

NOVEL REGULATORY PROTEINS AND INHIBITORS

TECHNICAL FIELD

[0001] The field relates to a previously uncharacterized protein that activates γ -secretase to produce β -amyloid protein ($A\beta$). Deposition of $A\beta$ has been associated with Alzheimer's disease and other pathologies. The invention provides, e.g., screening methods and novel research tools, inhibitors of this novel protein, and methods of treatment and control of Alzheimer's disease and other neurodegenerative conditions associated with deposition of $A\beta$.

BACKGROUND OF THE INVENTION

[0002] Currently, over twelve million people worldwide suffer from Alzheimer's Disease (AD). This number is predicted to quadruple over the next 40 years. As such, treatment of AD represents a major unmet medical need. Currently approved medicines to treat AD may help ameliorate symptoms, but are not effective to stop progression of the disease.

[0003] Without being bound by a particular theory, it is believed that the pathology of Alzheimer's disease ("AD") involves amyloid- β (" $A\beta$ ") peptides, which are metabolites of β -amyloid precursor protein (Alzheimer's disease-associated precursor protein or "APP"), and are believed to be major pathological determinants of AD. These peptides exist mainly in 40 or 42 amino acid forms, $A\beta$ 1-40 (" $A\beta$ 40") and $A\beta$ 1-42 (" $A\beta$ 42"), respectively. $A\beta$ 40 and $A\beta$ 42 are generated by two enzymatic cleavages occurring close to the C-terminus of APP, subsequent to cleavage by beta secretase. The enzymes responsible for the cleavage, β -secretase and γ -secretase, generate the N- and C-termini of $A\beta$, respectively. The amino terminus of $A\beta$ is formed by β -secretase cleavage between methionine residue 596 and aspartate residue 597 of APP (numbering based on APP 695 isoform). γ -secretase cleaves at varying positions 38-, 40- or 42-residues at the C-terminal of this β -secretase cleavage product to release the $A\beta$ peptides. A third enzyme, α -secretase, cleaves the precursor protein between the β - and γ -cleavage sites, thus precluding $A\beta$ production and releasing an approximately 3 kDa peptide known as P3, which is non-pathological. Both β - and α -secretase cleavage also result in soluble, secreted-terminal fragments of APP, known as sAPP β and sAPP α , respectively. The sAPP α fragment has been suggested to be

neuroprotective. For example, γ -secretase also cleaves Notch-1 protein and is believed to have other substrates. Direct acting gamma-secretase inhibitors have substantial and unwanted side effects due to the effects on development pathways requiring Notch cleavage. Little is known about the molecular mechanisms that confer substrate specificity on this potentially promiscuous enzyme. Gamma secretase enzyme is known to contain four subunits: presenilin, nicastrin, anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2).

[0004] In normal individuals, the A β peptide is found in two predominant forms, the majority A β -40 (also known as A β 1-40) form and the minority A β 42 (also known as A β 1-42) form, each having a distinct COOH-terminus. The major histological lesions of AD are neuritic plaques and neurofibrillary tangles occurring in affected brain regions. Neuritic plaques consist of A β peptides, primarily A β 40 and A β 42. Although healthy neurons produce at least ten times more A β 40 compared to A β 42, plaques contain a larger proportion of the less soluble A β 42. Patients with the most common form of familial Alzheimer's disease show an increase in the amount of the A β 42 form. The A β 40 form is not associated with early deposits of amyloid plaques. In contrast, the A β 42 form accumulates early and predominantly in the parenchymal plaques and there is strong evidence that A β 42 plays a major role in amyloid plaque deposits in familial Alzheimer's disease patients. Neurofibrillary tangles consist of aggregated tau protein and their role in AD pathology is less clear. AD symptoms are most closely correlated with total brain A β rather than plaques. About 10% of AD cases result from autosomal dominant inheritance of mutations in either the APP or the presenilin 1 and presenilin 2 genes. In both cases, increased production of total A β or A β 42 versus A β 40 results.

[0005] The N2a cell system has been extensively studied as a model system of A β production relevant to neurodegeneration in AD. Assays measuring production of A β in N2a cells are known, wherein A β -production activity is evaluated e.g., by A β ELISA assay and/or by Western blotting. Various agents including compounds such as Gleevec (Imatinib, STI571) have previously been shown to be capable of lowering A β levels in the N2a cell system at drug concentrations of below 10 μ M.

[0006] International Patent Publication No. WO 03/057165 discloses that certain previously known inhibitors of tyrosine kinases, such as imatinib, are useful to inhibit the production of and accumulation of A β . Netzer et al., *Proc Natl Acad Sci.*, 100(21):12444-9

(2003) showed that imatinib inhibits production of A β without affecting γ -secretase cleavage of Notch-1 and without unacceptable toxicity to the neurons. Imatinib is not an ideal drug for treating AD, however, because it does not penetrate the blood brain barrier very well, and it has other biological effects. The specific target of imatinib for inhibition of production of and accumulation of A β has not been defined, so finding improved derivatives presents challenges.

SUMMARY OF THE INVENTION

[0007] We have discovered a novel γ -secretase activating protein (gSAP) that selectively modulates A β production through a mechanism involving its interactions with both γ -secretase and its substrate, amyloid precursor protein C-terminal fragment (APP-CTF). gSAP does not interact with Notch nor affect its cleavage by γ -secretase. Recombinant gSAP stimulates A β production *in vitro* and in intact cells. Reducing gSAP in cell lines decreases A β levels. Knockdown of gSAP in mouse models of Alzheimer's disease reduces levels of A β and plaque development. gSAP represents a novel therapeutic target for the treatment of Alzheimer's disease and other A β -mediated conditions, as inhibition of gSAP results in significant reduction in A β formation.

[0008] Applicants have synthesized compounds that inhibit the gSAP and thus lead to decreased A β , but do not affect NOTCH metabolism, which is a potential side effect with use of some gamma-secretase inhibitors.

[0009] Accordingly, the invention provides, e.g., methods of screening compounds for potential utility in reducing A β comprising measuring their ability to selectively inhibit gSAP, and methods of treating A β -mediated conditions, such as AD, comprising administering effective amounts of a compound which selectively inhibit gSAP.

DESCRIPTION OF DRAWINGS

[0010] Figure 1 depicts a sequence alignment for gSAP from various animal species: human, canine, bovine, murine, rat, and chicken. Residues in bold are identical across the six species; residues in italics are conservatively substituted.

[0011] Figure 2 depicts expression levels of gSAP in different tissues. Tissues from 3 month old wild type BL/6 mice are harvested and gSAP levels are quantitated using real time

PCR. Both actin and GAPDH serve as internal controls (n=6). Tissue extracts are adjusted to the same protein levels prior to analysis.

[0012] Figure 3 shows inhibition of gSAP by shRNA reducing A β production and plaque development in an AD mice model.

5 [0013] Figure 4 shows effect of intrahippocampal injection of AAV2-carrying shRNA against gSAP in reducing amyloid plaque development in double transgenic AD mice.

[0014] Figure 5 shows gSAP action on APP processing. Ternary complex of gSAP, APP and γ -secretase (top) is associated with elevated γ -cleavage (A β -beta production) and
10 reduced ϵ -cleavage (AICD production). In the absence of gSAP (bottom), the binary complex of APP and ϵ -secretase is associated with decreased γ -cleavage and increased ϵ -cleavage.

[0015] Figure 6 shows truncation of APP-CTF and immunoprecipitation using gSAP antibody through gSAP, demonstrating that gSAP interacts with the juxtamembrane region of APP-CTF. APP-CTF-T1 is the truncated form of APP- β -CTF spanning from its N-terminus
15 to HHGV⁶⁴. APP-CTF-T2 is the truncated form of APP- β -CTF spanning from its N-terminus to VMLKK⁵⁵. Truncated forms are overexpressed in HEK293 cells and immunoprecipitated with gSAP antibody. 6E10 antibody is used for immuno-detection.

DETAILED DESCRIPTION

20 [0016] The examples and drawings provided in the detailed description are merely examples, which should not be used to limit the scope of the claims in any claim construction or interpretation.

[0017] Senile plaques composed predominantly of A β peptides are a hallmark of Alzheimer's disease. A β is derived from APP-CTF upon cleavage by γ -secretase. γ -Secretase
25 also cleaves many other type I membrane proteins (such as Notch), leading to the release of intracellular domains with critical cellular functions. As a result, non-selective γ -secretase inhibitors may have detrimental side effects that prevent their clinical use. This laboratory previously demonstrated that imatinib (STI571, Gleevec®) can inhibit production of all species of A β without influencing Notch cleavage. Netzer et al., *Proc Natl Acad Sci.*,
30 100(21):12444-9 (2003). We have now determined that the A β -lowering activity of imatinib

results from its interaction with a previously unknown factor, which we designate γ -secretase activating protein (gSAP).

[0018] To identify the target responsible for imatinib's selective A β -lowering activity, we synthesized a biotinylated derivative of imatinib, "biotin-imatinib". Solubilized γ -secretase components, including presenilin-1, PEN2, and nicastrin, are specifically captured by the immobilized biotin-imatinib. To identify the protein with which imatinib directly interacts, we synthesized a photoactivatable azido imatinib derivative, G01. When ^{125}I -G01 is incubated with a membrane preparation followed by photolysis, none of the four components of γ -secretase are labeled. Rather, ^{125}I -G01 labels a ~ 16 kDa protein, which co-immunoprecipitates with the more slowly migrating 18 kDa presenilin-1-CTF. This result is confirmed by intact cell photolabeling using cell permeable ^3H -G01. Similar to ^{125}I -G01, the ^3H -imatinib derivative does not bind to any of the four γ -secretase components, but does label a band of ~ 16 kDa that co-immunoprecipitated with PS1.

[0019] To purify the potential target protein, immobilized biotin-imatinib is incubated with solubilized membrane preparations and bound proteins are separated by SDS-PAGE. After silver staining, a ~ 16 kDa band is observed. Peptide fragments, derived from this band after trypsin digestion, and analyzed by tandem mass spectrometry, corresponded to the C-terminal region of an uncharacterized protein, pigeon homologue protein (PION) (human accession number: NP_059135). The identification is made based on two unique tryptic peptides ($^{766}\text{LWDHPMSSNIISR}^{778}$ and $^{779}\text{NHVTRLLQNYKK}^{790}$) covering approximately 20% of the 16 kDa fragment. Its sequence, especially the C-terminal region, is highly conserved among multiple species from chicken to human. Expression pattern analysis indicates that this gene is expressed in diverse tissues. We characterize PION as a gamma-secretase activating protein (gSAP).

[0020] Based on its predicted sequence, the full opening reading frame of human gSAP encodes a protein of 854 amino acids (~ 98 kDa). To determine whether the 16 kDa fragment might be derived from a high molecular weight precursor, the metabolism of endogenous gSAP in neuroblastoma cells is monitored by pulse-chase analysis. The results showed that gSAP is synthesized as a holo-protein (~ 98 kDa) and is rapidly processed into a ~ 16 kDa C-terminal fragment (gSAP-16K). In the steady state, the 16 kDa fragment is the predominant form. Incubation of neuroblastoma cells with photoactivatable ^3H -G01 followed by immunoprecipitation with anti-gSAP antibody confirms that imatinib directly

binds gSAP-16K. In presenilin 1/2 (-/-) embryonic stem cells, imatinib also binds gSAP, indicating that its binding to gSAP does not require presenilins. When gSAP levels are reduced using siRNA, the amount of γ -secretase associated with biotin-imatinib decreases significantly. This indicates that the affinity of imatinib for the γ -secretase complex depends on gSAP.

[0021] When siRNA is used to reduce gSAP level (by $72 \pm 15\%$) in neuroblastoma cells overexpressing APP695, the level of A β decreases about 50%. The addition of imatinib has little or no additional effect on A β levels. ShRNA-mediated gSAP knockdown (by $65 \pm 12\%$) in HEK293 cells expressing APP Swedish mutation also results in a decrease of A β 40 and A β 42 levels of 61% and 48%, respectively. Conversely, overexpression of gSAP in HEK293 cells expressing the APP Swedish mutation stimulates A β production by approximately 38%; the increase is abolished by imatinib treatment. Together, these findings indicate that gSAP is the molecule through which imatinib and related molecules lower A β .

[0022] One distinctive feature of imatinib is its selective inhibition of A β production while sparing Notch cleavage (Netzer et al., 2003). The effect of gSAP on Notch cleavage is evaluated using HEK293 cells stably expressing Notch ΔE (Notch without its extracellular domain), the Notch substrate for γ -secretase. The level of the γ -secretase cleavage product, the Notch intracellular domain (NICD), is not changed either by reducing gSAP levels using shRNA or by overexpressing gSAP. Thus, gSAP modulates the γ -secretase cleavage of APP, but not of Notch.

[0023] To further test whether gSAP can modulate γ -secretase activity, the effect of purified gSAP on A β production is examined in an *in vitro* γ -secretase assay. When recombinant gSAP-16K (aa 733-854 of full length human gSAP), isolated after expression in *E.coli*, is added to membrane preparations from HEK293 cells containing overexpressed APP- β -CTF, A β production is stimulated 2.4 ± 0.3 fold. These *in vitro* results suggest that gSAP stimulates A β production by direct regulation of γ -secretase activity.

[0024] To determine whether endogenous gSAP might be in a complex with γ -secretase, we use gel filtration analysis of membrane proteins from neuroblastoma cells solubilized in 1% CHAPSO. Endogenous gSAP-16K and γ -secretase co-migrate as a high molecular weight complex. In addition, endogenous gSAP co-immunoprecipitates with γ -secretase components, providing further evidence that these proteins exist in a complex in

cells. These results, together with those from the *in vitro* γ -secretase activity assay, strongly suggest that gSAP-16K is in a complex with γ -secretase and can activate the protease.

[0025] In contrast to some other regulators of γ -secretase activity, gSAP is selective and influences cleavage of APP but not of Notch. Although the mechanism of substrate selection by γ -secretase remains unclear, a number of other proteases and phosphatases with broad substrate recognition can achieve specificity through auxiliary factors that couple the core enzyme to a subset of substrates. To determine the mechanism by which gSAP might confer such specificity, we analyze its binding to specific substrates in HEK293 cells. gSAP-16K immunoprecipitates with APP-CTF but not with Notch ΔE . Addition of imatinib (10 μ M) reduces the interaction between gSAP and APP-CTF by $47 \pm 14\%$ ($n=3$). The binding of gSAP to APP-CTF, but not to Notch, may account for the selective effect of gSAP on APP processing. Disruption of this interaction by imatinib seems likely to explain its A β -lowering activity.

[0026] The site of interaction between gSAP and APP is determined to be in the juxtamembrane region of APP-CTF (Figure 6). APP-CTF is cleaved by γ -secretase in the middle of its transmembrane domain to generate A β (γ -cleavage) and near its cytosolic membrane boundary to generate APP intracellular domain (AICD) (ϵ -cleavage). The effect of gSAP on AICD production is examined in N2a cells overexpressing APP695. Both gSAP knockdown and imatinib treatment increases levels of AICD (supplementary Fig. 7a). gSAP overexpression in HEK293 cells reduces AICD production (supplementary Fig. 7b). These results indicate that gSAP differentially regulates γ - and ϵ -cleavage of APP-CTF to form A β and AICD respectively.

[0027] To determine whether our findings are relevant to AD pathology, the effects of gSAP on soluble A β levels and on plaque development are examined *in vivo*. A gSAP knockdown mouse line is generated by integration of tetracycline-inducible gSAP shRNA vector into the mouse genomic locus. Upon induction, gSAP mRNA level in the mice brain is reduced by $\sim 85\%$. To evaluate the effect of gSAP on A β levels *in vivo*, gSAP RNAi mice are cross-bred with an AD mouse model with APP^{swe} and PS1 $\Delta 9$ mutations (AD 2 $\times$ mice) (Jankowsky et al. 2001). After 1 month gSAP shRNA induction, A β 40 and A β 42 levels in the cross-bred mice are lowered by $\sim 28\%$ and $\sim 32\%$, respectively. To evaluate the effect of gSAP on plaque development in AD 2 $\times$ mice, recombinant adeno-associated virus 2 (AAV2) carrying gSAP shRNA is injected into plaque-developing hippocampus on one side of the

brain, while the contralateral side receives the AAV2 without shRNA. Amyloid plaque development on the ipsilateral side of gSAP knockdown is suppressed by $26 \pm 8\%$ ($p < 0.001$, $n=4$) as compared to the contralateral side after 1 month. These data indicate that gSAP plays a critical role in A β formation and plaque development *in vivo*.

5 **[0028]** To summarize, gamma-secretase processes diverse substrates with low homology at their cleavage sites. The various roles of γ -secretase during development and in tissue homeostasis require that its activity be tightly regulated. Recent reports have shown the existence of biological molecules that modify the selectivity of cleavage by γ -secretase. The discovery of gSAP, a novel protein the function of which is previously unknown, and its
10 ability to selectively stimulate A β formation, enables new strategies for the development of drugs to address AD and other A β -mediated diseases, such as Alzheimer's disease, progressive supranuclear palsy, Down Syndrome, memory and cognitive disorders, dementia, amyloid neuropathies, brain inflammation, nerve and brain trauma, vascular amyloidosis, cerebral hemorrhage with amyloidosis, Parkinson's disease, Huntington's disease, prion
15 disease and/or vascular, neurological, and/or neurodegenerative disorders related to the abnormal expression or accumulation of tau or amyloid proteins such as A β . Inhibitors of gSAP will selectively prevent β amyloid formation without affecting other key functions of γ -secretase. In support of this view, we demonstrate that a widely used anti-cancer drug, imatinib, achieves its A β -lowering effect by preventing gSAP activation of γ -secretase to
20 generate A β , but without affecting other γ -secretase functions. The discovery of potent and direct pharmacological inhibitors of gSAP should facilitate the development of novel therapeutic reagents for the treatment of Alzheimer's disease.

[0029] gSAP is found to exist in a variety of animal species, e.g., as depicted in Figure 1, and is highly conserved. A gSAP peptide is a peptide which is

- 25 a. at least 60%, preferably at least 70%, e.g., at least 80%, for example at least 90% similar to a sequence selected from the sequences depicted in Figure 1, using a BLAST algorithm (see e.g. <http://blast.ncbi.nlm.nih.gov/Blast.cgi>)
- b. a peptide having residues corresponding to the conserved residues in bold in Figure 1, or
- 30 c. a peptide comprising the sequence Seq ID No. 7 (LWDHPMSSNIISR) and/or Seq ID No. 8 (NHVTRLLQNYKK), or

- d. any of the peptides of Seq ID Nos. 1-6 as depicted in Figure 1, especially human gSAP, or
- e. a fragment of gSAP capable of forming a complex with gamma secretase, e.g., a fragment of approximately 16 kDa from the C-terminal of any of Seq ID Nos. 1-6 as depicted in Figure 1, especially human gSAP-16K.

5 [0030] In one aspect the invention provides a gSAP peptide as defined above isolated and purified from its natural environment, for example a transgenic gSAP peptide, e.g. produced by a bacterial, baculovirus or mammalian cell.

[0031] In another aspect, the invention provides a vector comprising a gene for a gSAP peptide operably linked to a heterologous promoter.

10 [0032] In another aspect, the invention provides a cell containing a heterologous gene expressing a gSAP peptide.

[0033] In another aspect the invention provides inhibitory RNA constructs capable of inhibiting gSAP expression in a mammalian cell.

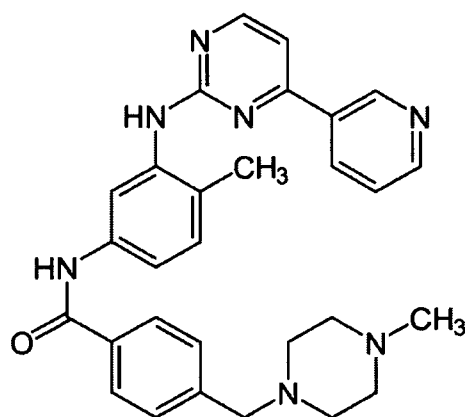
15 [0034] In another aspect, the invention provides

- a. monoclonal antibodies to gSAP, e.g., capable of inhibiting its interaction with gamma-secretase and so inhibiting or reducing as A β production and accumulation;
- b. vaccines comprising an immunogenic fragment of gSAP in combination with a suitable adjuvant and/or carrier; and
- c. immunogenic conjugates comprising an immunogenic fragment of gSAP linked to an immunogenic carrier.

20 [0035] In another aspect, the invention provides gSAP knockout mammals, e.g., mammals, for example mice, wherein their gene for gSAP is disrupted.

25 [0036] In another aspect, the invention provides the use of a gSAP peptide, e.g. as defined above, in an assay to identify compounds which inhibit A β -deposition, e.g., a method of identifying an inhibitor of A β -deposition, comprising one or more of the following:

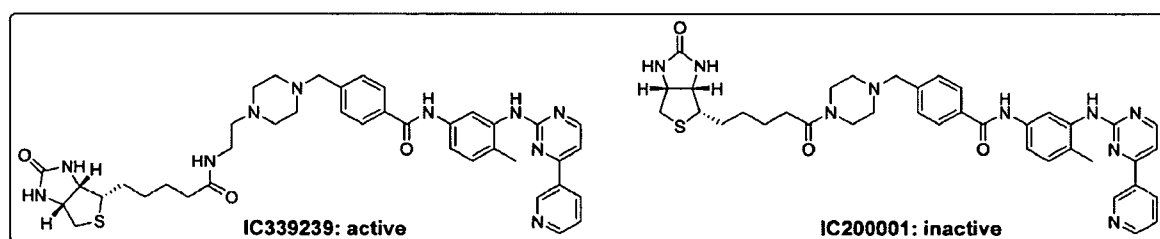
- a. measuring binding of test compounds to a gSAP peptide, e.g., as defined above,
 - i. e.g., in a competitive binding assay,
 - ii. e.g., using labeled derivatives of imatinib:



iii. e.g., labeled by substitution by or modification with a labeling group at the methylpiperizinyl moiety,

iv. e.g., selected from

1. photolabeled derivatives, e.g., 4-azido-2-hydroxy-*N*-(4-methyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)phenyl)benzamide,
2. radiolabeled derivatives, e.g., 4-azido-2-hydroxy-5-¹²⁵I-iodo-*N*-(4-methyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)phenyl)benzamide, or ³H-imatinib, and
3. biotinylated derivatives, e.g., selected from IC339239 and IC2000001 (an inactive control compound):



[0037] The invention thus additionally provides labelled derivatives of imatinib, e.g., as described above.

[0038] The invention additionally provides a method of identifying persons at risk of developing AD comprising checking for elevated expression levels and/or mutations in gSAP relative to normal values as identified using a control population.

[0039] According to a further feature of this aspect of the invention there is provided

a method for producing an inhibitory effect against the accumulation of abnormal protein aggregates in a warm-blooded animal, such as man, in need of such treatment which comprises administering to said animal an effective amount of a compound of formula (I), or a pharmaceutically acceptable salt thereof.

5 [0040] Furthermore, the compounds of this invention are useful in the treatment, control and management of diseases characterized by accumulation of abnormal protein aggregates, especially in the brain – for example, diseases such as Alzheimer’s disease, progressive supranuclear palsy, Down Syndrome, memory and cognitive disorders, dementia, amyloid neuropathies, brain inflammation, nerve and brain trauma, vascular amyloidosis, cerebral hemorrhage with amyloidosis, Parkinson’s disease, Huntington’s disease, prion disease and/or vascular, neurological, and/or neurodegenerative disorders related to the abnormal expression or accumulation of tau or amyloid proteins such as A β . Such abnormal protein aggregates include, for example, i) amyloid plaques and neurofibrillary tangles, and ii) precipitates of tau or amyloid proteins such as A β .

15 [0041] Accordingly, the present invention provides methods of treatment of Alzheimer’s disease, progressive supranuclear palsy, Down Syndrome, memory and cognitive disorders, dementia, amyloid neuropathies, brain inflammation, nerve and brain trauma, vascular amyloidosis, cerebral hemorrhage with amyloidosis, Parkinson’s disease, Huntington’s disease, prion disease and/or vascular, neurological, and/or neurodegenerative disorders related to the abnormal expression or accumulation of tau or amyloid proteins such as A β , comprising administering an effective amount of a compound to inhibit gSAP activity.

20 [0042] Compounds useful to inhibit gSAP activity include known small molecules, e.g.

- 25 a. Imatinib and other compounds as disclosed in International Patent Publication No. WO 03/057165 and in U.S. Patent No. 5,521,184, the contents of which are incorporated herein by reference;
- b. Compounds as described in WO 05/072826; J. Zimmermann et al., Bioorganic & Medicinal Chem. Lett., 7(2): 187- 192; EP 1 533 304; WO 04/005281; WO 05/039586; U.S. Patent No. 5,521,184; and WO 04/110452, the contents of which are incorporated herein by reference; and
- 30 c. Compounds as disclosed in WO/2008/153974, WO/2008/153959, and WO/2008/057599, the contents of which are incorporated herein by reference.

[0043] Compounds useful to inhibit gSAP activity also include novel biotherapeutics, as described herein, including

- a. Inhibitory RNA molecules, e.g., selected from double-stranded, hairpin, sense or antisense RNA corresponding to a portion of the mRNA for gSAP, and capable of inhibiting gSAP transcription or translation; for example,

- i. siRNAs comprising sense sequence e.g.,

AUGCAGAGCUGGACGACAUUU and antisense sequence e.g. 5'-
P.AUGUCGUCCAGCUCUGCAUUU; or

- ii. hairpin transcripts produced by a gSAP shRNA coding sequence, e.g.

TCCCGGAACTCCATGATTGACAAATTTCAAGAGAATTTGTCA
ATCATGGAGTTCC TTTTTA or
TGCTGTTGACAGTGAGCGCGGAAATAGAGTGGTGATTAAAT
AGTGAAGCCACAGATGTATTTAATCACCACTCTATTTCCATG
CCTACTGCCTCGGA;

- b. Vectors and cells producing inhibitory RNA molecules, e.g. recombinant adeno-associated virus 2 (AAV2) carrying gSAP shRNA;
- c. Antibodies to gSAP, especially monoclonal antibodies, for example antibodies raised against fragments from the C-terminal region, e.g., 16K-gSAP, for example antibodies raised against the peptide
CFEGHDNVDAEFVEEAALKHT (corresponding to aa 829-848 of human gSAP with an N-terminal cysteine attached for conjugation) as described more fully below;

- d. Vaccines for gSAP, comprising a fragment of gSAP in combination with an immunogenic adjuvant and/or carrier, e.g., conjugated to an immunogenic carrier, e.g., a bacterial toxoid, e.g. diphtheria or tetanus toxoid, keyhole limpet hemocyanin (KLH), blue carrier protein, ovalbumin, or bovine serum albumin, and/or delivered together with an adjuvant, e.g. Freund's adjuvant or alum adjuvant.

[0044] The invention provides, in a further embodiment, methods to identify candidates for treatment with gSAP-targeted therapy, e.g., , e.g. administration with gSAP inhibitors or vaccines as described above, the methods selected from, e.g.,

1. Measuring gSAP expression, e.g., using antibodies to gSAP or quantitative PCR for gSAP expression, wherein elevated expression is seen in candidates for treatment with gSAP targeted therapy; or

2. Identifying patients having mutations affecting gSAP or gSAP expression, e.g., having haplotypes comprising any of the following groups of SNPs

- a) rs6976567|rs1468682|rs1819814,
- b) rs1468682|rs1819814|rs4729535,
- c) rs1819814|rs4729535|rs4729540,
- d) rs7781642|rs6955503|rs7776973

wherein patients having such mutations or haplotypes are candidates for treatment with gSAP targeted therapy;

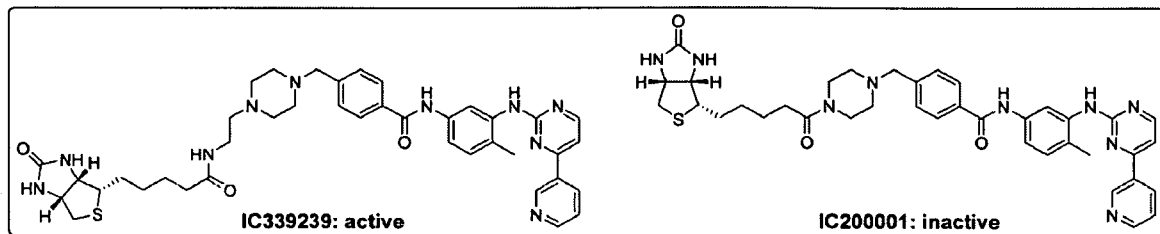
3. Identifying patients having mutations affecting gSAP activity, e.g., mutations at the sequence encoding the juxtamembrane region of APP-CTF, wherein patients having such mutations or haplotypes are candidates for treatment with gSAP targeted therapy.

[0045] The invention further provides diagnostic assay kits for use in such methods, e.g., comprising monoclonal antibodies to gSAP, or primers to the gSAP gene or fragments thereof, and oligonucleotide probes to detect mutations in the gSAP gene or in the juxtamembrane region of the gene for APP-CTF.

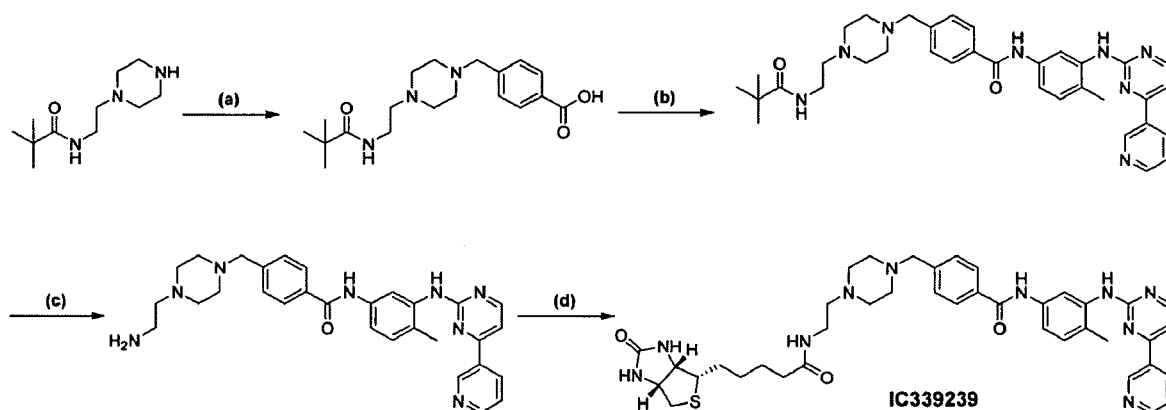
Example 1 – Synthesis of labeled imatinib derivatives

[0046] 2,5-dioxopyrrolidin-1-yl 4-azido-2-hydroxybenzoate (NHS-ASA) is purchased from ProChem. Inc (Rockford, IL). 6-Methyl-*N*¹-(4-(pyridin-3-yl)pyrimidin-2-yl)benzene-1,3-diamine and *N*-(4-methyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)phenyl)-4-(piperazin-1-ylmethyl)benzamide (*N*-desmethyl imatinib) are purchased from ChemPacific Inc (Baltimore, MD). 2,5-dioxopyrrolidin-1-yl 5-((3a*S*,4*S*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (Biotin-OSu), *N*-(chloro(dimethylamino)methylene)-*N*-methylmethanaminium hexafluorophosphate (TCFH), trifluoroacetic acid (TFA), 1*H*-benzo[*d*][1,2,3]triazol-1-ol (HOBt) and *N,N*-diisopropylethyl amine (DIPEA) are purchased from Sigma-Aldrich (St. Louis, MO). *Tert*-butyl 2-(piperazin-1-yl)ethylcarbamate is purchased from Astatech Inc (Bristol, PA).

[0047] (a) Synthesis and kinase profiling of biotin-imatinib (active and inactive form): Two forms of biotin imatinib are synthesized, one having the characteristic kinase activity of imatinib, and the other lacking kinase activity.



[0048] Inactive biotin-imatinib, (IC200001) is synthesized by reacting N-desmethyl imatinib with Biotin-OSu. Active biotin-imatinib, (IC339239) is synthesized from the key intermediates, tert-butyl 2-(piperazin-1-yl)ethylcarbamate and 6-methyl-*N*¹-(4-(pyridin-3-yl)pyrimidin-2-yl)benzene-1,3-diamine, via 4 steps:



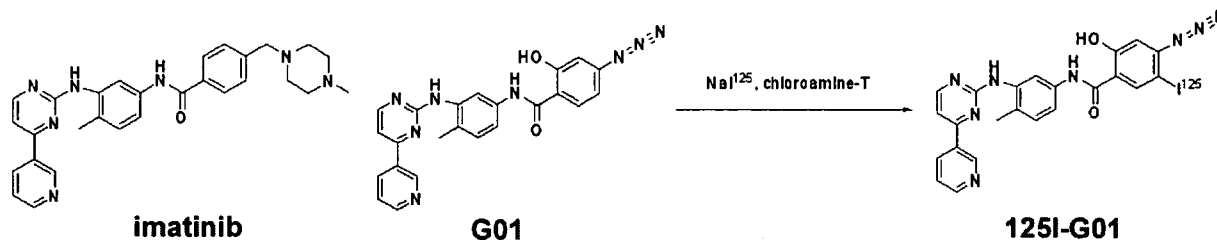
Reagents and Conditions: (a) 4-(bromomethyl) benzoic acid, K₂CO₃, DMF, r.t., 2 h. (b) 6-methyl-*N*¹-(4-(pyridin-3-yl)pyrimidin-2-yl)benzene-1,3-diamine, TCFH, DIPEA, DMF, r.t., overnight. (c) TFA, CH₂Cl₂, r.t., 30 min. (d) Biotin-OSu, HOBt, DIPEA, r.t., overnight, and then HPLC purification.

[0049] The kinase profiling is performed by Millipore Inc. using the standard assays for Abl kinase and PDGF receptor (ATP = 45 μM). Compound IC200001 shows no significant inhibitory activity toward either kinase, while compound IC339239 has an IC₅₀ of 146 nM against Abl kinase (imatinib has an IC₅₀ of 79 nM) and an IC₅₀ of 6.6 μM against

PDGF receptor (imatinib has an IC₅₀ of 4.8 μ M). Thus, we refer to IC200001 as "inactive biotin-imatinib" and IC339239 as "active biotin-imatinib".

[0050] (b) Synthesis of an imatinib derived photo-affinity label, G01: An imatinib derivative, 4-azido-2-hydroxy-N-(4-methyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)phenyl)benzamide, is capable of being photoactivated. *N,N*-Diisopropylethylamine (DIPEA) (63 μ l, 0.36 mmol) is added to a solution of NHS-ASA (50 mg, 0.18 mmol), HOBt (25 mg, 0.18 mmol), and 6-Methyl-*N*¹-(4-(pyridin-3-yl)pyrimidin-2-yl)benzene-1,3-diamine (50 mg, 0.18 mmol) in DMF (2 ml). The reaction mixture is stirred at room temperature overnight under argon atmosphere. The generated crude product is purified by a semi-preparative HPLC to give 54 mg of the titled compound with a yield of 68%. The product, **G01**, 4-azido-2-hydroxy-*N*-(4-methyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)phenyl)benzamide, is confirmed by mass spectral analysis using an ESI-MS in the positive mode $[M+H]^+$, demonstrating a m/z of 439.1.

[0051] (c) Radioiodination of G01 by ¹²⁵I is performed without carrier using a modification of a Chloramine-T procedure and the iodinated product is purified by HPLC. Specifically, in a UV protected "V" vial, total volume 0.9 ml, ~10 mCi of ¹²⁵I stock isotope (volume = 25 μ l) is added to 200 μ l of 0.2M phosphate buffer, pH 7.2. G01 is dissolved to 1 mg/ml in ethanol and 25 μ l of this solution is combined with chloramine-T at 1 mg/mL in water (50 μ l) and then added to the V-vial. The reaction proceeded for 1 min and is terminated by the addition of 50 μ L of 1 mg/ml meta-bisulfite. The reaction mixture is chromatographed on a 25 cm Waters RP-C18 column, using 0.1% TFA in water as the "A" solvent and 0.1% TFA in acetonitrile as the "B" solvent. A gradient is run at 1 ml/min from 0% B to 50% B for 45 minutes and held at 50% B for 15 minutes. The product demonstrated a retention time of 54.5 min as followed by radiochemical detection, and had a specific activity of 2000 Curies per millimole. The ¹²⁵I labeling experiment is performed by PerkinElmer Life and Analytical Sciences, Inc. The structures of G01, ¹²⁵I-G01, as compared to imatinib are:



³H-G01 is prepared by ViTrax Radiochemicals via catalytic tritium exchange of G01. The labeled product is purified by HPLC. The composition of the purified product is verified by co-injection of the tritium labeled product with its cold precursor and both compounds co-chromatographed on an analytical HPLC.

(d) Cellular A β production assays and incubation with G01.

[0052] Neuroblastoma 2a cells stably overexpressing human APP695 are treated with 10 μ M G01 for 3 hr. Cells treated with DMSO, or DMSO plus imatinib are used as controls. After 3 hr, conditioned medium is collected and A β immunoprecipitation is conducted using 4G8 antibody. The immunoprecipitated A β is separated on 10-20% Tris-tricine gel, transferred to PVDF membrane and detected by 6E10 antibody. Although not as potent as imatinib, G01 significantly reduces A β compared to the DMSO vehicle at levels of 10 μ M.

Example 2: Immobilization of imatinib and affinity purification.

[0053] For affinity purification, HEK293 cells are homogenized with 10 mM Hepes, 250 mM sucrose, pH 7.4 in the presence of protease inhibitors. After cell debris is cleared by centrifugation at 1,000 g for 5 mins, the supernatant is subjected to ultracentrifugation at 100,000 g for 1 h. The membrane pellet is then solubilized on ice in 50 mM Hepes, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, and 1% CHAPSO containing protease inhibitors (Roche Inc. cat#04 693 132 001) for 1 h and subject to ultracentrifugation for 1 h at 100,000g. The soluble membrane extracts are incubated with Myone™ streptavidin T1 beads (cat#656-01, Invitrogen) containing bound active biotin-imatinib for 3 hr at 4 °C. Subsequently, the beads are washed three times with lysate buffer. Bound proteins are eluted with tricine SDS-PAGE sample buffer and separated on 10-20% tris-tricine gels. For immunoblotting, the gel is then transferred to PVDF membrane and probed with γ -secretase antibodies: PS1 antibody (cat#529592) and Pen-2 antibody (cat#NE1008) are from EMD Biosciences and nicastrin

antibody is from BD Transduction Laboratories (cat # 612290). Silver staining is used to identify protein bands in SDS-PAGE gels. The ~ 16 kDa band is excised, trypsinized, and sequenced by tandem MS/MS mass spectrometry.

[0054] To identify the target responsible for imatinib's selective A β -lowering

activity, we synthesize a biotinylated derivative of imatinib, "biotin-imatinib, which specifically captures solubilized γ -secretase components, including presenilin-1, PEN2, and nicastrin. To identify the protein with which imatinib directly interacts, we synthesized a photoactivatable azido imatinib derivative, G01. When ^{125}I -G01 is incubated with a membrane preparation followed by photolysis, none of the four components of γ -secretase are labeled. Rather, ^{125}I -G01 labels a ~ 16 kDa protein, which co-immunoprecipitates with the more slowly migrating 18 kDa presenilin-1-CTF. This result is confirmed by intact cell photolabeling using cell permeable ^3H -G01. Similar to ^{125}I -G01, the ^3H -imatinib derivative does not bind to any of the four γ -secretase components, but does label a band of ~16 kDa that co-immunoprecipitated with PS1.

[0055] To purify the potential target protein, immobilized active biotin-imatinib is incubated with solubilized membrane preparations and bound proteins are separated by SDS-PAGE. After silver staining, a ~16 kDa band is observed. Peptide fragments, derived from this band after trypsin digestion, and analyzed by tandem mass spectrometry, corresponded to the C-terminal region of an uncharacterized protein, pigeon homologue protein (PION) (human accession number: NP_059135). The identification is made based on two unique tryptic peptides ($^{766}\text{LWDHPMSSNIISR}^{778}$ and $^{779}\text{NHVTRLLQNYKK}^{790}$) covering approximately 20% of the 16 kDa fragment. Its sequence, especially the C-terminal region, is highly conserved among multiple species from chicken to human. Expression pattern analysis indicates that this gene is expressed in diverse tissues. We characterize PION as a gamma-secretase activating protein (gSAP).

Example 3: *In vitro* and intact cell photolabeling.

[0056] For *in vitro* labeling, membrane pellets are prepared as described above and resuspended in 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl_2 , 5 mM CaCl_2 .

Resuspended membranes are incubated with 20 nM ^{125}I -G01 for 3 hr at 4 °C prior to

photolysis using a compact UV lamp (4 watt, model UVGL-25, UVP Inc.) at 254 nm for 2 mins. To examine labeling specificity, 50 μM imatinib is added to a parallel assay. After photolysis, membranes are pelleted by ultracentrifugation at 100,000 g for 1 h and solubilized

with 50 mM Hepes, 150 mM NaCl, 1% CHAPSO, 5 mM MgCl₂, 5 mM CaCl₂. The supernatant is pre-cleared with protein G plus/ protein A beads (EMD Biosciences, cat#IP05) for 30 min and proteins are precipitated using PS1 antibody (EMD Biosciences, cat#529592) coupled to protein G plus/protein A beads for 2 h before washing with lysate buffer 4 times.

5 The bound material is eluted in SDS-tricine sample buffer and separated using 10-20% Tris-tricine gels, followed by transfer to PVDF membrane. The membrane is dried and exposed to Kodak MS film for autoradiography. For intact cell labeling, human embryonic kidney cells (HEK293) grown to ~80% confluency (~ 10⁷ cells) are incubated with 0.1 μM ³H-G01 in Opti-MEM for 2 hours in an incubator at 37°C, with 5% CO₂ before being transferred to ice

10 for an additional hour. Medium is removed and cells are washed twice with cold phosphate buffered saline (PBS), pH 7.4. Photolysis is conducted on ice for 2 min using a compact UV lamp (4 watt, model UVGL-25, UVP Inc.) at 254 nm. As controls, cells are incubated either without UV crosslinking or in the presence of 50 μM unlabeled imatinib. After photolysis, cells (~10⁷ cells for each treatment) are immediately homogenized in 1 ml 50 mM Hepes, pH

15 7.4, 150 mM NaCl, 1% CHAPSO, 5 mM MgCl₂, 5 mM CaCl₂ with protease inhibitor mixture (Roche) on ice. After pre-clearing with protein G plus/ protein A beads, proteins are immunoprecipitated for 2 hr using 10 μl of the PS1-loop antibody (cat#529592 EMD Biosciences). The immunoprecipitate is washed 3 times with lysis buffer. The immuno-

20 purified (IP) material is eluted with SDS sample buffer and product is separated using a 10-20% Tris-Tricine SDS-PAGE gel, transferred to PVDF membrane, and the membrane dried and exposed to Kodak MS film for autoradiography.

Example 4: gSAP antibody production and metabolic labeling.

[0057] Rabbit polyclonal antiserum against gSAP is generated by injecting New

25 Zealand White rabbits with the peptide CFEGHDNVDAEFVEEAALKHT (corresponding to aa 829-848 of human gSAP with an N-terminal cysteine attached for conjugation) coupled to keyhole limpet hemocyanin (cat#PI-77563, Fisher Scientific). Rabbit injections, bleeds, and housing are performed by Cocalico Biologicals (Reamstown, PA). The antibody is purified by passing serum through a Sulfolink resin (Thermo Scientific, cat#44999) with the antigenic

30 peptide immobilized and eluted following the company instructions. For pulse-chase labeling, neuroblastoma 2a cells are incubated for 30 min with DME minimal essential medium deficient in methionine and cysteine (Met-Cys-DMEM). Cell proteins are labeled

with Met-Cys-DMEM containing EXPRESS ³⁵S Protein Labeling Mix (cat#NEG772014MC, Perkin Elmer) for 15 min at 37°C. The chase periods are initiated by replacing the medium with full culture medium 50% DMEM/ 50% Opti-MEM, 5% fetal bovine serum (FBS) and cells are incubated at 37°C for various times. For continuous labeling, cells are labeled with ³⁵S Protein Labeling Mix (Perkin Elmer) for 4 hrs without chase, and washed with Dulbecco's Phosphate Buffered Saline (DPBS). Cell monolayers are lysed in RIPA buffer (10 mM Tris, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, at pH 7.4) containing protease inhibitors. The lysates are clarified by centrifugation for 20 min at 13,000 rpm and the supernatant is pre-cleared with protein G plus/ protein A beads followed by immunoprecipitation using gSAP antibody for 2 hrs. The beads are incubated with Tris-tricine sample buffer to elute bound proteins which are then separated by 10-20% Tris-tricine gel, transferred to PVDF membrane, and exposed to Kodak MR film for autoradiography.

Example 5: Cellular knockdown and overexpression

[0058] For cellular gSAP knockdown experiments, small interfering RNA (siRNA) of

gSAP is purchased from Dharmacon Inc. The sequences of the siRNA used are as follow:

sense sequence: AUGCAGAGCUGGACGACAUUU; antisense sequence: 5'-

P.AUGUCGUCCAGCUCUGCAUUU. Neuroblastoma 2a cell line stably overexpressing APP695 is transfected with siRNA using DharmaFect 2 reagent at a concentration of 50 nM following instructions provided by the manufacturer. Non-targeting control siRNA (cat# D-

001810-01, Dharmacon Inc.) is transfected in parallel as control. Short hairpin RNA (shRNA) of gSAP is purchased from Open Biosystems and transfected into cells using lipofectamine 2000. The sequence of mouse gSAP shRNA in pGIPZ shRNAmir-GFP vector is follows:

TGCTGTTGACAGTGAGCGCGGGTATAGCCTTATTTGCATATAGTGAAGCCACAGA

TGTATATGCAAATAAGGCTATACCCATGCCTACTGCCTCGGA. The sequence of human gSAP shRNA in pGIPZ shRNAmir-GFP vector is as follows:

TGCTGTTGACAGTGAGCGCGGAAATAGAGTGGTGATTAAATAGTGAAGCCACAG

ATGTATTTAATCACCACTCTATTTCCATGCCTACTGCCTCGGA. The knockdown efficiencies are examined using a real time RT-PCR kit (cat#12183, Invitrogen).

[0059] For gSAP overexpression in cells, mammalian expression vector pReceiver-M07 with the full length and the 16 KDa C-terminal fragment (amino acid sequence 733-854) of gSAP coding a C-terminal HA tag is purchased from Genecopoeia Inc. The plasmid

is transformed into XL1 blue competent cells (cat#200249-11, Stratagene) and purified using an EndoFree Maxi preparation kit (cat#12362, Qiagen). Plasmid is transfected into a stable HEK293 cell line overexpressing APP695, containing the Swedish mutation, using Fugene 6 (cat# 11815091001, Roche).

5 **[0060]** To assay A β , the medium is removed after 48 h of transfection and replaced with Opti-MEM for 3 h incubation. A β is then immunoprecipitated from conditioned medium using the 4G8 antibody. Quantitation of A β levels in the conditioned medium is also assessed using A β 40 and A β 42 ELISA kits (Invitrogen). The procedures are carried out according to the manufacturer's instructions.

10 **[0061]** When siRNA is used to reduce gSAP level (by 72 $\pm$ 15%) in neuroblastoma cells overexpressing APP695, the level of A β decreases about 50%. The addition of imatinib has little or no additional effect on A β levels. ShRNA-mediated gSAP knockdown (by 65 $\pm$ 12%) in HEK293 cells expressing APP Swedish mutation also results in a decrease of A β 40 and A β 42 levels of 61% and 48%, respectively. Conversely, overexpression of gSAP
15 in HEK293 cells expressing the APP Swedish mutation stimulates A β production by approximately 38%; the increase is abolished by imatinib treatment.

Example 6: Co-immunoprecipitation.

[0062] For co-immunoprecipitation, $\sim 10^7$ cells are lysed with 1 ml of 50 mM Hepes, 150 mM NaCl, 1% CHAPSO, 5 mM MgCl₂, 5 mM CaCl₂, with protease inhibitors for 30
20 min on ice. Cell debris and nuclei are removed by centrifugation at 13,000 rpm for 20 min. After pre-clearing with protein G plus/ protein A beads for 30 min, immunoprecipitation is performed using the corresponding antibody and 30 μ l beads for 2 hr on ice. The beads are washed 4 times with the lysate buffer and eluted with 30 μ l of SDS sample buffer at 95 degree for 5 mins. Immunoprecipitated proteins are resolved by SDS-PAGE and analyzed by
25 immunoblot. Presenilin 1 loop antibody AB14 (EMD Biosciences #529594) is used to detect PS1-NTF, Pen-2 antibody is purchased from EMD Biosciences (#NE1008). Nicastrin antibody is from BD Biosciences (#612290). HA monoclonal antibody (#A0089) and Myc tag polyclonal antibody (#A00172) are from Genscript Inc. APP-CTF is detected using the 369 antibody (Xu et al. 1998). 6E10 (#SIG39320) and 4G8 (#SIG39220) antibodies from
30 Covance are used to detect A β .

[0063] Solubilized γ -secretase components from HEK293 cell membrane preparations are bound to the immobilized imatinib derivative, biotin-imatinib, and detected

by immunoblotting. Both biotin-coated beads and an inactive biotin-imatinib derivative served as controls. Endogenous γ -secretase components are detected by specific antibodies to Nicastrin, PS1-CTF and Pen-2. Photoactivatable ^{125}I and ^3H -G01 are used to label membrane preparations or intact HEK293 cells, respectively. After lysis and immunoprecipitation with PS1 antibody, bound proteins are separated by 10-20% Tris-tricine SDS-PAGE. A 16 kDa band is detected by autoradiography in both photolabeling conditions. This labeling is eliminated by co-incubation with 50 μM unlabeled imatinib before photolysis. The same membranes probed with PS1-CTF antibody show that PS1-CTF migrates with a slower mobility than the 16 kDa band and is not labeled by G01. Proteins in HEK293 cell lysates that bind biotin-imatinib beads are separated on SDS-PAGE and visualized with silver staining. A ~ 16 kDa band is detected (arrow and label "gSAP") that does not bind to biotin alone or to inactive biotin-imatinib. After trypsinization, the 16 kDa band is identified as the C-terminal domain of gSAP by MS/MS mass spectrometry.

[0064] ^{35}S -methionine pulse-chase labeling of endogenous gSAP in N2a cells is followed by immunoprecipitation using a polyclonal antibody raised against the C-terminus of gSAP. gSAP is synthesized as the full length 98kDa-precursor protein and rapidly processed into a C-terminal fragment migrating at 16 kDa. After 4 hrs of continuous ^{35}S -methionine labeling (steady-state conditions), the predominant cellular form of gSAP is the 16 kDa species. Intact N2a cell labeling is performed by incubation with ^3H -G01. Cells are lysed with RIPA buffer and proteins were immunoprecipitated with gSAP antibody. After separation on SDS-PAGE and radiography, gSAP-16K is found to be specifically labeled by ^3H -G01; this labeling is quenched by pre-incubation of cells with 50 μM imatinib. gSAP overexpressed with or without PS1 in presenilin null embryonic stem cells bound biotin-imatinib is detected by immunoblotting. Under gSAP siRNA knockdown conditions, PS1 no longer captures biotin-imatinib.

Example 7: Gel filtration chromatography.

[0065] N2a cells are labeled with ^{35}S Protein Labeling Mix (Perkin Elmer) for 4 hrs as described above. Solubilized membrane preparations (0.2 ml, ~ 1 mg of solubilized protein, in 50 mM Hepes, 150 mM NaCl, 1% CHAPSO, 5 mM MgCl_2 , 5 mM CaCl_2) are centrifuged for 1 h at 100,000 g to remove potentially aggregated material. The resulting supernatant is loaded onto a Superdex 200 10/300 GL column (Amersham Biosciences) of an AKTA fast performance liquid chromatography system (Amersham Biosciences). Fractionation is

performed in the lysate buffer at a flow rate of 0.5 ml/min and 1-ml fractions are collected for analysis. Each fraction is analyzed by western blot against γ -secretase antibodies. To detect endogenous gSAP, each fraction is immunoprecipitated with gSAP antibody. The immunoprecipitated materials are eluted with Tris-tricine sample buffer, separated by 10-20% Tris-tricine gel, transferred to PVDF membrane, and exposed to Kodak MR film for autoradiography.

Example 8: In vitro γ -secretase assay.

[0066] Membrane pellets are prepared from HEK293 cells transfected with APP- β -CTF (CT-100) as described above, and the membranes are washed with assay buffer (10 mM Hepes, 140 mM KOAc, 2.5 mM MgOAc, 0.1 mM CaCl_2 , 1 mM ATP, pH 7.2) and pelleted at 100,000 g for 30 min at 4 °C. Recombinant gSAP-16K (aa733-854 of the human gSAP) is expressed and purified from BL21 DE3 *E.coli*. The membranes are resuspended in 200 μ l of assay buffer with 2 μ g recombinant gSAP-16K or the same amount of BSA as control. A parallel system with 1 μ M L685,458 (γ -secretase inhibitor) is also used as a control. The membrane suspension is pre-incubated at 4 °C for 1 hr and then incubated for 2 hr at 37 °C to allow *in vitro* generation of A β . The membranes are solubilized in ¼ volume of 200 mM Tris, pH 7.8, 760 mM NaCl, 24 mM EDTA, 10% Triton X-100, and insoluble material is removed by centrifugation at 10,000 g for 20 min. A β is immunoprecipitated from the lysate using 4G8 antibody, separated on 10-20% Tris-tricine gel, transferred to PVDF membrane, and subjected to autoradiography using Kodak MR film.

Example 9: Notch cleavage analysis.

[0067] Plasmid coding Notch Δ E (truncated Notch-1, lacking most of the Notch extracellular domain, with a C-terminal myc tag) is described previously (Netzer et al. 2003). Cells transfected with Notch Δ E are co-transfected with gSAP-shRNA or gSAP plasmids. After two days of transfection, Notch expression and cleavage are detected with anti-myc antibody. The cleaved Notch intracellular domain (NICD) is detected with a cleavage-specific antibody (Notch1 Val-1744, Cell Signaling Inc.). Cells treated with L-685,458 serve as controls.

[0068] gSAP regulates A β production but does not influence Notch cleavage. siRNA-mediated knockdown of gSAP in N2a cells overexpressing APP695 results in lowered A β production. The A β -lowering effects of imatinib and of siRNA are not additive. Transfection with gSAP shRNA of HEK293 cells stably expressing human APP containing the Swedish

mutation reduces the levels of both A β 40 and A β 42. gSAP overexpression in HEK293 cells increases A β levels and this effect is blocked by imatinib. Under either gSAP knockdown or overexpression conditions, Notch processing is not affected in HEK293 cells overexpressing extracellular domain truncated Notch (Notch Δ E, with C-terminal myc tag). NICD is detected using a myc antibody and a cleavage-product specific antibody (Notch1 Val-1744).

Recombinant gSAP-16K purified from *E. coli* stimulates A β production in an *in vitro* γ -secretase assay. The γ -secretase inhibitor, L685,458 (1 μ M) abolishes A β production.

[0069] gSAP interacts with γ -secretase and APP-CTF, but not with Notch Δ E.

Membrane preparations from N2a cells are prepared, lysated with 1% CHAPSO, subjected to gel filtration, and detected by western blotting. Endogenous gSAP-16K co-migrates with γ -secretase components. Column void fraction = 6. Immunoprecipitation of endogenous gSAP results in co-immunoprecipitation of γ -secretase components. In HEK293 cells that co-express Notch Δ E-myc, APP-CTF, and gSAP-16K-HA, immunoprecipitation of gSAP-16K is associated with co-immunoprecipitation of APP-CTF but not of Notch Δ E.

Immunoprecipitation of APP-CTF, but not of Notch Δ E, is associated with co-immunoprecipitation of gSAP-16K. Treatment with imatinib reduces the association between gSAP-16K and APP-CTF.

Example 10: gSAP RNAi Mice line generation and A β level measurement.

[0070] RNAi mice are generated following the procedure as described before (Seibler et al. 2007). Specifically, exchange vector carrying gSAP shRNA coding sequence TCCCGGAACTCCATGATTGACAAATTTCAAGAGAATTTGTCAAT CATGGAGTTCCTTTTTA is under the control of a H1-Tet promoter. Using the recombinase-mediated cassette exchange (RMCE) technology, the vector is integrated into the mouse ES cell genome (B6/129S6 background). Subsequently, transfected ES cells are injected into tetraploid blastocyst to generate inducible RNAi mice. Heterozygous RNAi mice are then cross-bred with an AD mouse model with APP^{swe} and PS1 Δ 9 mutations (AD 2 $\times$ mice) to generate gSAP-RNAi AD mice. shRNA induction is conducted by introducing 2 mg/ml doxycycline (Sigma D-9891) in drinking water containing 10% sucrose. Control mice are fed with drinking water containing 10% sucrose. Drinking water is changed every second day and kept dark. gSAP knockdown efficiency in mice is assayed using quantitative real time RT-PCR. Total RNA is isolated following standard procedure and cDNA is synthesized using the Reverse Transcription Core Kit (Eurogentec). Real-time PCR reactions are

performed using an iCycler Thermal cycler instrument (Bio-Rad). For mouse brain A β level measurements, 2 month old gSAP-RNAi AD mice are induced with doxycycline for 1 month and brain tissue is extracted with formic acid for ELISA assay.

[0071] Knockdown of gSAP reduces A β production and plaque development in AD mice models. gSAP RNAi- AD mice are generated by cross-breeding of double transgenic AD mice with inducible gSAP RNAi mice. gSAP shRNA expression (under doxycycline induction) reduces both A β 40 and A β 42 levels in the mice brain by ~28% and ~32%, respectively (**: P< 0.01. n= 4) (Figure 3).

Example11: Intra-hippocampal injections of AAV2-gSAP shRNA to PS/APP

transgenic mice

[0072] Mouse GIPZ shRNA mir individual clone (V2LMM_88580: containing the hairpin sequence for mouse gSAP gene

TGCTGTTGACAGTGAGCGCGGGTATAGCCTTATTTGCATATAGTGAAGCCACAGATGTATATGCAAATAAGGCTATACCCATGCCTACTGCCTCGGA) is purchased from

Openbiosystems/Thermo Scientific. The hairpin region is excised and inserted into AAV2-siln4.1-MCS-EGFP vector (Vector biolabs) via BamHI and HindIII sites. The triple transgenic AD mice (6 months old) are analyzed for A β . The double transgenic AD mice (13 months old) are analyzed for plaques. For each group, mice are deeply anesthetized with a mixture of ketamine (100mg/kg) and xylazine (10mg/kg) and placed in a stereotaxic frame. AAV2 virus carrying AAV2-gSAP shRNA-GFP or AAV2-GFP is bilaterally injected into right or left hippocampus. Stereotaxic coordinates are determined according to Paxinos Atlas of the mouse brain: anteroposterior 2.18mm, mediolateral 1.97mm and dorso-ventral 2mm. One μ l of each AAV2 (shRNA for gSAP or GFP control) (3.3×10^{13} vg/ml) is injected for 5 min at a rate of 0.2 μ l/min with a 10ul Hamilton syringe equipped with a motorized injection pump. The injection needle is allowed to remain in the brain for an additional 5min to prevent fluid extravasation. Mice are sacrificed 4 weeks after injection.

[0073] To determine A β levels, the hippocampus is removed and solubilized in 2% SDS containing protease inhibitors. The lysates are centrifuged at 13,000 rpm for 20 mins and the supernatant is used for ELISA analysis using A β 40 and A β 42 assay kits (Invitrogen Inc.).

[0074] For immunolocalization studies, mice are subjected to intracardiac perfusion with 0.1M PBS followed by 4% paraformaldehyde/PBS. After perfusion, brains are removed

and post-fixed with 4% paraformaldehyde/PBS at 4°C overnight, followed by incubation with 15% sucrose and then 30% sucrose for 24 hours. Cryo-protected brains are cut into 25-50µM thick sections using a cryostat. Sections are labeled with the anti-Aβ antibody 6E10 (1:1000, Novus Biologicals) to visualize extracellular amyloid plaques and anti-EGFP antibody (1:500, Invitrogen) to visualize neurons positively transduced with the shRNA coding AAV virus using an M.O.M immunodetection kit (Vector laboratories, PK-2200). Imaging is performed using a Zeiss LSM510 confocal microscope.

[0075] Intrahippocampal injection of AAV2-carrying shRNA against gSAP reduces amyloid plaque development in double transgenic AD mice. Areas showing GFP staining indicate regions of AAV2 vector expression, while red fluorescence reveals amyloid plaques, showing that the vector expression coincides with the reduction in plaque formation. GFP-positive regions from five consecutive sections are analyzed by confocal microscopy. Data is expressed as plaques per mm² (n= 4. ***: P<0.001) (Figure 4).

Example 12- N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide binds gSAP and lowers Aβ.

[0076] N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide (WO/2008/153974, ex. 7) is selected as a representative imatinib analogue having little kinase inhibitory activity when tested against a panel of 40 kinases (data not shown). Compared to imatinib, which has a K_i versus Abl kinase (its principal target for approved anti-cancer indications) of roughly 100 nM, N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide has 100-fold weaker activity, approximately 12,000 nM K_i versus this kinase. This compound nevertheless inhibits Aβ in a manner similar to imatinib. This further supports that the kinase inhibitory activity of imatinib is not the basis for its activity against gSAP but rather that it has a specific effect on the interaction between gSAP and gamma secretase.

[0077] HEK293 cells are transfected with gSAP with a C-terminal Hemagglutinin (HA) tag. A membrane pellet is prepared the pellet and resuspended in 50 mM Hepes, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, and incubated with the indicated amount of either imatinib or N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide for 2 hrs, followed by addition of 2 µM Biotin-NCG and incubated for 1 hr. The membrane is pelleted down, solubilized in 50 mM Hepes, 150

mM NaCl, 1% octyl β -D-glucopyranoside, and bound to Myone streptavidin T1 beads for 1 hr, then washed 3 times. The captured protein is released by incubation with SDS sample buffer and detected by western blot.

[0078] Total human A β in the N2a cell system is measured in a standard sandwich ELISA by using a specific monoclonal antibody (6E10, Signet Laboratories) to capture the A β 40 and A β 42 and an antibody to A β 17–24 (4G8, Signet Laboratories, Dedham, MA) for detection. Cell culture medium (5 μ L/well) is diluted to 100 μ L in phosphate-buffered saline/0.2% Tween 20 and loaded onto ELISA plates. For Western blot assays, cell medium is diluted with 2x tricine sodium dodecyl sulfate sample buffer, and heated at 95°C for 5 min. A β is separated by electrophoresis on 16% tricine polyacrylamide gels (BioRad), proteins are transferred onto nitrocellulose membranes and blocked overnight with LiCor blocking buffer (LiCor, Lincoln, NE). A β is detected with antibody 4G8 and an Alexa 680-conjugated rabbit anti-mouse secondary and scanned with a LiCor Odyssey infrared scanner. N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide significantly lowers A β at concentrations of < 500nM.

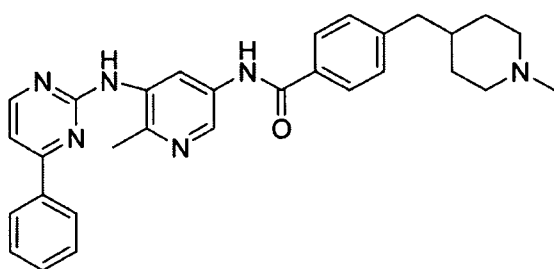
[0079] For detection of Notch cleavage, HEK293 human embryonic kidney cells are transfected with mouse Δ E-Notch cDNA with a C-terminal myc tag. For analysis of Δ E-Notch cleavage, cells are incubated with test compounds for 4 h. Cell extracts are prepared by lysing cells in cell lysis buffer (50mM Tris, pH7.5, 10mM NaCl, 5mM EGTA, 1mM EDTA and complete protease inhibitors (Roche Diagnostics, Indianapolis, IN). Protein concentration is determined using Bradford protein detection reagent. Volume is adjusted with cell lysis buffer, then 4X sample loading buffer (Tris-Tricine gel loading buffer) is added, samples are heated 5 min at 95°C, and equivalent amounts of cell protein for each treatment are loaded onto a 10% Bis-Tris gel. C-terminal Notch species are detected with monoclonal anti-c-myc antibody 9E10 (Roche Diagnostics) and fluorescent goat anti-mouse secondary antibody. Fluorescent antibody is quantitated via LiCor Odyssey infrared fluorescence detector.

[0080] N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide and imatinib do not influence NOTCH metabolism in the cell system outlined. The competitive and direct gamma-secretase inhibitor DAPT is used as a positive control in the assay. Since inhibition of NOTCH processing could potentially cause undesirable side-effects, compounds that prevent processing of NOTCH are

less desirable as drug candidates. N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide is comparable to imatinib in this assay.

[0081] Example 13 - Synthesis of N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide

[0082] N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide, depicted below, is disclosed in WO/2008/153974, but a synthesis is provided for convenience sake:



a) (2-Methyl-5-nitro-pyridin-3-yl)-(4-phenyl-pyrimidin-2-yl)-amine

[0083] To a mixture of 3-Bromo-2-methyl-5-nitro-pyridine (4.46 g, 2.10 mmol) and 4-Phenyl-2-pyrimidinamine (1.3 g, 1.75 mmol) in dry toluene (25 mL) are added Cs_2CO_3 (0.85 g, 2.62 mmol), $\text{Pd}_2(\text{dba})_3$ (32 mg, 0.035 mmol) and Xantphos (60 mg, 0.105 mmol). The mixture is evacuated and purged with N_2 , heated to 90 °C under nitrogen for 24 h. The reaction mixture is cooled to room temperature, diluted with EtOAc and filtered. The filtrate is concentrated under reduced pressure, and the residue is purified by silica gel column chromatography to afford product as yellow solids (320 mg, yield 59%). ^1H NMR (200 MHz, CDCl_3): δ 2.75 (s, 3H), 7.19 (s, 1H), 7.37 (d, $J = 4.0$ Hz, 1H), 7.57-7.54 (m, 3H), 8.17-8.12 (m, 2H), 8.59 (d, $J = 4.0$ Hz, 1H), 9.02 (d, $J = 2.0$ Hz, 1H), 9.87 (d, $J = 2.0$ Hz, 1H); MS ESI^+ m/z 308 $[\text{M}+\text{H}]^+$. **(b) 2-methyl- N^3 -(4-phenylpyrimidin-2-yl)pyridine-3,5-diamine**

[0084] A mixture of catalytic ferric chloride (12 mg), (2-Methyl-5-nitro-pyridin-3-yl)-(4-phenyl-pyrimidin-2-yl)-amine (320 mg, 1.04 mmol) in hydrazine hydrate (12 mL) and methanol (20 mL) is refluxed for 15 min. The reaction mixture is cooled to room temperature, concentrated under reduced pressure and the crude residue is dissolved in water and extracted with EtOAc. The combined extracts are dried over anhydrous Na_2SO_4 , filtered,

concentrated under reduced pressure. The residue is stirred with Et₂O for 5 minutes, the ether layer is decanted and the residue is dried under vacuum to give product as yellow solids (270 mg, yield 93%). Mp: 133.1-133.4 °C; ¹H NMR (200 MHz, CDCl₃): δ 2.51 (s, 3H), 3.62 (bs, 2H), 6.93 (s, 1H), 7.20 (d, J = 6.0 Hz, 1H), 7.52-7.49 (m, 3H), 7.74 (d, J = 2.0 Hz, 1H), 8.08-8.03 (m, 2H), 8.13 (d, J = 2.4 Hz, 1H), 8.48 (d, J = 4.0 Hz, 1H); MS (ESI⁺) m/z 278 [M+H]⁺.

(c) 4-((1-methylpiperidin-4-yl)methyl)benzoic acid

[0085] 4-(piperidin-4-ylmethyl)benzoic acid (114 mg, 0.342 mmol) is dissolved in 2 mL of methanol, and then 37% formaldehyde aqueous solution (56 µL, 0.685 mmol) is added. The reaction mixture is stirred at room temperature for 5 min, and then NaBH₃CN (26 mg, 0.41 mmol) is added. The mixture is stirred at room temperature for 2 h, quenched with a small amount of water, and then evaporated to dryness under high vacuum to give white foamy solids, which is used for the next reaction without further purification. MS (ESI⁺) m/z 234.1 [M+H]⁺.

d) N-(6-methyl-5-(4-phenylpyrimidin-2-ylamino)pyridin-3-yl)-4-((1-methylpiperidin-4-yl)methyl)benzamide

[0086] DIEA (149 µL, 0.86 mmol) is added into a suspension of 2-methyl-N³-(4-phenylpyrimidin-2-yl)pyridine-3,5-diamine (47 mg, 0.17 mmol), 4-((1-methylpiperidin-4-yl)methyl)benzoic acid (40 mg, 0.17 mmol), BOP (91 mg, 0.21 mmol) in DMF. The reaction mixture is stirred at room temperature under argon atmosphere overnight. The mixture is filtered through a 0.45 µm microfilter and the filtrate is purified by a Waters semi-preparative HPLC to give 16 mg of the final product as white powder. MS (ESI⁺) m/z 493.1 [M+H]⁺.

[0087] Temperatures are given in degrees Celsius (°C); operations are carried out at room or ambient temperature ("rt") are at a temperature in the range of 18-25°C. Organic solutions are dried over anhydrous sodium sulphate; evaporation of solvent is carried out using a rotary evaporator under reduced pressure (600-4000 Pascals; 4.5-30 mmHg) with a bath temperature of up to 60 °C. In general, the course of reactions is followed by TLC and reaction times are given for illustration only; final products had satisfactory proton nuclear magnetic resonance (NMR) spectra and/or mass spectral data. Yields are given for illustration only and are not necessarily those which can be obtained by diligent process development; preparations are repeated if more material is required. When given, NMR data is in the form of delta values for major diagnostic protons, given in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. Chemical symbols have their

usual meanings; SI units and symbols are used. The following abbreviations have been used:

Cs₂CO₃ cesium carbonate;

HPLC high performance liquid chromatography;

Na₂SO₄ sodium sulfate;

5 NaBH₃CN sodium cyanoborohydride

BOP benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate;

DMF *N,N*-dimethylformamide;

EtOAc ethyl acetate;

DIEA *N,N*-diisopropylethylamine;

10 Pd₂(dba)₃ tris(dibenzylideneacetone)dipalladium(0); and

Xantphos 4,5-Bis(diphenylphosphino)-9,9-dimethylxanthene

[0088] Example 15-Binding Assay

[0089] In one example, an assay may be used to test for an inhibitor of the gSAP protein. A selected sequence of the gSAP protein may be used to detect binding with a potential inhibitor.

[0090] Example 16-Diagnostic Assays

[0091] a. A diagnostic assay is used to measure levels of the gSAP protein. Elevated levels of the gSAP protein may be correlated with disease.

20 **[0092]** b. Genetic analysis of variants in the gSAP region is used to identify candidates for therapy targeting gSAP. Nine SNPs in the region of the gene for gSAP are identified (PION). In single marker analyses, none of the SNPs is associated with AD after correction for multiple testing. However, in sliding window haplotype analyses, 4 haplotypes are associated with AD:

25 a) rs6976567|rs1468682|rs1819814,

b) rs1468682|rs1819814|rs4729535,

c) rs1819814|rs4729535|rs4729540,

d) rs7781642|rs6955503|rs7776973

Two of the SNPs in these haplotypes (rs4729540 and rs7776973) are significantly associated with delayed recognition test in the single marker analyses also. Patients identified as having these haplotypes are thus candidates for treatment with gSAP inhibitors.

Example 17 – Identification of region of interaction between gSAP and APP

[0093] Alternative combinations and variations of the examples provided will become apparent based on this disclosure. It is not possible to provide specific examples for all of the many possible combinations and variations of the embodiments described, but such combinations and variations may be claims that eventually issue.

CLAIMS

1. A gSAP peptide selected from the following:
 - a. A peptide at least 60% similar to a sequence selected from the sequences depicted in Figure 1, wherein similarity is measured by a BLAST algorithm;
 - b. a peptide having residues corresponding to the conserved residues in bold in Figure 1,
 - c. a peptide comprising the sequence Seq ID No. 7 (LWDHPMSSNIISR) and/or Seq ID No. 8 (NHVTRLLQNYKK),
 - d. any of the peptides of Seq ID Nos. 1-6 as depicted in Figure 1,
 - e. gSAP from any mammalian species, for example mouse, rat or monkey,
 - f. a fragment of gSAP capable of forming a complex with gamma secretase,
 - g. a fragment of approximately 16 kDa from the C-terminal of any of Seq ID Nos. 1-6 as depicted in Figure 1,
- wherein the gSAP peptide is isolated or separated from its natural environment.
2. A gSAP peptide according to claim 1 which is produced by a recombinant cell, e.g., selected from a bacterial cell, a baculovirus or a mammalian cell.
3. A vector comprising a gene for a gSAP peptide operably linked to a heterologous promoter.
4. A transgenic cell containing a heterologous gene which expresses a gSAP peptide according to claim 1.
5. A nucleic acid which is or which expresses a RNA construct capable of inhibiting gSAP expression in a mammalian cell.
6. An antibody to a gSAP.
7. A vaccine comprising an immunogenic fragment of gSAP in combination with a suitable adjuvant and/or carrier.
8. A non-human gSAP knockout mammal, wherein its gene for gSAP is disrupted.
9. Use of a gSAP peptide according to claim 1 in an assay to identify compounds which inhibit A β -deposition
10. A method of identifying a compound inhibiting A β -deposition, comprising measuring binding of a test compounds to a gSAP peptide.

11. A derivative of imatinib wherein the methylpiperizinyl moiety of imatinib is substituted or modified with a labeling group.
12. The derivative of claim 11 selected from photolabeled derivatives, radiolabeled derivatives and biotinylated derivatives.
- 5 13. A method of identifying persons at risk of developing Alzheimer's disease comprising checking for elevated expression levels and/or mutations in gSAP relative to normal values as identified using a control population.
14. A method for inhibiting the accumulation of abnormal protein aggregates in a warm-blooded animal in need of such inhibition which comprises administering to said
10 animal an effective amount of a compound to inhibit gSAP activity.
15. A method of treating Alzheimer's disease, progressive supranuclear palsy, Down Syndrome, memory and cognitive disorders, dementia, amyloid neuropathies, brain inflammation, nerve and brain trauma, vascular amyloidosis, cerebral hemorrhage with amyloidosis, Parkinson's disease, Huntington's disease, prion disease and/or vascular,
15 neurological, and/or neurodegenerative disorders related to the abnormal expression or accumulation of tau or amyloid proteins such as A β , comprising administering an effective amount of a compound to inhibit gSAP activity.
16. The method of claim 14 or 15 wherein the compound to inhibit gSAP activity is selected from small molecules, inhibitory RNA molecules, vectors and cells producing inhibitory
20 RNA molecules, antibodies to gSAP, and vaccines for gSAP.
17. A gSAP peptide according to claim 1 which has been labeled.
18. Use of gSAP derivatives in biochemical or genetic screening systems to identify further proteins in the gSAP macromolecular complex that could function as alternative targets for disease intervention.
- 25 19. A method of identifying a candidate for treatment with gSAP-targeted therapy, comprising measuring gSAP expression, using antibodies to gSAP or quantitative PCR for gSAP expression, wherein elevated expression is seen in candidates for treatment with gSAP targeted therapy.
20. A method of identifying a candidate for treatment with gSAP-targeted therapy,
30 comprising identifying patients having mutations or genetic variations affecting gSAP or gSAP expression or gSAP activity, wherein patients having such mutations or haplotypes are candidates for treatment with gSAP targeted therapy.

21. The method of claim 20 wherein the variant is a haplotype selected from the following groups of SNPs:

- a) rs6976567|rs1468682|rs1819814,
- b) rs1468682|rs1819814|rs4729535,
- c) rs1819814|rs4729535|rs4729540,
- d) rs7781642|rs6955503|rs7776973

22. The method of claim 20 wherein the variant is a variant of the sequence encoding the juxtamembrane region of APP-CTF.