(11) Application No. AU 2004237408 C9

(19) AUSTRALIAN PATENT OFFICE

(54) Title

Medical use of inhibitors of glutaminyl and glutamate cyclases

(51)[6] International Patent Classification(s)

A61K 31/00 (2006.01) 20060101ALI2005100 8BMEP **A61K** 31/4188
A61K 31/4178 (2006.01) 20060101ALI2005100 8BMEP **A61K** 31/4192
A61K 31/4184 (2006.01) 20060101ALI2005100 8BMEP **A61K** 31/4745
A61K 31/4188 (2006.01) 20060101ALI2005100 8BMEP **C07D** 233/54
A61K 31/4192 (2006.01) 20060101ALI2005100 8BMEP **C07F** 9/572
A61K 31/4745 (2006.01) 20060101ALI2005100 8BMEP **C07F** 9/59
C07D 233/54 (2006.01) 20060101ALI2005100 8BMEP **C07F** 9/6506
C07F 9/572 (2006.01) 20060101ALI2005100 8BMEP **C07F** 9/6561
C07F 9/59 (2006.01) 20060101ALI2005100 8BMEP
C07F 9/6506 (2006.01) PCT/EP2004/004778
C07F 9/6561 (2006.01)
A61K 31/00 20060101AFI2005100 8BMEP **A61K** 31/4178 20060101ALI2005100 8BMEP **A61K** 31/4184 20060101ALI2005100 8BMEP

(21) Application No: 2004237408

(22) Application Date: 2004.05.05

(87) WIPO No: WO04/098625

(30) Priority Data

(31) Number	(32) Date	(33) Country
60/512,038	2003.10.15	US
60/468,043	2003.05.05	US
60/468,014	2003.05.05	US

20100415

(43) Publication Date : 2004.11.18

(71) Applicant(s)

Probiodrug AG

(72) Inventor(s)

Niestroj, Andre J.; Heiser, Ulrich; Demuth, Hans-Ulrich; Hoffmann, Torsten; Schilling, Stephan

(74) Agent/Attorney

Watermark Patent & Trademark Attorneys, Level 2 302 Burwood Road, Hawthorn, VIC, 3122

(56) Related Art

WO 2002/013821 A1
WO 2001/034594 A1
WO 1993/020061 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 November 2004 (18.11.2004)

PCT

(10) International Publication Number
WO 2004/098625 A3

(51) International Patent Classification[7]: A61K 31/00, 31/4745, A61P 25/00

(21) International Application Number:
PCT/EP2004/004778

(22) International Filing Date: 5 May 2004 (05.05.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/468,043	5 May 2003 (05.05.2003)	US
60/468,014	5 May 2003 (05.05.2003)	US
60/512,038	15 October 2003 (15.10.2003)	US

(71) Applicant *(for all designated States except US)*: **PROBIODRUG AG** [DE/DE]; Weinbergweg 22, 06120 Halle/Saale (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DEMUTH, Hans-Ulrich** [DE/DE]; Hegelstrasse 14, 06114 Halle/Saale (DE). **HOFFMANN, Torsten** [DE/DE]; Körnerstrasse 8, 06114 Halle/Saale (DE). **NIESTROJ, André, J.** [DE/DE]; Thälmannplatz 1, 06193 Sennewitz (DE). **SCHILLING, Stephan** [DE/DE]; Läuferweg 4, 06128 Halle/Saale (DE). **HEISER, Ulrich** [DE/DE]; Franz-Schubert-Strasse 5, 06108 Halle/Saale (DE).

(74) Agents: **FORSTMEYER, Dietmar** et al.; Boeters & Lieck, Bereiteranger 15, 81541 München (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
23 June 2005

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/098625 A3

(54) Title: MEDICAL USE OF INHIBITORS OF GLUTAMINYL AND GLUTAMATE CYCLASES

(57) Abstract: The present invention provides novel physiological substrates of mammalian glutaminyl cyclase (QC, EC 2.3.2.5), new effectors of QC, methods for screeing for such effectors, and the use of such effectors and pharmaceutical compositions comprising such effectors for the treatment of conditions that can be treated by modulation of QC-activity. Preferred compositions additionally comprise inhibitors of DP IV or DP IV-like enzymes for the treatment or alleviation of conditions that can be treated by modulation of QC- and DP IV-activity.

Use of Effectors of Glutaminyl and Glutamate Cyclases

Field of the invention

The invention relates to glutaminyl cyclase (QC, EC 2.3.2.5) that catalyzes the intramolecular cyclization of N-terminal glutamine residues into pyroglutamic acid (5-oxo-proline, pGlu*) under liberation of ammonia and the intramolecular cyclization of N-terminal glutamate residues into pyroglutamic acid under liberation of water.

The present invention identifies mammalian QCs as metalloenzymes, provides novel physiological substrates of QC in mammals and the use of effectors of QC and pharmaceutical compositions comprising effectors of QC for the treatment of conditions that can be treated by modulation of QC-activity. Additionally, it is shown that metal interaction is a useful approach for development of QC inhibitors.

In a preferred embodiment, the present invention provides the use of effectors of QC activity in combination with inhibitors of DP IV or DP IV-like enzymes for the treatment or alleviation of conditions that can be treated by modulation of QC- and/or DP IV-activity.

A screening method is also provided for the identification and selection of effectors of QC activity.

Background

Glutaminyl cyclase (QC, EC 2.3.2.5) catalyzes the intramolecular cyclization of N-terminal glutamine residues into pyroglutamic acid (pGlu*) liberating ammonia. A QC was first isolated by Messer from the latex of the tropical plant *Carica papaya* in 1963 (Messer, M. 1963 *Nature 4874*, 1299). 24 years later, a corresponding enzymatic activity was discovered in animal pituitary (Busby, W. H. J. et al. 1987 *J Biol Chem 262*, 8532-8536; Fischer, W. H. and Spiess, J. 1987 *Proc Natl Acad Sci U S A 84*, 3628-3632). For the mammalian QC, the conversion of Gln into pGlu by QC could be shown for the precursors of TRH and GnRH (Busby, W. H. J. et al. 1987 *J Biol Chem*

262, 8532-8536; Fischer, W. H. and Spiess, J. 1987 *Proc Natl Acad Sci U S A 84*, 3628-3632). In addition, initial localization experiments of QC revealed a co-localization with its putative products of catalysis in bovine pituitary, further improving the suggested function in peptide hormone synthesis (Bockers, T. M. et al. 1995 *J Neuroendocrinol 7*, 445-453). In contrast, the physiological function of the plant QC is less clear. In case of the enzyme from *C. papaya*, a role in the plant defense against pathogenic microorganisms was suggested (El Moussaoui, A. et al.2001 *Cell Mol Life Sci 58*, 556-570). Putative QCs from other plants were identified by sequence comparisons recently (Dahl, S. W. et al.2000 *Protein Expr Purif 20*, 27-36) The physiological function of these enzymes, however, is still ambiguous.

The QCs known from plants and animals show a strict specificity for L-Glutamine in the N-terminal position of the substrates and their kinetic behavior was found to obey the Michaelis-Menten equation (Pohl, T. et al. 1991 *Proc Natl Acad Sci U S A 88*, 10059-10063; Consalvo, A. P. et al. 1988 *Anal Biochem 175*, 131-138; Gololobov, M. Y. et al. 1996 *Biol Chem Hoppe Seyler 377*, 395-398). A comparison of the primary structures of the QCs from *C. papaya* and that of the highly conserved QC from mammals, however, did not reveal any sequence homology (Dahl, S. W. et al. 2000 *Protein Expr Purif 20*, 27-36). Whereas the plant QCs appear to belong to a new enzyme family (Dahl, S. W. et al. 2000 *Protein Expr Purif 20*, 27-36), the mammalian QCs were found to have a pronounced sequence homology to bacterial aminopeptidases (Bateman, R. C. et al. 2001 *Biochemistry 40*, 11246-11250), leading to the conclusion that the QCs from plants and animals have different evolutionary origins.

EP 02 011 349.4 discloses polynucletides encoding insect glutaminyl cyclase, as well as polypeptides encoded thereby. This application further provides host cells comprising expression vectors comprising polynucleotides of the invention. Isolated polypeptides and host cells comprising insect QC are useful in methods of screening for agents that reduce glutaminyl cyclase activity. Such agents are described as useful as pesticides.

Alzheimer's disease (AD) is characterized by abnormal accumulation of extracellular amyloidotic plaques closely associated with dystrophic neurones, reactive astrocytes and microglia (Terry, R. D. and Katzman, R. 1983 *Ann Neurol 14*, 497-506; Glenner, G. G. and Wong, C. W. 1984 *Biochem Biophys Res Comm 120*, 885-890; Intagaki, S. et al. 1989 *J Neuroimmunol 24*, 173-182; Funato, H. et al. 1998 *Am J Pathol* 152, 983-992; Selkoe, D. J. 2001 *Physiol Rev 81*, 741-766). Amyloid-β (Aβ) peptides are the primary components of senile plaques and are considered to be directly involved in the pathogenesis and progression of AD, a hypothesis supported by genetic studies (Glenner, G. G. and Wong, C. W. 1984 *Biochem Biophys Res Comm 120*, 885-890; Borchelt, D. R. et al. 1996 *Neuron 17*, 1005-1013; Lemere, C. A. et al. 1996 *Nat Med 2*, 1146-1150; Mann, D. M. and Iwatsubo, T. 1996 *Neurodegeneration 5*, 115-120; Citron, M. et al. 1997 *Nat Med 3*, 67-72; Selkoe, D. J. 2001 *Physiol Rev 81*, 741-766). Aβ is generated by proteolytic processing of the β-amyloid precursor protein (APP) (Kang, J. et al. 1987 *Nature 325*, 733-736; Selkoe, D. J. 1998 *Trends Cell Biol 8*, 447-453), which is sequentially cleaved by β-secretase at the N-terminus and by γ-secretase at the C-terminus of Aβ (Haass, C. and Selkoe, D. J. 1993 *Cell 75*, 1039-1042; Simons, M. et al. 1996 *J Neurosci* 16 899-908). In addition to the dominant Aβ peptides starting with L-Asp at the N-terminus (Aβ1-42/40), a great heterogeneity of N-terminally truncated forms occurs in senile plaques. Such shortened peptides are reported to be more neurotoxic *in vitro* and to aggregate more rapidly than the full-length isoforms (Pike, C. J. et al. 1995 *J Biol Chem* 270 23895-23898). N-truncated peptides are known to be overproduced in early onset familial AD (FAD) subjects (Saido, T. C. et al. 1995 *Neuron 14*, 457-466; Russo, C. et al. 2000 *Nature 405*, 531-532), to appear early and to increase with age in Down's syndrome (DS) brains (Russo, C. et al. 1997 *FEBS Lett 409*, 411-416, Russo, C. et al. 2001 *Neurobiol Dis 8*, 173-180; Tekirian, T. L. et al. 1998 *J Neuropathol Exp Neurol 57*, 76-94). Finally, their amount reflects the progressive severity of the disease (Russo, C. et al. 1997 *FEBS Lett 409*, 411-416). Additional post-translational processes may further modify the N-terminus by isomerization or racemization of the aspartate at position 1 and 7 and by cyclization of glutamate at residues 3 and 11. Pyroglutamate-containing isoforms at position 3 [pGlu^3]Aβ3-40/42] represent the prominent forms – approximately 50 % of the total Aβ amount – of the N-truncated

species in senile plaques (Mori, H. et al. 1992 *J Biol Chem 267*, 17082-17086, Saido, T. C. et al. 1995 *Neuron 14*, 457-466; Russo, C. et al. 1997 *FEBS Lett 409*, 411-416; Tekirian, T. L. et al. 1998 *J Neuropathol Exp Neurol 57*, 76-94; Geddes, J. W. et al. 1999 *Neurobiol Aging 20*, 75-79; Harigaya, Y. et al. 2000 *Biochem Biophys Res Commun 276*, 422-427) and they are also present in pre-amyloid lesions (Lalowski, M. et al. 1996 *J Biol Chem 271*, 33623-33631). The accumulation of AβN3(pE) peptides is likely due to the structural modification that enhances aggregation and confers resistance to most amino-peptidases (Saido, T. C. et al. 1995 *Neuron 14*, 457-466 ; Tekirian, T. L. et al. 1999 *J Neurochem 73*, 1584-1589). This evidence provides clues for a pivotal role of AβN3(pE) peptides in AD pathogenesis. However, relatively little is known about their neurotoxicity and aggregation properties (He, W. and Barrow, C. J. 1999 *Biochemistry 38*, 10871-10877; Tekirian, T. L. et al. 1999 *J Neurochem 73*, 1584-1589). Moreover, the action of these isoforms on glial cells and the glial response to these peptides are completely unknown, although activated glia is strictly associated to senile plaques and might actively contribute to the accumulation of amyloid deposits. In recent studies the toxicity, aggregation properties and catabolism of Aβ1-42, Aβ1-40, [pGlu^3]Aβ3-42 and [pGlu^3]Aβ3-40 peptides were investigated in neuronal and glial cell cultures, and it was shown that pyroglutamate modification exacerbates the toxic properties of Aβ-peptides and also inhibits their degradation by cultured astrocytes. Shirotani et al. investigated the generation of [pGlu^3]Aβ peptides in primary cortical neurons infected by Sindbis virus *in vitro*. They constructed amyloid precursor protein complementary DNAs, which encoded a potential precursor for [pGlu^3]Aβ by amino acid substitution and deletion. For one artificial precursor starting with a N-terminal glutamine residue instead of glutamate in the natural precursor, a spontaneous conversion or an enzymatic conversion by glutaminyl cyclase to pyroglutamate was suggested. The cyclization mechanism of N-terminal glutamate at position 3 in the natural precursor of [pGlu^3]Aβ was not determined *in vivo* (Shirotani, K., Tsubuki, S., Lee, H. J., Maruyama, K., and Saido, T. C. (2002) *Neurosci Lett* **327**, 25-28)

Dipeptidyl peptidase IV (DP IV) is a post-proline (to a lesser extent post-alanine, post-serine or post-glycine) cleaving serine protease found in various tissues of the

body including kidney, liver, and intestine and cleaves N-terminal dipeptides from a peptide chain. Recently it was shown that DP IV plays an important role in neuropeptide metabolism, T-cell activation, attachment of cancer cells to the endothelium and the entry of HIV into lymphoid cells. See therefore WO 02/34242, WO 02/34243, WO 03/002595 and WO 03/002596.

It is known that DP IV inhibitors may be useful for the treatment of impaired glucose tolerance and diabetes mellitus (International Patent Application, Publication Number WO 99/61431, Pederson, R. A. et al. 1998 *Diabetes 47*, 1253-1258 and Pauly, R. P. et al. 1999 *Metabolism* 48, 385-389). In particular WO 99/61431 discloses DP IV inhibitors comprising an amino acid residue and a thiazolidine or pyrrolidine group, and salts thereof, especially L-*threo*-isoleucyl thiazolidine, L-*allo*-isoleucyl thiazolidine, L-*threo*-isoleucyl pyrrolidine, L-*allo*-isoleucyl thiazolidine, L-*allo*-isoleucyl pyrrolidine, and salts thereof.

Further examples of low molecular weight dipeptidyl peptidase IV inhibitors are agents such as tetrahydroisoquinolin-3-carboxamide derivatives, N-substituted 2-cyanopyroles and –pyrrolidines, N-(N'-substituted glycyl)–2-cyanopyrrolidines, N-(substituted glycyl)-thiazolidines, N-(substituted glycyl)-4-cyanothiazolidines, amino-acyl-borono-prolyl-inhibitors, cyclopropyl-fused pyrrolidines and heterocyclic compounds. Inhibitors of dipeptidyl peptidase IV are described in US 6,380,398, US 6,011,155; US 6,107,317; US 6,110,949; US 6,124,305; US 6,172,081; WO 95/15309, WO 99/61431, WO 99/67278, WO 99/67279, DE 198 34 591, WO 97/40832, DE 196 16 486 C 2, WO 98/19998, WO 00/07617, WO 99/38501, WO 99/46272, WO 99/38501, WO 01/68603, WO 01/40180, WO 01/81337, WO 01/81304, WO 01/55105, WO 02/02560 and WO 02/14271, WO 02/04610, WO 02/051836, WO 02/068420, WO 02/076450; WO 02/083128, WO 02/38541, WO 03/000180, WO 03/000181, WO 03/000250, WO 03/002530, WO 03/002531, WO 03/002553, WO 03/002593, WO 03/004496, WO 03/024942 and WO 03/024965, the teachings of which are herein incorporated by reference in their entirety, especially concerning these inhibitors, their definition, uses and their production.

Summary of the invention

The present invention provides novel physiological substrates of QC in mammals, Aβ3-40/42, [Gln^3]Aβ3-40/42, [Glu^{11}]Aβ11-40/42, [Gln^{11}]Aβ11-40/42, [Gln^1]Gastrin, [Gln^1]Neurotensin, [Gln^1]FPP, [Gln^1]CCL 2, [Gln^1]CCL 7, [Gln^1]CCL 8, [Gln^1]CCL 16, [Gln^1]CCL 18, [Gln^1]Fractalkine, [Gln^1]Orexin A, [Gln^3]glucagon3-29 and [Gln^5]substance P5-11 and the use of effectors of QC and pharmaceutical compositions comprising effectors of QC for the treatment of conditions that can be treated by modulation of QC activity.
It was shown by inhibition studies that human QC is a metal-dependent transferase. QC apoenzyme could be reactivated most efficiently by zinc ions, and the metal-binding motif of zinc-dependent aminopeptidases is also present in human QC. Compounds interacting with the active-site bound metal are potent inhibitors.

Unexpectedly, it was shown that recombinant human QC as well as QC-activity from brain extracts catalyze both, the *N*-terminal glutaminyl as well as glutamate cyclization. Most striking is the finding, that cyclase-catalyzed Glu^1-conversion is favored around pH 6.0 while Gln^1-conversion to pGlu-derivatives occurs with a pH-optimum of around 8.0. Since the formation of pGlu-Aβ-related peptides can be suppressed by inhibition of recombinant human QC and QC-activity from pig pituitary extracts, the enzyme QC is a target in drug development for treatment of Alzheimer's disease.

By administering effectors of QC activity to a mammal it can be possible to prevent or alleviate or treat conditions selected from Alzheimer's disease, Down Syndrome, ulcer disease and gastric cancer with or w/o *Heliobacter pylori* infections, pathogenic psychotic conditions, schizophrenia, infertility, neoplasia, inflammatory host responses, cancer, psoriasis, rheumatoid arthritis, atherosclerosis, impaired humoral and cell-mediated immune responses, leukocyte adhesion and migration processes in the endothelium, impaired food intake, sleep-wakefulness, impaired homeostatic regulation of energy metabolism, impaired autonomic function, impaired hormonal balance and impaired regulation of body fluids.

Further, by administration of effectors of QC activity to a mammal it can be possible to stimulate gastrointestinal tract cell proliferation, preferably proliferation of gastric mucosal cells, epithelial cells, acute acid secretion and the differentiation of acid producing parietal cells and histamine-secreting enterochromaffin-like cells.

Furthermore, by administration of effectors of QC activity to a mammal it can be possible to suppress the proliferation of myeloid progenitor cells.

In addition, administration of QC inhibitors can lead to suppression of male fertility.

In a preferred embodiment, the present invention provides the use of effectors of QC activity in combination with inhibitors of DP IV or DP IV-like enzymes for the treatment or alleviation of conditions that can be treated by modulation of QC- and/or DP IV-activity.

The present invention provides pharmaceutical compositions for parenteral, enteral or oral administration, comprising at least one effector of QC optionally in combination with customary carriers and/or excipients; or comprising at least one effector of QC in combination with at least one DP IV-inhibitor, optionally in combination with customary carriers and/or excipients.

Screening methods are also provided for the identification and selection of effectors of QC.

Brief description of the drawings

Further understanding of these and other aspects of the present invention will be had by reference to the figures wherein:

Figure 1 shows progress curves of the cyclization of H-Gln-Ala-OH, catalyzed by human QC, monitoring the decrease in absorbance at 340 nm. The samples contained 0.3 mM $NADH/H^+$, 14 mM α-Ketoglutaric acid, 30 U/ml glutamic dehydrogenase and 1 mM H-Gln-Ala-OH. From curve A-D, varying concentrations of QC were applied: A, 10 mU/ml, B, 5 mU/ml, C, 2.5 mU/ml. In case of curve D, QC was omitted. A linear relationship was obtained between the QC concentration and the observed activity (inset).

Figure 2 shows the pH dependence of human and papaya (inset) QC, determined under first-order rate conditions using Gln-βNA as substrate. In case of human QC, a buffer system providing a constant ionic strength according to Ellis and Morrison was used, consisting of 25 mM MES, 25 mM acetic acid and 50 mM Tris (Ellis, K. J. and Morrison, J. F. 1982 *Methods Enzymol. 87*, 405-426). Due to a slightly inhibiting effect of Tris, papaya QC was investigated using a 50 mM Mops buffer. The ionic strength was adjusted to 0.05 M by addition of NaCl. The rate profiles were evaluated by fitting to a model that is based on dissociating groups. In case of papaya QC, a pK_a of 7.13 ±0.03 was obtained by fitting of the data to a single dissociation model.

Figure 3 shows the effect of the pH on the stability of the QC from Papaya latex and human QC. An enzyme stock solution was diluted 20-fold in 0.1 M buffer of various pH values (pH 4-7 sodium citrate, pH 7-10 sodium phosphate). Enzyme solutions were incubated at 30 °C for 30 min and subsequently enzymatic activity was analyzed according to the standard protocol.

Figure 4 shows the comparison of the specificity constant k_{cat}/K_M for a set of substrates containing glutamate in the second amino acid position. Whereas an increase in specificity of human QC was detected from the di- to the tetrapeptides, no change was observed in case of papaya QC. The data presented here is a replot of the parameters given in Table 3.

Figure 5 shows the formation of pGlu-Lys(pGlu)-Arg-Leu-Ala-NH_2 from H-Gln-Lys(Gln)-Arg-Leu-Ala-NH_2, catalyzed by human QC. Substrate conversion is

monitored by a time-dependent change in the m/z ratio due to the expulsion of ammonia. The sample composition was 0.5 mM substrate, 38 nM QC in 40 mM Tris/HCl, pH 7.7. At the times indicated, samples were removed from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded. A very similar dependence was observed in case of papaya QC.

Figure 6 shows the formation of pGlu-Phe-Lys-Ala-Glu-NH_2 from H-Gln(NMe)-Phe-Lys-Ala-Glu-NH_2 catalyzed by papaya QC. Substrate conversion is monitored by a time-dependent change in the m/z ratio due to the expulsion of methylamine. The sample composition was 0.5 mM substrate, 0.65 μM papaya QC in 40 mM Tris/HCl, pH 7.7. At the times indicated, samples were removed from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded. No substrate conversion was observed in samples without papaya QC or by applying up to 1.5 μM human QC to the substrate (not shown).

Figure 7 shows the formation of [Gln^3]-Aβ3-11 from [Gln^3]Aβ1-11 catalysed by DPIV. At the times indicated, samples were removed, from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 8 shows the prevention of the cleavage of [Gln^3]Aβ1-11 by the DP IV-inhibitor Val-Pyrrolidide (Val-Pyrr). At the times indicated, samples were removed from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 9 shows the formation of [$pGlu^3$]Aβ3-11 from [Gln^3]A β3-11 catalyzed by QC. At the times indicated, samples were removed from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 10 shows the inhibition of the formation of [$pGlu^3$]Aβ3-11 from [Gln^3]Aβ3-11 by the QC-inhibitor 1,10-phenanthroline. At the times indicated from the assay tube, samples were removed, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 11 shows the formation of [pGlu^3]Aβ3-11 from [Gln^3]Aβ1-11 after consecutive catalysis by DP IV and QC. At the times indicated, samples were removed from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 12 shows the inhibition of [pGlu^3]Aβ3-11 formation from [Gln^3]Aβ1-11 by the QC-inhibitor 1,10-phenanthroline in the presence of catalytically active DP IV and QC. At the times indicated, samples were removed from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 13 shows the reduction of [pGlu^3]Aβ3-11 formation from [Gln^3]Aβ1-11 by the DP IV-inhibitor Val-Pyrr in the presence of catalytically active DP IV and QC. At the times indicated, samples were removed from the assay mixture, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 14 shows the formation of [pGlu^3]Aβ-peptide3-11 from [Gln^3]Aβ1-11 after consecutive catalysis by aminopeptidase(s) and QC that are present in porcine pituitary homogenate. At the times indicated, samples were removed from the assay tube, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded.

Figure 15 A and **B** show Mass spectra of Aβ3-11a and Aβ3-21a incubated with recombinant human QC, that was boiled for 10 min before use. **C** and **D** show Mass spectra of Aβ3-11 and Aβ3-21a in presence of active human QC resulting in the formation of [pGlu^3]Aβ3-11a and [pGlu^3]Aβ3-21a, respectively. **E** and **F** show Mass spectra of Aβ3-11a and Aβ3-21a in presence of active QC and 5 mM Benzimidazole suppressing the formation of [pGlu^3]formation.

Figure 16 shows reaction rates of papaya QC- catalyzed Glu-βNA-conversion plotted against the substrate concentration. The initial rates were measured in 0.1 M pyrophosphate buffer, pH 6.1 (squares), 0.1 M phosphate buffer, pH 7.5 (circles) and

0.1 M borate buffer, pH 8.5 (triangles). The kinetic parameters were as follows: K_M= 1.13 ±0.07 mM, k_{cat}= 1.13 ±0.04 min^{-1} (pH 6.1); K_M= 1.45 ±0.03 mM, k_{cat}= 0.92 ±0.01 min^{-1} (pH 7.5); K_M= 1.76 ±0.06 mM, k_{cat}= 0.56 ±0.01 min^{-1} (pH 8.5).

Figure 17 shows the pH-dependence of the conversion of Gln-βNA (circles) and Glu-βNA (squares), determined under first-order rate-law conditions ($S<<K_M$). Substrate concentration was 0.01 mM and 0.25 mM, respectively. For both determinations, a three-component buffer system was applied consisting of 0.05 M acetic acid, 0.05 M pyrophosphoric acid and 0.05 M Tricine. All buffers were adjusted to equal conductivity by addition of NaCl, in order to avoid differences in ionic strength. The data were fitted to equations that account for two dissociating groups revealing pK_a-values of 6.91 ±0.02 and 9.5 ±0.1 for Gln-βNA and 4.6 ±0.1 and 7.55 ±0.02 for Glu-βNA. The pK_a-values of the respective substrate amino groups, determined by titration, were 6.97 ±0.01 (Gln-βNA) and 7.57 ±0.05 (Glu-βNA). All determinations were carried out at 30 °C.

Figure 18 shows progress curves of human QC-catalyzed cyclization of H-Gln-AMC in presence of imidazole, dipicolinic acid and in absence of an inhibitory compound. The hyperbolic shape of the curve in presence of dipicolinic acid indicates metal ion remove from the active site of QC.

Figure 19 shows the time-dependent inactivation of QC by the heterocyclic chelator 1,10-phenanthroline. After incubation of the QC-enzyme with the inhibitor in absence of substrate (continuous line), a reduced enzymatic activity was observed compared to samples that were not preincubated with inhibitor (dotted trace), indicating metal ion remove from the active site of QC.

Figure 20 shows the reactivation of human QC with monovalent- and divalent metal ions. QC was inactivated by addition of 2 mM dipicolinic acid in 50 mM Bis-Tris, pH 6.8. Subsequently, the enzyme was subjected to dialysis against 50 mM Bis-Tris, pH 6.8, containing 1.0 mM EDTA. Reactivation of the enzymes was achieved by incubation of the inactivated enzyme sample with metal ions at a concentration of 0.5 mM, in presence of 0.5 mM EDTA in order to avoid an unspecific reactivation by

2004237408 27 Aug 2009

traces of metal ions present in buffer solutions. Controls are given by enzyme samples that were not inactivated, but also dialyzed against EDTA solution as the inactivated enzyme (+EDTA) and enzyme samples that were dialyzed against buffer solutions without added EDTA (-EDTA).

Figure 21 Sequence alignment of human QC (hQC) and other M28 family members of the metallopeptidase Clan MH. Multiple sequence alignment was performed using ClustalW at ch.EMBnet.org with default settings. The conservation of the zinc-ion ligating residues is shown for human QC (hQC; GenBank X71125), the Zn-dependent aminopeptidase from *Streptomyces griseus* (SGAP; Swiss-Prot P80561), and within the N-acetylated-alpha-linked acidic dipeptidase (NAALADase I) domain (residues 274 - 587) of the human Glutamate carboxypeptidase II (hGCP II; Swiss-Prot Q04609). The amino acids involved in metal binding are typed in bold and underlined. In case of human QC, these residues are the putative counterparts to the peptidases.

Figure 22: A shows the mass spectra of human [Gln^3]Aβ1-11a incubated with recombinant human QC and DPIV resulting in generation of [pGlu]Aβ3-11a (asterisk). **B** mass spectra of human [Gln^3]Aβ1-11a incubated with recombinant human QC and DPIV in presence of the DPIV-inhibitor Val-Pyrr. **C** mass spectra of human [Gln^3]Aβ1-11a incubated with recombinant human QC and DPIV in presence of the QC-inhibitor 1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea **D** mass spectra of human [Gln^3]Aβ1-11a incubated with recombinant human QC and DPIV in presence of the QC-inhibitor 1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea and DPIV-inhibitor Val-Pyrr. Due to inhibition by either DPIV or QC, formation of [pGluAβ]3-11a is suppressed. Use of the combination of 1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea and Val-Pyrr inhibitors was most efficient because [pGluAβ]3-11a-formation was negligible, as shown in D.

Peptide Sequences

The peptides mentioned and used herein have the following sequences:

Aβ1 -42:

Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-

2004237408 27 Aug 2009

Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val Val-Ile-Ala

Aβ1-40:

Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val

Aβ3-42:

Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala

Aβ3-40:

Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val

Aβ1-11a:

Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-NH_2

Aβ3-11a:
Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-NH_2

Aβ1-21a:
Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-NH_2

Aβ3-21a:
Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-NH_2

Gln^3-Aβ3-40:
Gln-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val

Gln^3-Aβ3-21a:
Gln-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-NH_2

Gln^3-Aβ1-11a:
Asp-Ala-Gln-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-NH_2

Gln^3-Aβ3-11a:
Gln-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-NH_2

Detailed description of the invention

The present invention provides effectors of glutaminyl cyclase (QC) for

a) the treatment of diseases in mammals that can be treated by modulation of QC activity *in vivo* and/or
b) the modulation of physiological processes based on the action of pGlu-containing peptides caused by modulation of QC activity.

Furthermore, the present invention provides compounds for the inhibition of glutaminyl cyclase (QC, EC 2.3.2.5) and/or QC-like enzymes in a mammal and the use of inhibitors of QC activity for the treatment of pathological conditions related to QC activity.

The present invention also provides a new method for the treatment of Alzheimer's disease and Down Syndrome. The N-termini of amyloid β-peptides deposited in Alzheimer`s disease and Down syndrome brain bear pyroglutamic acid. The pGlu

formation is an important event in the development and progression in the disease, since the modified amyloid β-peptides show an enhanced tendency to β-amyloid aggregation and toxicity, likely worsening the onset and progression of the disease. (Russo, C. et al. 2002 *J Neurochem 82*,1480-1489).

In contrast, in the natural Aβ-peptides (3-40/42), glutamic acid is present as an N-terminal amino acid. There is no enzymic conversion of Glu to pGlu known to date. Moreover, spontaneous cyclization of Glu-peptides to pGlu-peptides has not been observed as yet. Therefore one aspect of the present invention was to determine the role of QC in Alzheimer's disease and Down Syndrome. This aspect was addressed by the synthesis of Aβ3-11 and Aβ1-11, containing the amino acid glutamine instead of glutamic acid at position three, the determination of the substrate characteristics of these modified amyloid β-peptides against QC, DP IV and DP IV-like enzymes and aminopeptidases and the use of inhibitors of QC to prevent the formation of pGlu from a N-terminal glutaminyl residue of the amyloid β-derived peptides 1-11 and 3-11. The results are shown in example 8. The applied method is described in example 3.

To date, there are no hints indicating an involvement of QC in the progression of the disease, because glutamic acid is the N-terminal amino acid in Aβ (3-40/42, or 11-40/42). But, QC is the only known enzyme capable of forming pGlu at the N-terminus of peptides. Other aspects of the present invention concern the following findings and discoveries:

a) In a side reaction, QC catalyzes the cyclization of glutamic acid to pyroglutamic acid at very low rates,
b) Glutamic acid of APP or its subsequently formed amyloid-β-peptides is converted into glutamine post-translationally by an unknown enzymatic activity and in a second step, QC catalyzes the cyclization of glutamine into pyroglutamic acid after processing of the amyloid β-peptide N-terminus,

c) Glutamic acid is converted into glutamine post-translationally by a chemical catalysis or autocatalysis and subsequently, QC catalyzes the cyclization of glutamine to pyroglutamic acid after processing of the amyloid β-peptide N-terminus,

d) There are mutations in the APP gene, which encode the amyloid β-protein, leading to Gln instead of Glu in position 3. After translation and processing of the N-terminus, QC catalyzes the cyclization of glutamine to pyroglutamic acid,

e) Glutamine is incorporated into the nascent peptide chain of APP, due to a malfunction of an unknown enzymatic activity and subsequently, QC catalyzes the cyclization of N-terminally glutamine to pyroglutamic acid after processing of the amyloid β-peptide N-terminus.

QC is involved in the critical step in all five cases listed above, namely the formation of pyroglutamic acid that favors the aggregation of amyloid β-peptides. Thus, an inhibition of QC leads to a prevention of the precipitation of the plaque-forming Aβ3-40/41/43 or Aβ11-40/41/43, causing the onset and progression of Alzheimer's disease and Down Syndrome, independently of the mechanism by which cyclization occurs.

Glutamate is found in positions 3, 11 and 22 of the amyloid β-peptide. Among them the mutation from glutamic acid (E) to glutamine (Q) in position 22 (corresponding to amyloid precursor protein APP 693, Swissprot P05067) has been described as the so called Dutch type cerebroarterial amyloidosis mutation.

The β-amyloid peptides with a pyroglutamic acid residue in position 3, 11 and/or 22 have been described to be more cytotoxic and hydrophobic than Aβ1-40/42/43 (Saido T.C. 2000 *Medical Hypotheses* 54(3): 427-429).

The multiple N-terminal variations can be generated by the β-secretase enzyme β-site amyloid precursor protein-cleaving enzyme (BACE) at different sites (Huse J.T. et al. 2002 *J. Biol. Chem.* 277 (18): 16278-16284), and/or by aminopeptidase

processing. In all cases, cyclization can take place according to a)-e) as described above.

So far, there was no experimental evidence supporting the enzymatic conversion of Glu^1-peptides into pGlu-peptides by an unknown glutamyl cyclase (EC) corresponding to pathway a) (Garden, R. W., Moroz, T. P., Gleeson, J. M., Floyd, P. D., Li, L. J., Rubakhin, S. S., and Sweedler, J. V. (1999) *J Neurochem* **72**, 676-681; Hosoda R. et al. (1998) *J Neuropathol Exp Neurol.* **57**, 1089-1095). To date, no such enzyme activity has been identified, capable to cyclize Glu^1-peptides which are protonated *N*-terminally and possess a negatively charged Glu^1 γ-carboxylate moiety under mildly alkaline pH-conditions.

QC-activity against Gln^1-substrates is dramatically reduced below pH 7.0. In contrast, it appears that Glu^1-conversion can occur at acidic reaction conditions (Iwatsubo, **T.**, Saido, T. C., Mann, D. M., Lee, V. M., and Trojanowski, J. Q. (1996) *Am J Pathol* **149,** 1823-1830; Russo, C., Saido, T. C., DeBusk, L. M., Tabaton, M., Gambetti, P., and Teller, J. K. (1977) *FEBS Lett* **409**, 411-416; Russo, C., Salis, S., Dolcini, V., Venezia, V., Song, X. H., Teller, J. K., and Schettini, G. (2001) *Neurobiol Dis* **8,** 173-180; Tekirian, T. L., Saido, T. C., Markesbery, W. R., Russell, M. J., Wekstein, D. R., Patel, E., and Geddes, J. W. (1998) *J Neuropathol Exp Neurol.* **57,** 76-94; Russo, C., Violani, E., Salis, S., Venezia, V., Dolcini, V., Damonte, G., Benatti, U., DArrigo, C., Patrone, E., Carlo, P., and Schettini, G. (2002) *J Neurochem* **82**, 1480-1489; Hosoda, R., Saido, T. C., Otvos, L., Jr., Arai, T., Mann, D. M., Lee, V. M., Trojanowski, J. Q., and Iwatsubo, T. (1998) *J Neuropathol Exp Neurol.* **57,** 1089-1095; Garden, R. W., Moroz, T. P., Gleeson, J. M., Floyd, P. D., Li, L. J., Rubakhin, S. S., and Sweedler, J. V. (1999) *J Neurochem* **72**, 676-681).

According to the present invention it was investigated whether QC is able to recognize and to turnover amyloid-β derived peptides under mild acidic conditions. Therefore, the peptides [Gln^3]Aβ1-11a, Aβ3-11a, [Gln^3]Aβ3-11a, Aβ3-21a, [Gln^3]Aβ3-21a and [Gln^3]Aβ3-40 as potential substrates of the enzyme were synthesized and investigated. These sequences were chosen for mimicking natural *N*-terminally and

C-terminally truncated [Glu^3]Aβ peptides and [Gln^3]Aβ peptides which could occur due to posttranslational Glu-amidation.

In the present invention it was shown that papaya and human QC catalyze both glutaminyl and glutamyl cyclization. Apparently, the primary physiological function of QC is to finish hormone maturation in endocrine cells by glutamine cyclization prior or during the hormone secretion process. Such secretory vesicles are known to be acidic in pH. Thus, a side activity of the enzyme in the narrow pH-range from 5.0 to 7.0 could be its newly discovered glutamyl cyclase activity transforming also Glu-Aβ peptides. However, due to the much slower occurring Glu-cyclization compared to Gln-conversion, it is questionable whether the glutamyl cyclization plays a significant physiological role. In the pathology of neurodegenerative disorders, however, the glutamyl cyclization is of relevance.

Investigating the pH-dependency of this enzymatic reaction, we found that the unprotonated *N*-terminus was essential for the cyclization of Gln^1-peptides and accordingly that the pK_a-value of the substrate was identical to the pK_a-value for QC-catalysis (see Figure 17). Thus, QC stabilizes the intramolecular nucleophilic attack of the unprotonated α-amino moiety on the γ-carbonyl carbon electrophilically activated by amidation (Scheme 1).

In contrast to the monovalent charge present on *N*-terminal glutamine containing peptides, the *N*-terminal Glu-residue in Glu-containing peptides is predominantly bivalently charged around neutral pH. Glutamate exhibits pK_a-values of about 4.2 and 7.5 for the γ-carboxylic and for the α-amino moiety, respectively. I.e. at neutral pH and above, although the α-amino nitrogen is in part or fully unprotonated and nucleophilic, the γ-carboxylic group is unprotonated, and so exercising no electrophilic carbonyl activity. Hence, intramolecular cyclization is impossible.

However, in the pH-range of about 5.2-6.5, between their respective pK_a-values, the two functional groups are present both in non-ionized forms, in concentrations of about 1-10% (-NH_2) or 10-1% (-COOH) of total *N*-terminal Glu-containing peptide. As a result, over a mildly acidic pH-range species of *N*-terminal Glu-peptides are present which carry both groups uncharged, and, therefore, it is possible that QC could stabilize the intermediate of intramolecular cyclization to pGlu-peptide. I.e. if the γ-

carboxylic group is protonated, the carbonyl carbon is electrophilic enough to allow nucleophilic attack by the unprotonated α-amino group. At this pH the hydroxyl ion functions as a leaving group (Scheme 3). These assumptions are corroborated by the pH-dependence data obtained for the QC catalyzed conversion of Glu-βNA (see example 11). In contrast to glutamine conversion of Gln-βNA by QC, the pH-optimum of catalysis shifts to the acidic range around pH 6.0, i.e. the pH-range, in which substrate molecule species are simultaneously abundant carrying a protonated γ-carboxyl and unprotonated α-amino group. Furthermore, the kinetically determined pK_a-value of 7.55 ±0.02 is in excellent agreement with that of the α-amino group of Glu-βNA, determined by titration (7.57 ±0.05).

Physiologically, at pH 6.0 the second-order rate constant (or specificity constant, k_{cat}/K_M) of the QC-catalyzed glutamate cyclization might be in the range of 8,000fold slower than the one for glutamine cyclization (Figure 17). However, the nonenzymatic turnover of both model substrates Glu-βNA and Gln-βNA is negligible, being conform with the observed negligible pGlu-peptide formation in the present invention. Hence, for the pGlu-formation by QC an acceleration of at least 10^8 can be estimated from the ratio of the enzymatic versus non-enzymatic rate constants (comparing the second-order rate constants for the enzyme catalysis with the respective nonenzymatic cyclization first-order rate constants the catalytic proficiency factor is 10^9-10^{10} M^{-1} for the Gln- and the Glu-conversion, respectively). The conclusion from these data is, that *in vivo* only an enzymatic path resulting pGlu-formations seems conceivable.

Since QC is highly abundant in the brain and taking into account the high turnover rate of 0.9 min^{-1} recently found for the maturation of 30 μM of (Gln-)TRH-like peptide (Prokai, L., Prokai-Tatrai, K., Ouyang, X., Kim, H. S., Wu, W. M., Zharikova, A., and Bodor, N. (1999) *J Med Chem* 42, 4563-4571), one can predict a cyclization half-life of about 100 hours for an appropriate glutamate-substrate, similar reaction conditions provided. Moreover, given compartmentalization and localization of brain QC/EC in the secretory pathway, the actual *in vivo* enzyme and substrate concentrations and reaction conditions might be even more favorable for the enzymatic cyclization in the intact cell. And, if *N*-terminal Glu is transformed to Gln a much more rapid pGlu-

formation mediated by QC could be expected. *In vitro*, both reactions were suppressed by applying inhibitors of QC/EC-activity (Figures 9, 10 and 15).

In summary, the present invention shows that human QC, which is highly abundant in the brain, is a likely catalyst to the formation of the amyloidogenic pGlu-Aβ peptides from Glu-Aβ and Gln-Aβ precursors which make up more than 50% of the plaque deposits found in Alzheimer's Disease. These findings identify QC/EC as a player in senile plaque formation and thus as a novel drug target in the treatment of Alzheimer's Disease.

In a second embodiment of the present invention, it was found that amyloid β-derived peptides are a substrate of dipeptidyl peptidase IV (DP IV) or DP IV-like enzymes, preferably dipeptidyl peptidase II (DPII). DP IV, DP II or other DP IV-like enzymes release a dipeptide from the N-terminus of the modified amyloid β-peptide (1-11) generating amyloid β-peptide (3-11) with glutamine as the N-terminal amino acid residue. The results are shown in example 8.

Prior to cleavage by DP II, DPIV or other DP IV-like enzymes, the peptide bond between aspartic acid (residue 1 of amyloid β-peptide) and alanine (residue 2 of amyloid β-peptide) may be isomerised yielding an isoaspartyl residue as described in the literature (Kuo, Y.-M., Emmerling, M. R., Woods, A. S., Cotter, R. J., Roher, A. E. (1997) *BBRC* 237, 188-191; Shimizu, T., Watanabe, A., Ogawara, M., Mori, H. and Shirasawa, T. (2000) *Arch. Biochem. Biophys.* 381, 225-234).

These isoaspartyl residues render the amyloid β-peptide resistant against aminopeptidase degradation and consequently the core plaques contain high amounts of isoAsp^1-amyloid β-peptides, which suggests a reduced turnover at the N-terminus.

However, in the present invention it is demonstrated for the first time, that the N-terminal dipeptide H-isoAsp^1-Ala^2-OH can be released by dipeptidyl peptidases especially under acidic conditions. Furthermore, it was shown that isomerization can precede also cleavage by β-secretase, and that isomerization may accelerate proteolytic processing, thus leading to liberation of an N-terminal isoaspartyl bond of isoAsp^1-amyloid β-peptides which subsequently is subject to turnover by DP II, DPIV or DP IV-like enzymes (Momand, J. and Clarke, S. (1987) *Biochemistry* 26, 7798-

7805; Kuo, Y.-M., Emmerling, M. R., Woods, A. S., Cotter, R. J., Roher, A. E. (1997) *BBRC* 237, 188-191). Accordingly, inhibition of isoaspartyl formation may lead to the reduction of cleavage by β-secretase and, in turn, to a reduced formation of amyloid β-peptides. In addition, blockage of the isoAsp[1]-amyloid β-peptide turnover by inhibition of DP II, DPIV or DP IV-like enzymes would prevent the exposure of [Glu[3]]Aβ to QC/EC-catalyzed formation of [pGlu[3]]Aβ.

In a third embodiment of the present invention, a combination of inhibitors of DP IV-activity and of inhibitors of QC can be used for the treatment of Alzheimer's disease and Down Syndrome.

The combined effect of DP IV and/or DP IV-like enzymes and of QC is illustrated as follows:

a) DP IV and/or DP IV-like enzymes cleave Aβ1-40/42, a dipeptide comprising H-Asp-Ala-OH and Aβ3-40/42 are released,

b) In a side reaction, QC catalyzes the cyclization of glutamic acid to pyroglutamic acid at very low rates,

c) Glutamic acid is converted into glutamine at the N-terminus post-translationally by an unknown enzymatic activity and subsequently, QC catalyzes the cyclization of glutamine into pyroglutamic acid after processing of the amyloid β-peptide N-terminus,

d) Glutamic acid is converted into glutamine post-translationally by a chemical catalysis or autocatalysis and in a second step, QC catalyzes the cyclization of glutamine into pyroglutamic acid after processing of the amyloid β-peptide N-terminus,

e) There are mutations in the APP gene, which encode the amyloid β-protein, leading to Gln instead of Glu in position 3 of Aβ, After translation and processing of the N-terminus, QC catalyzes the cyclization of glutamine to pyroglutamic acid,

f) Glutamine is incorporated into the nascent peptide chain of APP, due to a malfunction of an unknown enzymatic activity and subsequently, QC catalyzes the cyclization of N-terminally glutamine

to pyroglutamic acid after processing of the amyloid β-peptide N-terminus,

The N-terminal Gln-exposure to QC-activity can be also triggered by different peptidase activities. Aminopeptidases can remove sequentially Asp and Ala from the N-terminus of Aβ1-40/41/43, thus unmasking amino acid three that is prone to cyclization. Dipeptidyl peptidases, such as DP I, DP II, DP IV, DP 8, DP 9 and DP 10, remove the dipeptide Asp-Ala in one step. Hence, inhibition of aminopeptidase- or dipeptidylpeptidase-activity is useful to prevent the formation of Aβ3-40/41/43.

The combined effect of inhibitors of DP IV and/or DP IV-like enzymes and of activity lowering effectors of QC is illustrated in the following way:

a) The inhibitors of DP IV and/or DP IV-like enzymes inhibit the conversion of Aβ1-40/42 to Aβ3-40/42.
b) An N-terminal exposure of glutamic acid is thereby prevented and no conversion to glutamine, either by enzymatic or by chemical catalysis, subsequently leading to pyroglutamic acid formation, is possible.
c) Inhibitors of QC prevent in addition the formation pyroglutamic acid from any residual modified Aβ3-40/42 molecules and those modified Aβ3-40/42 molecules, which are generated by mutations of the APP gene.

Within the present invention, a similar combined action of DP IV or DP IV-like enzymes and QC was demonstrated for further peptide hormones, such as glucagon, CC chemokines and substance P.

Glucagon is a 29-amino acid polypeptide released from pancreatic islet alpha-cells that acts to maintain euglycemia by stimulating hepatic glycogenolysis and gluconeogenesis. Despite its importance, there remains controversy about the mechanisms responsible for glucagon clearance in the body. Pospisilik et al.

assessed the enzymatic metabolism of glucagon using sensitive mass spectrometric techniques to identify the molecular products. Incubation of glucagon with purified porcine dipeptidyl peptidase IV (DP IV) yielded sequential production of glucagon3-29 and glucagon5-29. In human serum, degradation to glucagon3-29 was rapidly followed by N-terminal cyclization of glucagon, preventing further DP IV-mediated hydrolysis. Bioassay of glucagon, following incubation with purified DP IV or normal rat serum demonstrated a significant loss of hyperglycemic activity, while a similar incubation in DP IV-deficient rat serum did not show any loss of glucagon bioactivity. Degradation, monitored by mass spectrometry and bioassay, was blocked by the specific DP IV inhibitor, isoleucyl thiazolidine. These results identify DP IV as a primary enzyme involved in the degradation and inactivation of glucagon. These findings have important implications for the determination of glucagon levels in human plasma (Pospisilik et al., *Regul Pept* 2001 Jan 12;96(3):133-41).

Human Monocyte Chemotactic Protein (MCP)-2 has originally been isolated from stimulated osteosarcoma cells as a chemokine coproduced with MCP-1 and MCP-3. Von Coillie et al. (Van Coillie, E. et al. 1998 *Biochemistry 37*, 12672-12680) cloned a 5'-end extended MCP-2 cDNA from a human testis cDNA library. It encoded a 76 residue MCP-2 protein, but differed from the reported bone marrow-derived MCP-2 cDNA sequence in codon 46, which coded for a Lys instead of a Gln. This MCP-2Lys46 variant, caused by a single nucleotide polymorphism (SNP), was biologically compared with MCP-2Gln46. The coding regions were sub-cloned into the bacterial expression vector pHEN1, and after transformation of Escherichia coli, the two MCP-2 protein variants were recovered from the periplasm. Edman degradation revealed a Gln residue at the NH_2 terminus instead of a pGlu. rMCP-2Gln46 and rMCP-2Lys46 and the NH_2-terminal cyclic counterparts were tested on monocytic cells in calcium mobilization and chemotaxis assays. No significant difference in biological activity was observed between the rMCP-2Gln46 and rMCP-2Lys46 isoforms. However, for both MCP-2 variants the NH_2-terminal pyroglutamate was shown to be essential for chemotaxis, but not for calcium mobilization. NH_2-terminal truncation of rMCP-2Lys46 by the serine protease CD26/dipeptidyl peptidase IV (CD26/DPP IV) resulted in the release of the NH_2-terminal Gln-Pro dipeptide, whereas synthetic MCP-2 with an

amino-terminal pGlu remained unaffected. CD26/DPP IV-clipped rMCP-2Lys46(3-76) was almost completely inactive in both chemotaxis and signaling assays. These observations indicated that the NH_2-terminal pGlu in MCP-2 is necessary for chemotactic activity but also that it protects the protein against degradation by CD26/DPP IV (van Coillie, E.. et al. *Biochemistry* 1998 37, 12672-80).

Within the present invention, it was determined by LC/MS-analysis that the formation of the N-terminal pyroglutamate residue determined in glucagon3-29 (Pospisilik et al., 2001), and in MCP-2 isoforms (van Coillie et al., 1998), is catalyzed by QC.

In addition, it was proven by LC/MS-investigation that after N-terminal DP IV-catalyzed removal of the two dipeptides Lys-Pro and Arg-Pro from substance P the remaining [Gln^5]substanceP5-11 is transformed by QC to [$pGlu^5$]substanceP5-11.

DP IV inhibitors are disclosed in WO 99/61431. In particular, DP IV inhibitors are disclosed comprising an amino acid residue and a thiazolidine or pyrrolidine group, and salts thereof, especially L-*threo*-isoleucyl thiazolidine, L-*allo*-isoleucyl thiazolidine, L-*threo*-isoleucyl pyrrolidine, L-*allo*-isoleucyl thiazolidine, L-*allo*-isoleucyl pyrrolidine, and salts thereof.

Further examples of low molecular weight dipeptidyl peptidase IV inhibitors are agents such as tetrahydroisoquinolin-3-carboxamide derivatives, N-substituted 2-cyanopyroles and –pyrrolidines, N-(N'-substituted glycyl)–2-cyanopyrrolidines, N-(substituted glycyl)-thiazolidines, N-(substituted glycyl)-4-cyanothiazolidines, amino-acyl-borono-prolyl-inhibitors, cyclopropyl-fused pyrrolidines and heterocyclic compounds. Inhibitors of dipeptidyl peptidase IV are described in US 6,380,398, US 6,011,155; US 6,107,317; US 6,110,949; US 6,124,305; US 6,172,081; WO 95/15309, WO 99/61431, WO 99/67278, WO 99/67279, DE 198 34 591, WO 97/40832, DE 196 16 486 C 2, WO 98/19998, WO 00/07617, WO 99/38501, WO 99/46272, WO 99/38501, WO 01/68603, WO 01/40180, WO 01/81337, WO 01/81304, WO 01/55105, WO 02/02560 and WO 02/14271, WO 02/04610, WO 02/051836, WO 02/068420, WO 02/076450; WO 02/083128, WO 02/38541, WO

03/000180, WO 03/000181, WO 03/000250, WO 03/002530, WO 03/002531, WO 03/002553, WO 03/002593, WO 03/004496, WO 03/024942 and WO 03/024965, the teachings of which are herein incorporated by reference in their entirety, especially concerning these inhibitors, their definition, uses and their production.

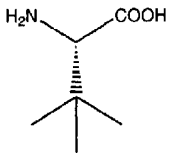
Preferred for the use in combination with effectors of QC are DPIV inhibitors such as NVP-DPP728A (1-[[[2-[{5-cyanopyridin-2-yl}amino]ethyl]amino]acetyl]-2-cyano-(S)-pyrrolidine) (Novartis) as disclosed by Hughes et al. 1999 *Biochemistry 38* 11597-11603, LAF-237 (1-[(3-hydroxy-adamant-1-ylamino)-acetyl]-pyrrolidine-2(S)-carbonitrile); disclosed by Hughes et al., Meeting of the American Diabetes Association 2002, Abstract no. 272 (Novartis), TSL-225 (tryptophyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), disclosed by Yamada et al. 1998 *Bioorg Med Chem Lett 8* , 1537-1540, 2-cyanopyrrolidides and 4-cyanopyrrolidides as disclosed by Asworth et al. 1996 *Bioorg Med Chem Lett 6*, , 1163-1166 and 2745-2748 , FE-999011, disclosed by Sudre et al. 2002 *Diabetes 51*, 1461-1469 (Ferring) and the compounds disclosed in WO 01/34594 (Guilford), employing dosages as set out in the above references.

More preferred DP IV inhibitors for the use in combination with effectors of QC are dipeptide compounds in which the amino acid is preferably selected from a natural amino acid, such as, for example, leucine, valine, glutamine, glutamic acid, proline, isoleucine, asparagines and aspartic acid. The dipeptide-like compounds used according to the invention exhibit at a concentration (of dipeptide compounds) of 10 μM, a reduction in the activity of plasma dipeptidyl peptidase IV or DPIV-analogous enzyme activities of at least 10 %, especially of at least 40 %. Frequently a reduction in activity of at least 60 % or at least 70 % is also desired *in vivo*. Preferred compounds may also exhibit a reduction in activity of a maximum of 20 % or 30 %.

Preferred dipeptide compounds are N-valyl prolyl, O-benzoyl hydroxylamine, alanyl pyrrolidine, isoleucyl thiazolidine like L-allo-isoleucyl thiazolidine, L-threo-isoleucyl pyrrolidine and salts thereof, especially the fumaric salts, and L-allo-isoleucyl pyrrolidine and salts thereof. Especially preferred compounds are glutaminyl

pyrrolidine and glutaminyl thiazolidine, H-Asn-pyrrolidine, H-Asn-thiazolidine, H-Asp-pyrrolidine, H-Asp-thiazolidine, H-Asp(NHOH)-pyrrolidine, H-Asp(NHOH)-thiazolidine, H-Glu-pyrrolidine, H-Glu-thiazolidine, H-Glu(NHOH)-pyrrolidine, H-Glu(NHOH)-thiazolidine, H-His-pyrrolidine, H-His-thiazolidine, H-Pro-pyrrolidine, H-Pro-thiazolidine, H-Ile-azididine, H-Ile-pyrrolidine, H-L-*allo*-Ile-thiazolidine, H-Val-pyrrolidine and H-Val-thiazolidine and pharmaceutically acceptable salts thereof. These compounds are described in WO 99/61431 and EP 1 304 327.

Furthermore, the present invention provides for the use of effectors of QC in combination with substrate-like peptide compounds useful for competitive modulation of dipeptidyl peptidase IV catalysis. Preferred peptide compounds are 2-Amino octanoic acid-Pro-Ile, Abu-Pro-Ile, Aib-Pro-Ile, Aze-Pro-Ile, Cha-Pro-Ile, Ile-Hyp-Ile, Ile-Pro-*allo*-Ile, Ile-Pro-*t*-butyl-Gly, Ile-Pro-Val, Nle-Pro-Ile, Nva-Pro-Ile, Orn-Pro-Ile, Phe-Pro-Ile, Phg-Pro-Ile, Pip-Pro-Ile, Ser(Bzl)-Pro-Ile, Ser(P)-Pro-Ile, Ser-Pro-Ile, *t*-butyl-Gly-Pro-D-Val, *t*-butyl-Gly-Pro-Gly, *t*-butyl-Gly-Pro-Ile, *t*-butyl-Gly-Pro-Ile-amide, *t*-butyl-Gly-Pro-*t*-butyl-Gly, *t*-butyl-Gly-Pro-Val, Thr-Pro-Ile, Tic-Pro-Ile, Trp-Pro-Ile, Tyr(P)-Pro-Ile, Tyr-Pro-*allo*-Ile, Val-Pro-*allo*-Ile, Val-Pro-*t*-butyl-Gly, Val-Pro-Val and pharmaceutically acceptable salts thereof, wherein *t*-butyl-Gly is defined as



and Ser(Bzl) and Ser(P) are defined as benzyl-serine and phosphoryl-serine, respectively. Tyr(P) is defined as phosphoryl-tyrosine. These compounds are dislcosed in WO 03/002593.

Further preferred DP IV-inhibitors, which can be used according to the present invention in combination with effectors of QC, are peptidylketones, e.g.

- 2-Methylcarbonyl-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide, 2-Methyl)carbonyl-1-N-[(L)-Valinyl-(L)-Prolyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,
- 2-[(Acetyl-oxy-methyl)carbonyl]-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,
- 2-[Benzoyl-oxy-methyl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,
- 2-{[(2,6-Dichlorbenzyl)thiomethyl]carbonyl}-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine,
- 2-[Benzoy-loxy-methyl)carbonyl]-1-N-[Glycyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,
- 2-[([1,3]-Thiazolethiazol-2-yl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate,
- 2-[(Benzothiazolethiazol-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidin trifluoracetate,
- 2-[(-Benzothiazolethiazol-2-yl)carbonyl]-1-N-[{(L)-Alanyl}-Glycyl]-(2S)-pyrrolidine trifluoracetate,
- 2-[(Pyridin-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate

and other pharmaceutically acceptable salts thereof. These compounds are disclosed in WO 03/033524.

Further, according to the present invention substituted aminoketones can be used in combination with effectors of QC. Preferred substituted aminoketones are

- 1-cyclopentyl-3-methyl-1-oxo-2-pentanaminium chloride,
- 1-cyclopentyl-3-methyl-1-oxo-2-butanaminium chloride,
- 1-cyclopentyl-3,3-dimethyl-1-oxo-2-butanaminium chloride,
- 1-cyclohexyl-3,3-dimethyl-1-oxo-2-butanaminium chloride,
- 3-(cyclopentylcarbonyl)-1,2,3,4-tetrahydroisoquinolinium chloride,
- *N*-(2-cyclopentyl-2-oxoethyl)cyclohexanaminium chloride

and other pharmaceutically acceptable salts thereof.

Among the rare group of proline-specific proteases, DP IV was originally believed to be the only membrane-bound enzyme specific for proline as the penultimate residue at the amino-terminus of the polypeptide chain. However, other molecules, even those structurally non-homologous with the DP IV but bearing corresponding enzyme activity, have been identified. DP IV-like enzymes, which have been identified so far, include e.g. fibroblast activation protein α, dipeptidyl peptidase IV β, dipeptidyl aminopeptidase-like protein, N-acetylated α-linked acidic dipeptidase, quiescent cell proline dipeptidase, dipeptidyl peptidase II, attractin and dipeptidyl peptidase IV related protein (DPP 8), DPL1 (DPX, DP6), DPL2 and DPP 9 described in review articles by Sedo & Malik (Sedo and Malik 2001, *Biochim Biophys Acta, 36506*, 1-10) and Abbott and Gorrell (Abbott, C.A. and Gorrell, M.D. 2002 In: Langner & Ansorge (ed.), Ectopeptidases. Kluwer Academic/Plenum Publishers, New York, 171-195). Recently, the cloning and characterization of dipeptidyl peptidase 10 (DPP 10) was reported (Qi, S.Y. et al., Biochemical Journal Immediate Publication. Published on 28 Mar 2003 as manuscript BJ20021914).

Effectors, as that term is used herein, are defined as molecules that bind to enzymes and increase or decrease their activity *in vitro* and/or *in vivo*. Some enzymes have binding sites for small molecules that affect their catalytic activity; a stimulator molecule is called an activator. Enzymes may even have multiple sites for recognizing more than one activator or inhibitor. Enzymes can detect concentrations of a variety of molecules and use that information to vary their own activities.

Effectors can modulate enzymatic activity because enzymes can assume both active and inactive conformations: activators are positive effectors, inhibitors are negative effectors. Effectors act not only at the active sites of enzymes, but also at regulatory sites, or allosteric sites, terms used to emphasize that the regulatory site is an element of the enzyme distinct from the catalytic site and to differentiate this form of regulation from competition between substrates and inhibitors at the catalytic site (Darnell, J., Lodish, H. and Baltimore, D. 1990, Molecular Cell Biology 2nd Edition, Scientific American Books, New York, page 63).

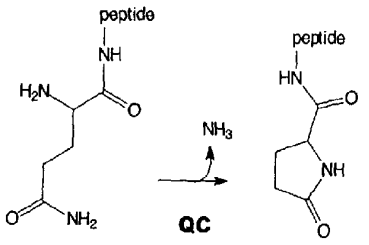
In the peptides of the present invention, each amino acid residue is represented by a one-letter or a three-letter designation, corresponding to the trivial name of the amino acid, in accordance with the following conventional list:

Amino Acid	One-Letter Symbol	Three-Letter Symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

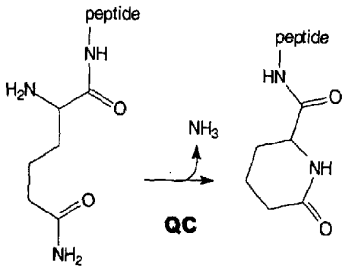
The term "QC" as used herein comprises glutaminyl cyclase (QC) and QC-like enzymes. QC and QC-like enzymes have identical or similar enzymatic activity, further defined as QC activity. In this regard, QC-like enzymes can fundamentally differ in their molecular structure from QC.

The term "QC activity" as used herein is defined as intramolecular cyclization of N-terminal glutamine residues into pyroglutamic acid (pGlu*) or of N-terminal L-homoglutamine or L-β-homoglutamine to a cyclic pyro-homoglutamine derivative under liberation of ammonia. See therefore schemes 1 and 2.

Scheme 1: Cyclization of glutamine by QC



Scheme 2: Cyclization of L-homoglutamine by QC



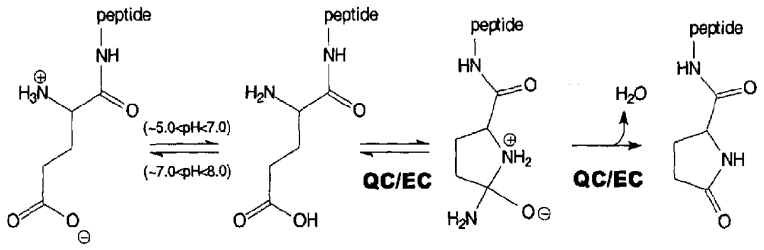
The term "EC" as used herein comprises the side activity of QC and QC-like enzymes as glutamate cyclase (EC), further defined as EC activity.

The term "EC activity" as used herein is defined as intramolecular cyclization of N-terminal glutamate residues into pyroglutamic acid (pGlu*) by QC. See therefore scheme 3.

The term "metal-dependent enzyme" as used herein is defined as enzyme(s) that require a bound metal ion in order to fulfill their catalytic function and/or require a bound metal ion in order to form the catalytically active structure.

Molecules that bind to enzymes and increase or decrease their activities are called "effectors". Effectors can modify enzymatic activity because enzymes can assume both active and inactive conformations: activators are positive effectors; inhibitors are negative effectors. Effectors bind at regulatory sites, or allosteric sites (from the Greek for "another shape"), a term used to emphasize that the regulatory site is an element of the enzyme distinct from the catalytic site and to differentiate this form of regulation from competition between substrates and inhibitors at the catalytic site. According to the individual embodiments of the present invention, either activators or inhibitors are preferred.

Scheme 3: *N*-terminal cyclization of uncharged glutamyl peptides by QC (EC)



Another aspect of the present invention is the identification of new physiological substrates of QC. These were identified by performing cyclization experiments with mammalian peptides as described in example 5. Prior, human QC and papaya QC

were isolated as described in example 1. The applied methods are described in example 2, and the peptide synthesis employed is outlined in example 6. The results of the study are shown in Table 1.

Table 1: New physiological substrates of glutaminyl cyclase (*, determined qualitatively by MALDI-TOF experiments)

Substrate	Human QC			Papaya QC		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)
[Gln^1]-Gastrin	31 ±1	54.1 ±0.6	**1745.2 ±36.9**	34 ±2	25.8 ±0.5	**759 ±30**
[Gln^1]-Neurotensin	37 ±1	48.8 ±0.4	**1318.9 ±24.8**	40 ±3	35.7 ±0.9	**893 ±44**
[Gln^1]-FPP	87 ±2	69.6 ±0.3	**800.0 ±14.9**	232 ±9	32.5 ±0.4	**140 ±4**
[Gln^1]-TRH	90 ±4	82.8 ±1.2	**920.0 ±27.6**	n.d.	n.d.	**n.d.**
[Gln^1]-GnRH	53 ±3	69.2 ±1.1	**1305.7 ±53.2**	169 ±9	82.5 ±1.9	**488.2 ±14.8**
[Gln^3]-glucagon(3-29)			*			*
[Gln^5]-substance P(5-11)			*			*

All analyses were performed in the optimal range of activity and stability of either human or plant QC, as demonstrated in example 4.

The amino acid sequences of physiological active peptides having an N-terminal glutamine residue and being therefore substrates for the QC enzyme are listed in Table 2:

Table 2: Amino acid sequences of physiological active peptides with an N-terminal glutamine residue

Peptide	Amino acid sequence	Function
Gastrin 17 Swiss-Prot: P01350	QGPWL EEEEEAYGWM DF (amide)	Gastrin stimulates the stomach mucosa to produce and secrete hydrochloric acid and the pancreas to secrete its digestive enzymes. It also stimulates smooth muscle contraction and increases blood circulation and water secretion in the stomach and intestine.

Neurotensin Swiss-Prot: P30990	QLYENKPRRP YIL	Neurotensin plays an endocrine or paracrine role in the regulation of fat metabolism. It causes contraction of smooth muscle.
FPP	QEP amide	A tripeptide related to thyrotrophin releasing hormone (TRH), is found in seminal plasma. Recent evidence obtained *in vitro* and *in vivo* showed that FPP plays an important role in regulating sperm fertility.
TRH Swiss-Prot: P20396	QHP amide	TRH functions as a regulator of the biosynthesis of TSH in the anterior pituitary gland and as a neurotransmitter/ neuromodulator in the central and peripheral nervous systems.
GnRH Swiss-Prot: P01148	QHWSYGL RP(G) amide	Stimulates the secretion of gonadotropins; it stimulates the secretion of both luteinizing and follicle-stimulating hormones.
CCL16 (small inducible cytokine A16) Swiss-Prot: O15467	QPKVPEW VNTPSTCCLK YYEKVLPRRL VVGYRKALNC HLPAIIFVTK RNREVCTNPN DDWVQEYIKD PNLPLLPTRN LSTVKIITAK NGQPQLLNSQ	Shows chemotactic activity for lymphocytes and monocytes but not neutrophils. Also shows potent myelosuppressive activity, suppresses proliferation of myeloid progenitor cells. Recombinant SCYA16 shows chemotactic activity for monocytes and THP-1 monocytes, but not for resting lymphocytes and neutrophils. Induces a calcium flux in THP-1 cells that were desensitized by prior expression to RANTES.
CCL8 (small	QPDSVSI PITCCFNVIN	Chemotactic factor that

inducible cytokine A8) Swiss-Prot: P80075	RKIPIQRLES YTRITNIQCP KEAVIFKTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP	attracts monocytes, lymphocytes, basophils and eosinophils. May play a role in neoplasia and inflammatory host responses. This protein can bind heparin.
CCL2 (small inducible cytokine A2) Swiss-Prot: P13500	QPDAINA PVTCCYNFTN RKISVQRLAS YRRITSSKCP KEAVIFKTIV AKEICADPKQ KWVQDSMDHL DKQTQTPKT	Chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils. Augments monocyte anti-tumor activity. Has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis or atherosclerosis. May be involved in the recruitment of monocytes into the arterial wall during the disease process of atherosclerosis. Binds to CCR2 and CCR4.
CCL18 (small inducible cytokine A18) Swiss-Prot: P55774	QVGTNKELC CLVYTSWQIP QKFIVDYSET SPQCPKPGVI LLTKRGRQIC ADPNKKWVQK YISDLKLNA	Chemotactic factor that attracts lymphocytes but not monocytes or granulocytes. May be involved in B cell migration into B cell follicles in lymph nodes. Attracts naive T lymphocytes toward dendritic cells and activated macrophages in lymph nodes, has chemotactic activity for naive T cells, CD4+ and CD8+ T cells and thus may play a role in both humoral and cell-mediated immunity responses.
Fractalkine (neurotactin) Swiss-Prot: P78423	QHHGVT KCNITCSKMT SKIPVALLIH YQQNQASCGK RAIILETRQH RLFCADPKEQ WVKDAMQHLD RQAAALTRNG GTFEKQIGEV KPRTTPAAGG MDESVVLEPE ATGESSSLEP TPSSQEAQRA LGTSPELPTG	The soluble form is chemotactic for T cells and monocytes, but not for neutrophils. The membrane-bound form promotes adhesion of those leukocytes to endothelial cells. May play

	VTGSSGTRLP PTPKAQDGGP VGTELFRVPP VSTAATWQSS APHQPGPSLW AEAKTSEAPS TQDPSTQAST ASSPAPEENA PSEGQRVWGQ GQSPRPENSL EREEMGPVPA HTDAFQDWGP GSMAHVSVVP VSSEGTPSRE PVASGSWTPK AEEPIHATMD PQRLGVLITP VPDAQAATRR QAVGLLAFLG LLFCLGVAMF TYQSLQGCPR KMAGEMAEGL RYIPRSCGSN SYVLVPV	a role in regulating leukocyte adhesion and migration processes at the endothelium. Binds to cx3cr1.
CCL7 (small inducible cytokine A7) Swiss-Prot: P80098	QPVGINT STTCCYRFIN KKIPKQRLES YRRTTSSHCP REAVIFKTKL DKEICADPTQ KWVQDFMKHL DKKTQTPKL	Chemotactic factor that attracts monocytes and eosinophils, but not neutrophils. Augments monocyte anti-tumor activity. Also induces the release of gelatinase B. This protein can bind heparin. Binds to CCR1, CCR2 and CCR3.
Orexin A (Hypocretin-1) Swiss-Prot O43612	QPLPDCCRQK TCSCRLYELL HGAGNHAAGI LTL	Neuropeptide that plays a significant role in the regulation of food intake and sleep-wakefulness, possibly by coordinating the complex behavioral and physiologic responses of these complementary homeostatic functions. It plays also a broader role in the homeostatic regulation of energy metabolism, autonomic function, hormonal balance and the regulation of body fluids. Orexin-A binds to both OX1R and OX2R with a high 7affinity.
Substance P	RPK PQQFFGLM	Belongs to the tachykinins. Tachykinins are active peptides which excite neurons, evoke behavioral responses, are potent vasodilators and

		secretagogues, and contract (directly or indirectly) many smooth muscles.

In a fourth embodiment, the peptides [Gln^1]Gastrin (17 and 34 amino acids in length), [Gln^1]Neurotensin and [Gln^1]FPP were identified as new physiological substrates of QC. Gastrin, Neurotensin and FPP comprise a pGlu residue in their N-terminal position. This N-terminal pGlu residue was shown to be formed from N-terminal glutamine by QC catalysis for all peptides. As a result, these peptides are activated in terms of their biological function upon conversion of the N-terminal glutamine residue to pGlu.

Transepithelial transducing cells, particularly the gastrin (G) cell, co-ordinate gastric acid secretion with the arrival of food in the stomach. Recent work showed that multiple active products are generated from the gastrin precursor, and that there are multiple control points in gastrin biosynthesis. Biosynthetic precursors and intermediates (progastrin and Gly-gastrins) are putative growth factors; their products, the amidated gastrins, regulate epithelial cell proliferation, the differentiation of acid-producing parietal cells and histamine-secreting enterochromaffin-like (ECL) cells, and the expression of genes associated with histamine synthesis and storage in ECL cells, as well as acutely stimulating acid secretion. Gastrin also stimulates the production of members of the epidermal growth factor (EGF) family, which in turn inhibit parietal cell function but stimulate the growth of surface epithelial cells. Plasma gastrin concentrations are elevated in subjects with *Helicobacter pylori*, who are known to have increased risk of duodenal ulcer disease and gastric cancer (Dockray, G.J. 1999 *J Physiol 15* 315-324).

The peptide hormone gastrin, released from antral G cells, is known to stimulate the synthesis and release of histamine from ECL cells in the oxyntic mucosa via CCK-2 receptors. The mobilized histamine induces acid secretion by binding to the H(2) receptors located on parietal cells. Recent studies suggest that gastrin, in both its fully amidated and less processed forms (progastrin and glycine-extended gastrin), is

also a growth factor for the gastrointestinal tract. It has been established that the major trophic effect of amidated gastrin is for the oxyntic mucosa of stomach, where it causes increased proliferation of gastric stem cells and ECL cells, resulting in increased parietal and ECL cell mass. On the other hand, the major trophic target of the less processed gastrin (e.g. glycine-extended gastrin) appears to be the colonic mucosa (Koh, T.J. and Chen, D. 2000 *Regul Pept* 9337-44).

In a fifth embodiment, the present invention provides the use of activity increasing effectors of QC for the stimulation of gastrointestinal tract cell proliferation, especially gastric mucosal cell proliferation, epithelial cell proliferation, the differentiation of acid-producing parietal cells and histamine-secreting enterochromaffin-like (ECL) cells, and the expression of genes associated with histamine synthesis and storage in ECL cells, as well as for the stimulation of acute acid secretion in mammals by maintaining or increasing the concentration of active [pGlu^1]-Gastrin.

In a sixth embodiment, the present invention provides the use of activity decreasing effectors of QC for the treatment of duodenal ulcer disease and gastric cancer with or w/o *Heliobacter pylori* in mammals by decreasing the conversion rate of inactive [Gln^1]Gastrin to active [pGlu^1]Gastrin.

Neurotensin (NT) is a neuropeptide implicated in the pathophysiology of schizophrenia that specifically modulates neurotransmitter systems previously demonstrated to be misregulated in this disorder. Clinical studies in which cerebrospinal fluid (CSF) NT concentrations have been measured revealed a subset of schizophrenic patients with decreased CSF NT concentrations that are restored by effective antipsychotic drug treatment. Considerable evidence also exists concordant with the involvement of NT systems in the mechanism of action of antipsychotic drugs. The behavioral and biochemical effects of centrally administered NT remarkably resemble those of systemically administered antipsychotic drugs, and antipsychotic drugs increase NT neurotransmission. This concatenation of findings led to the hypothesis that NT functions as an endogenous antipsychotic. Moreover, typical and atypical antipsychotic drugs differentially alter NT neurotransmission in

nigrostriatal and mesolimbic dopamine terminal regions, and these effects are predictive of side effect liability and efficacy, respectively (Binder, E. B. et al. 2001 *Biol Psychiatry 50* 856-872).

In a seventh embodiment, the present invention provides the use of activity increasing effectors of QC for the preparation of antipsychotic drugs and/or for the treatment of schizophrenia in mammals. The effectors of QC either maintain or increase the concentration of active [pGlu^1]neurotensin.

Fertilization promoting peptide (FPP), a tripeptide related to thyrotrophin releasing hormone (TRH), is found in seminal plasma. Recent evidence obtained *in vitro* and *in vivo* showed that FPP plays an important role in regulating sperm fertility. Specifically, FPP initially stimulates nonfertilizing (uncapacitated) spermatozoa to "switch on" and become fertile more quickly, but then arrests capacitation so that spermatozoa do not undergo spontaneous acrosome loss and therefore do not lose fertilizing potential. These responses are mimicked, and indeed augmented, by adenosine, known to regulate the adenylyl cyclase (AC)/cAMP signal transduction pathway. Both FPP and adenosine have been shown to stimulate cAMP production in uncapacitated cells but inhibit it in capacitated cells, with FPP receptors somehow interacting with adenosine receptors and G proteins to achieve regulation of AC. These events affect the tyrosine phosphorylation state of various proteins, some being important in the initial "switching on," others possibly being involved in the acrosome reaction itself. Calcitonin and angiotensin II, also found in seminal plasma, have similar effects *in vitro* on uncapacitated spermatozoa and can augment responses to FPP. These molecules have similar effects *in vivo*, affecting fertility by stimulating and then maintaining fertilizing potential. Either reductions in the availability of FPP, adenosine, calcitonin, and angiotensin II or defects in their receptors contribute to male infertility (Fraser, L.R. and Adeoya-Osiguwa, S. A. 2001 *Vitam Horm 63*, 1-28).

In an eight embodiment, the present invention provides the use of activity lowering effectors of QC for the preparation of fertilization prohibitive drugs and/or to reduce

the fertility in mammals. The activity lowering effectors of QC decrease the concentration of active [pGlu^1]FPP, leading to a prevention of sperm capacitation and deactivation of sperm cells. In contrast it could be shown that activity increasing effectors of QC are able to stimulate fertility in males and to treat infertility.

In a ninth embodiment, further physiological substrates of QC were identified within the present invention. These are [Gln^1]CCL2, [Gln^1]CCL7, [Gln^1]CCL8, [Gln^1]CCL16, [Gln^1]CCL18 and [Gln^1]fractalkine. For details see Table 2. These polypeptides play an important role in pathophysiological conditions, such as suppression of proliferation of myeloid progenitor cells, neoplasia, inflammatory host responses, cancer, psoriasis, rheumatoid arthritis, atherosclerosis, humoral and cell-mediated immunity responses, leukocyte adhesion and migration processes at the endothelium.

Several cytotoxic T lymphocyte peptide-based vaccines against hepatitis B, human immunodeficiency virus and melanoma were recently studied in clinical trials. One interesting melanoma vaccine candidate alone or in combination with other tumor antigens, is the decapeptide ELA. This peptide is a Melan-A/MART-1 antigen immunodominant peptide analog, with an N-terminal glutamic acid. It has been reported that the amino group and gamma-carboxylic group of glutamic acids, as well as the amino group and gamma-carboxamide group of glutamines, condense easily to form pyroglutamic derivatives. To overcome this stability problem, several peptides of pharmaceutical interest have been developed with a pyroglutamic acid instead of N-terminal glutamine or glutamic acid, without loss of pharmacological properties. Unfortunately compared with ELA, the pyroglutamic acid derivative (PyrELA) and also the N-terminal acetyl-capped derivative (AcELA) failed to elicit cytotoxic T lymphocyte (CTL) activity. Despite the apparent minor modifications introduced in PyrELA and AcELA, these two derivatives probably have lower affinity than ELA for the specific class I major histocompatibility complex. Consequently, in order to conserve full activity of ELA, the formation of PyrELA must be avoided (Beck A. et al. 2001, *J Pept Res* 57(6):528-38.). Recently, it was found that also the enzyme

glutaminyl cyclase (QC) is overexpressed in melanomas (Ross D. T et al., 2000, *Nat Genet* 24:227-35.).

In a tenth embodiment, the present invention provides the use of effectors of QC for the preparation of a medicament for the treatment of pathophysiological conditions, such as suppression of proliferation of myeloid progenitor cells, neoplasia, inflammatory host responses, cancer, malign metastasis, melanoma, psoriasis, rheumatoid arthritis, atherosclerosis, impaired humoral and cell-mediated immunity responses, leukocyte adhesion and migration processes at the endothelium.

In an eleventh embodiment, [Gln^1]orexin A was identified as a physiological substrate of QC within the present invention. Orexin A is a neuropeptide that plays a significant role in the regulation of food intake and sleep-wakefulness, possibly by coordinating the complex behavioral and physiologic responses of these complementary homeostatic functions. It plays also a role in the homeostatic regulation of energy metabolism, autonomic function, hormonal balance and the regulation of body fluids.

In a twelfth embodiment, the present invention provides the use of effectors of QC for the preparation of a medicament for the treatment of impaired food intake and sleep-wakefulness, impaired homeostatic regulation of energy metabolism, impaired autonomic function, impaired hormonal balance and impaired regulation of body fluids.

Polyglutamine expansions in several proteins lead to neurodegenerative disorders, such as Parkinson disease and Kennedy's disease. The mechanism therefore remains largely unknown. The biochemical properties of polyglutamine repeats suggest one possible explanation: endolytic cleavage at a glutaminyl–glutaminyl bond followed by pyroglutamate formation may contribute to the pathogenesis through augmenting the catabolic stability, hydrophobicity, amyloidogenicity, and neurotoxicity of the polyglutaminyl proteins (Saido, T; Med Hypotheses (2000) Mar;54(3):427-9). In a thirteenth embodiment, the present invention provides

therefore the use of effectors of QC for the preparation of a medicament for the treatment of Parkinson disease and Huntington's disease.

In a fourteenth embodiment, the present invention provides a general way to reduce or inhibit the enzymatic activity of QC. Examples of inhibitory compounds are also provided.

Inhibition of a mammalian QC was only detected initially for 1,10-phenanthroline and reduced 6-methylpterin (Busby, W. H. J. et al. 1987 *J Biol Chem 262*, 8532-8536). EDTA did not inhibit QC, thus it was concluded that QC is not a metal-dependent enzyme (Busby, W. H. J. et al. 1987 *J Biol Chem 262*, 8532-8536, Bateman, R.C.J. et al. 2001 *Biochemistry 40*, 11246-11250, Booth, R.E. et al. 2004 *BMC Biology 2*). In the present invention, however, it is shown that human QC and other animal QCs are metal-dependent enzymes, as revealed by the inhibition characteristics of QC by 1,10-phenanthroline, dipicolinic acid, 8-hydroxy-quinoline and other chelators (Figures 18,19) and by the reactivation of QC by transition metal ions (Figure 20). Finally, the metal dependence is outlined by a sequence comparison to other metal-dependent enzymes, showing a conservation of the chelating amino acid residues also in human QC (Figure 21). The interaction of compounds with the active-site bound metal ion represents a general way to reduce or inhibit QC activity.
In the present invention it is shown that imidazole derivatives are potent inhibitors of QC. Using the continuous assay (for details see example 2), many imidazole derivatives were analyzed concerning their ability to inhibit the human QC as a member of the highly conserved mammalian QCs.

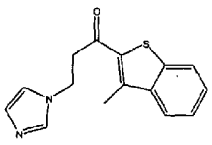
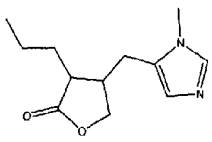
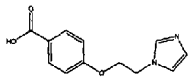
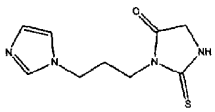
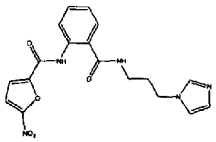
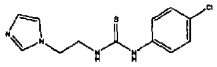
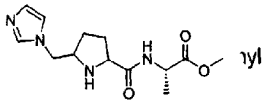
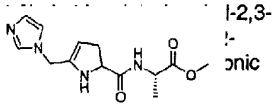
Thus, the present invention provides imidazole derivatives and histidine and its derivatives as activity reducing effectors of QC and their characteristics in terms of inhibition type and potency. Structures and K_i-values are shown in tables 3 and 4. The results are described in detail in example 7.

Table 3: Inhibitory constants of imidazole derivatives in the human QC catalyzed reaction. Determinations were performed at 30 °C in 0.05 M

Tris-HCl pH 8.0, containing 5 mM EDTA.

Compound	K_i-value (mM)	Structure
core structures		
imidazole	0.103 ±0.004	
benzimidazole	0.138 ±0.005	
N-1 derivatives		
1-benzylimidazole	0.0071 ±0.0003	
1-methylimidazole	0.030 ±0.001	
1-vinylimidazole	0.049 ±0.002	
oxalic acid diimidazolidide	0.078 ±0.002	
N-acetylimidazole	0.107 ±0.003	
N-(trimethylsilyl)-imidazole	0.167 ±0.007	
N-benzoylimidazole	0.174 ±0.007	
1-(2-oxo-2-phenyl-ethyl)-imidazole	0.184 ±0.005	
1-(3-aminopropyl)-imidazole	0.41 ±0.01	
1-phenylimidazole	no inhibition	
1,1´-sulfonyldiimidazole	no inhibition	
C-4(5) derivatives		
N-omega-acetylhistamine	0.017 ±0.001	
L-histidinamide	0.56 ±0.04	
H-His-Trp-OH	0.60 ±0.03	
L-histidinol	1.53 ±0.12	
L-histidine	4.4 ±0.2	
4-imidazole-carboxaldehyde	7.6 ±0.7	
imidazole-4-carbonic acid methylester	14.5 ±0.6	
L-histamine	0.85 ±0.04	
C-4,5 derivatives		
5-hydroxymethyl-4-methyl-imidazole	0.129 ±0.005	
4-amino-imidazole-5-carbonic acid amide	15.5 ±0.5	
4,5-diphenyl-imidazole	no inhibition	
4,5-dicyanoimidazole	no inhibition	
C-2 derivatives		
2-methyl-benzylimidazole	0.165 ±0.004	
2-ethyl-4-methyl-imidazole	0.58 ±0.04	
2-aminobenzimidazole	1.8 ±0.1	
2-chloro-1H-benzimidazole	no inhibition	

Others

3-(1H-imidazol-1-yl)-1-(3-methylbenzo[b]thiophene-2-yl)propan-1-one	0.0025 ±0.0001	
4-[(1-methyl-1H-imidazol-5-yl)methyl]-3-propyldihydrofuran-2-(3H)-one	0.0067± 0.0003	
4-[2-(1*H*-imidazol-1-yl)-ethoxy]benzoic acid	0.0034 ±0,0001	
3-[3-(1H-imidazol-1-yl)propyl]-2-thioxoimidazolidin-4-one	0.00041 ±0.00001	
5-nitro-2-[2-([{3-(1H-imidazol-1-yl-)propyl}amino] carbonyl)phenyl]furamide	0.0066 ±0,0004	
N-(4-chlorophenyl)-N´-[2-(1H-imidazol-1-yl)ethyl]thiourea	0.00165 ±0.00007	
 yl	0,0322 ±0,0007	
 l-2,3- :- onic	n.d.	

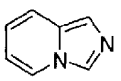
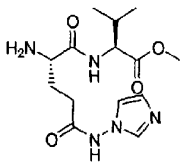
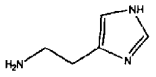
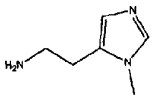
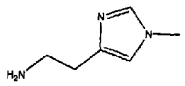
Imidazo<1.5a>pyridine	0.0356 ±0.0005	
Methyl (2*S*)-2-{[(2*S*)-2-amino-5-(1*H*-imidazol-1-ylamino)-5-oxopentanoyl]amino}-3-methylbutanoate	0.164 ±0.004	

Table 4: QC inhibition by L-histamine and its two biological metabolites (also known as *tele*-methylhistamine).

Compound	K_i value (mM)	Structure
L-histamine	0.85 ±0.04	
3-methyl-4-(β-aminoethyl)-imidazole	0.120 ±0.004	
1-methyl-4-(β-aminoethyl)-imidazole	n.i.	

Surprisingly, during characterization of the enzymatic activity it was discovered that besides an N-terminal glutaminyl residue, N-terminal β-homo-glutaminyl residues also fulfill properties as substrates of QCs from plants and mammals. The N-terminal β-homo-glutaminyl residue was converted into a five-membered lactam ring by

catalysis of human and papaya QC, respectively. The results are described in example 5. The applied method is illustrated in example 2 and the peptide synthesis was performed as described in example 6.

Another preferred embodiment of the present invention comprises screening methods for effectors of QC.

A preferred screening method for identifying activity modifying effectors of QC from a group of compounds comprises the steps of:

a) Contacting said compounds with QC under conditions which would permit binding therebetween;
b) Adding a substrate of QC;
c) Monitoring the conversion of the substrate or optionally measuring the residual QC activity; and
d) Calculating changes in the substrate conversion and/or enzyme activity of QC to identify an activity modifying effector.

Another preferred screening method relates to a method for the identification and selection of effectors which interact directly or indirectly with the active-site bound metal ion of QC and comprises the following steps:

a) Contacting said compounds with QC under conditions which would permit binding therebetween;
b) Adding a substrate of QC which is subject to conversion by QC;
c) Monitoring the conversion of the substrate or optionally measuring the residual QC activity; and
d) Calculating changes in the substrate conversion and/or enzyme activity of QC wherein changes may be used to identify an activity modifying effector of QC.

Preferred for the use in the above described screening methods are mammalian QC or *Papaya* QC. Especially preferred is mammalian QC, since the effectors identified

by these screening methods shall be used for the treatment of diseases in mammals, especially in humans.

The agents selected by the above described screening methods can work by decreasing the conversion of at least one substrate of QC (negative effectors, inhibitors), or by increasing the conversion of at least one substrate of QC (positive effectors, activators).

The compounds of the present invention can be converted into acid addition salts, especially pharmaceutically acceptable acid addition salts.

The salts of the compounds of the invention may be in the form of inorganic or organic salts.

The compounds of the present invention can be converted into and used as acid addition salts, especially pharmaceutically acceptable acid addition salts. The pharmaceutically acceptable salt generally takes a form in which a basic side chain is protonated with an inorganic or organic acid. Representative organic or inorganic acids include hydrochloric, hydrobromic, perchloric, sulfuric, nitric, phosphoric, acetic, propionic, glycolic, lactic, succinic, maleic, fumaric, malic, tartaric, citric, benzoic, mandelic, methanesulfonic, hydroxyethanesulfonic, benzenesulfonic, oxalic, pamoic, 2-naphthalenesulfonic, p-toulenesulfonic, cyclohexanesulfamic, salicylic, saccharinic or trifluoroacetic acid. All pharmaceutically acceptable acid addition salt forms of the compounds of the present invention are intended to be embraced by the scope of this invention.

In view of the close relationship between the free compounds and the compounds in the form of their salts, whenever a compound is referred to in this context, a corresponding salt is also intended, provided such is possible or appropriate under the circumstances.

Where the compounds according to this invention have at least one chiral center, they may accordingly exist as enantiomers. Where the compounds possess two or more chiral centers, they may additionally exist as diastereomers. It is to be understood that all such isomers and mixtures thereof are encompassed within the scope of the present invention. Furthermore, some of the crystalline forms of the compounds may exist as polymorphs and as such are intended to be included in the present invention. In addition, some of the compounds may form solvates with water (i.e. hydrates) or common organic solvents, and such solvates are also intended to be encompassed within the scope of this invention.

The compounds, including their salts, can also be obtained in the form of their hydrates, or include other solvents used for their crystallization.

In a further embodiment, the present invention provides a method of preventing or treating a condition mediated by modulation of the QC enzyme activity in a subject in need thereof which comprises administering any of the compounds of the present invention or pharmaceutical compositions thereof in a quantity and dosing regimen therapeutically effective to treat the condition. Additionally, the present invention includes the use of the compounds of this invention, and their corresponding pharmaceutically acceptable acid addition salt forms, for the preparation of a medicament for the prevention or treatment of a condition mediated by modulation of the QC activity in a subject. The compound may be administered to a patient by any conventional route of administration, including, but not limited to, intravenous, oral, subcutaneous, intramuscular, intradermal, parenteral and combinations thereof.

In a further preferred form of implementation, the invention relates to pharmaceutical compositions, that is to say, medicaments, that contain at least one compound of the invention or salts thereof, optionally in combination with one or more pharmaceutically acceptable carriers and/or solvents.

The pharmaceutical compositions may, for example, be in the form of parenteral or

enteral formulations and contain appropriate carriers, or they may be in the form of oral formulations that may contain appropriate carriers suitable for oral administration. Preferably, they are in the form of oral formulations.

The effectors of QC activity administered according to the invention may be employed in pharmaceutically administrable formulations or formulation complexes as inhibitors or in combination with inhibitors, substrates, pseudosubstrates, inhibitors of QC expression, binding proteins or antibodies of those enzyme proteins that reduce the QC protein concentration in mammals. The compounds of the invention make it possible to adjust treatment individually to patients and diseases, it being possible, in particular, to avoid individual intolerances, allergies and side-effects.

The compounds also exhibit differing degrees of activity as a function of time. The doctor providing treatment is thereby given the opportunity to respond differently to the individual situation of patients: he is able to adjust precisely, on the one hand, the speed of the onset of action and, on the other hand, the duration of action and especially the intensity of action.

A preferred treatment method according to the invention represents a new approach for the prevention or treatment of a condition mediated by modulation of the QC enzyme activity in mammals. It is advantageously simple, susceptible of commercial application and suitable for use, especially in the treatment of diseases that are based on unbalanced concentration of physiological active QC substrates, e.g. listed in Tables 1 and 2, in mammals and especially in human medicine.

The compounds may be advantageously administered, for example, in the form of pharmaceutical preparations that contain the active ingredient in combination with customary additives like diluents, excipients and/or carriers known from the prior art. For example, they can be administered parenterally (for example *i.v.* in physiological saline solution) or enterally (for example orally, formulated with customary carriers).

Depending upon their endogenous stability and their bioavailability, one or more

doses of the compounds can be given per day in order to achieve the desired normalisation of the blood glucose values. For example, such a dosage range in humans may be in the range of from about 0.01 mg to 250.0 mg per day, preferably in the range of from about 0.01 to 100 mg of compound per kilogram of body weight.

By administering effectors of QC activity to a mammal it could be possible to prevent or alleviate or treat conditions selected from Alzheimer's disease, Down Syndrome, ulcer disease and gastric cancer with or w/o *Heliobacter pylori* infections, pathogenic psychotic conditions, schizophrenia, infertility, neoplasia, inflammatory host responses, cancer, psoriasis, rheumatoid arthritis, atherosclerosis, impaired humoral and cell-mediated immune responses, leukocyte adhesion and migration processes in the endothelium, impaired food intake, sleep-wakefulness, impaired homeostatic regulation of energy metabolism, impaired autonomic function, impaired hormonal balance and impaired regulation of body fluids.

Further, by administering effectors of QC activity to a mammal it could be possible to stimulate gastrointestinal tract cell proliferation, preferably proliferation of gastric mucosal cells, epithelial cells, acute acid secretion and the differentiation of acid producing parietal cells and histamine-secreting enterochromaffin-like cells.

In addition, administration of QC inhibitors to mammals may lead to a loss of sperm cell function thus suppressing male fertility. Thus, the present invention provides a method for the regulation and control of male fertility and the use of activity lowering effectors of QC for the preparation of contraceptive medicaments for males.

Furthermore, by administering effectors of QC activity to a mammal it may be possible to suppress the proliferation of myeloid progenitor cells.

The compounds used according to the invention can accordingly be converted in a manner known *per se* into conventional formulations, such as, for example, tablets, capsules, dragées, pills, suppositories, granules, aerosols, syrups, liquid, solid and cream-like emulsions and suspensions and solutions, using inert, non-toxic,

pharmaceutically suitable carriers and additives or solvents. In each of those formulations, the therapeutically effective compounds are preferably present in a concentration of approximately from 0.1 to 80 % by weight, more preferably from 1 to 50 % by weight, of the total mixture, that is to say, in amounts sufficient for the mentioned dosage latitude to be obtained.

The substances can be used as medicaments in the form of dragées, capsules, bitable capsules, tablets, drops, syrups or also as suppositories or as nasal sprays.

The formulations may be advantageously prepared, for example, by extending the active ingredient with solvents and/or carriers, optionally with the use of emulsifiers and/or dispersants, it being possible, for example, in the case where water is used as diluent, for organic solvents to be optionally used as auxiliary solvents.

Examples of excipients useful in connection with the present invention include: water, non-toxic organic solvents, such as paraffins (for example natural oil fractions), vegetable oils (for example rapeseed oil, groundnut oil, sesame oil), alcohols (for example ethyl alcohol, glycerol), glycols (for example propylene glycol, polyethylene glycol); solid carriers, such as, for example, natural powdered minerals (for example highly disperse silica, silicates), sugars (for example raw sugar, lactose and dextrose); emulsifiers, such as non-ionic and anionic emulsifiers (for example polyoxyethylene fatty acid esters, polyoxyethylene fatty alcohol ethers, alkylsulphonates and arylsulphonates), dispersants (for example lignin, sulphite liquors, methylcellulose, starch and polyvinylpyrrolidone) and lubricants (for example magnesium stearate, talcum, stearic acid and sodium lauryl sulphate) and optionally flavourings.

Administration may be carried out in the usual manner, preferably enterally or parenterally, especially orally. In the case of enteral administration, tablets may contain in addition to the mentioned carriers further additives such as sodium citrate, calcium carbonate and calcium phosphate, together with various additives, such as starch, preferably potato starch, gelatin and the like. Furthermore, lubricants, such

as magnesium stearate, sodium lauryl sulphate and talcum, can be used concomitantly for tabletting. In the case of aqueous suspensions and/or elixirs intended for oral administration, various taste correctives or colourings can be added to the active ingredients in addition to the above-mentioned excipients.

In the case of parenteral administration, solutions of the active ingredients using suitable liquid carriers can be employed. In general, it has been found advantageous to administer, in the case of intravenous administration, amounts of approximately from 0.01 to 2.0 mg/kg, preferably approximately from 0.01 to 1.0 mg/kg, of body weight per day to obtain effective results and, in the case of enteral administration, the dosage is approximately from 0.01 to 2 mg/kg, preferably approximately from 0.01 to 1 mg/kg, of body weight per day.

It may nevertheless be necessary in some cases to deviate from the stated amounts, depending upon the body weight of the experimental animal or the patient or upon the type of administration route, but also on the basis of the species of animal and its individual response to the medicament or the interval at which administration is carried out. Accordingly, it may be sufficient in some cases to use less than the above-mentioned minimum amount, while, in other cases, the mentioned upper limit will have to be exceeded. In cases where relatively large amounts are being administered, it may be advisable to divide those amounts into several single doses over the day. For administration in human medicine, the same dosage latitude is provided. The above remarks apply analogously in that case.

Examples of pharmaceutical formulations

1. Capsules containing 100 mg of a compound of the invention per capsule:

For approximately 10,000 capsules a solution of the following composition is prepared:

compound of the invention	1.0 kg
glycerol	0.5 kg
polyethylene glycol	3.0 kg
water	0.5 kg
	5.0 kg

The solution is introduced into soft gelatin capsules in a manner known *per se*. The capsules are suitable for chewing or swallowing.

2. Tablets or coated tables or dragées containing 100 mg of a compound of the invention:

The following amounts refer to the preparation of 100,000 tablets:

compound of the invention, finely ground	10.0 kg
glucose	4.35 kg
lactose	4.35 kg
starch	4.50 kg
cellulose, finely ground	4.50 kg

The above constituents are mixed and then provided with a solution prepared from

polyvinylpyrrolidone		2.0 kg
polysorbate		0.1 kg
and water	approx.	5.0 kg

and granulated in a manner known *per se* by grating the moist mass and, after the addition of 0.2 kg of magnesium stearate, drying it. The finished tablet mixture of 30.0 kg is processed to form convex tablets weighing 300 mg. Ideally, the tablets can be coated or sugar-coated in a manner known *per se*.

The pharmaceutical compositions defined throughout the specification advantageously contain a combination of at least one effector of QC activity and at least one DP IV inhibitor. Such pharmaceutical compositions are especially useful for the treatment of Alzheimer's Disease and Down Syndrome.

Example 1: Preparation of Human and Papaya QC

Host strains and media

Pichia pastoris strain X33 (*AOX1*, *AOX2*), used for the expression of human QC was grown, transformed and analyzed according to the manufacturer's instructions (Invitrogen). The media required for *P. pastoris*, i.e. buffered glycerol (BMGY) complex or methanol (BMMY) complex medium, and the fermentation basal salts medium were prepared according to the manufacturer's recommendations.

Molecular cloning of plasmid vectors encoding the human QC

All cloning procedures were done applying standard molecular biology techniques. For expression in yeast, the vector pPICZαB (Invitrogen) was used. The pQE-31 vector (Qiagen) was used to express the human QC in *E. coli*. The cDNA of the mature QC starting with codon 38 was fused in frame with the plasmid encoded 6xhistidine tag. After amplification utilizing the primers pQCyc-1 and pQCyc-2 (Table 1) and subcloning, the fragment was inserted into the expression vector employing the restriction sites of *Sph*I and *Hin*dIII.

Transformation of P. pastoris and mini-scale expression

Plasmid DNA was amplified in *E. coli* JM109 and purified according to the recommendations of the manufacturer (Qiagen). In the expression plasmid used, pPICZαB, three restriction sites are provided for linearization. Since *Sac*I and *Bst*XI cut within the QC cDNA, *Pme*I was chosen for linearization. 20-30 μg plasmid DNA

was linearized with *PmeI*, precipitated by ethanol, and dissolved in sterile, deionized water. 10 µg of the DNA was then applied for transformation of competent *P. pastoris* cells by electroporation according to the manufacturer's instructions (BioRad). Selection was done on plates containing 150 µg/ml Zeocin. One transformation using the linearized plasmid yielded several hundred transformants.

In order to test the recombinant yeast clones for QC expression, recombinants were grown for 24 h in 10 ml conical tubes containing 2 ml BMGY. Afterwards, the yeast was centrifuged and resuspended in 2 ml BMMY containing 0.5 % methanol. This concentration was maintained by addition of methanol every 24 h up to 72 h. Subsequently, QC activity in the supernatant was determined. The presence of the fusion protein was confirmed by western blot analysis using an antibody directed against the 6×histidine tag (Qiagen). Clones that displayed the highest QC activity were chosen for further experiments and fermentation.

Large-scale expression in a fermenter

Expression of the QC was performed in a 5 l reactor (Biostat B, B. Braun biotech), essentially as described in the "*Pichia* fermentation process guidelines" (Invitrogen). Briefly, the cells were grown in the fermentation basal salts medium supplemented with trace salts, and with glycerol as the sole carbon source (pH 5.5). During an initial batch phase for about 24 h and a subsequent fed-batch phase for about 5 h, cell mass was accumulated. Once a cell wet weight of 200 g/l was achieved, induction of QC expression was performed using methanol applying a three-step feeding profile for an entire fermentation time of approximately 60 h. Subsequently, cells were removed from the QC-containing supernatant by centrifugation at 6000×*g*, 4 °C for 15 min. The pH was adjusted to 6.8 by addition of NaOH, and the resultant turbid solution was centrifuged again at 37000×*g*, 4 °C for 40 min. In cases of continued turbidity, an additional filtration step was applied using a cellulose membrane (pore width 0.45 µm).

Purification of 6xhistidine tagged QC expressed in P. pastoris

The His-tagged QC was first purified by immobilized metal affinity chromatography (IMAC). In a typical purification, 1000 ml of culture supernatant were applied to a Ni^{2+}-loaded Chelating Sepharose FF column (1.6 x 20 cm, Pharmacia), that was equilibrated with 50 mM phosphate buffer, pH 6.8, containing 750 mM NaCl, at a flow rate of 5 ml/min. After washing with 10 column volumes of equilibration buffer and 5 column volumes of equilibration buffer containing 5 mM histidine, the bound protein was eluted by a shift to 50 mM phosphate buffer, pH 6.8, containing 150 mM NaCl and 100 mM histidine. The resulting eluate was dialyzed against 20 mM Bis-Tris/HCl, pH 6.8, at 4 °C overnight. Subsequently, the QC was further purified by anion exchange chromatography on a Mono Q6 column (BioRad), equilibrated with dialysis buffer. The QC-containing fraction was loaded onto the column using a flow rate of 4 ml/min. The column was then washed with equilibration buffer containing 100 mM NaCl. The elution was performed by two gradients resulting in equilibration buffer containing 240 mM and 360 mM NaCl in 30 or 5 column volumes, respectively. Fractions of 6 ml were collected and the purity was analyzed by SDS-PAGE. Fractions containing homogenous QC were pooled and concentrated by ultrafiltration. For long-term storage (–20 °C), glycerol was added to a final concentration of 50 %. Protein was quantified according to the methods of Bradford or Gill and von Hippel (Bradford, M. M. 1976 *Anal Biochem 72*, 248-254; Gill, S.C. and von Hippel, P.H. 1989 *Anal Biochem 182*, 319-326.).

Expression and purification of QC in E. coli

The construct encoding the QC was transformed into M15 cells (Qiagen) and grown on selective LB agar plates at 37 °C. Protein expression was carried out in LB medium containing 1 % glucose and 1 % ethanol at room temperature. When the culture reached an OD_{600} of approximately 0.8, expression was induced with 0.1 mM IPTG overnight. After one cycle of freezing and thawing, cells were lysed at 4 °C by addition of 2.5 mg/ml lysozyme in 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl and 2 mM histidine for approximately 30 min. The solution was clarified by centrifugation at 37000×*g*, 4 °C for 30 min, followed by a filtration applying a glass frit (DNA separation) and two additional filtration steps applying cellulose filters for crude

and fine precipitates. The supernatant (approx. 500 ml) was applied onto a Ni^{2+}-affinity column (1.6 x 20 cm) at a flow rate of 1 ml/ min. Elution of QC was carried out with 50 mM phosphate buffer containing 150 mM NaCl and 100 mM histidine. The QC-containing fraction was concentrated by ultrafiltration.

Purification of QC from papaya latex

QC from papaya latex was prepared using the BioCAD 700E (Perseptive Biosystems, Wiesbaden, Germany) with a modified version of a previously reported method (Zerhouni, S. et al. 1989 *Biochim Biophys Acta 138*, 275-290). 50 g latex was dissolved in water and centrifugated as described therein. Inactivation of proteases was performed with S-methyl methanethiosulfonate, and the resultant crude extract was dialyzed. After dialysis, the entire supernatant was loaded onto a (21X2.5 cm i.d.) SP Sepharose Fast Flow column, equilibrated with 100 mM sodium acetate buffer, pH 5.0 (flow rate 3 ml/min). Elution was performed in three steps by increasing sodium acetate buffer concentration at a flow rate of 2 ml/min. The first step was a linear gradient from 0.1 to 0.5 M acetate buffer in 0.5 column volumes. The second step was a linear increase in buffer concentration from 0.5 to 0.68 M in four column volumes. During the last elution step, one column volume of 0.85 M buffer was applied. Fractions (6 ml) containing the highest enzymatic activity were pooled. Concentration and buffer changes to 0.02 M Tris/HCl, pH 8.0 were performed via ultrafiltration (Amicon; molecular mass cut-off of the membrane 10 kDa).

Ammonium sulfate was added to the concentrated papaya enzyme, obtained from the ion exchange chromatography step to a final concentration of 2 M. This solution was applied onto a (21X2.5 cm i.d.) Butyl Sepharose 4 Fast Flow column (flow rate 1.3 ml/min), equilibrated with 2 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0. Elution was performed in three steps with decreasing concentrations of ammonium sulfate. During the first step a linear gradient from 2 to 0.6 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0 was applied for 0.5 column volumes at a flow rate of 1.3 ml/min. The second step was a linear gradient from 0.6 to 0 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0, in 5 column volumes at a flow rate of 1.5 ml/min. The last elution

step was carried out by applying 0.02 M Tris/HCl at pH 8.0 for 2 column volumes at a flow rate of 1.5 ml/min. All fractions containing QC activity were pooled and concentrated by ultrafiltration. The resultant homogenous QC was stored at –70°C. Final protein concentrations were determined using the method of Bradford, compared to a standard curve obtained with bovine serum albumin.

Example 2: Assays for glutaminyl cyclase activity

Fluorometric assays

All measurements were performed with a BioAssay Reader HTS-7000Plus for microplates (Perkin Elmer) at 30 °C. QC activity was evaluated fluorometrically using H-Gln-βNA. The samples consisted of 0.2 mM fluorogenic substrate, 0.25 U pyroglutamyl aminopeptidase (Unizyme, Hørsholm, Denmark) in 0.2 M Tris/HCl, pH 8.0 containing 20 mM EDTA and an appropriately diluted aliquot of QC in a final volume of 250 µl. Excitation/emission wavelengths were 320/410 nm. The assay reactions were initiated by addition of glutaminyl cyclase. QC activity was determined from a standard curve of β-naphthylamine under assay conditions. One unit is defined as the amount of QC catalyzing the formation of 1 µmol pGlu-βNA from H-Gln-βNA per minute under the described conditions.

In a second fluorometric assay, QC was activity was determined using H-Gln-AMC as substrate. Reactions were carried out at 30°C utilizing the NOVOStar reader for microplates (BMG labtechnologies). The samples consisted of varying concentrations of the fluorogenic substrate, 0.1 U pyroglutamyl aminopeptidase (Qiagen) in 0.05 M Tris/HCl, pH 8.0 containing 5 mM EDTA and an appropriately diluted aliquot of QC in a final volume of 250 µl. Excitation/emission wavelengths were 380/460 nm. The assay reactions were initiated by addition of glutaminyl cyclase. QC activity was determined from a standard curve of 7-amino-4-methylcoumarin under assay conditions. The kinetic data were evaluated using GraFit sofware.

Spectrophotometric assay of QC

This novel assay was used to determine the kinetic parameters for most of the QC substrates. QC activity was analyzed spectrophotometrically using a continuous method, that was derived by adapting a previous discontinuous assay (Bateman, R. C. J. 1989 *J Neurosci Methods 30*, 23-28) utilizing glutamate dehydrogenase as auxiliary enzyme. Samples consisted of the respective QC substrate, 0.3 mM NADH, 14 mM α-Ketoglutaric acid and 30 U/ml glutamate dehydrogenase in a final volume of 250 µl. Reactions were started by addition of QC and persued by monitoring of the decrease in absorbance at 340 nm for 8-15 min. Typical time courses of product formation are presented in Figure 1.

The initial velocities were evaluated and the enzymatic activity was determined from a standard curve of ammonia under assay conditions. All samples were measured at 30°C, using either the SPECTRAFluor Plus or the Sunrise (both from TECAN) reader for microplates. Kinetic data was evaluated using GraFit software.

Inhibitor assay

For inhibitor testing, the sample composition was the same as described above, except of the putative inhibitory compound added. For a rapid test of QC-inhibition, samples contained 4 mM of the respective inhibitor and a substrate concentration at 1 K_M. For detailed investigations of the inhibition and determination of K_i-values, influence of the inhibitor on the auxiliary enzymes was investigated first. In every case, there was no influence on either enzyme detected, thus enabling the reliable determination of the QC inhibition. The inhibitory constant was evaluated by fitting the set of progress curves to the general equation for competitive inhibition using GraFit software.

Example 3: MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry was carried out using the Hewlett-Packard G2025 LD-TOF System with a linear time of flight analyzer. The

instrument was equipped with a 337 nm nitrogen laser, a potential acceleration source (5 kV) and a 1.0 m flight tube. Detector operation was in the positive-ion mode and signals were recorded and filtered using LeCroy 9350M digital storage oscilloscope linked to a personal computer. Samples (5 μl) were mixed with equal volumes of the matrix solution. For matrix solution we used DHAP/DAHC, prepared by solving 30 mg 2´,6´-dihydroxyacetophenone (Aldrich) and 44 mg diammonium hydrogen citrate (Fluka) in 1 ml acetonitrile/0.1% TFA in water (1/1, v/v). A small volume (≈ 1 μl) of the matrix-analyte-mixture was transferred to a probe tip and immediately evaporated in a vacuum chamber (Hewlett-Packard G2024A sample prep accessory) to ensure rapid and homogeneous sample crystallization.

For long-term testing of Glu^1-cyclization, Aβ-derived peptides were incubated in 100μl 0.1 M sodium acetate buffer, pH 5.2 or 0.1 M Bis-Tris buffer, pH 6.5 at 30°C. Peptides were applied in 0.5 mM [Aβ3-11a] or 0.15 mM [Aβ3-21a] concentrations, and 0.2 U QC was added all 24 hours. In case of Aβ3-21a, the assays contained 1 % DMSO. At different times, samples were removed from the assay tube, peptides extracted using ZipTips (Millipore) according to the manufacturer`s recommendations, mixed with matrix solution (1:1 v/v) and subsequently the mass spectra recorded. Negative controls did either contain no QC or heat deactivated enzyme. For the inhibitor studies the sample composition was the same as described above, with exception of the inhibitory compound added (5 mM benzimidazole or 2 mM 1,10-phenanthroline).

Example 4: pH dependence

The pH-dependence of catalysis of human and papaya QC was investigated under first-order rate conditions, thus reflecting the impact of the proton concentration on the specificity constant k_{cat}/K_M. For this purpose, the coupled enzymatic assay using pyroglutamyl aminopeptidase as auxiliary enzyme and Gln-βNA as substrate was used. Pyroglutamyl aminopeptidase was shown to be active and stable between pH 5.5-8.5 (Tsuru, D. et al. 1978 *J Biochem (Tokyo) 84*, 467-476). Hence, the assay enabled the study of QC catalysis in this pH-region. The rate profiles obtained were

fit to classical bell shaped curves, as shown in Figure 2. The human QC bears a very narrow pH-dependence with an optimum at about pH 7.8-8.0. The rate tended to decrease at more basic pH. This is in contrast to the rate profile observed with papaya QC, which showed no drop in activity up to pH 8.5 (Figure 2, inset). However, both enzymes had their optimum of specificity at pH 8. Surprisingly, evaluation of the curves revealed identical pK_a-values in the acidic range of 7.17 ±0.02 and 7.15 ±0.02 for human and papaya QC, respectively.

The reduction of the activity of human QC at basic pH-values was obviously due to dissociation of a group with a pK_a of approximately 8.5. In case of papaya QC, there was no excessive data point collection in the basic pH-range possible to enable a reliable determination of the second pK_a value. This is supported by fitting of the data to a single dissociation model, resulting in an almost identical pK_a-value (pK_a 7.13 ±0.03) compared to fitting the data to a double disssociation model. This indicates that both pK_a–values are fairly separated..

pH stability

The stability of the glutaminyl cyclases was investigated by incubating the plant and animal enzymes at 30 °C for 30 min at different pH values between pH 4-10. Afterwards, QC activity was determined under standard conditions. The results are shown in Figure 3.

The QC from papaya latex was stable in the pH-range studied, without an obvious trend for instability in the acidic or basic range. In contrast, human QC only showed a comparable stability in the pH-range between 7 and 8.5, exhibiting a remarkable instability at pH values above pH 8.5 and below pH 6. Thus, the region around pH 8 seems to be optimal for activity and stability of plant and human QC and a suitable pH-value for performing a substrate specificity comparison of the QCs.

Example 5: Determination of substrate specificity of QC

Spectrophotometric assay

The continuous spectrophotometric assay was performed as described in example 2. Accordingly, QC activity is reflected by a decrease in absorbance at 340 nm caused by the ammonia release and subsequent consumption of $NADH/H^+$ due to formation of glutamate from α-ketoglutaric acid. As shown in Figure 1, linear progress curves were monitored and there was a linear relationship between the measured activity and the concentration of QC. Furthermore, the kinetic parameters obtained for H-Gln-Gln-OH using the continuous assay presented here (Table 1) were in good agreement with those obtained using the discontinuous method (K_M= 175 ±18 μM, k_{cat}= 21.3 ±0.6 s^{-1}). In addition, the kinetic parameters for conversion of the substrates H-Gln-Ala-OH, H-Gln-Glu-OH, H-Gln-Gln-OH, H-Gln-OtBu and H-Gln-NH_2 by papaya QC shown in Table 1 correspond well to those determined using a direct method at pH 8.8 and 37 °C (Gololobov, M. Y. et al. 1996 *Biol Chem Hoppe Seyler 377*, 395-398). Hence, it is quite obvious that the novel continuous assay provides reliable results.

Di-, Tri- and Dipeptide-surrogates

Utilizing the novel continuous assay described above, about 30 compounds were tested as potential substrates of QC from *C. papaya* and human. The results are displayed in Table 5. By comparison of the specificities it was shown, that nearly all of the short peptide substrates are more efficiently converted by papaya QC compared to the human enzyme. Interestingly, for both enzymes substrates with large hydrophobic residues in the second position are the most potent ones, as shown by the specificities of H-Gln-Tyr-Ala-OH, H-Gln-Phe-Ala-NH_2 and H-Gln-Trp-Ala-NH_2 compared to those of other tripeptides or by the reactivities of the chromophoric substrates H-Gln-AMC, H-Gln-βNA and H-Gln-Tyr-OH in comparison to dipeptide substrates. For papaya QC, this finding is in agreement with earlier results showing that the specificity is in correlation with the size of the second amino acid residue (Gololobov, M. Y. et al. 1996 *Biol Chem Hoppe Seyler 377*, 395-398). The only striking difference in specificity of the plant and animal QC was observed in case of H-Gln-OtBu . Whereas the ester was converted by papaya QC with similar

specificity compared to dipeptide substrates, it was converted about one order of magnitude slower by human QC.

Oligopeptides

Besides several dipeptides and tripeptides, a number of oligopeptides was tested upon conversion by papaya and human QC (Table 5). Interestingly, the overall difference in the specificities between human and plant QC for a set of tetrapeptides was not that large as it was observed for dipeptide and tripeptide substrates. This indicates that the amino acids in the 3rd and 4th position still affect the kinetic behavior especially of human QC. An exception, however, comprise the peptides with a proline residue in the second amino acid position which show noticeably reduced k_{cat}/K_M values in a set of tetrapeptides of the structure H-Gln-X_{aa}-Tyr-Phe-NH_2 (Table 5). The reduction in specificity was more pronounced for human QC, leading to an approximately 8-fold difference in the k_{cat}/K_M-value as compared to papaya QC.

Slightly reduced specificities of human QC were also observed for conversion of substrates with a positively charged amino acid C-terminal of glutamine, as indicated by the specificities for H-Gln-Arg-Tyr-Phe-NH_2, H-Gln-Arg-Tyr-Phe-NH_2 and H-Gln-Lys-Arg-Leu-NH_2 as compared to other tetrapeptides. Apparently, the reduced specificity was mainly due to a smaller turnover number. This effect was not the case for the plant enzyme.

Table 5: Kinetic evaluation of peptide substrates of human and Papaya QC (n.r., not reactive; n.i., no inhibition; n.d., not determined; *, for substrate inhibition)

Substrate	Human QC				Papaya QC			
	K_M (μM)	k_{cat} (s^{-1})	K_i* (mM)	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	K_i* (mM)	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)
H-Gln-OH	n.r.	n.r.	n.d.	n.r.	n.d.	n.d.	n.d.	**0.23 ±0.1**
H-Gln-AMC	54 ±2	5.3 ±0.1	n.d.	**98 ±2**	42 ±1	39.4 ±0.4	n.d.	**938 ±13**
H-Gln-βNA	70 ±3	20.6 ±0.5	1.21 ±0.07	**294 ±6**	38 ±3	51.4 ±1.4	1.20 ±0.08	**1353 ±70**
H-Gln-OtBu	1235 ±74	6.7 ±0.2	n.i.	**5.4 ±0.2**	223 ±9	49.4 ±0.6	n.i.	**222 ±6**

H-Gln-NH_2	409 ±40	12.8 ±0.5	n.i.	31 ±2	433 ±13	44.8 ±0.4	n.i.	103±2
H-Gln-Gly-OH	247 ±10	13.2 ±0.2	n.i.	53 ±1	641 ±20	45.8 ±0.4	n.i.	71 ±2
H-Gln-Ala-OH	232 ±5	57.2 ±0.4	n.i.	247 ±4	158 ±8	69.8 ±1.0	n.i.	442 ±16
H-Gln-Gln-OH	148 ±5	20.7 ±0.2	n.i.	140 ±2	44 ±3	43.2 ±0.7	n.i.	982 ±51
H-Gln-Glu-OH	359 ±10	24.7 ±0.2	n.i.	58 ±1	106 ±5	50.3±0.6	n.i.	475 ±17
H-Gln-Val-OH	196 ±5	17.2 ±0.1	n.i.	88 ±2	n.d.	n.d.	n.i.	n.d.
H-Gln-Tyr-OH	211 ±5	94 ±1	n.i.	446±6	n.d.	n.d.	n.i.	n.d.
H-Gln-Glu-Tyr-NH_2	79 ±2	45.1 ±0.4	n.i.	524±8	103 ±4	53.6 ±0.7	n.i.	520 ±13
H-Gln-Gly-Pro-OH	130 ±5	25.3 ±0.2	n.i.	195 ±7	333 ±15	41.7 ±0.5	n.i.	125 ±4
H-Gln-Tyr-Ala-OH	101 ±4	125 ±1	n.i.	930 ±27	63 ±3	104.0 ±1.0	n.i.	1650 ±63
H-Gln-Phe-Ala-NH_2	69 ±3	109 ±1	n.i.	1811 ±64	111 ±5	132.1 ±0.6	n.i.	1190 ±48
H-Gln-Trp-Ala-NH_2	50 ±2	47.0 ±0.7	n.i.	940 ±24	78 ±5	151.8 ±2.6	n.i.	1946 ±91
H-Gln-Arg-Gly-Ile-NH_2	143 ±4	33.5 ±0.4	n.i.	234 ±4	123 ±10	49.2 ±1.7	n.i.	400 ±19
H-Gln-Asn-Gly-Ile-NH_2	172 ±5	56.6 ±0.5	n.i.	329 ±7	153 ±9	51.4 ±0.9	n.i.	336 ±14
H-Gln-Ser-Tyr-Phe-NH_2	55 ±3	52.8 ±0.8	n.i.	960 ±38	135 ±6	64.9 ±1.0	n.i.	481 ±14
H-Gln-Arg-Tyr-Phe-NH_2	55 ±2	29.6 ±0.3	n.i.	538 ±14	124 ±6	48.9 ±0.7	n.i.	394 ±13
H-Gln-Pro-Tyr-Phe-NH_2	1889 ±152	31.7 ±1.2	n.i.	17 ±1	149 ±14	18.8 ±0.6	n.i.	126 ±8
H-Gln-His-Tyr-Phe-NH_2	68 ±3	55.4 ±0.7	n.i.	815 ±26	92 ±7	75.9 ±1.4	n.i.	825 ±48
H-Gln-Gln-Tyr-Phe-NH_2	41 ±2	41.4 ±0.4	n.i.	1010 ±40	45 ±2	52.9 ±0.7	n.i.	1176 ±37
H-Gln-Glu-Tyr-Phe-NH_2	47 ±4	46 ±1	n.i.	979 ±62	100 ±4	54.6 ±0.6	n.i.	546 ±16
H-Gln-Glu-Ala-Ala-NH_2	77 ±4	46 ±1	n.i.	597 ±18	102 ±4	53.7 ±0.6	n.i.	526 ±15
H-Gln-Glu-Tyr-Ala-NH_2	69 ±2	42.1 ±0.4	n.i.	610 ±12	113 ±5	44.7 ±0.5	n.i.	396 ±13
H-Gln-Glu-Ala-Phe-NH_2	39 ±3	39 ±1	n.i.	1000 ±51	81 ±3	48.5 ±0.45	n.i.	599 ±17
H-Gln-Glu-Asp-Leu-NH_2	55 ±2	45.8 ±0.5	n.i.	833 ±21	107 ±6	58.5 ±0.4	n.i.	547 ±27
H-Gln-Lys-Arg-Leu-NH_2	54 ±3	33.4 ±0.5	n.i.	619 ±25	118 ±6	48.2 ±0.8	n.i.	408 ±14

The results obtained with the tetrapeptides give also rise to another conclusion. As already pointed out, papaya QC showed a higher selectivity for dipeptides. For some of the tetrapeptides, however, higher specificity-constants were observed with human QC, as shown in Figure 4 providing a plot of the data given in Table 5, for a set of peptides containing glutamate in the second amino acid position. Furthermore, as the chain length increases from di- to tetrapeptides, the selectivity of human QC increases, in contrast to the results obtained with papaya QC. Additionally, the highest selectivity of human QC was recorded for the peptides containing bulky hydrophobic residues in the 3rd and 4th amino acid position, which indicate hydrophobic interactions with the enzyme. By comparison of the kinetic parameters for the respective peptides, the changes seem to be mainly due to lower K_M-values, the turnover numbers for conversion of the peptides were found to be similar. Thus, the higher selectivity of human QC for longer peptides is considered to be the result of tighter binding of the more hydrophobic substrates to the enzyme.

The differences between human and plant QC observed with peptides containing hydrophobic amino acids in the 3rd and 4th position becomes also evident by a comparison of the specificity constants of the enzymes towards H-Gln-Arg-Gly-Ile-NH_2 and H-Gln-Arg-Tyr-Phe-NH_2 or H-Gln-Gln-OH and H-Gln-Gln-Tyr-Phe-OH.

Human QC was also found to be more selective for homologous substrates containing N-terminal Gln and an increasing number of C-terminal Ala residues (Table 6). While the selectivity of human QC increased with substrate length, there was no such a trend with the papaya QC. Since human QC was less specific for a peptide containing a Ser residue in the sequence, also the nature of the side chain seems to be of importance (table 6).

Table 6: Influence of substrate length on the activity of human and Papaya QC

Substrate	Human QC			Papaya QC		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
H-Gln-Ala -NH_2	155 ±9	40.1 ±0.9	**259 ±9**	212 ±21	62.8 ±3.0	**296 ±15**
H-Gln-Ala-Ala-NH_2	87 ±3	76.3 ±0.7	**877 ±22**	164 ±6	83.2 ±1.0	**507 ±12**
H-Gln-Ala-Ala-Ala-Ala-NH_2	65 ±3	60.5 ±0.7	**1174 ±43**	197 ±8	74.6 ±1.0	**379 ±10**
H-Gln-Ala-Ala-Ser-Ala-Ala-NH_2	79 ±6	55.3 ±1.6	**700 ±33**	216 ±6	78.5 ±1.0	**363 ±5**

Influence of ionic strength on catalysis

Another parameter that was investigated concerning its influence on the substrate specificity was ionic strength. For that purpose, the kinetic parameters for cyclization of several substrates were determined in presence and absence of 0.5 M KCl (Table 7). Surprisingly, the selectivity for substrates with uncharged backbone did not change significantly by addition of the salt in case of QC from papaya latex and human QC. The specificity constants of the human QC for H-Gln-Ala-OH and H-Gln-Glu-OH, however, decreased by addition of KCl. As indicated by the individual kinetic parameters, this effect was due to an increasing K_M and an only slightly decreasing k_{cat}-value. In case of papaya QC, there was no effect on either parameter detected. The effect seemed not to be due to the negatively charged substrate as such, since

unchanged parameters were found for the negatively charged peptide H-Gln-Glu-Asp-Leu-NH_2. An interesting effect of the salt addition was found for the positively charged substrates H-Gln-Arg-Gly-Ile-NH_2 and H-Gln-Lys-Arg-Leu-NH_2. In case of plant and human QC, a positive effect on catalysis was determined mainly due to a smaller K_M value and a slightly higher turnover number.

Table 7: Influence of ionic strength on catalysis of human and Papaya QC

	Substrate	0.05 M Tricine-NaOH, pH 8.0				0.05 M Tricine-NaOH, pH 8.0, 0.5 M KCl			
		K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	K_i (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	K_i (mM)
Papaya QC	H-Gln-NH_2	0.434 ±0.015	43.4 ±0.4	100 ±3	n.i.	0.446 ±0.010	45.2 ±0.3	101 ±2	n.i.
	H-Gln-βNA	0.036 ±0.002	48.8 ±1.0	1356 ±50	1.14 ±0.05	0.032 ±0.002	47.2 ±0.8	1475 ±70	1.33 ±0.07
	H-Gln-Ala-OH	0.137 ±0.007	69.7 ±.9	509 ±19	n.i.	0.143 ±0.005	68.1 ±0.6	480 ±12	n.i.
	H-Gln-Glu-OH	0.098 ±0.005	45.0 ±0.5	459 ±18	n.i.	0.094 ±0.003	44.4 ±0.3	472 ±12	n.i.
	H-Gln-Trp-Ala-NH_2	0.079 ±0.005	138 ±3	1747 ±73	n.i.	0.072 ±0.004	133 ±3	1847 ±61	n.i.
	H-Gln-Arg-Gly-Ile-NH_2	0.106 ±0.008	52.9 ±1.2	499 ±26	n.i.	0.065 ±0.005	48.4 ±1.0	745 ±42	n.i.
	H-Gln-Lys-Arg-Leu-NH_2	0.102 ±0.007	50 ±1	493 ±22	n.i.	0.053 ±0.002	58.1 ±0.7	1096 ±28	n.i.
	H-Gln-Glu-Asp-Leu-NH_2	0.109 ±0.005	52.4 ±0.7	481 ±16	n.i.	0.094 ±0.003	53.6 ±0.5	570 ±13	n.i.

		0.05 M Tris-HCl, pH 8.0				0.05 M Tris-HCl, pH 8.0, 0.5 M KCl			
Human QC	H-Gln-NH_2	0.442 ±0.030	12.8 ±0.3	29±1	n.i.	0.401 ±0.014	12.2 ±0.1	30±1	n.i.
	H-Gln-βNA	0.076 ±0.004	21.7 ±0.5	285±8	1.39 ±0.08	0.063 ±0.003	20.0 ±0.4	318 ±9	0.97 ±0.04
	H-Gln-Ala-OH	0.269 ±0.007	54.4 ±0.5	202±3	n.i.	0.357 ±0.012	47.6 ±0.6	133 ±3	n.i.
	H-Gln-Glu-OH	0.373 ±0.015	21.4 ±0.3	57±2	n.i.	0.607 ±0.036	18.9 ±0.5	31 ±1	n.i.
	H-Gln-Trp-Ala-NH_2	0.054 ±0.003	50.8 ±0.6	941 ±41	n.i.	0.056 ±0.002	50.0 ±0.4	893±25	n.i.
	H-Gln-Arg-Gly-Ile-NH_2	0.166 ±0.013	31 ±1	187 ±9	n.i.	0.091 ±0.005	29.8 ±0.5	327 ±12	n.i.
	H-Gln-Lys-Arg-Leu-NH_2	0.051 ±0.003	29.4 ±0.5	577 ±24	n.i.	0.034 ±0.001	31.6 ±0.3	929 ±19	n.i.
	H-Gln-Glu-Asp-Leu-NH_2	0.060 ±0.002	46.6 ±0.5	777 ±18	n.i.	0.061 ±0.002	45.6 ±0.5	748 ±16	n.i.

Physiological substrates

In earlier studies, conversion of [Gln^1]-TRH and [Gln^1]-GnRH by QC was already shown for the QC from bovine and porcine pituitary (Busby, W. H. J. et al. 1987 *J*

Biol Chem 262, 8532-8536; Fischer, W. H. and Spiess, J. 1987 *Proc Natl Acad Sci U S A 84*, 3628-3632). In addition to these already investigated pituitary hormones, three potential physiological substrates of human QC were synthesized and tested upon conversion, namely [Gln^1]Gastrin, [Gln^1]Neurotensin, and [Gln^1]FPP. The kinetic parameters for their conversion are listed in Table 1. Interestingly, the glutaminyl peptides are converted to the respective pyroglutamyl peptides with increasing specificity constants depending on their size, i.e., the first largest peptide pro-gastrin with 17 amino acids followed by pro-neurotensin, pro-GnRH, pro-TRH and pro-FPP. These findings correspond to the data obtained with the synthetic peptides.

Surprisingly, the longer substrates are also converted with higher selectivity by the plant enzyme, a result that contrasts in part with the findings for the shorter oligopeptides. Possibly, there are secondary binding interactions between substrate and enzyme far distant from the active site.

Peptides comprising modified amino acids

In order to further investigate the specificity and selectivity of the QCs, peptides were synthesized comprising either a modified N-terminal glutaminyl residue or a modified amino acid in the second position. The conversion of these peptides was investigated qualitatively utilizing MALDI-TOF mass spectrometry (see also example 3). Due to the cyclization of the glutaminyl residue or its analog, respectively, a mass difference of the substrate and the product of catalysis is detected. In cases of ammonia liberation of one mole per mole of substrate, the conversion was also analyzed quantitatively using the spectrophotometric assay.

H-Gln-Lys(Gln)-Arg-Leu-Ala-NH$_2$. This N-terminally branched peptide, comprising two glutaminyl residues at the N-terminus that are bound to a lysyl residue via a peptide- and partial isopeptide bond, was converted by human (Figure 5) and papaya QC (not shown) in an apparently identical manner. Both glutaminyl residues were converted into pyroglutamic acid, without any detectable preference for a distinct residue, as indicated by the consistent substrate conversion (Figure 5).

Thus, the selectivity of the QCs for the differently bound glutaminyl residues differs not fundamentally.

H-Gln(NMe)-Phe-Lys-Ala-Glu-NH_2. The methylated glutaminyl residue was only converted into a pyroglutamyl residue by papaya QC (Figure 6). Additionally, an inhibition of the human QC by the peptide was not detected, indicating that the methylated residue is not recognized by human QC.

H-Glu(OMe)-βNA and H-Glu-βNA. Neither of these compounds were converted by papaya or human QC. These fluorogenic substrates were analyzed fluorometrically, utilizing pyroglutamyl aminopeptidase as auxiliary enzyme. The O-methylated glutamate residue, however, showed a remarkable instability in both, Tris and Tricine buffers tending to a non-enzymatically catalyzed cyclization. Furthermore, activity of both QCs against H-Gln-AMC as substrate was not inhibited by the longer peptides H-Glu(OMe)-Phe-Lys-Arg-Leu-Ala-NH_2 or H-Glu-Phe-Lys-Arg-Leu-Ala-NH_2, indicating that glutamic acid or derivates are not recognized by both QC forms. Furthermore, the result implies that not only the negative charge of the glutamic acid residue is the reason for the repulsion of the peptide from the active site.

H-Gln-cyclo(Nε-Lys-Arg-Pro-Ala-Gly-Phe). The conversion of *H-Gln-cyclo(Nε-Lys-Arg-Pro-Ala-Gly-Phe)*, which contains an intramolecular partial isopeptide bond was analyzed quantitatively, revealing K_M-values of 240 ±14 μM and 133 ±5 μM for human and papaya QC, respectively. Due to the higher turnover number of conversion by papaya QC (49.4 ±0.6 s^{-1}) compared to human QC (22.8 ±0.6 s^{-1}), the plant enzyme exhibits with 372 ±9 mM^{-1} min^{-1} an approximately 4-fold higher k_{cat}/K_M-value than the human QC. Thus, the specificity constant is in case of the papaya QC only slightly smaller compared to substrates having a similar size, such as H-Gln-Ala-Ala-Ser-Ala-Ala-NH_2. The k_{cat}/K_M-value for human QC, however, was found with 95 ±3 $mM^{-1}s^{-1}$ to be approximately one order of magnitude smaller in comparison with substrates of similar size (Table 5).

H-βhomoGln-Phe-Lys-Arg-Leu-Ala-NH_2. The N-terminal β-homoglutaminyl residue was converted into a five-membered lactam ring by catalysis of human and papaya QC, respectively. The concomitant liberation of ammonia was analyzed spectrophotometrically and by MALDI-tof analysis as described before. There was no liberation of ammonia detected when QC was omitted or boiled, indicating a specific catalysis of the cyclization. Interestingly, the QC from *C. papaya* (K_M= 3.1 ±0.3 mM, k_{cat}= 4.0 ±0.4 s^{-1}) and human (K_M= 2.5 ±0.2 mM, k_{cat}= 3.5 ±0.1 s^{-1}) catalyze the conversion of this peptide with almost identical k_{cat}/K_M values of 1.4 ±0.1 and 1.3 ±0.1 $mM^{-1}s^{-1}$, respectively. Thus, the cyclization of the β-homoglutamine residue is catalyzed with an approximately 1000-fold reduced efficiency compared to peptides of similar size containing a glutaminyl residue at their N-terminus. This shows that the constitution of the α-carbon of the substrate is important for substrate recognition by the QC forms, but not essential. The essential requirement for being a substrate is a γ-amide group and an unprotonated N-terminal amino group in distance and angle prone for cyclization, a requirement that is fulfilled by N-terminal glutaminyl and β-homo-glutaminyl residues.

Example 6: Synthesis of the QC substrates

Oligopeptides. Peptides were synthesized semiautomatically in 0.5 mmol scale using a peptide synthesizer (Labortec SP650, Bachem, Switzerland) as previously described (Schilling, S. et al. 2002 *Biochemistry 41*, 10849-10857). Longer peptides were synthesized in 25 µmol scale using the automated Symphony peptide synthesizer (Rainin Instrument Co.) as described (Manhart, S. et al. 2003 *Biochemistry 42*, 3081-3088). For all peptide couplings modified Fmoc-protocols of solid-phase peptide synthesis were employed using 2-(1H-Benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate (TBTU; Novabiochem)/base (diisopropyl ethylamine or N-methyl- morpholine; Merck) or in case of difficult couplings *N*-[(Dimethylamino)-1*H*-1,2,3,-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanamminium hexafluorophosphate *N*-oxide (4,5) (HATU; Applied Biosystems)/ diisopropyl ethylamine as activating reagents were used. After cleavage from the resin by trifluoroacetic acid (TFA; Merck) containing cocktail, the crude peptides were purified by preparative HPLC with acid free solvents in order to avoid further cyclization of the N-terminal glutamine. Preparative HPLC was performed with a linear gradient of acetonitrile (Merck) in water (5-40 % or 65 % acetonitrile over 40 min) on a 250-21 Luna RP18 column (Phenomenex). To confirm peptide purity and identity analytical HPLC and ESI-MS was employed.

Glu(NH-NH_2)-Ser-Pro-Thr-Ala-NH_2. The linear precursor peptide (Fmoc-Glu-Ser-Pro-Thr-Ala-NH_2) was synthesized according to standard Fmoc-procedures (Schilling, S. et al. 2002 *Biochemistry 41*, 10849-10857) on Rink amide MBHA resin (Novabiochem). After cleavage of the Fmoc-protected peptide from the resin, the peptide was precipitated with diethyl ether (Merck), filtered and dried. HMBA-AM resin (1.16 mmol/g, Novabiochem) was used for coupling of the γ-carboxylic acid group of glutamic acid of the precursor peptide (3 eq.) in dichloromethane (DCM, Merck). Dicyclohexylcarbodiimide (DCC, Serva) (4 eq.) and dimethylaminopyridine (DMAP, Aldrich) (0.1 eq) were used as coupling reagents. After 12 hours the resin was filtered, washed with DCM and the reaction was repeated. After deprotection of the N-terminal Fmoc-group by employing 20% piperidine in DMF (3 × 5 min) the

peptide resin was treated with a 5% hydrazine solution (20 ml/g) for 1.5 hours. The resin was filtered and washed with dimethylformamide (DMF, Roth, Germany) and TFA. Following evaporation, the crude peptide was precipitated with ether giving 76 % yield.

H-Gln-Lys(Gln)-Arg-Leu-Ala-NH_2. The linear peptide was synthesized according to standard Fmoc/^{t}Bu-procedure on Rink amide MBHA (Schilling, S. et al. 2002 *Biochemistry 41*, 10849-10857) using Fmoc-Lys(Fmoc)-OH as penultimate amino acid coupling. After deprotection of the two amino protecting groups of lysine with 20 % piperidine (Merck) in DMF, 4 eq. Fmoc-Gln(Trt)-OH were coupled. Standard cleavage procedure resulted in 95% yield.

H-Gln(NMe)-Phe-Lys-Ala-Glu-NH_2. Fmoc-Gln(NMe)-OH was synthesized starting from Fmoc-Glu-OtBu loaded on Fmoc-MI-AM (Novabiochem) resin. After swelling with DCM, the resin (0.5 g) was washed with DMF and deprotected with 20% piperidine solution in DMF. The resin was given into 5 ml DMF and 5 eq. Fmoc-Glu-OtBu, 5 eq. HATU and 10 eq. DIPEA were added subsequently and shaked for 6 hours. After filtration and washing, the product was cleaved according to standard TFA cleavage conditions. The peptide H-Gln(NMe)-Phe-Lys-Ala-Glu-NH_2 was synthesized as described (Schilling, S. et al. 2002 *Biochemistry 41*, 10849-10857). Fmoc-Gln(NMe)-OH was coupled with HATU/DIPEA overnight. Standard cleavage procedure resulted in 78% of the crude peptide.

H-Glu(OMe)-β-naphthylamide, H-Gln-Val-OH, H-Gln-Tyr-OH. Boc-protected dipeptides were synthesized applying standard mixed anhydride procedure by using isobutyl chlorocarbonate (Merck). The C-terminal methylesters Boc-Gln-Tyr-OMe and Boc-Gln-Val-OMe were saponified by 1N NaOH in dioxane. The Boc-protected peptides were deprotected by HCl/dioxane solution for 10 min. After evaporation the residue was crystallized with several solvents giving 60-70% of a solid compound.

H-Gln-cyclo(Nε-Lys-Arg-Pro-Ala-Gly-Phe). The linear precursor Boc-Gln(Trt)-Lys-Arg(Pmc)-Ala-Gly-Phe-OH was synthesized on acid sensitive 2-chlorotrityl resin.

Coupling was carried out using a standard protocol of Fmoc/^{t}Bu-strategy using Fmoc-Lys(Mtt)-OH. After cleavage with 3% TFA solution in DCM (10 times 5 min), the solution was neutralized with 10% pyridine (Merck) in methanol (MeOH; Merck), washed 3 times with DCM and MeOH, evaporated to 5 % of the volume and the crude peptide was precipitated with icecold water. Following, the crude peptide was cyclized using DCC/*N*-hydroxybenzotriazole (HOBt; Aldrich) activation. The crude peptide was dissolved in dry dichloromethane (0.2 mmol/50 ml), 0.2 mmol N-methylmorpholine and 0.4 mmol 1-hydroxybenzotriazole were added. This solution was added dropwise to a solution of 0.4 mmol dicyclohexylcarbodiimide in 250 ml dichloromethane at 0°C. The reaction was completed by stirring overnight at room temperature. After filtration of *N,N'*-dicyclohexylurea, the solvent was removed by evaporation. The residue was dissolved in ethyl acetate and washed several times with 1N HCl, saturated solution of $NaHCO_3$ and water. The solution was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness in vacuo.

Example 7: Characterization of effectors of QC

Imidazole derivatives

Imidazole and benzimidazole derivatives carrying substituents in different positions of the 5-membered ring were tested as inhibitors of QC (Table 3). The constitution of the numbers refers to the imidazole ring. The applied methods are described in example 2.

C-4(5) and C-4,5 derivatives. The compounds carrying substitutions in either in the constitutionally equivalent 4- or 5- position of the imidazole ring or in both positions showed a diminished potency for inhibition of human QC. The only exception, however, comprised N-ω-acetylated histamine that proved to be one of the most potent inhibitory compounds. Small substituents in these positions had only little effect on binding as indicated by the similar inhibition constant of 5-hydroxymethyl-4-methyl-imidazole compared to imidazole. Larger and more bulky groups attached to

these sites diminished or abolished binding of the compound by the enzyme. Some of the other substituents tested are known to exert negative inductive or mesomeric effects that are capable to reduce the electron density in the imidazole ring, which also contributes to poorer binding constants. The difference in the K_i-values of L-histidine and histidinamide also indicate some influence of the charge on binding. Evidence for electrostatic repulsion of charged substrates were already shown in the substrate specificity studies, i.e. glutaminamide was readily converted to products by human QC, but no reactivity was observed for free glutamine as substrate.

C-2 derivatives. All derivatives tested inhibited QC more weakly as imidazole. Any substitution bigger than a proton hinders proper QC-binding. Only due to the methyl group in 2-methyl-benzimidazole, the inhibition constant drops about one order of magnitude. A very similar relation was shown by comparison of the K_i-values for benzimidazole and 2-amino-benzimidazole. Additionally, the results indicate that the influence is not related to electronic alterations.

N-1 derivatives. Among the imidazole derivatives tested on inhibition of human QC, most compounds that had improved K_i-values compared to imidazole showed alterations at one nitrogen atom. These compounds also contained one of the most effective QC inhibitors, 1-benzylimidazole. Interestingly, only little alterations of this structure led to a loss of inhibitory quality, as can be seen for 1-benzoylimidazole and phenylimidazole, which was inactive under the experimental conditions. Also in this case, the observed changes seemed not to be only caused by a reduced electron density of the imidazole ring due to the negative mesomeric effect of the Phenyl group, because also the bulky trimethyl-silyl group, exhibiting a positive inductive effect showed reduced binding compared to other residues. Interestingly, one of the less effective compounds of this group was 1-aminopropyl-imidazole. The small efficacy of this compound is caused by the basic amino group, since the sterically similar compounds 1-methylimidazole and 1-vinylimidazole showed improved binding to the active site. Thus, the positively charged amino group accounts for the smaller K_i-value, a result that is corroborated by a comparison of the K_i-values of N-ω-acetylated histamine (Table 3) and histamin (Table 4).

Effect of 3,4 and 3,5 derivatization. The imidazole derivatives that contained substituents in postions 4(5) or both were shown to have a restricted efficiency for binding to the enzyme. The effect of the specific substitutions were specified by comparison of the inhibitory constants of L-histamine and the two intermediates in the biological degradation of histamine, 3-methyl-4-histamine and 3-methyl-5-histamine (Table 4). L-Histamine revealed a K_i value that was about one order of magnitude smaller compared to its acetylated counterpart. Methylation of one nitrogen resulted in a considerable improvement of efficacy in case of 3-methyl-4-histamine. Methylation leading to 3-methyl-5-histamine, however, resulted in a complete loss of inhibitory activity. Thus, the observed effect seems to be mainly caused by a sterical hindrance of binding due to the derivatisation of the carbon adjacent to the basic nitrogen. Presumably, the basic nitrogen plays a key role for binding to the enzyme.

Example 8: Formation of Aβ3-40/42 derivatives

The measurements were carried out with two short N-terminal peptide sequences of Aβ3-40/42, [Gln^3]-Aβ1-11 (sequence: DAQFRHDSGYE) and [Gln^3]Aβ3-11, which contain a glutamine instead of an glutamic acid residue in the third position. Cleavage by DP IV and cyclization of the N-terminal glutamine residue by QC of the two peptides was tested using MALDI-TOF mass spectrometry. Measurements were carried out using purified DP IV (porcine kidney) or crude porcine pituitary homogenate as sources of QC as well as for both enzymes for measurements of consecutive catalysis.

Results

1. *Formation of [Gln^3]Aβ3-11a from [Gln^3]Aβ1-11a catalysed by DPIV and its prevention by the DP IV-inhibitor Val-Pyrrolidide (Val-Pyrr)*

DPIV or DPIV-like activity is cleaving [Gln^3]Aβ1-11a under formation of [Gln^3]Aβ3-11a (Figure 7). The residue in the third position is uncovered by this cleavage and

becomes therefore accessible for modification by other enzymes, i.e. QC. As expected, catalysis can be completely prevented by Val-Pyrr (Figure 8).

2. *Formation of [pGlu^3]Aβ3-11a from [Gln^3]Aβ3-11a by catalysis of QC in pituitary homogenate and prevention by 1,10-phenanthroline*

Glutaminyl cyclase present in the homogenate of porcine pituitary catalyzes conversion of [Gln^3]Aβ3-11a to [pGlu^3]Aβ3-11a (Figure 9). Formation of [pGlu^3]Aβ3-11a was inhibited by addition of 1,10-phenanthroline (Figure 10).

3. *Consecutive catalysis of DPIV and QC resulting in formation of [pGlu^3]Aβ3-11a and prevention by Val-Pyrr and 1,10-phenanthroline*

Formation of [pGlu^3]Aβ3-11a from [Gln^3]Aβ1-11a takes place after consecutive catalysis by DP IV and QC, measured in crude homogenate of porcine pituitary with added DPIV from porcine kidney (Figure 11). [pGlu^3]Aβ3-11a was not formed when the QC-inhibitor 1,10-phenanthroline (Figure 12) or the DP IV-inhibitor Val-Pyrr was added (Figure 13). The slight appearance of [pGlu^3]Aβ3-11a is due to aminopeptidase cleavage and following cyclization of the glutamine residue, also indicated by formation of [Gln^3]Aβ2-11a.

4. *Formation of [pGlu^3]Aβ3-11a in crude pituitary homogenate by catalysis of aminopeptidase(s)*

Due to the formation of [pGlu^3]Aβ3-11a that was not dependent on DPIV catalysis, degradation of [Gln^3]Aβ1-11a was investigated in crude pituitary homogenate without added DPIV (Figure 14). As expected from the data in section 4, formation of [pGlu^3]Aβ3-11a was observed. The data show that the degradation of [Gln^3]Aβ1-11a may also be catalyzed by aminopeptidase(s), resulting in [pGlu^3]Aβ3-11a. Hence, the results show that pyroglutamyl formation is an endpoint of N-terminal peptide degradation in this tissue, further supporting the role of QC in plaque formation.

Example 9: Turnover of [Gln^3]Aβ3-11a; 3-21a and 3-40 by recombinant human QC

All [Gln^3]Aβ derived peptides tested were efficiently converted by human QC into the corresponding pyroglutamyl forms (Table 8). Due to the poor solubility of [Gln^3]Aβ3-21a and [Gln^3]Aβ3-40 in aqueous solution, the determinations were carried out in presence of 1% DMSO. The better solubility of [Gln^3]Aβ3-11a, however, allowed the kinetic analysis of the QC-catalyzed turnover in presence and absence of DMSO (Table 8). Taken together, the investigation of the Aβ peptides as QC-substrates with chain-length of 8, 18 and 37 amino acids (see Table 8) confirmed the observation that human QC-activity increases with the length of its substrates. Accordingly, Gln^1-gastrin, Gln^1-neurotensin, Gln^1-GnRH are among the best QC-substrates taking the specificity constants into account. Similarly, [Gln^3]Aβ3-40 and glucagon, the largest QC-substrates investigated thus far, exhibited high second order rate constants (449 $mM^{-1}s^{-1}$ and 526 $mM^{-1}s^{-1}$ respectively) even in presence of 1% DMSO (Table 8). Interestingly, the kinetic parameters for the conversion of the investigated amyloid peptides did not change dramatically with increasing size, suggesting only moderate effects of the *C*-terminal part of Aβ on QC catalysis. Therefore, due to better solubility and experimental handling, the further investigations concerning *N*-terminal aminopeptidase processing of these peptides were performed using the smaller fragments of Aβ, [Gln^3]Aβ1-11a, [Gln^3]Aβ3-11a and Aβ3-11a.

Table 8: Kinetic parameters for conversion of N-terminally Gln-containing peptides by recombinant human QC in buffer solution containing 1% DMSO

Peptide	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)
[Gln^3]Aβ3-11a	87 ±3$^{\#}$	55 ±1$^{\#}$	632 ±10$^{\#}$
[Gln^3]Aβ3-11a	155 ±4	41.4 ±0.4	267 ±4
[Gln^3]Aβ3-21a	162 ±12	62 ±3	383 ±10
[Gln^3]Aβ3-40	89 ±10	40 ±2	449 ±28
Glucagon(3-29)	19 ±1	10.0 ±0.2	526 ±17

Determined in absence of DMSO

Example 10: Turnover of Aβ3-11a and Aβ3-21a by recombinant human QC

The incubation of Aβ3-11a and Aβ3-21a in presence of QC revealed that in contrast to previous work, glutamate-containing peptides can also serve as QC-substrates (Figures 15C and D). The QC-catalyzed formation of [pGlu^3]Aβ3-11a and [pGlu^3]Aβ3-21a was investigated at pH 5.2 and 6.5, respectively. If the QC-inhibitor benzimidazole was added to the solution before starting the assay by addition of QC, substrate conversion resulting in [pGlu^3]Aβ3-11a or [pGlu^3]Aβ3-21a was suppressed (Figures 15E and F). If QC was boiled before addition, formation of the pGlu-peptides was negligible (Figures 15A and B).

Example 11: pH-dependency of the papaya QC-catalyzed cyclization of Gln-βNA and Glu-βNA

Papaya QC converted Glu-*β*NA in a concentration range up to 2 mM (which was limited by substrate solubility) in accordance with Michaelis-Menten kinetics (Figure 16). Inspection of turnover versus substrate concentration diagrams for the QC-catalyzed conversion of Glu-*β*NA, studied between pH 6.1 and 8.5, revealed that for this Glu-substrate both parameters, K_M and k_{cat}, changed in a pH-dependent manner (Figure 16). This is in contrast to the previously described QC-catalyzed glutamine cyclization, for which only changes in K_M were observed over the given pH range (Gololobov, M. Y., Song, I., Wang, W., and Bateman, R. C. (1994) *Arch Biochem Biophys* **309,** 300-307).

Subsequently, to study the impact of the proton concentration during Glu- and Gln-cyclization, the pH-dependence of cyclization of Glu-*β*NA and Gln-*β*NA under first-order rate-law conditions (i.e. substrate concentrations far below K_M-values) was investigated (Figure 17). The cyclization of glutamine has a pH-optimum at pH 8.0, in contrast to the cyclization of glutamic acid which showed a pH-optimum of pH 6.0.

While the specificity constants at the respective pH-optima differ approximately 80,000-fold, the ratio of QC versus EC activity around pH 6.0, is only about 8,000.
The nonenzymatic pGlu-formation from Gln-βNA investigated at pH 6.0, was followed for 4 weeks and revealed a first-order rate constant of $1.2*10^{-7}$ s^{-1}. However, during the same time period, no pGlu-βNA was formed from Glu-βNA, allowing to estimate a limiting rate constant for turnover of $1.0*10^{-9}$ s^{-1}.

Example 12: Enzyme Inactivation/Reactivation Procedures

An aliquot of human QC (0.1-0.5 mg, 1 mg/ml) was inactivated overnight by dialysis against a 3000-fold excess of 5 mM 1,10-phenanthroline or 5 mM dipicolinic acid in 0.05 M Bis-Tris/HCl, pH 6.8. Subsequently, the inactivating agent was carefully removed by dialysis (3 cycles, 2000-fold excess) of the samples against 0.05 M Bis-Tris/HCl, pH 6.8, containing 1 mM EDTA. Reactivation experiments were performed at room temperature for 15 minutes using Zn^{++}, Mn^{++}, Ni^{++}, Ca^{++}, K^{+} and Co^{++} ions at concentrations of 1.0, 0.5, 0.25 mM in 0.025 M Bis-Tris, pH 6.8 containing 0.5 mM EDTA. QC activity assays were performed in 0.05 M Tris/HCl, pH 8.0, containing 2 mM EDTA, in order to avoid a rapid reactivation by traces of metal ions present in buffer solutions.
The inhibition of porcine QC by 1,10-phenanthroline has already been described (Busby, W. H. J. et al. 1987 *J Biol Chem 262*, 8532-8536, Bateman, R.C.J. et al. 2001 *Biochemistry 40*). However, the fact that EDTA has been shown to have an activating effect on QC catalysis suggested that inhibition by phenanthroline is not due to metal chelation (Busby, W. H. J. et al. 1987 *J Biol Chem 262*, 8532-8536, Bateman, R.C.J. et al. 2001 *Biochemistry 40*). Also, in addition to being inhibited by 1,10-phenanthroline, human QC catalyzed substrate cyclization was abolished in presence of dipicolinic acid and 8-hydroxyquinoline, other inhibitors of metalloenzymes. These chelators inhibited QC in a competitive and time-dependent manner, i.e., already competitively inhibited initial activity was found to be further reduced after prolonged incubation with the compounds (Figure 18,19). Interestingly, EDTA did not show remarkable inhibition regardless of incubation time or under any conditions.

2004237408 27 Aug 2009

Human QC was almost completely inactivated after extensive dialysis against 5 mM 1,10-phenanthroline or 5 mM dipicolinic acid. After repeated dialysis overnight against chelator-free buffer solutions, QC activity was partially reactivated up to 50- 60%. However, when dialyzed against buffers containing 1 mM EDTA, no reactivation was observed.

Near-total restoration of QC activity after inactivation by either dipicolinic acid or 1,10-phenanthroline was achieved by incubating the protein for 10 minutes with 0.5 mM $ZnSO_4$ in presence of 0.5 mM EDTA (Figure 20). Partial restoration of QC activity was similarly obtained using Co^{++} and Mn^{++} ions for reactivation. Even in the presence of 0.25 mM Zn^{++} a reactivation up to 25% of the original activity was possible. No reactivation was observed applying Ni^{++}, Ca^{++} or K^{+} ions. Similarly, incubation of fully active QC with these ions had no effect on the enzyme activity.

Example 13: effect of a combination of a QC inhibitor and a DPIV inhibitor

1. Assay

In accordance with example 8 of the present invention (p. 72), the measurements were carried out with two short N-terminal peptide sequences of Aβ3-40/42, [Gln^3]-Aβ1-11 (sequence: DAQFRHDSGYE) and [Gln^3]Aβ3-11, which contain a glutamine instead of an glutamic acid residue in the third position. Cleavage by DPIV and cyclization of the N-terminal glutamine residue by QC of the two peptides was tested using MALDI-TOF mass spectrometry as described in example 3 (pp. 57-58). Measurements were carried out using recombinant human DPIV and QC.

2. Results

As shown in Figures 5 to 15 of the present invention, the inhibition of DPIV and QC is a strategy to reduce either the generation of the QC-substrate (Aβ3-11a) or the cyclization of the N-terminus to produce [pGlu]Aβ(3-11), respectively.

In Figure 22, the combined approach is exemplified shown with two highly potent inhibitors of QC (1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea) and DPIV (Valyl-Pyrrolidine, Val-Pyrr). Again, incubation of [Gln^3]Aβ(1-11)a (m/z 1323) with DPIV and QC results in intermediate production of [Gln^3]Aβ(3-11)a (m/z 1137) and

2004237408 27 Aug 2009

in formation of [pGlu]Aβ(3-11)a (m/z 1120) by consecutive catalysis of both enzymes (A). Inhibition of DPIV alone reduces the velocity of formation of [Gln^3]Aβ(3-11)a (the substrate of QC), and therefore, also [pGlu]Aβ(3-11)a is slightly reduced, comparing Figure 1 (A) and (B). The same was observed, if the QC-mediated cyclization of [Gln^3]Aβ(3-11)a was inhibited by applying the QC inhibitor 1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea (C): the formation of [pGlu]Aβ(3-11)a was diminished. The appearance of [pGlu]Aβ(3-11)a was, however, almost abolished, if both inhibitors were present. Under these conditions both the generation of the substrate of QC ([Gln^3]Aβ(3-11)a, m/z 1137) and the QC catalysis were inhibited, resulting in a highest efficacy to suppress the generation of [pGlu]Aβ(3-11)a (marked by an asterisk). Thus, combination of both DPIV and QC inhibiting strategies clearly displays highest efficacy to diminish the pGlu-formation.

2004237408 18 Nov 2009

CLAIMS:

1. A method of treatment, including preventive treatment, of Alzheimer's disease, comprising administering to a mammalian subject in need thereof a composition comprising an inhibitor of glutaminyl cyclase, or a pharmaceutically acceptable salt thereof.

2. The method according to claim 1, wherein said inhibitor of glutaminyl cyclase is administered in a pharmaceutical composition for parenteral, enteral or oral administration, which further comprises at least one pharmaceutically acceptable carrier or excipient.

3. The method according to claim 2, wherein said pharmaceutical composition additionally comprises at least one agent selected from the group consisting of:

i) inhibitors of DP IV or DP IV-like enzymes, and

ii) substrates, pseudosubstrates, inhibitors of glutaminyl cyclase expression, binding proteins or antibodies of enzyme proteins that reduce the glutaminyl cyclase protein concentration.

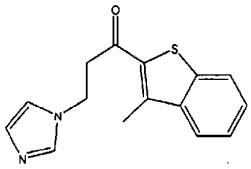
4. The method according to any one of claims 1 to 3, wherein the glutaminyl cyclase inhibitor is a competitive inhibitor.

5. The method according to claim 1, wherein the glutaminyl cyclase inhibitor has a Ki-value of about 0.0071 mM or below.

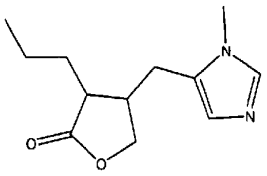
6. The method according to claim 1, wherein the glutaminyl cyclase inhibitor has a Ki-value in the range of from about 0.00041 mM to about 0.0071 mM.

7. The method according to claim 6, wherein the glutaminyl cyclase inhibitor is selected from the group consisting of:

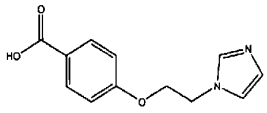
3-(1H-imidazol-1-yl)-1-(3-methylbenzo[b]thiophene-2-yl)propan-1-one



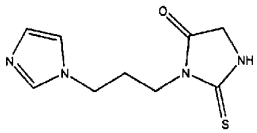
4-[(1-methyl-1H-imidazol-5-yl)methyl]-3-propyldihydrofuran-2-(3H)-one



4-[2-(1*H*-imidazol-1-yl)-ethoxy]benzoic acid

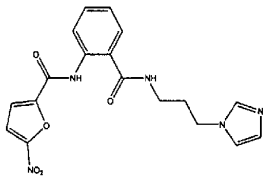


3-[3-(1H-imidazol-1-yl)propyl]-2-thioxoimidazolidin-4-one



2004237408 30 May 2008

5-nitro-2-[2-({[3-(1H-imidazol-1-yl-)propyl}amino] carbonyl)phenyl]furamide



1-benzylimidazole

8. The method according to claim 1, wherein said inhibitor of glutaminyl cyclase inhibits the conversion of N-terminal glutamic acid or glutamine residues to pyroglutamic acid residues of at least one substrate of glutaminyl cyclase selected from the group consisting of Aβ3-40/42, [Gln^{3}]Aβ3-40/42, [Glu^{11}]Aβ11-40/42, [Gln^{11}]Aβ11-40/42, [Gln^{1}]Gastrins (17 and 34), [Gln^{1}]Neurotensin, [Gln^{1}]FPP, [Gln^{1}]TRH, [Gln^{1}]GnRH, [Gln^{1}]CCL 2, [Gln^{1}]CCL 7, [Gln^{1}]CCL 8, [Gln^{1}]CCL 16, [Gln^{1}]CCL 18, [Gln^{1}]ELA, [Gln^{1}]Fractalkine, and [Gln^{1}]Orexin A.

9. The method according to claim 1, wherein the administration of said inhibitor of glutaminyl cyclase inhibits the conversion of N-terminal glutamic acid or glutamine residues to pyroglutamic acid residues of at least one substrate of glutaminyl cyclase selected from the group consisting of Aβ3-40/42, [Gln^{3}]Aβ3-40/42, [Glu^{11}]Aβ11-40/42 and [Gln^{11}]Aβ11-40/42.

10. The method according to claim 3, wherein the H-isoAsp-Ala-OH generating activity of the DP IV-like enzyme is blocked.

11. The method according to claim 10, wherein the DP IV-like enzyme is DP II.

12. The method according to claim 3, wherein the DP IV-inhibitor is selected from the group consisting of L-*threo*-isoleucyl thiazolidine, L-*allo*-isoleucyl thiazolidine, L-*threo*-isoleucyl pyrrolidine, L-*allo*-isoleucyl pyrrolidine, NVP-DPP728A (1-[[[2-[{5-cyanopyridin-2-yl}amino]ethyl]amino]acetyl]-2-cyano-(S)-pyrrolidine), LAF-237

2004237408 30 May 2008

(1-[(3-hydroxy-adamant-1-ylamino)-acetyl]-pyrrolidine-2(S)-carbonitrile); TSL-225 (tryptophyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), FE-999011, N-valyl prolyl, O-benzoyl hydroxylamine, alanyl pyrrolidine, H-Asn-pyrrolidine, H-Asn-thiazolidine, H-Asp-pyrrolidine, H-Asp-thiazolidine, H-Asp(NHOH)-pyrrolidine, H-Asp(NHOH)-thiazolidine, H-Glu-pyrrolidine, H-Glu-thiazolidine, H-Glu(NHOH)-pyrrolidine, H-Glu(NHOH)-thiazolidine, H-His-pyrrolidine, H-His-thiazolidine, H-Pro-pyrrolidine, H-Pro-thiazolidine, H-Ile-azididine, H-Ile-pyrrolidine, H-L-*allo*-Ile-thiazolidine, H-Val-pyrrolidine and H-Val-thiazolidine, 2-Amino octanoic acid-Pro-Ile, Abu-Pro-Ile, Aib-Pro-Ile, Aze-Pro-Ile, Cha-Pro-Ile, Ile-Hyp-Ile, Ile-Pro-*allo*-Ile, Ile-Pro-*t*-butyl-Gly, Ile-Pro-Val, Nle-Pro-Ile, Nva-Pro-Ile, Orn-Pro-Ile, Phe-Pro-Ile, Phg-Pro-Ile, Pip-Pro-Ile, Ser(Bzl)-Pro-Ile, Ser(P)-Pro-Ile, Ser-Pro-Ile, *t*-butyl-Gly-Pro-D-Val, *t*-butyl-Gly-Pro-Gly, *t*-butyl-Gly-Pro-Ile, *t*-butyl-Gly-Pro-Ile-amide, *t*-butyl-Gly-Pro-*t*-butyl-Gly, *t*-butyl-Gly-Pro-Val, Thr-Pro-Ile, Tic-Pro-Ile, Trp-Pro-Ile, Tyr(P)-Pro-Ile, Tyr-Pro-*allo*-Ile, Val-Pro-*allo*-Ile, Val-Pro-*t*-butyl-Gly, Val-Pro-Val, and pharmaceutically acceptable salts thereof.

13. The method according to claim 3, wherein the DP IV-inhibitor is selected from the group consisting of:

2-Methylcarbonyl-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-Methyl)carbonyl-1-N-[(L)-Valinyl-(L)-Prolyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-[(Acetyl-oxy-methyl)carbonyl]-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-[Benzoyl-oxy-methyl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-{[(2,6-Dichlorbenzyl)thiomethyl]carbonyl}-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine,

2-[Benzoy-loxy-methyl)carbonyl]-1-N-[Glycyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-[([1,3]-Thiazolethiazol-2-yl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate,

2004237408 30 May 2008

2-[(Benzothiazolethiazol-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate,

2-[(-Benzothiazolethiazol-2-yl)carbonyl]-1-N-[{(L)-Alanyl}-Glycyl]-(2S)-pyrrolidine trifluoracetate,

2-[(Pyridin-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate,

1-cyclopentyl-3-methyl-1-oxo-2-pentanaminium chloride,

1-cyclopentyl-3-methyl-1-oxo-2-butanaminium chloride,

1-cyclopentyl-3,3-dimethyl-1-oxo-2-butanaminium chloride,

1-cyclohexyl-3,3-dimethyl-1-oxo-2-butanaminium chloride,

3-(cyclopentylcarbonyl)-1,2,3,4-tetrahydroisoquinolinium chloride, and

N-(2-cyclopentyl-2-oxoethyl)cyclohexanaminium chloride, and

other pharmaceutically acceptable salts thereof.

14. The method according to claim 1, wherein said inhibitor of glutaminyl cyclase binds to the active-site bound metal ion of glutaminyl cyclase.

15. A method of inhibiting conversion of N-terminal glutamic acid or glutamine residues to pyroglutamic acid residues of at least one substrate of glutaminyl cyclase in a mammalian subject comprising:

administering to a mammalian subject in need thereof a composition comprising an inhibitor of glutaminyl cyclase, or a pharmaceutically acceptable salt thereof;

wherein the inhibitor of glutaminyl cyclase inhibits the conversion of N-terminal glutamic acid or glutamine residues to pyroglutamic acid residues of at least one substrate of glutaminyl cyclase selected from the group consisting of Aβ3-40/42, [Gln^3]Aβ3-40/42, [Glu^{11}]Aβ11-40/42, [Gln^{11}]Aβ11-40/42, [Gln^1]Gastrins (17 and 34), [Gln^1]Neurotensin, [Gln^1]FPP, [Gln^1]TRH, [Gln^1]GnRH, [Gln^1]CCL 2, [Gln^1]CCL 7, [Gln^1]CCL 8, [Gln^1]CCL 16, [Gln^1]CCL 18, [Gln^1]ELA, [Gln^1]Fractalkine, and [Gln^1]Orexin A in the mammalian subject; and

the mammalian subject is diagnosed with Alzheimer's disease.

2004237408 27 Aug 2009

16. The method according to claim 15, wherein the administration of said inhibitor of glutaminyl cyclase inhibits the conversion of N-terminal glutamic acid or glutamine residues to pyroglutamic acid residues of at least one substrate of glutaminyl cyclase selected from the group consisting of Aβ3-40/42, [Gln^3]Aβ3-40/42, [Glu^{11}]Aβ11-40/42 and [Gln^{11}]Aβ11-40/42.

17. The method according to claim 15, wherein said inhibitor of glutaminyl cyclase is administered in a pharmaceutical composition for parenteral, enteral or oral administration, which further comprises at least one pharmaceutically acceptable carrier or excipient.

18. The method according to claim 17, wherein said pharmaceutical composition additionally comprises at least one agent selected from the group consisting of:

i) inhibitors of DP IV or DP IV-like enzymes, and

ii) substrates, pseudosubstrates, inhibitors of glutaminyl cyclase expression, binding proteins or antibodies of enzyme proteins that reduce glutaminyl cyclase protein concentration.

19. The method according to claim 15, wherein the glutaminyl cyclase inhibitor is a competitive inhibitor.

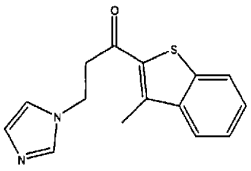
20. The method according to claim 15, wherein the glutaminyl cyclase inhibitor has a Ki-value of about 0.0071 mM or below.

21. The method according to claim 20, wherein the glutaminyl cyclase inhibitor has a Ki-value in the range of from about 0.00041 mM to about 0.0071 mM.

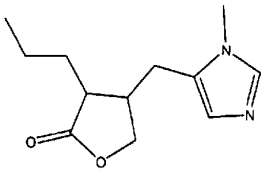
22. The method according to claim 15, wherein the glutaminyl cyclase inhibitor is selected from the group consisting of:

2004237408 30 May 2008

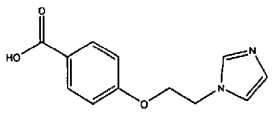
3-(1H-imidazol-1-yl)-1-(3-methylbenzo[b]thiophene-2-yl)propan-1-one



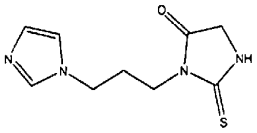
4-[(1-methyl-1H-imidazol-5-yl)methyl]-3-propyldihydrofuran-2-(3H)-one



4-[2-(1*H*-imidazol-1-yl)-ethoxy]benzoic acid

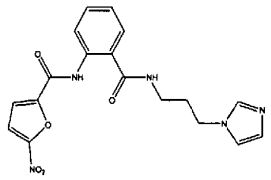


3-[3-(1H-imidazol-1-yl)propyl]-2-thioxoimidazolidin-4-one



2004237408 30 May 2008

5-nitro-2-[2-([[3-(1H-imidazol-1-yl-)propyl}amino] carbonyl)phenyl]furamide



1-benzylimidazole

23. The method according to claim 18, wherein the H-isoAsp-Ala-OH generating activity of the DP IV-like enzyme is blocked.

24. The method according to claim 23, wherein the DP IV-like enzyme is DP II.

25. The method according to claim 18, wherein the DP IV-inhibitor is selected from the group consisting of L-*threo*-isoleucyl thiazolidine, L-*allo*-isoleucyl thiazolidine, L-*threo*-isoleucyl pyrrolidine, L-*allo*-isoleucyl pyrrolidine, NVP-DPP728A (1-[[[2-[{5-cyanopyridin-2-yl}amino]ethyl]amino]acetyl]-2-cyano-(S)-pyrrolidine), LAF-237 (1-[(3-hydroxy-adamant-1-ylamino)-acetyl]-pyrrolidine-2(S)-carbonitrile); TSL-225 (tryptophyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), FE-999011, N-valyl prolyl, O-benzoylhydroxylamine, alanyl pyrrolidine, H-Asn-pyrrolidine, H-Asn-thiazolidine, H-Asppyrrolidine, H-Asp-thiazolidine, H-Asp(NHOH)-pyrrolidine, H-Asp(NHOH)-thiazolidine, H-Glu-pyrrolidine, H-Glu-thiazolidine, H-Glu(NHOH)-pyrrolidine, H-Glu(NHOH)-thiazolidine, H-His-pyrrolidine, H-His-thiazolidine, H-Pro-pyrrolidine, H-Pro-thiazolidine, H-Ile-azididine, H-Ile-pyrrolidine, H-L-*allo*-Ile-thiazolidine, H-Val-pyrrolidine and H-Val-thiazolidine, 2-Amino octanoic acid-Pro-Ile, Abu-Pro-Ile, Aib-Pro-Ile, Aze-Pro-Ile, Cha-Pro-Ile, Ile-Hyp-Ile, Ile-Pro-*allo*-Ile, Ile-Pro-*t*-butyl-Gly, Ile-Pro-Val, Nle-Pro-Ile, Nva-Pro-Ile, Orn-Pro-Ile, Phe-Pro-Ile, Phg-Pro-Ile, Pip-Pro-Ile, Ser(Bzl)-Pro-Ile, Ser(P)-Pro-Ile, Ser-Pro-Ile, *t*-butyl-Gly-Pro-D-Val, *t*-butyl-Gly-Pro-Gly, *t*-butyl-Gly-Pro-Ile, *t*-butyl-Gly-Pro-Ile-amide, *t*-butyl-Gly-Pro-*t*-butyl-Gly, *t*-butyl-Gly-Pro-Val, Thr-Pro-Ile, Tic-Pro-Ile, Trp-Pro-Ile, Tyr(P)-Pro-Ile, Tyr-Pro-*allo*-Ile, Val-Pro-

2004237408 30 May 2008

allo-Ile, Val-Pro-*t*-butyl-Gly, Val-Pro-Val, and pharmaceutically acceptable salts thereof.

26. The method according to claim 18, wherein the DP IV-inhibitor is selected from the group consisting of:

2-Methylcarbonyl-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-Methyl)carbonyl-1-N-[(L)-Valinyl-(L)-Prolyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-[(Acetyl-oxy-methyl)carbonyl]-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-[Benzoyl-oxy-methyl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-{[(2,6-Dichlorbenzyl)thiomethyl]carbonyl}-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine,

2-[Benzoyl-oxy-methyl)carbonyl]-1-N-[Glycyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide,

2-[([1,3]-Thiazolethiazol-2-yl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate,

2-[(Benzothiazolethiazol-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate,

2-[(-Benzothiazolethiazol-2-yl)carbonyl]-1-N-[{(L)-Alanyl}-Glycyl]-(2S)-pyrrolidine trifluoracetate,

2-[(Pyridin-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetate,

1-cyclopentyl-3-methyl-1-oxo-2-pentanaminium chloride,

1-cyclopentyl-3-methyl-1-oxo-2-butanaminium chloride,

1-cyclopentyl-3,3-dimethyl-1-oxo-2-butanaminium chloride,

1-cyclohexyl-3,3-dimethyl-1-oxo-2-butanaminium chloride,

3-(cyclopentylcarbonyl)-1,2,3,4-tetrahydroisoquinolinium chloride, and

N-(2-cyclopentyl-2-oxoethyl)cyclohexanaminium chloride, and

other pharmaceutically acceptable salts thereof.

2004237408 18 Nov 2009

27. The method according to claim 15, wherein said inhibitor of glutaminyl cyclase binds to the active-site bound metal ion of glutaminyl cyclase.

28. Use of an inhibitor of glutaminyl cyclase (QC), or a pharmaceutically acceptable salt thereof, for the preparation of a medicament for the treatment, including preventive treatment, of Alzheimer's disease.

29. The use according to claim 28, wherein said inhibitor of glutaminyl cyclase inhibits the conversion of the glutamic acid to a pyroglutamic acid residue at the N-terminus of at least one QC-substrate.

30. The use according to claim 29, wherein said at least one QC substrate is selected from the group consisting of Aβ3-40/42, [Gln^3]Aβ3-40/42, [Glu^{11}]Aβ11-40/42, [Gln^{11}]Aβ11-40/42, [Gln^1]Gastrins (17 and 34), [Gln^1]Neurotensin, [Gln^1]FPP, [Gln^1]TRH, [Gln^1]GnRH, [Gln^1]CCL 2, [Gln^1]CCL 7, [Gln^1]CCL 8, [Gln^1]CCL 16, [Gln^1]CCL 18, [Gln^1]ELA, [Gln^1]Fractalkine, and [Gln^1]Orexin A.

31. The use according to any one of claims 28 to 30, wherein said medicament additionally comprises at least one agent selected from the group
consisting of:

i) inhibitors of DP IV or DP IV-like enzymes, and

ii) substrates, pseudosubstrates, inhibitors of glutaminyl cyclase expression, binding proteins or antibodies of enzyme proteins that reduce the glutaminyl cyclase protein concentration.

32. The use according to any one of claims 28 to 31, wherein the glutaminyl cyclase inhibitor is selected from the group as claimed in claim 7.

2004237408 30 May 2008

33. The use according to claim 31 or 32, wherein said inhibitor of DP IV is selected from the group as claimed in claim 12 or 13.

5

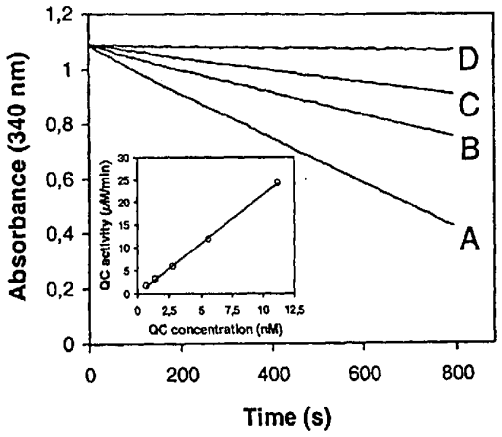
PROBIODRUG AG

WATERMARK PATENT & TRADE MARK ATTORNEYS

P26193AU00

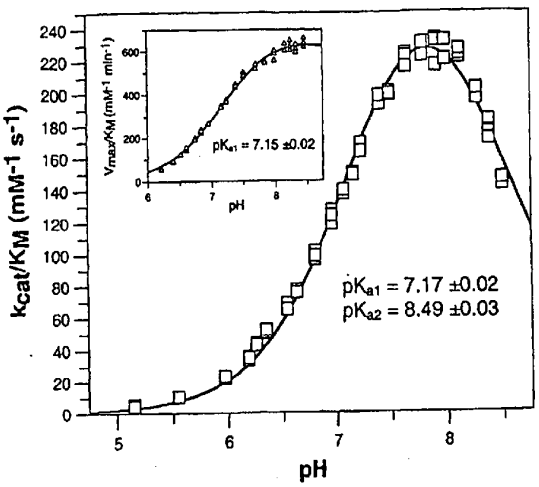
2004237408 27 Aug 2009

Figure 1



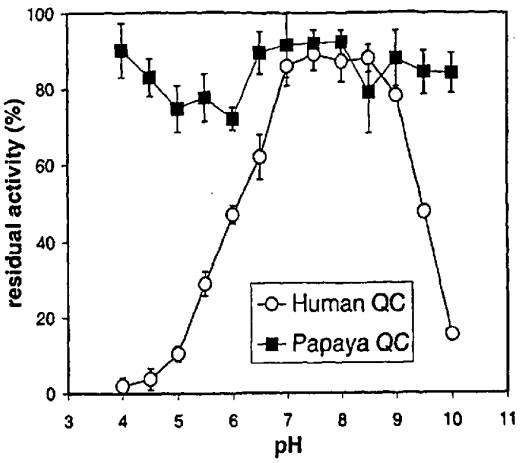
2004237408 27 Aug 2009

Figure 2



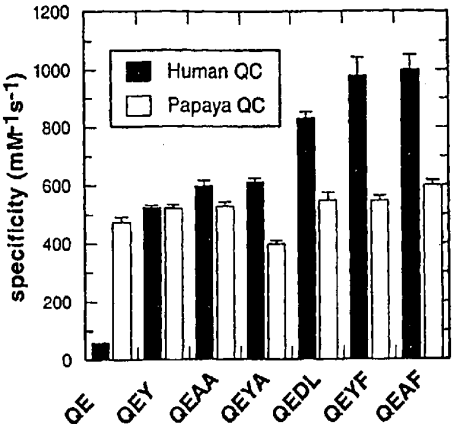
2004237408 27 Aug 2009

Figure 3



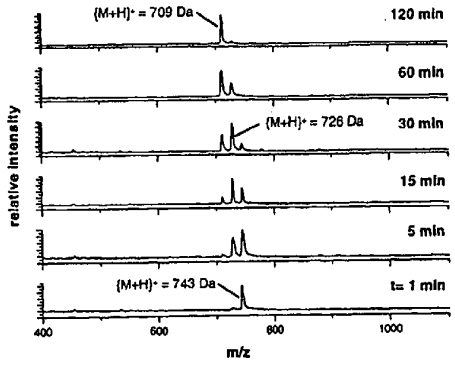
2004237408 27 Aug 2009

Figure 4



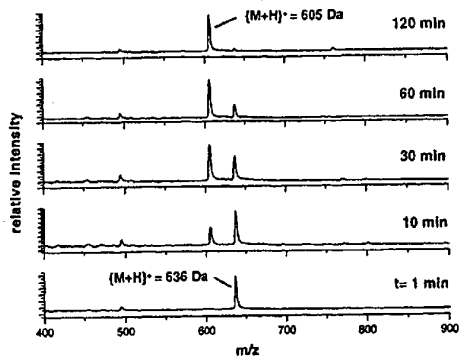
2004237408 27 Aug 2009

Figure 5



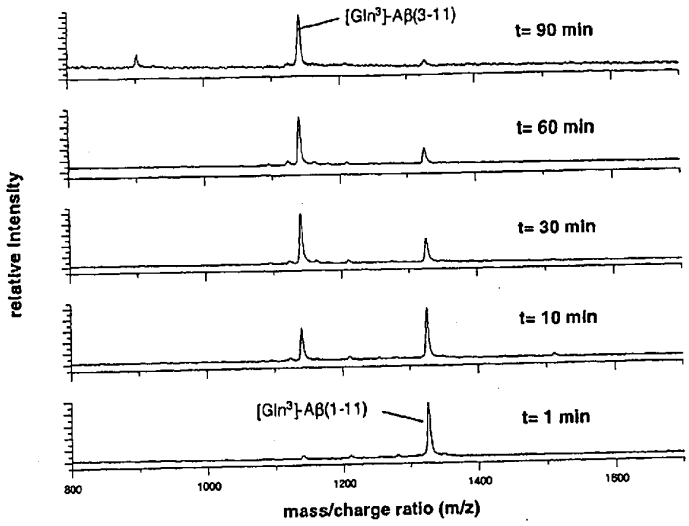
2004237408 27 Aug 2009

Figure 6



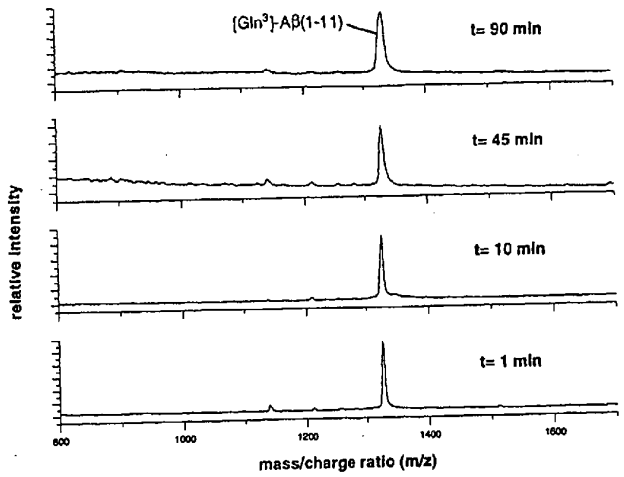
2004237408 27 Aug 2009

Figure 7



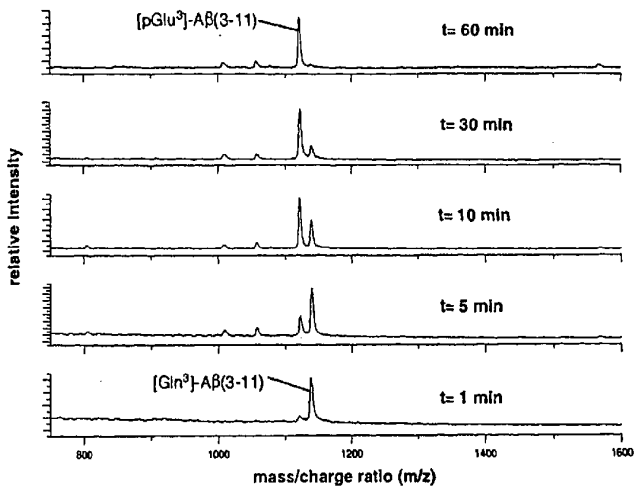
2004237408 27 Aug 2009

Figure 8



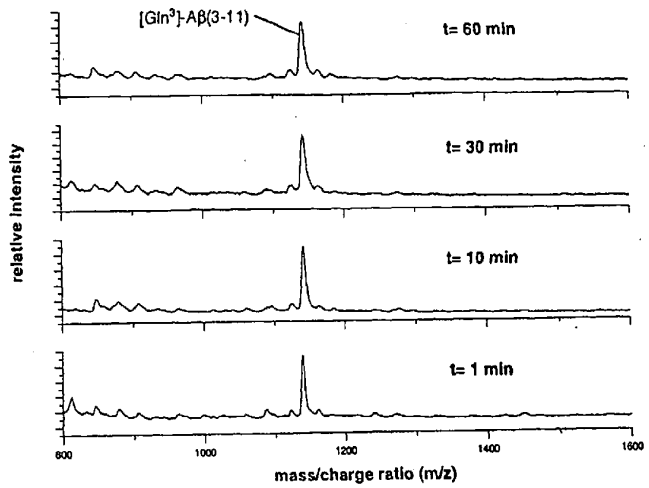
2004237408 27 Aug 2009

Figure 9



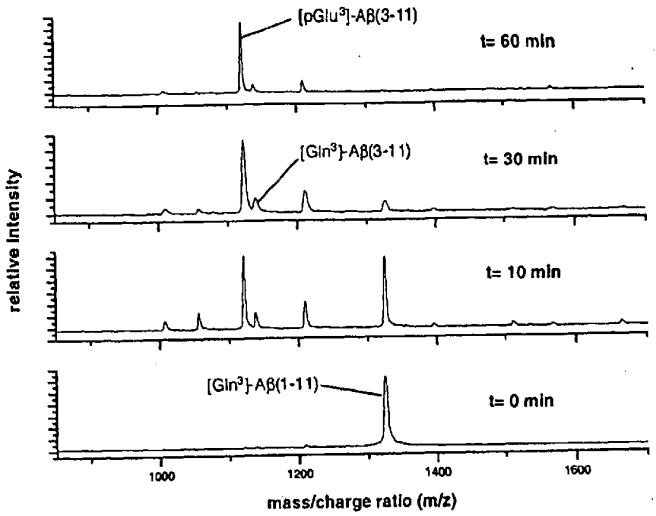
2004237408 27 Aug 2009

Figure 10



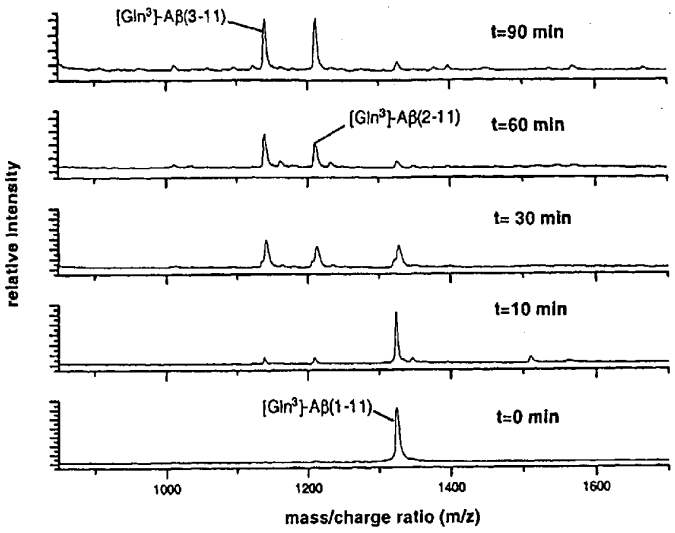
2004237408 27 Aug 2009

Figure 11



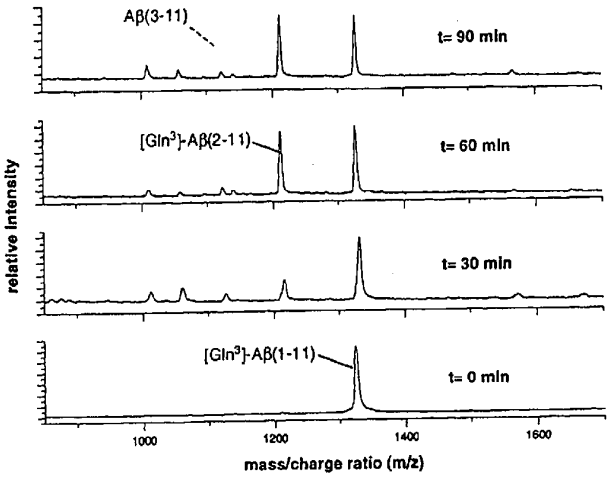
2004237408 27 Aug 2009

Figure 12



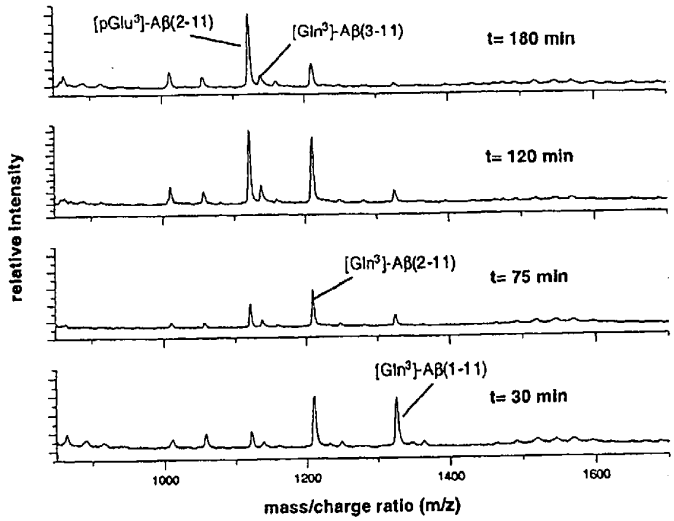
2004237408 27 Aug 2009

Figure 13



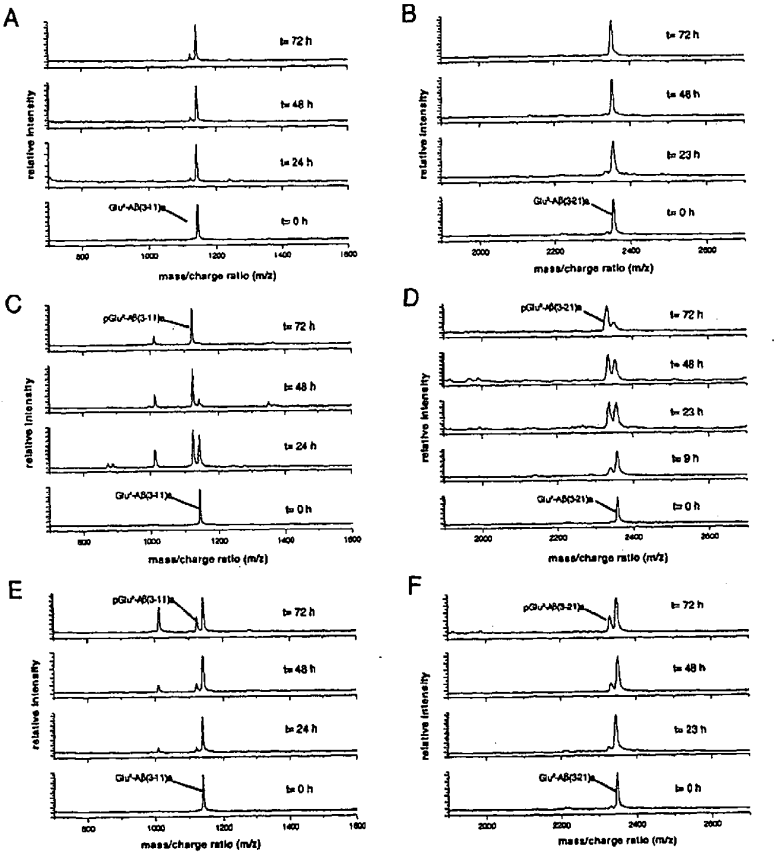
2004237408 27 Aug 2009

Figure 14



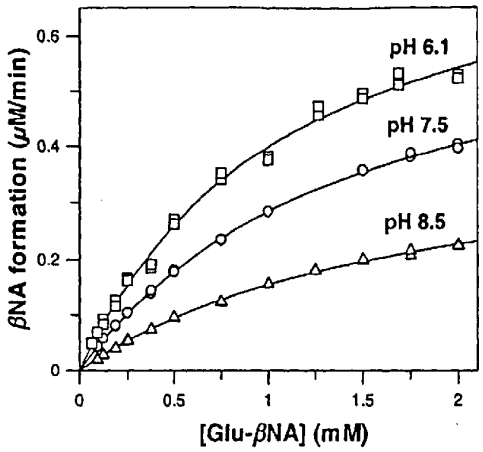
2004237408 27 Aug 2009

Figure 15



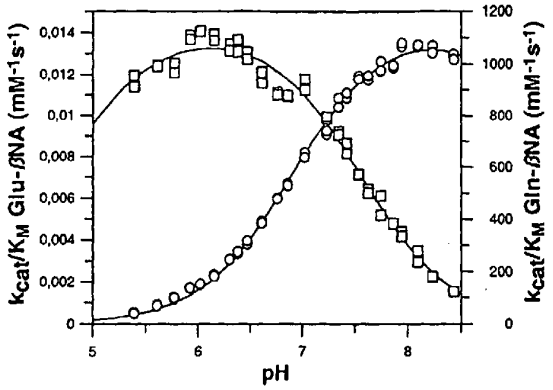
2004237408 27 Aug 2009

Figure 16



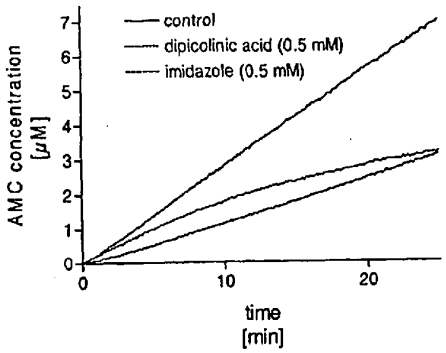
2004237408 27 Aug 2009

Figure 17



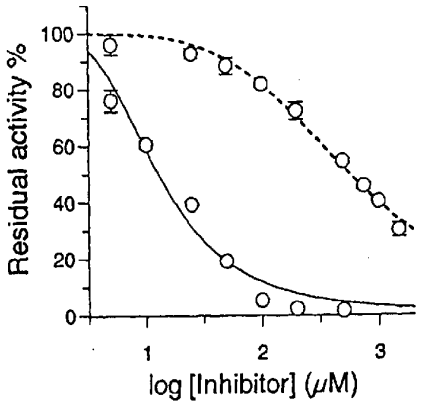
2004237408 27 Aug 2009

Figure 18



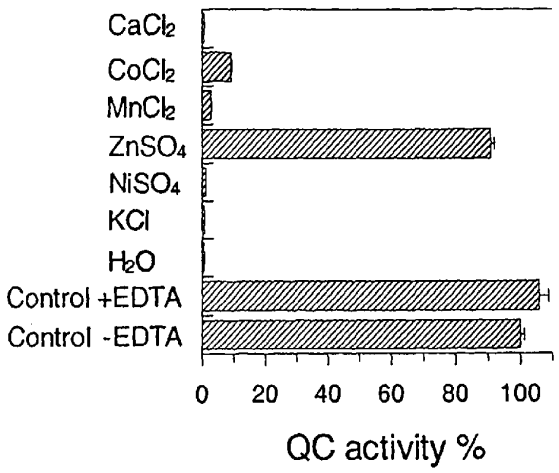
2004237408 27 Aug 2009

Figure 19



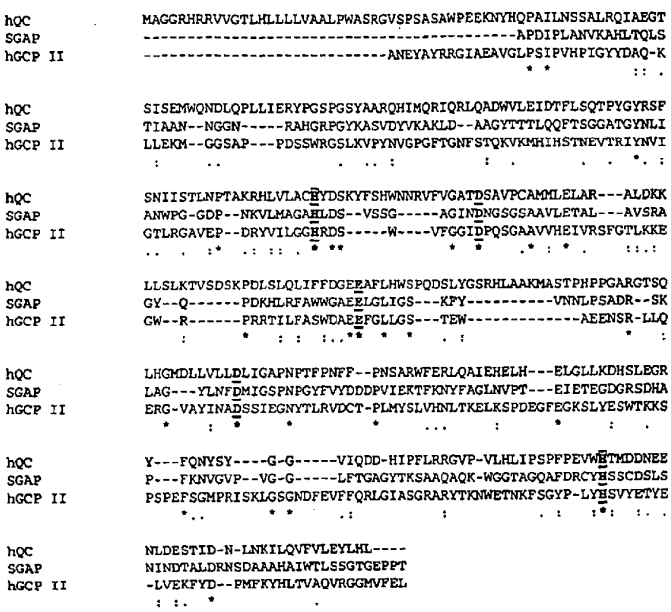
2004237408 27 Aug 2009

Figure 20



2004237408 27 Aug 2009

Figure 21



2004237408 27 Aug 2009

Figure 22

