Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2811454 A1 2012/03/15

(21) 2 811 454

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2011/08/30
(87) Date publication PCT/PCT Publication Date: 2012/03/15
(85) Entrée phase nationale/National Entry: 2013/03/15
(86) N° demande PCT/PCT Application No.: EP 2011/064893
(87) N° publication PCT/PCT Publication No.: 2012/031941
(30) Priorité/Priority: 2010/09/07 (DE10 2010 044 561.4)

(51) Cl.Int./Int.Cl. *C07D 417/06* (2006.01), *A61K 31/5415* (2006.01), *A61P 25/28* (2006.01), *C07D 417/14* (2006.01), *G01N 33/68* (2006.01)

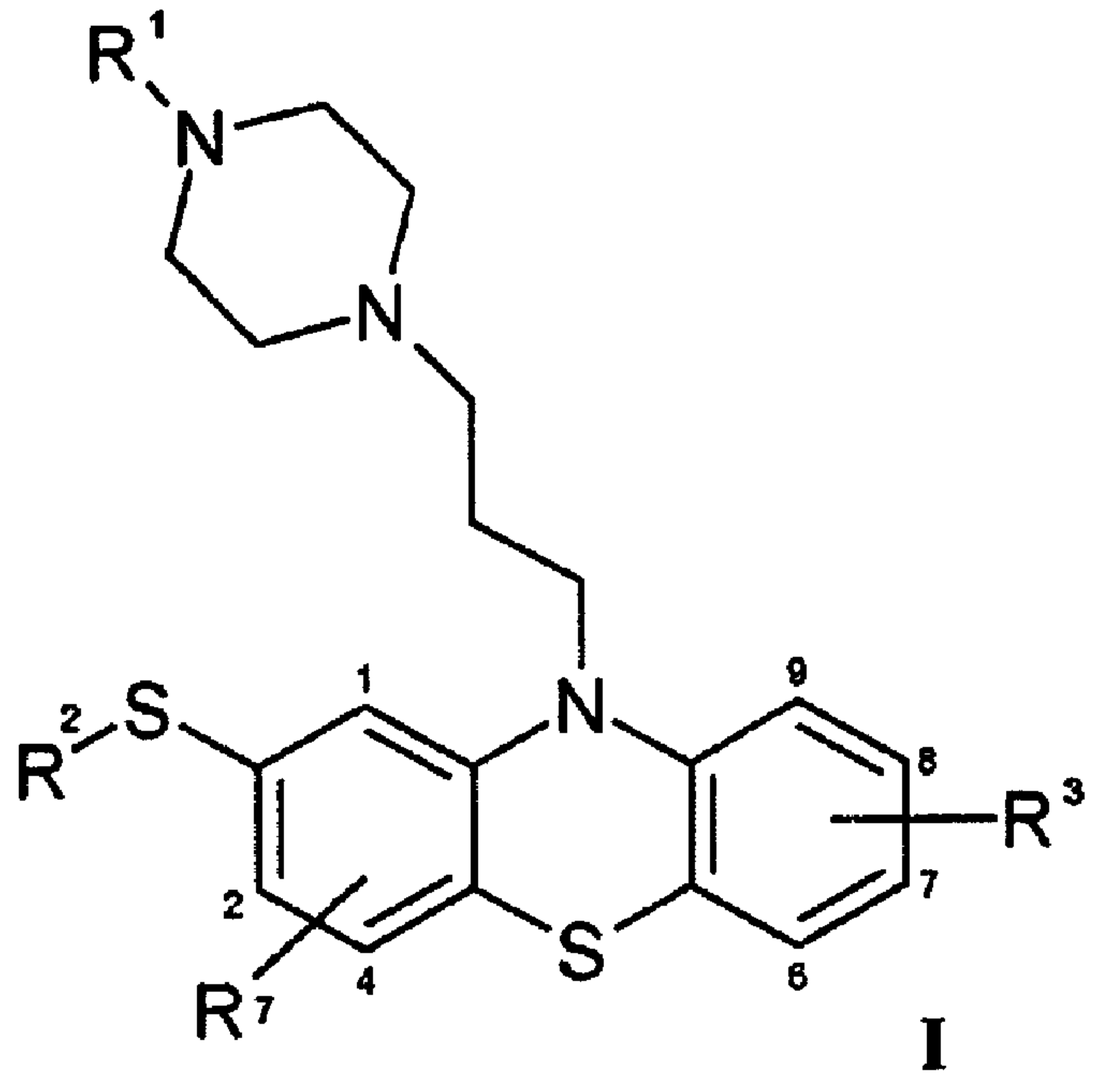
(71) Demandeur/Applicant:
UNIVERSITAET ROSTOCK, DE

(72) Inventeur/Inventor:
PAHNKE, JENS, DE

(74) Agent: ADAMS PATENT & TRADEMARK AGENCY

(54) Titre : 2-(R^2-THIO)-10-[3-(4-R^1-PIPERAZIN-1-YL)PROPYL]-10H-PHENOTHIAZINE POUR LE TRAITEMENT D'UNE PATHOLOGIE β-AMYLOIDE OU D'UNE ALPHA-SYNUCLEOPATHIE, ET PROCEDE POUR EN FAIRE LE DIAGNOSTIC OU LE PRE-DIAGNOSTIC

(54) Title: 2-(R^2-THIO)-10-[3-(4-R^1-PIPERAZIN-1-YL)PROPYL]-10H-PHENOTHIAZINE FOR TREATING A β-AMYLOIDOPATHY OR AN ALPHA-SYNUCLEOPATHY, AND METHOD FOR THE DIAGNOSIS OR PREDIAGNOSIS THEREOF



I

(57) Abrégé/Abstract:
The invention relates to 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10H-phenothiazine according to general formula I, for treating a β-amyloidopathy or an α-synucleinopathy accompanied by a cerebral protein deposit and a reduced activity of the cerebral ABCC1-transporter. The invention also relates to a method for the diagnosis or prediagnosis of a β-amyloidopathy or an α-synucleopathy accompanied by a cerebral protein deposit and a reduced activity of the cerebral ABCC1-transporter, or for determining the risk of a proband suffering from such an illness, the proband already having accumulated substances transported by the cerebral ABCC1 transporter.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
15. März 2012 (15.03.2012)

WIPO | PCT

(10) Internationale Veröffentlichungsnummer
WO 2012/031941 A3

(51) Internationale Patentklassifikation:
C07D 417/06 (2006.01) *A61P 25/28* (2006.01)
C07D 417/14 (2006.01) *G01N 33/68* (2006.01)
A61K 31/5415 (2006.01)

(21) Internationales Aktenzeichen: PCT/EP2011/064893

(22) Internationales Anmeldedatum:
30. August 2011 (30.08.2011)

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
10 2010 044 561.4
7. September 2010 (07.09.2010) DE

(71) Anmelder *(für alle Bestimmungsstaaten mit Ausnahme von US)*: **UNIVERSITÄT ROSTOCK** [DE/DE]; Universitätsplatz 1, 18055 Rostock (DE).

(72) Erfinder; und
(75) Erfinder/Anmelder *(nur für US)*: **PAHNKE, Jens** [DE/DE]; Zum Fuchsbau 6, 18147 Rostock (DE).

(74) Anwalt: **WABLAT LANGE KARTHAUS**; Potsdamer Chaussee 48, 14129 Berlin (DE).

(81) Bestimmungsstaaten *(soweit nicht anders angegeben, für jede verfügbare nationale Schutzrechtsart)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Bestimmungsstaaten *(soweit nicht anders angegeben, für jede verfügbare regionale Schutzrechtsart)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), eurasisches (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

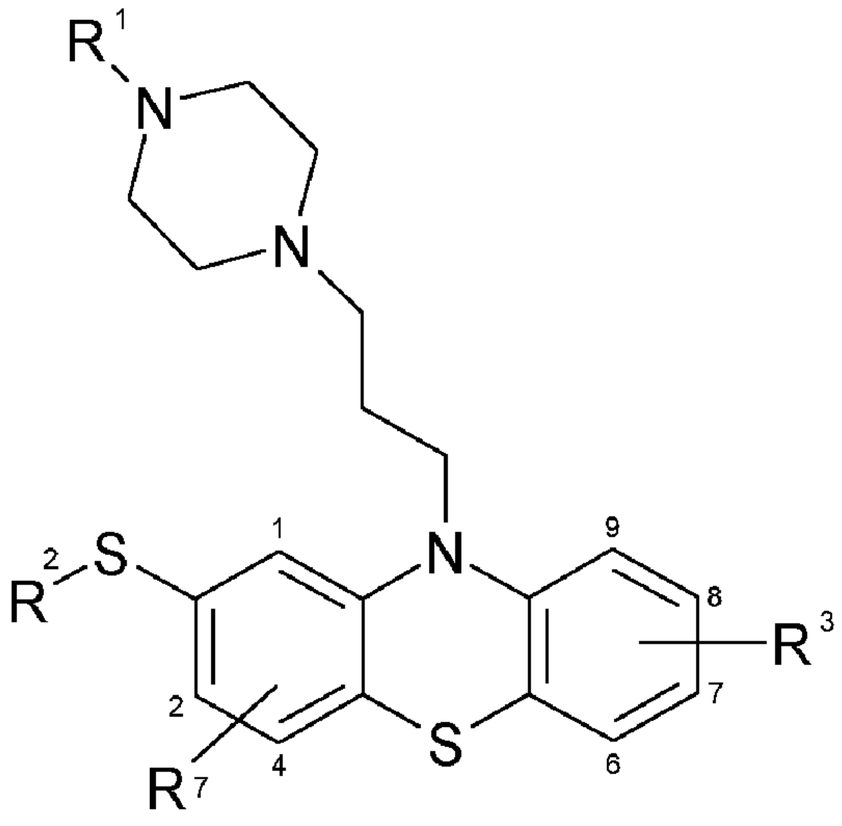
Erklärungen gemäß Regel 4.17:

— *Erfindererklärung (Regel 4.17 Ziffer iv)*

[Fortsetzung auf der nächsten Seite]

(54) Title: 2-(R²-THIO)-10-[3-(4-R¹-PIPERAZIN-1-YL)PROPYL]-10*H*-PHENOTHIAZINE FOR TREATING A β-AMYLOIDOPATHY OR AN ALPHA-SYNUCLEOPATHY, AND METHOD FOR THE DIAGNOSIS OR PREDIAGNOSIS THEREOF

(54) Bezeichnung : 2-(R²-THIO)-10-[3-(4-R¹-PIPERAZIN-1-YL)PROPYL]-10*H*-PHENOTHIAZINE ZUR BEHANDLUNG EINER β-AMYLOIDOPATHIE ODER ALPHA-SYNUCLEOPATHIE SOWIE VERFAHREN ZU DEREN DIAGNOSE ODER PRÄDIAGNOSE



(I)

(57) Abstract: The invention relates to 2-(R²-thio)-10-[3-(4-R¹-piperazin-1-yl)propyl]-10*H*-phenothiazine according to general formula I, for treating a β-amyloidopathy or an α-synucleinopathy accompanied by a cerebral protein deposit and a reduced activity of the cerebral ABCC1-transporter. The invention also relates to a method for the diagnosis or prediagnosis of a β-amyloidopathy or an α-synucleopathy accompanied by a cerebral protein deposit and a reduced activity of the cerebral ABCC1-transporter, or for determining the risk of a proband suffering from such an illness, the proband already having accumulated substances transported by the cerebral ABCC1 transporter.

(57) Zusammenfassung: Die Erfindung betrifft 2-(R²-Thio)-10-[3-(4-R¹-piperazin-1-yl)propyl]-10*H*-phenothiazine gemäss der allgemeinen Formel I zur Behandlung einer β-Amyloidopathie oder einer α-Synucleinopathie, welche mit einer zerebralen Proteinablagerung und einer verminderten Aktivität des zerebralen ABCC1- Transporters einhergehen. Ebenso betrifft die Erfindung ein Verfahren

[Fortsetzung auf der nächsten Seite]

WO 2012/031941 A3

WO 2012/031941 A3

Veröffentlicht:

— *mit internationalem Recherchenbericht (Artikel 21 Absatz 3)*

— *vor Ablauf der für Änderungen der Ansprüche geltenden Frist; Veröffentlichung wird wiederholt, falls Änderungen eingehen (Regel 48 Absatz 2 Buchstabe h)*

(88) Veröffentlichungsdatum des internationalen Recherchenberichts:

20. September 2012

zur Diagnose oder Prädiagnose einer β-Amyloidopathie oder α-Synucleopathie, welche mit einer zerebralen Proteinablagerung und einer verminderten Aktivität des zerebralen ABCC1-Transporters einhergehen oder zur Ermittlung des Risikos eines Probanden, an einer solchen Krankheit zu erkranken, wobei der Proband bereits Substanzen zu sich nimmt, welche über den zerebralen ABCC1-Transporter transportiert werden.

2-(R^2-THIO)-10-[3-(4-R^1-PIPERAZIN-1-YL)PROPYL]-10*H*-PHENOTHIAZINE FOR TREATING A B-AMYLOIDOPATHY OR AN ALPHA-SYNUCLEOPATHY, AND METHOD FOR THE DIAGNOSIS OR PREDIAGNOSIS THEREOF

The accumulation of proteins or protein fragments (peptides) in the brain is a significant feature of age-dependent neurodegenerative diseases. In Alzheimer's dementia (Alzheimer's disease, AD) and cerebral β-amyloidopathy (CAA) the aggregation of β-amyloid peptides (Aβ) is a trigger factor, the basic mechanism being unknown. The Aβ proteostasis, i.e. the equilibrium of production and degradation/removal by means of receptors or proteases is disturbed in AD and CAA. However, so far little attention has been paid to the removal of Aβ peptides by cellular transporters (ABC transporters). In Parkinson's disease, the protein α-synuclein accumulates, which inter alia regulates the dopamine release in the substantia nigra. In Parkinson's disease α-synucleinopathy it is known that ABC transporters play a crucial role for transport (Kortekaaset *al.*, Ann Neural 2005, 57, 176-179). Here there are several subfamilies A-G which can alternatingly transport various substrates(metabolites, medicaments, peptides, proteins, ions) and are even able to replace each other in the transport function (e.g. ABCB1 and ABCC1, Tao *et al.* Cancer Chemotherapy and Pharmacology, 64, 5, 961-969).

It has been shown by means of various genetically modified mouse models that the ABC transporter (a common structural element of the ABC transporter is an ATP-binding cassette and a transport pore) ABCC1 is an important protein/peptide transporter, in particular Aβ transporter, which has extraordinary functional effects on the cerebral protein accumulation. ABCC1 is also an important α-synuclein transporter.

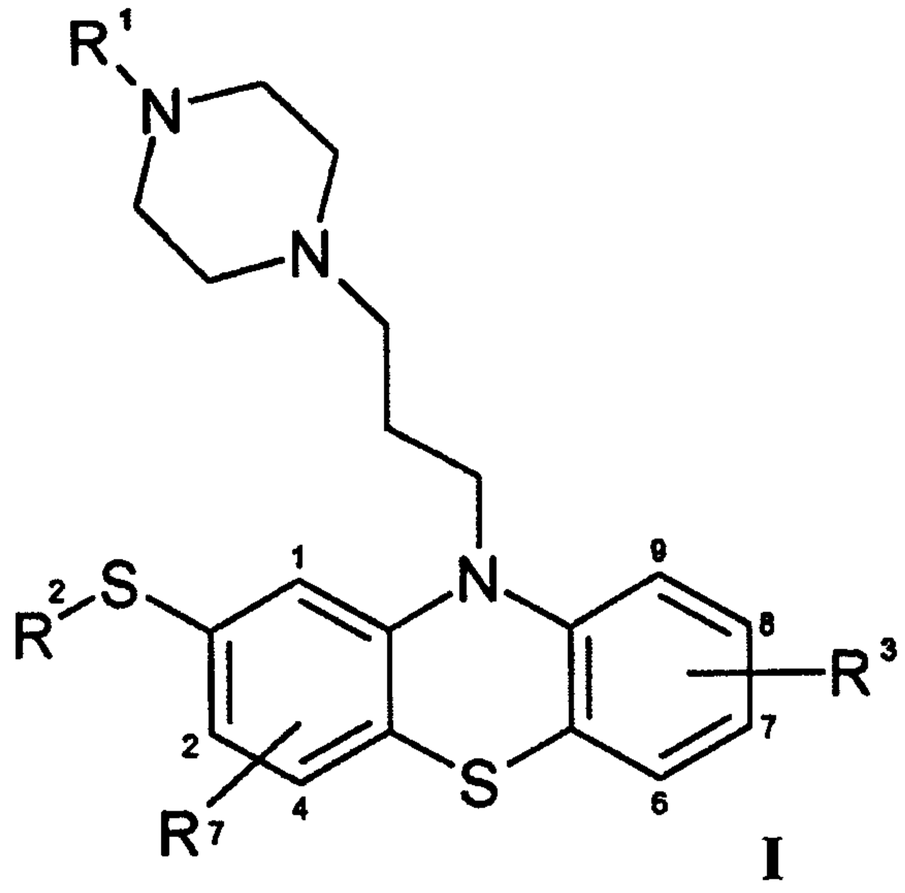
The investigations of the transporter activity are shown as an example hereinafter for Aβ transport.

In order to determine the ABCC1 activity in vivo, in APP-expressing, transgenic mice, the ABCB1, ABCG2 or ABCC1 transporter was removed genetically (knockout mice) in each case.
Here it was found that:

i) the quantity of Aβ in the mice lacking the ABCC1 transporter was increased by a factor of 12,
ii) loss of the ABCB1transporter only results in a three-fold increase and
iii) loss of ABCG2 has no Aβ-accumulating effect.

It was therefore the object of the present invention to provide substances which suitably influence the ABCC1 transporter in order to thus be able to treat neurodegenerative diseases, in particular β-amyloidopathiesor α-synucleopathies. This object was solved by 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines according to claim 1. Further preferred embodiments are obtained from the dependent claims.

In other words, the object was solved by2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines according to the general formula I



I

wherein the residues

R^1 and R^2 are the same or different and each independently of one another are C_1-C_6 alkyl groups, which independently of one another optionally comprise another substituent selected from alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio groups and halogen atoms, wherein the respective alkyl groups optionally comprise at least one further halogen atom and the residue

R^3 is located at one of the positions 6-9 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio or alkylthio group or a halogen atom, wherein the respective alkyl groups optionally comprise at least one further halogen atom or an NR^4R^5 or OR^6 group, wherein R^4, R^5 and R^6 are the same or different and each independently of one another are selected from hydrogen and C_1-C_3 alkyl groups and the residue

R^7 is located at one of the positions 1, 2 or 4 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio or alkylthio group or a halogen atom, wherein the respective alkyl

groups optionally comprise at least one further halogen atom or an NR^8R^9 or OR^{10} group, wherein R^8, R^9 and R^{10} are the same or different and each independently of one another are selected from hydrogen and C_1-C_3 alkyl group, for treating a β-amyloidopathy or an α-synucleinopathy accompanied by a cerebral protein deposit.

Furthermore, both in the case of α-synucleinopathies and in the case of β-amyloidopathies, there is a need to identify or to diagnose or prediagnose these diseases.

It was also the object of the invention to provide a method with which α-synucleinopathies and also β-amyloidopathies can be diagnosed or prediagnosed. This object is solved by a method according to claim 11. Preferred embodiments are obtained from the dependent claims. In other words the object is solved by a method for the diagnosis or prediagnosis of a β-amyloidopathy or α-synucleopathy or for determining the risk of a proband to develop such an illness, wherein the proband already takes substances transported by the cerebral ABCC1 transporter, consisting of the following steps:

a) determining the quantity of ingested substance in body fluid samples of the proband at a specific time point;
b) repeating the determination of step a) at at least one further later time point;
c) comparing the quantities determined in step a) and b) with quantities which had been defined as characteristic at the same time points for probands who at the time of the sampling showed no clinical symptoms of a β-amyloidopathy or an α-synucleopathy.

The fact that the proband already takes at least one substance which is transported via the cerebral ABCC1 transporter means that this substance needs not to be administered. On the contrary it is already present in the

body of the proband, for example, as a result of a drug treatment of another disease. The body fluid samples of the proband which are studied are preferably samples from blood plasma, blood serum and/or cerebral spinal fluid.

The β-amyloidopathy is preferably an Alzheimer's dementia, the α-synucleinopathy is preferably Parkinson's disease. Optionally, the α-synucleinopathy can also be a dementia with Lewy bodies (DLB). Substances which are transported via the cerebral ABCC1 transporter are preferably selected from antibiotics (e.g. difloxacin, grepafloxacin), virostatics/antiviral medicaments(e.g.saquinavir, ritonavir), anti-allergics/antihistamines (e.g.cimetidine), cardio-vascular medicaments (e.g. verapamil), antidepressants (e.g. citalopram), antihyperuricemics (e.g. probenecid), cytostatics (e.g. methotrexate, etoposit, edatrexate, ZD1694), vitamins/vitaminanalogues (e.g. methotrexate, folic acid, L-leucovorin), antiphlogistics (e.g. indomethacin), anti-epileptics (e.g. valproic acid), hormones/hormone derivatives (e.g. 17β-estradiol), leukotrienes (e.g. LTC4), fluorescent samples (e.g. calcein, Fluo-3, BCECF, SNARF), GSH-, sulphate-orglucuronide-coupledmetabolites of natural substances (endogenously produced), toxinsor of medicaments (e.g. 2,4-dinitrophenyl-SG, bimane-SG, N-ethylmaleimide-SG, doxorubicin-SG, thiotepa-SG, cyclophosphamide-SG, melphalan-SG, chlorambucil-SG, ethacrynic acid-SG, metolachlor-SG, atrazine-SG, sulforaphan-SG, aflatoxin B1-epoxide-SG, 4-nitroquinolin 1-oxide-SG, As(SG)3, etoposide-gluc, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)-3β-0-gluc, SN-38-gluc, 4-methylumbelliferyl-β-d-gluc, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)-benzothiazolsulfate (E3040S)-gluc, leukotriene C4, prostaglandin A2-SG, 15-deoxy-Δ12,14 prostaglandin J2-SG, hydroxynonenal-SG, 17β-estradiol-17-β-d-gluc, glucuronosylbilirubin, bis-lucuronosylbilirubin, hyodeoxycholate-6-α-gluc, estron-3-sulfate,

dehydroepiandrosteronesulfate, sulfatolithocholate) (see alsoDeeley RG et al.: Substrate recognition and transport by multi drug resistance protein 1 (ABCC1), FEBS letters 2006, 580 (4), pp. 1103-1111.)

This indirect analysis of the transport activity of ABCC1 transporters can be used for the diagnosis/prediagnosis of a corresponding disease. In probands who already take ABCC1-transportable substances by other routes, the profile of the active substance concentration in body fluids, preferably blood plasma, serum and/or cerebrospinal fluid can be investigated. A time-dependentmeasurement for probands in whom there is a reduced ABCC1 transport activity compared with healthy probands shows a delayed or shifted substance concentration curve (concentration c plotted over time t), i.e. the maximum of the curve varies with time.

When a shifted curve is obtained compared with the healthy case, this is an indication of a changed ABCC1 transport activity. This means that substances such as Aβ or α-synuclein are transported less efficiently and is therefore an indication of a corresponding disease.

Both the mouse model and the pharmacological influencing of the ABCC1 show that this is an important cellular transmembrane transporter for the Aβ protein and imply that the blood-brain barrier and the plexus choroideus occupy a key position for the Aβ release from the brain. It could be shown that the selective pharmacological activation of the ABCC1 transporter significantly reduces the cerebral loading with Aβ and can thus be used therapeutically for the treatment of diseases with disturbed brain proteostasis. Furthermore, the analysis of the transporter activity of the ABCC1 transporter as described above can be used for the indirect or direct diagnosis /prediagnosis of a corresponding disease. Direct analysis would be possible

via the administration of substances which are transported via the ABCC1 transporter, and the determination thereof. The indirect analysis has already been described further above.

Changes to export mechanisms which are related to ABC transporters can substantially influence the temporal aggregation profile of Aβ and other brain proteins. Consequently an influencing of the function of the ABCC1 transporter has a positive effect on the risk of developing neurodegenerative diseases, particularly Alzheimer's disease. "Treatment of neurodegenerative diseases" in this sense comprises the prevention and also the treatment of pre-existing diseases.

The role of the ABC transporter in the Aβ release was initially studied in such a manner that it was demonstrated that ABCC1 is able to transport Aβ.For this purpose in vitro transwell assays with endothelial cells (endothelialcelltranswellassay, ECTA) of primary cultivated capillary endothelial cells from mouse brains (cell culture approach) were used.

Primary cultures of endothelial cells from brain capillaries of ABCB1-deficient, ABCC1-deficient (knock out) mice and control mice (C57BI/6, FVB/N) were used to study the Aβ-specific transport activity. The transport of Aβ from the abluminal (brain) into the luminal (blood) compartment is impaired in ABCB1-deficient and ABCC1-deficient endothelial cells. The mean Aβ transport rate during the first six hours after administration of Aβ peptides (Aβ42) was 2.2 pg/min for the control cells. In contrast to this, the ABCC1-deficient cells only reached half the transport capacity (1.0 pg/min). In the ABCB1-deficient cells the Aβ transport was almost non-existent (0.3 pg/min). Further investigations of capillary endothelial cells and cells from the plexus choroideus

revealed that the ABCB1 transporter is strongly expressed in brain capillary endothelial cells whereas the endothelial ABCC1 expression in brain capillaries is lower.

The relative significance of members of the ABC transporter family was then investigated in vivo using newly generated ABC transporter-deficient Alzheimer mouse models. The genetically modified mice each exhibit a deficiency (knock out) at specific ABC transporters ABCG2, ABCB1 or ABCC1.

The Aβ immunohistochemistry of brain sections showed:
i) significant increases in the cortical number and the size of Aβ-positive plaques in ABCC1-deficient mice compared to control mice (see Fig. 1 and 2a-c).
ii) ABCB1-deficient mice showed a smaller increase in the number and size of Aβ-plaques than ABCC1-deficient mice.
iii) No significant difference could be determined between control mice and ABCG2-deficient mice (Fig. 2a-c).

In order to determine the quantity of buffer-soluble Aβ (mostly monomers and smaller oligomers) and of guanidine-soluble Aβ (mostly fibrillar or aggregated material), enzyme-coupled immune adsorption tests (enzyme-linkedimmunoabsorbentassays, ELISAs) were used for Aβ.

In agreement with the morphological results from the immunohistochemistry, the ABCC1-deficient mice showed a significant increase in aggregated Aβ compared to the control mice at all measurement time points. The cerebral loading with Aβ was greatest at an age of 25 weeks. At this time point, the Aβ values (Aβ42) were 12 times higher than in the control mice. Buffer-soluble Aβ also increased with age but after 25 weeks, at the time of the highest plaque loading, the values of the soluble Aβ in the ABCC1-deficient group decreased substantially.

Further investigations were carried out which provided further proof for the relationship between the possibly lacking removal by ABCC1 and the aggregation of Aβ.

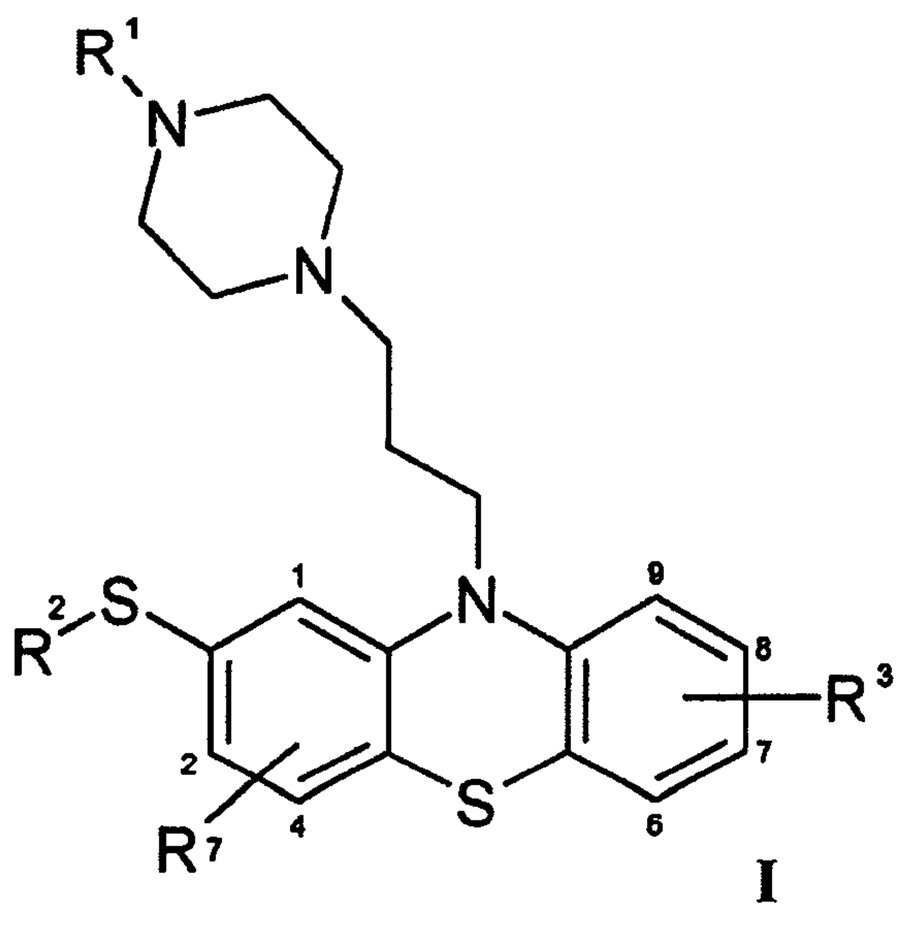
The transport kinetics of ABC transporters depend inter alia on specific protein/peptide characteristics such as the specific charge. The Dutch-type variant of the amyloid precursor protein (Dutch mutant, APP_{dt}) which introduces an additional negative charge near the interface of the α-secretase of the APP and thus results in a severe cerebral amyloidangiopathy(CAA) influence the elimination of $A\beta_{dt}$ via the blood-brain barrier. The Western blot analyses of brain capillaries and plexuschoroideus (CP) from control mice showed a strong expression of ABCB1 in cerebral capillary endothelial cells (BC) and of ABCC1 in CP (Fig. 3d). Since ABC transporters play an important role in the elimination of Aβ, it was assumed that ABC-transporter-deficient (at the blood-brain barrier and at the blood plexus choroideus barrier) APP_{dt}-transgenic mice exhibit an increased accumulation of $A\beta_{dt}$ in meningeal vessels. The degree of CAA in the ABC-deficient APP_{dt} mice was quantified at the age of 24 months. In agreement with the assumption, at least 51% of the vessels were severely impaired (>75% of the vessel wall loaded with Aβ) in the ABCC1-deficient animals compared to 23% in the controls (Fig. 3c).

On the basis of these results, it was investigated how far the content of soluble Aβ in the brain could be reduced/influenced by active-substance-mediated activation of ABC transporters. Mice with amyloid deposits were treated for 30 days with the anti-emeticthiethylperazine(torecan®,2-(ethylthio)-10-[3-(4-methylpiperazin-1-yl)propyl]-10*H*-phenothiazine). 3mg/kg body weight was administered intramuscularly twice daily. The preventative treatment began before the mice exhibited senile plaques. ELISA measurements of the treated animals

showed a reduction in the quantity of Aβ of at least 31% in the treated mice compared to vehicle-treated animals (vehicle = water) (Fig. 3e). The results are reproduced graphically in Fig. 3.

The capacity to remove Aβ proved to be a key factor in the regulation of the intracerebral accumulation of Aβ.

Thiethylperazine (Torecan®) proved to be a particularly efficient activator of the ABCC1 transporter. Other derivatives starting from the same scaffold also showed good results in the activation of the ABCC1 transporter. The corresponding derivatives are represented in the general formula I



I

wherein the residues

R^1 and R^2 are the same or different and each independently of one another are C_1-C_6 alkyl groups, which independently of one another optionally comprise another substituent selected from alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio groups and halogen atoms, wherein the respective

alkyl groups optionally comprise at least one further halogen atom and the residue

R^3 is located at one of the positions 6-9 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio group or a halogen atom, wherein the respective alkyl groups optionally comprise at least one further halogen atom or an NR^4R^5 or OR^6 group, wherein R^4, R^5 and R^6 are the same or different and each independently of one another are selected from hydrogen and C_1-C_3 alkyl groups and the residue

R^7 is located at one of the positions 1, 2 or 4 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio group or a halogen atom, wherein the respective alkyl groups optionally comprise at least one further halogen atom or an NR^8R^9 or OR^{10} group, wherein R^8, R^9 and R^{10} are the same or different and each independently of one another are selected from hydrogen and C_1-C_3 alkyl group

These derivatives are accordingly well suited to the treatment of neurodegenerative diseases, in particular β-amyloidopathies or α-synucleinopathies where the treatment, as already mentioned, comprises both the prevention and the treatment of pre-existing diseases. The halogen atom/the halogen atoms are preferably selected from fluorine and chlorine. The acyl groups (-(C=0)-R) of the residues $R^{1,2,3,7}$ are preferably acetyl groups (-C(=0)CH_3). preferably the residues R^1 and R^2 are the same or different and each independently of one another are a C_1-C_6 alkyl group or a C_1-C_6 alkyl group (preferably C_1 alkyl) substituted with an acetyl group and the residues R^3 and R^7 are hydrogen or an acetyl group. In a preferred embodiment the residues R^1 and R^2 are the same or different and each independently of one another are a C_1-C_3 alkyl group. It is further preferred

that the residues R^3 and R^7 are hydrogen. It is particularly preferred that the residue R^1 is a methyl group, the residue R^2 is an ethyl group and the residues R^3 and R^7 are hydrogen (thiethylperazine, Torecan®). When used for the treatment of neurodegenerative diseases, it has proved advantageous to add further active substances, preferably 1-benzohydrylpiperazines, most preferably 1-benzohydryl-4-cinnamyl piperazine (cinnarizine).

Various neurodegenerative diseases can be treated with the 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazine derivatives according to the invention or can be diagnosed by means of the indirect analysis described above.In a particularly preferred embodiment, the neurodegenerative disease is a β-amyloidopathy, in particular Alzheimer's dementia (AD). Another embodiment relates to the case that the neurodegenerative disease is anα-synucleinopathy, in particular Parkinson's disease (PD). Both diseases, i.e. β-amyloidopathy and α-synucleinopathies are characterized by cerebral protein deposits which can be treated by means of an activation of the ABCC1 transporter or can be diagnosed by means of its activity.

Other diseases which can also be treated by activation of the ABCC1 transporter or which can be diagnosed by means of the ABCC1 transporter activity are mentioned hereinafter. Another treatable disease is thus Lewy body dementia (LBD). This is also characterized by cerebral protein aggregation, i.e. is anα-synucleinopathy like Parkinson's disease.

Another embodiment relates to the case that the neurodegenerative disease is Huntington's disease (HD). Another embodiment relates to the case that the neurodegenerative disease is a prion disease, in particular Creutzfeld-Jacob disease (CJD) orfatalfamilialinsomnia(FFI). Another embodiment relates to

the case that the neurodegenerative disease is a tauopathy, in particular cortico-basal degeneration (CBD), Steel-Richardson-Olszewski syndrome (PSP, progressivesupranuclearpalsy) or Pick's disease (PiD). Another embodiment relates to the case that the neurodegenerative disease frontotemporaldegeneration (FTLD), in particular ubiquitin-positive degeneration, TDP43-positive degeneration or forubiquitin and TDP43-negative degenerations. Another embodiment relates to the case that the neurodegenerative disease is an amyotrophiclateralsclerosis (ALS). Another embodiment relates to the case that the neurodegenerative disease is a spinocerebellarataxia (SCA) or spasticparaparesis (SPG). Another embodiment relates to the case that the neurodegenerative/neuroimmunological disease ismultiple sclerosis (MS) or an MS-related syndrome, in particular ADEM or Devic's syndrome.

Description of the figures

In the figures

Fig. 1a shows that the cortical density of neuritic plaques in ABCC1-deficient mice (ABCCI ko) is increased by ~75%;

Fig. 1b,c shows that the mean plaque size is increased (+34%) as a result of the larger number of plaques (+63%) having a size of more than 700 μm^2 and a lower frequency of smaller plaques (-24%). Error bars, standard error (n ≥3);

Fig. 1d shows that the IHC staining in ABCG2-deficient (ABCG2ko), ABCB1-deficient(ABCBIko), ABCC1-deficient (ABCCIko) miceand in control mice shows a higher surface density of Aβ in ABCC1-deficient animals. Typical plaques of the same size are

shown in section, scaling bars represent500 µm (overview) and 50 µm (section) (*p<0.05);

Fig.2a shows that the plaque density in the cortex (coverage) in specific ABC-transporterknockout mice is increased. In particular ABCC1-deficient(ABCCIko) mice show an increased Aβ-amyloid loading (light-grey bars, in each case on the outside right in the individual groupings), w = week on the abscissa;

Fig. 2b shows that the total plaque size in ABCC1-deficient(ABCCIko) and ABCB1-deficient (ABCBIko) mice at the age of 25 weeks is increased, w = week on the abscissa;

Fig. 2c shows that the total increase in the plaque size is associated with fewer smaller plaques and more larger plaques (>700 μm^2) whereas the number of medium-size plaques remains at the same value, error bars, standard error (n ≥ 5), *p<0.05;

Fig. 3 shows that the deficiency of ABCC1 promotes the accumulation of Aβ and $A\beta_{dt}$ and that the activation of ABCC1 (by administration of Torecan) reduces the Aβ values; where

Fig. 3a shows that at an age of 25 weeks ABCC1 deficiency leads to a marked increase (~12 times) in insoluble Aβ; and

Fig. 3b shows that the quantity of buffer-soluble Aβ42 at an age of 25 weeks is noticeably reduced compared with 22 weeks (-56%). This is probably due to the deposition in insoluble deposits. At the same age the area covered by Aβ deposits which is measured

in the immunohistochemistry is increased by 83% (error bars, standard error n ≥ 5, p< 0.05);

Fig. 3c shows that 53% of the blood vessels are severely impaired by CAA (>75% of the vessel walls exhibit Aβ). This relates to ABCC1-deficient mice (ABCC1ko) compared to 23% in the controls (n = 3);

Fig. 3d shows that the expression of ABCC1 can be seen predominantly in the plexus choroideus (CP) whereas ABCD1 is principally expressed in the capillaries of the brain (BP);

Fig. 3e shows that the activation of ABCC1 by thiethylperazine (Torecan) lowers the Aβ values in mice (-28%), error bars, standard error (n = 4, *p < 0.05).

Examples

Animals

APP-transgenic mice (APP, APP_{dt}) were obtained from The Jackson Laboratory (Bar Harbor, USA) and the University of Tübingen (Tübingen, Germany). The NEP-deficient mice were obtained from the Riken Brain Research Institute (Saitama, Japan). ABCG2-, ABCB1-, and ABCC1-deficient mice were obtained from Taconic-Farms (Denmark). All transgenic and knockout mouse lines were hybridised for at least 9 generations in the geneticFVB-background. The mice were held in a 12h/12h light/dark cycle at 23°C with free access to food and water.

Methods

Tissue preparation

For the tissue preparation the mice were killed by cervical

dislocation and perfused transcardially with PBS (phosphate-buffered, physiological saline solution). The brain was removed and one hemisphere was stored in buffered 4% paraformaldehydefor paraffin embedding andimmunohistochemistry. The other hemisphere was shock-frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

ELISA

ELISA kits (TH40HS, TK42HS) from The Genetics Company (TGC, Schlieren, Switzerland) were used for the quantification of Aβ. Brain hemispheres were homogenised using PreCellys24 (12 s, 6,500 rpm). After addingcarbonate buffer (pH 8.0) the homogenisates were mixed using PreCellys (5 s, 5,000 rpm) and centrifuged for 90 min at 4°C and 24,000 g, in order to separate insoluble from soluble Aβ species. The remaining supernatant (buffer-soluble fraction) was mixed with 8M guanidine hydrochloride in a ratio of 1:1.6. To extract the aggregated Aβspecies, the pellet was dissolved in 8 volumes of 5M guanidine hydrochloride, agitated at room temperature for 3h and centrifuged at 24,000 g for 20 min at 4°C. The remaining supernatant formed the guanidine-soluble fraction (GuaHCI). Protein contents of all the samples were measured three times, using a Nanodrop1000 spectrophotometer (ThermoFisher Scientific, Wilmington, USA). The ELISAswere carried out according to the manufacturer's instructions using suitable dilutions.

Western Blots

Tissue homogenisates were prepared for the WesternBlots. The total protein concentrations of the extracts were determined using a BCA assay (Pierce, part of Thermo Fisher Scientific, Rockford, USA). After electrophoresis of 10 µgtotal protein per trace, the proteins were blotted onto PVDF membranes. After blocking in 5% dry milk in TBST-buffer (50 mMTris pH 7.4, 150 mMNaCI, 0.1 % Tween20) for 1

h at room temperature, the blots were studied either on ABCB1 (1:500, D-11, Santa Cruz), ABCC1 (1:200, Alexis Bio) orβ-actin (1:20.000, Sigma) overnight at 4°C. Anti-mouse-HRP, anti-rat-HRP oranti-hare-HRP were used as detection antibodies. An Amersham ECL Plus Detectionkitand a RoperCoolSnap HQ^2 camera were used for visualisation.

Immunohistochemistry (IHC)

Formalin-fixed brains were embedded in paraffin and cut into 4 µm thick sections. After removing the paraffin, the sections were further treated with a BondMax(TM) Autostainer (Menarini/Leica, Germany). Immunostaining was initiated after blocking of endogenic peroxidase (5 min) and epitoperetrieval for 5 min using 95% formic acid (for antibody 6F3D, Dako, Germany) and 70% formic acid (for antibody 4G8, Millipore, Germany). Primary antibodies were routinely incubated at room temperature for 30 min with the following dilutions: 6F3D (1:100), 4G8 (1:500). Primary antibodies were detected with theBondMax(TM) Bond Polymer Refinedetectionkit and according to the DAB R30 standard protocol. The sections were completely digitised with a resolution of 230nm using a MiraxDesk/MiraxMidi scanner and then analysed automatically using the AxioVision software package (Zeiss, Germany).

Assessment of the severity of the CAA

Brain sections of APP_{dt} were stained with 4G8-antibody. At least two non-consecutive sections were studied for CAA of the meningeal vessels in a masked manner. All meningeal vessels were counted manually and the severity of the CAA was categorised as follows:

Category I: not adversely affected
Category II: ≤25% of the periphery positively stained
Category III: ≤50% of the periphery positively stained
Category IV: ≤75% of the periphery positively stained
Category V: ≤100% of the periphery positively stained

The average number of vessels for each category was calculated relative to the total number of identified vessels.

Endothelial cell transwell assay (ECTA)

Endothelial cells of mouse brain capillaries were prepared as describedin Coisne et al. (Coisne, C. et al.Mouse syngenic*in vitro* blood-brain barrier model: a new tool to examine inflammatory events in cerebral endothelium. Laboratory Investigation; 85, 734-746 (2005)).At least 3-4 week old mice were beheaded and the brains removed. Following dissection of the brain stem, the white matter and the meninges, the tissue was homogenised in two volumes of wash buffer B (WBB) (Hanks bufferedsaltsolution (HBBS), 10 mM HEPES, 0.1 % BSA) using a 15 ml glassdouncer (Wheaton Industries, Millville, NJ; USA). One volume of 30% dextran solution was added to the homogenisate. This was centrifuged twice at 3,000 g and 4°C. The pellet containing the vessels was resuspended in WBB and large vessels were broken up manually by harsh pipetting of the solution. Vacuum filtration through 60 µm membranes (SEFAR, Switzerland) was used to separate large vessels from the capillaries. After combined treatment withcollagenase/dispase (HBSS, 10 mM HEPES, 0.15 g/ml TCLK, 10 µg/ml DNAse-1, 1 mg/ml collagenase/dispase (Roche) single cell suspension was achieved by further harsh pipetting of the solution. Endothelial cells wereinserted intoMatrigel-coatedTranswellinserts (0.4 µmpores, Greiner Bio-One, Germany) having a density of 120,000 cells per insert and allowed to grow on a supporting glial culture.

Sulphur yellow was used to determine the paracellular flux during the assay. The culture medium of the abluminal compartment was replaced with a solution containing 10 ng Ass42 (1.6 nM final concentration). Samplesfrom the luminal compartment were then taken after 2h, 6h or 24h and the Aβ

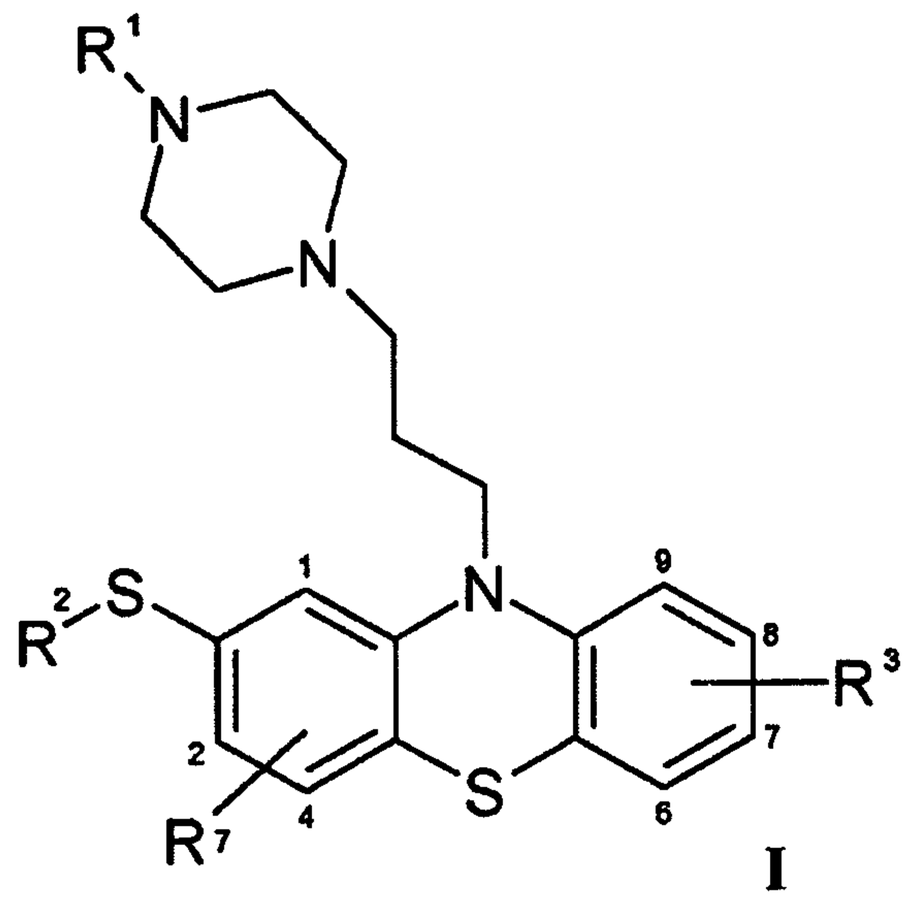
content was determined with ELISA (TK42-highsense, TGC, Switzerland). The transport rate was described in Coisne*et al.* (Coisne, C. *et al.* Mouse syngenic*in vitro* blood-brain barrier model: a new tool to examine inflammatory events in cerebral endothelium. Laboratory Investigation; 85, 734-746 (2005)).

ELISA statistics

TheLillieforsgoodness-of-fit test (alpha=0.05) was applied to the ELISA data and to the log-transformed ELISA data to distinguish between the assumption of normally distributed sample data and the assumption of log-normally distributed sample data. Despite the small sample size, the null hypothesis (H_0) was dismissed for both sets of data for 5 of 44 samples. In agreement with the observation of predominantly positive (skew) and strictly positive sample data, the assumption of normally distributed data was rejected. Mean confidence intervals were calculated assuming a basic log-normal distribution. The Wilcoxon rank-sum test was applied to compare the ELISA data of the various mouse strains for each time point.

CLAIMS

1. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines according to the general formula I



I

wherein the residues

R^1 and R^2 are the same or different and each independently of one another are C_1-C_6 alkyl groups, which independently of one another optionally comprise another substituent selected from alkyl, aryl, acyl, amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio groups and halogen atoms, wherein the respective alkyl groups optionally comprise at least one further halogen atom and the residue

R^3 is located at one of the positions 6-9 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl, amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio group or a halogen atom, wherein the respective alkyl groups optionally comprise at least one further halogen atom or an NR^4R^5 or OR^6 group, wherein R^4, R^5 and R^6 are the

same or different and each independently of one another are selected from hydrogen and C_1-C_3 alkyl groups and the residue

R^7 is located at one of the positions 1, 2 or 4 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl, amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio group or a halogen atom, wherein the respective alkyl groups optionally comprise at least one further halogen atom or an NR^8R^9 or OR^{10} group, wherein R^8, R^9 and R^{10} are the same or different and each independently of one another are selected from hydrogen and C_1-C_3 alkyl group, for treating a β-amyloidopathy or an α-synucleinopathy accompanied by a cerebral protein deposit and a reduced activity of the cerebral ABCC1-transporter.

2. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to claim 1, characterized in that the halogen atom/the halogen atoms are selected from fluorine and chlorine.

3. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to claim 1 or 2, characterized in that R^1 and R^2 are the same or different and each independently of one another are a C_1-C_3 alkyl group.

4. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to any one of claims 1 to 3, characterized in that the residues R^3 and R^7 are hydrogen.

5. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to any one of claims 1 to 4, characterized in that the residue R^1 is

a methyl group, the residue R^2 is an ethyl group and the residues R^3 and R^7 are hydrogen.

6. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to any one of claims 1 to 5, characterized in that further active substances are added.

7. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to claim 6, characterized in that 1-benzohydrylpiperazines are added as further active substances.

8. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to claim 6 or 7, characterized in that 1-benzohydryl-4-cinnamyl piperazine is added as 1-benzohydrylpiperazine.

9. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to any one of claims 1 to 8, characterized in that the β-amyloidopathy is Alzheimer's dementia.

10. 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines for the use according to any one of claims 1 to 8, characterized in that the α-synucleinopathy is Parkinson's disease or Lewy body dementia.

11. Method for the diagnosis or prediagnosis of a β-amyloidopathy or α-synucleopathy accompanied by a cerebral protein deposit and a reduced activity of the cerebral ABCC1-transporter, or for determining the risk of a proband to develop such an illness, wherein the proband has already taken substances transported by the cerebral ABCC1 transporter, consisting of the following steps:

a) determining the quantity of ingested substance in body fluid samples of the proband at a specific time point;

b) repeating the determination of step a) at at least one further later time point;

c) comparing the quantities determined in step a) and b) with quantities which had been defined as characteristic at the same time points for probands who at the time of the sampling showed no clinical symptoms of a β-amyloidopathy or an α-synucleopathy.

12. Method for the diagnosis or prediagnosis of a β-amyloidopathy or α-synucleopathy or for determining the risk of a proband to develop such a disease according to claim 11, characterized in that the body fluid samples of the proband are samples of blood plasma, blood serum and/or cerebrospinal fluid.

13. Method for the diagnosis or prediagnosis of a β-amyloidopathy or α-synucleopathy or for determining the risk of a proband to develop such a disease according to claim 11 or 12, characterized in that the β-amyloidopathy is an Alzheimer's dementia.

14. Method for the diagnosis or prediagnosis of a β-amyloidopathy or α-synucleopathy or for determining the risk of a proband to develop such a disease according to claim 11 or 12, characterized in that the α-synucleopathy is Parkinson's disease or Lewy body dementia.

15. Method for the diagnosis or prediagnosis of a β-amyloidopathy or an α-synucleopathy or for determining

the risk of a proband to develop such a disease according to any one of claims 11-14, characterized in that the substances transported via the cerebral ABCC1 transporter are selected from antibiotics, virostatics/antiviral medicaments, anti-allergics/antihistamines, cardio-vascular medicaments, antidepressants, antihyperuricemics, cytostatics, vitamins/vitamin analogues, antiphlogistics, anti-epileptics, hormones/hormone derivatives, leukotrienes, fluorescent samples, GSH-, sulphate or glucoronide-coupled metabolites of natural substances (endogenously produced), toxins, or from medicaments.

Application number / Numéro de demande: EP 2011 064893

Figures: 1d et 3d

Pages: 4/12 et 11/12

Unscannable items received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de la préparation
des dossiers au 10ième étage)

Fig. 1a

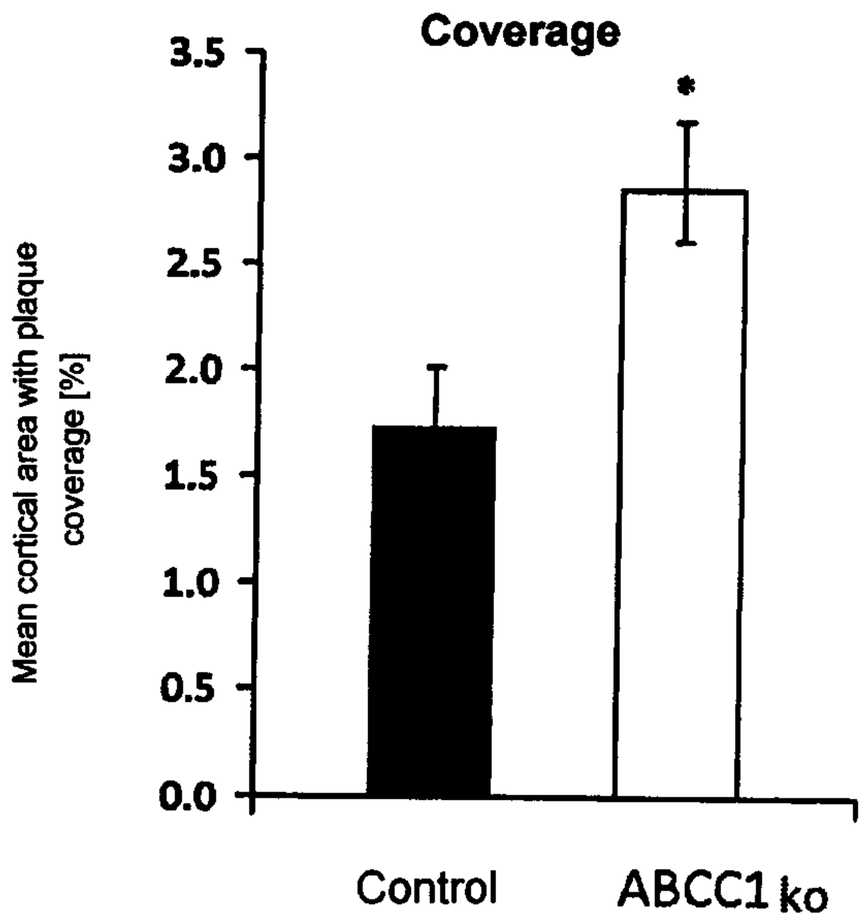

Coverage
Mean cortical area with plaque coverage [%]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
ABCC1 ko

Fig. 1b

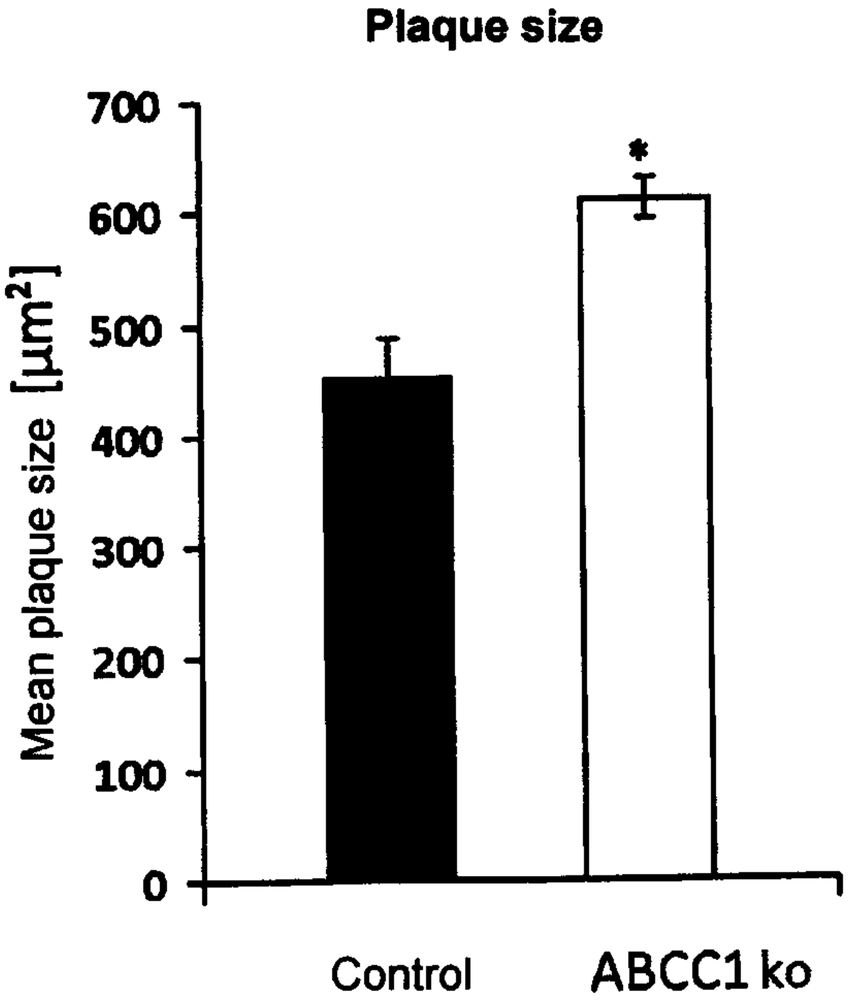

Plaque size
Mean plaque size [µm²]
700
600
500
400
300
200
100
0
*
Control
ABCC1 ko

Fig. 1c

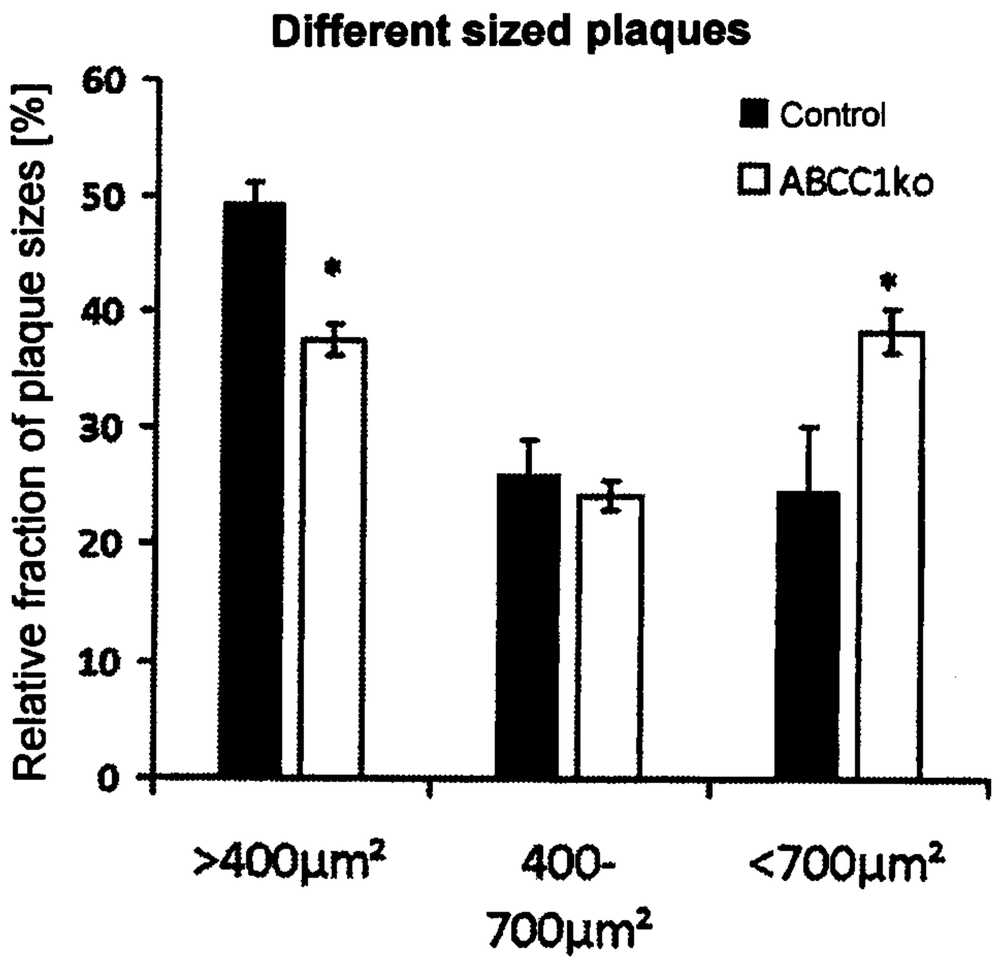

Different sized plaques
Control
ABCC1ko
Relative fraction of plaque sizes [%]
60
50
40
30
20
10
0
*
*
>400µm²
400-
700µm²
<700µm²

Fig. 2a

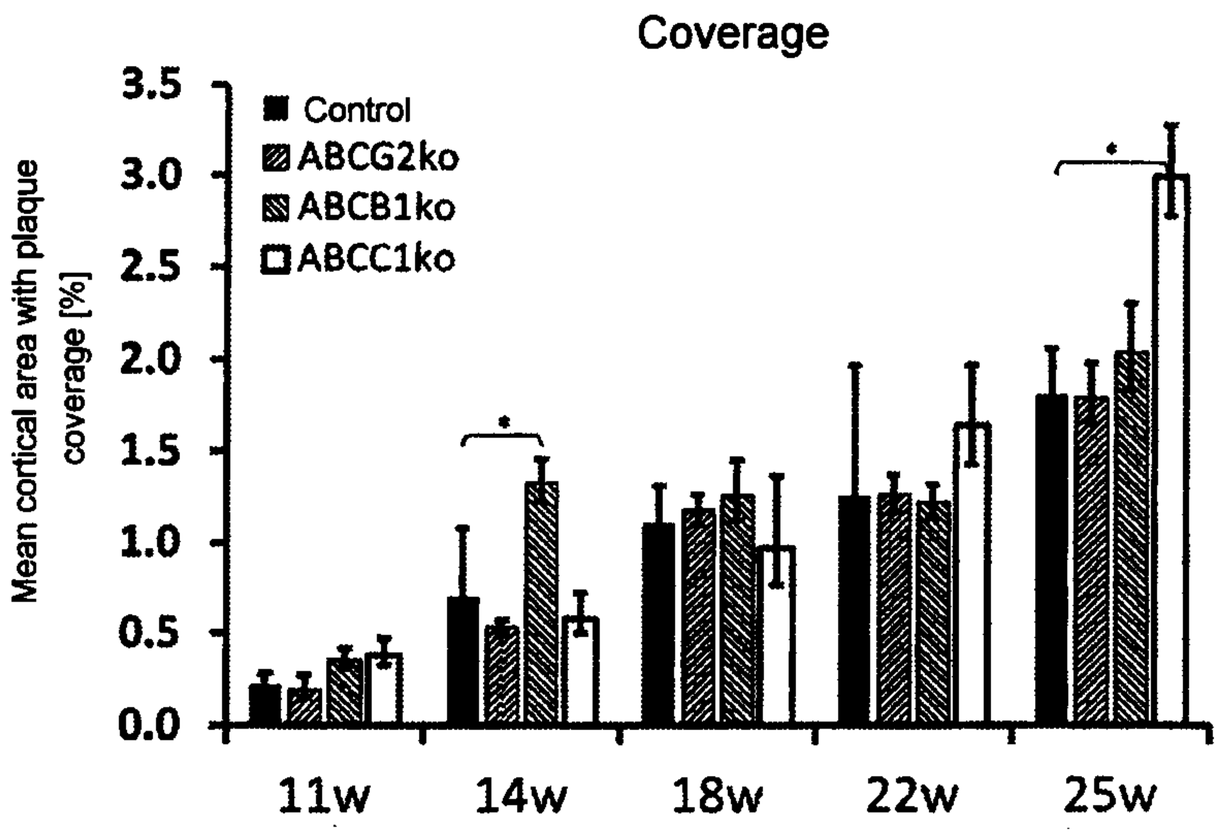

Coverage
Control
ABCG2ko
ABCB1ko
ABCC1ko
Mean cortical area with plaque coverage [%]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
11w
14w
18w
22w
25w

Fig. 2b

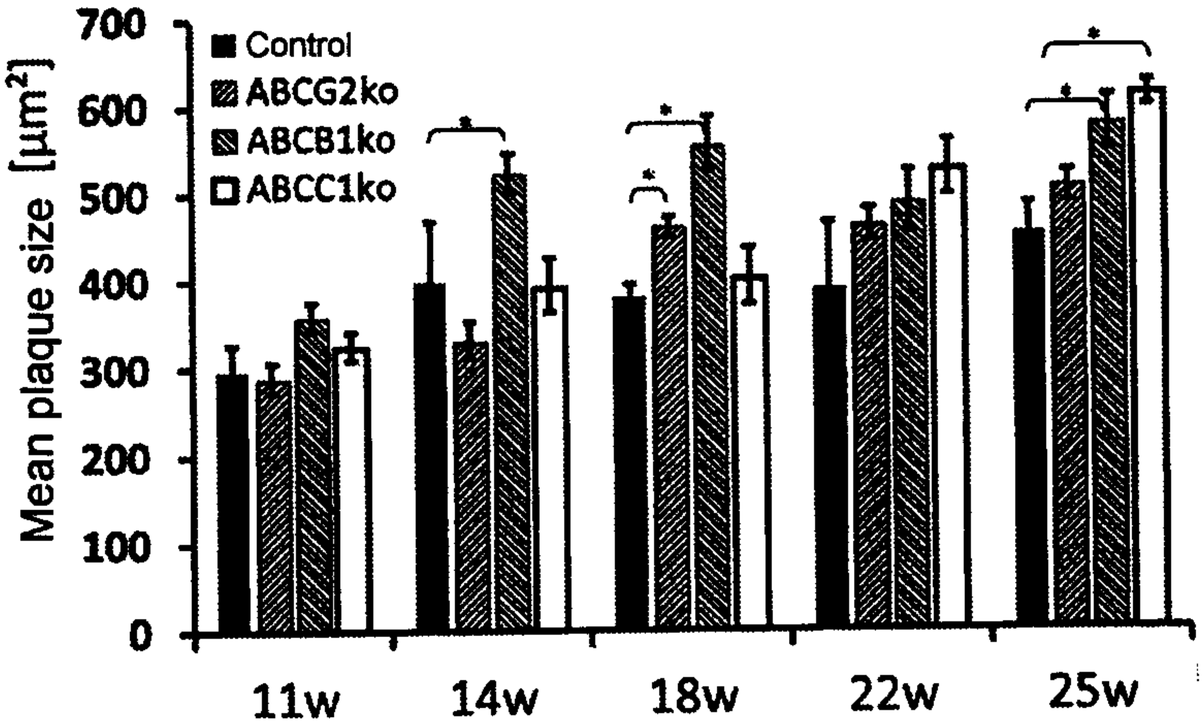

Plaque size
Mean plaque size [µm²]
700
600
500
400
300
200
100
0
Control
ABCG2ko
ABCB1ko
ABCC1ko
11w
14w
18w
22w
25w
*

Fig. 2c

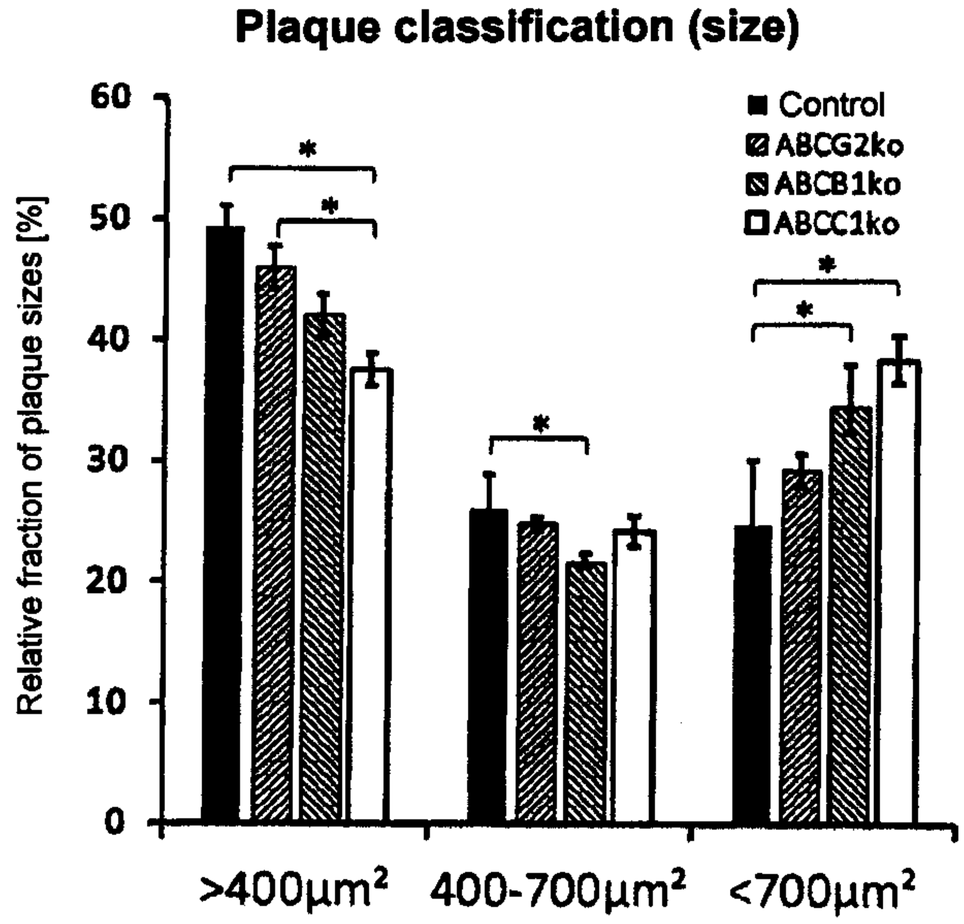

Plaque classification (size)
Relative fraction of plaque sizes [%]
60
50
40
30
20
10
0
Control
ABCG2ko
ABCB1ko
ABCC1ko
*
*
*
*
*
>400µm²
400-700µm²
<700µm²

Fig. 3a

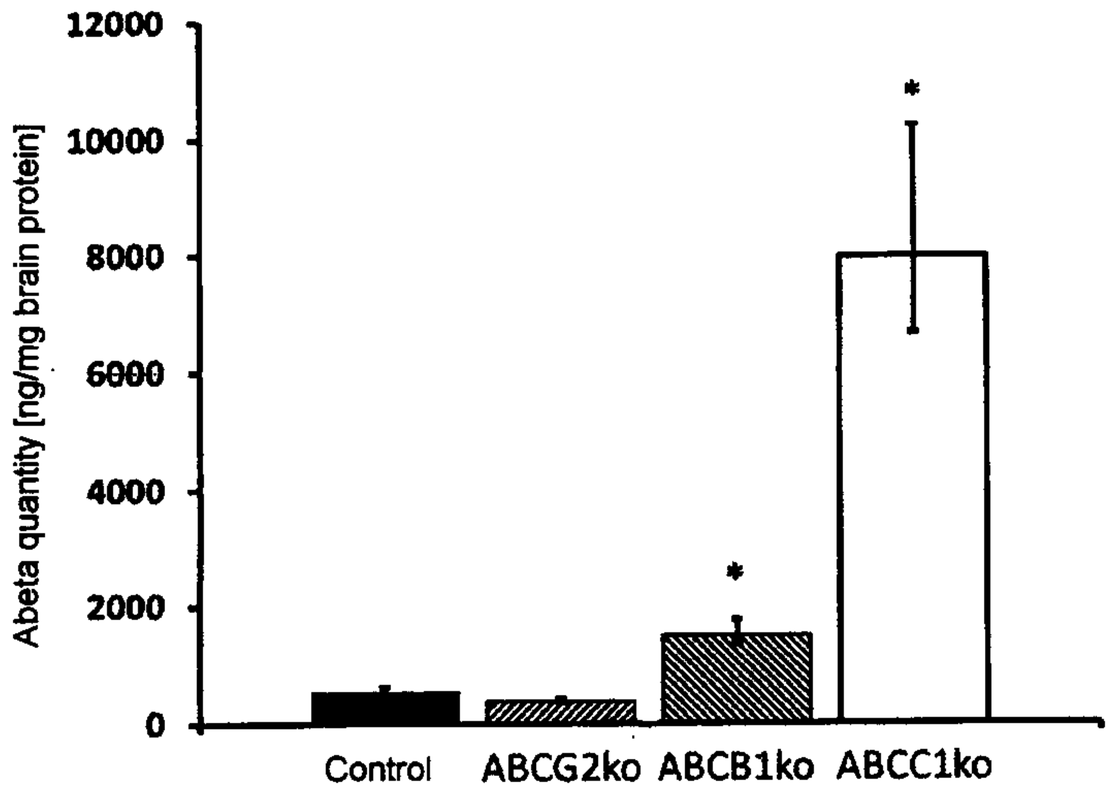

12000
10000
8000
6000
4000
2000
0
Abeta quantity [ng/mg brain protein]
*
*
Control
ABCG2ko
ABCB1ko
ABCC1ko

Fig. 3b

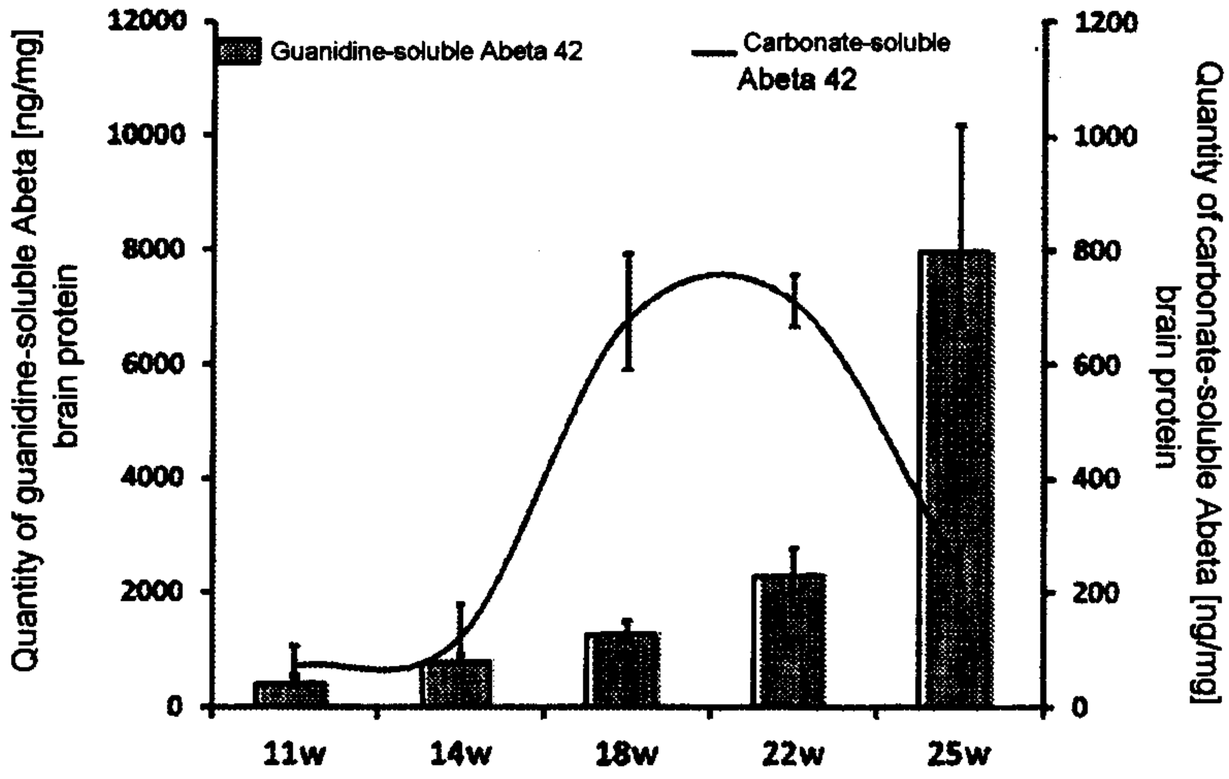

Guanidine-soluble Abeta 42
Carbonate-soluble Abeta 42
Quantity of guanidine-soluble Abeta [ng/mg] brain protein
Quantity of carbonate-soluble Abeta [ng/mg] brain protein
12000
10000
8000
6000
4000
2000
0
1200
1000
800
600
400
200
0
11w
14w
18w
22w
25w

Fig. 3c

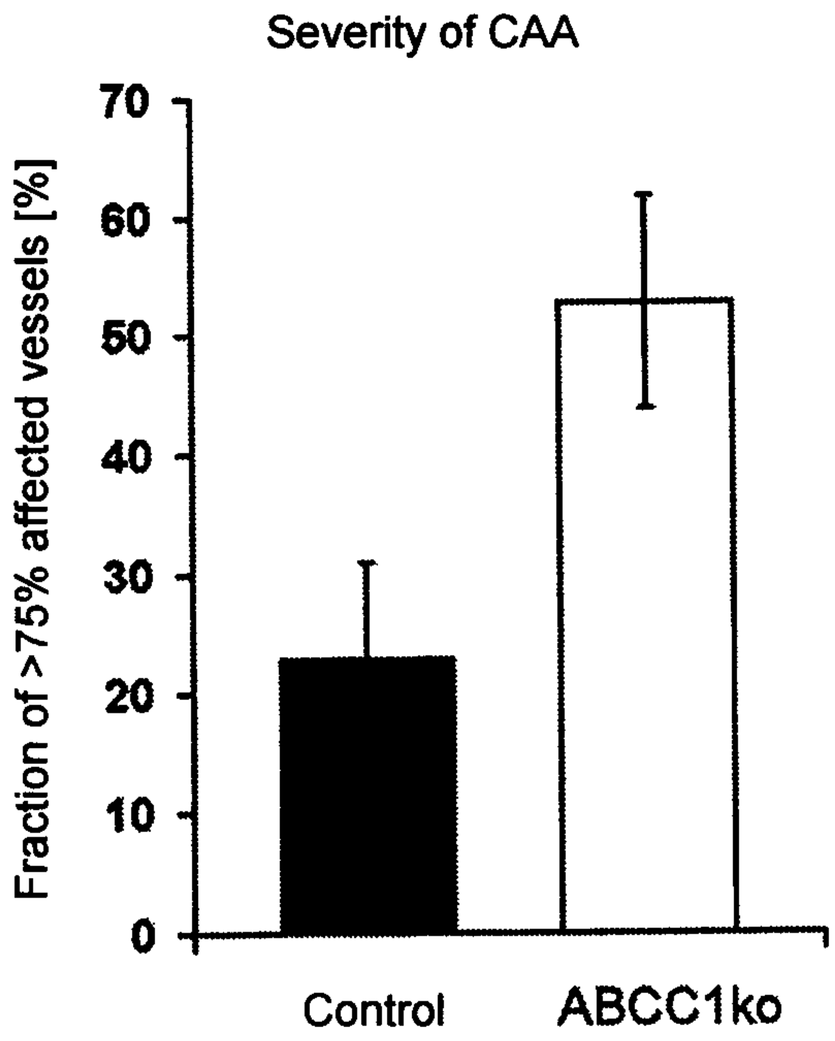
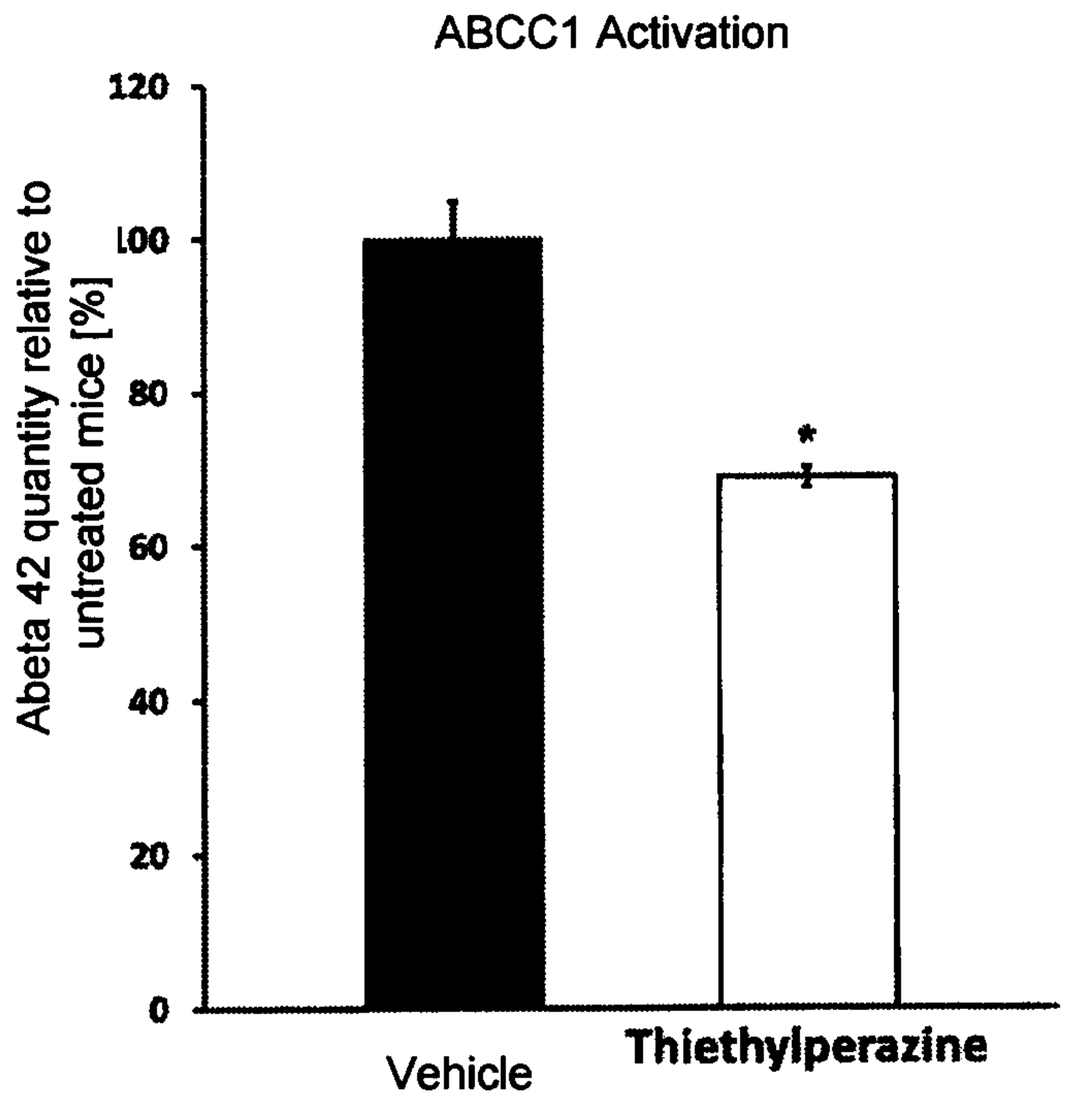
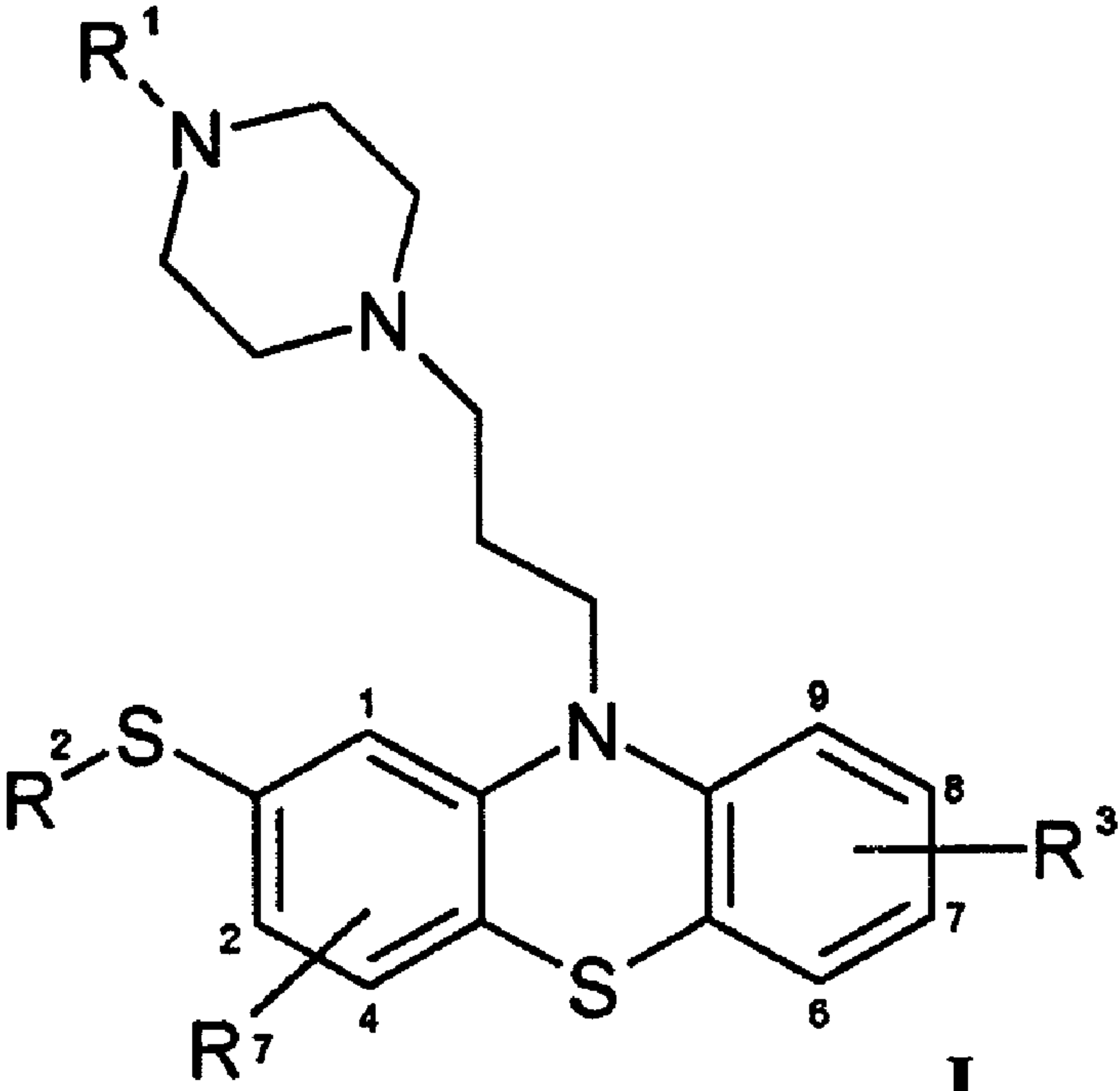

Severity of CAA
Fraction of >75% affected vessels [%]
70
60
50
40
30
20
10
0
Control
ABCC1ko

Fig. 3e


ABCC1 Activation
Abeta 42 quantity relative to untreated mice [%]
120
100
80
60
40
20
0
*
Vehicle
Thiethylperazine



I