. For the cleavage of FS-IV there was substantial inhibition observed by some of the compounds (see table 2), but cleavage of FS-VI was never affected. Autofluorescence or quenching effects of the test compounds can be excluded to explain these differences, since this was tested before the measurements. Furthermore, the positive control, the peptidic transition state mimetic inhibitor P10-P4'StatVal revealed IC_{50} values of 80 nM with both substrates, which is in the range of the published IC_{50} values of 30 nM as determined in the original publication (Sinha et al., 1999) and of 200 nM as determined by Grüninger-Leitch et al. (Grüninger-Leitch et al., 2002). The same was true for a second positive control, the peptidic transition state mimetic inhibitor OM99-2 (Ghosh et al., 2000), for which we also observed comparable inhibition with both substrates.

3.2.2. Discussion

The screening of 15'000 small molecules by our novel yeast growth selection assay revealed 10 compounds, which stimulated the growth of yeast cells in a BACE-dependent manner. Six of these compounds caused a significant reduction of Aβ secretion from HEK 293 APP (Sw) cells and five compounds significantly inhibited BACE1 activity in an isolated enzyme assay. An overview of the performance of these 10 compounds in the different systems is shown in table 3. Four compounds are active in all three systems, two only in yeast and cell culture, one in yeast and *in vitro*, and three compounds are only active in yeast. There is no obvious correlation between the performance of the compounds in yeast and their performance in the other systems. The two compounds performing best in yeast only show additional activity in either the cell culture assay (101C3) or the *in vitro* assay (77H7) and the compounds performing next best in yeast (48E8 and 90C10), both do not show any activity in another system than yeast. The compounds being active in all three systems (36D10, 102H6, 103B3 and 128D2) show a low to medium performance in yeast. We assume that Biscre11 cells, which are deficient for the drug efflux pumps Pdr5p, Snq2p and Yor1p display an increased sensitivity towards a broad spectrum of compounds compared to wildtype yeast cells, but are still less sensitive than cultured mammalian cells. This could explain, how a compound

producing a relatively small growth stimulation in yeast already provokes an effect in mammalian cells, but not why a compound producing a high and BACE-dependent signal in yeast, does not respond in the other systems. This lack of clear correlation between the yeast system and the other systems is not necessarily a drawback for the yeast selection as a tool for primary screenings, since its good statistical performance allows for setting a low threshold of 5% growth stimulation, which should not be a bottleneck for true positives. However, due to the lack of correlation and the artificial character of the yeast system, it is not suitable as a tool for hit validation or to rank candidates of a hit expansion.

In *part 3.1.* it was stated that compounds identified by the yeast system would be pre-selected for membrane-permeability and against general toxicity. This seems to hold true, since none of the compounds tested in cell culture revealed significant toxicity and six out of ten caused a significant reduction of Aβ secretion. The mode of action of the six compounds affecting Aβ secretion, however, remains to be elucidated. Since both, the yeast readout, as well as the quantification of Aβ peptide in the conditioned medium, involve the secretory pathway, a compound interfering with the secretory pathway of yeast and of mammalian cells would produce a positive signal with both readouts. Although the compounds seem to be BACE-dependent in yeast as determined by the use of constitutively secreted invertase and Yps1p-dependent secretion of invertase it is still important to demonstrate their mode of action in another system than the one with which they were discovered. For this purpose we performed *in vitro* assays using two different fluorescence resonance energy transfer (FRET) assays. The results from these experiments however were not conclusive, since the significant and reproducible inhibition by five compounds as determined with the first assay substrate could never be reproduced with the second assay, whereas the positive controls worked well with both substrates. Up to the present we could not track down the factors, which are responsible for the observed differences and we cannot yet be sure, whether the identified compounds are bona fide BACE1 inhibitors. Further investigations will be necessary to answer this question. There are alternative possibilities to determine the mode of action of putative BACE1 inhibitors. Beside alternative *in vitro* readouts involving detection of cleavage products by high performance liquid chromatography, mass spectrometry or ELISA, the

extent of β-site cleavage could also be assessed *in vivo*. This could be achieved by quantifying the N-terminal cleavage products of APP sAPPα and sAPPβ as described for example by Steinhilb et al. (Steinhilb et al., 2000).

A conclusive evaluation of the yeast growth selection is not yet possible, since i) it is not yet clear, whether it identified bona fide BACE1 inhibitors, ii) the number of screened compounds is relatively small and iii) a direct comparison with other screening systems is not possible, since this particular library was only screened with the yeast system. As discussed in previous sections of this thesis, BACE1 is a potentially ideal drug target from a biological point of view, and consequently there is a huge interest to find potent inhibitors. However, from a pharmacological point of view, BACE1 proofed to be a challenging target (see also *part 2.4*). Despite the effort of many academic groups and several big pharma companies to identify potent small molecule inhibitors of BACE1, there are no publications describing successful screenings for BACE1 inhibitors. Since already the screening of our small library revealed a considerable number of promising candidates, we believe that it would be worth applying the yeast selection system to screen a larger library.

3.2.3. Materials and methods

Yeast media and transformation

All media were prepared according to Burke et al. (Burke et al., 2000). Growth selection media: 5% sucrose 2% galactose –his –ura –trp; the sucrose was dissolved in 2% galactose –his –ura –trp liquid medium and sterile filtered immediately before use. Transformation of yeast cells was performed following the lithium acetate method (Gietz et al., 1992).

Yeast strains and plasmids

The yeast strain Biscre11 is a derivative of Biscre1, which is described in materials and methods of *part 3.1.*. To generate Biscre11 the genes *PDR5*, *SNQ2* and *YOR1* were sequentially deleted using *in vivo* site-directed mutagenesis as described by Storici et al. and referred to as “delitto perfetto” (Storici et al., 2001). The expression plasmids used for this study were the same as described in *part 3.1.*, with the addition of the plasmid

encoding for natural invertase (Suc2p). The ORF of *SUC2* was subcloned (BstBI → blunted/SalI) from the plasmid encoding the full-length version expressed from the *ACT1* promoter as described by Lüthi et al. (Lüthi et al., 2003) into the multiple cloning site (StuI/SalI) of pMH28 (described by Urech et al. (Urech et al., 2003)), which drives expression from a truncated *ADH1* promoter.

The chemical library

The chemical library was designed so as to maximize structural diversity among the molecules. Furthermore the molecular weight (MW) of the compounds was kept below 500 g/mol. The resulting selection, which revealed a high score with the Lipinski rules (MW ≤ 500 g/mol, logP ≤ 5, H-acceptors ≤ 10, H-donors ≤ 5, rotable bonds ≤ 8), was ordered at ChemDiv and was delivered as white and colored powders in 96 deep well plates. 80 wells of each plate were filled with approximately 1 mg of different compounds, which were dissolved in DMSO to an approximate concentration of 5 mM generating the so-called "grandmother plates". 100 µl aliquots of the "grandmother plates" were transferred to V-shaped polypropylene 96 well plates (Greiner bio-one 651201) to generate the so-called "mother plates". The wells A1-H1 of the mother plates were filled with 100 µl of DMSO, the wells A12-H12 were empty. The grandmother plates were stored at –20°C, the mother plates at 4°C.

The screening procedure

The library was subdivided into 5 batches of 38 plates (batch 5 contained only 36 plates). The screening capacity was one batch in 2 days, which gives a screening throughput of 1520 compounds per day. To screen one batch, a 50 ml preculture was inoculated with Biscre11 cells transformed with BACE-ACT1 and Suc2pAPP^{Sw} and grown overnight in 2% galactose –trp –ura medium at 30°C. After about 14 hours incubation, it was diluted with 2% galactose –trp –ura medium to obtain a cell density corresponding to $OD_{600\ nm} = 0.25$ and further incubated at 30°C to reach $OD_{600\ nm} \cong 1$ (determined for a 1/10 dilution in a Shimadzu UV mini-1240 spectrometer, l = 1 cm). This exponentially growing culture was washed with 50 ml H_2O and resuspended in 50 ml growth selection medium before it was used to inoculate 900 ml of growth selection

medium to reach a starting $OD_{600\ nm}$ of 0.05. The screening plates were prepared as follows: 1.5 µl aliquots from the mother plates were replica plated into flat bottom polystyrene 96 well plates (Greiner bio-one 656101) by the use of a Tomtec 96 needle pipeting robot. The positive control, 1.5 µl of 1 mM MG132 was added by hand to the wells C12-F12. The following steps were performed in a fully automated manner by a Tecan robot: 150 µl of yeast suspension were added to each well of the screening plates, which were shaken 4 times for 10 seconds with alternating directions at 1100 rpm before the $OD_{595\ nm}$ was measured (= blank values, t = 0) in a Tecan Genios reader. After 20, 26 and 32 hours incubation without shaking at 30°C 100% humidity, the cell densities were determined as described for measuring the blank value. For the evaluation, the blank value was subtracted from the values obtained after 20, 26 and 32 hours. The statistical performances of the screening plates were assessed by calculating the Z' factor:

$$Z' = 1\text{-}3 \times (SD_{positive\ control} + SD_{negative\ control}) / \left| AV_{positive\ control} - AV_{negative\ control} \right|$$

(SD: Standard deviation; AV: Average)

For the BACE dependency tests in yeast, there were new mother plates composed by picking and diluting the selected compounds from the original mother plates, or, for the second round of BACE dependency test, with the reordered and freshly dissolved compounds. Otherwise, the growth assays were performed as described above.

Cell culture, XTT reduction assay and Aβ ELISA

HEK 293 cells overexpressing the Swedish mutation of APP (Citron et al., 1992) (a kink gift from Christian Haass) were grown in 10 cm tissue culture dishes with 10 ml Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and 200 µg/ml G418 (herein after referred to as cell culture medium) to near-confluence before they were trypsinized. After washing the cells with cell culture medium lacking phenol red, the cell density of the cell suspension was determined with the Neubaur counter chamber. After dilution with cell culture medium lacking phenol red, the cells were seeded into 96 well plates containing the test substances to reach 50'000 cells per well resuspended in 150 µl cell culture medium lacking phenol red. The final concentration of the test substances was 20 µM, the final DMSO concentration was 0.1%. Every compound was tested in triplicates. After 2 days of incubation at 37°C, 5% CO_2 and

100% humidity 30 µl of conditioned medium were removed. The residual 120 µl were subjected to the XTT reduction assay: 40 µl of XTT/phenazine methosulfate (PMS) solution (1:20 dilution of 500 µM PMS in 1 mg/ml XTT dissolved in cell culture medium lacking phenol red) were added to each well, and after 45 minutes incubation at 37°C, 5% CO_2 and 100% humidity, the $OD_{450\ nm}$ was determined in a microtiter plate reader. For the Aβ40 ELISA, a commercially available kit (hAmyloid β40 ELISA (HS) from The Genetics Company) was used. The conditioned medium was diluted 1:50 in sample dilution buffer and the samples were proceeded according to the manual.

FRET assays

All FRET assays were performed in triplicates in 384 well plates in an assay volume of 50 µl. The reaction was performed in 100 mM NaOAc pH 4.5 with 20 nM recombinant human BACE1 ectodomain (R&D systems, 931-AS-050) at a substrate concentration of 5 µM and a final DMSO concentration of 2.5%. The test substances were pre-incubated at indicated concentrations with the enzyme for 30 minutes at 37°C or 30°C before addition of the substrate and starting the measurement in a microtiter plate reader (Tecan Genios). For experiments using FS-IV (H-Arg-Glu(EDANS)Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys(DABCYL) Arg-OH; Calbiochem 565758) as a substrate the measurements were performed in white 384 well plates (Nunc 3911932) at 37°C, at an excitation wavelength of 360 nm and an emission wavelength of 485 nm. For experiments using FS-VI (H-Lys(DABSYL)-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Arg-Gin-(lucifer yellow); Calbiochem 565781) as a substrate the measurements were performed in black 384 well plates (Corning #3654) at 30°C, at an excitation wavelength of 430 nm and an emission wavelength of 520 nm. The intial velocities were calculated using the values obtained between the 2nd and the 10th minute of the reaction. Enzymatic activity in the presence of a test compound was calculated as percentage of the initial velocity compared to initial velocity of the negative control, which was set to 100%. The BACE1 inhibitors OM99-2 and P10-P4'StatVal were acquired by purchase from Bachem (H5108) and Calbiochem (171601), respectively.

3.2.4. Figures and tables

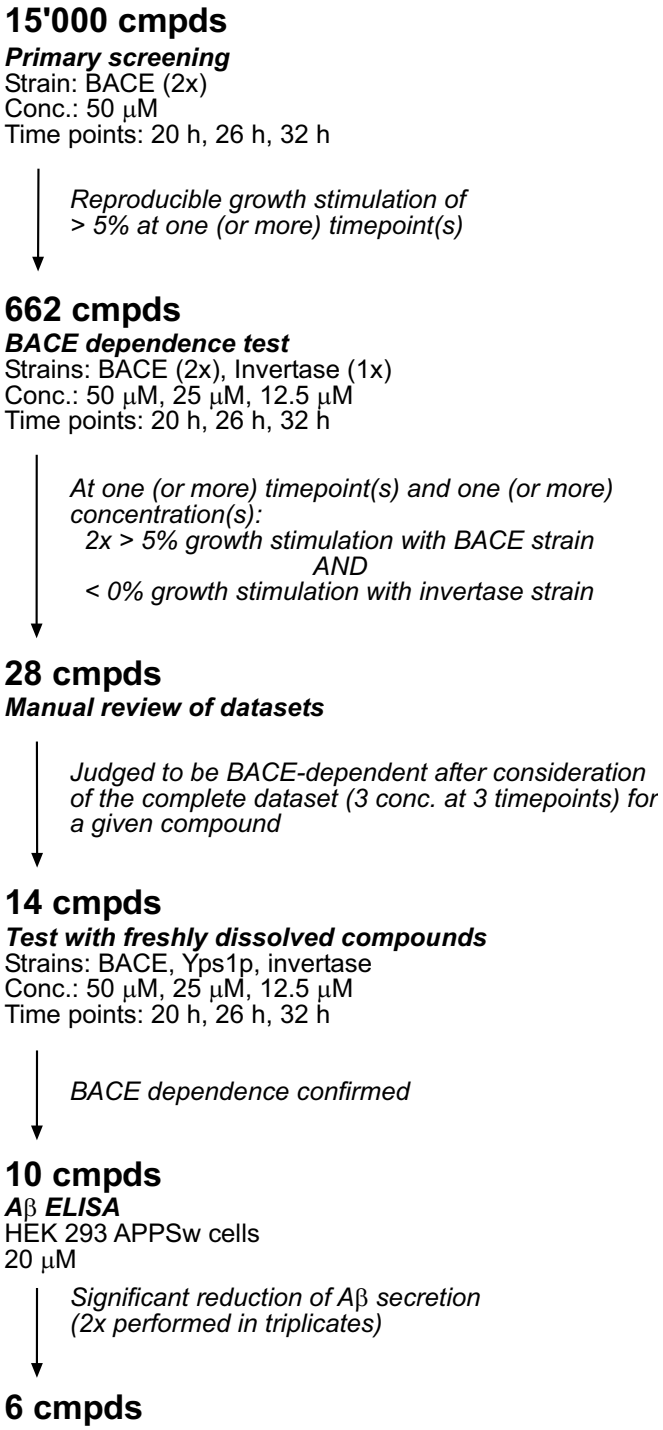
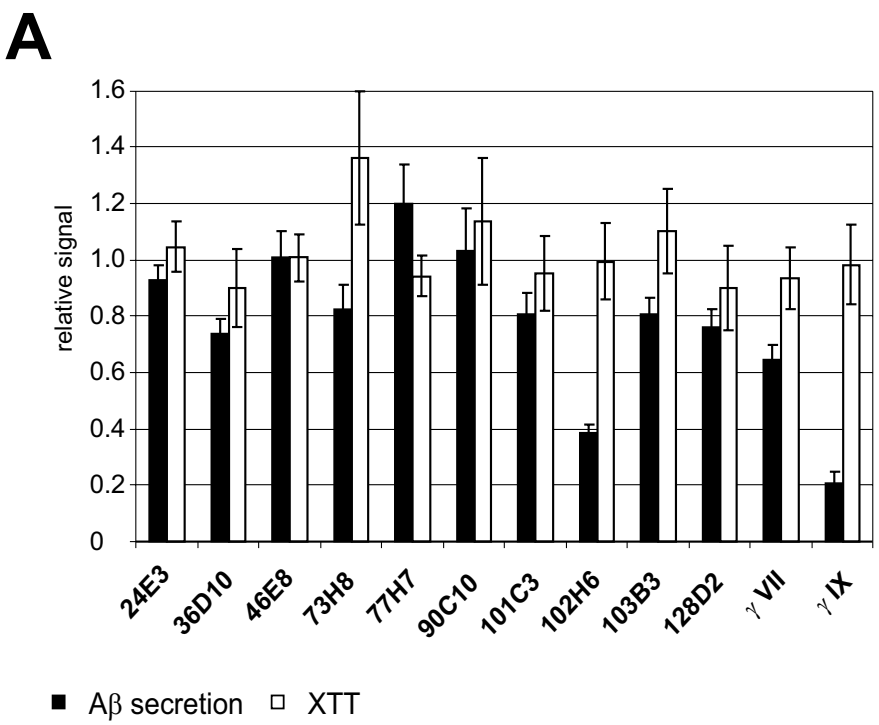


Figure 1 Overview of the selection of putative BACE1 inhibitors

Figure 1 Overview of the selection of putative BACE1 inhibitors. **(1)** 15'000 compounds were screened twice with the BACE strain (Biscre11 transformed with BACE-ACT1 and Suc2pAPP^{Sw}). 662 compounds that stimulated growth repeatedly >5% after 20, 26 or 32 hours incubation were selected and subjected to (**2)** the BACE dependency test: 28 compounds stimulating the growth of the BACE strain twice >5% and the growth of the invertase strain (Biscre11 transformed with a plasmid encoding natural invertase) <0% at a concentration of 12.5 µM, 25 µM or 50 µM after 20, 26 or 32 hours incubation were selected as being BACE-dependent. **(3)** The complete datasets obtained for the 28 compounds during the BACE dependency test were manually reviewed. After this procedure there were 14 compounds remaining, which were reordered and dissolved in DMSO immediately before they were subjected to the final test in yeast **(4)**. The freshly dissolved compounds were tested with the BACE strain, the invertase strain and the Yps1p strain (Biscre11 transformed with Suc2pAPP^{Sw} and a plasmid encoding Yps1p). This test confirmed BACE dependency of 10 compounds, which were subjected to cell culture experiments with HEK 293 cells overexpressing the Swedish mutation of APP **(5)**. Among these 10 compounds there were 6, which significantly reduced the amount of Aβ peptide in the conditioned medium.



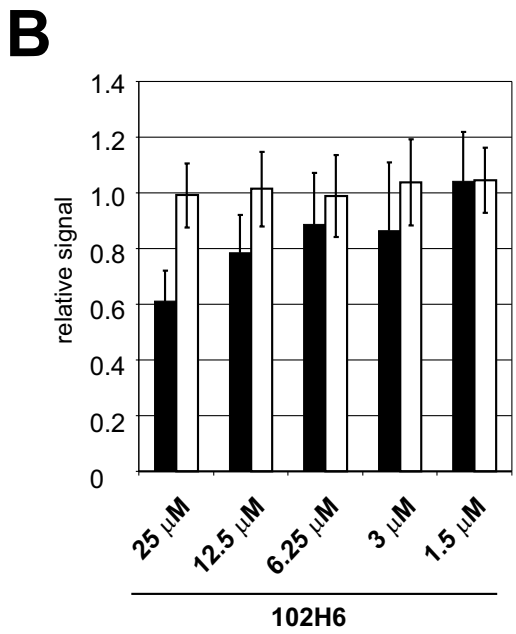


Figure 2 Effects of selected compounds on Aβ secretion. HEK 293 cells overexpressing the Swedish mutation of APP were incubated with in the presence of the test compounds. After two days of incubation the amount of soluble Ab40 peptide in the conditioned medium was quantified by the use of a sandwich ELISA (black columns) and the cell

viability was monitored with an XTT reduction assay (white columns). All experiments were performed in triplicates. The results are normalized to the negative control (DMSO), which is set to 1 (not shown). **A** Results for 10 compounds, which were tested at a concentration of 20 µM. The γ-secretase inhibitors VII (γVII) and IX (γIX) were added at concentrations of 5 µM and 0.1 µM and served as positive controls for inhibition of Aβ secretion. **B** Dose-dependence of compound 102H6.

Table 1

Confirmation of BACE dependence of freshly dissolved compounds in yeast

	BACE			Yps1p			Invertase		
	50 µM	25 µM	12.5 µM	50 µM	25 µM	12.5 µM	50 µM	25 µM	12.5 µM
24E3	**1.059**	1.046	1.022	1.024	0.998	0.996	1.044	1.018	1.039
36D10	**1.076**	1.028	**1.058**	0.934	0.875	0.870	0.903	0.889	0.921
46E8	**1.265**	**1.151**	**1.143**	0.834	0.833	0.847	0.919	0.964	**1.122**
48E7*	**1.172**	**1.115**	**1.108**	**1.119**	**1.109**	**1.133**	**1.080**	**1.062**	1.024
73H8**	1.043	1.008	0.972	1.000	0.902	0.952	1.044	0.965	1.012
77H7	**2.107**	**1.971**	**1.911**	0.900	0.903	0.880	**1.053**	1.013	1.037
82F10*	0.998	0.994	0.954	**1.078**	1.032	1.009	**1.113**	**1.091**	1.021
90C10	**1.291**	**1.152**	**1.080**	0.865	0.847	0.875	0.934	0.964	0.983
101C3	**1.908**	**2.210**	**1.738**	0.787	0.825	0.811	0.940	0.903	0.927
102H6**	**1.071**	1.027	1.047	**1.094**	0.972	0.977	**1.087**	1.027	**1.058**
103B3	**1.233**	**1.165**	**1.160**	**1.098**	1.003	1.027	**1.071**	0.995	0.970
104G4*	0.966	**1.233**	0.836	0.863	**1.454**	1.044	0.960	**1.181**	0.960
124C3*	1.004	1.023	1.031	0.977	0.974	0.955	0.987	**1.051**	1.033
128D2	**1.146**	**1.104**	**1.051**	0.878	0.839	0.879	0.946	0.897	0.926

Depicted values represent relative cell densities compared to the negative control (1% DMSO, set to 1) of the corresponding strain (BACE1, Yps1p or invertase). Values > 1.05 are heavy typed

* BACE dependence not confirmed

** BACE dependence not confirmed, but not rejected

Table 2

***In vitro* inhibition of BACE1 activity**

Compound	Inhibition
24E3	0% (10 μM)
36D10	40% (50 μM)
46E8	0% (20 μM)
73H8	0% (50 μM)
77H7	40% (20 μM)
90C10	0% (50 μM)
101C3	0% (50 μM)
102H6	45% (50 μM)
103B3	20% (50 μM)
128D2	25% (50 μM)

BACE1 activity was assessed in the presence of the test compound (concentration in brackets) by the use of FS-IV as a substrate.

Table 3

Performance of selected compounds in the different systems

	Potenial from yeast data			
	Growth stimulation of BACE strain	BACE-dependent	Reduction of Aβ secretion in HEK 293 APP(Sw) cells	In vitro Inhibition of BACE cleavage (FS-IV)
24E3	+	Yes	0	0
36D10	+	Yes	+	++
46E8	++	Yes	0	0
73H8	+	Yes/No*	+	0
77H7	+++	Yes	0	++
90C10	++	Yes	0	0
101C3	+++	Yes	+	0
102H6	+	Yes/No*	+++	++
103B3	++	Yes	+	+
128D2	++	Yes	+	+

+ 5-10%	+ 10-25%	+ 20-30%
++ 10-30%	++ 25-50%	++ 30-50%
+++ 30-100%	+++ 50-100%	

* BACE-dependent in first, but not in second test

3.2.5. References

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Part 3.3.

Selection of ankyrin repeat protein-based inhibitors of β-site cleavage

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3.3. Selection of ankyrin repeat protein-based inhibitors of β-site cleavage

3.3.1 Abstract

We performed a screening to identify ankyrin repeat (AR) protein-based inhibitors of BACE1. A combination of *in vitro* selection for BACE1 binding AR proteins by ribosome display with a subsequent functional selection in yeast yielded BACE1 binding AR proteins that inhibit the cleavage of a BACE1 substrate both *in vivo* and *in vitro*. Further analysis however revealed that the observed inhibition by selected proteins rather relied on the interference with the substrate than on impairing BACE1 function directly.

3.3.2. Introduction

Ankyrin repeat (AR) proteins are found in virtually all phyla and are expressed as nuclear, cytosolic, secreted or transmembrane proteins (Bork, 1993). Variable numbers of the usually 33 amino acid ARs build topologically well defined stacks, so-called AR domains, the function of which is to mediate specific protein-protein interactions. This evolutionary approved concept was exploited to generate combinatorial libraries of consensus-designed AR proteins (Binz et al., 2003). By sequence and structure analyses, a AR module was designed with invariable framework residues and randomized potential interaction residues. This module was cloned in varying numbers between terminal capping ARs, leading to combinatorial libraries of AR proteins of varying repeat numbers. Determination of the crystal structure of the unselected library member E3_5 and analysis of several randomly picked library members revealed an AR domain fold and favorable thermodynamic stabilities of the designed AR proteins (Binz et al., 2003; Kohl et al., 2003). Binding AR proteins with nanomolar affinities against several globular proteins were selected from the consensus AR protein libraries by using ribosome display (Binz et al., 2004).

In an attempt to identify AR protein-based inhibitors of BACE1 we combined *in vitro* ribosome-display selection for BACE1 binding AR proteins with a subsequent functional selection for proteins that inhibit the cleavage of a BACE1 substrate. The advantage of this combination is that the *in vitro* selection step decreases the library complexity to a

size, which is suitable for the subsequent *in vivo* screening. At the same time, the *in vitro* selection increases the chance of finding functional AR protein inhibitors, since the selected proteins already show BACE1 binding. The functional selection was performed in yeast using the "Yeast growth selection system for the identification of cell-active inhibitors of β-secretase" (Middendorp et al., 2004), which is described in *part 3.1.* of this thesis.

3.3.3. Results

In vitro selection of BACE1 binders

The AR protein libraries we used in our experiment were termed N2C and N3C, reflecting the content of an N-terminal capping AR, two or three randomized AR modules and a C-terminal capping AR. The estimated diversity of the libraries is at least 10^{10} (Binz et al., 2004). Three independent selections comprising four rounds of ribosome display each were performed against the BACE1 ectodomain using twice the N3C library and once the N2C library. We observed a rapid and specific enrichment of binding throughout the selection process. After four selection rounds, 16 clones from each selection were analyzed with an enzyme-linked immunosorbent assay (ELISA) for BACE1 ectodomain binding using *Escherichia coli (E. coli)* crude cell extract. Figure 1 shows that of 16 clones from the N2C pool, 11 specifically (as compared to binding to neutravidin) bound BACE1; one bound neutravidin as well and 4 gave no signal above background. For the two N3C selections there were 15 and 16 specific BACE1 binding AR proteins among the analyzed clones. This result indicates that the *in vitro* selections indeed enriched for proteins with BACE1-binding properties.

Subcloning of BACE1 binding AR proteins into an ER targeted yeast expression library

To select for BACE1 binding AR proteins with inhibitory properties using the yeast selection system, they had to be targeted to the secretory pathway, where the β-secretase cleavage of the invertase-APP reporter fusion protein occurs (Lüthi et al., 2003). In order to find an efficient way to direct AR proteins to the yeast ER, the amino acids 1-35 of the yeast invertase (Suc2p), which comprise the signal peptide (amino acids 1-19), were

fused N-terminally to the RGSHis$_6$-tagged AR protein E3_5 (Figure 2A), which is an unselected member of the N3C library. This construct, named Suc2p(1-35)-RGSHis$_6$-E3_5, was expressed in yeast and detected by the use of an αRGSHis$_6$-antibody in a Western blot analysis of yeast crude cell extract. This expression analysis revealed two bands of comparable intensity (figure 2B, lane 1). Analysis of an endoH glycosylase-treated aliquot of the same crude cell extract revealed a decrease of the intensity of the upper band compared to the untreated sample (figure 2B, lane 2), indicating that the upper band could represent a glycosylated molecule population and consequently that the fusion protein reached the ER, since this is the organelle, where N-glycosylation occurs. In fact, the 35 amino acid Suc2p peptide contain a potential N-glycosylation site at position 23. As a further control, we expressed a version of Suc2p(1-35)-RGSHis$_6$-E3_5, which is deleted for the first 19 amino acids (named Suc2p(20-35)-RGSHis$_6$-E3_5; figure 2A). Expression of this construct revealed one band on the Western blot, which migrated at the same level as the lower band of the doublets observed for Suc2p(1-35)-RGSHis$_6$-E3_5 (figure 2B, lane 3). Therefore, this lower band could represent Suc2p(1-35)-RGSHis$_6$-E3_5, from which the signal peptide was removed during the translocation to the ER. Taken together, the results of this Western blot analysis provide good evidence that the AR protein E3_5 was indeed translocated to the yeast ER by the Suc2p(1-35) leader sequence.

We hence subcloned the DNA pools originating from the three independent *in vitro* selections against the BACE1 ectodomain into this plasmid (replacing E3_5). Like this, libraries named L4, L5 and L6, with diversities of about $1.5 \cdot 10^5$ were generated, suitable for *in vivo* selections in yeast.

Functional selection of putative BACE1 inhibitors

In the yeast selection system, active BACE1 liberates the invertase moiety from an invertase-APP fusion reporter protein (Lüthi et al., 2003). The liberated invertase activity is secreted to the periplasmic space, where it encounters and hydrolyzes sucrose, thereby generating fructose and glucose (the selection medium contains 5% sucrose, 2% galactose and lacks histidine). The freshly generated glucose dominantly blocks the transcription of the growth selection marker *HIS3*, which is under the control of the

GAL1-10 promoter ($P_{GAL1-10}$-*HIS3*). Expression of the *HIS3* gene, however, is required for growth on histidine-depleted medium. If no invertase activity is secreted, and consequently no glucose is generated, galactose induces the expression of *HIS3* from the *GAL1-10* promoter and cells can grow on histidine-depleted medium. Hence, in the presence of active BACE1, invertase activity is secreted and growth on histidine-depleted medium is repressed by the freshly generated glucose. If a yeast cell is transformed with an inhibitory AR protein, the secretion of invertase activity is impaired and the cell can form a colony. One limitation of this selection system is the so-called cross-repression, which means that diffusing glucose generated by neighboring cells can repress the growth of a positive clone. This produces the paradox situation, in which a high transformation efficiency can yield less positives than a low transformation efficiency. Ideally, the transformation efficiency should be about 5000 evenly spread clones per 15 cm plate (data not shown).

The growth selection marker $P_{GAL1-10}$-*HIS3* was stably integrated into the genome of the selection yeast strain (Biscre1), whereas the invertase-APP fusion protein, BACE1 and the libraries were expressed from episomal plasmids (see materials and methods). We performed three screening experiments, one for each library. The screening of the L4 library had a scale of 11 plates; the screenings of the L5 and the L6 library were performed with 5 plates each. For the individual performances of the three screenings see Table1. In total we screened about $1.7 \cdot 10^5$ transformants, which revealed 266 putative positive clones after three days of incubation at 30°C. We picked 127 colonies and restreaked them on plates containing selection medium - all of them grew up again. From 60 clones out of these 127, we could rescue a plasmid with a restriction digest pattern corresponding to the one of a library plasmid. After retransformation of the rescued plasmids, 42 gave rise to colony formation again. Figure 3A shows a representative example of a retransformation of a selected AR protein (L4_36), which gives rise to colony formation. The sizes of the colonies are comparable to the ones formed by cells expressing an inactive version of BACE1 (D93N mutant) and no AR protein. The colonies formed by cells expressing active BACE1 and no AR protein, or active BACE1 and an unselected AR protein (E3_5), are both hardly visible (small size).

Selected ankyrin repeat proteins stimulate growth of yeast cells in a BACE1-dependent manner

In order to produce a provisional ranking of the selected AR proteins, their growth restoration capability was quantified by means of a liquid growth assay. Pre-cultures with unselective medium were inoculated with Biscre1 cells expressing invertase-APP, BACE1 and the selected AR proteins. Equal amounts of exponentially growing pre-cultures were then used to inoculate cultures with selective medium (histidine-depleted medium containing 5% sucrose and 2% galactose). After 26 hours incubation the cell densities of the different cultures were determined by measuring $OD_{595\ nm}$ and normalized to the negative control being a sample that expresses no AR protein. The values obtained ranged from 1 to 6. The growth of a control expressing inactive BACE1 (D93N mutant) or an empty vector instead of active BACE1 is usually enhanced by a factor of 7 to 8 compared with the negative control. 37 clones that markedly stimulated growth in this experiment were sequenced.

Although the AR proteins were pre-selected for binding to the BACE1 ectodomain, it could still be that the observed growth stimulation in the presence of selected AR proteins was caused by a different mode of action than directly impairing BACE1 function. Therefore, we examined, whether selected AR proteins would have a similar effect on cultures expressing an alternative secretase instead of BACE1. The yeast protease Yps1p cleaves APP at the α-site (Zhang et al., 1997) and liberates the invertase moiety from the invertase-APP fusion protein (Lüthi et al., 2003). Replacing BACE1 by Yps1p in the described liquid growth assay system produces a comparable growth retardation in the absence of selected AR proteins. Importantly, the selected AR proteins, which were tested, could only rescue the growth of BACE1-expressing cultures, whereas they had no effect on Yps1p-expressing cultures (figure 3B).

In vitro analysis of selected ankyrin repeat proteins

On the basis of the sequence information of the 37 clones (see supplementary data) we chose which candidates to characterize further *in vitro*. The sequences from the N2C library (L5) could be divided into two groups. The first contained eight members, whereof two are identical and the remaining differ by only one amino acid from the

others. The second group contains only one member (L5_23), which differs in most randomized positions from the consensus of the first group. However, we could only subclone L5_23 in a bacterial expression vector. For all members of the first group the cloning never worked, probably due to a toxic effect for the bacteria. The sequences of the N3C (L4 and L6) selections revealed four pairs of identical (or almost identical) sequences each, whereas the residual 20 sequences were remarkably different from each other. We chose one of the clones from each pair as well as eight further candidates, which were chosen due to a good performance in the liquid growth assay or due to mutations at framework positions of the AR protein. For one of these 12 candidates, the subcloning failed similar as described for the L5 clones. Finally, proteins of 12 candidates were purified and subjected to further *in vitro* characterization.

The proteins were expressed in soluble form in the cytoplasm of *E. coli* and could be purified to homogeneity in a single purification step via binding of the RGSHis$_6$-tag to an immobilized metal ion affinity chromatography (IMAC) column (data not shown).

ELISA analysis of the purified proteins revealed that out of 12 there were eight, which specifically (as compared to binding to neutravidin) bound to the BACE1 ectodomain at pH 7.4 and at pH 4.5 (figure 4A), which is the pH that was used for BACE1 enzymatic activity assays. The inhibitory potentials of the selected AR proteins were assessed by a FRET assay, in which the substrate is a modified peptide harboring the Swedish mutation (Citron et al., 1992) of the APP β-site. This peptide is coupled to a fluorescence group and a quenching group, which become separated upon cleavage resulting in an increase of fluorescence signal. Figure 4B shows an overview of BACE1 kinetic activities in the presence of 1 μM of selected AR proteins. Whereas for the unselected AR proteins E2_5 and E3_5 there was no significant inhibition observed, most selected proteins revealed a modest, but significant reduction of signal gain per time (initial velocity). This reduction was about 15% in average. The highest reduction was observed for L4_65, for which a concentration dependence experiment was performed (figure 4C). The initial velocity of the reaction was indeed decreasing with increasing concentrations of L4_65, but in addition we also observed a concentration dependent increase of the fluorescence signal measured at the beginning of the reaction (figure 4D). Also the other selected AR proteins caused an increased start signal in the FRET assay at a concentration of 1 μM

when compared to samples with buffer alone or 1 μM of the unselected library members E2_5 and E3_5 (data not shown).

We further examined this phenomenon by the use of a product standard, which consists of the fluorescence group coupled to the N-terminal half of the substrate and thereby mimics the signal originating from the cleaved substrate. The outcome of this experiment was that the selected AR proteins enhanced the fluorescence signal, when incubated with the uncleaved substrate, but not, when incubated with the product standard. An explanation for this would be that the selected AR proteins somehow interfere with the intramolecular quenching of the substrate. However, from these results we could not conclude whether the observed interference of selected AR proteins with the substrate would also influence the increase of signal over time. Therefore we developed a cathepsin D assay. Cathepsin D is like BACE1 an aspartic protease and it is known that it cleaves the Swedish mutation of APP at the β-site (Grüninger-Leitch et al., 2000). Therefore we tested our BACE1 FRET substrate for cleavage by cathepsin D and found that it was cleaved at the same conditions as used for cleavage by BACE1. In order to obtain a comparable velocity of substrate turnover for the BACE1 and the cathepsin D reaction, we used the double substrate concentration for the cathepsin D assay (this was necessary because we performed the reaction at pH 4.5, which is optimal for BACE1 *in vitro* cleavage, and not at pH 3.5, which would have been optimal for cathepsin D). Figure 4D shows that in the presence of 1 and 5 μM L4_65 the cathepsin D-mediated increase of fluorescence signal over time is inhibited in a concentration-dependent manner. We observed also a small, but significant concentration-dependent inhibition in the presence of 1 and 5 μM of the unselected E3_5 protein. In addition we tested for inhibition of cathepsin D-cleavage by 5 μM L4_37 and 4 μM L6_11, which were found to be 70%, and 34%, respectively (data not shown).

Is the growth stimulation in yeast by selected ankyrin repeat proteins dependent on the sequence of the substrate?

We wanted to test the hypothesis whether the growth stimulation of yeast cultures by selected AR proteins was dependent on a putative interaction of the AR protein with the invertase-APP fusion protein rather than on the binding of the AR proteins to BACE1.

Therefore we made use of a modified invertase-APP fusion protein, in which the residues p4-p4' of the β-site were substituted by an optimized artificial BACE1 cleavage site (Turner et al., 2001). We compared this construct named invertase-APP(art) in a growth assay to the construct invertase-APP(Sw), which was used in all the previously described yeast experiments and which harbors the Swedish mutation at the β-site (figure 5A). Whereas in the absence of AR proteins the two construct performed in a comparable manner – with BACE1 as well as without BACE1 – the growth stimulation by selected AR proteins was more pronounced with invertase-APP(Sw) (figure 5B). This result indicates that the growth stimulation by selected AR proteins is not completely dependent on the exact sequence of invertase-APP(Sw). Unexpectedly, in the presence of the unselected AR proteins E2_5 and E3_5 the growth of cultures expressing invertase-APP(art) was reduced compared to cultures expressing invertase-APP(Sw), while there was no difference in the absence of AR proteins (Although small, this seems to be a true effect, since the repetition of the experiment produced exactly the same picture).

In order to visualize the cleavage of the two different invertase-APP constructs in the presence or absence of selected AR proteins, we performed Western blot analysis to stain the APP C-terminal fragments (CTFβ) that were produced upon BACE1 cleavage. Figure 5C shows that the stainings of CTFβs originating from invertase-APP(art) are generally more pronounced compared the CTFβs from invertase-APP(Sw). However, the comparison of bands from invertase-APP(Sw) and invertase-APP(art) does not necessarily reflect different cleavage efficiencies, since it could well be that these cleavage products have different half-life times in yeast as well. The comparison of bands originating from the same construct, however, is valid if the intensities of the bands migrating immediately below the 35 kDa marker are taken into account, since these are unspecific bands and reflect the relative amounts of samples loaded. This kind of evaluation reveals that the cleavage of both, the invertase-APP(Sw) and invertase-APP(art) is reduced in the presence of the analyzed selected AR proteins, but not in the presence of E3_5.

3.3.4. Discussion

The aim of the present work was the identification of AR protein-based inhibitors of BACE1. To achieve this, we pursued a stepwise selection approach, in which we first selected for a pool of BACE1 binding AR proteins *in vitro* and then subjected this pool to a functional selection in yeast to identify AR proteins that inhibit β-secretase cleavage. Our data indicate that the *in vitro* selection worked efficiently; as among 48 clones analyzed before the yeast selection there were 42 binding specifically to BACE1. Surprisingly, this ratio was worse after the yeast selection, out of 12 purified proteins there were no more than two third specifically binding to BACE1. Further *in vitro* analysis of purified proteins revealed a modest, but significant inhibition of BACE1-mediated FRET substrate cleavage by all the selected AR proteins. However, at least for the selected AR proteins L4_37, L4_65 and L6_11, this inhibition was not BACE1 specific, as they inhibited also cathepsin D cleavage of the same substrate. Although not tested, we believe that this is also the case for the other proteins purified. The reason for this is, that in the FRET assay all proteins caused an increase of the fluorescent start signal, which was in the case of L4_65 concentration-dependent, indicating that the proteins interfere with the FRET substrate. Using two alternative FRET substrates, we observed for both unspecific inhibition of the cleavage by unselected AR proteins (data not shown).

These *in vitro* results give rise to the speculation that also in yeast, the growth stimulatory effect by the selected proteins is rather dependent on an interaction of the AR protein with the substrate rather than on the interaction with BACE1. The strongest argument in favor of this hypothesis is that one third of the purified proteins did not bind BACE1 *in vitro*. However, so far we have no prove for this hypothesis. Replacing BACE1 by Yps1p, which cleaves 16 amino acids C-terminal of the β-site, abolished the growth stimulatory effect of the tested AR proteins, indicating, that the effect would be β-site cleavage-specific. Exchanging invertase-APP(Sw) by invertase-APP(art), in which eight amino acid residues around the β-site are replaced by an artificial BACE1 cleavage sequence, resulted in growth stimulation by selected AR proteins as well - even though reduced. This data is difficult to interpret. It can be interpreted in favor of our hypothesis, with the explanation that two (maybe critical) amino acid residues of the two cleavage

sequences are identical and that also adjacent residues might be important for binding of the AR protein to the β-site. Therefore, the selected AR proteins could still, but with lower affinity, bind to the artificial β-site. Another suitable explanation for the impaired growth restoration of invertase-APP(art) expressing cultures by selected AR proteins, is that the artificial sequence is simply more efficiently cleaved, which is the case *in vitro* (Turner et al., 2001). In the presence of the unselected proteins E2_5 and E3_5 the growth of cultures expressing invertase-APP(art) is also reduced compared to cultures expressing invertase-APP(Sw), which is in accordance with the second explanation; however, in the absence of any AR proteins, there is no more growth difference. The data obtained from Western blot analysis (figure 5C) could indicate as well that invertase-APP(art) is more efficiently cleave than invertase-APP(Sw), but, as mentioned above, this data could also reflect different half-life times of the C-terminal cleavage products in yeast.

The bottom line of aforesaid Western blot analysis is that the analyzed selected AR proteins indeed seem to prevent β-site cleavage of both substrates. The clones analyzed by Western blot are all competent of binding BACE1 ectodomain *in vitro*. If the AR proteins fused to the Suc2p leader sequence are still competent of binding membrane-bound BACE1 expressed in yeast, we do not know. Further experiments, such as co-immunoprecipitations or GST pull down assays, are necessary to investigate the mechanism by which the selected AR proteins prevent the cleavage of invertase-APP in yeast.

Although the actual yeast data does not provide evidence for the hypothesis that β-site cleavage is prevented via binding of selected proteins to the substrate, it does not exclude this possibility either and the *in vitro* analysis of purified proteins supports this hypothesis. How could it come that all the purified proteins seem to interfere with the substrate? Maybe the AR protein scaffold has an inherent tendency for binding to the amino acid sequence around the β-site. Furthermore, the character of the amino acid residues at certain randomized positions would modulate this binding tendency and the yeast system would have selected for those with enhanced binding properties. A possible hint for this scenario is that E3_5 also weakly interferes with the FRET assay and that this interference is more evident in the cathepsin D assay, in which we used the double

substrate concentration. A high abundance of proteins with enhanced affinities for the substrate would titrate out the BACE1 inhibitors from the pool of analyzed positives. This putative problem could be overcome by analyzing more positives, which is quite laborious since they all have to be at least partially purified, or alternatively by finding conditions that no more allow for growth of cultures expressing AR proteins, which were selected with the described conditions, but still allow for growth in the absence of BACE1.

In summary, the *in vitro* selection worked, as there were specific BACE1 binders identified – the *in vivo* selection worked as well, since it revealed inhibitors of β-site cleavage. The combination of the two systems, however, did not facilitate the preferred identification of BACE1 inhibitors, possibly due to a predisposition of the used AR protein format to bind to the β-secretase cleavage site of APP.

3.3.5. Materials and methods

Ribosome-display selection of BACE1 binding AR proteins

Human BACE1 was purchased from oncogene research products (San Diego, CA, USA). The ribosome-display selections were essentially performed as described (Binz et al., 2004) with the following modifications: For the panning, 100 µl/well 65 nM BACE1 was immobilized directly on a maxisorp surface (Nunc) at 4°C overnight. All wells were blocked with 300 µl 0.5% BSA in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20 for 1 hour at room temperature prior to selection. The selection comprised two prepanning steps (1 hour in a neutravidin coated well; 45 minutes in a phage lambda protein D coated well) (Binz et al., 2004) followed by the 45 minutes selection step in a BACE1 coated well. Four ribosome-display selection rounds were performed with the N2C and N3C AR protein libraries (Binz et al., 2004) prior to analyzing single selected library members for BACE1 binding. Two independent selections were performed with the N3C library. The selected DNA pools were subcloned into pQE30 (QIAgen), followed by a crude extract ELISA screening for BACE1 binding (see ref. (Binz et al., 2004); **Fig. 1**). Quantitative ELISAs were performed basically as described (see ref. (Binz et al., 2004); **Fig. 4**), with the following modifications: Two different binding

buffers were used during the 40 minutes binding step at 4°C {PBS pH 7.4 (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) or 100 mM NaOAc pH 4.5}. Apart from this, all blocking, washing and incubation steps were in PBS supplemented with 0.05% Tween 20. AR protein concentration was 100 nM. BACE1 was immobilized as described above.

Yeast expression plasmids

The yeast expression plasmids for BACE1 (BACE-ACT1), invertase-APP(Sw) (Suc2pAPP^{Sw}) and Yps1p, as well as the yeast strain Biscre1 have been described in *part 3.1.* of this thesis. The plasmid expressing invertase-APP(art) is a Suc2pAPP^{Sw} derivative, which was cloned as follows: A DNA fragment encoding the APP amino acids 590-695 harboring the alternative cleavage site was amplified by means of PCR. The primers used for this PCR reaction were oligo #5651 (gctgcggccgctgagatctctgaaatt gatttgatggttttggatcgacatgactcaggatatgaag), which contains the DNA code for the alternative cleavage site as well as homologies to the APP nucleotide sequence, and the oligo #5458 (tcacctacttagtcgacttaagg), which anneals 3' of the APP nucleotide sequence of Suc2pAPP^{Sw}. The template was Suc2pAPP^{Sw}. The resulting DNA fragment was cloned into the NotI/SalI sites of Suc2pAPP^{Sw}. Suc2p(1-35)-RGSHis$_6$-AR fusion proteins are expressed from an *ACT1* promoter on a 2 micron plasmid with a *LEU2* marker, which was constructed the following: The 1175 bp EcoRI/SphI cassette of pDU10 (Urech et al., 2003) was ligated into the EcoRI/SphI sites of YEplac181 (NCBI database: X75460) producing plasmid#21. The DNA sequence coding for Suc2p(1-35)-RGSHis$_6$ was amplified by a PCR reaction of partially self-complementary oligos (#6639: ttttctagaatgcttttgcaagctttccttttccttttggctggttttgcagccaaaatatctgcatcaatgacaaacgaaactagcgat, #6640: aaaagatctgtgatggtgatggtgatgcgatcctctcatgggtgtgaagtggaccaaaggtctatcgctagtttcgtttg tcattgatgc) and cloned into the BglII/XbaI sites of plasmid#21 thereby producing plasmid#370 into which the DNA coding for the AR proteins was cloned by means of BglII and SpeI sites. For the construction of plasmids expressing fusions with Suc2p(20-35)-RGSHis$_6$ the self complementary oligos #6641 (ctagaatgtcaatgacaaacgaaactagcga tagacctttggtccacttcacacccatgagaggatcgcatcaccatcaccatcaca) and #6642 (gatctgtgatggtgat ggtgatgcgatcctctcatgggtgtgaagtggaccaaaggtctatcgctagtttcgtttgtcattgacatt) were cloned into

the BglII/XbaI sites of plasmid#370 derivatives, into which the DNA coding for the AR proteins was already inserted.

Construction of the yeast expression libraries: 2 kbp of spacer DNA were inserted into the BglII/SpeI sites of plasmid#370 to produce a vector, the digestion of which by BglII/SpeI can be readily controlled. For the generation of the library, the BglII/SpeI spacer fragment was replaced by the DNA fragments resulting from ribosome-display selections to generate the libraries L4 (N3C(2) selection), L5 (N2C selection) and L6 (N3C(1) selection). Transformation into *E. coli* (KC8) yielded $1.64 \cdot 10^5$ colonies for L4, $1.5 \cdot 10^5$ colonies for L5 and $1.45 \cdot 10^5$ colonies for L6, from which the plasmids were prepared using the QIAgen "Hispeed Plasmid Maxi Kit".

Yeast growth selections

The yeast strain Biscre1, the construction of which has been described in *part 3.1.* of this thesis, was used for all yeast experiments described in this section. Transformation of yeast cells was performed following the lithium acetate method (Gietz et al., 1992). Media were prepared according to Burke et al. (Burke et al., 2000). For the yeast selection, Biscre1 cells pre-transformed with BACE-ACT1 were supertransformed with Suc2pAPP^{Sw} and one of the libraries L4, L5 or L6. The transformed yeast cells were resuspended in YPAD, incubated for three hours at 30°C and washed with H_2O before they were plated on 14.5 cm dishes with –his –leu –ura –trp 5% sucrose 2% galactose medium. After 3-4 days incubation at 30°C upcoming colonies were picked and restreaked on –his –leu –ura –trp 5% sucrose 2% galactose medium. From these restreaks there were single clones isolated und subjected to the plasmid rescue protocol.

Growth assays in liquid medium were performed in microtiter plates basically as described in *part 3.1.* of this thesis with the following modifications: The selection medium was –his –leu –ura –trp 5% sucrose 2% galactose, the culture volume was 200 µl.

Plasmid rescue

1.5 ml –leu 2% glucose yeast cultures were grown over night at 30°C and spun down in microfuge tubes. After decanting the supernatant and addition of glass beads, the samples

were vigorously vortexed for 3-5 minutes. The following steps were performed using components of the Promega Wizard miniprep kit. After addition of 250 µl lysis solution, the samples were gently mixed and incubated for 4 minutes at room temperature before 350 µl of neutralization solution were added to the samples, which were then centrifuged in a microfuge for 10 minutes a full speed. The supernatants were transferred to the Promega miniprep spin columns and the plasmid DNA was purified following the Promega protocol. The plasmids were eluted with 50 µl TE, of which 10 µl were transformed into chemo-competent MC1066 *E. coli* cells. Plating the transformations on M9 –leu plates (Sambrook et al., 1989) allowed for selection of clones harboring yeast *LEU2* plasmids.

Western blot analysis

Whole cell extracts from transformed yeast were prepared as described by Burke et al. (Burke et al., 2000). Proteins were separated by SDS PAGE and Western blot analysis was performed according to standard procedures. A mouse monoclonal antibody from Sigma (A8717) that recognizes residues 676-695 of human APP695 was used at a final concentration of 25 ng/ml to detect expression of the CTFβ fragment. RGSHis$_6$-tagged AR proteins were detected using 133 ng/ml of an αRGSHis$_6$ antibody (QIAgen, 34650).

FRET assays

BACE1 FRET assays were performed using a PanVera kit (distributed by Invitrogen, P2985). The AR proteins were dissolved in 250 mM NaCl 25 mM Tris·HCl pH 8.0 10% glycerol and were added as 25 times concentrated solutions to the reaction. BACE1 was pre-incubated with the AR proteins for 30 minutes at 30°C before addition of the substrate and measuring fluorescence in a microtiter plate reader (Tecan Genios) at an excitation wavelength of 535 nm and an emission wavelength of 580 nm. The intial velocities were calculated using the values obtained between the 2nd and the 10th minute of the reaction. Cathepsin D FRET assays were performed using the same reaction buffer and the same FRET substrate as for the BACE1 FRET assay. Recombinant cathepsin D was obtained from Sigma (C8696) and used at a final concentration of 10 nM. The FRET

substrate was used at a final concentration of 500 nM (which is double as much as for the BACE1 assay).

3.3.6. Figures and tables

Table 1

Overview of yeast screenings				
Library	L4 (N3C)	L5 (N2C)	L6 (N3C)	**total**
Scale	11 plates	5 plates	5 plates	**21 plates**
Transformants	9900/ plate	6500/ plate	5900/ plate	**170'000**
Putative positives	113	72	81	**266**
average per plate	10.3	14.4	16.2	
Colonies picked	79	24	24	**127**
Growth after retransformation	24	9	9	**42**
Proteins purified	9	1	2	**12**

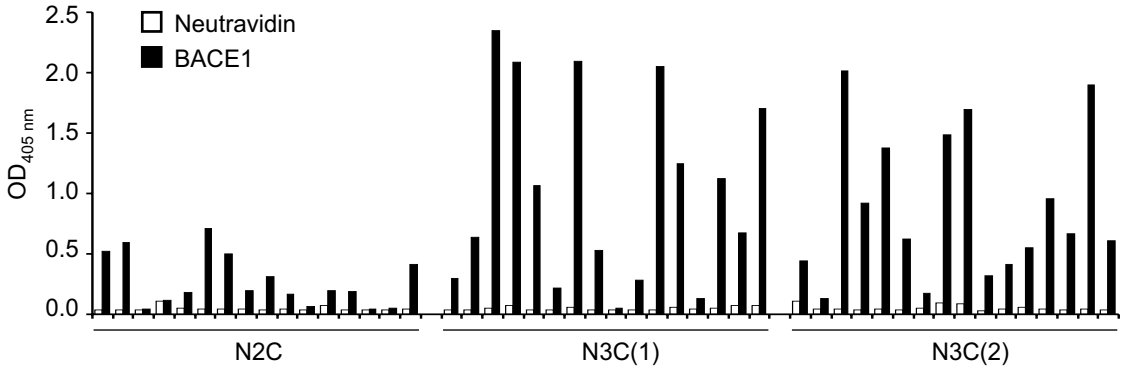

Figure 1. Crude cell extract ELISA for the identification of BACE1 binding AR proteins of the pools of selected AR proteins from the three independent ribosome display selections N2C, N3C(1) and N3C(2). Crude cell extracts of *E. coli* expressing *in vitro* selected AR proteins were probed for binding to neutravidin (white bars) and to BACE1 ectodomain (black bars). The background signal of the detection antibodies has not been subtracted in this representation.

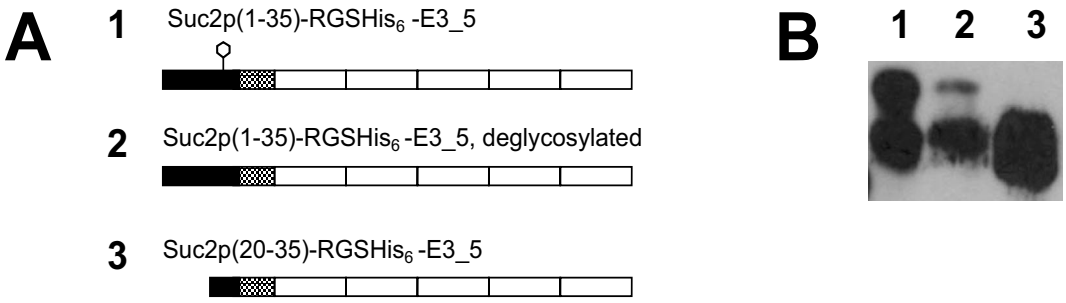

Figure 4. Western blot analysis of crude cell extracts of yeast cells expressing an ER targeted AR fusion protein. **A** Schematic drawings of analyzed samples. A portion of the Suc2p protein (represented in black, numbers in brackets indicate amino acid positions) was fused N-terminally to the $RGSHis_6$-tagged (represented in gray) unselected N3C library member E3_5 (represented by 5 white boxes). **1** Putatively glycosylated and ER targeted protein. **2** Putatively ER targeted protein, subjected to deglycosylation by endoH glycosylase. **3** Truncated protein lacking amino acids 1-19 of the Suc2p portion, putatively cytoplasm-resident. **B** Western blot analysis of the samples described in A. The bands were stained by using an $\alpha RGSHis_6$-antibody.

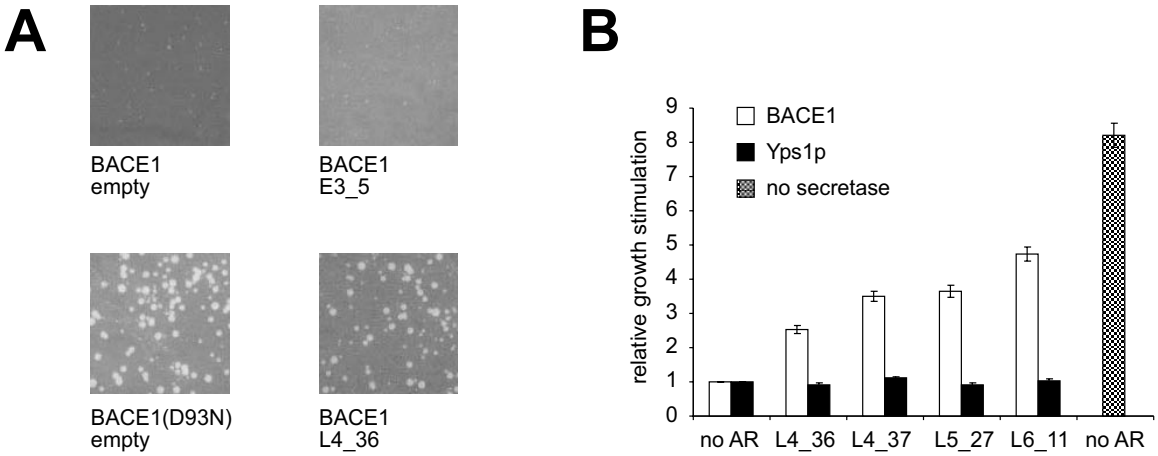

Figure 3. Selected AR proteins stimulate growth of Biscre1 cells in a BACE1-specific manner. **A** Growth of differentially transformed Biscre1 cells on selective plates after three days incubation at 30°C. All transformants express invertase-APP(Sw). Additionally, they express: BACE1 and no AR protein (empty), inactive BACE1 (D93N mutant) and no AR protein (empty), BACE1 and the unselected AR protein E3_5 or BACE1 and the selected AR protein L4_36. The colony formation induced by L4_36 is representative for all the selected AR proteins, which gave again rise to colony formation after plasmid rescue. **B** Growth of differentially transformed Biscre1 cells in selective liquid medium after 26 hours incubation at 30°C. All transformants express invertase-APP(Sw) together with BACE1 (with bars), Yps1p (black bars) or no secretase (gray bar). In addition, the yeast cells express one of four different selected AR proteins (L4_36, L4_37, L5_27, L6_11) or no AR protein. Growth was determined by measuring $OD_{595\ nm}$ and normalized to the samples expressing no AR and the respective secretase. The sample expressing no secretase was normalized to the growth of the culture expressing no AR protein and BACE1.

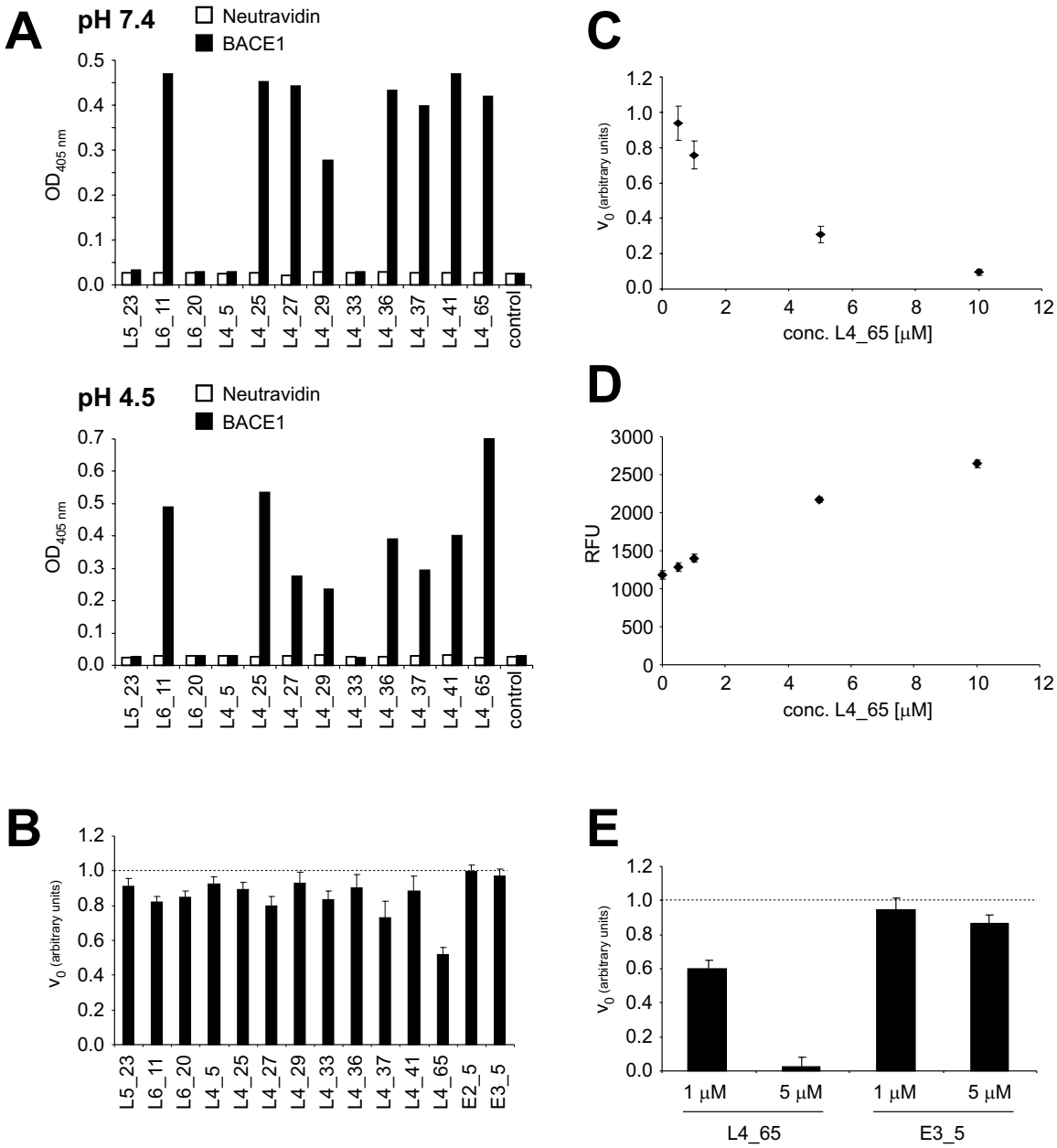

Figure 4. *In vitro* analysis of purified *in vivo* selected AR proteins. **A** BACE1 binding of selected AR proteins at pH 7.4 (upper panel) and pH 4.5 (lower panel). 100 nM of AR protein each was tested for BACE1 ectodomain (black bars) or neutravidin (white bars) binding. The background signal of the detection antibodies has not been subtracted in this representation. **B** Kinetic study of FRET substrate hydrolysis by BACE1 in the presence of 1 μM selected or unselected AR proteins. Displayed initial velocity (v_0) values of the

different reactions were normalized to v_0 values obtained by control reactions in the absence of AR proteins (negative control; dashed line) and represent averages of three samples. **C** Kinetic study of FRET substrate hydrolysis by BACE1 in the presence of increasing concentrations (as indicated) L4_65 protein. **D** Fluorescence signal measured at the beginning of the kinetic study shown in C; RFU: Relative fluorescence units. **E** Kinetic study of FRET substrate hydrolysis by cathepsin D in the presence of 1 and 5 µM L4_65 or E3_5 protein. Displayed v_0 values were normalized to the negative control (dashed line).

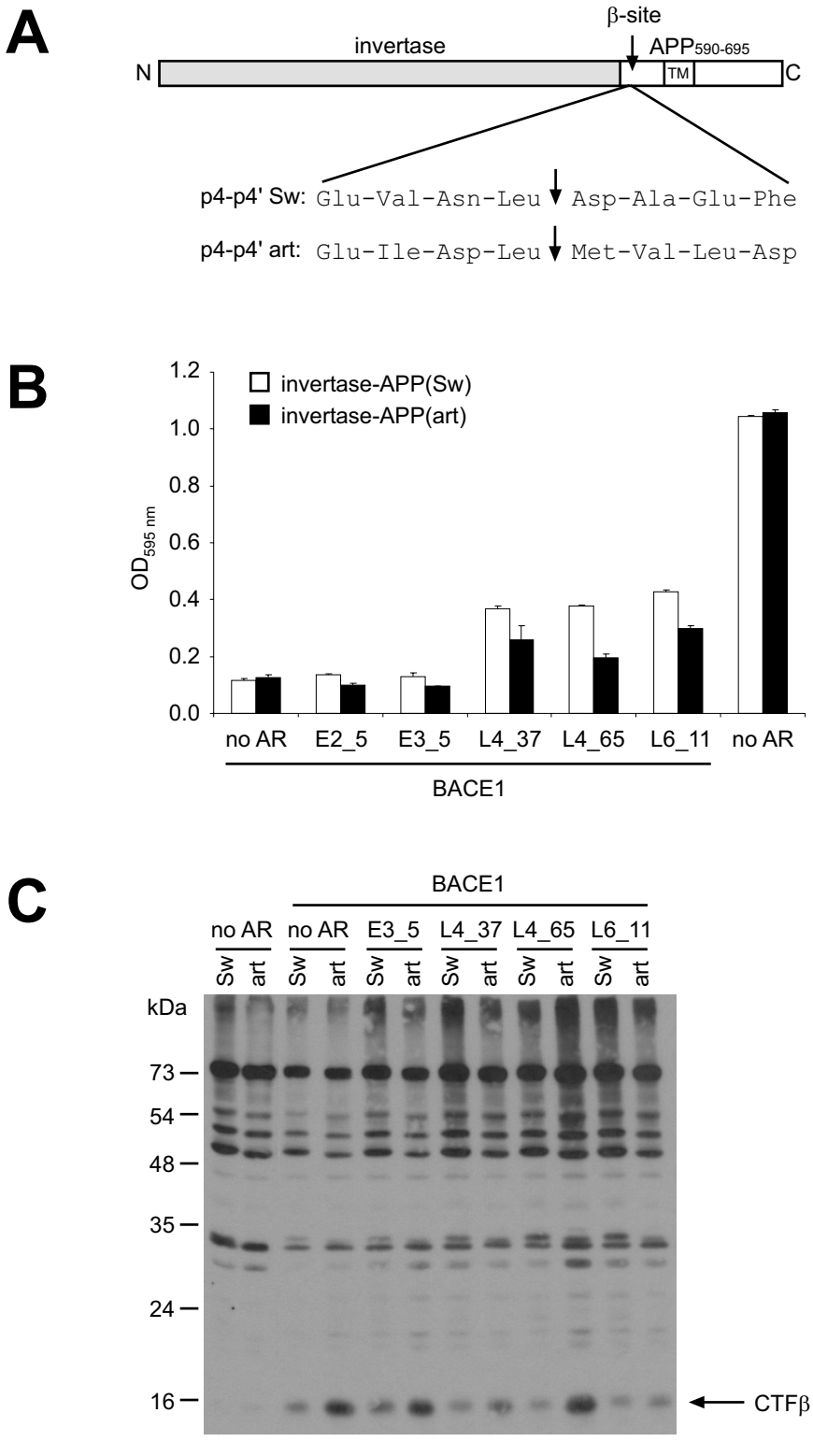
A
β-site
invertase
APP590-695
N
TM
C
p4-p4' Sw: Glu-Val-Asn-Leu Asp-Ala-Glu-Phe
p4-p4' art: Glu-Ile-Asp-Leu Met-Val-Leu-Asp
B
invertase-APP(Sw)
invertase-APP(art)
OD595 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
no AR
E2_5
E3_5
L4_37
L4_65
L6_11
no AR
BACE1
C
BACE1
no AR
no AR
E3_5
L4_37
L4_65
L6_11
Sw
art
kDa
73
54
48
35
24
16
CTFβ

Figure 5

Figure 5. Examinations of an artificial BACE1 cleavage site introduced into the invertase-APP fusion protein. **A** Schematic drawings of the two different invertase-APP fusion constructs that were used. The two constructs with the invertase moiety represented as gray bar and the APP portion represented as white bar are identical except for the p4-p4' sequence of the β-site. The p4-p4' (Sw) sequence originates from the Swedish mutation of APP and was inserted into the invertase-APP(Sw) construct. The p4-p4' (art) is an optimized artificial cleavage site for BACE and was inserted into the invertase-APP(art) construct. The vertical arrows indicate the cleavage site; TM: Transmembrane domain. **B** Growth of differentially transformed Biscre1 cells in selective liquid medium after 26 hours incubation at 30°C. The transformants contain either invertase-APP(Sw) (white bars) or invertase-APP(art) (black bars) together with BACE1 (where indicated) or empty vector. In addition, the cells express an unselected AR proteins (E2_5, E3_5), a selected AR protein (L4_37, L4_65, L6_11) or no AR protein. In this representation there is absolute growth displayed as determined by measuring $OD_{595\ nm}$. **C** Detection of the APP C-terminal fragments (CTFβ) produced by β-site cleavage in differentially transformed Biscre1 one cells by Western blot analysis. Yeast crude cell extract was produced from the same transformants as described in B. The samples were separated on 12% SDS polyacrylamid gel and stained with an antibody directed against the APP C terminus. Sw: invertase-APP(Sw); art: invertase-APP(art). An arrow indicates the position of the CTFβ on the membrane.

3.3.7. Supplementary data

Alignment of sequenced AR protein clones

Note that the N-terminal MLLQAFLFLLAGFAAKISASMTNETSDRPLVHFTPMRGSHHHHHHRSGS sequence was identical in all sequences and has thus been removed in this representation. L4_59 is a N2C protein that was obtained from an N3C selection. The sequence of L4_33 was not well defined.

N3C:

```
        50.................7071.............................105106.............................140141.............................175176.........................206
Input   DLGKKLLEAARAGQDDEVRILMANGADVNAxDxxGxTPLHLAAxxGhLEIvEVLLKzGADVNAxDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNAxDxxGxTPLHLAAxx.GHLEIVEVLLKzGADVNAQDKFGKTAFDISIDNGNEDLAEILQ
L6_18   ..............................m.ty.l.......rh...........h......t.tr.s.......wn...........y......y..i.m.....a.fn............n.............................
L6_20   ..............................m.ty.l.......rh...........h......t.tr.s.......wn...........y......y..i.m.....a.fn............n.............................
L4_5    ..............................k.in.s.......ni...........y......i.nw.w.......iq........m..h......v.in.h.....a.nn............h.............................
L4_7    ..............................k.in.s.......ni...........y......i.nw.w.......iq........m..h......v.in.h.....a.nn............h.............................
L4_65   ..............................q.my.m.......yy...........y......n.tw.v.......yd...........y......e.wl.n.....a.yn............h.............................
L4_67   ..............................q.my.m.......yy...........y......n.tw.v.......yd...........y......e.wl.n.....a.yn............h.............................
L4_44   ..............................w.hy.f.......yy...........y......s.nw.v.......ye...........y......a.fl.t.....a.sl............h.............................
L4_56   ..............................k.vf.m.......hf...........y......d.hw.v..v....fed..........n......n.wl.n.....a.dr............h.............................
L4_41   ..............................f.ea.l.....v.va...........y......t.yw.v.......yv...........n......t.wl.n.....a.fs............h.............................
L4_31   ..............................e.yn.f.......vy...........y......a.hw.i.......yl...........n......e.wldn.....a.tn............y.............................
L4_37   ..............................y.ry.f.......fn...........n......q.yw.v.......ys...........n......n.wl.n.....a.av............n.............................
L6_26   ..............g..............s.wy.f........i...........h......y.rw.v.......ya...........h......m.wi.t.....a.wi............n.............................
L6_16   ..............................f.dn.f.......wy...........y......f.nw.i.......yk...........y......f.ai.t.....a.fl............n.............................
L4_29   ..............................h.te.f.......td...........h......w.nw.i.......fd...........y......v.wl.t.....a.fer...........h.....................s.......
L6_23   ..............................l.ev.e.......fn.....i.....y......l.iy.v.......fd...........h......v.wl.n.....a.ve............n.............................
L4_26   .m.............d.............s.hh.m.......fn...........h......m.yw.i......gye......d....y......n.li.t.....a.ws....d.......h.............................
L4_27   ..............................s.hh.m.......fn.........m.h.....ds.dy.i.......yn...........h......i.hf.t.....a.fn............y.............................
L6_11   ..............................f.al.d.......yi...........h......y.fh.i.......ym...........h......m.af.t.....a.fd............y.............................
L4_25   ..............................k.il.d.......yr...........n......e.ey.i.......yt...........y......k.iy.t.....v.fi............y.............................
L4_36   ..............................s.nl.f.......vy..........nn......l.df.v.......yf...........h......s.fl.t.....a.fd............y.............................
L4_58   ..............................s.yh.m.......vy...........n......t.yw.v.......yf...........h......s.fl.ts....a.fd............y.............................
L6_14   ..............................l.nw.f.......ty...........n......k.sw.v.......ys...........h......k.if.t.....a.fd............n.............................
L4_30   ..............................v.hy.n.......vm...........y......n.dw.v.s.....yt...........n......i.ym.t.....a.fd............y.............................
L4_22   ..............................v.qf.s.......vm...........y......s.hw.v.......yv...........h......k.ii.t.....a.fd............y.............................
L4_8    ......................t.......t.hy.s.......ir.q.........n......i.nw.i.......yt...........n......m.yi.t.....a.fd............n.............................
L6_22   ..............................y.im.m.......hf...........n......r.dw.i.......yv..q.......nh......d.yi.t.....a.ft............y.............................
L4_50   ........................d.....k.dl.n.......vw...........y......k.wy.i.......is...........n......e.yi.t.....a.fi............h.............................
L4_33   ..............................x.xx.x.......xx...........y......x.yw.x.......xx...........x......x.dx.t.....a.yx............y.............................
```

N2C:

```
        50.................7071.............................105106.............................140141.........................
Input   DLGKKLLEAARAGQDDEVRILMANGADVNAxDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNAxDxxGxTPLHLAAxxGHLEIVEVLLKzGADVNAQDKFGKTAFDISIDNGNEDLAEILQ
L5_12   ..............................m.iw.l.......dl...........y...i..d.mf.v.......fw...........h.............................
L5_30   ..............................m.iw.l.......dl...........y...i..d.mf.v.......fw...........h.............................
L5_27   ..............................m.iw.l.......dl...........y......d.mf.v.......fw...........h.............................
L5_28   ..............................v.iw.l.......dl...........y......d.mf.v.......fw...........h.............................
L5_32   ..............................m.iw.l.......dl...........y.t....d.mf.v.......fw...........h.............................
L4_59   ............................tm.iw.l.......dl...........y......d.mf.v.......fw...........h.............................
L5_21   ......................k......m.iw.l.......dl...........y......d.mf.v.......fw...........h.............................
L5_29   ..............................m.iw.l.......dl...........yc.....d.mf.v.......fw...........h.............................
L5_23   ..............................m.gl.d.......le...........y......a.mf.mi......yi...........y.............................
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3.3.8. References

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4. Conclusions

The present thesis describes a unique growth readout in yeast for the identification of BACE1 inhibitors. From a biological point of view BACE1 is widely considered as an ideal drug target for AD therapy. The best progress towards therapeutic inhibition of BACE1 has so far been achieved by the use of peptidomimetic compounds. No lead structures originating from successful screenings of small molecule libraries have been published so far. A potential problem of *in vitro* screenings might be the artificial nature of the reconstituted reaction, in which the naturally membrane-bound substrate is represented by a short peptide and the naturally membrane-bound enzyme by the soluble BACE1 ectodomain. The difficulties of conventional cell-based assays are demanding liquid handling requirements, low throughput and a high rate of false-positive compounds that interfere in an unspecific manner with the readout.

As in the natural situation in mammalian cells, the reconstituted β-site cleavage in yeast takes place in topologically extracellular compartments and employs membrane-bound substrate and membrane-bound BACE1. In these aspects, the yeast assay is comparable to conventional cell-based assays. In a screening for BACE1 inhibitors, however, the yeast assay displays considerable advantages over conventional cell-based assays being minimized liquid handling, the possibility to rapidly exclude false positives by the use of simple controls and the positive growth readout, which allows to excludes toxic compounds.

The system was initially validated with two bona fide BACE1 inhibitors, which were developed by Novartis Pharma. These inhibitors stimulated the growth of BACE1-expressing yeast cultures in a concentration-dependent manner, whereas they did not affect the growth of Yps1p-expressing cultures. In order to further validate the system and to identify BACE1 inhibitors, the yeast selection system was applied in a screening of 15'000 small molecules. The screening revealed six compounds which significantly reduced the secretion of Aβ from HEK 293 APP(Sw) cells. The final proof that they indeed affect BACE1 activity by an *in vitro* assay, however could not yet unambiguously be presented. Therefore the identified compounds await further characterization to demonstrate inhibition of β-site cleavage in cell culture by Western blot analysis.

Due to the limited size of the screened library, the evaluation of the system should not be reduced to the potential value of identified compounds. It can be stated that the screening did not yield toxic compounds, and a high number of compounds that were activating the system independent of BACE1 inhibition could be eliminated due to simple specificity controls - as this was predicted. The statistical performance as well as the practicability of the system can be considered superior as compared to conventional cell-based assay.

In an attempt to further validate the yeast selection system, it was applied for a selection on plates to identify artificial ankyrin repeat (AR) protein-based inhibitors of BACE1. This selection yielded a number of proteins, which stimulated the growth of yeast cells in a β-site specific manner, which means that they did not stimulate the growth of Yps1p-expressing cultures, but leaves the possibility that cultures expressing an alternative β-secretase would also be stimulated by the selected AR proteins. Further experiments would be necessary to determine the exact mode of action of the selected AR proteins in yeast.

In conclusion the data presented in this thesis demonstrate that the yeast selection system can be a powerful tool to identify BACE1 inhibitors and therefore the screening of larger small molecule libraries with this system would be a valuable approach to identify additional small molecule lead structures.

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I thank my girlfriend for loving me.

6. Attachments

6.1. Curriculum vitae

Name	Middendorp
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Geburtsdatum	7. August 1973 in Winterthur ZH
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Ausbildung

1980-1986	Primarschule Oberwinterthur	
1986-1989	Sekundarschule Oberwinterthur	
1989-1994	Kantonsschule Im Lee Winterthur, Typus Lehramt	
1994-1999	Studium der Biologie, Universität Zürich	
	1994-1995	1. Vordiplom
	1995-1996	2. Vordiplom
	1996-1999	Diplomstudiom der Molekularbiologie mit den Nebenfächern Biochemie und Immunologie
	Diplomarbeit: *"Generation of Mouse Embryonic Stem Cells with Mutated p300"* unter der Leitung von Dr. Richard Eckner, begutachted von Prof. Dr. Walter Schaffner	
1999-2000	Fachdidaktik Biologie, Universität Zürich	
2001-jetzt	Dissertation in der Biotechnologie-Firma ESBATech AG, Schlieren, unter der Leitung von Dr. Alcide Barberis (ESBATech AG) und Prof. Howard Riezman (Biozentrum, Basel)	
	Titel der Dissertation: *"Yeast growth selection system for the identification of cell-active inhibitors of β-secretase"*	

6.2. Publication list

Middendorp, O., Ortler, C., Neumann, U., Paganetti, P., Lüthi, U., and Barberis, A. (2004). Yeast growth selection system for the identification of cell-active inhibitors of β-secretase. Biochim. Biophys. Acta *1674,* 29-39

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Lüthi, U., Schaerer-Brodbeck, C., Tanner, S., Middendorp, O., Edler, K., and Barberis, A. (2003). Human β-secretase activity in yeast detected by a novel cellular growth selection system. Biochim. Biophys. Acta *1620*, 167-178

6.3. Scientific conferences

Growth and Death in the nervous system, St. Mortitz, March 2004

Yeast 2003 Conference, Göteborg, June 2003