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(54) Title: NOVEL GENES RELATED TO GLUTAMINYL CYCLASE

(57) Abstract: The present invention relates to novel glutaminyl-peptide cyclotransferase-like proteins (QPCTLs), which are isoenzymes of glutaminyl cyclase (QC, EC 2.3.2.5), and to isolated nucleic acids coding for these isoenzymes, all of which are useful for the discovery of new therapeutic agents, for measuring cyclase activity, and for determining the inhibitory activity of compounds against these glutaminyl cyclase isoenzymes.

Novel Genes Related to Glutaminyl Cyclase

Field of the Invention

The present invention relates to novel glutaminyl-peptide cyclotransferase-like proteins (QPCTLs), which are isoenzymes of glutaminyl cyclase (QC, EC 2.3.2.5), and to isolated nucleic acids coding for these isoenzymes, all of which are useful for the discovery of new therapeutic agents, for measuring cyclase activity, and for determining the inhibitory activity of compounds against these glutaminyl cyclase isoenzymes.

Background of the Invention

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 USA 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 the 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.

Recently, 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.

EP 02 011 349.4 discloses polynucleotides 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 useful as pesticides.

Inhibitors of QC, which also could be useful as inhibitors of QC isoenzymes, are described in WO 2004/098625, WO 2004/098591, WO 2005/039548 and WO 2005/075436, which are incorporated herein in their entirety, especially with regard to the structure of the inhibitors, their use and their production.

Definitions

Enzyme inhibitors

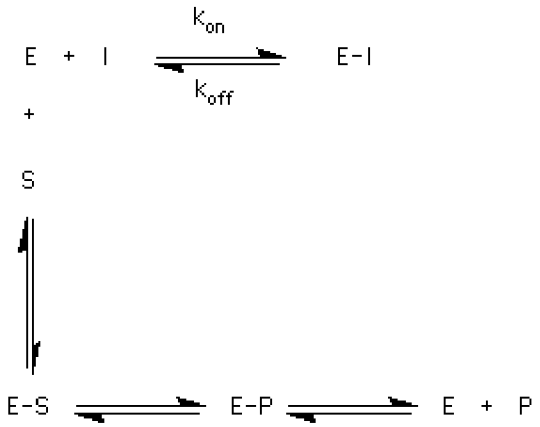
Reversible enzyme inhibitors: comprise competitive inhibitors, non-competitive reversible inhibitors, slow-binding or tight-binding inhibitors, transition state analogs and multisubstrate analogs.

Competitive inhibitors show

i) non-covalent interactions with the enzyme,

ii) compete with substrate for the enzyme active site,

The principal mechanism of action of a reversible enzyme inhibitor and the definition of the dissociation constant can be visualized as follows:



$$K_D = K_i = \frac{k_{off}}{k_{on}}$$

The formation of the enzyme-inhibitor [E-I] complex prevents binding of substrates, therefore the reaction cannot proceed to the normal physiological product, P. A larger inhibitor concentration [I] leads to larger [E-I], leaving less free enzyme to which the substrate can bind.

Non-competitive reversible inhibitors

i) bind at a site other than active site (*allosteric binding site)*

ii) cause a conformational change in the enzyme which decreases or stops catalytic activity.

Slow-binding or tight-binding inhibitors

i) are competitive inhibitors where the equilibrium between inhibitor and enzyme is reached slowly,

ii) (k_{on} is slow), possibly due to conformational changes that must occur in the enzyme or inhibitor

a) are often transition state analogs

b) are effective at concentrations similar to the enzyme conc. (subnanomolar K_D values)

c) due to k_{off} values being so low these types of inhibitors are "almost" irreversible

Transition state analogs

are competitive inhibitors which mimic the transition state of an enzyme catalyzed reaction. Enzyme catalysis occurs due to a lowering of the energy of the transition state, therefore, transition state binding is favored over substrate binding.

Multisubstrate Analogs

For a reaction involving two or more substrates, a competitive inhibitor or transition state analog can be designed which contains structural characteristics resembling two or more of the substrates.

Irreversible enzyme inhibitors: drive the equilibrium between the unbound enzyme and inhibitor and enzyme inhibitor complex (E + I <---> E-I) all the way to the right with a covalent bond (~100 kcal/mole), making the inhibition irreversible.

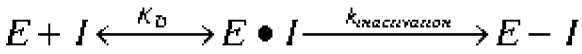
Affinity labeling agents

- Active-site directed irreversible inhibitors (competitive irreversible inhibitor) are recognized by the enzyme (reversible, specific binding) followed by covalent bond formation, and

i) are structurally similar to substrate, transition state or product allowing for specific interaction between drug and target enzyme,

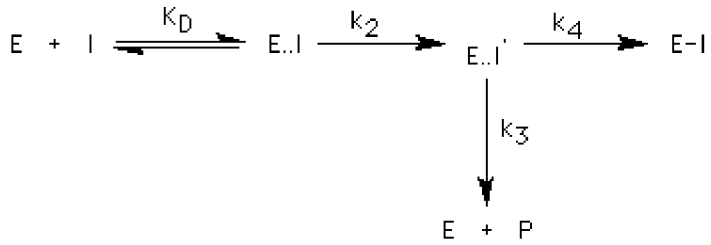
ii) contain reactive functional group (e.g. a nucleophile, $-COCH_2Br$) allowing for covalent bond formation

The reaction scheme below describes an active-site directed reagent with its target enzyme where K_D is the dissociation constant and $k_{inactivation}$ is the rate of covalent bond formation.



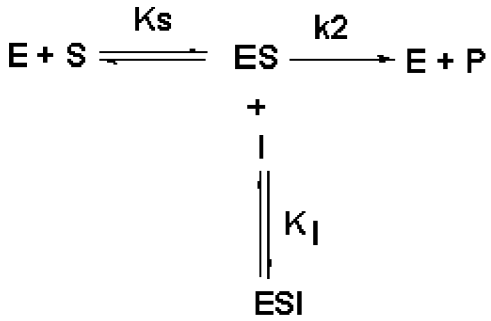
- Mechanism-based enzyme inactivators (also called suicide inhibitors) are active-site directed reagents (unreactive) which binds to the enzyme active site where it is transformed to a reactive form (activated) by the enzyme's catalytic capabilities. Once activated, a covalent bond between the inhibitor and the enzyme is formed.

The reaction scheme below shows the mechanism of action of a mechanism based enzyme inactivator, where K_D is the dissociation complex, k_2 is the rate of activation of the inhibitor once bound to the enzyme, k_3 is the rate of dissociation of the activated inhibitor, P, from the enzyme (product can still be reactive) from the enzyme and k_4 is the rate of covalent bond formation between the activated inhibitor and the enzyme.



Inactivation (covalent bond formation, k_4) must occur prior to dissociation (k_3) otherwise the now reactive inhibitor is released into the environment. Partition ratio, k_3/k_4: ratio of released product to inactivation should be minimized for efficient inactivation of the system and minimal undesirable side reactions.
A large partition ratio (favors dissocation) leads to nonspecific reactions.

Uncompetitive enzyme inhibitors: From the definition of uncompetitive inhibitor (an inhibitor which binds only to ES complexes) the following equilibria can be written:



The ES complex dissociates the substrate with a dissociation constant equal to Ks, whereas the ESI complex does not dissociate it (i.e has a Ks value equal to zero). The K_m's of Michaelis-Menten type enzymes are expected to be reduced. Increasing substrate concentration leads to increasing ESI concentration (a complex incapable of progressing to reaction products), therefore the inhibition can not be removed.

Preferred according to the present invention are competitive enzyme inhibitors.
Most preferred are competitive reversible enzyme inhibitors.

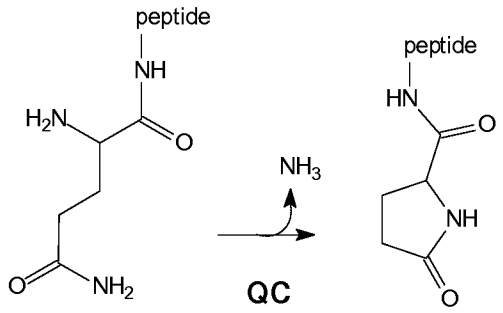
The terms "k_i" or "K_I" and "K_D" are binding constants, which describe the binding of an inhibitor to and the subsequent release from an enzyme. Another measure is the "IC_{50}" value, which reflects the inhibitor concentration, which at a given substrate concentration results in 50 % enzyme activity.

The term "QC" as used herein comprises glutaminyl cyclase (QC), which is synonymous to glutaminyl-peptide cyclotransferase (QPCT); and QC-like enzymes, which are synonymous to glutaminyl-peptide cyclotransferase-like proteins (QPCTLs). 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.

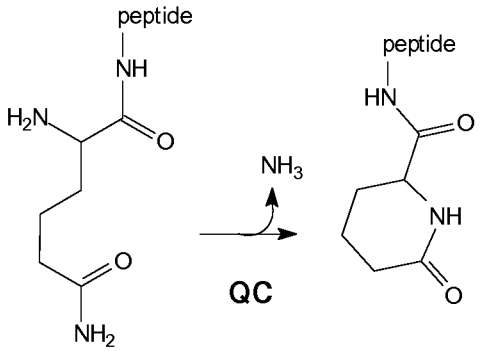
"QC-activity" is defined as the catalytic activity of glutaminyl cyclase (QC, QPCT) and QC-like enzymes (QPCTLs). These enzymes are found in various tissues of the body of a mammal including kidney, liver, intestine, brain and body fluids such as CSF, where they cyclize glutamine or glutamate at the N-terminus of biologically active peptides with a high specificity.

In particular, 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



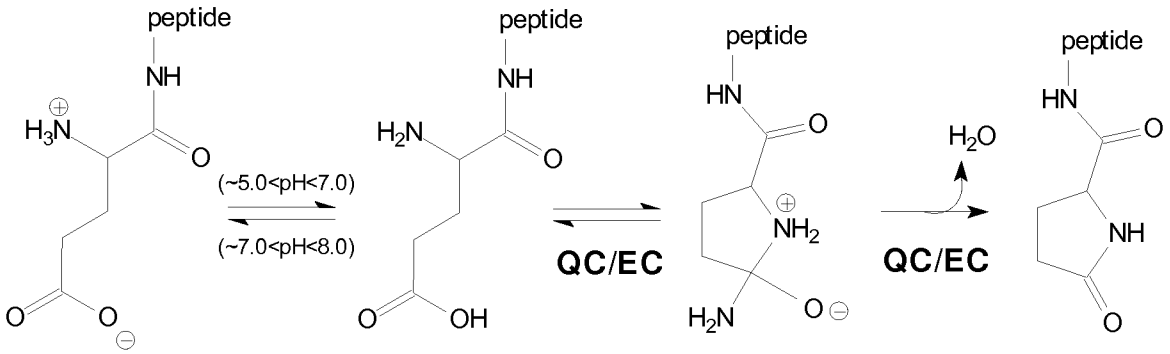
Scheme 2: Cyclization of L-homoglutamine by QC



The term "EC" as used herein comprises the side activity of glutaminyl cyclase (QC, QPCT) and QC-like enzymes (QPCTLs) 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 glutaminyl cyclase (QC, QPCT) and QC-like enzymes (QPCTLs). See therefore scheme 3.

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



The term “QC-inhibitor” or “glutaminyl cyclase inhibitor” is generally known to a person skilled in the art and means enzyme inhibitors, which inhibit the catalytic activity of glutaminyl cyclase (QC, QPCT) or QC-like enzymes (QPCTLs) or their glutamyl cyclase (EC) activity, preferably by direct interaction of the inhibitor with the enzyme.

Ther term “selective QC-inhibitor” as defined herein means enzyme inhibitors, which inhibit the catalytic activity of glutaminyl cyclase (QC, QPCT) but do not or with a lower potency inhibit at least one QC-like enzymes (QPCTLs). Preferred are selective QC-inhibitors, which inhibit glutaminyl cyclase (QC, QPCT) with an ki-value, which is one order of magnitude lower than its ki-value for the inhibition of at least one QC-like enzyme (QPCTL). More preferably, the ki-value of said selective QC-inhibitor for the inhibition of glutaminyl cyclase (QC, QPCT) is two orders of magnitude lower than its ki-value for the inhibition of at least one QC-like enzyme (QPCTL). Even more preferred are selective QC-inhibitors, wherein their ki-value for the inhibition of glutaminyl cyclase (QC, QPCT) is three orders of magnitude lower than their ki-value for the inhibition of at least one QC-like enzyme (QPCTL). Most preferred are selective QC-inhibitors, which do not inhibit QC-like enzymes (QPCTLs).

Ther term "selective QPCTL-inhibitor" as defined herein means enzyme inhibitors, which inhibit the catalytic activity of at least one QC-like enzyme (QPCTL), but do not or with a lower potency inhibit the activity of glutaminyl cyclase (QC, QPCT). Preferred are selective QPCTL-inhibitors, which inhibit at least one QC-like enzyme (QPCTL) with an ki-value, which is one order of magnitude lower than its ki-value for the inhibition of glutaminyl cyclase (QC, QPCT). More preferably, the ki-value of said selective QPCTL-inhibitor for the inhibition of at least one QC-like enzyme (QPCTL) is two orders of magnitude lower than its ki-value for the inhibition of of glutaminyl cyclase (QC, QPCT). Even more preferred are selective QPCTL-inhibitors, wherein their ki-value for the inhibition of at least one QC-like enzyme (QPCTL) is three orders of magnitude lower than their ki-value for the inhibition of glutaminyl cyclase (QC, QPCT). Most preferred are selective QPCTL-inhibitors, which do not inhibit the activity of glutaminyl cyclase (QC, QPCT).

Potency of QC inhibition

In light of the correlation with QC inhibition, in preferred embodiments, the subject method and medical use utilize an agent with a K_i for QC inhibition of 10 μM or less, more preferably of 1 μM or less, even more preferably of 0.1 μM or less or 0.01 μM or less, or most preferably 0.01 μM or less. Indeed, inhibitors with K_i values in the lower micromolar, preferably the nanomolar and even more preferably the picomolar range are contemplated. Thus, while the active agents are described herein, for convience, as "QC inhibitors", it will be understood that such nomenclature is not intending to limit the subject of the invention to a particular mechanism of action.

Molecular weight of QC inhibitors

In general, the QC inhibitors of the subject method or medical use will be small molecules, e.g., with molecular weights of 1000 g/mole or less, 500 g/mole or less, preferably of 400 g/mole or less, and even more preferably of 350 g/mole or less and even of 300 g/mole or less.

The term "subject" as used herein, refers to an animal, preferably a mammal, most preferably a human, who has been the object of treatment, observation or experiment.

The term “therapeutically effective amount” as used herein, means that amount of active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue system, animal or human being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the disease or disorder being treated.

As used herein, the term “pharmaceutically acceptable” embraces both human and veterinary use: for example the term “pharmaceutically acceptable” embraces a veterinarily acceptable compound or a compound acceptable in human medicine and health care.

Guillain-Barré syndrome (GBS)

Alternative names are Landry-Guillain-Barré syndrome, Acute idiopathic polyneuritis, Infectious polyneuritis or Acute inflammatory polyneuropathy.

Guillain-Barré syndrome is a serious disorder that occurs when the body's defense (immune) system mistakenly attacks part of the nervous system. This leads to nerve inflammation that causes muscle weakness, which continues to get worse.

Guillain-Barré syndrome is an autoimmune disorder. The exact cause of Guillain-Barré syndrome is unknown. The syndrome may occur at any age, but is most common in people of both sexes between the ages 30 and 50. It often follows a minor infection, usually a respiratory (lung) infection or gastrointestinal (gut) infection. Usually, signs of the original infection have disappeared before the symptoms of Guillain-Barré begin. Guillain-Barré syndrome causes inflammation that damages parts of nerves. This nerve damage causes tingling, muscle weakness, and paralysis. The inflammation usually affects the nerve's covering (myelin sheath). Such damage is called demyelination. Demyelination slows nerve signaling. Damage to other parts of the nerve can cause the nerve to stop working.

Symptoms of Guillain-Barré get worse very quickly. It may take only a few hours to reach the most severe symptoms. Muscle weakness or the loss of muscle function (paralysis) affects both sides of the body. If the muscle weakness starts in the legs and then spreads to the arms, it is called ascending paralysis.

Patients may notice tingling, foot or hand pain, and clumsiness. As the loss of muscle function gets worse, the patient may need breathing assistance.

There is no cure for Guillain-Barré syndrome. However, many treatments are available to help reduce symptoms, treat complications, and speed up recovery. When symptoms are severe, the patient will need to go to the hospital for breathing help, treatment, and physical therapy. A method called plasmaphoresis is used to remove a person's blood and replace it with intravenous fluids or donated blood that is free of antibodies. High-dose immunoglobulin therapy is another procedure used to reduce the severity and length of Guillain-Barré symptoms. Other treatments are directed at preventing complications.

Chronic inflammatory demyelinizing polyradiculoneuropathy (CIDP)

A disease, which resembles GBS but is characterized by a chronic course is called chronic inflammatory demyelinizing polyradiculoneuropathy (CIDP). There is as yet no generally applicable definition for CIDP with the exception of the observation that in contrast to GBS, the progressive phase lasts longer than four weeks, often longer than six months, and that deficiencies often remain in the patient. The mechanism, which causes the severe paresis with GBS and CIDP possibly includes an immune reaction and inflammation mediated by T lymphocytes, which follows demyelinization of peripheral neurons. This assumption is confirmed by increased amounts of complement compounds and cytokines observed in the serum and cerebrospinal fluid of GBS patients. The process of demyelinization, especially in the region of the nerve roots, is currently regarded as the decisive mechanism in the development of nerve conduction block. One theory is based on a disorder of the blood/cerebrospinal fluid (CSF) barrier as a relatively early important step in the development of the disease. Another theory claims that leaks develop in the blood/CSF barrier as a consequence of the disease and cause the increased protein content in the CSF. At any rate, non-specific serum constituents without direct reference to the immune system could penetrate into the CSF from the blood, cause neuronal or glial dysfunctions and/or modify neuronal activity. An alternative mechanism is a reduced flow rate of the CSF, which could explain the increased protein content of the CSF. This interpretation requires no impairment or modified selectivity of the blood/CSF barrier. Although all the effects mentioned could be of importance for the course of GBS and CIDP, their actual contribution to the symptoms has not yet been clarified. It has not been possible to

establish a connection between the increased protein concentrations in the CSF and specific electrophysiological findings or the clinical picture. Factors in the CSF of GBS patients and multiple sclerosis patients, which interact with potential-dependent sodium channels have recently been described (Wüz et al. 1995, Muscle and Nerve *18*, 772-781). Brinkmeier (Brinkmeier et al. 1996, Muscle and Nerve *19*, 54-62) report that the factors have a molecular weight of less than three kDa, and under more stringent test conditions of less than one kDa. On the basis of this observation and the fact that the activity of the factors was not substantially reduced even after incubation of CSF with proteases, the authors concluded that the factors were neither antibodies nor cytokines.

Multiple Sclerosis (MS)

Multiple sclerosis is an autoimmune disease that affects the central nervous system (the brain and spinal cord). Multiple sclerosis usually affects woman more than men. The disorder most commonly begins between ages 20 and 40, but can strike at any age. The exact cause is not known, but MS is believed to result from damage to the myelin sheath, the protective material, which surrounds nerve cells. It is a progressive disease, meaning the damage gets worse over time. Inflammation destroys the myelin, leaving multiple areas of scar tissue (sclerosis). The inflammation occurs when the body's own immune cells attack the nervous system. The inflammation causes nerve impulses to slow down or become blocked, leading to the symptoms of MS. Repeated episodes, or flare ups, of inflammation can occur along any area of the brain and spinal cord. Symptoms vary because the location and extent of each attack varies. Usually episodes that last days, weeks, or months alternate with times of reduced or no symptoms (remission). Recurrence (relapse) is common although non-stop progression without periods of remission may also occur.

It is not clear what triggers an attack. Patients with MS typically have a higher number of immune cells than a healthy person, which suggests that an immune response might play a role. The most common theories point to a virus or genetic defect, or a combination of both. There also appears to be a genetic link to the disease. MS is more likely to occur in northern Europe, the northern United States, southern Australia, and New Zealand than in other areas. Geographic studies indicate there may be an environmental factor involved. People with a family history of MS and those who live in a geographical area with a higher incidence rate for MS have a higher risk of the disease.

There is no known cure for multiple sclerosis at this time. However, there are a number of therapies that may slow the disease. The goal of treatment is to control symptoms and maintain a normal quality of life.

Summary of the Invention

The present invention provides proteins with glutaminyl cyclase activities that constitute novel members of a family of proteins related to glutaminyl cyclase, including the full-length proteins, alternative splice forms, subunits, and mutants, as well as nucleotide sequences encoding the same. The present invention also provides methods of screening for substrates, interacting proteins, agonists, antagonists or inhibitors of the above proteins, and furthermore to pharmaceutical compositions comprising the proteins and/or mutants, derivatives and/or analogues thereof and/or ligands thereto.

These novel proteins having significant sequence similarity to glutaminyl cyclase (nucleic acid sequence of SEQ ID NO 1, protein sequence of SEQ ID NO 10) are proteins (QPCTLs) from human (further named as human isoQC) (GenBank accession no. NM_017659), mouse (GenBank accession no. NM_027455), *Macaca fascicularis* (GenBank accession no. AB168255), *Macaca mulatta* (GenBank accession no. XM_001110995), cat (GenBank accession no. XM_541552), rat (GenBank accession no. XM_001066591), cow (GenBank accession no. BT026254) or an analogue thereof having at least 50% / 75% sequence identity / similarity, preferably 70% / 85% sequence identity / similarity, most preferably 90% / 95% sequence identity / similarity.

The protein sequences are given in SEQ. ID NOS: 11 to 18. Further disclosed are nucleic acid sequences coding for these proteins (SEQ. ID NOS: 2 to 9). Table 1 illustrates the similarity between the novel proteins and the known glutaminyl cyclase. Table 2 illustrates the identity between the novel proteins and the known glutaminyl cyclase.

Table 1: Similarity of the protein sequences of the novel glutaminyl-peptide cyclotransferase-like proteins with glutaminyl cyclase

QPCTL source	human isoQC (SEQ ID NO 11)	human QC (SEQ ID NO 10)
human isoQC	-	71.98%

(SEQ ID NO 11)		
M_fascicularis (SEQ ID NO 13)	99.48%	72.24%
M_mulatta (SEQ ID NO 14)	99.48%	72.24%
C_familiaris (SEQ ID NO 15)	95.82%	72.31%
R_norvegicus (SEQ ID NO 16)	95.30%	70.77%
M_musculus (SEQ ID NO 17)	95.04%	70.77%
B_taurus (SEQ ID NO 18)	96.08%	72.31%

Table 2: Identity of the protein sequences of the novel glutaminyl-peptide cyclotransferase-like proteins with glutaminyl cyclase

QPCTL source	**human isoQC (SEQ ID NO 11)**	**human QC (SEQ ID NO 10)**
human isoQC (SEQ ID NO 11)	-	45.24%
M_fascicularis (SEQ ID NO 13)	98.17%	44.99%
M_mulatta (SEQ ID NO 14)	98.17%	44.99%
C_familiaris (SEQ ID NO 15)	88.51%	45.13%
R_norvegicus (SEQ ID NO 16)	84.33%	45.38%
M_musculus (SEQ ID NO 17)	84.07%	44.62%
B_taurus (SEQ ID NO 18)	84.60%	45.64%

There is a high similarity of 95 to 99 % and a high identity of 84 to 98 % between the QPCTLs from different sources (see figure 2). On the basis of sequence similarity with human and murine glutaminyl cyclase (see Figure 1), one might predict that these QPCTLs would have functions that include, but are not limited to, roles as enzymes. Cloning, expression, biochemical and molecular characterization have confirmed this hypothesis.

The expression pattern of the QPCTLs in brain, prostate and lung tissue is consistent with a role in the diseases described below. The enzymatic activity as glutaminyl cyclase demonstrates that QPCTLs-activating or inhibiting molecules will have numerous therapeutic applications as described below.

QPCTL activities described herein and their expression patterns are compatible with their functional roles as physiological regulators of the immune and neuroendocrine systems through the enzymatic modification of biochemical mediators like hormones, peptides and chemokines. The numerous functions previously described for QC based upon the use of inhibitors may be due in part to its action and that of similar proteins, like the QPCTLs. Therefore, the discovery of selective and potent inhibitors of QC, of the QPCTLs and of other related enzymes is considered central to achieving effective and safe pharmaceutical use of these and any newly identified glutaminyl-peptide cyclotransferases, as well as other active compounds that modify the function(s) of such proteins.

The invention thus provides novel proteins or polypeptides, the nucleic acids coding therefore, cells which have been modified with the nucleic acid so as to express these proteins, antibodies to these proteins, a screening method for the discovery of new therapeutic agents which are inhibitors of the activity of these proteins (or which are inhibitors of QC and not of the proteins), and therapeutic agents discovered by such screening methods. The novel proteins and the nucleic acids coding therefore can be used to discover new therapeutic agents for the treatment of certain diseases, such as for example, neurodegenerative, reproductive, inflammatory and metabolic disorders and also in the preparation of antibodies with therapeutic or diagnostic value.

In accordance with one aspect of the present invention, there are provided novel, mature, biologically active proteins, preferably of human origin. Such proteins may be isolated in small quantities from suitable animal (including human) tissue or biological fluids by standard techniques; however, larger quantities are more conveniently prepared in cultures of cells genetically modified so as to express the protein.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding polypeptides of the present invention including mRNAs, DNAs, cDNAs, genomic DNAs thereof.

In accordance with a further aspect of the present invention, nucleic acid probes are also provided comprising nucleic acid molecules of sufficient length to specifically hybridize to a nucleic acid sequence of the present invention.

In accordance with a still further aspect of the present invention, processes utilizing recombinant techniques are provided for producing such polypeptides useful for *in vitro* scientific research, for example, synthesis of DNA and manufacture of DNA vectors. Processes for producing such polypeptides include culturing recombinant prokaryotic and/or eukaryotic host cells that have been transfected with DNA vectors containing a nucleic acid sequence encoding such a polypeptide and/or the mature protein under conditions promoting expression of such protein and subsequent recovery of such protein or a fragment of the expressed product.

In accordance with still another aspect, the invention provides methods for using QPCTL polypeptides and polynucleotides for the treatment of diseases.

In accordance with yet another aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for the discovery of compounds that inhibit the biological activity of the mature proteins, e.g. the QC activity or the EC activity, and such inhibitors are thus also provided.

In accordance with a more specific aspect, the invention provides an isolated nucleic acid which encodes (a) a QPCTL polypeptide, selected from SEQ ID NOS: 11 to 18, or (b) having an amino acid sequence that is at least about 75% similar thereto and exhibits the same biological function, or which is an alternative splice variant of one of SEQ ID NOS: 2 to 9, or which is a probe comprising at least 14 contiguous nucleotides from said nucleic acid encoding (a) or (b), or which is complementary to any one of the foregoing.

In accordance with another specific aspect, the invention provides a polypeptide which may be optionally glycosylated, and which (a) has the amino acid sequence of a mature protein set forth in any one of SEQ ID NOS: 10 to 18; preferably of a mature protein set

forth in any one of SEQ ID NOS: 11 to 18 (b) has the amino acid sequence of a mature protein having at least about 75% similarity to one of the mature proteins of (a) and which exhibits the same biological function; (c) has the amino acid sequence of a mature protein having at least about 50% identity with a mature protein of any of SEQ ID NOS: 10 to 18; preferably of a mature protein set forth in any one of SEQ ID NOS: 11 to 18 or (d) is an immunologically reactive fragment of (a).

In accordance with still another specific aspect, the invention provides a method of screening for a compound capable of inhibiting the enzymatic activity of at least one mature protein according to the present invention, preferably selected from the proteins of SEQ ID NOS: 11 to 18, which method comprises incubating said mature protein and a suitable substrate for said mature protein in the presence of one or more test compounds or salts thereof, measuring the enzymatic activity of said mature protein, comparing said activity with comparable activity determined in the absence of a test compound, and selecting the test compound or compounds that reduce the enzymatic activity.

Further, the present invention pertains to diagnostic kits and methods based on the use of a QC-inhibitor, selective QC-inhibitor or selective QPCTL-inhibitor.

These and other aspects of the present invention should be apparent to those skilled in the art from the detailed description, which follows.

Brief Description of the Drawings

Figure 1 shows the sequence alignment of human QC (hQC), human isoQC (hisoQC), murine QC (mQC) and murine isoQC (misoQC). Multiple sequence alignment was performed using ClustalW at PBIL (Pôle Bioinformatique Lyonnais) (http://npsa-pbil.ibcp.fr) with default settings. The conservation of the zinc-ion ligating residues is shown for human QC (hQC; GenBank X71125, SEQ ID NO: 10), human isoQC (hisoQC, GenBank NM_017659, SEQ ID NO: 11), murine QC (mQC, GenBank NM_027455, SEQ ID NO: 79) and murine isoQC (misoQC, GenBank BC058181, SEQ ID NO: 17) in bold and underlined.

Figure 2 shows the sequence alignment of isoQC from *Homo sapiens* (hisoQC, GenBank NM_017659, SEQ ID NO: 11), *Macaca fascicularis* (M_fascicularis, GenBank AB168255, SEQ ID NO: 13), *Macaca mulatta* (M_mulatta, GenBank XM_001110995, SEQ ID NO: 14), *Canis familiaris* (C_familiaris, GenBank XM_541552, SEQ ID NO: 15), *Rattus norvegicus* (R_norvegicus, GenBank XM_001066591, SEQ ID NO: 16), *Mus musculus* (M_musculus, GenBank BC058181, SEQ ID NO: 17) and *Bos taurus* (B_taurus, GenBank BT026254, SEQ ID NO: 18). Multiple sequence alignment was performed using ClustalW at PBIL (Pôle Bioinformatique Lyonnais) (http://npsa-pbil.ibcp.fr) with default settings. The amino acids of the conserved zinc-ion ligating residues are underlined and typed in bold.

Figure 3 shows the sequence alignment of human QC (hQC, SEQ ID NO: 10) and human isoQC (hisoQC, SEQ ID NO: 12) 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 amino acid residues ligating the single zinc-ion within the human QC (hQC; Swiss-Prot Q16769, SEQ ID NO: 10), is shown for the human isoQC (isoQC; Swiss-Prot Q53HE4, SEQ ID NO: 12) (residues 19-382), the Zn-dependent aminopeptidase from *Streptomyces griseus* (SGAP; Swiss-Prot P80561, SEQ ID NO: 80) and the mature Zn-dependent leucyl-aminopeptidase from *Vibrio proteolyticus* (VpAP; Swiss-Prot Q01693, SEQ ID NO: 81). The respective amino acid residues are underlined and typed in bold.

Figure 4 shows the sequence alignment of human QC (hQC, SEQ ID NO: 10) and human isoQC (hisoQC, SEQ ID NO: 11), showing two putative tranlational starts (methionine I – bold, underlined; methionine II – bold). Multiple sequence alignment was performed using ClustalW at PBIL (Pôle Bioinformatique Lyonnais) http://npsa-pbil.ibcp.fr with default settings. The transmembrane domain, present in human isoQC, is indicated by the black bar.

Figure 5 shows the sequence alignment of human QC (hQC, SEQ ID NO: 10) and human isoQC (hisoQC, SEQ ID NO: 12), starting with methionine II (bold). Multiple sequence alignment was performed using ClustalW at ch.EMBnet.org with default

settings. The amino acids involved in metal binding are underlined and typed in bold. The transmembrane domain, present in human isoQC, is indicated by the black bar.

Figure 6 shows the analysis of isoQC expression by RT-PCR. Detection in SH-SY5Y, LN405, HaCaT and Hep-G2.
Lanes: bp, DNA standard; 1, amplified PCR product of human isoQC from SH-SY5Y; 2, amplified PCR product of human isoQC from LN405; 3, amplified PCR product of human isoQC from HaCaT; 4, amplified PCR product of human isoQC from Hep-G2.

Figure 7 shows the analysis of isoQC (Met I, SEQ ID NO: 11) subcellular localization by immunhistochemistry. Human isoQC starting at methionine I (see Figure 5) was expressed as a fusion protein with EGFP (isoQC (MetI) EGFP) in LN 405. Mannosidase II counterstaining was performed using AB3712 (Chemicon). Merge represents the overlay of isoQC (MetI)-EGFP and Mannosidase II staining.

Figure 8 shows the analysis of isoQC (Met I, SEQ ID NO: 11) subcellular localization by immunhistochemistry. Human isoQC starting at methionine I was expressed as a fusion protein with EGFP (isoQC (MetI) EGFP) in LN 405. Mitochondrial counterstaining was performed using MAB1273 (Chemicon). Merge represents the overlay of isoQC (MetI)-EGFP and mitochondrial staining.

Figure 9 shows the analysis of isoQC (Met II, SEQ ID NO: 12) subcellular localization by immunhistochemistry. Human isoQC starting at methionine II was expressed as a fusion protein with EGFP (isoQC (MetII) EGFP) in LN 405. Mannosidase II counterstaining was performed using AB3712 (Chemicon). Merge represents the overlay of isoQC (MetII)-EGFP and Mannosidase II staining.

Figure 10 shows the analysis of isoQC (Met II, SEQ ID NO: 12) subcellular localization by immunhistochemistry. Human isoQC starting at methionine II was expressed as a fusion protein with EGFP (isoQC (MetII) EGFP) in LN 405. Mitochondrial counterstaining was performed using MAB1273 (Chemicon). Merge represents the overlay of isoQC (MetII)-EGFP and mitochondrial staining.

Figure 11 shows the analysis of the subcellular localization of isoQC (Met I, SEQ ID NO: 11) by immunhistochemistry. Human isoQC starting at methionine I was expressed as a fusion protein with EGFP (isoQC (MetI) EGFP) in COS-7. Mannosidase II counterstaining was performed using AB3712 (Chemicon). Merge represents the overlay of isoQC (MetI)-EGFP and Mannosidase II staining.

Figure 12 shows the analysis of isoQC (Met I, SEQ ID NO: 11) subcellular localization by immunhistochemistry. Human isoQC starting at methionine I was expressed as a fusion protein with EGFP (isoQC (MetI) EGFP) in COS-7. Mitochondrial counterstaining was performed using MAB1273 (Chemicon). Merge represents the overlay of isoQC (MetI)-EGFP and mitochondrial staining.

Figure 13 shows the analysis of isoQC (Met II, SEQ ID NO: 12) subcellular localization by immunhistochemistry. Human isoQC starting at methionine II was expressed as a fusion protein with EGFP (isoQC (MetII) EGFP) in COS-7. Mannosidase II counterstaining was performed using AB3712 (Chemicon). Merge represents the overlay of isoQC (MetII)-EGFP and Mannosidase II staining.

Figure 14 shows the analysis of isoQC (Met II, SEQ ID NO: 12) subcellular localization by immunhistochemistry. Expression of human isoQC starting at methionine II as a fusion protein with EGFP (isoQC (MetII) EGFP) in COS-7. Mitochondrial counterstaining was performed using MAB1273 (Chemicon). Merge represents the overlay of isoQC (MetII)-EGFP and mitochondrial staining.

Figure 15 shows the inhibition of human isoQC-catalyzed conversion of H-Gln-AMC into pGlu-AMC by the inhibitor P150/03. The data were evaluated according to the Michaelis-Menten kinetic model considering linear competitive inhibition. Inhibitor concentrations were as follows:

—▽—	0 µM
—▲—	0.3125 µM
—△—	0.625 µM
—■—	1.25 µM
—□—	2.5 µM
—●—	5 µM

The determined K_i-value was 240 ± 8 nM.

Figure 16 shows the human isoQC-catalyzed conversion of H-Gln-Ala-OH into pGlu-Ala-OH determined using a spectrophotometric assay. The data were evaluated according to Michaelis-Menten kinetics. The kinetic parameters were 324 ± 28 µM and 7.4 ± 0.2 nM/min for the K_M and V_{max}-value, respectively.

Figure 17 provides a schematic representation of the human isoQC protein constructs that were expressed hetereologously in the yeast *P. pastoris*. Two mutations were introduced in some proteins, leading to a glycosylation site at position 55 (I55N) and a mutated cystein residue at position 351 (C351A). For expression, the N-terminus including the transmembrane domain was replaced by a secretion signal of yeast (YSS). The constructs containing the N-terminal secretion signal should be efficiently secreted into the medium.

Figure 18 shows the QC activity, which was determined in the medium of expressing yeast cells. Due to the transmembrane domain, the native constructs were not secreted into the medium (not implemented). Caused by glycosylation (I55N), proteins are most efficiently secreted. The mutation C351A resulted also in higher QC activity detected in the medium. The constructs are described in Figure 17.

Figure 19 shows the purification of the human isoQC, based on construct YSShisoQCI55NC351A C-His, from the medium of a transgenic *P.pastoris* strain. The QC was purified by a combination of IMAC (immobilized metal affinity chromatography, lane 3), HIC (hydrophobic interaction chromatography, lane 4) and desalting (lane 5). The glycosylation of the enzyme was evidence by enzymatic deglycosalytion, which results in a shift in migration of the protein (lane 6). Lane 1, protein standard: Lane 2, medium prior to purification.

Figure 20 shows the purification of the human isoQC, based on construct GST-hisoQC C-His, from the cell homogenate of transformed *E. coli*. The isoQC was purified by a combination of IMAC (immobilized metal affinity chromatography, lane 3), GST-affinity (lane 4), desalting (lane 5) and ion exchange chromatography (lane 6). Lane 1, protein standard: Lane 2, cell homogenate prior to purification. The difference in the molecular mass between the hisoQC which was expressed in yeast and *E. coli* is caused by the N-terminal GST-tag fusion. The expressed construct is provided schematically in the upper part of the figure.

Figure 21 shows the specificity constants for conversion of dipeptide-surrogates, dipeptides and oligopeptides by human isoQC (YSShisoQCI55NC351A C-His; compare Figure 17), GST-hisoQC and human QC. The specificity of GST-hisoQC was the lowest, followed by YSShisoQCI55NC351A C-His. The highest specificity displayed human QC, indicating a higher overall enzymatic activity.

Figure 22 shows the pH-dependency of catalysis, investigated with human isoQC (hisoQC), which was expressed in yeast, and human QC (hQC). Both proteins display a pH-optimum between pH 7 and 8.The fitted curve is based on three dissociating groups that influence catalysis, one at acidic pH, two at basic pH.

Figure 23 shows the analysis of conversion of glutamic acid, which is present at the N-terminus of the amyloid-β related peptide Aβ(3-11). The analysis was performed using Maldi-Tof mass spectrometry, the substrate and product differ in their molecular mass/charge ratio of the single chared molecule by about 18Da, which is the mass of the released water. In both cases, the same protein concentration was present in the

samples, clearly suggesting that human isoQC also converts N-terminal glutamic acid, but slower than the human QC.

Figure 24 shows the tissue distribution of murine QC (mQC, SEQ ID NO: 79) and its isoenzyme misoQC (SEQ ID NO: 17), analyzed using real-time PCR. Bothe enzymes are expressed in the tested organs. However, the expression level of mQC was higher in the brain compared with the peripheral organs. In contrats, misoQC was expressed in all tested organs and tisssues at a more similar level, indicating a ubiquitous, "house-keeping" protein.

Figure 25 shows the time-dependent inhibition of human isoQC (hisoQC) by metal-chelating compounds 1,10-phenanthroline (circles) and EDTA (squares). Residual hisoQC activity was determined directly after addition (closed symbols) or preincubation of hisoQC with respective reagent for 15 min at 30 °C (open symbols).

Figure 26 shows the biochemical analysis of the subcellular localization of QC activity after expression of pcDNA and the native enzymes hisoQC (Met I, SEQ ID NO: 11), hisoQC (Met II, SEQ ID NO: 12) and hQC (SEQ ID NO: 10) in HEK293 cells. (**A**) specific activity within the cell fractions in µmole / min / g. (**B**) absolute activity in nM /min. (**C**) Expression of h-isoQC (Met I, SEQ ID NO: 11), h-isoQC (Met II, SEQ ID NO: 12) and hQC (SEQ ID NO: 10) possessing a C-terminal FLAG-tag in HEK293 in comparison to vector-transfected control (pcDNA), followed by Western Blot analysis applying specific antibodies detecting either the FLAG-epitope (anti-DYKDDDDK-antibody, Cell Signaling), a 65 kDa protein of human mitochondria (anti-human mitochondria, Chemicon) or human Sialyltransferase ST1GAL3 (Abnova).

Figure 27 shows the subcellular localization of human isoQC (hisoQC) signal sequences (**27A**) methionine I – serine 53 and (**27B**) methionine II – serine 53, fused to EGFP (1, 4). Golgi complex was stained using an anti-mannosidase II antibody (2) and mitochondria were stained using an antibody detecting a 65 kDA protein of human mitochondria (5). Co-localization is shown by superimposition of EGFP fluorescence and Red X fluorescence (3, 6).

Figure 28 shows the domain structure of human isoQC (hisoQC) and murine isoQC (misoQC) in comparison to published sequences of human glycosyltransferases: alpha-N-acetylgalactosaminide alpha-2,6-sialyl transferase 1 (ST6GalNAC1; E.C. 2.4.99.3); beta-1,4-galactosyltransferase 1 (b4Gal-T1, E.C. 2.4.1.-); Galactoside 3(4)-L-fucosyltransferase (FucT-III; E.C. 2.4.1.65) and Glycoprotein-fucosylgalactoside alpha-N-acetylgalactosaminyl transferase (NAGAT, E.C.2.4.1.40). The number of amino acids as listed below the cloumns. The cytosolic part is shaded, the transmembrane helix is black and luminal part is illustrated in white.

Figure 29 shows the quantification of human isoQC (QPCTL) mRNA in different carcinoma cell lines. The QPCTL expression was normalized to 50 ng total-RNA. The black bar within the boxes represents the respective median.

Figure 30 shows the quantification of human isoQC (QPCTL) mRNA expression in different melanoma cell lines. The QPCTL expression was normalized to 50 ng total-RNA.

Figure 31 shows the quantification of human isoQC (QPCTL) mRNA expression in samples from soft tissue carcinoma, gastric carcinoma and thyroid carcinoma from different patients. The QPCTL expression was normalized to 50 ng total-RNA. The black bar within the boxes represents the respective median.

Figure 32 shows the human isoQC (QPCTL) mRNA expression in different gastric carcinomas against their stage of differentiation. QPCTL expression was normalized to 50 ng total-RNA. The black bar within the boxes represents the respective median.

Figure 33 shows a comparison of human QC (QPCT) mRNA expression in different thyroid carcinomas. QPCT expression was normalized to 50 ng total-RNA. The black bar within the boxes represents the respective median. (FTC: folicular thyroid carcinoma; PTC: papillary thyroid carcinoma; UTC: undifferentiated thyroid carcinoma).

Figure 34 shows a comparison of human isoQC (QPCTL) mRNA expression in different thyroid carcinomas. QPCTL expression was normalized to 50 ng total-RNA. The black

bar within the boxes represents the respective median. (FTC: folicular thyroid carcinoma; PTC: papillary thyroid carcinoma; UTC: undifferentiated thyroid carcinoma).

Figure 35 shows the influence of different stimuli on mRNA expression of human QC (QPCT), human isoQC (QPCTL) and CCL2 in HEK293 cells. The amount of transcripts is depicted relating to basal expression without stimulus. The used concentration of stimulus is stated on the x-axis drawing.

Figure 36 shows the influence of different stimuli on mRNA expression of human QC (QPCT), human isoQC (QPCTL) and CCL2 in FTC-133 cells. The amount of transcripts is depicted relating to basal expression without stimulus. The used concentration of stimulus is stated on the x-axis drawing.

Figure 37 shows the influence of different stimuli on mRNA expression of human QC (QPCT), human isoQC (QPCTL) and CCL2 in THP-1 cells. The amount of transcripts is depicted relating to basal expression without stimulus. The used concentration of stimulus is stated on the x-axis drawing.

Figure 38 shows the influence of different stimuli on mRNA expression of human QC (QPCT), CCL2, CCL7, CCL8 and CCL13 in THP-1 cells. The amount of transcripts is depicted relating to basal expression without stimulus. The used concentration of stimulus is stated on the x-axis drawing.

Figure 39 shows the influence of hypoxia on the mRNA level of human QC (QPCT), human isoQC (QPCTL) and HIF1α in HEK293 (A), FTC-133 (b) and THP-1 (C).

List of Sequences

SEQ ID NO	Description
1	human QC, nucleic acid
2	human isoQC Met I, nucleic acid
3	human isoQC Met II, nucleic acid
4	*Macaca fascicularis* QPCTL, nucleic acid
5	*Macaca mulatta* QPCTL, nucleic acid
6	*Canis familiaris* QPCTL, nucleic acid
7	rat QPCTL, nucleic acid
8	mouse QPCTL, nucleic acid
9	bovine QPCTL, nucleic acid
10	human QC, protein
11	human isoQC Met I, protein
12	human isoQC Met II, protein
13	*Macaca fascicularis* QPCTL, protein
14	*Macaca mulatta* QPCTL, protein
15	*Canis familiaris* QPCTL, protein
16	rat QPCTL, protein
17	mouse QPCTL, protein
18	bovine QPCTL, protein
19	human isoQC splice form 1, nucleic acid
20	human isoQC splice form 2, nucleic acid
21	human isoQC splice form 1, protein
22	human isoQC splice form 2, protein
23	Amyloid beta peptide (Abeta) (1-42)
24	Abeta (1-40)
25	Abeta (3-42)
26	Abeta (3-40)
27	Abeta (11-42)
28	Abeta (11-40)

29	$pGlu^3$-Abeta (3-42)
30	$pGlu^3$-Abeta (3-40)
31	$pGlu^3$-Abeta (11-42)
32	$pGlu^3$-Abeta (11-40)
33	ABri
34	ADan
35	Gastrin 17
36	Gastrin 34
37	pGlu-Abri
38	pGlu-ADan
39	pGlu-Gastrin 17
40	pGlu-Gastrin 34
41	Neurotensin
42	GnRH
43	CCL16
44	CCL8
45	CCL2
46	CCL18
47	Fractalkine
48	CCL7
49	Orexin A
50	Substance P
51	QYNAD
52	pGlu-YNAD
53	human isoQC forward primer used for cell line screening
54	human isoQC reverse primer used for cell line screening
55	forward primer used for isolation of human isoQC
56	reverse primer used for isolation of human isoQC
57	forward primer used for cloning of human isoQC (isoform Met I) into vector pEGFP-N3
58	forward primer used for cloning of human isoQC (isoform Met II) into vector pEGFP-N3

59	reverse primer used for cloning of human isoQC (isoforms Met I and Met II) into vector pEGFP-N3
60	forward primer used for cloning of human isoQC into vector pET41a
61	reverse primer used for cloning of human isoQC into vector pET41a
62	forward primer for cloning human isoQC into vector pPICZαA with a C-terminal histidine tag
63	forward primer for cloning human isoQC into vector pPICZαA with a N-terminal histidine tag
64	reverse primer for cloning human isoQC into vector pPICZαA with a N-terminal histidine tag
65	forward primer for real-time PCR analysis of isoQC
66	reverse primer for cloning human isoQC into vector pPICZαA with a C-terminal histidine tag
67	reverse primer for real-time PCR analysis of isoQC
68	Forward primer for cloning of murine isoQC cDNA
69	Reverse primer for cloning of murine isoQC cDNA
70	Forward primer for cloning of murine isoQC cDNA
71	forward primer for real-time PCR analysis of murine QC
72	reverse primer for real-time PCR analysis of murine QC
73	forward primer for real-time PCR analysis of murine QC
74	reverse primer for real-time PCR analysis of murine QC
75	forward primer for site-directed mutagenesis hisoQC I55N
76	reverse primer for site-directed mutagenesis hisoQC I55N
77	forward primer for site-directed mutagenesis hisoQC C351A
78	reverse primer for site-directed mutagenesis hisoQC C351A
79	Mouse glutaminyl cyclase protein
80	Streptomyces griseus SGAP
81	Vibrio proteolyticus VpAP
82	forward primer for insertion of native hQC into pcDNA 3.1
83	reverse primer for insertion of native hQC into pcDNA 3.1
84	reverse primer for amplification of hisoQC including the stop codon for insertion into pcDNA 3.1
85	forward primer for amplification EGFP

86	reverse primer for amplification EGFP
87	Reverse primer for amplification of hisoQC N-terminal sequence for fusion with EGFP
88	Reverse primer for amplification hQC C-FLAG for insertion into pcDNA 3.1
89	Reverse primer for amplification hisoQC C-FLAG for insertion into pcDNA 3.1

Detailed Description of the Invention

In accordance with an aspect of the present invention, there are provided isolated nucleic acid sequences (polynucleotides) of SEQ ID NOS: 2 to 9, 19 and 20, which encode the mature polypeptides having the deduced amino acid sequences of the QPCTLs from different sources (SEQ ID NOS: 11 to 18, 21 and 22).

Preferred according to the present invention are isolated nucleic acid sequences (polynucleotides) of SEQ ID NOS: 2 and 3, 19 and 20, which encode the mature polypeptides having the deduced amino acid sequences of the QPCTLs from human (SEQ ID NOS: 11 and 12, 21 and 22).

More preferred according to the present invention are isolated nucleic acid sequences (polynucleotides) of SEQ ID NOS: 2 and 3, which encode the mature polypeptides having the deduced amino acid sequences of the human QPCTLs of SEQ ID NOS: 11 and 12.

Even preferred according to the present invention are isolated nucleic acid sequences (polynucleotides) of SEQ ID NOS: 19 and 20, which encode the mature polypeptides having the deduced amino acid sequences of alternative spliceforms of human QPCTLs of SEQ ID NOS: 21 and 22.

Most preferred according to the present invention is the isolated nucleic acid sequence (polynucleotide) of SEQ ID NO: 2, which encodes the mature polypeptide having the deduced amino acid sequence of the human QPCTL of SEQ ID NOS: 11.

Even most preferred according to the present invention is the isolated nucleic acid sequence (polynucleotide) of SEQ ID NO: 3, which encodes the mature polypeptide having the deduced amino acid sequence of the human QPCTL of SEQ ID NOS: 12.

The aforementioned embodiments and preferences apply to the QPCTL nucleic acids as well as QPCTL proteins and any desired method of use, diagnosing, treatment, screening, effectors, inhibitors and other uses and methods according to the present invention.

The polynucleotides of this invention were discovered by similarity search using Nucleotide BLAST at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) applying human QC as template. The search resulted in discovery of a putative QPCTL on chromosome 19, which is encoded in region 19q13.32. On basis of the search, primers for a cell line screening of human isoQC were designed (Table 4). The isolated cDNA for human QPCTL contains an open reading frame encoding a protein of 382 amino acids in length, which is related to human QC displaying 45.24% sequence identity, and 71.98% similarity. Applying different bioinformatic algorithms (www.expasy.ch) for prediction of the subcellular localization did not result in a reliable result. The prognosis, depending on the prediction program, was transfer to golgi-apparatus or mitochondria.

Amino acid sequence alignments of human QPCTL with other members of the M28 family members of the metallopeptidase Clan MH shows that human QPCTL protein has overall sequence and structural homology to human and murine QC (Figure 1) and bacterial aminopeptidases (Figure 3). A database search for additional human QPCTL-related genes revealed the presence of rodent, simian, cattle and dog QPCTLs. Alignment of these sequences with the novel human QPCTL shows that they display considerable homology with its human counterpart. The zinc-complexing residues of human QC (Asp-Glu-His) are conserved within QPCTLs from the different orgins (Figure 2).

The human isoQC gene contains at least 8 exons. The sequence coding for the human isoQC protein is located on exons 1 to 7. Human isoQC maps to chromosome 19 at position 19q13.32. A cell line screening for human isoQC revealed transcripts in cells

origin from liver (Hep-G2, hepatocellular carcinoma), skin (HaCaT, keratinocyte) and neuronal tissues (LN405, astrocytoma; SH-SY5Y, neuroblastoma) (Figure 6).

The isolated QPCTL-cDNA was tested on functional expression in several expression hosts. Expression in *P. pastoris*, which was successfully applied for human QC, did not result in an enzymatically active protein. Expression in mammalian cells resulted in detection of activity, however, expression levels were very low. Thus, the isolation of an enzymatically active protein was not possible with the knowledge of the skilled artisan. Enzymatically active protein was isolated only following expression of a GST-QPCTL fusion protein in *E. coli*, applying very unusual expression conditions: Expression for 4 h at 37°C in presence of 1% Glucose, induction of expression using 20 µM IPTG.
The expression conditions result in a low-level expression in *E. coli*, which is necessary for functional folding of the peptide chain.

In another embodiment, the present invention relates to QPCTL knockout animals, preferably rats or mice. The use of knockout mice in further analysis of the function of QPCTL genes is a valuable tool.

The polynucleotides of the present invention may be in the form of RNA or in the form of DNA; DNA should be understood to include cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded and, if single stranded, may be the coding strand or non-coding (antisense) strand. The coding sequence, which encodes the mature polypeptide may be identical to the coding sequence shown in SEQ ID NOS 2 to 9, or it may be a different coding sequence encoding the same mature polypeptide, as a result of the redundancy or degeneracy of the genetic code or a single nucleotide polymorphism. For example, it may also be an RNA transcript which includes the entire length of any one of SEQ ID NOS 11 to 18.

The polynucleotides which encode the mature proteins of SEQ ID NOS 2 to 9 may include but are not limited to the coding sequence for the mature protein alone; the coding sequence for the mature polypeptide plus additional coding sequence, such as a leader or secretory sequence or a proprotein sequence; and the coding sequence for the mature protein (and optionally additional coding sequence) plus non-coding

sequence, such as introns or a non-coding sequence 5' and/or 3' of the coding sequence for the mature protein.

Thus, the term "polynucleotide encoding a polypeptide" or the term "nucleic acid encoding a polypeptide" should be understood to encompass a polynucleotide or nucleic acid which includes only coding sequence for the mature protein as well as one which includes additional coding and/or non-coding sequence. The terms polynucleotides and nucleic acid are used interchangeably.

The present invention also includes polynucleotides where the coding sequence for the mature protein may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell; for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell may be so fused. The polypeptide having such a leader sequence is termed a preprotein or a preproprotein and may have the leader sequence cleaved, by the host cell to form the mature form of the protein. These polynucleotides may have a 5' extended region so that it encodes a proprotein, which is the mature protein plus additional amino acid residues at the N-terminus. The expression product having such a prosequence is termed a proprotein, which is an inactive form of the mature protein; however, once the prosequence is cleaved an active mature protein remains. Thus, for example, the polynucleotides of the present invention may encode mature proteins, or proteins having a prosequence, or proteins having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptides of the present invention. The marker sequence may be a polyhistidine tag, a hemagglutinin (HA) tag, a c-myc tag or a V5 tag when a mammalian host, e. g. COS-1 cells, is used.

The HA tag would correspond to an epitope derived from the influenza hemagglutinin protein (Wilson, I., etal., Cell, 37: 767 (1984)), and the c-myc tag may be an epitope from human Myc protein (Evans, G. I. et al., Mol. Cell. Biol.5 : 3610-3616(1985)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

The term "significant sequence homology" is intended to denote that at least 25%, preferably at least 40%, of the amino acid residues are conserved, and that, of the nonconserved residues, at least 40% are conservative substitutions.

Fragments of the full-length genes of the present invention may be used as a hybridization probe for a cDNA library to isolate full-length cDNA as well as to isolate other cDNAs, which have significant sequence homology to the gene and will encode proteins or polypeptides having similar biological activity or function. By similar biological activity or function, for purposes of this application, is meant the ability to form pyroglutamate from a N-terminal glutamine or glutamic acid of peptides, proteins, hormones or other substrates, defined as QC- and EC-activity, respectively. Such a probe of this type has at least 14 bases (at least 14 contiguous nucleotides from one of SEQ ID NOS: 2 to 9), preferably at least 30 bases, and such may contain, for example, 50 or more bases. Preferred are the probes of SEQ ID NOS 53 to 61. Such probe may also be used to identify a cDNA clone corresponding to a full-length transcript and/or a genomic clone or clones that contains the complete gene, including regulatory and promoter regions, exons, and introns. Labelled oligonucleotides having a sequence complementary to that of the gene of the present invention are useful to screen a library of human cDNA, genomic DNA or mRNA or similar libraries from other sources or animals to locate members of the library to which the probe hybridizes. As an example, a known DNA sequence may be used to synthesize an oligonucleotide probe, which is then used in screening a library to isolate the coding region of a gene of interest.

The present invention is considered to further provide polynucleotides which hybridize to the hereinabove-described sequences wherein there is at least 70%, preferably at least 90%, and more preferably at least 95% identity or similarity between the sequences, and thus encode proteins having similar biological activity. Moreover, as known in the art, there is "similarity" between two polypeptides when the amino acid sequences contain the same or conserved amino acid substitutes for each individual

residue in the sequence. Identity and similarity may be measured using sequence analysis software (e. g., ClustalW at PBIL (Pôle Bioinformatique Lyonnais) http://npsa-pbil.ibcp.fr). The present invention particularly provides such polynucleotides, which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means conditions which permit hybridization between polynucleotides sequences and the polynucleotide sequences of SEQ ID NOS: 2 to 9 where there is at least about 70% identity.

Suitably stringent conditions can be defined by, e. g., the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, by increasing the concentration of formamide, and/or by raising the hybridization temperature.

For example, hybridization under high stringency conditions may employ about 50% formamide at about 37°C to 42°C, whereas hybridization under reduced stringency conditions might employ about 35% to 25% formamide at about 30°C to 35°C. One particular set of conditions for hybridization under high stringency conditions employs 42°C, 50% formamide, 5x. SSPE, 0.3% SDS, and 200 µg/ml sheared and denatured salmon sperm DNA. For hybridization under reduced stringency, similar conditions as described above may be used in 35% formamide at a reduced temperature of 35°C. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art. Preferably, hybridization should occur only if there is at least 95%, and more preferably at least 97%, identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which exhibit substantially the same biological function or activity as the mature protein encoded by one of the cDNAs of SEQ ID NOS : 2 to 9.

As mentioned, a suitable polynucleotide probe may have at least 14 bases, preferably 30 bases, and more preferably at least 50 bases, and will hybridize to a polynucleotide

of the present invention, which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as a probe for hybridizing to the polynucleotides of SEQ ID NOS: 2 to 9 respectively, for example, for recovery of such a polynucleotide, or as a diagnostic probe, or as a PCR primer. Thus, the present invention includes polynucleotides having at least a 70% identity, preferably at least a 90% identity, and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptides of SEQ ID NOS: 11 to 18 respectively, as well as fragments thereof, which fragments preferably have at least 30 bases and more preferably at least 50 bases, and to polypeptides encoded by such polynucleotides.

As is well known in the art, the genetic code is redundant in that certain amino acids are coded for by more than one nucleotide triplet (codon), and the invention includes those polynucleotide sequences which encode the same amino acids using a different codon from that specifically exemplified in the sequences herein. Such a polynucleotide sequence is referred to herein as an "equivalent" polynucleotide sequence. The present invention further includes variants of the hereinabove described polynucleotides which encode for fragments, such as part or all of the mature protein, analogs and derivatives of one of the polypeptides having the deduced amino acid sequence of any one of SEQ ID NOS: 11 to 18. The variant forms of the polynucleotides may be a naturally occurring allelic variant of the polynucleotides or a non-naturally occurring variant of the polynucleotides. For example, the variant in the nucleic acid may simply be a difference in codon sequence for the amino acid resulting from the degeneracy of the genetic code, or there may be deletion variants, substitution variants and addition or insertion variants. As known in the art, an allelic variant is an alternative form of a polynucleotide sequence, which may have a substitution, deletion or addition of one or more nucleotides that does not substantially alter the biological function of the encoded polypeptide.

The present invention further includes polypeptides, which have the deduced amino acid sequence of SEQ ID NOS: 11 to 18, as well as fragments, analogs and derivatives of such polypeptides. The terms "fragment", "derivative" and "analog", when referring to the polypeptides of SEQ ID NOS: 11 to 18, means polypeptides that retain essentially

the same biological function or activity as such polypeptides. An analog might, for example, include a proprotein, which can be activated by cleavage of the proprotein portion to produce an active mature protein. The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides or synthetic polypeptide; however, they are preferably recombinant polypeptides, glycosylated or unglycosylated.

The fragment, derivative or analog of a polypeptide of any one of SEQ ID NOS 11 to 18, may be (i) one in which one or more of the amino acid residues is substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which additional amino acids are fused to the mature protein, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art to provide upon the basis of the teachings herein.

The polypeptides and polynucleotides of the present invention should be in an isolated form, and preferably they are purified to substantial homogeneity or purity. By substantial homogeneity is meant a purity of at least about 85%.

The term "isolated" is used to mean that the material has been removed from its original environment (e. g., the natural environment if it is naturally occurring). For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not considered to be isolated, but the same polynucleotide or polypeptide, when separated from substantially all of the coexisting materials in the natural system, is considered isolated. For DNA, the term includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e. g., a cDNA or a genomic or cDNA fragment produced by polymerase chain reaction (PCR) or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA, which is part of a hybrid gene encoding additional polypeptide sequence, e.g., a fusion protein. Further included is recombinant DNA

which includes a portion of the nucleotides shown in one of SEQ ID NOS 2 to 9 which encodes an alternative splice variant of the QPCTLs. Various alternative splice variants are exemplified in SEQ ID NOS: 19-22.

The polypeptides of the present invention include any one of the polypeptides of SEQ ID NOS 11 to 18 (in particular the mature proteins), as well as polypeptides which have at least 75% similarity (e. g. preferably at least 50% and more preferably at least 70% identity) to one of the polypeptides of SEQ ID NOS 11 to 18, more preferably at least 85% similarity (e. g. preferably at least 70% identity) to one of the polypeptides of SEQ ID NOS 11 to 18, and most preferably at least 95% similarity (e. g. preferably at least 90% identity) to any one of the polypeptides of SEQ ID NOS 11 to 18. Moreover, they should preferably include exact portions of such polypeptides containing a sequence of at least 30 amino acids, and more preferably at least 50 amino acids.

Fragments or portions of the polypeptides of the present invention may be employed as intermediates for producing the corresponding full-length polypeptides by peptide synthesis. Fragments or portions of the polynucleotides of the present invention may also be used to synthesize full-length polynucleotides of the present invention.

The present invention also includes vectors, which include such polynucleotides, host cells which are genetically engineered with such vectors and the production of polypeptides by recombinant techniques using the foregoing. Host cells are genetically engineered (transduced or transformed or transfected) with such vectors, which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those commonly used with the host cell selected for expression, as well known to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotides may be included in any one of a variety of expression vectors for expressing polypeptides.

Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e. g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by any of a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site (s) by procedures well known in the art, which procedures are deemed to be within the scope of those skilled in this art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence (s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli* lac or trp, the phage lambda P.sub.L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses.

The expression vector should also contain a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells, such as dihydrofolate reductase or neomycin-resistance for eukaryotic cell culture, or such as tetracycline-or ampicillin-resistance in *E. coli*.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli, Streptomyces, Salmonella typhimurium*; fungal cells, such as yeast; insect cells, such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells, such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

Synthetic production of nucleic acid sequences is well known in the art as is apparent from CLONTECH 95/96 Catalogue, pages 215-216, CLONTECH, 1020 East Meadow Circle, Palo Alto, Calif. 94303. Thus, the present invention also includes expression vectors useful for the production of the proteins of the present invention. The present invention further includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs may comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example: Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNHI8A, pNH46A (Stratagene), ptrc99a, pKK223-3, pKK233-3, pDR540 and pRIT5 (Pharmacia); and Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXTI, pSG (Stratagene), pSVK3, pBPV, pMSG, and pSVL (Pharmacia). However, any other suitable plasmid or vector may be used as long as it is replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol acetyl transferase) vectors or other vectors with selectable markers.

Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P.sub.R, P.sub.L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

Components of the expression vector may generally include: 1) a neomycin phosphotransferase (G418), or hygromycin B phosphotransferase (hyg) gene as a selection marker, 2) an *E. coli* origin of replication, 3) a T7 and SP6 phage promoter sequence, 4) lac operator sequences, 5) the lactose operon repressor gene (lacIq) and 6) a multiple cloning site linker region. Such an origin of replication (oriC) may be derived from pUC19 (LTI, Gaithersburg, Md.).

A nucleotide sequence encoding one of the polypeptides of SEQ ID NOS: 2 to 9 having the appropriate restriction sites is generated, for example, according to the PCR protocol described in Examples 1 and 2 hereinafter, using PCR primers having restriction sites for EcoR I (as the 5' primer) and Sal I (as the 3'primer) for cloning of isoQC Met I and Met II into vector EGFP-N3, or sites for Spe I (as the 5' primer) and EcoR I (as the 3' primer) for cloning of isoQC into vector pET41a. The PCR inserts are gel-purified and digested with compatible restriction enzymes. The insert and vector are ligated according to standard protocols.

In a further embodiment, the present invention provides host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

Such constructs in host cells are preferably used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers or by chemical ligation of suitable fragments thus prepared.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N. Y., (1989).

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector.

Enhancers include cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, acytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e. g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes, such as 3-phosphoglycerate kinase (PGK), alpha-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter.

The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desired, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli, Bacillus subtilis, Salmonella typhimurium* and various species within the genera Pseudomonas, Streptomyces and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from

commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, Wis., U. S. A.). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e. g., temperature shift or chemical induction), and cells are cultured for an additional period.

Cells are typically harvested by centrifugation and then disrupted by physical or chemical means, with the resulting crude extract being retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption and use of cell-lysing agents; such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express a recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23: 175 (1981). Other cell lines capable of expressing a compatible vector include, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will generally comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Recovery can be facilitated if the polypeptide is expressed

at the surface of the cells, but such is not a prerequisite. Recovery may also be desirable of cleavage products that are cleaved following expression of a longer form of the polypeptide. Protein refolding steps as known in this art can be used, as necessary, to complete configuration of the mature protein. High performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be purified natural products, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect or mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

In a preferred embodiment, the proteins of the invention are isolated and purified so as to be substantially free of contamination from other proteins. For example, the proteins of the invention should constitute at least 80% by weight of the total protein present in a sample, more preferably at least 90%, even more preferably at least 95%, and most preferably at least 98% by weight of the total protein.

These proteins may be in the form of a solution in water, another suitable solvent, such as dimethyl sulphoxide (DMSO) or ethanol, or a mixture of suitable solvents.

Examples of mixtures of solvents include 10% (by weight) ethanol in water and 2% (by weight) DMSO in water. A solution may further comprise salts, buffering agents, chaotropic agents, detergents, preservatives and the like. Alternatively, the proteins may be in the form of a solid, such as a lyophilised powder or a crystalline solid, which may also comprise a residual solvent, a salt or the like.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as F (ab')2 and Fab'proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included.

Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans should be reduced.

Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to human isoQC protein or a peptide therefrom, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled human isoQC protein or peptide).

Genes encoding polypeptides having potential human isoQC polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding such polypeptides can be obtained in a number of ways well known in the art.

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from inoculating a variety of warm-blooded animals, such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice and rats, with a human isoQC polypeptide or a fragment thereof. The immunogenicity of a human isoQC polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant, or surface active substances, such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, KLH or dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum* are especially preferable. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of isoQC or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier, such as keyhole limpet hemocyanin (KLH), bovine serum

albumin (BSA) or tetanus toxoid, for immunization. Antibodies to isoQC may also be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, and single chain antibodies, Fab fragments, and fragments produced by a Fab expression library.

Neutralizing antibodies (i. e., those which block or modify interactions at the active sites) are especially preferred for therapeutic use.

For the production of antibodies, binding proteins, or peptides which bind specifically to QPCTL, libraries of single chain antibodies, Fab fragments, other antibody fragments, non-antibody protein domains, or peptides may be screened. The libraries could be generated using phage display, other recombinant DNA methods, or peptide synthesis (Vaughan, T. J. et al. Nature Biotechnology 14: 309-314 (1966)). Such libraries would commonly be screened using methods, which are well known in the art to identify sequences which demonstrate specific binding to QPCTL.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to QPCTL have an amino acid sequence consisting of at least about 5 amino acids and, more preferably, of at least about 10 amino acids. It is also preferable that these oligopeptides, peptides, or fragments are identical to a portion of the amino acid sequence of the natural protein. Short stretches of QPCTL amino acids may also be fused with those of another protein, such as KLH, and antibodies to the chimeric molecule may be produced.

Monoclonal antibodies to QPCTL may be prepared using any well known technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique, although monoclonal antibodies produced by hybridoma cells may be preferred.

In addition, techniques developed for the production of "chimeric antibodies", such as the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used, see Neuberger,

M. S. et al. Nature 312: 604-608 (1984). Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce QPCTL-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be generated by chain shuffling from random combinatorial immunoglobulin libraries. (Burton D. R. Proc. Natl. Acad. Sci. 88 : 11120-11123 (1991)).

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature. (Orlandi, R. et al. Proc. Natl. Acad. Sci. 86: 3833-3837 (1989)).

Antibody fragments, which contain specific binding sites for QPCTL may also be generated. For example, such fragments include, but are not limited to, $F(ab')_2$ fragments produced by pepsin digestion of the antibody molecule and Fab fragments generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (Huse, W. D. et al. Science 254: 1275-1281(1989)).

Various immunoassays may be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between QPCTL and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering QPCTL epitopes is preferred, but a competitive binding assay may also be employed.

As earlier mentioned, the QPCTLs can be used in treatment of the Diseases.

Pharmaceutical compositions suitable for use in this aspect of the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose relating to one of the Diseases. The determination of a

therapeutically effective dose is well within the capability of those skilled in the art and can be estimated initially either in cell culture assays, e. g. of neoplastic cells, or in animal models, usually mice, rats, rabbits, dogs, or pigs. An animal model may also be used to determine the appropriate concentration range and route of administration, which information is then commonly used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, e.g. a QPCTL or fragment thereof, antibodies of DPRP, or an agonist, antagonist or inhibitor of QPCTL, which ameliorates particular symptoms or conditions of the disease. For example, the amount to be administered may be effective to cyclise N-terminal Glu or Gln of a desired target substrate upon contact therewith. Therapeutic efficacy and toxicity may likewise be determined by standard pharmaceutical procedures in cell cultures or with experimental animals, such as by calculating the ED50 (the dose therapeutically effective in 50% of the population) or LD50 (the dose lethal to 50% of the population) statistics. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the LD50/ED50 ratio. Pharmaceutical compositions, which exhibit large therapeutic indices, are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, the sensitivity of the patient, and the route of administration.

An exact dosage will normally be determined by the medical practitioner in light of factors related to the subject requiring treatment, with dosage and administration being adjusted to provide a sufficient level of the active moiety or to maintain a desired effect. Factors to be taken into account include the severity of the disease state, the general health of the subject, the age, weight, and gender of the subject, diet, time and frequency of administration, drug combination (s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or even once every two weeks, depending on the half-life and clearance rate of the particular formulation.

Yet another aspect of the invention provides polynucleotide molecules having sequences that are antisense to mRNA transcripts of a polynucleotide of SEQ ID NOS 2 to 9. Administration of an antisense polynucleotide molecule can block the production of the protein encoded by the QPCTL genes of SEQ ID NOS 2 to 9. The techniques for preparing antisense polynucleotide molecules and administering such molecules are known in the art. For example, antisense polynucleotide molecules can be encapsulated into liposomes for fusion with cells.

In particular, the expression of the QPCTL genes of SEQ ID NOS 2 to 9 in brain, prostate, lung, heart, liver, spleen and kidney tissue provides evidence for a potential role in the pathophysiology of the diseases described below. Therefore in a further aspect, the invention relates to diagnostic assays for detecting diseases associated with inappropriate QPCTL activity or expression levels. Antibodies that specifically bind QPCTL may be used for the diagnosis of disorders characterized by expression of QPCTL, or in assays to monitor patients being treated with QPCTL or with agonists or antagonists (inhibitors) of QPCTL. Antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for QPCTL include methods that utilize the antibody and a label to detect QPCTL in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and they may be labeled by covalent or non-covalent joining with a reporter molecule. A wide variety of reporter molecules are known in the art. Recombinant QPCTL proteins that have been modified so as to be catalytically inactive can also be used as dominant negative inhibitors. Such modifications include, for example, mutation of the active site.

A variety of protocols for measuring QPCTL, including ELISAs, RIAs and FACS, are known in the art and provide a basis for diagnosing altered or abnormal levels of QPCTL expression. Normal or standard values for QPCTL expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to QPCTL under conditions suitable for complex formation. The method for detecting QPCTL in a biological sample would comprise the steps of a) providing a biological sample; b) combining the biological sample and an

anti-QPCTL antibody under conditions which are suitable for complex formation to occur between QPCTL and the antibody; and c) detecting complex formation between QPCTL and the antibody, thereby establishing the presence of QPCTL in the biological sample.

The amount of complex formation then may be quantified by various methods, preferably by photometric means. Quantities of QPCTL expressed in a subject, control, and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding QPCTL are used for diagnostic purposes, which polynucleotides may include oligonucleotide sequences, complementary RNA and DNA molecules, and PNAs. These polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of QPCTL may be correlated with one of the diseases. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of QPCTL and to monitor regulation of QPCTL levels during therapeutic intervention. Moreover, pharmacogenomic, single nucleotide polymorphisms (SNP) analysis of the QPCTL genes can be used as a method to screen for mutations that indicate predisposition to disease or modified response to drugs.

QPCTL polynucleotide and polypeptide sequences, fragments thereof, antibodies of QPCTLs, and agonists, antagonists or inhibitors of QPCTLs can be used as discovery tools to identify molecular recognition events and therefore proteins, polypeptides and peptides that interact with QPCTL proteins. A specific example is phage display peptide libraries where greater than 108 peptide sequences can be screened in a single round of panning. Such methods as well as others are known within the art and can be utilized to identify compounds that inhibit or enhance the activity of any one of the QPCTLs of SEQ ID NOS 11-18.

Coupled links represent functional interactions such as complexes or pathways, and proteins that interact with QPCTLs can be identified by a yeast two-hybrid system, proteomics (differential 2D gel analysis and mass spectrometry) and genomics

(differential gene expression by microarray or serial analysis of gene expression SAGE).

Proteins identified as functionally linked to QPCTLs and the process of interaction form the basis of methods of screening for inhibitors, agonists and antagonists and modulators of these QPCTL-protein interactions.

The term "antagonist", as it is used herein, refers to an inhibitor molecule which, when bound to QPCTL, decreases the amount or the duration of the effect of the biological or immunological activity of QPCTL, e. g. decreasing the enzymatic activity of the peptidase to cyclise Glu- or Gln-residues at the N-termini of the QPCTL substrates. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, or any other molecules which decrease the effect of QPCTL; for example, they may include small molecules and organic compounds that bind to and inactivate QPCTLs by a competitive or non-competitive type mechanism. Preferred are small molecule inhibitors of QPCTL. Most preferred are competitive small molecule inhibitors of QPCTL.

Specific examples of QPCTL enzyme activity inhibitors are described in Example 4. Inhibitors can be, for example, inhibitors of the QPCTL cyclase activity, or alternatively inhibitors of the binding activity of the QPCTL to proteins with which they interact. Specific examples of such inhibitors can include, for example, anti-QPCTL antibodies, peptides, protein fragments, or small peptidyl protease inhibitors, or small non-peptide, organic molecule inhibitors which are formulated in a medium that allows introduction into the desired cell type. Alternatively, such inhibitors can be attached to targeting ligands for introduction by cell-mediated endocytosis and other receptor mediated events. Such methods are described further below and can be practiced by those skilled in the art given the QPCTL nucleotide and amino acid sequences described herein.

A further use of QPCTLs is for the screening of potential antagonists for use as therapeutic agents, for example, for inhibiting binding to QPCTL, as well as for screening for agonists. QPCTL, its immunogenic fragments, or oligopeptides thereof can be used for screening libraries of compoundswhich are prospective agonists or antagonists in any of a variety of drug screening techniques. The fragment employed in

such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes between QPCTL and the agent being tested is then measured. Other assays to discover antagonists that will inhibit QPCTL are apparent from the disclosures of Patents Nos. WO 2004/098625, WO 2004/098591 and WO 2005/075436, which describe inhibitors of QC and which are incorporated herein in their entirety. Another worthwhile use of these QPCTLs is the screening of inhibitors of QC to show that they will not have undesired side effects by also inhibiting one or more of the QPCTLs.

A method provided for screening a library of small molecules to identify a molecule which binds QPCTL generally comprises: a) providing a library of small molecules; b) combining the library of small molecules with the polypeptide of either SEQ ID NOS 11 to 18, or with a fragment thereof, under conditions which are suitable for complex formation; and c) detecting complex formation, wherein the presence of such a complex identifies a small molecule, which binds to the QPCTL.

One method for identifying an antagonist comprises delivering a small molecule which binds QPCTL into extracts from cells transformed with a vector expressing QPCTL along with a chromogenic substrate (e. g. Ala-Pro-AFC or Ala-Pro-AMC) under conditions where cleavage would normally occur, and then assaying for inhibition of cleavage by the enzyme by monitoring changes in fluorescence, or UV light absorption, by spectrophotometry to identify molecules that inhibit cleavage. A reduced rate of reaction or total amount of fluorescence or UV light absorption, in the presence of the molecule, establishes that the small molecule is an antagonist, which reduces QPCTL catalytic/enzymatic activity. Once such molecules are identified, they may be administered to reduce or inhibit cyclisation of N-terminal Glu- or Gln-residues by a QPCTL.

In accordance with still another specific aspect, the invention provides a method of screening for a compound capable of inhibiting the enzymatic activity of at least one mature protein according to the present invention, preferably selected from the proteins of SEQ ID NOS: 11 to 18, which method comprises incubating said mature protein and a suitable substrate for said mature protein in the presence of one or more test

compounds or salts thereof, measuring the enzymatic activity of said mature protein, comparing said activity with comparable activity determined in the absence of a test compound, and selecting the test compound or compounds that reduce the enzymatic activity.

Furthermore, the invention also provides a method of screening for a selective QC-inhibitor, i.e. a compound capable of inhibiting the enzymatic activity of QC, wherein said QC is preferably the protein of SQ ID NO: 10, that does not inhibit the enzymatic activity of at least one mature protein according to the present invention, preferably selected from the proteins of SEQ ID NOS: 11 to 18, which method comprises incubating said mature protein and a suitable substrate in the presence of one or more inhibitors or salts thereof of QC, measuring the enzymatic activity of said mature protein, comparing said activity with comparable activity determined in the absence of the QC inhibitor, and selecting a compound that does not reduce the enzymatic activity of said mature protein.

Furthermore, the invention also provides a method of screening for a selective QPCTL-inhibitor, i.e. a compound capable of inhibiting the enzymatic activity of at least one QPCTL protein, which is preferably selected from the proteins of SEQ ID NOS: 11 to 18; that does not inhibit the enzymatic activity of QC, wherein said QC is preferably the protein of SQ ID NO: 10, which method comprises incubating said QC in the presence of one or more inhibitors or salts thereof of a QPCTL, measuring the enzymatic activity of QC, comparing said activity with comparable activity determined in the absence of the QPCTL inhibitor, and selecting a compound that does not reduce the enzymatic activity of said QPCTL protein.

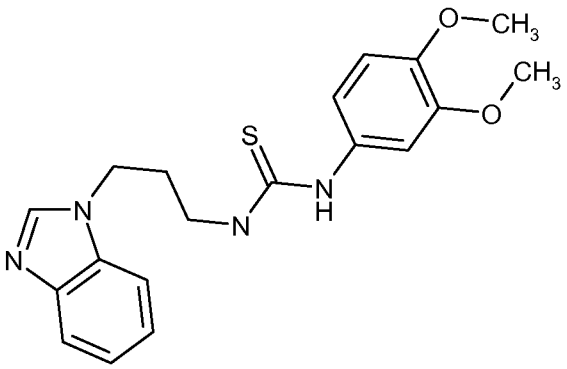
Useful inhibitors of QC, which also could be useful as inhibitors of QPCTLs, are described in WO 2004/098625, WO 2004/098591, WO 2005/039548 and WO 2005/075436, which are incorporated herein in their entirety, especially with regard to the structure of the inhibitors and their production.

Examples of QPCTL-inhibitors

Potential QPCTL-inhibitors, which are suitable for uses and methods according to the present invention are disclosed in WO 2005/075436, which is incorporated herein in its entirety with regard to the structure, synthesis and methods of use of the QC-inhibitors.

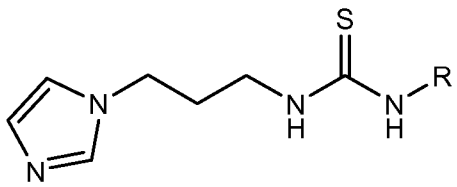
In particular:

A suitable compound is that of formula 1*:



formula 1*

In a further embodiment, the inhibitors of QPCTL may be those of formula 1a,



(1a)

wherein R is defined in examples 1 to 53.

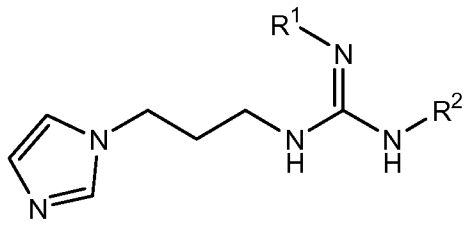
Example	R	ESI-MS (M+H)
1	Methyl	199.3
2	*tert*-Butyl	241.4
3	Benzyl	275.4
4	Phenyl	261.4
5	4-(fluoro)-phenyl	279.35
6	4-(chloro)-phenyl	295.80
7	4-(ethyl)-phenyl	289.41
8	4-(trifluoromethyl)-phenyl	329.4

Example	R	ESI-MS (M+H)
9	4-(methoxy-carbonyl)-Phenyl	319.4
10	4-(acetyl)-phenyl	303.4
11	4-(methoxy)-phenyl	291.4
12	bicyclo[2.2.1]hept-5-en-2-yl	277.5
13	3,4-(dimethoxy)-phenyl	321.5
14	2,4-(dimethoxy)-phenyl	321.5
15	3,5-(dimethoxy)-phenyl	321.5
16	2-(methoxy-carbonyl)-Phenyl	319.4
17	4-(oxazol-5-y)-phenyl	328.5
18	4-(pyrazol-1-yl)-phenyl	327.4
19	4-(isopropyl)-phenyl	303.5
20	4-(piperidine-1-sulfonyl)-Phenyl	408.6
21	4-(morpholin-4-yl)-phenyl	346.5
22	4-(cyano)-phenyl	286.4
23	2,3-dihydro-benzo[1,4] dioxin-6-yl	319.4
24	benzo[1,3]dioxol-5-yl	305.4
25	3,4,5(trimethoxy)-phenyl	351.5
26	3-(methoxy)-phenyl	291.4
27	4-(ethoxy)-phenyl	305.5
28	4-(benzyloxy)-phenyl	367.5
29	4-(methoxy)-benzyl	305.5
30	3,4-(dimethoxy)-benzyl	335.5
31	2-(methoxy-carbonyl)-thiophene-3-yl	325.5

Example	R	ESI-MS (M+H)
32	3-(ethoxy-carbonyl)-4,5,6,7-tetrahydrobenzo[b]thio-phene2-yl	392.6
33	2-(methoxy-carbonyl)-4-(methyl)-thiophene-3-yl	339.5
34	Benzo[c][1,2,5]thiazol-4-yl	319.5
35	Benzo[c][1,2,5]thiazol-5-yl	319.5
36	5-(methyl)-3-(phenyl)-isooxazol-4-yl	342.5
37	3,5-(dimethyl)-isooxazol-4-yl	280.4
38	4-(iodo)-phenyl	387.3
39	4-(bromo)-phenyl	340.3
40	4-(methyl)-phenyl	275.4
41	Naphthalen-1-yl	311.5
42	4-(nitro)-phenyl	306.4
43	Butyl	241.4
44	Cyclooctyl	295.5
45	Furan-2-ylmethyl	265.4
46	Tetrahydrofuran-2-ylmethyl	269.4
47	Benzo[1,3]dioxol-5-ylmethyl	319.4
48	2-(morpholin-4-yl)-ethyl	298.5
49	4-(methylsulfanyl)-phenyl	307.5
50	4-(dimethylamino)-phenyl	304.5
51	4-(trifluoromethoxy)-phenyl	345.4

Example	R	ESI-MS (M+H)
52	Benzoyl	288.3
53	Pyridin-4-yl	261.1

Further suitable inhibitors of QPCTL may be those of formula 1b,



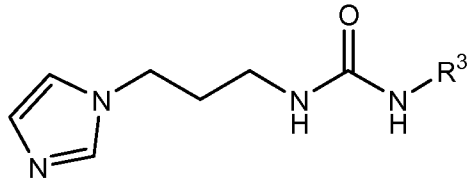
(1b)

wherein R^1 and R^2 are defined in examples 54 to 95.

Example	R^1	R^2
54	Cyano	Methyl
55	Cyano	3,4-(dimethoxy)-phenyl
56	Cyano	2,4-(dimethoxy)-phenyl
57	Cyano	3,5-(dimethoxy)-phenyl
58	Cyano	2,3-dihydrobenzo[b][1,4]dioxin-7-yl
59	Cyano	Benzo[d][1,3]dioxol-6-yl
60	Cyano	3,4,5-(trimethoxy)-phenyl
61	Cyano	3-(methoxy)-phenyl
62	Cyano	4-(ethoxy)-phenyl
63	Cyano	4-(benzyloxy)-phenyl
64	Cyano	Phenyl
65	Cyano	4-(methoxy)-phenyl
66	Cyano	4-(acetyl)-phenyl
67	Cyano	4-(nitro)-phenyl
68	Cyano	Benzyl
69	Cyano	Naphthalen-1-yl

Example	R^1	R^2
70	Cyano	4-(fluoro)-phenyl
71	Cyano	4-(iodo)-phenyl
72	Cyano	4-(bromo)-phenyl
73	Cyano	Cyclooctyl
74	Cyano	*tert*-butyl
75	Cyano	4-(methyl)-phenyl
76	Cyano	4-(methylthio)-phenyl
77	Cyano	4-(ethyl)-phenyl
78	Cyano	4-(dimethylamino)-phenyl
79	Cyano	Butyl
80	Cyano	Trityl
81	Cyano	(Benzo[d][1,3]dioxol-6yl)methyl
82	Cyano	(tetrahydrofuran-2yl)methyl
83	Cyano	4-(trifluoromethyl)-phenyl
84	Cyano	(furan-2-yl)methyl
85	Cyano	2-(morpholin-4-yl)-ethyl
86	Cyano	4-(oxazol-5yl)-phenyl
87	Cyano	Pyridin-3-yl
88	Cyano	4-(cyano)-phenyl
89	Cyano	4-(trifluoromethoxy)-phenyl
90	Cyano	4-(piperidinosulfonyl)-phenyl
91	Cyano	4-(1H-pyrazol-1-yl)phenyl
92	H	3,4-(dimethoxy)-phenyl
93	Methyl	3,4-(dimethoxy)-phenyl
94	Cyano	2,3,4-(trimethoxy)-phenyl
95	Cyano	Cycloheptyl

Further suitable inhibitors of QPCTL may be those of formula 1c,

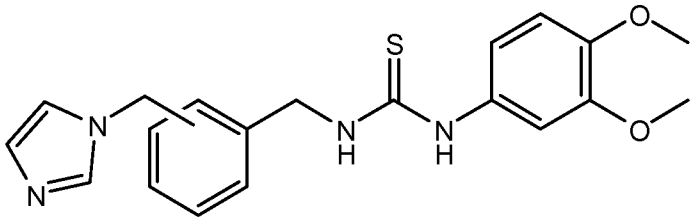


(1c)

wherein R^3 is defined in examples 96 to 102.

Example	R^3	ESI-MS (M+H)
96	Ethyl	197.3
97	6-fluoro-4H-benzo[d] [1,3]dioxin-8-yl	321.4
98	3-(cylopentyloxy)-4-(methoxy)-phenyl	359.4
99	4-(heptyloxy)-phenyl	359.5
100	3,4-dihydro-2H-benzo[b] [1,4]dioxepin-7-yl	317.4
101	4-(butoxy)-phenyl	317.4
102	3,4-(dimethoxy)-phenyl	305.4

Further suitable inhibitors of QPCTL may be those of formula 1d,

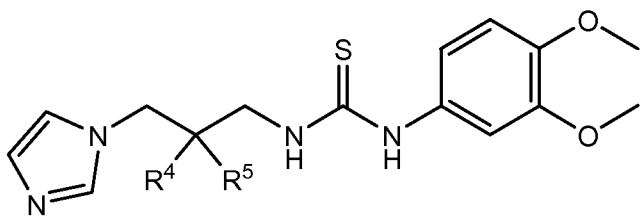


(1d)

wherein the position on the ring is defined in examples 103 to 105.

Example	Position of the Benzyl-substitution	ESI-MS (M+H)
103	2	383.5
104	3	383.5
105	4	383.5

Further suitable inhibitors of QPCTL may be those of formula 1e,

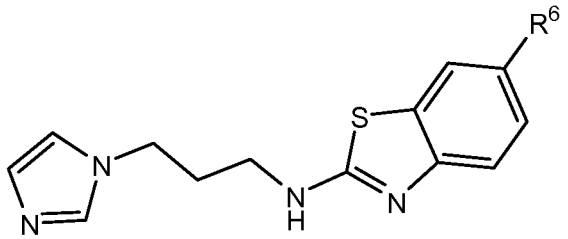


(1e)

wherein R^4 and R^5 are defined in examples 106 to 109.

Example	R^4	R^5	ESI-MS (M+H)
106(S)	H	Methyl	335.5
107(R)	Methyl	H	335.5
108	Methyl	Methyl	349.5
109	$-CH_2-CH_2-$		347.5

Further suitable inhibitors of QPCTL may be those of formula 1f,

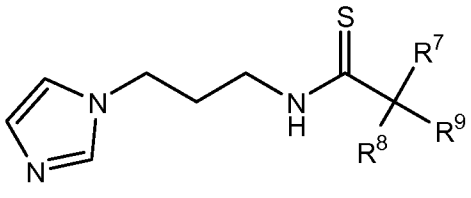


(1f)

wherein R^6 is defined in examples 110 to 112.

Example	R^6	ESI-MS (M+H)
110	H	259.4
111	Chloro	293.8
112	Methoxy	289.4

Further suitable inhibitors of QPCTL may be those of formula 1g,

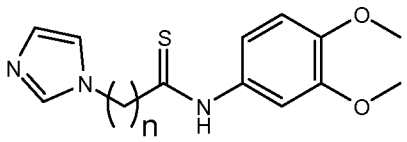


(1g)

wherein R^7, R^8 and R^9 are defined in examples 113 to 132.

Example	R^7	R^8	R^9	ESI-MS (M+H)
113	Phenyl	H	H	260.4
114	Thiophen-2-yl	H	H	266.5
115(R)	Phenyl	Methyl	H	274.5
116(S)	Phenyl	H	Methyl	274.5
117	Phenyl	H	Ethyl	288.5
118	Phenyl	H	Phenyl	336.5
119	3,4-(dimethoxy)-Phenyl	H	H	320.5
120	3,4-(dimethoxy)-Phenyl	Methyl	Methyl	347.2
121	4-(chloro)-phenyl	$-CH_2-CH_2-CH_2-$		334.9
122	4-(chloro)-phenyl	$-CH_2-C_2H_4-CH_2-$		349.0
123	4-(methoxy)-phenyl	$-CH_2-C_3H_6-CH_2-$		358.6
124	4-(methoxy)-phenyl	$-CH_2-CH_2-$		316.5
125	3,4-(dimethoxy)-Phenyl	$-CH_2-CH_2-$		346.5
126	3,4,5-(trimethoxy)-Phenyl	$-CH_2-CH_2-$		376.6
127	2,3,4-(trimethoxy)-Phenyl	$-CH_2-CH_2-$		376.6
128	2-(methoxy)-phenyl	$-CH_2-CH_2-$		316.5
129	3-(methoxy)-phenyl	$-CH_2-CH_2-$		316.5
130	2,3-(dimethoxy)-Phenyl	$-CH_2-CH_2-$		346.5
131	3,5-(dimethoxy)-Phenyl	$-CH_2-CH_2-$		346.5
132	2,5-(dimethoxy)-Phenyl	$-CH_2-CH_2-$		346.5

Further suitable inhibitors of QPCTL may be are those of formula 1h,

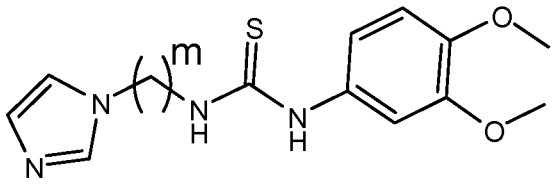


(1h)

wherein n is defined in examples 133 to 135.

Example	N	ESI-MS (M+H)
133	3	306.4
134	4	320.5
135	5	334.5

Further suitable inhibitors of QPCTL may be those of formula 1i,

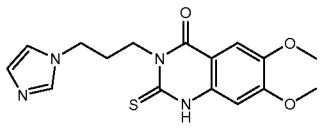
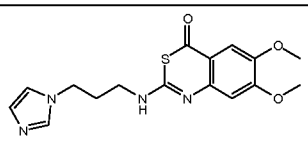


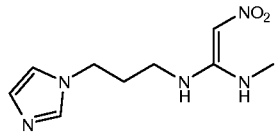
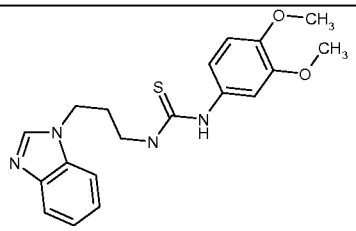
(1i)

wherein m is defined in examples 136 and 137.

Example	m	ESI-MS (M+H)
136	2	307.4
137	4	335.5

Further suitable inhibitors of QPCTL may be those of formula 138 to 141.

Example	Structure	ESI-MS (M+H)
138		347.5
139		347.2

Example	Structure	ESI-MS (M+H)
140		226.3
141		370.4

The term "agonist", as used herein, refers to a molecule which, when bound to QPCTL, increases or prolongs the duration of the effect of QPCTL. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules that bind to and modulate the effect of QPCTL. Although it is less likely that small molecules will prove to be effective QPCTL agonists, a method for identifying such a small molecule, which binds QPCTL as an agonist, comprises delivering a chromogenic form of a small molecule that binds QPCTL into cells transformed with a vector expressing QPCTL and assaying for fluorescence or UV light absorption changes by spectrophotometry. An increased amount of UV absorption or fluorescence would establish that the small molecule is an agonist that increases QPCTL activity.

Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application W0 84/03564. In this method, large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with QPCTL, or with fragments thereof, and then washed. Bound QPCTL is then detected by methods well known in the art. Purified QPCTL can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding QPCTL specifically compete with a test compound for binding QPCTL. In this manner, antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants with QPCTL.

As indicated above, by investigating the binding sites, ligands may be designed that, for example, have more interactions with QPCTL than do its natural ligands. Such antagonist ligands will bind to QPCTL with higher affinity and so function as competitive ligands. Alternatively, synthetic or recombinant proteins homologous or analogous to the ligand binding site of native QPCTL may be designed, as may other molecules having high affinity for QPCTL. Such molecules should also be capable of displacing QPCTL and provide a protective effect.

As indicated above, the knowledge of the structures of QPCTL enables synthetic binding site homologues and analogues to be designed. Such molecules will facilitate greatly the use of the binding properties to target potential therapeutic agents, and they may also be used to screen potential therapeutic agents. Furthermore, they may be used as immunogens in the production of monoclonal antibodies, which antibodies may themselves be used in diagnosis and/or therapy as described hereinbefore.

Therapeutic applications

It is known in the art that amyloid peptides, e.g. Abeta 1-42 (SEQ ID NO 23) and Abeta 1-40 (SEQ ID NO 24) become N-terminally truncated by proteolytic enzymes such as for example aminopeptidases or dipeptidyl aminopeptidases, resulting in the Abeta-peptides 3-42 (SEQ ID NO 25), 3-40 (SEQ ID NO 26), 11-42 (SEQ ID NO 27) and 11-40 (SEQ ID NO 28). These truncated Abeta peptides start with a glutamate residue at the N-terminus and are thus substrates for QC (see also WO 2004/09862) and possibly also for the QPCTLs of SEQ ID NOS 11-18, 21 and 22, preferably the human isoQCs of SEQ ID NOS 11, 12, 21 and 22, most preferably the human isoQCs of SEQ ID NOS 11 and 12. The resulting pGlu-Abeta peptides of SED ID NOS 29-32 are much more hydrophobic than the non-pyroglutamted peptides, are much more prone to form A-beta peptide aggregates, such as oligomers and fibrills, and were shown to by highly

neurotoxic. Finally, the Abeta-peptides of SEQ ID NOS 29-32 play a crucial role in the development of Alzheimer's disease and Down Syndrome.

Accordingly, inhibitors of the QPCTLs of SEQ ID NOS 11-18, 21 and 22, preferably the human isoQCs of SEQ ID NOS 11, 12, 21 and 22, most preferably the human isoQCs of SEQ ID NOS 11 and 12, may be used for the treatment of amyloid peptide related diseases, especially neurodegenerative diseases, in particular Alzheimer's disease and Down Syndrome.

Other potential physiological substrates of QPTCLs in mammals are selected from the group consisting of Glu^1-ABri (SEQ ID NO 33), Glu^1-ADan (SEQ ID NO 34), and Gln^1-Gastrins (17 and 34) (SEQ ID NOS 35 and 36). Their pyroglutamated forms (SEQ ID NOS 37-40) cause pathologies such as those selected from the group consisting of duodenal cancer with or w/o *Helicobacter pylori* infections, colorectal cancer, Zolliger-Ellison syndrome, Familial British Dementia (FBD) and Familial Danish Dementia (FDD). Accordingly, inhibitors of QPCTLs can be used to treat these pathologies.

Further potential physiological substrates of QPCTLs are shown in table 3.

Table 3: Amino acid sequences of physiological active peptides with an N-terminal glutamine residue

Peptide	Amino acid sequence	Function
Gastrin 17 (SEQ ID NO 35) 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 (SEQ ID NO 41) Swiss-Prot: P30990	QLYENKPRRP YIL	Neurotensin plays an endocrine or paracrine role in the regulation of fat metabolism. It causes contraction of smooth muscle.

Peptide	Amino acid sequence	Function
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 (SEQ ID NO 42) 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) (SEQ ID NO 43) 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 inducible cytokine A8) (SEQ ID NO 44) Swiss-Prot: P80075	QPDSVSI PITCCFNVIN RKIPIQRLES YTRITNIQCP KEAVIFKTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP	Chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils. May play a role in neoplasia and inflammatory host responses. This protein can bind heparin.

Peptide	Amino acid sequence	Function
CCL2 (small inducible cytokine A2) (SEQ ID NO 45) 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) (SEQ ID NO 46) 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) (SEQ ID NO 47) Swiss-Prot: P78423	QHHGVT KCNITCSKMT SKIPVALLIH YQQNQASCGK RAIILETRQH RLFCADPKEQ WVKDAMQHLD RQAAALTRNG GTFEKQIGEV KPRTTPAAGG MDESVVLEPE ATGESSSLEP TPSSQEAQRA LGTSPELPTG VTGSSGTRLP PTPKAQDGGP VGTELFRVPP VSTAATWQSS APHQPGPSLW AEAKTSEAPS TQDPSTQAST ASSPAPEENA PSEGQRVWGQ GQSPRPENSL EREEMGPVPA HTDAFQDWGP GSMAHVSVVP VSSEGTPSRE PVASGSWTPK AEEPIHATMD PQRLGVLITP VPDAQAATRR	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 a role in regulating leukocyte adhesion and migration processes at the endothelium. Binds to cx3cr1.

Peptide	Amino acid sequence	Function
	QAVGLLAFLG LLFCLGVAMF TYQSLQGCPR KMAGEMAEGL RYIPRSCGSN SYVLVPV	
CCL7 (small inducible cytokine A7) (SEQ ID NO 48) 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) (SEQ ID NO 49) 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 (SEQ ID NO 50)	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.

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 QPCTLs. Gastrin, Neurotensin and FPP comprise a pGlu residue in their N-terminal position. This N-

terminal pGlu residue may be formed from N-terminal glutamine by QPCTL 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 93*37-44).

In a further embodiment, the present invention provides the use of activity increasing effectors of QPCTLs 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 (SEQ ID NOS 39 and 40).

In a further embodiment, the present invention provides the use of inhibitors of QPCTLs for the treatment of duodenal ulcer disease and gastric cancer with or w/o *Helicobacter pylori* infections in mammals by decreasing the conversion rate of inactive Gln^1-Gastrin (SEQ ID NOS 35 and 36) to active $pGlu^1$-Gastrin (SEQ ID NOS 39 and 40).

Neurotensin (NT) (SEQ ID NO 41) 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).

Accordingly, the present invention provides the use of activity increasing effectors of QPCTLs for the preparation of antipsychotic drugs and/or for the treatment of schizophrenia in mammals. The effectors of QPCTLs 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 a further embodiment, the present invention provides the use of inhibitors of QPCTLs for the preparation of fertilization prohibitive drugs and/or to reduce the fertility in mammals. The inhibitors of QPCTLs 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 further embodiment, further physiological substrates of QPCTLs were identified within the present invention. These are Gln^1-CCL2 (SEQ ID NO 45), Gln^1-CCL7 (SEQ ID NO 48), Gln^1-CCL8 (SEQ ID NO 44), Gln^1-CCL16 (SEQ ID NO 43), Gln^1-CCL18 (SEQ ID NO 46) and Gln^1-fractalkine (SEQ ID NO 47). For details see Table 3. 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.).

Accordingly, the present invention provides the use of inhibitors of QPCTLs 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.

Furthermore, Gln^1-orexin A (SEQ ID NO 49) was identified as a physiological substrate of QPCTLs 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 further embodiment, the present invention provides the use of inhibitors of QPCTLs 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). Accordingly, the present invention provides therefore the use of inhibitors of QPCTLs for the preparation of a medicament for the treatment of Parkinson disease and Huntington's disease.

A further substrate of QPTCLs is the peptide QYNAD (SEQ ID NO 51). Its pyroglutamated form pGlu-Tyr-Asn-Ala-Asp (pEYNAD) (SEQ ID NO 52) is the effective agent with blocking activity of voltage-gated sodium channels. Sodium channels are expressed at high density in myelinated axons and play an obligatory role in conducting action potentials along axons within the mammalian brain and spinal cord. Therefore, it is speculated that they are involved in several aspects of the pathophysiology of multiple sclerosis (MS), the Guillain-Barré syndrome and chronic inflammatory demyelinizing polyradiculoneuropathy.

In a further embodyment, the present invention provides the use of inhibitors of QPCTLs for the preparation of a medicament for the treatment of inflammatory autoimmune diseases, especially for multiple sclerosis, the Guillain-Barré syndrome and chronic

inflammatory demyelinizing polyradiculoneuropathy, wherein the formation of the voltage-gated sodium channel blocking peptide pEYNAD is inhibited.

Furthermore, the present invention provides a diagnostic assay, comprising a QC-inhibitor.

In another embodiment, the present invention provides a method of diagnosing any one of the aforementioned diseases and/or conditions, comprising the steps of

- collecting a sample from a subject who is suspected to be afflicted with said disease and/or condition,
- contacting said sample with a QC-inhibitor, and
- determining whether or not said subject is afflicted by said disease and/or condition.

Preferably, the sample in said diagnosing method is a blood sample, a serum sample, a sample of cerebrospinal liquor or a urine sample.
Preferably, the subject in said diagnosing method is a human being.
Preferably, the QC inhibitor in said diagnosing method is a selective QC inhibitor.
Further preferred for the use in said diganostic assay are selective QPCTL inhibitors.

The present invention further pertains to a diagnostic kit for carrying out the dignosing method comprising as detection means the aforementioned diagnostic assay and a determination means.

Example 1: Preparation of human isoQC

Cell lines and media

African green monkey kidney cell line COS-7, human neuroblastoma cell line SH-SY5Y, human asatrocytoma cell line LN405, human keratinocytoma cell line HaCaT and human hepatocellular carcinoma cell line Hep-G2 were cultured in appropriate cell culture media (DMEM, 10 % FBS for Cos-7, SH-SY5Y, LN405, HaCaT), (RPMI1640, 10% FBS for Hep-G2), in a humidified atmosphere of 5% CO_2 (HaCaT, Hep-G2, COS-7) or 10% CO_2 (SH-SY5Y, LN405) at 37°C.

Analysis of human isoQC Expression using RT-PCR

Total RNA was isolated from SH-SY5Y, LN405, HaCaT and Hep-G2 cells using the RNeasy Mini Kit (Qiagen) and reversely transcribed by SuperScript II (Invitrogen). Subsequently, human isoQC was amplified on a 1:12,5 dilution of generated cDNA product in a 25 µl reaction with Herculase Enhanced DNA-Polymerase (Stratagene) using primers isoQCh-1 (sense, SEQ ID NO: 53) and isoQCh-2 (antisense, SEQ ID NO: 54). The PCR product of Hep-G2 was purified utilizing the Strataprep PCR Purification Kit (Stratagene) and confirmed by sequencing.

Results

Analysis of human isoQC Expression using RT-PCR

Transcripts of human isoQC were found to be present in cell lines SH-SY5Y (Figure 6, lane 1), LN405 (Figure 6, lane 2), HaCaT (Figure 6, lane 3) and Hep-G2 (Figure 6, lane 4). The PCR product of Hep-G2 was confirmed by sequencing.

Isolation of human isoQC

Full-length cDNA of human isoQC was isolated from Hep-G2 cells using RT-PCR. Briefly, total RNA of Hep-G2 cells was reversely transcribed by SuperScript II (Invitrogen). Subsequently, human isoQC was amplified on a 1:12,5 dilution of generated cDNA product in a 25 µl reaction with Herculase Enhanced DNA-Polymerase (Stratagene) using primers isoQChu-1 (sense, SEQ ID NO: 55) and isoQChu-2 (antisense, SEQ ID NO: 56). The resulting PCR-product was subcloned into vector pPCRScript CAM SK (+) (Stratagene) and confirmed by sequencing.

Example 2: Preparation and Expression of human isoQC in mammalian cell culture

Molecular cloning of plasmid vectors encoding a human isoQC-EGFP fusion protein

All cloning procedures were done applying standard molecular biology techniques. For expression of human isoQC-EGFP fusion protein in human cells, the vector pEGFP-N3

(Invitrogen) was used. The cDNA of the native human isoQC starting either at methionine I or at methionine II was fused N-terminally in frame with the plasmid encoded enhanced green fluorescent protein (EGFP). The primers isoQC EGFP-1 Met I (SEQ ID NO: 57) and isoQC EGFP-3 (SEQ ID NO: 59) were used for amplification of human isoQC starting with methionine I and primers isoQC EGFP-2 Met II (SEQ ID NO: 58) and isoQC EGFP-3 (SEQ ID NO: 59) were used for amplification of human isoQC starting with methionine II. The fragments were inserted into vector pEGFP-N3 (Invitrogen) employing the restriction sites of *EcoR*I and *Sal*I and the correct insertion was confirmed by sequencing. Subsequently, the vectors were isolated for cell culture purposes using the EndoFree Maxi Kit (Qiagen).

Cloning procedure of the N-terminal sequences of hisoQC

In addition, the EGFP sequence of vector pEGFP-N3 (Invitrogen) was introduced into vector pcDNA 3.1 (Invitrogen) using EGFP-1 (sense) (SEQ ID NO: 85) and EGFP-2 (antisense) (SEQ ID NO: 86) for amplification. The fragment was introduced into *Xho*I site of pcDNA 3.1. The N-terminal sequences of hisoQC beginning with methionine I and II each ending at serine 53 were fused C-terminally with EGFP in vector pcDNA 3.1 using isoQC EGFP-1 Met I (sense, SEQ ID NO: 57) and hisoQC SS EGFP pcDNA as (antisense) (SEQ ID NO: 87) for the N-terminal fragment of hisoQC beginning with methionine I and isoQC EGFP-2 Met II (sense, SEQ ID NO: 58) and hisoQC SS EGFP pcDNA as (antisense) (SEQ ID NO: 87) for the N-terminal fragment of hisoQC beginning with methionine II. Fragments were inserted into *Eco*RI and *Not*I restrictione sites of vector pcDNA 3.1. Subsequently, the vectors were isolated for cell culture purposes using the EndoFree Maxi Kit (Qiagen).

Cloning procedure for native expression of hisoQC and hQC

Native hQC was inserted into *Hind*III and *Not*I restriction sites and native hisoQC was inserted into *Eco*RI and *Not*I restriction sites of vector pcDNA 3.1 (+) (Invitrogen) after amplification utilizing primers hQC-1 (sense) (SEQ ID NO: 82) and hQC-2 (antisense) (SEQ ID NO: 83) for hQC, isoQC EGFP-1 Met I (sense) (SEQ ID NO: 57) and hisoQC pcDNA as (antisense) (SEQ ID NO: 84) for hisoQC starting with methionine I and isoQC EGFP-2 Met II (sense) (SEQ ID NO: 58) and hisoQC pcDNA as (antisense) (SEQ ID NO: 84) for hisoQC starting with methionine II.

Cloning procedure for FLAG-tagged hisoQC and hQC

Human QC was cloned with a C-terminal FLAG-tag after amplification applying primers hQC-1 (sense) (SEQ ID NO: 82) and hQC C-FLAG pcDNA as (antisense) (SEQ ID NO: 88) into HindIII and NotI restriction sits of vector pcDNA 3.1. Human isoQC was inserted with a C-terminal FLAG-tag into pcDNA 3.1 after amplification using primers isoQC EGFP-1 Met I (sense) (SEQ ID NO: 57) and hisoQC C-FLAG pcDNA as (antisense) (SEQ ID NO: 89) for hisoQC starting with methionine 1 and primers isoQC EGFP-2 Met II (sense) (SEQ ID NO: 58) and hisoQC C-FLAG pcDNA as (antisense) (SEQ ID NO: 89) for hisoQC starting with methionine 2.

Example 3: Immunhistochemical staining of human isoQC in mammalian cells

Transfection and histochemical staining of COS-7 and LN405

For expression of human isoQC-EGFP fusion proteins starting either with methionine I or methionine II, COS-7 and LN405 were cultured in 6-well dishes containing a cover slip. Cells were grown until 80% confluency, transfected using Lipofectamin2000 (Invitrogen) according to manufacturer's manual and incubated in the transfection solution for 5 hours. Afterwards, the solution was replaced by appropriate growth media and cells were grown over night.

The next day, cells were washed twice with D-PBS (Invitrogen) and fixed using ice-cold methanol for 10 min at –20°C, followed by 3 washing steps using D-PBS for 10 min at room temperature. For staining of the golgi-zone, COS-7 and LN405 were incubated with rabbit anti-mannosidase II polyclonal antibody (Chemicon) in a 1:50 dilution of antibody in D-PBS for 3 h. For staining of mitochondria in COS-7 and LN405, cells were incubated with mouse anti-human mitochondria monoclonal antibody (Chemicon) in a 1:100 dilution of antibody in D-PBS for 3 h at room temperature. Subsequently, the cells were washed 3 times with D-PBS for 10 min. Cells stained for golgi-zone were incubated with goat anti-rabbit IgG secondary antibody conjugated with Rhodamin-RedX (Dianova) for 45 min at room temperature in the dark. Cells stained for mitochondria were incubated with goat anti-mouse IgG secondary antibody conjugated with Rhodamin-RedX (Dianova) for 45 min at room temperature in the dark. Afterwards, cells were washed 3 times with D-PBS for 5 min at room temperature and at least, the

cover slips were mounted on a microscope slide with citiflour. Cells were observed under a fluorescence microscope (Carl-Zeiss).

Results

1. Transfection and histochemical staining of LN405

The expression of human isoQC-EGFP fusion protein starting with methionine I and methionine II in cell line LN405 (green fluorescence) leads to a compartmentalization of the resulting protein. Counterstaining of the golgi-zone of LN405 using mannosidase II antibody (red fluorescence) and subsequent superimposition of human isoQC-EGFP with mannosidase II suggests a localization of human isoQC-EGFP fusion protein within the golgi-compartment (yellow coloration of the merged images) (Figure 7,9). Thereby, it is evident that human isoQC starting at methionine II is sufficient to generate a golgi-localization of the human isoQC fusion protein.

The expression of human isoQC-EGFP fusion protein starting with methionine I and II (green fluorescence) and counterstaining for mitochondria (red fluorescence) did not reveal a localization of human isoQC-EGFP fusion protein starting with methionine I or II within the mitochondria due to the absence of a yellow coloration of the merged images after superimposition (Figure 8, 10).

2. Transfection and histochemical staining of COS-7

In analogy to the expression of human isoQC-EGFP fusion protein starting with methionine I and methionine II in cell line LN405, leads the expression of human isoQC-EGFP fusion protein starting with methionine I and methionine II in COS-7 to a compartmentalization of the resulting protein (green fluorescence). Counterstaining of the golgi-zone of COS-7 cells using mannosidase II antibody (red fluorescence) and subsequent superimposition of human isoQC-EGFP with mannosidase II suggests a localization of human isoQC-EGFP fusion protein within the golgi-compartment of COS-7 (yellow coloration of the merged images) (Figure 11,13). Again, in COS-7 cells the expression of human isoQC-EGFP fusion protein starting at methionine II is sufficient to cause a golgi-localization.

As expected, the expression of human isoQC-EGFP fusion protein starting with methionine I and II in COS-7 (green fluorescence) and counterstaining for mitochondria (red fluorescence) did not result in a localization of human isoQC-EGFP fusion protein

starting with methionine I or II within the mitochondria due to the absence of a yellow coloration of the merged images after superimposition (Figure 12,14).

Example 4: Expression and purification of human isoQC in E. coli

Host strains and media

Escherichia coli strain DH5α was used for propagation of plasmids and *E. coli* strain BL21 was used for the expression of human isoQC. *E. coli* strains were grown, transformed and analyzed according to the manufacturer's instructions (Qiagen(DH5α) Stratagene (BL21)). The media required for *E. coli*, i.e. Luria-Bertani (LB) medium, was 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 *E. coli* BL21, the vector pET41a (Novagen) was used. The cDNA of the mature human isoQC starting with codon 30 (counting from methionine II) was fused in frame with the plasmid encoded GST-tag. After amplification utilizing the primers hisoQC pET41a-1 (SEQ ID NO: 60) and hisoQC pET41a-2 (SEQ ID NO: 61) (Table 4) a N-terminal protease cleavage site for Enterokinase and a C-terminal $(His)_6$-tag was introduced. After subcloning, the fragment was inserted into the expression vector employing the restriction sites of *Spe* I and *EcoR* I.

Expression and purification in *E. coli* BL21

The construct encoding the human isoQC was transformed into BL21 cells (Stratagene) and grown on selective LB agar plates at 37°C. Protein expression was carried out in LB medium containing 1% glucose at 37°C. After reaching an OD_{600} of approximately 0.8, isoQC expression was induced with 20 µM IPTG for 4 h at 37°C. Cells were separated from the medium by centrigugation (4000xg, 20 min), resuspended in PBS (140mM NaCl, 2,7mM KCl, 10mM Na_2HPO_4, 1,8mM KH_2PO_4, pH 7,3) and lysed by one cycle of freezing and thawing followed by one cycle of French Press. The cell lysate was diluted to a final volume of 1.5 l using phosphate-containing buffer (50mM Na_2HPO_4, 500mM NaCl. pH 7,3) and centrifuged at 13.400 x g at 4°C for 1 h. After centrifugation, the protein concentration of the resulting supernatant was determined

using the mothod of Bradford. If necessary, the solution was diluted again to obtain a final total protein concentration of 0.6 mg/ml. The GST-isoQC fusion protein was purified utilizing a 4-step protocol (Table 5). The purfication is illustrated by SDS-PAGE analysis in Figure 20.

Example 5: Assays for glutaminyl cyclase activity

Fluorometric assays

All measurements were performed with a NovoStar reader for microplates (BMG Labtechnologies) 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 (Qiagen, Hilden, Germany) in 0.05 M Tris/HCl, pH 8.0 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 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 isoQC

This assay was used to determine the kinetic parameters for most of the QC substrates. QC activity was analyzed spectrophotometrically using a continuous method (Schilling, S. et al., 2003 Biol Chem 384, 1583-1592) utilizing glutamic dehydrogenase as auxiliary

enzyme. Samples consisted of the respective QC substrate, 0.3 mM NADH, 14 mM α-Ketoglutaric acid and 30 U/ml glutamic dehydrogenase in a final volume of 250 µl. Reactions were started by addition of QC and pursued by monitoring of the decrease in absorbance at 340 nm for 8-15 min. 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 the Sunrise reader for microplates. Kinetic data were 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.

Results

A variety of different substrates was evaluated on conversion by human isoQC (Table 3). All analyzed substrates were converted by isoQC, indicating a relatively relaxed overall specificity similar to human QC (Schilling, S. et al., 2003 Biol Chem 384, 1583-1592). As observed previously for human QC (Schilling, S. et al., 2003 Biol Chem 384, 1583-1592), highest specificity constants (k_{cat}/K_M) were observed for substrates carrying large hydrophobic amino acids adjacent to the N-terminal glutaminyl residue, e.g. Gln-AMC. In contrast, negatively charged residues in that very position led to a drastic drop in specificity, as observed for Gln-Glu, indicating a negatively charged active site of isoQC. Compared to human QC, both recombinant iosQCs exerted a lower enzymatic activity (Figure 21). The difference was up to one order of magnitude. According to the specificity of isoQC, it reasonable to assume that the enzyme is responsible for conversion of different substrates *in vivo*, i.e. isoQC is involved in the generation of many different physiological substrates.

Human isoQC activity was competitively inhibited by imidazole derivatives (table 6, figure 15). The inhibition constants K_i for imidazole and benzimidazole was very similar to the value which was obtained for human QC previously. A 10-fold drop in K_i, however, was observed for the potent QC inhibitor P150/03. Thus, the binding mode of the chelating part, i.e. the imidazole ring, appears to be very similar. Presumably, this results from complexation of the active site zinc ion of QC and isoQC by the imidazole basic nitrogen. The differences in the K_i-values for P150/03 clearly demonstrates that the active sites of both enzymes display subtle differences. Therefore, it is possible to generate inhibitors that exert selectivity for one enzymic isoform. Selective inhibitors are beneficial for the treatment of the diseases.

Table 3: Kinetic evaluation of peptide substrates of human QC and human isoQC. Human isoQC was expressed in *E. coli* BL21 (hisoQCdt) or *P. pastoris* (YSShisoQC). The substrates are displayed in the one-letter code of amino acids.

Substrate	K_M (mM) hisoQCdt	K_M (mM) YSShisoQC	k_{cat} (s^{-1}) hisoQCdt	k_{cat} (s^{-1}) YSShisoQC	k_{cat}/K_M ($mM^{-1}*s^{-1}$) hisoQCdt	k_{cat}/K_M ($mM^{-1}*s^{-1}$) YSShisoQC
Q-βNA	0,03±0,002	0,035±0,0005	3,37±0,12	8,16±0,87	93,26±6,68	228,70±22,22
QAMC	0,01±0,0009	0,03±0,0064	1,07±0,03	3,72±0,44	62,57±5,68	102,87±29,22
QQ	0,11±0,027	0,11±0,007	2,72±0,25	6,08±0,17	24,50±4,009	54,32±4,61
QE	0,7±0,13	0,61±0,064	2,64±0,21	5,33±0,43	3,85±0,56	8,75±0,87
QG	0,42±0,04	0,36±0,047	1,65±0,04	3,24±0,18	3,93±0,31	9,01±1,75
QGP	0,21±0,016	0,23±0,02	4,01±0,14	8,98±0,07	18,82±1,26	38,42±3,55
QYA	0,22±0,01	0,08±0,022	7,7±0,4	16,47±0,72	66,48±13,07	206,9±57,54
QFA	0,11±0,016	0,104±0,025	7,49±0,28	11,68±2,39	33,03±2,38	116,99±34,37
QEYF	0,03±0,004	0,04±0,004	3,34±0,15	5,64±0,39	109,57±21,03	122,56±5,6
QEDL	0,63±0,052	0,16±0,01	6,41±0,15	9,24±0,65	10,2±0,84	55,04±5,14

Table 4: Utilized primers

Primer	Sequence 5' → 3'	Application
IsoQCh-1 (SEQ ID NO : 53)	GGTCTACACCATTTGGAGCGGCTGGC	Cell Line Screening
IsoQCh-2 (SEQ ID NO : 54)	GGGTTGGAAGTACATCACTTCCTGGGG	Cell Line Screening
IsoQChu-1 (SEQ ID NO : 55)	ACCATGCGTTCCGGGGGCCGCGGG	Isolation of hisoQC
IsoQChu-2 (SEQ ID NO : 56)	ACGCTAGAGCCCCAGGTATTCAGCCAG	Isolation of hisoQC
IsoQC EGFP-1 Met	ATATATGAATTCATGCGTTCCGGGGGCCGC	Cloning human

Primer	Sequence 5' ➔ 3'	Application
I (SEQ ID NO : 57)		isoQC (Met I) into vector pEGFP-N3
IsoQC EGFP-2 Met II (SEQ ID NO : 58)	ATATATGAATTCATGGAGCCACTCTTGCCGCCG	Cloning human isoQC (Met II) into vector pEGFP-N3
IsoQC EGFP-3 (SEQ ID NO : 59)	ATATATGTCGACGAGCCCCAGGTATTCAGCCAG	Cloning human isoQC (Met I and Met II) into vector pEGFP-N3
HisoQC pET41a-1 (SEQ ID NO : 60)	ATATACTAGTGATGACGAC GACAAGTTCTACACCATTTGGAGCG	Cloning human isoQC into vector pET41a
HisoQC pET41a-2 (SEQ ID NO : 61)	TATAGAATTCCTAGTGATGGT GATGGTGATGGAGCCCCAGGTATTCAGC	Cloning human isoQC into vector pET41a
hisoQC HIS C-Term pPICZAA-1 (SEQ ID NO : 62)	ATA TGA ATT CTT CTA CAC CAT TTG GAG C	Cloning human isoQC into vector PPICZαA
hisoQC HIS N-Term pPICZAA-1 (SEQ ID NO : 63)	ATA TGA ATT CCA TCA CCA TCA CCA TCA CTT CTA CAC CAT TTG GAG CGG C	Cloning human isoQC into vector PPICZαA
hisoQC HIS N-Term pPICZAA-2 (SEQ ID NO : 64)	5'- ATA TAT GCG GCC GCC TAG AGC CCC AGG TAT TCA GC-3'	Cloning human isoQC into vector PPICZαA
isoQCm RT s (SEQ ID NO : 65)	CCA GGA TCC AGG CTA TTG AG	Real-time PCR analysis of isoQC
hisoQC HIS C-Term pPICZAA-2 (SEQ ID NO : 66)	ATA TAT GCG GCC GCC TAG TGA TGG TGA TGG TGA TGG AGC CCC AGG TAT TCA GCC AG	Cloning human isoQC into vector PPICZαA
isoQCm RT as (SEQ ID NO : 67)	TTC CAC AGG GCC GGG GGG C	Real-time PCR analysis of isoQC
isoQCm MetI s	ATG AGT CCC GGG AGC CGC	Cloning of

Primer	Sequence 5' ➔ 3'	Application
(SEQ ID NO : 68)		murine isoQC cDNA
isoQCm MetI as (SEQ ID NO : 69)	CTA GAG TCC CAG GTA CTC	Cloning of murine isoQC cDNA
isoQCm kurz s (SEQ ID NO : 70)	AGT TCC TGC CCC TGC TGC TG	Cloning of murine isoQC cDNA
mQC RT s (SEQ ID NO : 71)	ATC AAG AGG CAC CAA CCA AC	Real-time PCR analysis of mQC
mQC RT as (SEQ ID NO : 72)	CTG GAT AAT ATT TCC ATA G	Real-time PCR analysis of mQC
mQC RT N-terminal s (SEQ ID NO : 73)	ACA GCT GGG AAT CTG AGT C	Real-time PCR analysis of mQC
mQC RT N-terminal as (SEQ ID NO : 74)	GAG CAG AAT AGC TTC CGG GCG	Real-time PCR analysis of mQC
Iso-I55Ns (SEQ ID NO : 75)	CTG CGG GTC CCA TTG AAC GGA AGC CTC CCC GAA	Site-directed mutagenesis hisoQC I55N
Iso-I55Nas (SEQ ID NO : 76)	TTC GGG GAG GCT TCC GTT CAA TGG GAC CCG CAG	Site-directed mutagenesis hisoQC I55N
Iso-C351As (SEQ ID NO : 77)	ACG GTA CAC AAC TTG GCC CGC ATT CTC GCT GTG	Site-directed mutagenesis hisoQC C351A
Iso-C351Aas (SEQ ID NO : 78)	CAC AGC GAG AAT GCG GGC CAA GTT GTG TAC CGT	Site-directed mutagenesis hisoQC C351A
hQC-1 (SEQ ID NO : 82)	ATATATAAGCTTATGGCAGGCGGAAGACAC	Insertion of native hQC into pcDNA 3.1
hQC-2 (SEQ ID NO : 83)	ATATGCGGCCGCTTACAAATGAAGATATTCC	Insertion of native hQC into pcDNA 3.1
hisoQC pcDNA as	ATATATGCGGCCGCCTAGAGCCCCAGGTATTCAGC	Amplification

Primer	Sequence 5' → 3'	Application
(SEQ ID NO : 84)		hisoQC including the stop codon for insertion into pcDNA 3.1
EGFP-1 (SEQ ID NO : 85)	ATATCTCGAGTCCATCGCCACCATGGTGAGC	Amplification EGFP
EGFP-2 (SEQ ID NO : 86)	ATATCTCGAGTTACTTGTACA GCTCGTCCAT	Amplification EGFP
hisoQC SS EGFP pcDNA as (SEQ ID NO : 87)	ATATGCGGCCGCATGTCGACGCTCCAAATGGTGTAGAACGC	Amplification hisoQC N-terminal sequence
hQC C-FLAG pcDNA as (SEQ ID NO : 88)	ATATGCGGCCGCTTACTTGTCATCGTCATCCTTGTAATC CAAATGAAGATATTCCAA	Amplification hQC C-FLAG
hisoQC C-FLAG pcDNA as (SEQ ID NO : 89)	ATATGCGGCCGCCTACTTGTCATCGTCATCCTTGTA ATCGAGCCCCAGGTATTCAGC	Amplification h-isoQC C-Flag
Hs_QPCT_1_SG	QuantiTect Primer Assay (200), Qiagen, Hilden	qPCR hQC
Hs_QPCTL_1_SG	QuantiTect Primer Assay (200), Qiagen, Hilden	qPCR h-isoQC
CCL2-F (SEQ ID NO : 90) CCL2-R (SEQ ID NO : 91)	GCCTCCAGCATGAAAGTCTC CAGATCTCCTTGGCCACAAT	qPCR CCL2
CCL7-F (SEQ ID NO : 92) CCL7-R (SEQ ID NO : 93)	ATGAAAGCCTCTGCAGCACT TGGCTACTGGTGGTCCTTCT	qPCR CCL7
CCL8-F (SEQ ID NO : 94) CCL8-R (SEQ ID NO : 95)	TCACCTGCTGCTTTAACGTG ATCCCTGACCCATCTCTCCT	qPCR CCL8
CCL13-F (SEQ ID NO : 96) CCL13-R (SEQ ID NO : 97)	ATCTCCTTGCAGAGGCTGAA AGAAGAGGAGGCCAGAGGAG	qPCR CCL13
HIF1α-F (SEQ ID NO : 98) HIF1α-R (SEQ ID NO : 99)	CACAGAAATGGCCTTGTGAA CCAAGCAGGTCATAGGTGGT	qPCR HIF1α
AIM1-F (SEQ ID NO : 100)	TCCTTTCATCCTGGAACCTG	qPCR AIM1

Primer	Sequence 5' ➔ 3'	Application
AIM1-R (SEQ ID NO : 101)	CGCCTCTTCTGTTTCACCTC	
AIM2-F (SEQ ID NO : 102) AIM2-R (SEQ ID NO : 103)	AAGCGCTGTTTGCCAGTTAT CACACGTGAGGCGCTATTTA	qPCR AIM2
MAGEA1-F (SEQ ID NO : 104) MAGEA1-R (SEQ ID NO : 105)	GTCAACAGATCCTCCCCAGA CAGCATTTCTGCCTTTGTGA	qPCR MAGEA1
MAGEA2-F (SEQ ID NO : 106) MAGEA2-R (SEQ ID NO : 107)	AGGTGGAGAGCCTGAGGAAT CTCGGGTCCTACTTGTCAGC	qPCR MAGEA2
MAGEA10-F (SEQ ID NO : 108) MAGEA10-R (SEQ ID NO : 109)	AAGCGAGGTTCTCGTTCTGA TGACCTCTTGCTCTCCCTGT	qPCR MAGEA10
MAGEB2-F (SEQ ID NO : 110) MAGEB2-R (SEQ ID NO : 111)	CTTCAAGCTCTCCTGCTGCT CGACCCTGACTTCCTGGTTA	qPCR MAGEB2
MART1-F (SEQ ID NO : 112) MART1-R (SEQ ID NO : 113)	GCTCATCGGCTGTTGGTATT ATAAGCAGGTGGAGCATTGG	qPCR MART1
MCL1-F (SEQ ID NO : 114) MCL1-R (SEQ ID NO : 115)	ATGCTTCGGAAACTGGACAT ATGGTTCGATGCAGCTTTCT	qPCR MCL1
TYR-F (SEQ ID NO : 116) TYR-R (SEQ ID NO : 117)	TACGGCGTAATCCTGGAAAC ATTGTGCATGCTGCTTTGAG	qPCR TYR
TYRP1-F (SEQ ID NO : 118) TYRP1-R (SEQ ID NO : 119)	CCGAAACACAGTGGAAGGTT TCTGTGAAGGTGTGCAGGAG	qPCR TYRP1
TYRP2-F (SEQ ID NO : 120) TYRP2-R (SEQ ID NO : 121)	GGTTCCTTTCTTCCCTCCAG AACCAAAGCCACCAGTGTTC	qPCR TYRP2

Table 5: Purification of GST-isoQC fusion protein following Expression in *E. coli*. The purified fusion protein was used for determination of QC activity.

Purification Step	**1**	**2**	**3**	**4**
Method	**Ni^{2+}-IMAC** (EBA)	**GST-TAG AC**	**GF (Desalting)**	**IEX (UNO S)**
Column type (Amersham Biosciences AB, Sweden)	Chelating Sepharose Fast Flow	Glutathion Sepharose 4 Fast Flow	Sephadex G-25 Fine	"continuous bed" matrix BIO-Rad
Column size	d=2,5cm l=42cm CV=206cm^3	d=1,6cm l=10cm CV=20cm^3	d=2,6cm l=10cm CV=53cm^3	d=1,2cm l=5,3cm CV=6cm^3
Equilibration Buffer pH Volume	 PBS 7,3 10CV	 PBS 7,3 10CV	 25mM Mes 6,0 10CV	 25mM Mes 6,0 10CV
Intermediate (Wash) Buffer pH Volume	 PBS 0,5mM Histidin 7,3 10CV	 PBS 7,3 10CV	 - 	 25mM Mes 6,0 10CV
Elution Buffer pH Volume	 PBS 100mM Histidin 7,3 1,5 CV	 50mM Tris 10mM Glutathion (reduced) 8,0 (reverse flow)	 25mM Mes 6,0 1 CV	 25mM Mes Gradient elution NaCl 6,0 CV

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Table 6: K_I-values for competitive inhibition of human QC and human isoQC by imidazole derivatives. Human isoQC was expressed in *E. coli* BL21 (hisoQCdt) or *P. pastoris* (YSShisoQC).

Inhibitor	Ki (µM) hisoQCdt	Ki (µM) YSShisoQC	Ki (µM) hQC
Imidazole	220 ± 1	235± 13	103 ± 2
Benzimidazole	200 ± 8	250 ± 5	138 ± 4
1-Benzylimidazole	7,3 ± 0,5	6,2 ± 0,2	7,1 ± 0,1
1-Methylimidazole	80 ± 5	82 ± 3	39,7 ± 0,2
PBD150 1-(3,4-Dimethoxy-phenyl)-3-(3-imidazole-1-yl-propyl)-thiourea	0,48 ± 0,03	0,519 ± 0,001	0,0584 ± 0,0002

Example 6: Expression and purification of human isoQC in P. pastoris

Host strains and media

Escherichia coli strain DH5α was used for propagation of plasmids and *P. pastoris* strain X-33 was used for the expression of human isoQC in yeast. *E. coli and P. pastoris* strains were grown, transformed and analyzed according to the manufacturer's instructions (Qiagen (DH5α), invitrogen (X-33)). The media required for *E. coli*, i.e. Luria-Bertani (LB) medium, was prepared according to the manufacturer's recommendations. The media required for *Pichia pastoris*, i.e. BMMY, BMGY, YPD, YPDS and the concentration of the antibiotics, i.e. Zeocin, were prepared as described in the Pichia manual (invitrogen, catalog. No. K1740-01). The manual also includes all relevant descriptions for the handling of yeast.

Molecular cloning of plasmid vectors encoding the human QC

All cloning procedures were done applying standard molecular biology techniques. For expression in *Pichia pastoris* X-33, the pPiCZαA (invitrogen) was used. The cDNA of the mature human isoQC starting with codon 30 (counting from methionine II) was fused in frame with the plasmid encoded α-factor, directing the protein into the secretory pathway. After amplification utilizing the primers hisoQC HIS C-Term pPICZAA-1 (SEQ ID NO: 62) or hisoQC HIS N-Term pPICZAA-1 (SEQ ID NO: 63) as sense-Primers and

hisoQC HIS N-Term pPICZAA-2 (SEQ ID NO: 64) and hisoQC HIS C-Term pPICZAA-2 (SEQ ID NO: 66) (Table 4) as antisense Primers, the fragment was inserted into the expression vector employing the restriction sites of *Not*I and *EcoR* I. Depending on the construct, Mutations were introduced in codons 55 (Ile) and 351 (Cys). The mutagenesis was performed according to standard PCR techniques followed by digestion of the parent DNA using *Dpn*I (quik-change II site-directed mutagenesis kit, Stratagene, Catalog No. 200524). The generated constructs are illustrated schematically in Figure 17.

Transformation of P. pastoris and Mini-Scale Expression

1-2 µg of plasmid DNA were applied for transformation of competent *P. pastoris* cells by electroporation according to the manufacturer`s instructions (BioRad). Selection was done on plates containing 100 µg/ml Zeocin. In order to test the recombinant yeast clones upon isQC 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 for about 72 h. Subsequently, QC activity in the supernatant was determined. Clones that displayed the highest activity were chosen for further experiments and fermentation. Depending on the expressed construct, the isoQC-activity in the medium differed (Figure 18).

Expression and purification of hisoQC in P. pastoris

For large scale-Expression of isoQC in *Pichia pasoris*, the condition were kept as described in the mini-scale expression, however, the total volume was 8L. The expression was performed in shake-flasks. After expression, cells were separated from the medium by centrigugation (1500xg, 20 min), and the pellet discarded. The pH-value of the supernatant was adjusted to neutrality, centrifuged again and applied for the first purification step. The isoQC protein was purified utilizing a 3-step protocol (Table 7). The purfication is illustrated by SDS-PAGE analysis in Figure 19.

Table 7: Purification of hisoQC (YSShisoQCN55IC351A C-His) following Expression in *P. pastoris.* The purified fusion protein was used for determination of QC activity and pH-dependence.

Purification Step	**1**	**2**	**3**
Method	**Ni^{2+}-IMAC**	**HIC**	**GF (Desalting)**
Column type (Amersham Biosciences AB, Sweden)	Chelating Sepharose Fast Flow	Butyl Sepharose 4Fast Flow	Sephadex G-25 Fine
Column size	d=2,5cm l=42cm CV=206cm^3	d=1,6cm l=15,5cm CV=23cm^3	d=2,6cm l=10cm CV=53cm^3
Equilibration Buffer pH Volume	 50mM NaH_2PO_4 7,0 10CV	 30mM NaH_2PO_4 1M $(NH_4)_2SO_4$ 7,0 10CV	 50mM Bis-Tris 100mM NaCl 6,8 10CV
Intermediate (Wash) Buffer pH Volume	 50mM NaH_2PO_4 0,5mM Histidin 7,0 10CV	 30mM NaH_2PO_4 1M $(NH_4)_2SO_4$ 7,0 6CV	 -
Elution Buffer pH Volume	 50mM NaH_2PO_4 100mM Histidin 7,0 1,5 CV	 30mM NaH_2PO_4 7,0 5 CV	 50mM Bis-Tris 100mM NaCl 6,8 1CV

Results

Human isoQC was expressed in the methylotrophic yeast *P. pastoris* successfully. Several diffrent constructs were generated, in order to select the best expression conditions in yeast (Figure 17). As illustrated in figure 18, the QC activity that is expressed and present in the medium of the expressing cells, varies depending on the expressed construct. Introduction of a glycosylation site resulted in proper secretion, as can be observed from constructs YSShisoQCN55IC351A C-His and YSShisoQCN55I C-His. Due to the highest activity in the medium, construct YSShisoQCN55IC351A C-His was expressed in large-scale and purified. The purification was carried out as described in Table 7, the yield of purification was 59%. The apparent homogeneous protein was glycosylated, as evidenced by a shift in migration to lower molecular mass (Figure 19). Glycosylation did not influence the catalytic activity of the enzyme.

Example 7: The pH-dependence of hisoQC

The fluorometric assay using H-Gln-βNA (described in example 5) was applied to investigate the pH-dependence of the catalytic specificity. The reactions were carried out at substarte concentrations of 7 µM, i.e. at $[S]<<K_M$. Therefore, the the observed specificity constants could be directly deduced from the initial velocity of the progress curves of substrate conversion. In these studies the reaction buffer consisted of 0.075 M acetic acid, 0.075 M Mes and 0.15 M Tris, adjusted to the desired pH using HCl or NaOH. The buffer assures a constant ionic strength over a very broad pH-range. Evaluation of the acquired enzyme kinetic data was performed using the following equation:

$$k_{cat}/K_M(pH) = k_{cat}/K_M(limit)*1/(1 + [H^+]/K_{HS} + K_{E1}/[H^+] + K_{E1}/[H^+]*K_{E2}/[H^+]),$$

in which $k_{cat}/K_M(pH)$ denotes the pH-dependent (observed) kinetic parameter. $k_{cat}/K_M(limit)$ denotes the pH-independent ("limiting") value. K_{HS}, K_{E1} and K_{E2} denote the dissociation constants of an dissociating group in the acidic pH-range, and two dissociating groups of the enzyme, respectively. Evaluation of all kinetic data was performed using GraFit software (version 5.0.4. for windows, ERITHACUS SOFTWARE Ltd., Horley, UK).

Results

The hisoQC displays a pH-optimum of specificity at pH 7-8. Thus, the pH-optimum of catalysis is very similar to human QC. Fitting of the data according to a model which is based on three dissociating groups resulted in a well interpretation of the pH-dependence of hisoQC and hQC (Figure 22). Thus, the catalysis of both enzymatic reactions is influenced by similar dissociating groups, suggesting a similar catalytic mechanism in general.

The determined pKa-values are displayed in Table 8. It is obvious, that only one pKa differs between hisoQC and hQC significantly. In hQC, the pKa corresponds to the pKa of the dissociation constant of the substrate. Possibly, the subtle difference between hQC and hisoQC is caused by structural changes occurring in isoQC catalysis (induced fit), influencing the pH-dependence.

Example 8: Investigation of Glutamyl Cyclase activity

It has been described for human QC, that the enzyme catalyses the cyclization of N-terminal glutamic acid into pyroglutamic acid. Therefore, QC is involved inteo the generation of pGlu-modified amyloid peptides.

In order to investigate the cyclization of glutamic acid, human QC and human isoQC were purified and the formation of pGlu-modified amyloid β(3-11) [pGlu-Aβ(3-11)] from Aβ(3-11) was monitored. Reactions consisted of 20µl substrate (Aβ(3-11), 2,5mM stock solution in 50mM Mes buffer, pH 6,5) and 80µl enzyme (0,62 mg/ml hQC stock solution; 0,61mg/ml hisoQC stock solution in 50mM Mes pH 6,5). Samples (15 µl) were removed after 0h, 6h, 24h, 48h und 72h and boiled for 5min in order to terminate the reaction. The analysis of substrate conversion was monitored by Maldi-Tof mass spectrometry. Substrate and product differ in their molecular mass by 18 Da, the mass of water, which is released during cyclization.

As shown in figure 23, human QC and human isoQC (YSShisoQCI55NC351A C-His) catalyze the conversion of Aβ(3-11) into pGlu-Aβ(3-11). However, based on equal protein concentrations in both samples, one can conclude that the conversion of N-terminal glutamic acid by hisoQC is much slower compared with hQC. Thus, the lower specificity constants for conversion of glutaminyl substrates is also observed with glutamyl substrates. No cyclization was observed under these conditions with inactivated enzyme (Schilling, S. et al., 2004 FEBS Lett. 563, 191-196).

Example 9: Tissue specificity of Murine isoQC

The tissue distribution of murine QC and murine isoQC was investigated using quantitative real time PCR techniques. Prior to analysis of cDNA from several different organs and tissues, the murine isoQC open reading frame was isolated applying specific primers (isoQCm MetI s (SEQ ID NO: 68), isoQCm MetI as (SEQ ID NO: 69) (table 4), which were deduced from the chromosomal coding region of murine isoQC.

The open reading frame was cloned into vector pPCR-Script CAM SK (+) (*PCR-Script CAM Cloning Kit*, Stratagene) and used as a positive control in the real-time PCR determinations and for preparation of a standard curve under assay conditions.

The characterization of the tissue specificity of misoQC expression was achieved applying cDNA from 3-6 month old mice. Total RNA was isolated from 30 mg tissue, using the RNA-isolation kit II (Macherey and Nagel). The RNA concentration and purity was assessed by gelelectrophoresis (agarose gel) and spectrophotometry. For synthesis of cDNA, 1 µg of RNA was used. The reaction was done applying the reverse Transcriptase *Superscript II RT* (Invitrogen) according to the recommendations of the supplier, the cDNA was stored at –80°C.

The quantitative analysis of the transcript concentration in different tissues was analysed using the „Light Cycler" (Corbett research), applying the „QuantiTect SYBR Green PCR" (Qiagen). The DNA standard (cloned cDNA isoQC mouse) was used for quantification. The copy number was calculated according to the following equation: ($X^{g}/_{\mu l}$ DNA) / (Plasmid length in bp*660)*6.022*1023 = Y $^{Molecules}/_{\mu l}$. The DNA standard contained 4 concentrations in the range of 10^{7}-10^{1} $^{Molecules}/_{\mu l}$, and an limiting concentration (10^{0}). The reaction protocoll is displayed in Table 8. The results are displayed in Figure 24.

For amplification of murine QC, the same protocol was used, applying the primers mQC RT N-terminal s (SEQ ID NO: 73) and mQC RT N-terminal as (SEQ ID NO: 74).

Table 8: Reaction protocol of the quantitative real-time-PCR using the Roto-Gene RG 3000 (Corbett Research)

PCR-Cycles		
step	**T in °C**	**t in sec.**
0 Denaturation	95	900
1 Denaturation	95	15
2 Primer Annealing	55	20
3 Elongation	72	20
Cycles	45	

Results

As shown in Figure 24, murine QC and murine isoQC are expressed in all organs tested. In contrast to murine QC, the variances in expression of murine isoQC between different organs are smaller, indicating a lower stringency of regulation of transcription. The data for expression of mQC correspond to previous analyses of bovine QC, which

was analyzed using Northern-Blot (Pohl, T. et al. 1991 Proc Natl Acad Sci U S *A 88*, 10059-10063). Highest expression of QC was observed in Thalamus, Hippocampus and Cortex. Thus, QC-expression is primarily detected in neuronal tissue. Little QC-expression is detected in peripheral organs as spleen and kidney. Also misoQC is expressed in neuronal tissue, but at lower levels compared with mQC. In contrast, expresssion levels in peripheral organs is very similar between isoQC and QC.

Concluding, based on the results of transcript concentration, the combined activity (isoQC and QC) should be highest in brain. Thus, highest QC-protein levels are present in organs that are afflicted by amyloidoses like Alzheimers Disease, familial british dementia and familial danish dementia.

Example 10: Inhibition of human isoQC by heterocyclic chelators

Results

The time-dependent inhibition of QCs from different sources using heterocyclic chelators, such as 1,10-phenanthroline and dipicolinic acid has been investigated previously (6, 9). In analogy, h-isoQC is also time-dependently inactivated by the heterocyclic chelators 1,10-phenanthroline (Figure 25) and dipicolinic acid (not shown), clearly pointing to a metal-dependent activity. Furthermore, EDTA also inhibited h-isoQC (Figure 25). This is in sharp contrast to QCs, since neither human QC, porcine QC nor murine QC has shown discernible inhibition by EDTA. However, inhibition of hisoQC by EDTA even stronger suggests a metal-dependent catalysis.

Example 11: Subcellular localization of hisoQC investigated using cell fractionation

Cell fractionation

The day following transfection, expressing HEK293 cells were washed with D-PBS and collected by centrifugation at 500 x g for 5 min at 4 °C. Subsequently, D-PBS was discarded and the cells were resuspended in 1 ml of disruption buffer (50 mM Tris, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, pH 7.6 adjusted with HCl) and cracked by 30 crushes in a Potter cell homogenisator. The suspension was centrifuged at 700 x g for 10 min at 4 °C. The obtained pellet was resuspended in 300 µl disruption buffer and

designated as debris fraction (D). The resulting supernatant was further centrifuged at 20.000 x g for 30 min at 4 °C. The pellet illustrated the heavy membrane fraction (HM) and was resuspended in 200 µl disruption buffer. The resulting supernatant was centrifuged at 100.000 x g for 1h at 4 °C using an ultracentrifuge (Beckmann). The obtained pellet was resuspended in 200 µl disruption buffer and was termed as light membrane fraction (LM). The supernatant was designated as soluble fraction (S). Debris, heavy membrane and light membrane fractions were sonicated for 10 sec and. the protein content of all fractions was determined using the method of Bradford. Subsequently, fractions were analyzed for QC activity and stained for marker proteins using Western Blot.

Results

For further corroboration, biochemical analysis of QC activity distribution, derived from hisoQC and hQC expression were performed. The native hisoQC beginning with methionine I and II and hQC were expressed in HEK293 cells, respectively. After cell fractionation the QC activity in the each fraction was determined using the fluorescence assay applying H-Gln-βNA as substrate. In cells, transfected with the empty vector (pcDNA), specific QC activity is hardly measurable. When expressing native hisoQC (MetI) and hisoQC (MetII), QC activity was readily detectable with the highest specific activity in the heavy membrane fraction (MetI: 40 ± 2 µmole/min/g; MetII: 36 ± 1.5 µmole/min/g) and the medium (MetI: 30 ± 2 µmole/min/g; MetII: 54 ± 3 µmole/min/g). In contrast, hQC shows the highest specific QC activity within the medium (1339 ± 76 µmole/min/g) followed by the heavy membrane fraction (251 ± 21 µmole/min/g) (Figure 26A).

In addition the absolute activities were calculated, illustrating that the expression of hisoQC (MetI) and hisoQC (MetII) led mainly to an increase in the intracellular QC activity, namely within the debris (MetI: 1032 ± 9 nM/min; MetII: 1110 ± 10 nM/min) and heavy membrane fraction (MetI: 374 ± 20 nM/min; MetII: 281 ± 12 nM/min). Only little QC activity was found within the medium (MetI: 27 ± 2 nM/min; MetII: 53 ± 3 nM/min). In contrast, QC activity deduced by hQC expression shows high activity within the medium (1138 ± 65 nM/min) and within intracellular compartements (debris: 1089 ± 14 nM/min; heavy membrane fraction: 583 ± 38 nM/min) supporting an Golgi localization of hisoQC as shown by histochemical analysis (Figure 26B).

The data obtained by the expression of the native enzymes was further supported by expression of hisoQC (MetI and MetII) and hQC possessing a C-terminal FLAG-tag (Figure 26C). Western Blot analysis of the resulting FLAG-tagged proteins in comparison to marker proteins of the Golgi complex and mitochondria revealed a mainly intracellular localization of hisoQC(MetI) and hisoQC (MetII) within the debris and heavy membrane fraction, whereas hQC is enriched within the medium but also found within the debris and heavy membrane fraction. Visualization of marker proteins of the Golgi complex (ST1GAL3) and mitochondria revealed the presence of these compartments within the debris and heavy membrane fraction. In addition the 65 kDa mitochondrial protein was also found to a smaller portion within the soluble fraction.

Example 12 Analysis on the Golgi retention signal of hisoQC

In order to clarify, whether the predicted N-terminal transmembrane helix is responsible for the retention of hisoQC within the Golgi complex, the signal peptides starting at MetI and MetII, including the transmembrane helix, were cloned in frame with EGFP. The resulting vectors hisoQC (MetI) SS EGFP and hisoQC (MetII) SS EGFP were expressed in LN405 cells and examined in analogy to the full-length hisoQC EGFP fusion proteins using confocal laserscanning microscopy. The expression of hisoQC (MetI) SS EGFP led to the same Golgi complex localization observed for the full-length hisoQC (MetI) EGFP fusion protein. Again, a transport of hisoQC (MetI) SS EGFP to the mitochondria was not observed (Figure 27A). In addition, the expression of the N-terminal truncated peptide hisoQC (MetII) SS EGFP also led to a enrichment of the protein within the Golgi complex. In analogy to hisoQC (MetI) SS EGFP, no mitochondrial EGFP fluorescence could be recorded (Figure 27B). Consequently, the N-terminal sequence of hisoQC leads to the co-translational translocation of the protein to the ER membrane and to the retention within the Golgi complex. Furthermore, due to the expression of hisoQC (MetII) SS EGFP, the Golgi retention signal was grossly mapped to reside between methionine 19 and serine 53 (counting of amino acids beginning at MetI).

Additional topology analysis revealed the possibility for a functional homology of the hisoQC N-terminus to glycosyltransferases. Glycosyltransferases are type II transmembrane proteins, possessing a short cytoplasmatic sequence, followed by the

transmembrane helix and a large luminal catalytic domain. Clearly, this is essentially the same domain structure as found for misoQC and hisoQC (Figure 28). For a number of glycosyltransferases, the Golgi retention signal was identified to reside within the transmembrane domain. Furthermore, for some of these enzymes truncation of the cytoplasmatic sequence was found to have no influence on the activity or the localization of the protein. In summary, evidence was provided, that hisoQC is a type II transmembrane protein showing a retention within the Golgi complex similar to glycosyltransferases.

Example 12 Detection of QPCTL mRNA in different human carcinoma cell lines and tissues

qPCR analysis

Analysis of human QPCTL expression in human carcinoma cell lines were performed using the quatitative real time PCR (qPCR) technique, essentially as described in example 9. For determining QPCTL mRNA, primers of the QuantiTect® primer assay were applied covering an exon/exon region for exclusion of co-amplification of genomic DNA. QPCR was performed following the manufacturers recommendations. The reaction mixture is depicted in Table 9 and the PCR program is illustrated in Table 8.

Table 9: Composition of the qPCR mixture

component	Volume in µl
2x QuantiTect SYBR Green PCR Master Mix (2,5 mM $MgCl_2$)	7,5
10x QuantiTect Primer Assay	1,5
cDNA (≤100 ng/Reaktion)	1
Aqua bidest.	5

The quantitative analysis of the transcript concentration in different tissues was analysed using the „Light Cycler“ (Corbett research), applying the „QuantiTect SYBR Green PCR“ (Qiagen). The DNA standard (cloned cDNA isoQC human) was used for quantification. The copy number was calculated according to the following equation: ($X^{g}/_{\mu l}$ DNA) / (Plasmid length in bp*660)*6.022*1023 = Y $^{Molecules}/_{\mu l}$. The DNA standard contained 4 concentrations in the range of 10^{7}-10^{1} $^{Molecules}/_{\mu l}$, and an limiting concentration (10^{0}).

The results of qPCR were evaluated using the rotor-gene operating software (Corbett research).

Results

Expression of QPCTL in different carcinoma cell lines

Among the tested cancer cell lines, human melanoma cells show the highest expression of QPCTL transcripts (approx. 7000 copies / 50 ng total-RNA), whereas the human soft tissue sarcoma cell lines show the lowest expression of QPCTL (365 copies / 50 ng total-RNA). Pancreas carcinoma shows 2100 copies, thyroid carcinoma 3500 copies and gastric carcinoma possesses 4100 copies in the median (Figure 29).

Expression of QPCTL in different melanoma cell lines

Recently it has been shown, that melanoma cells possess comparable high QPCT expression (Gillis, J.S., J. Transl. Med. 4 (2006), 4:27). Therefore, QPCTL expression in different melanoma cell lines was analyzed. As depicted in Figure 30, QPCTL expression was detected in all melanoma cell lines, tested. The variation among the cell lines varied from 2025 copies / 50 ng total-RNA in line Mel_ZL_11 to 18043 copies / 50 ng total-RNA in line Mel_ZL12.

Table 10: Correlation of QPCT and QPCTL to tumor-associates antigens (taa) and correlation of taa among each other

correlation	significance	correlation	significance
QPCT - MAGEB2	0,0436	AIM1 - MCL1	0,0163
QPCT - MART1	0,0020	MAGEA1 - MAGEA2	0,00002
QPCT - TYR	0,0023	MAGEA1 - MAGEB2	0,0058
QPCT - MAGEA1	0,0591	TYRP2 - MART1	0,0042
QPCTL - MART1	0,0008	TYR - MART1	0,0335
		TYR - TYRP2	0,0408
AIM1 - AIM2	0,0082	TYR - MCL-1	0,0151

Furthermore, QPCT and QPCTL expression was correlated to the expression of tumor-associated antigens (taa). The melanoma-specific tumor-associated antigens were selected by data base mining and published results. Among others, AIM1 and AIM2 (absent in melanoma), MAGEA1, -A2, -A10 and MAGEB2 (melanoma antigen familiy A and B), MART1 (melanoma antigen recognized by T-cells), TYR (tyrosinase), TYRP1

and TYRP2 (tyrosinase related protein) and MCL-1 (myeloid cell leukemia) are tumor-associated antigens in melanoma. Data were compared using SPSS statistic software. Correlation between QPCT and MAGEB2 was significant ($p = 0.0436$). Furthermore, correlation between QPCT and MART1 ($p = 0.002$), QPCTL and MART1 ($p = 0.008$) and QPCT and TYR ($p = 0.0023$) was also statistically highly significant. The correlations show a direct dependence, which implies: the higher QPCT/QPCTL expression, the higher the expression of tumor-associated antigens. The only exception is the correlation between TYR and MCL1, which shows an indirect dependence.

Expression of QPCT and QPCTL in different tumor tissues

The expression of QPCT and QPCTL was evaluated in tumor tissues of soft tissue sarcoma, gastric carcinoma and thyroid carcinoma. Highest expression of QPCT has been found in thyroid carcinoma followed by gastric carcinoma and soft tissue carcinoma (Table 11). The same order was observed for QPCTL expression, however, the copy number of QPCTL transcripts was always lower, than observed for QPCT transcripts as revealed by Student's t-test ($p_{\text{soft tissues carcinoma}} = 0.001$; $p_{\text{gastric carcinoma}} = 4.8E\text{-}7$; $p_{\text{thyroid carcinoma}} = 0.04$) (Table 11; Figure 31).

Table 11: Comparison of QPCT and QPCTL expression in different tumor tissues

	soft tissue sarcoma (119 samples)	**gastric carcinoma (47 samples)**	**thyroid carcinoma (29 samples)**
QPCT	1293	2985	8303
QPCTL	170	469	2540

Further investigations on the expression level of QPCT and QPCTL revealed a two-sided significant correlation by Pearson in soft tissue sarcoma ($p = 2E\text{-}31$) and gastric carcinoma ($p = 0.015$). No correlation has been observed for QPCT and QPCTL expression level in thyroid carcinoma ($p = 0.46$).

Expression of QPCTL dependent on the stage of differentiation in gastric carcinoma

For gastric carcinomas, QPCTL expression in samples representing different stages of tumor differentiation were investigated. As control served tumor-surrounding normal tissue. The comparison of normal with tumor tissue revealed a significantly higher QPCTL expression ($p = 0.04$) in tumor tissues. Undifferentiated gastric carcinomas show lower QPCTL expression, than normal tissue. Poorly and well to moderate

differentiated gastric carcinomas show no differences in the median compared to normal tissue (Figure 32).

Expression of QPCT and QPCTL in different stages of thyroid carcinoma

Different stages of thyroid carcinoma were inverstigated concerning QPCT and QPCTL expression. The stages were classified according to nomenclature of the world health organisation (WHO) as follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC) and undifferentiated thyroid carcinoma (UTC). Samples from patients possessing goiter served as control.

The QPCT mRNA level (median) in differentiated thyroid carcinomas FTC (6700 copies / 50 ng total-RNA) and PTC (16000 copies / 50 ng total-RNA) were higher than in non-tumor tissue (goiter: 2100 copies / 50 ng total-RNA). UTC possesses 5400 copies / 50 ng total-RNA and is 2.5 times higher than observed in goiter. The mRNA copy number of QPCT is in all thyroid tumors significantly higher than in goiter ($p = 0.04$, Student's t-test) (Figure 33).

The QPCTL mRNA level in thyroid carcinoma is homogeneous. The samples from FTC (2600 copies / 50 ng total-RNA) and UTC (2500 copies / 50 ng total-RNA) are similar to goiter (2500 copies / 50 ng total-RNA). The expression of QPCTL in PTC is slightly decreased to 1900 copies / 50 ng total-RNA (Figure 34).

In conclusion, QPCT and QPCTL are equally expressed in goiter. However, in tumor tissues the expression of QPCT increases, whereas the expression of QPCTL remains stable.

Example 13 Investigations on the QPCT and QPCTL expression in human cell lines after incubation with different stimuli

Cell lines and media

The stimulation experiments were performed using the human embryonal kidney cell line HEK293, human acute monocytic leukemia cell line THP-1 and the follicular thyroid carcinoma cell line FTC-133. Cells were grown in appropriate culture media (DMEM, 10 % FBS for HEK293, RPMI1640, 10 % FBS for THP-1 and DMEM/F12, 10 % FBS for FTC-133) in a humidified atmosphere at 37 °C and 5 % CO_2.

Stimulation using bioactive peptides, chemicals or LPS

HEK293 and FTC-133 cells were cultivated as adherent cultures and THP-1 cells were grown in suspension. For stimulation assay $2x10^5$ cells of FTC-133 and HEK293 cells were transferred to 24well plates. In case of HEK293, plates were coated with collagen I for ensuring proper adherence. In addition, $2x10^6$ cells of THP-1 were grown in 24well suspension plates. All stimulation experiments were applied under serum-free conditions. FTC-133 was grown over night. Afterwards, cells were adapted to serum-free media for another 24 h and the stimulation was started by replacing the conditioned media by fresh serum-free media. HEK293 cells were grown over night and afterwards the stimulation using respective agents was started without an adaption to serum-free conditions due to morphological changes in case of cultivation of HEK293 under serum-free conditions for more than 24 h. THP-1 cells were plated in serum-free media together with respective agent. The applied stimuli and final concentrations are listed in Table 12.

Table 12: Stimuli for investigations on the regulation of hQC and hisoQC in human cell lines

Name	Final concentration
butyric acid (BA)	2 mM
hepatocyte growth factor (HGF)	10 ng/ml
lipopolysaccharide (LPS)	1, 10 µg/ml
transforming growth factor β (TGFβ)	10, 100 ng/ml
tumor necrosis factor α (TNFα)	10, 100 ng/ml

Cells were incubated with the respective stimulus for 24 h. Afterwards, total-RNA from the cells was isolated using the Nucleo-Spin® RNA II Kit (Macherey-Nagel) and stored until qPCR assay.

Stimulation using hypoxia

THP-1, HEK293 and FTC-133 cells were plated into two 25 cm^2 tissue culture flasks, respectively. Thereby, one flask of each cell line served as negative control, cultivated under normal growth conditions for 24 h. The other flasks were placed in a anaerobic bag together with an anaerobic reagent (Anaerocult® P, Merck) and an indicator. The bag was sealed to ensure air tight conditions. Cells were also grown for 24 h and

subsequently, total-RNA was isolated using the Nucleo-Spin® RNA II Kit (Macherey-Nagel) and stored until qPCR assay.

Results

Basal expression of QPCT and QPCTL in HEK293, FTC-133 and THP-1

The basal expression in the used cell lines HEK293, FTC-133 and THP-1 was evaluated in preparation for the following stimulation experiments. The copy number of QPCT and QPCTL transcripts is summarized in Table 13.

Table 13: Basal expression of QPCT and QPCTL in different cell lines

cell line	Absolute mRNA copy numbers per 50 ng total RNA	
	QPCT	QPCTL
HEK-293 (8 samples)	37196 ± 18928	3206 ± 855
FTC-133 (8 samples)	24790 ± 7605	10262 ± 1899
THP-1 (8 samples)	3588 ± 853	6725 ± 1763

Influence of selected stimuli on expression of QPCT and QPCTL

Regulational binding sites of the promotors of QPCT and QPCTL and signal transduction pathways leading to their regulation are not decribed so far. Therefore, stimulation experiments using different cell lines and stimuli were conducted. QPCT mRNA levels in HEK293 cells were increased by stimulation using TNF-α, HGF and butyric acid. In addition the regulation of CCL2 as QPCT/QPCTL substrate has been inverstigated. TNF-α and butyric acid increased the amount of CCL2 transcripts in HEK293. HGF had no influence in CCL2 expression. In contrast QPCTL was not regulated by TNF-α, HGF and butyric acid (Figure 35).

In addition FTC-133 was stimulated using LPS and TGF-β and the regulation of QPCT, QPCTL and CCL2 was monitored. In FTC-133, LPS and TGF-β stimulated the expression of QPCT mRNA, but failed to induce QPCTL and CCL2 expression (Figure 36).

This experiments were further coroborated by stimulation of THP-1 cells using LPS (1 µg/ml), LPS (10 µg/ml), TGF-β and TNF-α. As observed for FTC-133 and HEK293, QPCT expression could be induced using different stimuli. In addition CCL2 expression

was induced using LPS and TNF-α. Again, no induction or repression of QPCTL mRNA could be observed (Figure 37).

In conclusion, the experiments revealed, that QPCT can be regulated by a set of stimuli in different cell lines (LPS, TNF-α, HGF, butyric acid and others). In contrast, QPCTL could neither stimulated nor repressed by the tested stimuli suggesting a house-keeping function of QPCTL.

Influence of selected stimuli on expression of QPCT its substrates

Since QPCT expression was induced by a number of stimuli, the question was raised, whether QPCT induction takes place in combination with an induction of the QPCT substrates CCL2, CCL7, CCL8 and CCL13. Therefore, the stimulation using LPS (1 µg/ml), LPS (10 µg/ml), TGF-β (100ng/ml) and TNF-α (100 ng/ml), respectively, was performed using THP-1 monocytes. THP-1 expresses all chemokines at a basal level, important for comparison of stimulated cells with the negative control.

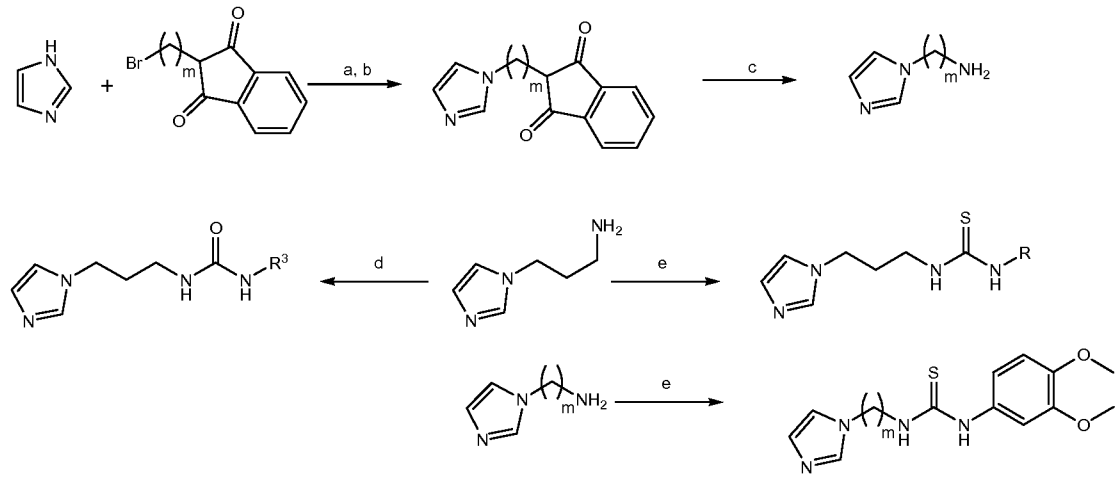
LPS and TNF-α led to the reliable induction of all tested chemokines and QPCT in THP-1 cells. TGF-β was less effective as stimulus and induced the expression of QPCT, CCL2, CCL7 and CCL8 maximum 2fold. CCL13 was repressed by TGF-β stimulation (Figure 38).

Stimulation of QPCT and QPCTL expression by hypoxia

QPCTL expression could not be regulated by chemical agents, bioactive pepitides or LPS. Therefore, we tested, whether QPCTL expression is regulated by hypoxia. As summarized in Figure 39. Hypoxia selectively induced the expression of QPCTL but not of QPCT. In comparison, hypoxia induced factor 1a (HIF1a) was repressed by 15 % (Figure 39A) and 45 % (Figure 39C). The data suggest a connection of QPCTL to hypoxia.

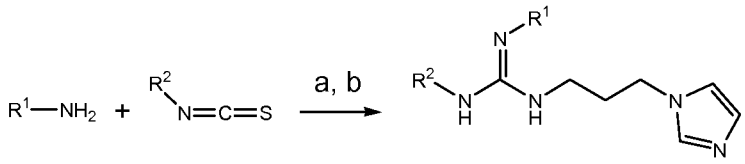
Synthesis of the inhibitors

Synthesis scheme 1: Synthesis of the examples 1-53, 96-102, 136-137



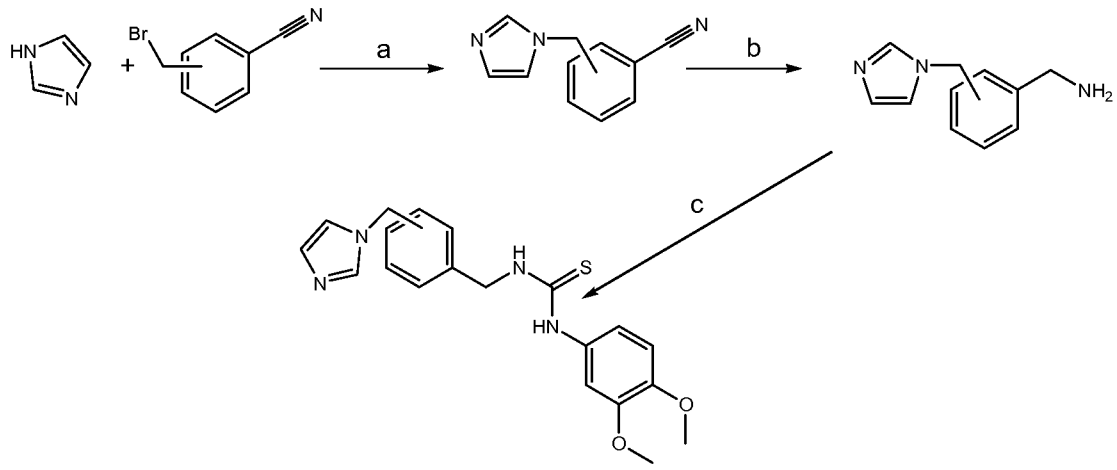
Reagents and conditions: (a) NaH, DMF, 4h, rt.; (b) , 8h, 100°C; (c) H_2N-NH_2,EtOH, 8h, reflux then 4N HCl, 6h, reflux, (d) R^3-NCO, EtOH, 6h, reflux, (e) 3,4 dimethoxy-phenyl-isothiocyanate,

Synthesis scheme 2: Synthesis of the examples 54-95



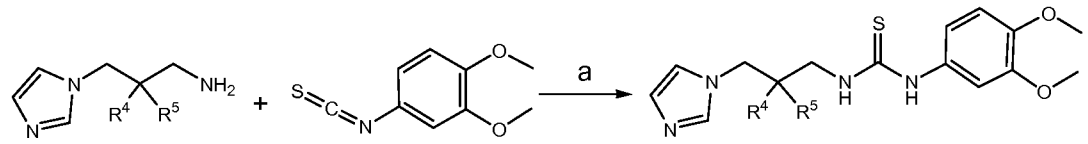
Reagents and conditions: (a) R-NCS, EtOH, 6h, reflux; (b) WSCD, 1*H*-imidazole-1-propanamine, DMF, 2h, r.t.

Synthesis scheme 3: Synthesis of the examples 103-105



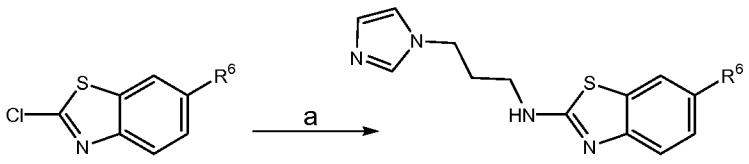
Reagents and conditions: (a) NaH, DMF, rt.,3h; (b) $LiAlH_4$, dioxane, reflux, 1h; (c) R-NCS, EtOH, reflux 6h,

Synthesis scheme 4: Synthesis of the examples 106 - 109



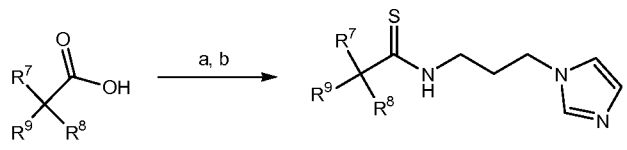
Reagents and conditions: (a) EtOH, 2 h, reflux

Synthesis scheme 5: Synthesis of the examples 110 - 112



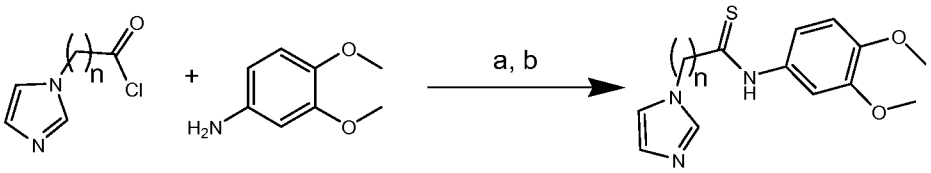
Reagents and conditions: (a) 1*H*-imidazole-1-propanamine, Triethylamine, Toluene, 12 h, reflux

Synthesis scheme 6: Synthesis of the examples 113 -132



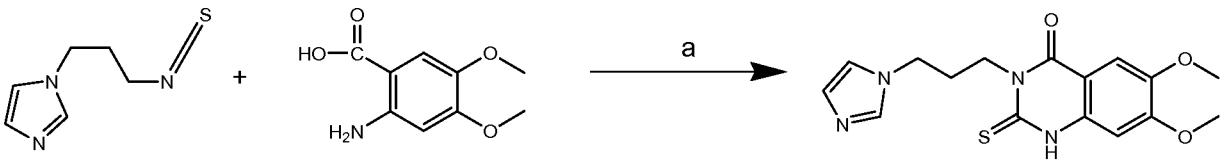
Reagents and conditions: (a) CAIBE, 1*H*-imidazole-1-propanamine, Dioxan, 0°C, 12h; (b) Laweson's Reaent, EtOH, reflux, 8h

Synthesis scheme 7: Synthesis of the examples 133 - 135



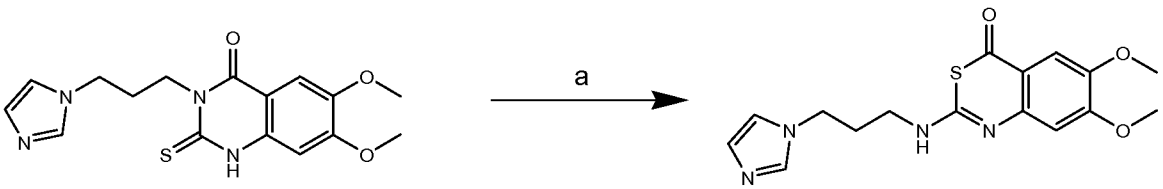
Reagents and conditions: (a) 1*H*-imidazole-1-propan acidic chloride, CH_2Cl_2, - 10°C., 1 h; (b) Lawesson's Reagent, Dioxane, reflux, 8 h

Synthesis scheme 8: Synthesis of the example 138



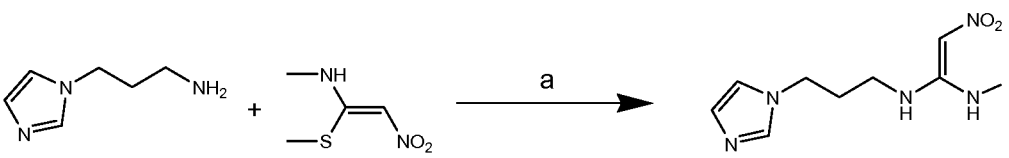
Reagents and conditions: (a) EtOH, reflux, 8 h

Synthesis scheme 9: Synthesis of the example 139



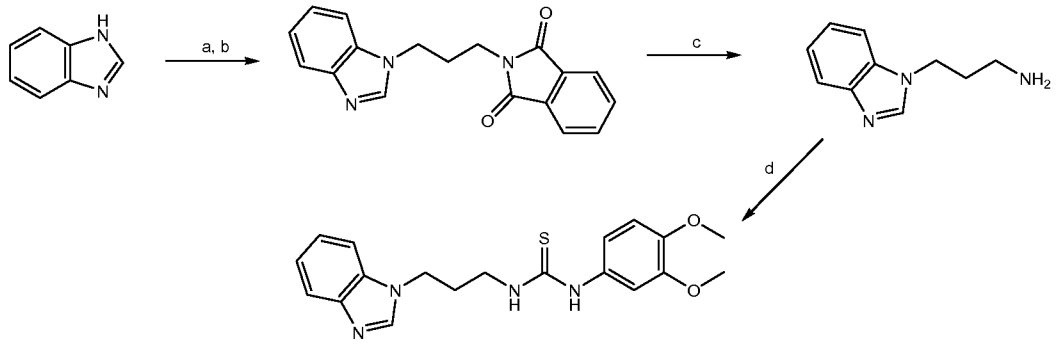
Reagents and conditions: (a) 75% conc. H_2SO_4 , 4h

Synthesis scheme 10: Synthesis of the example 140



Reagents and conditions: (a) Acetonitrile, reflux 2h

Synthesis scheme 11: Synthesis of the example 141



Reagents and conditions: (a) NaH, DMF, 4h, rt.; (b) , 8h, 100°C; (c) H_2N-NH_2,EtOH, 8h, reflux then 4N HCl, 6h, reflux, (d) 3,4 dimethoxy-phenyl-isothiocyanate, EtOH, 6h, reflux

Analytical conditions

ESI-Mass spectra were obtained with a SCIEX API 365 spectrometer (Perkin Elmer). The ^{1}H-NMR (500 MHz) data was recorded on a BRUKER AC 500, using DMSO-D_6 as solvent. Chemical shifts are expressed as parts per million downfield from tetramethylsilane. Splitting patterns have been designated as follows: s (singulet), d (doublet), dd (doublet of doublet), t (triplet), m (multiplet), and br (broad signal).

Detailed synthesis description

Examples **1 - 12 and 14 - 53**

1*H*-imidazole-1-propanamine was reacted with the corresponding isothiocyanate in ethanol under reflux for 8h. After that the solvent was removed and the remaining oil was dissolved in methylene chloride. The organic layer was washed twice with a saturated solution of $NaHCO_3$ followed by $NaHSO_4$ and brine, dried then evaporated. The remaining solid was re-crystallized from ethyl acetate, yielding the example thiourea in yields of 80 - 98%.

Example **13**

1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea

4.0 mmol of 3,4-dimethoxyphenyl isothiocyanate and 4.0 mmol of 3-(1*H*-imidazol-1-yl)alkyl-1-amine were dissolved in 10 mL of absolute ethanol. After stirring for 2 h under reflux, the solvent was evaporated and the resulting solid was recrystallized from ethanol.

Yield: 0.66 g (51.3 %); mp: 160.0 - 161.0°C

^{1}H NMR δ 1.8 - 2.0 (m, 2H), 3.4 - 3.5 (m, 2H), 3.75 (s, 6H), 3.9 - 4.0 (m, 2H), 6.7 - 6.8 (m, 1H), 6.9 (br m, 2H), 6.95 (s, 1H), 7.15 (s, 1H), 7.55 (br s, 1H), 7.6 (s, 1H), 9.3 (s, 1H); MS m/z 321.2 (M+H), 253.3 (M-$C_3H_3N_2$•)

Examples **96 – 102**

1*H*-imidazole-1-propanamine was reacted with the corresponding isocyanate in ethanol under reflux for 8h. After that the solvent was removed and the remaining oil was dissolved in methylene chloride. The organic layer was washed twice with a saturated solution of $NaHCO_3$ followed by $NaHSO_4$ and brine, dried then evaporated. The remaining solid was re-crystallized from ethyl acetate, yielding the example urea in yields of 85 - 90%.

Examples **136, 137**

The 1*H*-imidazole-1-alkylamines were prepared according to the literature from □-brom-alkyl-phtalimides and imidazolium salt and subsequent hydrazinolysis. The resulting products were transformed into the thioureas according to example 1 - 53 giving a 88% (example 136) and 95% (example 137) yield.

Examples **54 – 95**

All examples were made from the corresponding thioureas by reacting with Water-soluble-carbodiimide (WSCD) and 1*H*-imidazole-1-propanamine in dry dimethyl formamide for 2h at r.t. giving the trisubstituted guanidines with yields from 40 – 87%.

Examples **103 - 105**

Imidazole was reacted with the corresponding brommethylphenylcyanide in DMF, utilizing 1 equivalent of NaH for 3h under rt., giving the 1*H*-imidazole-1-

methylphenylcyanides. The solvent was removed and the resulting oil was re-dissolved in dioxane. The cyanides were converted in the corresponding amines using 1 equivalent of $LiAlH_4$. After adding a saturated solution of $KHSO_4$, dioxane was evaporated and the aqueous layer was extracted by means of $CHCl_3$. The organic layer was concentrated in *vacuo* and the amine was converted in the corresponding thioureas according to example **1 – 53** giving a 78% (example 103) and 65% (example 104) and 81% (example 105) yield.

Examples **106 – 109**

Starting from the corresponding methansulfonate-2-methylpropyl-phthalimides the amines were synthesized as described for the amines in example **136 – 137**. The resulting products were transformed into the thioureas according to example 1 – 53 giving example **106 – 109** in total yields of 25 – 30%.

Examples **110 – 112**

1*H*-imidazole-1-propanamine was reacted with the corresponding 2-chlorobenzo[d] thiazole in toluol for 24h at a temperature of 130°C. After removing the solvent and recristallization from methanol example 110 – 112 was yielded in an amount of 55 – 65%.

Examples **113 - 118, 120 - 124 and 126 - 132**

1*H*-imidazole-1-propanamine was reacted with the corresponding 2-phenyl acetic acid in dry dioxane by adding one equivalent of CAIBE and N-methylmorpholine at a temperature of 0°C. After 2h the mixture was allowed to warm to r.t. and the mixture was stirred for 12h. After removing the solvent the resulting oil was redissolved in methylene chloride and the organic layer was washed by means of an aqueous solution of $NaHCO_3$ and water, dried and the solvent was evaporated. The remaining oil was dissolved in dioxane adding Laweson's Reagent. After stirring for 12h a saturated solution of $NaHCO_3$ was added. Dioxane was evaporated and the aqueous layer was extracted by means of ethyl acetate. The organic layer was separated, dried and the solvent was evaporated. The remainig solid was crystallized from acetyl acetate/ether, giving **113 - 118, 120 - 124 and 126 - 132** with total yields of 62 – 85%.

Example **119**

N-(3-(1H-imidazol-1-yl)propyl)-2-(3,4-dimethoxyphenyl)ethanethioamide

A mixture of 4.0 mmol triethylamine and 4.0 mmol of 3-(1*H*-imidazol-1-yl)alkyl-1-amine 20 mL of dioxane was added drop wise to an ice cooled, stirred solution of 4.0 mmol of 2-(3,4-dimethoxyphenyl)acetyl chloride in 30 mL of dioxane. The mixture was allowed to warm to r.t., and then stirred for 1 h. After removing the solvent by reduced pressure, the residue was redissolved in 50 mL of dichloromethane. The organic layer was washed by means of 30 mL of saturated aqueous solution of $NaHCO_3$, and water. The organic solution was dried, filtered, and the solvent was removed under reduced pressure. After redissolving in 50 mL of dry dioxane 2.2 mmol of Lawesson's reagent was added, and the mixture was heated to 90°C and stirred for 8 h. The solvent was removed by reduced pressure, and the residue was redissolved in 50 mL of dichloromethane. The organic layer was washed three times by means of a saturated aqueous solution of $NaHCO_3$, followed three times by water, dried, filtered, and then the organic solvent was removed. The compound was purified by chromatography using a centrifugal-force-chromatography device, (Harrison Research Ltd.) utilizing silica plates of a layer thickness of 2 mm, and a $CHCl_3$/MeOH gradient as eluting system.

Yield: 0.14 g (10.6 %); melting point: 148.0 - 150.0°C

^{1}H NMR δ 2.0 - 2.15 (br m, 2H), 3.4 - 3.5 (m, 2H), 3.7 (s, 6H), 6.75 - 6.8 (m, 2H), 4.1 - 4.2 (m, 2H), 6.8 - 6.9 (m, 2H), 6.95 - 7.0 (m, 1H), 7.4 (s, 1H), 7.75 - 7.85 (br m, 1H), 8.6 (s, 1H), 10.2 (s, 1H); MS m/z 320.2 (M+H), 252.2 (M-$C_3H_3N_2$•)

Example **125**

N-(3-(1H-imidazol-1-yl)propyl)-1-(3,4-dimethoxyphenyl)cyclopropanecarbothioamide

11.06 mmol of 3,4-dimethoxyphenyl acetonitrile, 34.8 mmol of 2-Bromo-1-chloroethanole and 1.16 mmol of triethylbenzylammonium hydrochloride were dissolved in 10 mL of an aqueous solution of KOH (60%). The mixture was transferred into an ultrasonic bath and vigorously stirred for 3h at room temperature. The resulting suspension was diluted with 40 mL of water and extracted three times by means of 20 mL of dichloromethane. The combined organic layers where washed by means of an aqueous solution of hydrochloric acid (1N), dried over Na_2SO_4 and the solvent was removed under reduced pressure. The remaining oil was purified by flash-

chromatography using silica gel and ethyl acetate/heptane as eluting system, resulting in 0.81 g (34.4 %) of 1-(3,4-dimethoxyphenyl)cyclopropanecarbonitrile

3.9 mmol of 1-(3,4-dimethoxyphenyl)cyclopropanecarbonitrile and 11.2 mmol of KOH were suspended in 80 mL of ethylene glycol. The mixture was stirred for 12 h under reflux. Then 80 mL of water were added and the aqueous layer was extracted two times with ether. After pH adjustment to a value of pH = 4 – 5 using HCl (1N) the aqueous layer was extracted three times by means of ether, then the combined organic layers were dried over Na_2SO_4 and the solvent was removed, resulting in 0.81 g (93.5%) of 1-(3,4-dimethoxyphenyl)cyclopropanecarboxylic acid.

3.44 mmol of 1-(3,4-dimethoxyphenyl)cyclopropanecarboxylic acid, 3.5 mmol of N-Methyl morpholine, and 3.5 mmol of isobutyl chloroformiat were dissolved in dry tetrahydrofurane and stirred for 15 min at –15°C. Then 3.5 mmol of 3-(1H-imidazol-1-yl)alkyl-1-amine was added and the mixture was allowed to warm to 0°C and was stirred for 12h. The solvent was removed under reduced pressure and the remaining oil was redissolved in chloroform. Then the organic layer was washed two times by means of a saturated aqueous solution of $NaHCO_3$, then dried over Na_2SO_4 and the solvent was removed. Purification was performed by means of centrifugal forced chromatography using a chromatotron® device (Harrison Research Ltd.) utilizing silica plates of a layer thickness of 2 mm, and a $CHCl_3$/MeOH gradient as eluting system resulting in 0.671 g (59.3%) of N-(3-(1H-imidazol-1-yl)propyl)-1-(3,4-dimethoxyphenyl)cyclopropane-carboxamide.

After redissolving in 30 mL of dry dioxane 1.43 mmol of Lawesson's reagent were added, and the mixture was heated to 90°C and stirred for 8h. The solvent was removed by reduced pressure, and the residue was remains were dissolved in 50 mL of dichloromethane. The organic layer was washed three times by means of a saturated aqueous solution of $NaHCO_3$, followed three times by water, dried, filtered, and then the organic solvent was removed. The compound was purified by chromatography using a centrifugal-force-chromatography device, (Harrison Research Ltd.) utilizing silica plates of a layer thickness of 2 mm, and a $CHCl_3$/MeOH gradient as eluting system.

Yield: 0.33 g (46.2 %); melting point: 127.0 - 127.5°C

^{1}H NMR δ 1.1 - 1.2 (t, 2H), 1.55 - 1.6 (t, 2H), 2.0 - 2.1 (m, 2H), 3.5 - 3.6 (m, 2H), 3.7 - 3.8 (s, 6H), 4.1 - 4.2 (t, 2H), 6.8 - 6.9 (m, 3H), 7.65 (s, 1H), 7.75 (s, 1H), 8.8 (m, 1H), 9.05 (s, 1H; MS m/z 346.0 (M+H), 278.2 (M-$C_3H_3N_2$•), 177.1 (M-$C_6H_8N_3S$•)

Examples **133 - 135**

A mixture of 1 equivalent triethylamine and 3,4-dimethoxyaniline in dioxane was added to an stirred solution of the corresponding ω-bromoalkyl acidic chloride at a temperature of 0°C. The solution was allowed to warm to r.t. and stirred for 2h. The solvent was evaporated, and the remaining oil was redissolved in dichloromethane. The organic layer was washed by means of water, dried, filtered, and the solvent was removed under reduced pressure.

Imidazole and sodium hydride were suspended in and the mixture was stirred under inert conditions at r.t. for 3 h. ω-Bromo-N-(3,4-dimethoxy-phenyl)alkylamide was added and the mixture was heated to 100°C and stirred for 8 h. After that, the solvent was evaporated, hot toluene were added and the solution was filtered. Then the solvent was removed under reduced pressure. The transformation into the thioamides was performed as described for example 113 – 132 by means of Laweson's reagent, giving **133 – 135** in total yields of 13 – 20 %.

The analytical data for further examples, which were syntesized according to the general synthesis schemes described above, are as follows:

Example 1: *1-(3-(1H-imidazol-1-yl)propyl)-3-methylthiourea*

melting point: 122 - 122.5°C

^{1}H NMR δ 1.85 - 1.95 (m, 2H), 2.8 (s, 3H), 3.2 - 3.5 (br d, 2H), 3.8 - 3.9 (m, 2H), 6.85 (d, 1H), 7.15 (d, 1H), 7.3 - 7.5 (br d, 2H), 7.65 (s, 1H); MS m/z 199.1 (M+H), 221.3 (M+Na), 131.0 (M-$C_3H_3N_2$•)

Example 2: *1-(3-(1H-imidazol-1-yl)propyl)-3-tert-butylthiourea*

melting point: 147.0 - 147.5°C

^{1}H NMR δ 1.3 - 1.4 (s, 9H), 1.85 -1.95 (m, 2H), 3.5 (t, 2H), 3.8 (t, 2H), 6.85 (d, 1H), 7.15 (d, 1H), 7.3 - 7.5 (br d, 2H), 7.65 (s, 1H); MS m/z 241.1 (M+H), 173.1 (M-$C_3H_3N_2$•)

Example 3: *1-(3-(1H-imidazol-1-yl)propyl)-3-benzylthiourea*

melting point: 127.0 - 128.0°C

^{1}H NMR δ 1.85 - 1.95 (m, 2H), 3.2 - 3.5 (br d, 2H), 3.8 - 3.9 (m, 2H), 4.6 (s, 2H), 6.8 (d, 1H), 7.15 (d, 1H), 7.19 - 7.35 (m, 5H), 7.5 - 7.6 (br d, 2H), 7.85 (s, 1H); MS m/z 275.3 (M+H), 207.1 (M-$C_3H_3N_2$•)

Example 5:*1-(3-(1H-imidazol-1-yl)propyl)-3-phenylthiourea*

melting point: 166.5 - 167.0°C

^{1}H NMR δ 1.95 - 2.05 (m, 2H), 3.3 - 3.5 (br d, 2H), 3.9 - 4.0 (m, 2H), 6.85 (d, 1H), 7.05 (m, 1H) 7.15 (d, 1H), 7.25 (m, 2H), 7.35 (m, 2H), 7.6 (s, 1H), 7.8 (br s, 1H), 9.5 (br s, 1H); MS m/z 261.1 (M+H), 193.2 (M-$C_3H_3N_2$•)

Example 6: *1-(3-(1H-imidazol-1-yl)propyl)-3-(4-fluorophenyl)thiourea*

melting point: 147.0 - 148.0°C

^{1}H NMR δ 1.95 - 2.05 (m, 2H), 3.3 - 3.5 (br d, 2H), 3.9 - 4.05 (m, 2H), 6.85 (d, 1H), 7.05 - 7.15 (m, 3H), 7.3 - 7.4 (m, 2H), 7.6 (s, 1H), 7.7 - 7.8 (br s, 1H), 9.4 (br s, 1H); MS m/z 279.3 (M+H), 211.2 (M-$C_3H_3N_2$•)

Example 7: *1-(3-(1H-imidazol-1-yl)propyl)-3-(4-ethylphenyl)thiourea*

melting point: 100.0 - 100.5°C

^{1}H NMR δ 1.15 - 1.2 (t, 3H), 1.9 - 2.0 (m, 2H), 2.5 - 2.6 (m, 2H), 3.3 - 3.5 (br d, 2H), 3.9 - 4.05 (m, 2H), 6.85 (d, 1H), 7.1 - 7.2 (m, 3H), 7.25 - 7.3 (m, 2H), 7.6 (s, 1H), 7.7 - 7.8 (br s, 1H), 9.4 (br s, 1H); MS m/z 289.3 (M+H), 221.1 (M-$C_3H_3N_2$•)

Example 8: *1-(3-(1H-imidazol-1-yl)propyl)-3-(4-(trifluoromethyl)phenyl)thiourea*

melting point: 154.5 - 155.0°C

^{1}H NMR δ 1.9 - 2.1 (br m, 2H), 3.4 - 3.6 (br d, 2H), 3.95 - 4.1 (br m, 2H), 6.85 (d, 1H), 7.2 (d, 1H), 7.6 - 7.8 (m, 5H), 8.2 (br s, 1H), 9.9 (br s, 1H); MS m/z 329.3 (M+H), 261.2 (M-$C_3H_3N_2$•)

Example 10: *1-(3-(1H-imidazol-1-yl)propyl)-3-(4-acetylphenyl)thiourea*

melting point: 170.0 - 171.0°C

^{1}H NMR δ 1.9 - 2.1 (br m, 2H), 2.4 - 2.5 (s, 3H), 3.2 – 3.5 (br m, 2H), 3.9 - 4.1 (m, 2H), 6.85 (d, 1H), 7.15 (d, 1H), 7.5 - 7.65 (br m, 3H), 7.8 - 7.9 (m, 2H), 8.1 (m, 2H), 9.8 (br s, 1H); MS m/z 303.2 (M+H), 235.1 (M-$C_3H_3N_2$•)

Example 11: *1-(3-(1H-imidazol-1-yl)propyl)-3-(4-methoxyphenyl)thiourea*
melting point: 125.0 - 125.5°C
^{1}H NMR δ 1.8 - 2.0 (br m, 2H), 3.2 - 3.5 (br m, 2H), 3.7 (s, 3H), 3.9 - 4.0 (m, 2H), 6.7 - 6.9 (m, 3H), 7.1 - 7.2 (m, 3H), 7.5 (s, 1H), 7.6 (s, 1H), 9.2 (s, 1H); MS m/z 291.1 (M+H), 223.2 (M-$C_3H_3N_2$•)

Example 14: *1-(3-(1H-imidazol-1-yl)propyl)-3-(2,4-dimethoxyphenyl)thiourea*
melting point: 120.0 - 120.5°C
^{1}H NMR δ 1.8 - 2.0 (br m, 2H), 3.4 - 3.5 (br m, 2H), 3.75 (s, 6H), 3.9 - 4.0 (m, 2H), 6.5 (d, 1H), 6.6 (s, 1H), 6.9 (s, 1H), 7.15 (s, 1H), 7.3 (d, 1H), 7.5 (br s, 1H), 7.6 (s, 1H), 9.75 (s, 1H); MS m/z 321.2 (M+H), 253.3 (M-$C_3H_3N_2$•)

Example 15: *1-(3-(1H-imidazol-1-yl)propyl)-3-(3,5-dimethoxyphenyl)thiourea*
melting point: 142.0 - 143.0°C
^{1}H NMR δ 1.8 - 2.0 (br m, 2H), 3.4 - 3.5 (br m, 2H), 3.6 (s, 6H), 3.95 - 4.0 (m, 2H), 6.25 (m, 1H), 6.6 (m, 2H), 6.9 (s, 1H), 7.2 (s, 1H), 7.6 (s, 1H), 7.8 (s, 1H), 9.5 (s, 1H); MS m/z 321.2 (M+H), 253.3 (M-$C_3H_3N_2$•)

Example 23: *1-(3-(1H-imidazol-1-yl)propyl)-3-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-thiourea*
melting point: 103.0 - 103.5°C
^{1}H NMR δ 1.9 - 2.0 (br m, 2H), 3.3 - 3.5 (br d, 2H), 3.9 - 4.0 (m, 2H), 4.2 - 4.3 (m, 4H), 6.7 (m, 1H), 6.8 - 6.8 (m, 1H), 6.9 (m, 2H), 7.2 (s, 1H), 7.6 (m, 2H), 9.3 (s, 1H); MS m/z 319.3 (M+H), 251.3 (M-$C_3H_3N_2$•)

Example 24: *1-(3-(1H-imidazol-1-yl)propyl)-3-(benzo[d][1,3]dioxol-6-yl)thiourea*
melting point: 115.0 - 115.6°C

^{1}H NMR δ 1.9 - 2.1 (br m, 2H), 3.4 - 3.5 (br d, 2H), 4.05 - 4.15 (m, 2H), 6.0 (s, 2H), 6.7 (m, 1H), 6.8 - 6.85 (m, 1H), 6.95 (d, 1H), 7.25 (s, 1H), 7.45 (s, 1H), 7.7 (br s, 1H), 8.5 (br s, 1H), 9.4 (br s, 1H); MS m/z 305.2 (M+H), 237.2 (M-$C_3H_3N_2$•)

Example 25: *1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4,5-trimethoxyphenyl)thiourea*
melting point: 124.5 - 125.5°C
^{1}H NMR δ 1.8 - 2.0 (m, 2H), 3.4 - 3.5 (br m, 2H), 3.6 (s, 3H), 3.7 (s, 6H), 3.9 - 4.0 (m, 2H), 6.65 (m, 2H), 6.85 (s, 1H), 7.2 (s, 1H), 7.6 (s, 1H), 7.7 (br s, 1H), 9.4 (s, 1H); MS m/z 351.3 (M+H), 283.2 (M-$C_3H_3N_2$•)

Example 26: *1-(3-(1H-imidazol-1-yl)propyl)-3-(3-methoxyphenyl)thiourea*
melting point: 89.5 - 90.0°C
^{1}H NMR δ 1.9 - 2.1 (br m, 2H), 3.4 - 3.5 (br m, 2H), 3.7 (s, 3H), 3.9 - 4.0 (m, 2H), 6.6 - 6.7 (m, 1H), 6.8 - 6.9 (m, 2H), 7.1 (m, 2H), 7.15 - 7.25 (br m, 1H), 7.6 (s, 1H), 7.8 (br s, 1H), 9.5 (s, 1H); MS m/z 291.1 (M+H), 223.2 (M-$C_3H_3N_2$•)

Example 27: 1-*(3-(1H-imidazol-1-yl)propyl)-3-(4-ethoxyphenyl)thiourea*
melting point: 126.0 - 126.5°C
^{1}H NMR δ 1.5 (br m, 3H), 1.9 - 2.0 (br m, 2H), 3.4 - 3.5 (br m, 2H), 3.9 - 4.0 (br m, 4H), 6.8 - 6.9 (m, 2H), 6.95 (s, 1H), 7.15 - 7.2 (m, 2H), 7.25 (s, 1H), 7.55 - 7.6 (br s, 1H), 7.8 (s, 1H), 9.3 (s, 1H); MS m/z 305.2 (M+H), 237.2 (M-$C_3H_3N_2$•)

Example 33:*1-(3-(1H-imidazol-1-yl)propyl)-3-(4-(methylthio)phenyl)thiourea*
melting point: 140.0 - 140.5°C
^{1}H NMR δ 1.8 - 2.05 (br m, 2H), 2.5 (s, 3H), 3.3 - 3.5 (br m, 2H), 3.9 - 4.1 (m, 2H), 6.9 (m, 1H), 7.1 - 7.3 (br m, 5H), 7.6 (s, 1H), 7.75 (br s, 1H), 9.4 (s, 1H); MS m/z 307.2 (M+H), 239.2 (M-$C_3H_3N_2$•)

Example 42: *1-(3-(1H-imidazol-1-yl)propyl)-3-(4-nitrophenyl)thiourea*
melting point: 165.0 . 166.0°C
^{1}H NMR δ 1.9 - 2.05 (m, 2H), 3.3 - 3.5 (br d, 2H), 3.95 - 4.05 (m, 2H), 6.85 (d, 1H), 7.15 (d, 1H), 7.6 (d, 1H), 7.7 (m, 2H), 8.1 (m, 2H), 8.3 (br s, 1H), 10.1 (br s, 1H); MS m/z 306.2 (M+H), 237.9 (M-$C_3H_3N_2$•)

Example 50: *1-(3-(1H-imidazol-1-yl)propyl)-3-(4-(dimethylamino)phenyl)thiourea*
melting point: 146.5 - 147.0°C
^{1}H NMR δ 1.9 - 2.0 (m, 2H), 2.9 (s, 6H), 3.4 (m, 2H), 3.9 - 4.0 (m, 2H), 6.7 (m, 2H), 6.9 (s, 1H), 7.05 - 7.1 (m, 2H), 7.15 (s, 1H), 7.4 (br s, 1H), 7.6 (s, 1H), 9.2 (s, 1H); MS m/z 304.2 (M+H), 236.0 (M-$C_3H_3N_2$•)

Example 102: *1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)urea*
melting point: 114.5 - 115.0°C
^{1}H NMR δ 1.7 - 1.9 (m, 2H), 2.9 - 3.1 (m, 2H), 3.7 (2s, 6H), 3.9 - 4.0 (m, 2H), 6.1 (t, 1H), 6.7 (s, 2H), 6.8 (s, 1H), 7.15 (d, 2H), 7.6 (s, 1H), 8.2 (s, 1H); MS m/z 321.2 (M+H), 253.3 (M-$C_3H_3N_2$•)

Example 106: *1-((S)-3-(1H-imidazol-1-yl)-2-methylpropyl)-3-(3,4-dimethoxyphenyl)-thiourea*
melting point:: 150.5 - 151.5°C
^{1}H NMR δ 0.9 (d, 3H), 2.3 - 2.4 (m, 2H), 2.5 (s, 1H), 3.7 (d, 6H), 4.0 - 4.1 (br m, 1H), 4.15 - 4.25 (br m, 1H), 6.75 - 6.8 (m, 1H), 6.85 (m, 1H), 6.9 - 7.0 (m, 1H), 7.65 (s, 1 H), 7.75 (s, 2H), 9.1 (s,1H), 9.5 (s, 1H); MS m/z 335.6 (M+H), 267.1 (M-$C_3H_3N_2$•)

Example 107: *1-((R)-3-(1H-imidazol-1-yl)-2-methylpropyl)-3-(3,4-dimethoxyphenyl)-thiourea*
melting point: 155.0 - 157.5 °C
^{1}H NMR δ 0.9 (d, 3H), 2.3 - 2.4 (m, 2H), 2.5 (s, 1H), 3.7 (d, 6H), 4.0 - 4.1 (br m, 1H), 4.15 - 4.25 (br m, 1H), 6.75 - 6.8 (m, 1H), 6.85 (m, 1H), 6.9 - 7.0 (m, 1H), 7.65 (s, 1 H), 7.75 (s, 2H), 9.1 (s,1H), 9.5 (s, 1H); MS m/z 335.4 (M+H), 267.2 (M-$C_3H_3N_2$•)

Example 109: *1-((1-((1H-imidazol-1-yl)methyl)cyclopropyl)methyl)-3-(3,4-dimethoxy-phenyl)thiourea*
melting point: 166.5 - 168.5°C
^{1}H NMR δ 0.7 - 0.8 (br m, 2H), 1.85 - 1.9 (m, 1H), 2.15 - 2.2 (m, 1H), 2.2 - 2.3 (m, 1H), 3.4 - 3.5 (m, 1H), 3.7 (d, 6H), 4.2 (s, 1H), 4.95 (s, 1H), 6.75 - 6.8 (br m, 1H), 6.85 - 6.9 (br m, 1H), 7.0 (s, 1H), 7.5 (m, 1H), 7.6 (m, 1H), 7.7 (s, 0.5H), 7.8 (s, 0.5H), 8.85 (s, 0.5

H), 9.1 (s, 0.5H), 9.35 (s, 0.5H), 9.45 (s, 0.5H); MS m/z 347.2 (M+H), 279.2 (M-$C_3H_3N_2$•), 137.5 (M-$C_9H_{13}N_4S$•)

Example 110: *N-(3-(1H-imidazol-1-yl)propyl)benzo[d]thiazol-2-amine*

^{1}H NMR δ 1.95 - 2.15 (m, 2H), 3.25 - 3. 35 (m, 2H), 4.0 - 4.1 (t, 2H), 6.9 (s, 1H), 6.95 - 7.05 (t, 1H), 7.15 – 7.2 (m, 2H), 7.35 – 7.4 (d, 1H), 7.60 – 7.70 (m, 2H), 8.0 – 8.1 (br s, 1H); MS m/z 259.4 (M+H), 191.3 (M-$C_3H_3N_2$•)

Example 111: *N-(3-(1H-imidazol-1-yl)propyl)-6-chlorobenzo[d]thiazol-2-amine*

^{1}H NMR δ 1.95 - 2.15 (m, 2H), 3.25 - 3. 35 (m, 2H), 4.0 - 4.1 (t, 2H), 6.9 (s, 1H), 7.1 – 7.2 (d, 2H), 7.3 – 7.4 (d, 1H), 7.65 (s, 1H), 7.8 (s, 1H), 8.2 (s, 1H); MS m/z 293.3 (M+H), 225.3 (M-$C_3H_3N_2$•)

Example 112: *N-(3-(1H-imidazol-1-yl)propyl)-6-methoxybenzo[d]thiazol-2-amine*

^{1}H NMR δ 1.9 - 2.05 (m, 2H), 3.2 - 3. 3 (m, 2H), 3.7 (s, 3H), 4.0 - 4.1 (t, 2H), 6.7 – 6.8 (d, 1H), 6.9 (s, 1H), 7.15 – 7.2 (s, 1H), 7.2 – 7.3 (m, 2H), 7.65 (s, 1H), 7.8 (s, 1H); MS m/z 289.1 (M+H), 221.4 (M-$C_3H_3N_2$•)

Example 115: *(R)-N-(3-(1H-imidazol-1-yl)propyl)-2-phenylpropanethioamide*

melting point: 82.0 - 82.5°C

^{1}H NMR δ 1.4 - 1.55 (d, 3H), 1.9 - 2.0 (m, 2H), 3.4 - 3.5 (m, 2H), 3.85 - 3.95 (m, 2H), 4.0 - 4.1 (q, 1H), 6.8 - 6.9 (s, 1H), 7.1 (s, 1H), 7.15 - 7.2 (m, 1H), 7.2 - 7.3 (m, 2H), 7.35 - 7.4 (m, 2H), 7.55 (s, 1H), 10.1 (s, 1H); MS m/z 274.4 (M+H), 206.3 (M-$C_3H_3N_2$•)

Example 116: *(S)-N-(3-(1H-imidazol-1-yl)propyl)-2-phenylpropanethioamide*

melting point: 82.5 - 83.5°C

^{1}H NMR δ 1.4 - 1.55 (d, 3H), 1.9 - 2.0 (m, 2H), 3.4 - 3.5 (m, 2H), 3.85 - 3.95 (m, 2H), 4.0 - 4.1 (q, 1H), 6.8 - 6.9 (s, 1H), 7.1 (s, 1H), 7.15 - 7.2 (m, 1H), 7.2 - 7.3 (m, 2H), 7.35 - 7.4 (m, 2H), 7.55 (s, 1H), 10.1 (s, 1H); MS m/z 274.4 (M+H), 206.3 (M-$C_3H_3N_2$•)

Example 121: *N-(3-(1H-imidazol-1-yl)propyl)-1-(4-chlorophenyl)cyclobutanecarbothioamide*

melting point: 137.5 - 139.0°C

^{1}H NMR δ 1.55 - 1.75 (br m, 2H), 1.85 - 1.95 (br m, 2H), 2.4 - 2.5 (br m, 2H), 2.7 - 2.85 (br m, 2H), 3.3 - 3.5 (br m, 2H), 3.8 (m, 2H), 6.9 (s, 1H), 7.0 (s, 1H), 7.3 (m, 2H), 7.45 (s, 1H), 7.5 (m, 2H), 9.6 (t, 1H); MS m/z 334.3 (M+H), 266.1 (M-$C_3H_3N_2$•)

Example 122: *N-(3-(1H-imidazol-1-yl)propyl)-1-(4-chlorophenyl)cyclopentanecarbothioamide*

melting point: 140.0 - 141.0°C

^{1}H NMR δ 1.5 - 1.65 (br m, 4H), 1.8 - 1.9 (m, 2H), 2.0 - 2.1 (m, 2H), 2.6 (m, 2H), 3.4 - 3.5 (m, 2H), 3.7 - 3.8 (m, 2H), 6.85 (s, 1H), 7.0 (s, 1H), 7.35 (m, 2H), 7.4 (m, 2H), 7.5 (s, 1H), 9.4 (t, 1H); MS m/z 348.2 (M+H), 280.2 (M-$C_3H_3N_2$•)

Example 123: *N-(3-(1H-imidazol-1-yl)propyl)-1-(4-methoxyphenyl)cyclohexanecarbothioamide*

melting point: 162.5 - 164.0°C

^{1}H NMR δ 1.2 - 1.3 (m, 1H), 1.35 - 1.5 (br m, 5H), 1.85 - 2.0 (br m, 4H), 2.4 - 2.6 (br m, 2H), 3.4 - 3.5 (m, 2H), 3.7 (s, 3H), 3.8 (m, 2H), 6.8 (m, 3H), 7.0 (s, 1H), 7.3 (m, 2H), 7.5 (s, 1H), 9.2 (t, 1H); MS m/z 358.3 (M+H), 290.3 (M-$C_3H_3N_2$•)

Example 124: *N-(3-(1H-imidazol-1-yl)propyl)-1-(4-methoxyphenyl)cyclopropanecarbothioamide*

melting point:: 129.0 - 129.5°C

^{1}H NMR δ 1.0 - 1.1 (m, 2H), 1.5 - 1.6 (m, 2H), 1.9 - 2.0 (br m, 2H), 3.4 - 3.5 (m, 2H), 3.7 (s, 3H), 3.9 (m, 2H), 6.9 (m, 3H), 7.1 (s, 1H), 7.2 - 7.3 (m, 2H), 7.6 (s, 1H), 8.9 (br s, 1H); MS m/z 316.0 (M+H), 248.4 (M-$C_3H_3N_2$•)

Example 134: *5-(1H-imidazol-1-yl)-N-(3,4-dimethoxyphenyl)pentanethioamide*

melting point:: 128.0 - 128.5°C

^{1}H NMR δ 1.65 - 1.70 (m, 2H), 1.75 - 1.80 (m, 2H), 2.7 - 2.75 (m, 2H), 3.7 (s, 3H), 3.75 (s, 3H), 4.0 - 4.05 (t, 2H), 6.9 - 7.0 (m, 2H), 7.2 (s, 1H), 7.3 (d, 1H), 7.5 (s, 1H), 7.75 (s, 1H), 11.0 (s, 1H); MS m/z 320.2 (M+H), 252.2 (M-$C_3H_3N_2$•)

Example 136: *1-(2-(1H-imidazol-1-yl)ethyl)-3-(3,4-dimethoxyphenyl)thiourea*

melting point: 157.5 - 159.0°C

^{1}H NMR δ 3.7 (2 s, 6H), 3.8 (m, 2H), 4.2 (m, 2H), 6.7 (m, 1H), 6.85 (m, 1H), 6.9 (m, 2H), 7.15 (s, 1H), 7.5 (br s, 1H), 7.6 (s, 1H), 9.5 (s, 1H); MS m/z 307.2 (M+H), 239.1 (M-$C_3H_3N_2$•)

Abbreviations

°C	degree Celsius
A, Ala	alanine
Aβ	amyloid-β peptide
ABri	amyloid peptide in familial british dementia
AC	adenylyl cyclase
ADan	amyloid peptide in familial danish dementia
AIM	absent in melanoma
AMC	aminio methyl coumarine
as	antisense
Asp	aspartate
βNA	beta-naphtylamine
BA	butyric acid
bp	basepair
BSA	bovine serum albumin
C	cysteine
CAT	chloramphenicol acetyl transferase
cAMP	cyclic adenosine monophsphate
CCL2	MCP-1, monocyte chemoattractant protein 1
CCL7	MCP-3, monocyte chemoattractant protein 3
CCL8	MCP-2, monocyte chemoattractant protein 2
CCL13	MCP-4, monocyte chemoattractant protein 4
cDNA	copy-DNA
C-His	C-terminal histidine tag
CIDP	Chronic inflammatory demyelinizing polyradiculoneuropathy
Cl	chlorine
CSF	cerebor-spinal fluid (*liquor cerebrospinalis*)
C-terminus	carboxy-terminus
CTL	cytotoxic T-lymphocyte
CV	column volume
d	diameter
Da	Dalton

DMSO	dimethyl sulphoxide
DNA	desoxyribonucleic acid
E	enzyme
EBV	Epstein Barr virus
ECL	enterochromaffin-like
E. coli	*Escherichia coli*
EC	glutamyl cyclase
ED	effective dose
EGFP	enhanced green fluorescent protein
ES	enzyme-substrate complex
FPP	fertilization promoting peptide
FTC	follicular thyroid carcinoma
g	relative centrifugal force
GBS	Guillain-Barré syndrome
GF	gel filtration
Gln	glutamine
Glu	glutamic acid
GnRH	gonadotropin-releasing hormone (gonadoliberin)
GST	glutathion S-transferase
H	hydrogen
h	human, hour
HGF	hepatocyte growth factor
HIC	hydrophobic interaction chromatography
HIF1a	hypoxia induced factor 1a
His	histidine
HPLC	high performance liquid chromatography
I	inhibitor, isoleucine
ID	identification
IMAC	immobilized metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranosid
K	potassium
k	constant
kDA	kilo-dalton

k_i	inhibitor constant
KLH	Keyhole limpet hemocyanin
l	length
LB	Luria-Bertani
LD	lethal dose
LPS	lipopolysaccharide
M	molar
µl	micro-liter
µM	micro-molar
MAGEA	melanoma antigen family A
MAGEB	melanoma antigen family B
Maldi-tof	matrix assisted laser desorption/ionization time-of-flight
MART1	melanoma antigen recognized by T-cells 1
max	maximum
MCL-1	myeloid cell leukemia 1
Met	methionine
min	minutes
mM	milli-molar
MS	Multiple Sclerosis
mRNA	messenger-RNA
N	asparagine
Na	sodium
NADH	nicotinamide adenine dinucleotide
nm	nanometer
NO	number
NT	Neurotensin
N-terminus	amino terminus
O	oxygen
OD	optical density
P	product, phosphor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pGlu	pyroglutamic acid

pH	pondus hydrogenii
Pro	proline
PTC	papillary thyroid carcinoma
Pyr	pyroglutamate
QC	glutaminyl cyclase (glutaminyl-peptide cyclotransferase)
qPCR	quantitative real-time polymerase chain reaction
QPCTL	glutaminyl-peptide cyclotransferase – like
RNA	ribonucleic acid
RT	reverse transcription; reverse transcriptase
S	substrate
s	sense
SAGE	serial analysis of gene expression
SDS	sodium dodecly sulfate
SDS-PAGE	SDS-polyacrylamid gelelectrophoresis
SGAP	*Streptomyces griseus* amino peptidase
SEQ	sequence
SNP	single nucleotide polymorphism
taa	tumor-associated antigen
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
TRH	thyreotropin-realeasing hormone (thyreoliberin)
TSH	thyroidea-stimulating-hormone
TYR	tyrosinase
TYRP	tyrosinase related protein
U	unit
UTC	undifferentiated thyroid carcinoma
UV	ultraviolet
V	velocity
VpAP	*Vibrio proteolytica* amino peptidase
YSS	yeast signal sequence
Zn	zinc

Claims

1. Isolated nucleic acid which encodes (a) a polypeptide, which includes the amino acid sequence of one of SEQ ID NOS: 11 to 18, or (b) a polypeptide having an amino acid sequence that is at least about 75% similar thereto and exhibits the same biological function; or which is an alternative splice variant of one of SEQ ID NOS: 2 to 9; or which is a probe comprising at least 14 contiguous nucleotides from said nucleic acid encoding (a) or (b); or which is complementary to any one of the foregoing.

2. The isolated nucleic acid of claim 1 which is DNA or RNA.

3. The isolated nucleic acid of claim 1 which is a DNA transcript that includes the entire length of any one of SEQ ID NOS: 2 to 9 or which is complementary to the entire coding region of one of SEQ ID NOS: 2 to 9.

4. An antisense oligonucleotide directed against the DNA of claim 3.

5. The isolated nucleic acid of claim 1 which is an RNA transcript, which includes the entire length of any one of SEQ ID NOS: 2 to 9.

6. The isolated nucleic acid of claim 1 which is an alternative splice variant of one of SEQ ID NOS: 2 to 9.

7. The splice variant of claim 6 corresponding to SEQ ID NOS: 19 or 20.

8. A polypeptide encoded by a nucleic acid of claim 6 or 7.

9. The isolated nucleic acid of claim 1 which encodes a polypeptide having an amino acid sequence that is at least about 85% similar to one of SEQ ID NOS: 11 to 18.

10. The isolated nucleic acid of claim 1 which encodes a polypeptide having an amino acid sequence that is at least about 95% similar to one of SEQ ID NOS: 11 to 18.

11. The isolated nucleic acid of claim 1 which encodes a polypeptide that has at least about 50% identity with one of SEQ ID NOS: 11 to 18.

12. A nucleic acid probe according to claim 1 comprising at least 14 contiguous nucleotides from one of SEQ ID NOS: 2 to 9.

13. The nucleic acid probe of claim 12 corresponding to any one of SEQ ID NOS: 53 to 61.

14. An isolated recombinant polynucleotide molecule comprising the nucleic acid according to claim 1 plus expression-controlling elements operably linked with said nucleic acid to drive expression thereof.

15. An expression vector comprising the nucleic acid of claim 1 encoding a polypeptide having the entire amino acid sequence set forth in any one of SEQ ID NOS: 11 to 18 operably linked to a promoter, said expression vector being present in a compatible host cell.

16. A mammalian, insect or bacterial host cell that has been genetically engineered by the insertion of nucleic acid according to claim 1 which codes for at least the mature protein portion of one the amino acid sequences of SEQ ID NOS: 11 to 18.

17. A process for producing a polypeptide which includes the mature protein portion of one of SEQ ID NOS: 11 to 18, which process comprises culturing the host cell of claim 16 under conditions sufficient for the production of said polypeptide.

18. The process of claim 17 wherein said polypeptide is expressed at the surface of said cell and further includes the step of recovering the polypeptide or a fragment thereof from the culture.

19. A polypeptide which may be optionally glycosylated, and which (a) has the amino acid sequence of a mature protein set forth in any one of SEQ ID NOS: 11 to 18; (b) has the amino acid sequence of a mature protein having at least about 75% similarity to one of the mature proteins of (a) and which exhibits the same biological function; (c) has the

amino acid sequence of a mature protein having at least about 50% identity with a mature protein of any of SEQ ID NOS: 11 to 18; or (d) is an immunologically reactive fragment of (a).

20. The polypeptide according to claim 19 which is a mature protein having at least about 85% similarity to a mature protein of (a).

21. The polypeptide according to claim 19 which is a mature protein having at least about 95% similarity to a mature protein of (a).

22. The polypeptide according to claim 19 having the amino acid sequence of the mature protein of one of SEQ ID NOS: 11 to 18, or is a fragment thereof which exhibits the same biological function as the respective mature protein.

23. An antibody that recognizes a polypeptide or a fragment according to any one of claims 19 to 22.

24. The antibody of claim 23, which recognizes a polypeptide having an amino acid sequence of one of SEQ ID NOS: 11 to 18.

25. A method of screening for a compound capable of inhibiting the enzymatic activity of at least one mature protein of claim 19, which method comprises incubating said mature protein and a suitable substrate for said mature protein in the presence of one or more test compounds or salts thereof, measuring the enzymatic activity of said mature protein, comparing said activity with comparable activity determined in the absence of a test compound, and selecting the test compound or compounds that reduce the enzymatic activity.

26. A method of screening for a selective QC-inhibitor that does not inhibit the enzymatic activity of at least one mature protein of claim 19, which method comprises incubating said mature protein and a suitable substrate in the presence of one or more inhibitors or salts thereof of QC, measuring the enzymatic activity of said mature protein, comparing said activity with comparable activity determined in the absence of

the QC inhibitor, and selecting a compound that does not reduce the enzymatic activity of said mature protein.

27. A method of screening for a selective QPCTL-inhibitor that does not inhibit the enzymatic activity of QC, which method comprises incubating said QC in the presence of one or more inhibitors or salts thereof of a QPCTL, measuring the enzymatic activity of QC, comparing said activity with comparable activity determined in the absence of the QPCTL inhibitor, and selecting a compound that does not reduce the enzymatic activity of said QPCTL protein.

28. A QPCTL antagonist, which inhibits the biological function of one of said mature proteins of any one of claims 19 to 22.

29. The QPCTL antagonist of claim 28, which is a small molecule inhibitor.

30. The inhibitor of claim 29, which has been identified by the screening method of claim 25 or 27.

31. A selective QC inhibitor, which has been identified by the screening method of claim 26.

32. A pharmaceutical composition for parenteral, enteral or oral administration, comprising at least one QPCTL inhibitor, or a pharmaceutical acceptable salt thereof, optionally in combination with customary carriers and/or excipients.

33. Use of a pharmaceutical composition according to claim 32 for the preparation of a medicament for the prevention or treatment of a disease selected from Alzheimer's disease, Familial British Dementia, Familial Danish Dementia, Down Syndrome, Huntington's disease, Kennedy's disease, ulcer disease, duodenal cancer with or w/o *Helicobacter pylori* infections, colorectal cancer, Zolliger-Ellison syndrome, gastric cancer with or without *Helicobacter pylori* infections, pathogenic psychotic conditions, schizophrenia, infertility, neoplasia, inflammatory host responses, cancer, malign metastasis, melanoma, psoriasis, rheumatoid arthritis, atherosclerosis, impaired humoral and cell-mediated immune responses, leukocyte adhesion and migration

processes in the endothelium, impaired food intake, impaired sleep-wakefulness, impaired homeostatic regulation of energy metabolism, impaired autonomic function, impaired hormonal balance or impaired regulation of body fluids, multiple sclerosis, the Guillain-Barré syndrome and chronic inflammatory demyelinizing polyradiculoneuropathy.

34. Use of an QPCTL inhibitor, or a pharmaceutical acceptable salt thereof, for the preparation of a medicament for the prevention or treatment of a disease selected from Alzheimer's disease, Familial British Dementia, Familial Danish Dementia, Down Syndrome, Huntington's disease, Kennedy's disease, ulcer disease, duodenal cancer with or w/o *Helicobacter pylori* infections, colorectal cancer, Zolliger-Ellison syndrome, gastric cancer with or without *Helicobacter pylori* infections, pathogenic psychotic conditions, schizophrenia, infertility, neoplasia, inflammatory host responses, cancer, malign metastasis, melanoma, psoriasis, rheumatoid arthritis, atherosclerosis, impaired humoral and cell-mediated immune responses, leukocyte adhesion and migration processes in the endothelium, impaired food intake, impaired sleep-wakefulness, impaired homeostatic regulation of energy metabolism, impaired autonomic function, impaired hormonal balance or impaired regulation of body fluids, multiple sclerosis, the Guillain-Barré syndrome and chronic inflammatory demyelinizing polyradiculoneuropathy.

35. The pharmaceutical composition or the use according to any one of claims 32 to 34, wherein the QPCTL inhibitor is a competitive inhibitor.

36. The pharmaceutical composition or the use according to any one of claims 32 to 35, wherein the QPCTL inhibitor binds to the active-site bound metal ion of the QPCTL.

37. The pharmaceutical composition or the use according to any one of claims 32 to 36, wherein the QPCTL is selected from one of SEQ ID NOS: 11 to 18, 21 and 22.

38. The pharmaceutical composition or the use according to claim 37, wherein the QPCTL is selected from one of SEQ ID NOS: 11 and 12.

39. The pharmaceutical composition or the use according to any one of claims 32 to 38, wherein the inhibitor of any one of claims 28 to 30 is used.

40. Diagnostic assay, comprising a QPCTL inhibitor.

41. Method of diagnosing any one of the diseases and/or conditions as defined in claim 33 or 34, comprising the steps of

- collecting a sample from a subject who is suspected to be afflicted with said disease and/or condition,
- contacting said sample with an inhibitor of a glutaminyl peptide cyclotransferase, and
- determining whether or not said subject is afflicted by said disease and/or condition.

42. The method of claim 41, wherein said subject is a human being.

43. The method of claim 41 or 42, wherein said sample is a blood sample, a serum sample, a sample of cerebrospinal liquor or a urine sample.

44. Diagnostic kit for carrying out the method of claims 41 to 43 comprising as detection means the diagnostic assay of claim 40 and a determination means.

Figure 1

```
hQC             --------------MAGGRHRRVVGTLHLL----LLVAALPWASRG--VSPSASAWPE--
hisoQC          MRSGGRGRPRLRLGERGLMEPLLPPKRRLLPRVRLLP-LLLALAVGSAFYTIWSGWHRRT
mQC             --------------MAGSEDKLVVGTLHLL----LLQATVLSLTAGN-LSLVSAAWTQ--
misoQC          MSPGSRGRPRQRLEDRGLMKPPSLSKRRLLPRVQFLPLLLLALAMGLAFYIVWNSWHPGV
                              *  .     . :**    :*   :   : *  .     .*

hQC             -----EKNYHQPAI--LNSSALRQIAEGTSISEMWQNDLQPLLIERYPGSPGSYAARQHI
hisoQC          EELPLGRELRVPLIGSLPEARLRRVVGQLDPQRLWSTYLRPLLVVRTPGSPGNLQVRKFL
mouse           -----EKNHHQPAH--LNSSSLQQVAEGTSISEMWQNDLRPLLIERYPGSPGSYSARQHI
misoQC          EEMSRSRDLRVPLIGSLSEAKLRLVVGQLDPQRLWGTFLRPLLIVRPPGSSGNLQVRKFL
                      :: : *    * .: *: :.   . ..:* . *:***: * ***.*.  .*:.:

hQC             MQRIQRLQADWVLEIDTFLSQTPYGYRSFSNIISTLNPTAKRHLVLACHYDSKYFSHWNN
hisoQC          EATLRSLTAGWHVELDPFTASTPLGPVDFGNVVATLDPRAARHLTLACHYDSKLFPPG-S
mouse           MQRIQRLQAEWVVEVDTFLSRTPYGYRSFSNIISTLNPEAKRHLVLACHYDSKYFPRWDS
misoQC          EATLQSLSAGWHVELDPFTASTPLGPLDFGNVVATLDPGAARHLTLACHYDSKFFPPG-L
                   :: * * * :*:*.* : ** *  .*.*:::**:* * ***.******** *.

hQC             RVFVGATDSAVPCAMMLELARALDKKLLSLKTVSDSKPDLSLQLIFFDGEEAFLHWSPQD
hisoQC          TPFVGATDSAVPCALLLELAQALDLELSRAK--KQAAP-VTLQLLFLDGEEALKEWGPKD
mouse           RVFVGATDSAVPCAMMLELARALDKKLHSLKDVSGSKPDLSLRLIFFDGEEAFHHWSPQD
misoQC          PPFVGATDSAVPCALLLELVQALDAMLSRIK--QQAAP-VTLQLLFL-GEEALKEWGPKD
                  *************::***.:***  *   *  . : * ::*:*:*: **:**: .*.*:*

hQC             SLYGSRHLAAKMASTPHPPGARGTSQLHGMDLLVLLDLIGAPNPTFPNFFPNSARWFERL
hisoQC          SLYGSRHLAQLMESIPHSPG---PTRIQAIELFMLLDLLGAPNPTFYSHFPRTVRWFHRL
mouse           TLYGSRHLAQKMASSPHPPGSRGTNQLDGMDLLVLLDLIGAANPTFPNFFPKTTRWFNRL
misoQC          SLYGSRHLAQIMESIPHSPG---PTRIQAIELFVLLDLLGASSPIFFSHFPRTARWFQRL
                :******** * * **.**   ..::..::*::****:**..* * ..**.:.***.**

hQC             QAIEHELHELGLLKDHSLEGRYFQNYSYGGVIQDDHIPFLRRGVPVLHLIPSPFPEVWHT
hisoQC          RSIEKRLHRLNLLQSHPQEVMYFQPGEPSGSVEDDHIPFLRRGVPVLHLISTPFPAVWHT
mouse           QAIEKELYELGLLKDHSLERKYFQNFGYGNIIQDDHIPFLRKGVPVLHLIASPSPEVWHT
misoQC          RSIEKRLHRLNLLQSHPQEVMYFQPGEPPGPVEDDHIPFLRRGVPVLHLIATPFPAVLHT
                ::**:.*:.*.**:.*. *  ***     . ::*********:********.:* * * **

hQC             MDDNEENLDESTIDNLNKILQVFVLEYLHL
hisoQC          PADTEVNLHPPTVHNLCRILAVFLAEYLGL
mouse           MDDNEENLHASTIDNLNKIIQVFVLEYLHL
misoQC          PADTEANLHPPTVHNLSRILAVFLAEYLGL
                  *.* **. .*:.** :*: **: *** *
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Identity: 38.97%

Similarity 65.64%

Figure 2

```
hisoQC              MRSGGRGRPRLRLGERGLMEPLLPPKRRLLPRVRLLP-LLLALAVGSAFYTIWSGWHRRT
M_fascicularis      MRSGGRGRPRLRLGERGVMEPLLPPKRRLLPRVRLLP-LLLALAVGSAFYTIWSGWHRRT
M_mulatta           MRSGGRGRPRLRLGERGVMEPLLPPKRRLLPRVRLLP-LLLALAVGSAFYTIWSGWHRRT
C_familiaris        MPSGGRGRSRLRLGERGLLEPPSPPKRRLLPRAHFLPLLLLALALASATYTIWSGWHHQT
R_norvegicus        MSPASRGRSRQRLGDRGLMKPPSLSKRRLLPRVQLLPLLLLALALGLAFYIVWNSWHPGV
M_musculus          MSPGSRGRPRQRLEDRGLMKPPSLSKRRLLPRVQFLPLLLLALAMGLAFYIVWNSWHPGV
B_taurus            MPSGGRGRPRLQVGERSLLERPSPPKRRLIPRAQLLPQLLLALTVASVFYTIWRIWHSQT
                    * ...***.* :: :*.:::    .****:**.::** *****::. . * :*  **  .

hisoQC              EELPLGRELRVPLIGSLPEARLRRVVGQLDPQRLWSTYLRPLLVVRTPGSPGNLQVRKFL
M_fascicularis      EELPLGRELRVPLIGSLPEARLRRVVGQLDPQRLWGTYLRPLLVVRTPGSPGNLQVRKFL
M_mulatta           EELPLGRELRVPLIGSLPEARLRRVVGQLDPQRLWGTYLRPLLVVRTPGSPGNLQVRKFL
C_familiaris        EELPRGRELRGRLIGSLSEARLRRVVGQLDPHRLWNTYLRPLLVVRTPGSPGNLQVRKFL
R_norvegicus        EEVSRSRDLRVPLIGSLSEAKLRLVVGQLDPQRLWGTFLRPLLIVRPPGSPGNLQVRKFL
M_musculus          EEMSRSRDLRVPLIGSLSEAKLRLVVGQLDPQRLWGTFLRPLLIVRPPGSSGNLQVRKFL
B_taurus            EELPLGRELRGPLIGSLPEARVRRVVGQLDPHRLWNTFLRPLLVVRTPGSPGNLQVRKFL
                    **:. .*:**  *****.**::* *******:***.*:*****:**.***.*********

hisoQC              EATLRSLTAGWHVELDPFTASTPLGPVDFGNVVATLDPRAARHLTLACHYDSKLFPPGST
M_fascicularis      EATLRSLTAGWHVELDPFTASTPLGPVDFGNVVATLDPGAARHLTLACHYDSKLFPPGST
M_mulatta           EATLRSLTAGWHVELDPFTASTPLGPVDFGNVVATLDPGAARHLTLACHYDSKLFPPGST
C_familiaris        EATLRTLTAGWHVELDPFTALTPLGPLDFGNVVATLDPGAARHLTLACHYDSKLFASESV
R_norvegicus        EATLQSLSAGWHVELDPFTASTPLGPLDFGNVVATLDPGAARHLTLACHYDSKFFPPGLP
M_musculus          EATLQSLSAGWHVELDPFTASTPLGPLDFGNVVATLDPGAARHLTLACHYDSKFFPPGLP
B_taurus            EATLRTLSAGWHIELDSFTASTPVGPLDFSNVVATLDPGAARHLTLACHYDSKLFPSDSA
                    ****::*:****:***.*** **:**:**.******** **************:*..

hisoQC              PFVGATDSAVPCALLLELAQALDLELSRAKKQAAPVTLQLLFLDGEEALKEWGPKDSLYG
M_fascicularis      PFVGATDSAVPCALLLELAQALDLELSRAKEQAAPVTLQLLFLDGEEALKEWGPKDSLYG
M_mulatta           PFVGATDSAVPCALLLELAQALDLELSRAKEQAAPVTLQLLFLDGEEALKEWGPKDSLYG
C_familiaris        PFVGATDSAVPCALLLELAQALDRELSRAKEQEAPVTLQLLFLDGEEALKEWGPTDSLYG
R_norvegicus        PFVGATDSAVPCALLLELVQALDVMLSRIKQQAAPVTLQLLFLDGEEALKEWGPKDSLYG
M_musculus          PFVGATDSAVPCALLLELVQALDAMLSRIKQQAAPVTLQLLFLDGEEALKEWGPKDSLYG
B_taurus            PFVGATDSAVPCSLLLELAQALDQELGKAKERAAPMTLQLIFLDGEEALKQWGPKDSLYG
                    *************:*****.****  *.: *:: **:*****:******:****:***.*****

hisoQC              SRHLAQLMESIPHSPGPTRIQAIELFMLLDLLGAPNPTFYSHFPRTVRWFHRLRSIEKRL
M_fascicularis      SRHLAQLMESIPHSPGPTRIQAIELFMLLDLLGAPNPTFYSHFPRTVRWFHRLRSIEKRL
M_mulatta           SRHLAQLMESIPHSPGPTRIQAIELFMLLDLLGAPNPTFYSHFPRTVRWFHRLRSIEKRL
C_familiaris        SRHLAQLMESAPHSPGPTRIQAIELFMLLDLLGAPNPNFYSHFPHTARWFHRLRSIEKRL
R_norvegicus        SRHLAQIMESIPHSPGPTRIQAIELFVLLDLLGAPSPIFFSHFPRTARWFQRLRSIEKRL
M_musculus          SRHLAQIMESIPHSPGPTRIQAIELFVLLDLLGASSPIFFSHFPRTARWFQRLRSIEKRL
B_taurus            SRHLAQLMESTPHGLGSTRIQAIELFMLLDLLGAPNPTFYSHFPRTARWFHRLRSIEKRL
                    ******:*** **. *.*********:********..* *:****:*.***:*********

hisoQC              HRLNLLQSHPQEVMYFQPGEPSGSVEDDHIPFLRRGVPVLHLISTPFPAVWHTPADTEVN
M_fascicularis      HRLNLLQSHPQEVMYFQPGEPFGSVEDDHIPFLRRGVPVLHLISTPFPAVWHTPADTEAN
M_mulatta           HRLNLLQSHPQEVMYFQPGEPFGSVEDDHIPFLRRGVPVLHLISTPFPAVWHTPADTEAN
C_familiaris        HRMNLLQSHPQEVMYFQPGEPPGSVEDDHIPFLRRGVPVLHLISMPFPSVWHTPDDSEAN
R_norvegicus        HRLNLLQSHPQEVMYFQPGEPPGPVEDDHIPFLRRGVPVLHLIAMPFPAVWHTPADTEAN
M_musculus          HRLNLLQSHPQEVMYFQPGEPPGPVEDDHIPFLRRGVPVLHLIATPFPAVWHTPADTEAN
B_taurus            HRLNLLQSHPWEVMYFQTGEPPGSVEDDHIPFLRRGVPVLHLIATPFPSVWHTSDDSEAN
                    **:******* ******.*** *.******************: ***:****. *:*.*

hisoQC              LHPPTVHNLCRILAVFLAEYLGL
M_fascicularis      LHPPTVHNLSRILAVFLAEYLGL
M_mulatta           LHPPTVHNLSRILAVFLAEYLGL
C_familiaris        LHPPTVHNLSRILAVFLAEYLGL
R_norvegicus        LHPPTVHNLSRILAVFLAEYLGL
M_musculus          LHPPTVHNLSRILAVFLAEYLGL
B_taurus            LHPPTVHNLSRILAVFLAEYLGL
                    *********.*************
```

Identity: 71.28%

Similarity 90.86%

Figure 3

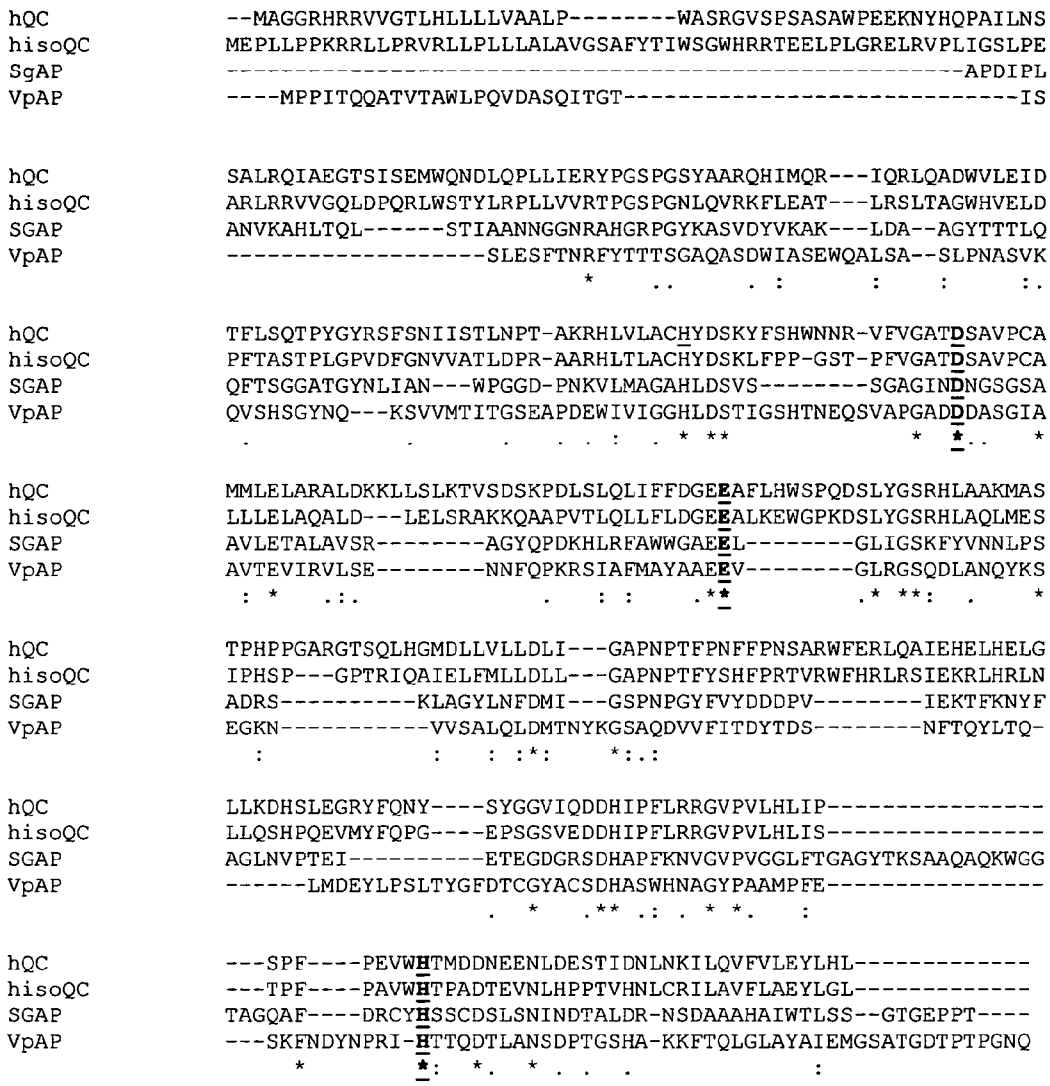

hQC --MAGGRHRRVVGTLHLLLLVAALP--------WASRGVSPSASAWPEEKNYHQPAILNS
hisoQC MEPLLPPKRRLLPRVRLLPLLLALAVGSAFYTIWSGWHRRTEELPLGRELRVPLIGSLPE
SgAP ----------APDIPL
VpAP ----MPPITQQATVTAWLPQVDASQITGT----------IS
hQC SALRQIAEGTSISEMWQNDLQPLLIERYPGSPGSYAARQHIMQR---IQRLQADWVLEID
hisoQC ARLRRVVGQLDPQRLWSTYLRPLLVVRTPGSPGNLQVRKFLEAT---LRSLTAGWHVELD
SGAP ANVKAHLTQL------STIAANNGGNRAHGRPGYKASVDYVKAK---LDA--AGYTTTLQ
VpAP ----------SLESFTNRFYTTTSGAQASDWIASEWQALSA--SLPNASVK
hQC TFLSQTPYGYRSFSNIISTLNPT-AKRHLVLACHYDSKYFSHWNNR-VFVGATDSAVPCA
hisoQC PFTASTPLGPVDFGNVVATLDPR-AARHLTLACHYDSKLFPP-GST-PFVGATDSAVPCA
SGAP QFTSGGATGYNLIAN---WPGGD-PNKVLMAGAHLDSVS--------SGAGINDNGSGSA
VpAP QVSHSGYNQ---KSVVMTITGSEAPDEWIVIGGHLDSTIGSHTNEQSVAPGADDDASGIA
hQC MMLELARALDKKLLSLKTVSDSKPDLSLQLIFFDGEEAFLHWSPQDSLYGSRHLAAKMAS
hisoQC LLLELAQALD---LELSRAKKQAAPVTLQLLFLDGEEALKEWGPKDSLYGSRHLAQLMES
SGAP AVLETALAVSR--------AGYQPDKHLRFAWWGAEEL--------GLIGSKFYVNNLPS
VpAP AVTEVIRVLSE--------NNFQPKRSIAFMAYAAEEV--------GLRGSQDLANQYKS
hQC TPHPPGARGTSQLHGMDLLVLLDLI---GAPNPTFPNFFPNSARWFERLQAIEHELHELG
hisoQC IPHSP---GPTRIQAIELFMLLDLL---GAPNPTFYSHFPRTVRWFHRLRSIEKRLHRLN
SGAP ADRS----------KLAGYLNFDMI---GSPNPGYFVYDDDPV--------IEKTFKNYF
VpAP EGKN----------VVSALQLDMTNYKGSAQDVVFITDYTDS--------NFTQYLTQ-
hQC LLKDHSLEGRYFQNY----SYGGVIQDDHIPFLRRGVPVLHLIP----------
hisoQC LLQSHPQEVMYFQPG----EPSGSVEDDHIPFLRRGVPVLHLIS----------
SGAP AGLNVPTEI----------ETEGDGRSDHAPFKNVGVPVGGLFTGAGYTKSAAQAQKWGG
VpAP ------LMDEYLPSLTYGFDTCGYACSDHASWHNAGYPAAMPFE----------
hQC ---SPF----PEVWHTMDDNEENLDESTIDNLNKILQVFVLEYLHL----------
hisoQC ---TPF----PAVWHTPADTEVNLHPPTVHNLCRILAVFLAEYLGL----------
SGAP TAGQAF----DRCYHSSCDSLSNINDTALDR-NSDAAAHAIWTLSS--GTGEPPT----
VpAP ---SKFNDYNPRI-HTTQDTLANSDPTGSHA-KKFTQLGLAYAIEMGSATGDTPTPGNQ

Figure 4

```
hisoQC      MRSGGRGRPRLRLGERGLMEPLLPPKRRLLPRVRLLPLLLALAVGSAFYTIWSGWHRRTE
hQC         MAGG-------R-------------HRRVVGTLHLLLLVAALPWAS--R----GVSPSAS
             * .*       *             :**::  ::** *: **. .*       *    :.

hisoQC      ELPLGRELRVPLIGSLPEARLRRVVGQLDPQRLWSTYLRPLLVVRTPGSPGNLQVRKFLE
hQC         AWPEEKNYHQPAI--LNSSALRQIAEGTSISEMWQNDLQPLLIERYPGSPGSYAARQHIM
              *  :: : * *  * .: **::.   . ..:*.. *:***: * *****.  .*:.:

hisoQC      ATLRSLTAGWHVELDPFTASTPLGPVDFGNVVATLDPRAARHLTLACHYDSKLFPPGS-T
hQC         QRIQRLQADWVLEIDTFLSQTPYGYRSFSNIISTLNPTAKRHLVLACHYDSKYFSHWNNR
              :: * *.* :*:*.* :.** *  .*.*:::**:* * ***.******** *.  .

hisoQC      PFVGATDSAVPCALLLELAQALDLELSRAKKQAAP---VTLQLLFLDGEEALKEWGPKDS
hQ          VFVGATDSAVPCAMMLELARALDKKLLSLKTVSDSKPDLSLQLIFFDGEEAFLHWSPQDS
             ************::****:*** :*   *. : .   ::***:*:*****: .*.*:**

hisoQC      LYGSRHLAQLMESIPHSP---GPTRIQAIELFMLLDLLGAPNPTFYSHFPRTVRWFHRLR
hQC         LYGSRHLAAKMASTPHPPGARGTSQLHGMDLLVLLDLIGAPNPTFPNFFPNSARWFERLQ
            ********  * * **.*   *.:::::.::*::****:******* ..**.:.***.**:

hisoQC      SIEKRLHRLNLLQSHPQEVMYFQPGEPSGSVEDDHIPFLRRGVPVLHLISTPFPAVWHTP
hQC         AIEHELHELGLLKDHSLEGRYFQNYSYGGVIQDDHIPFLRRGVPVLHLIPSPFPEVWHTM
            :**:.**.*.**:.*. *  ***  . .* ::*****************.:*** ****

hisoQC      ADTEVNLHPPTVHNLCRILAVFLAEYLGL
hQC         DDNEENLDESTIDNLNKILQVFVLEYLHL
             *.* **. .*:.** :** **: *** *
```

Identity: 45.24%

Similarity 71.98%

Figure 5


hQC ---MAGGRHRRVVGTLHLLLLVAALP-------WASRGVSPSASAWPEEKNYHQPAILNS
hisoQC MEPLLPPKRRLLPRVRLLPLLLALAVGSAFYTIWSGWHRRTEELPLGRELRVPLIGSLPE
hQC SALRQIAEGTSISEMWQNDLQPLLIERYPGSPGSYAARQHIMQRIQRLQADWVLEIDTFL
hisoQC ARLRRVVGQLDPQRLWSTYLRPLLVVRTPGSPGNLQVRKFLEATLRSLTAGWHVELDPFT
hQC SQTPYGYRSFSNIISTLNPTAKRHLVLACHYDSKYFSHWNNRVFVGATDSAVPCAMMLEL
hisoQC ASTPLGPVDFGNVVATLDPRAARHLTLACHYDSKLFPPGS-TPFVGATDSAVPCALLLEL
hQC ARALDKKLLSLKTVSDSKPDLSLQLIFFDGEEAFLHWSPQDSLYGSRHLAAKMASTPHPP
hisoQC AQALDLELSRAKKQAAP---VTLQLLFLDGEEALKEWGPKDSLYGSRHLAQLMESIPHSP
hQC GARGTSQLHGMDLLVLLDLIGAPNPTFPNFFPNSARWFERLQAIEHELHELGLLKDHSLE
hisoQC ---GPTRIQAIELFMLLDLLGAPNPTFYSHFPRTVRWFHRLRSIEKRLHRLNLLQSHPQE
hQC GRYFQNYSYGGVIQDDHIPFLRRGVPVLHLIPSPFPEVWHTMDDNEENLDESTIDNLNKI
hisoQC VMYFQPGEPSGSVEDDHIPFLRRGVPVLHLISTPFPAVWHTPADTEVNLHPPTVHNLCRI
hQC LQVFVLEYLHL
hisoQC LAVFLAEYLGL

Figure 6

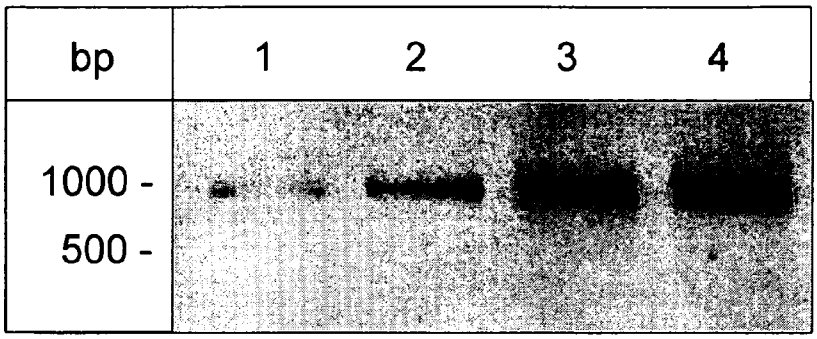


Figure 7

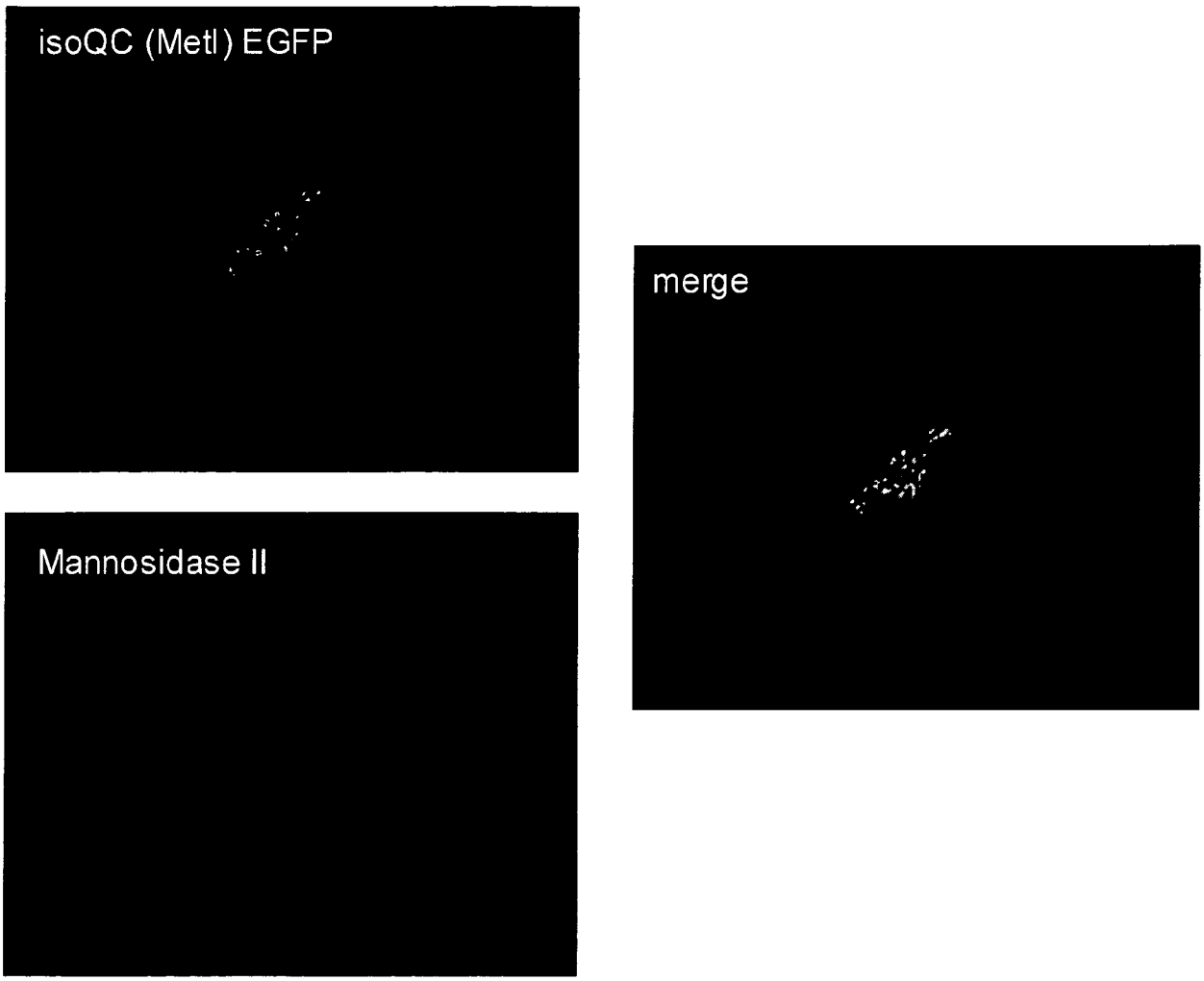


Figure 8

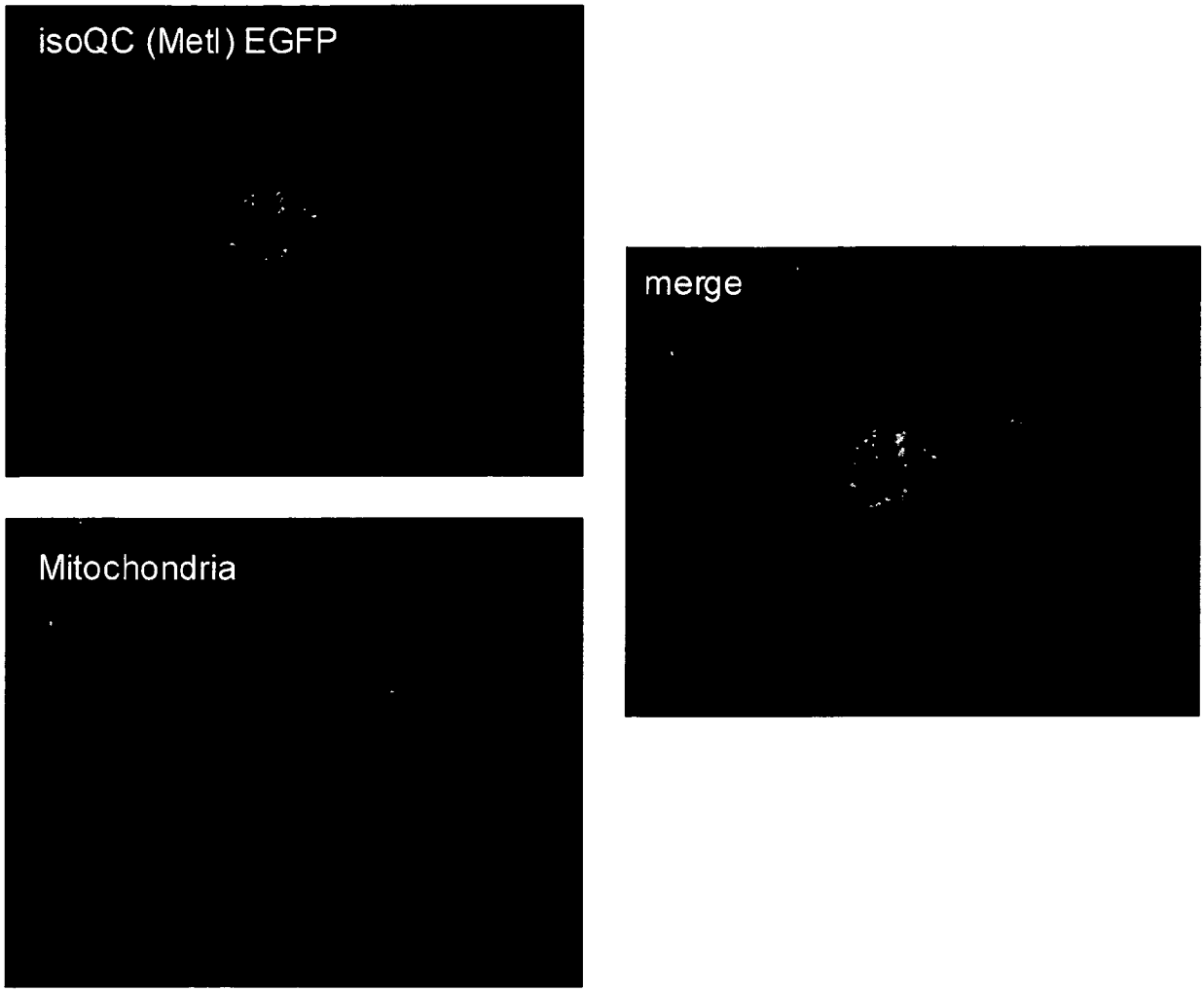


Figure 9

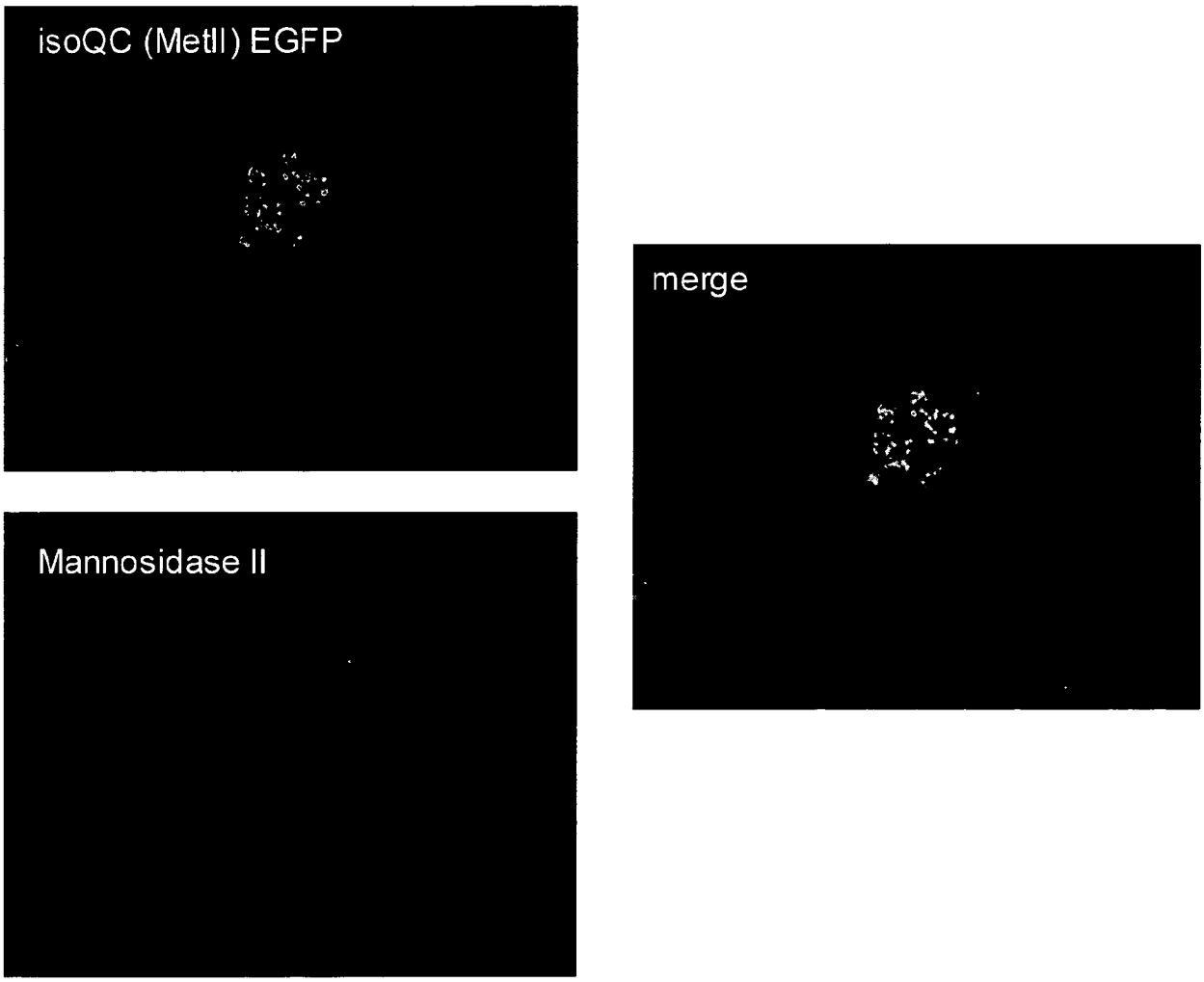


Figure 10

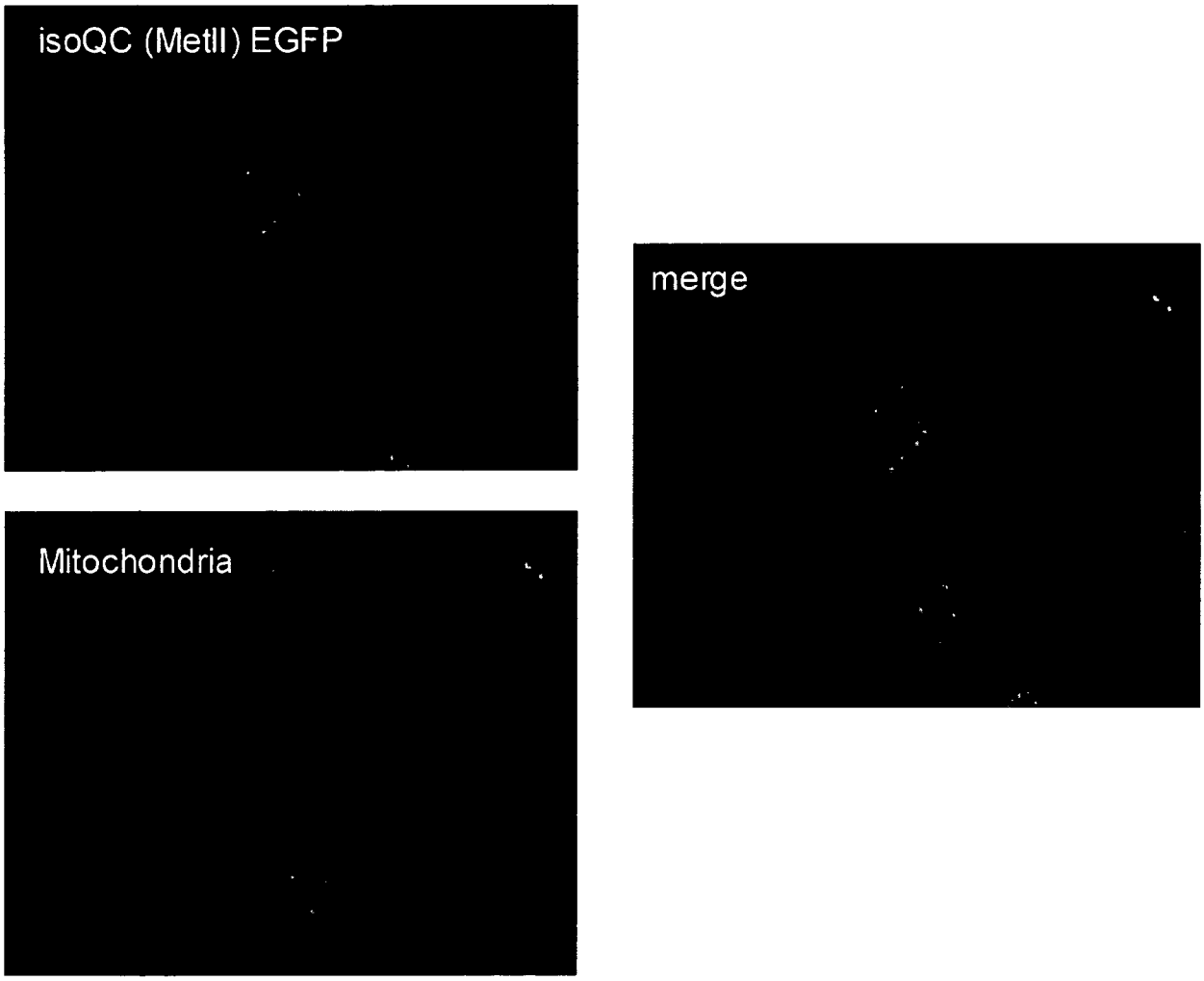


Figure 11

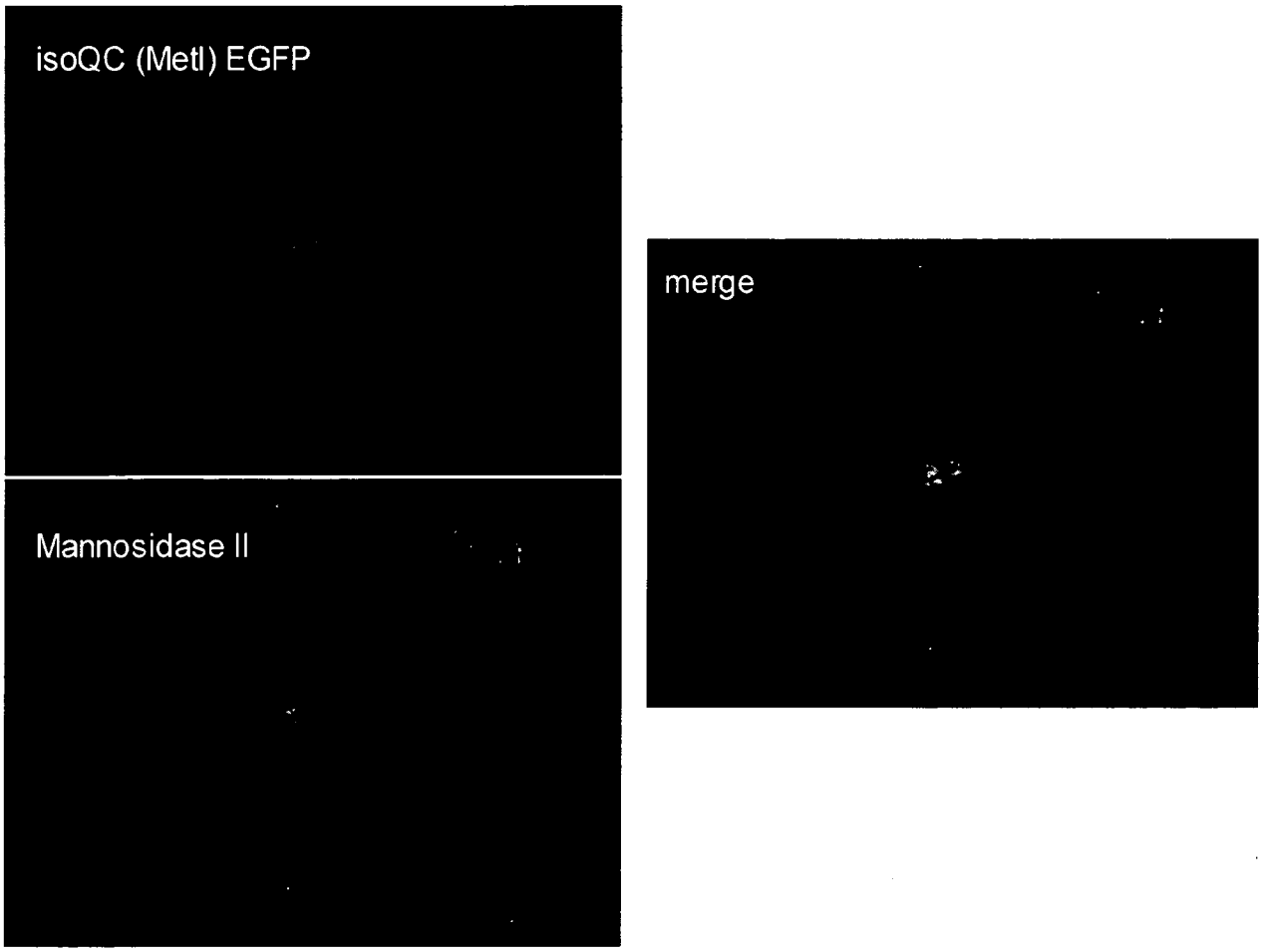


Figure 12

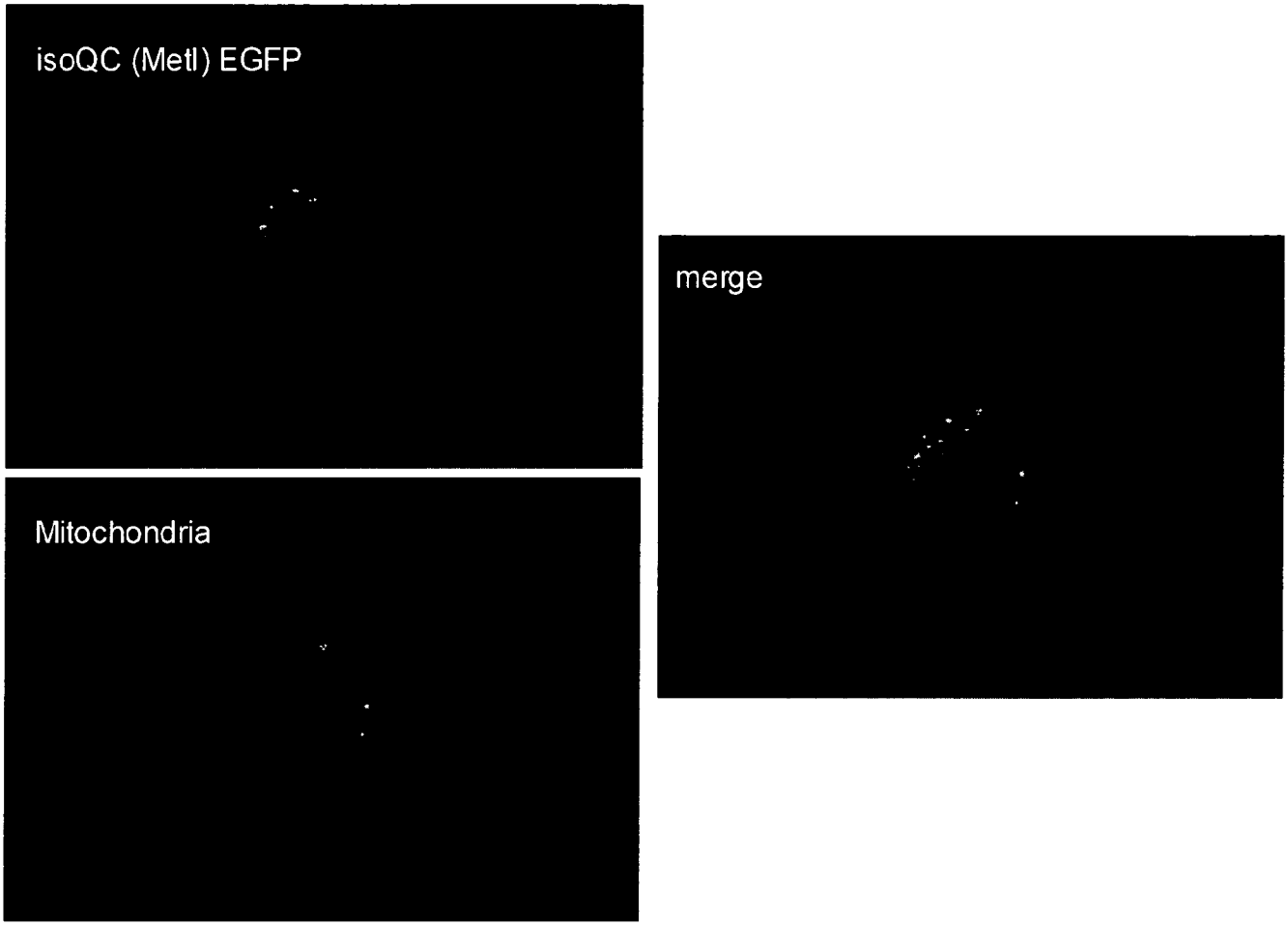


Figure 13

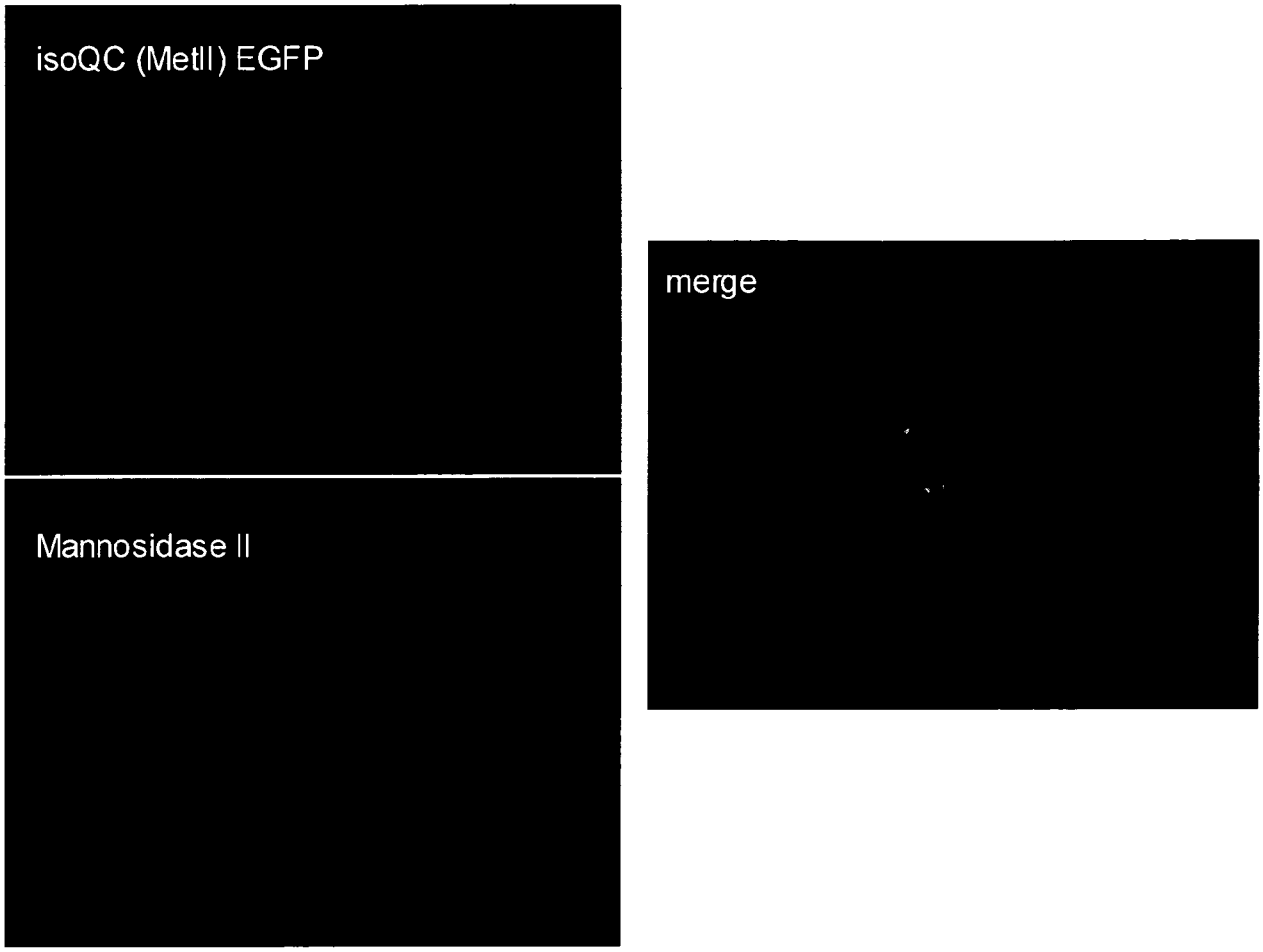


Figure 14

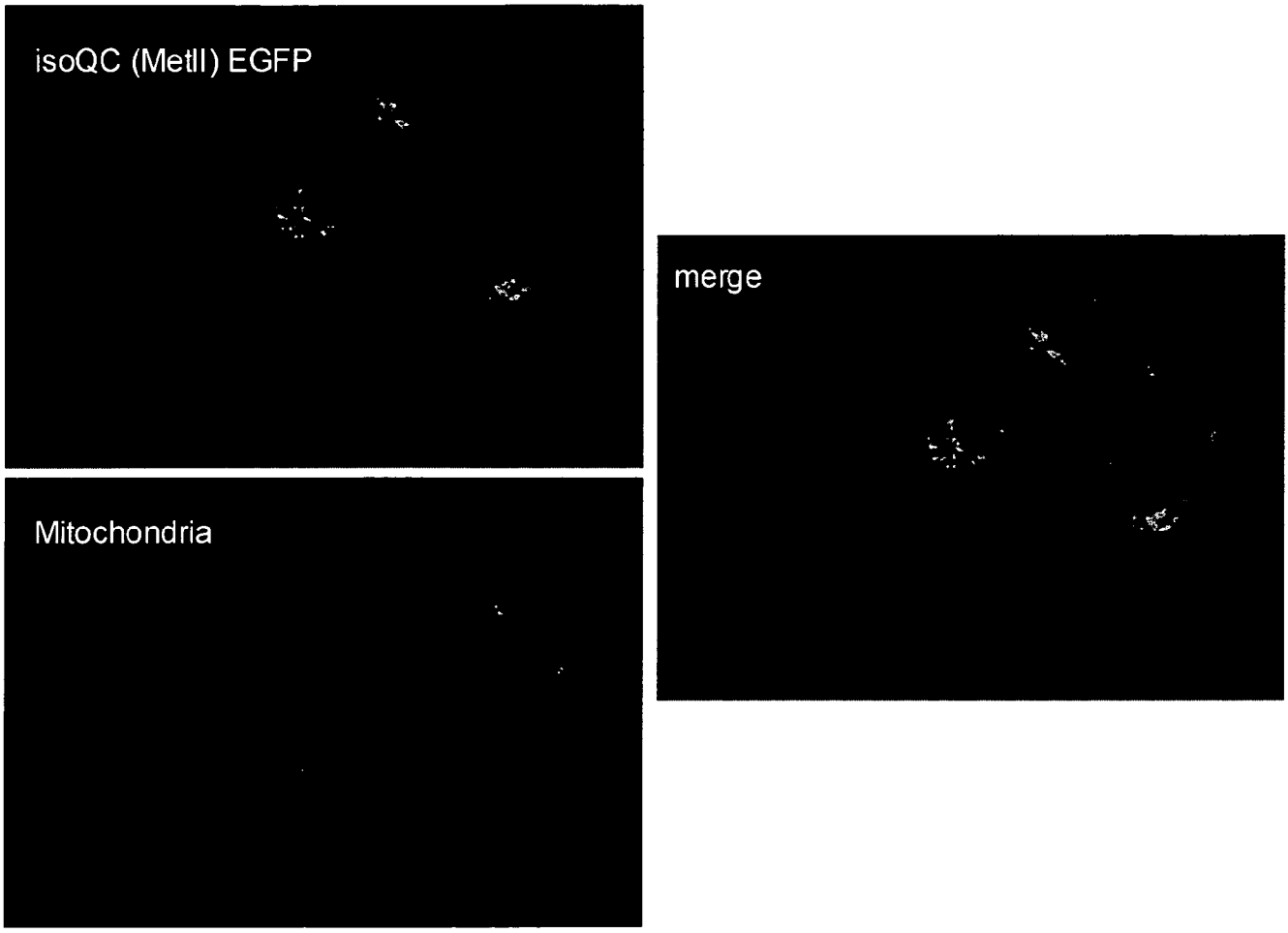


Figure 15

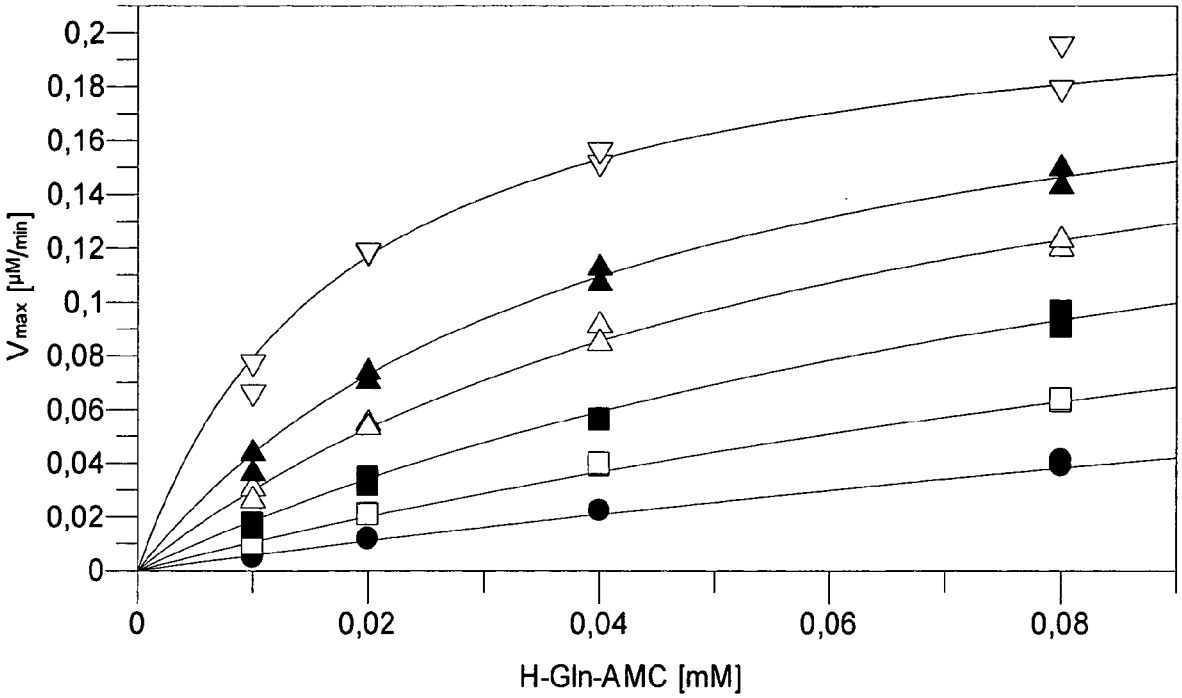

0,2
0,18
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
Vmax [µM/min]
0
0,02
0,04
0,06
0,08
H-Gln-AMC [mM]

Figure 16

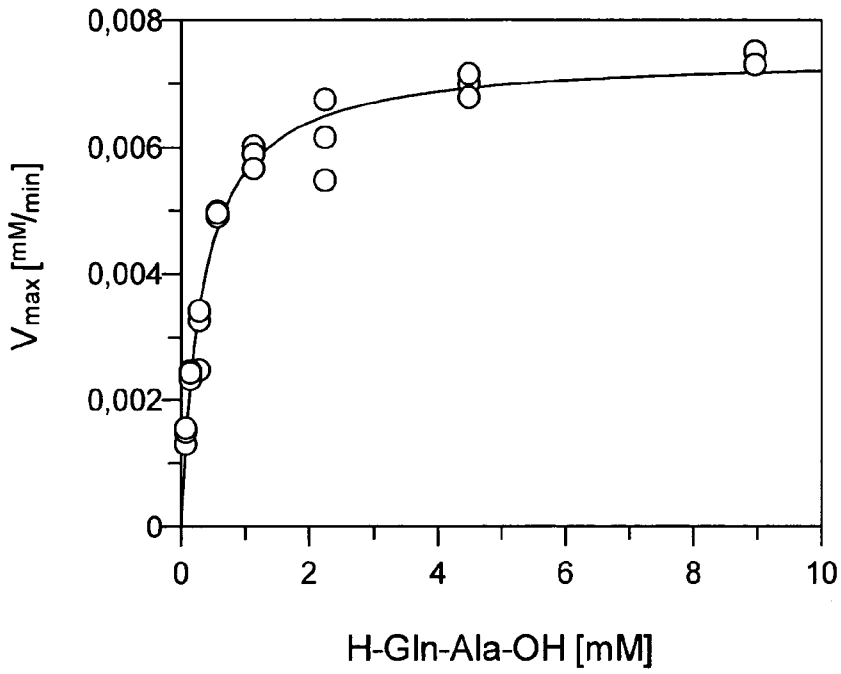


Figure 17

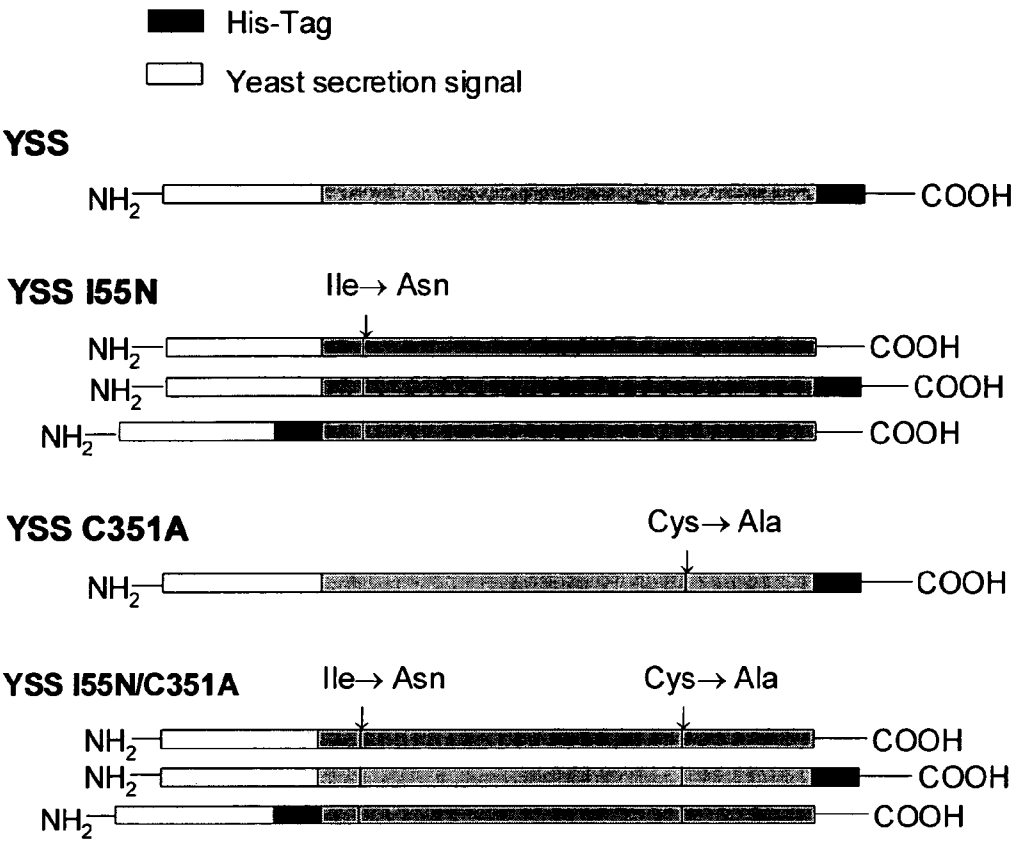

His-Tag
Yeast secretion signal
YSS
NH2
COOH
YSS I55N
Ile→ Asn
YSS C351A
Cys→ Ala
YSS I55N/C351A

Figure 18

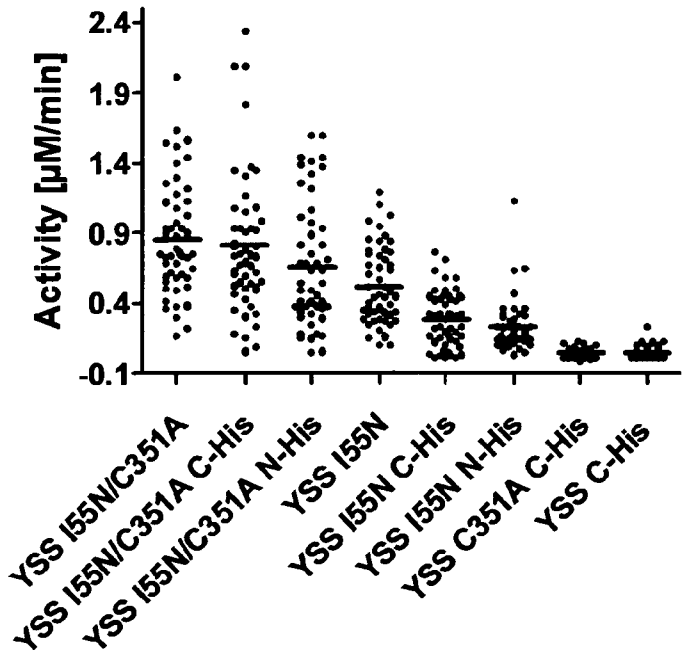

Activity [µM/min]
2.4
1.9
1.4
0.9
0.4
-0.1
YSS I55N/C351A
YSS I55N/C351A C-His
YSS I55N/C351A N-His
YSS I55N
YSS I55N C-His
YSS I55N N-His
YSS C351A C-His
YSS C-His

Figure 19

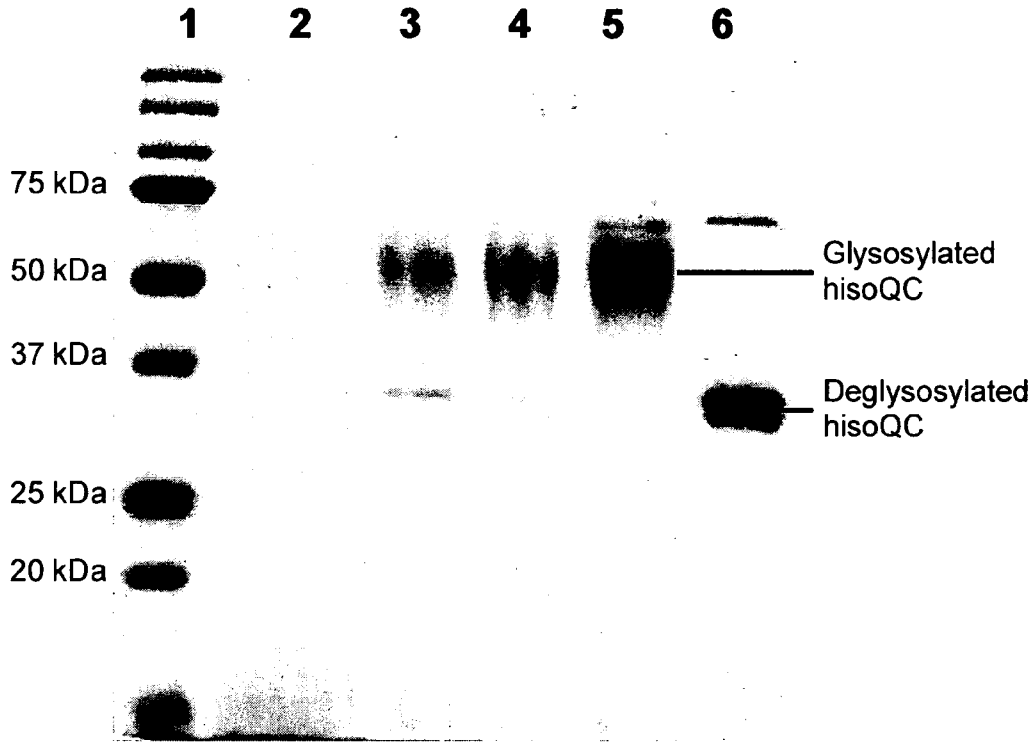

1
2
3
4
5
6
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
Glysosylated hisoQC
Deglysosylated hisoQC

Figure 20

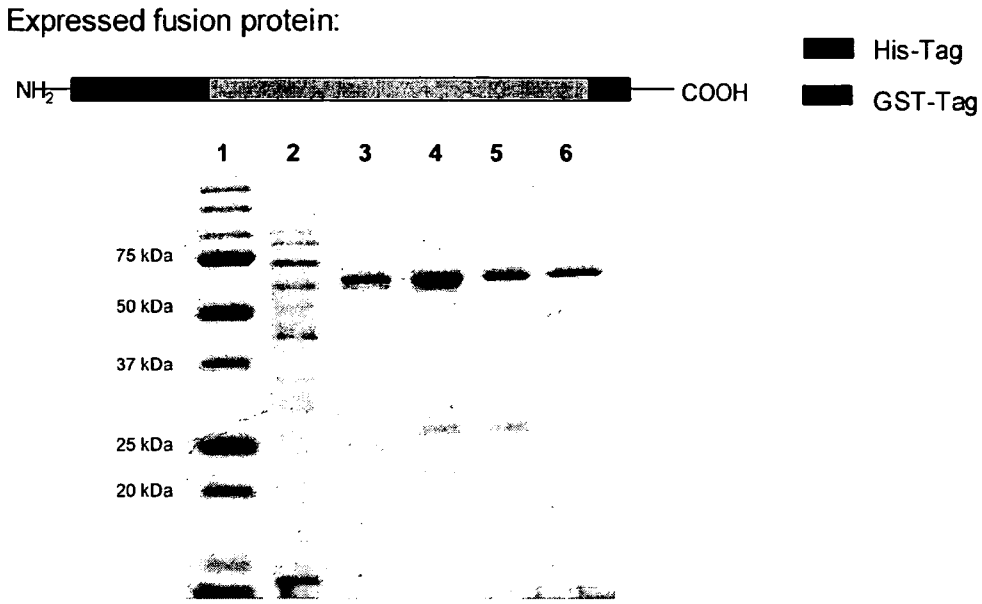

Expressed fusion protein:
NH2
COOH
His-Tag
GST-Tag
1
2
3
4
5
6
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa

Figure 21

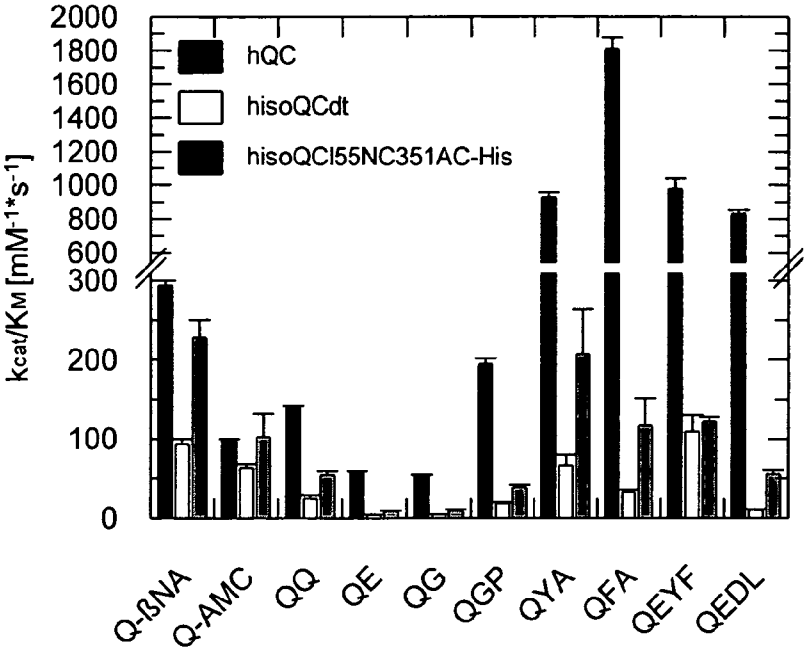

2000
1800
1600
1400
1200
1000
800
600
300
200
100
0
kcat/KM [mM-1*s-1]
hQC
hisoQCdt
hisoQCl55NC351AC-His
Q-ßNA
Q-AMC
QQ
QE
QG
QGP
QYA
QFA
QEYF
QEDL

Figure 22

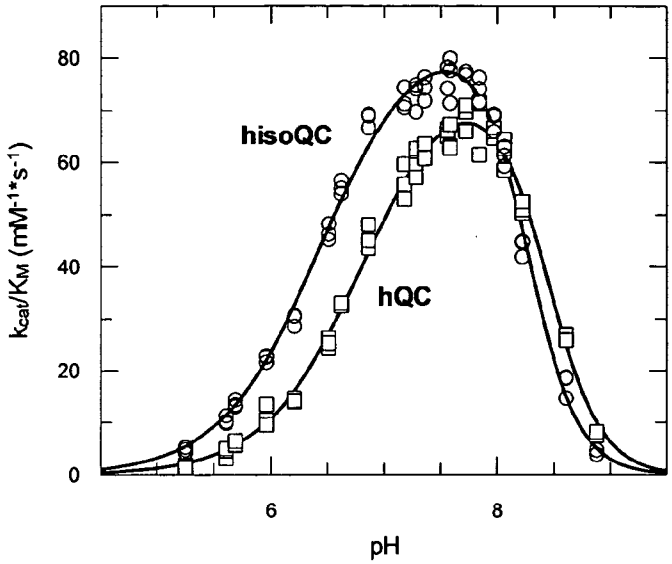
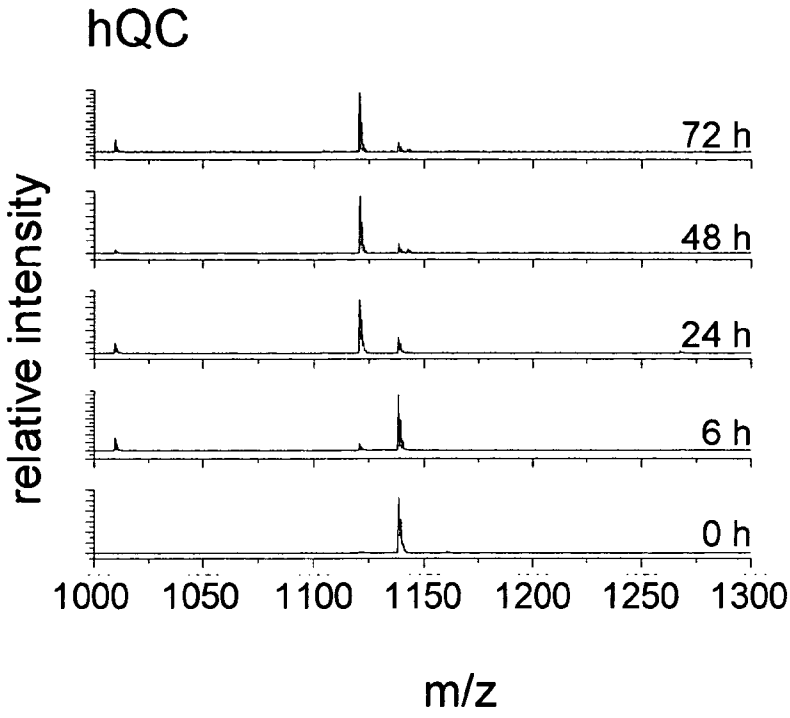


Figure 23


hQC
relative intensity
72 h
48 h
24 h
6 h
0 h
1000 1050 1100 1150 1200 1250 1300
m/z

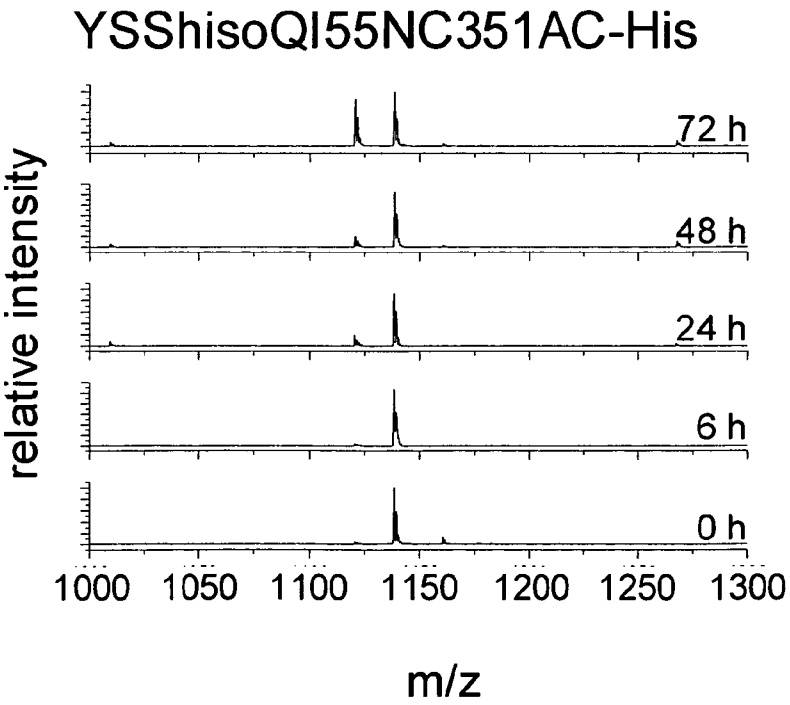

YSShisoQI55NC351AC-His
relative intensity
72 h
48 h
24 h
6 h
0 h
1000 1050 1100 1150 1200 1250 1300
m/z

Figure 24

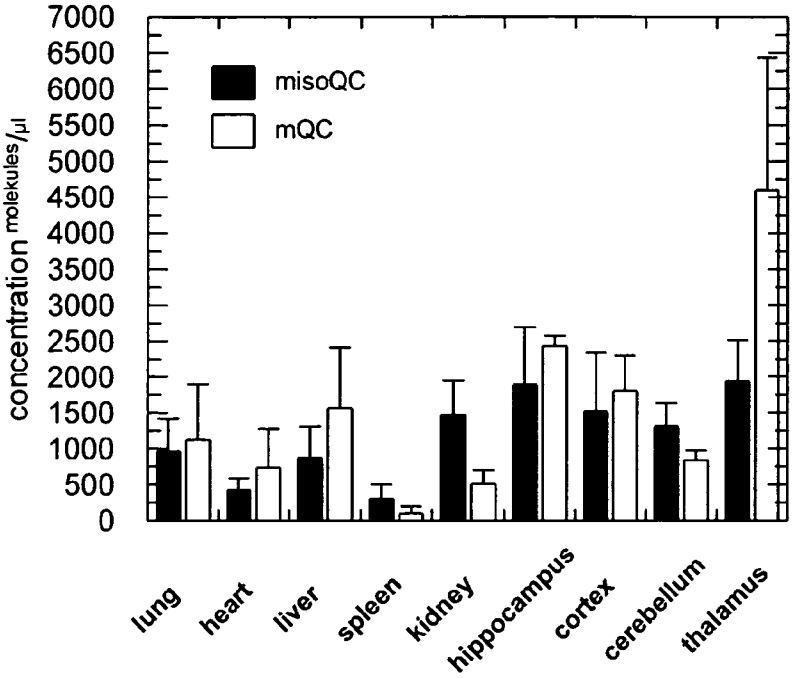

7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
concentration molekules/µl
misoQC
mQC
lung
heart
liver
spleen
kidney
hippocampus
cortex
cerebellum
thalamus

Figure 25

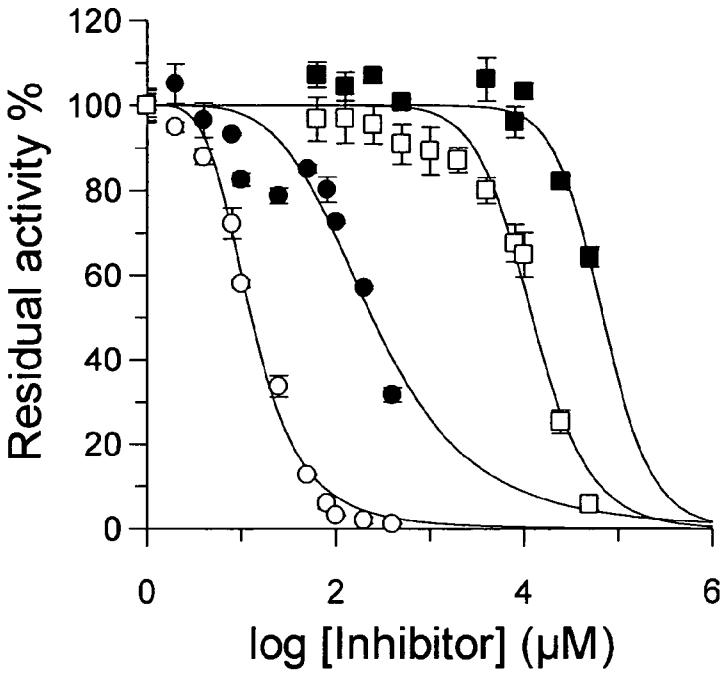


Figure 26A

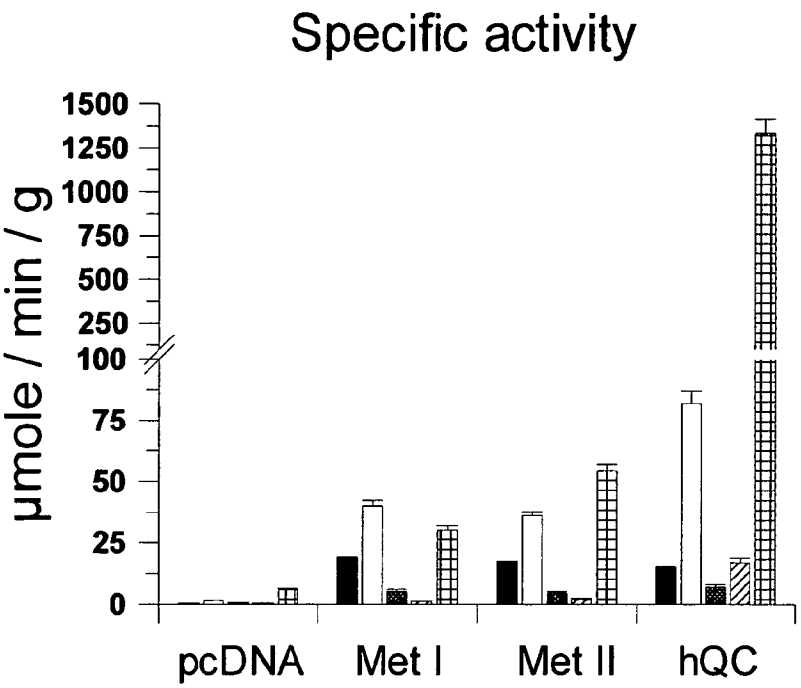


Figure 26B

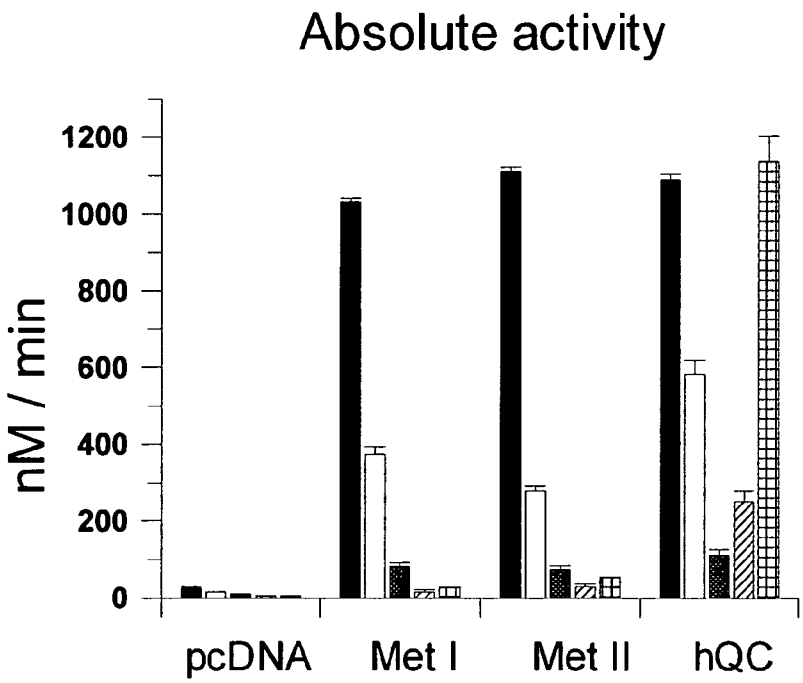


Figure 26C

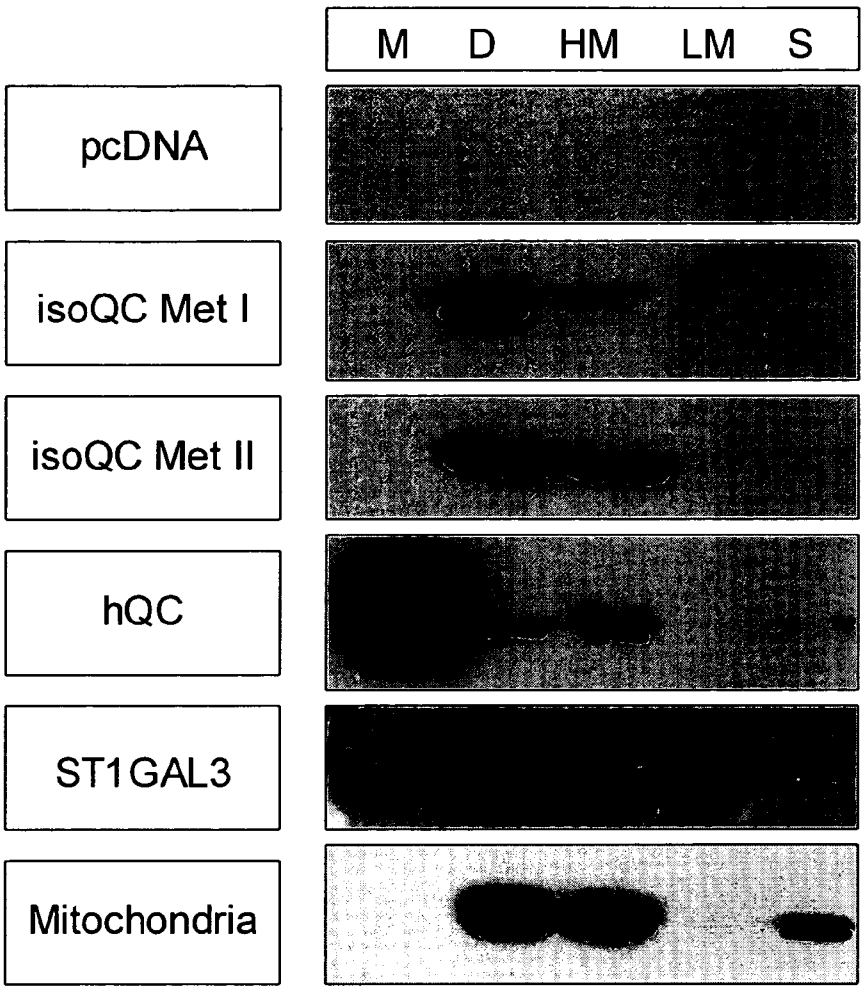

M
D
HM
LM
S
pcDNA
isoQC Met I
isoQC Met II
hQC
ST1GAL3
Mitochondria

Figure 27A

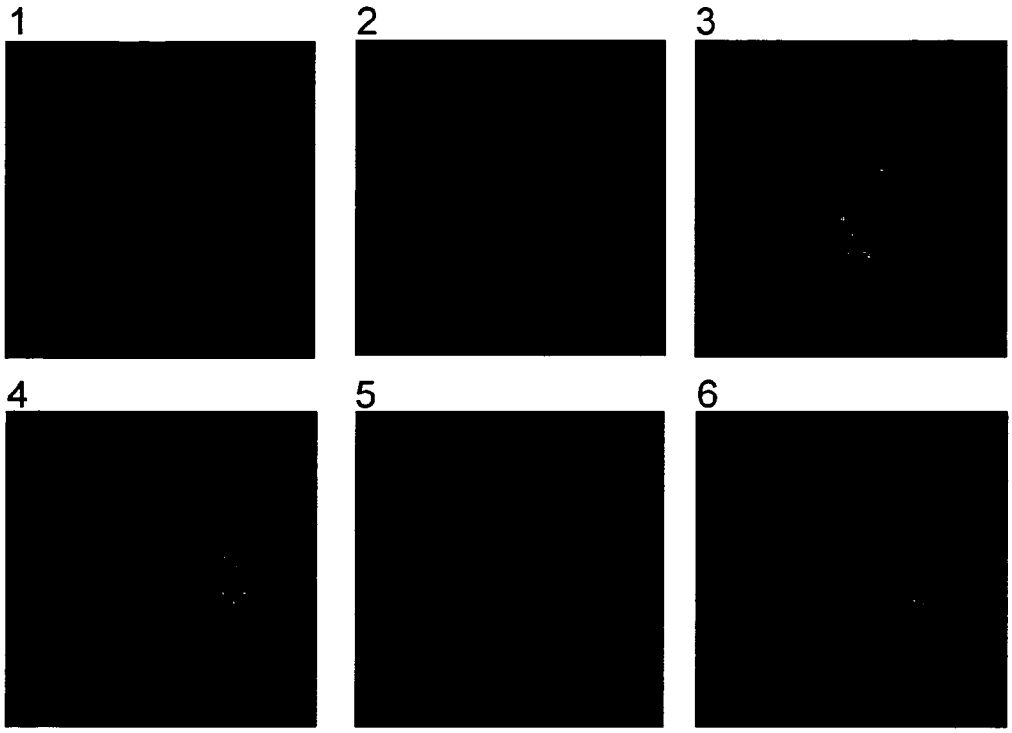


Figure 27B

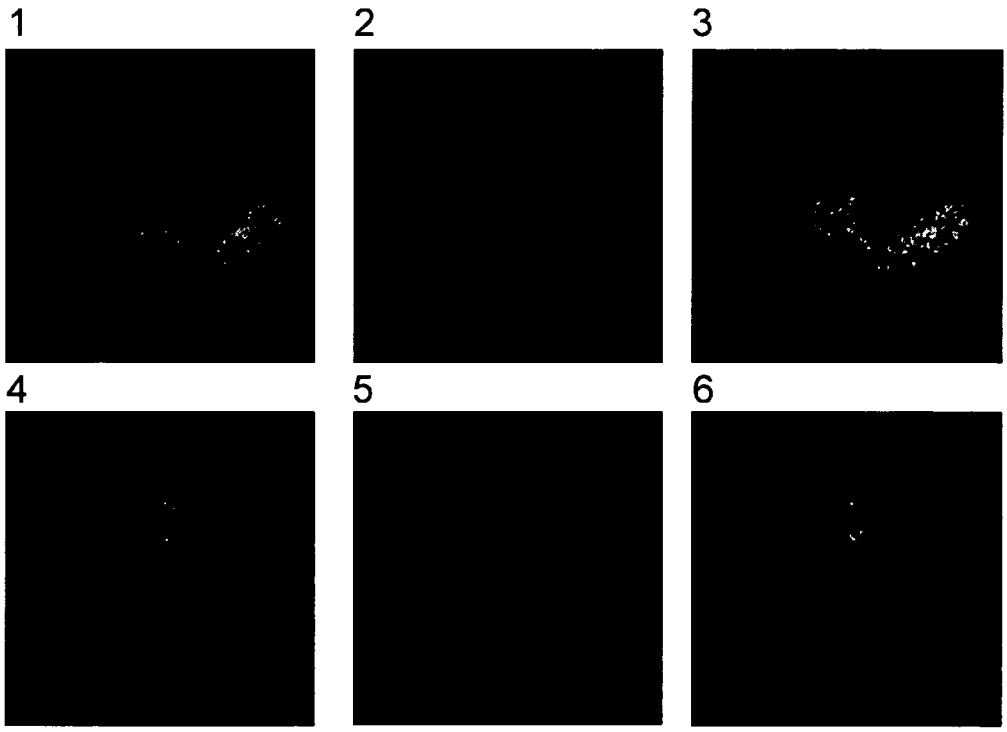


Figure 28

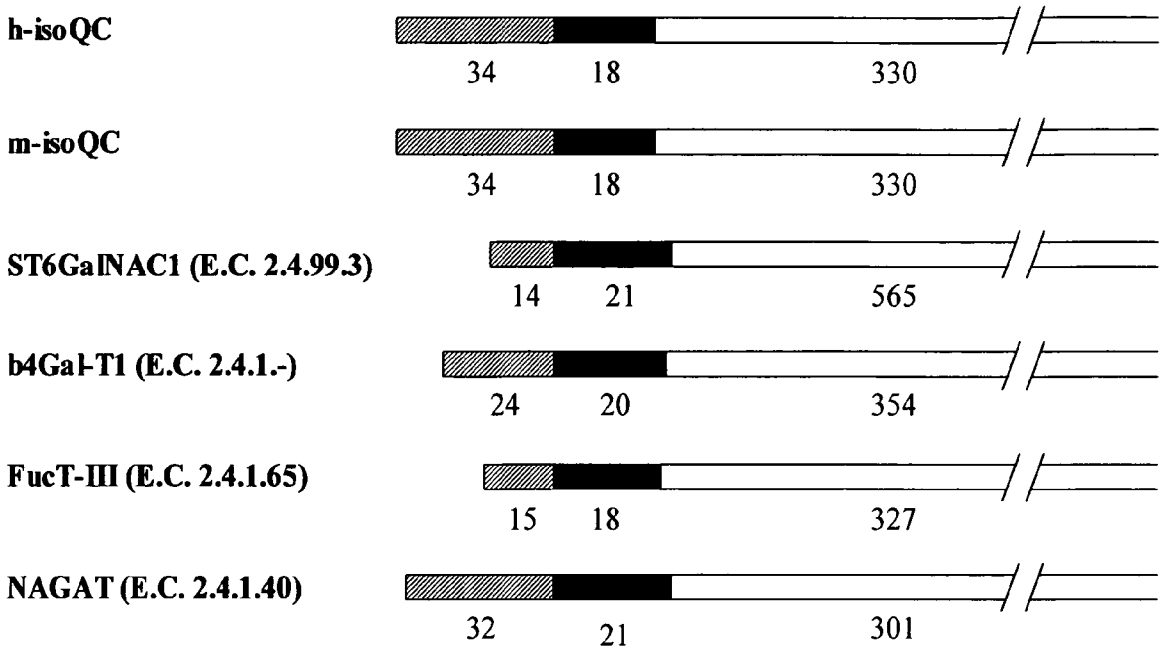

h-isoQC
34
18
330
m-isoQC
34
18
330
ST6GalNAC1 (E.C. 2.4.99.3)
14
21
565
b4Gal-T1 (E.C. 2.4.1.-)
24
20
354
FucT-III (E.C. 2.4.1.65)
15
18
327
NAGAT (E.C. 2.4.1.40)
32
21
301

Figure 29

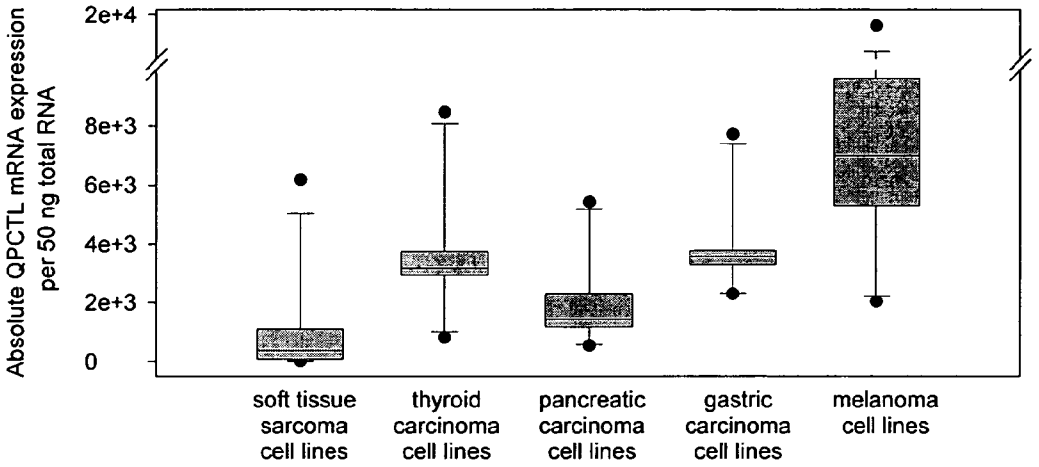


Figure 30

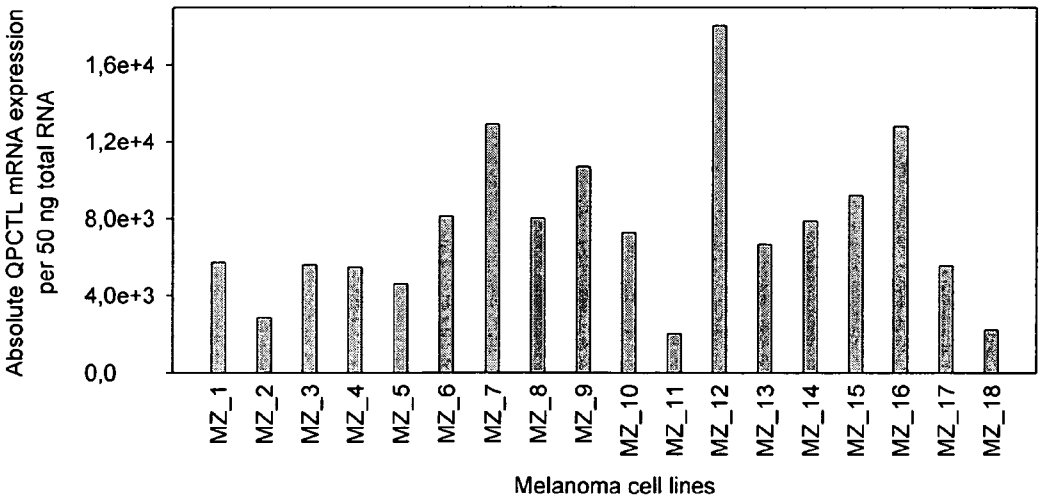


Figure 31

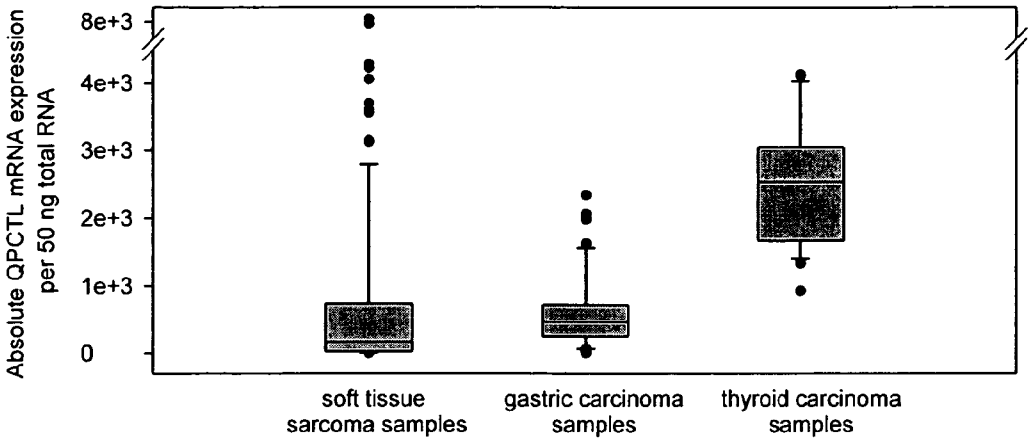


Figure 32

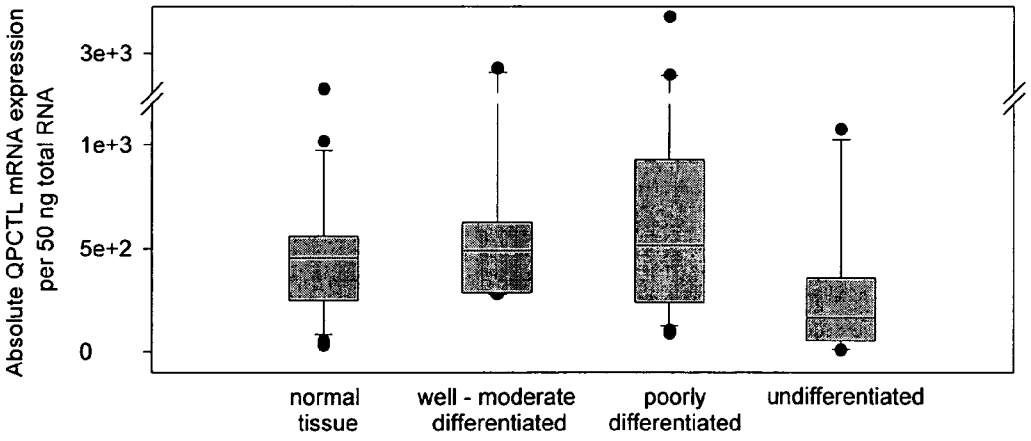


Figure 33

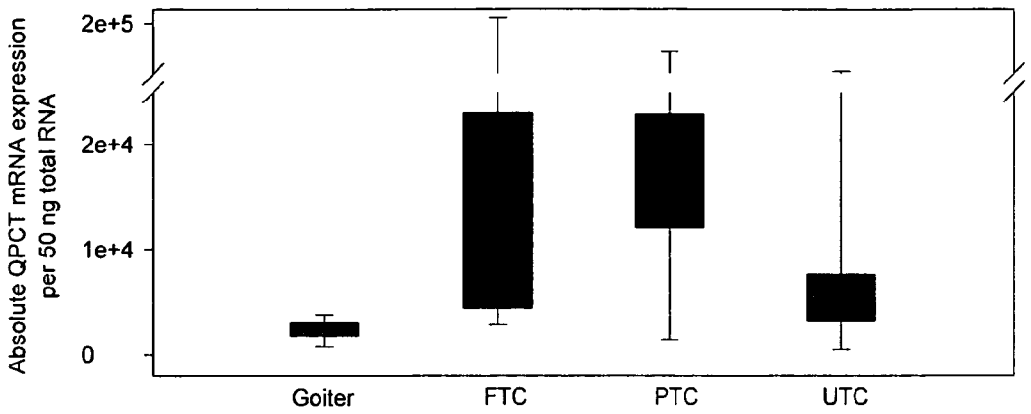


Figure 34

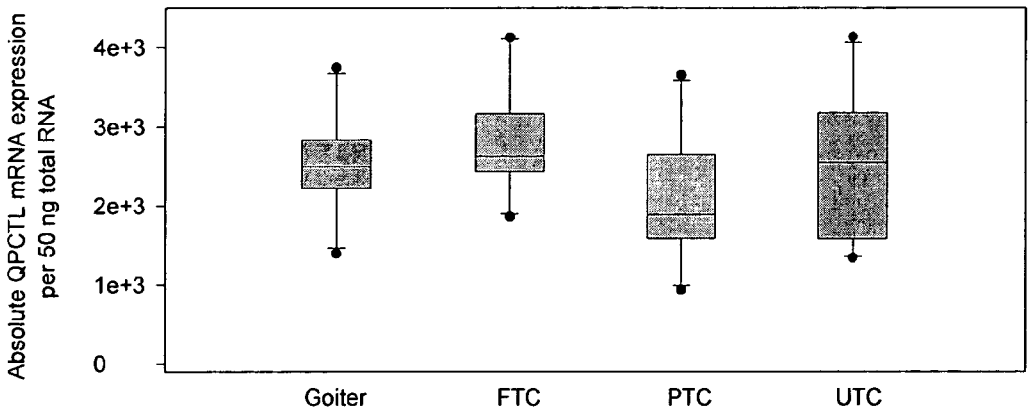


Figure 35

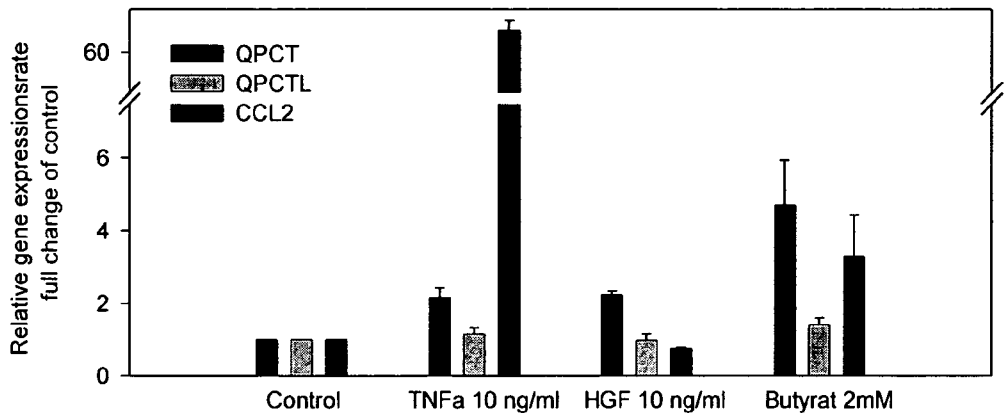


Figure 36

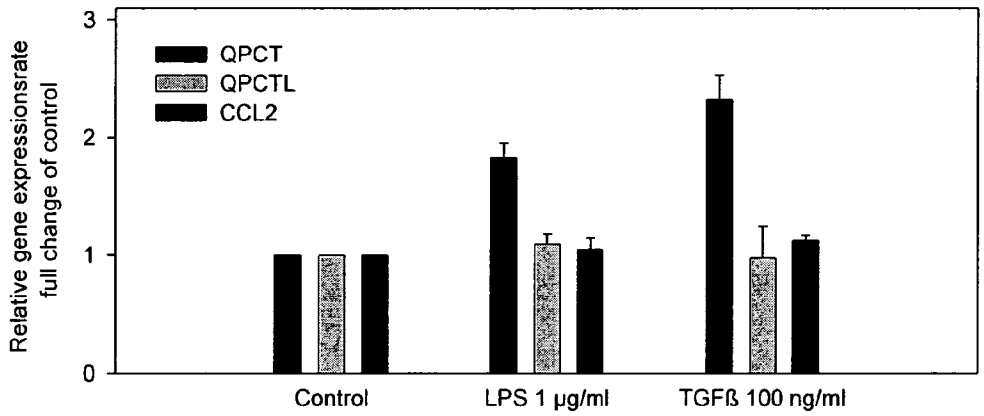


Figure 37

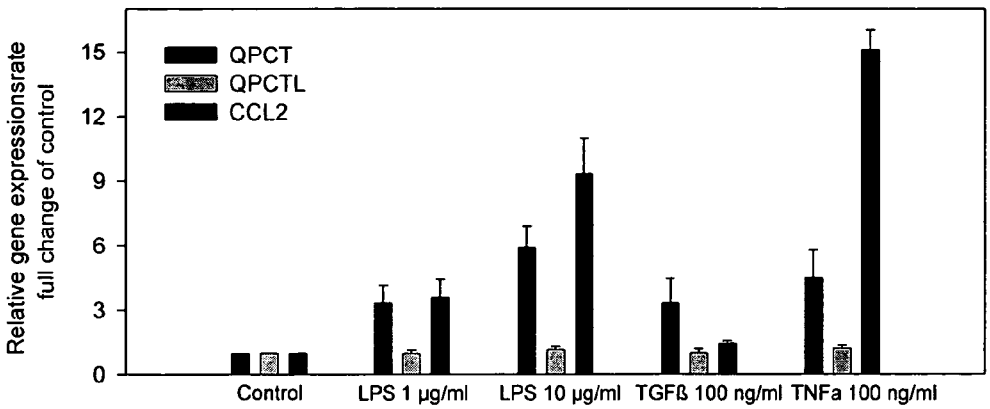


Figure 38

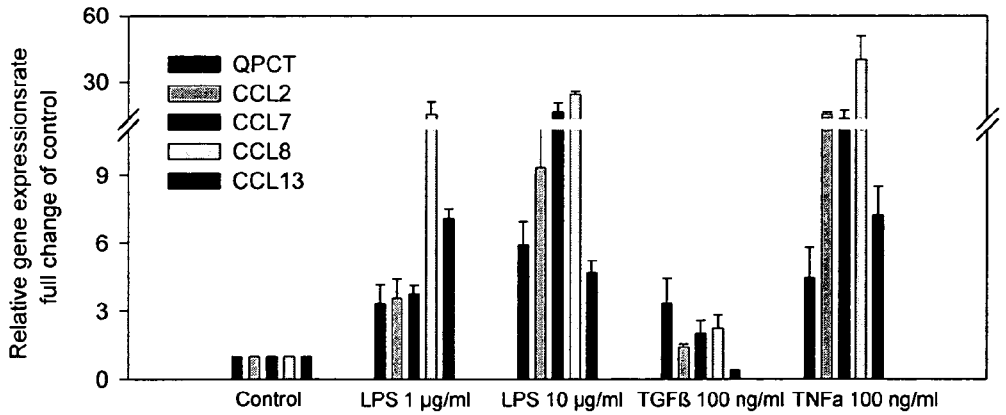
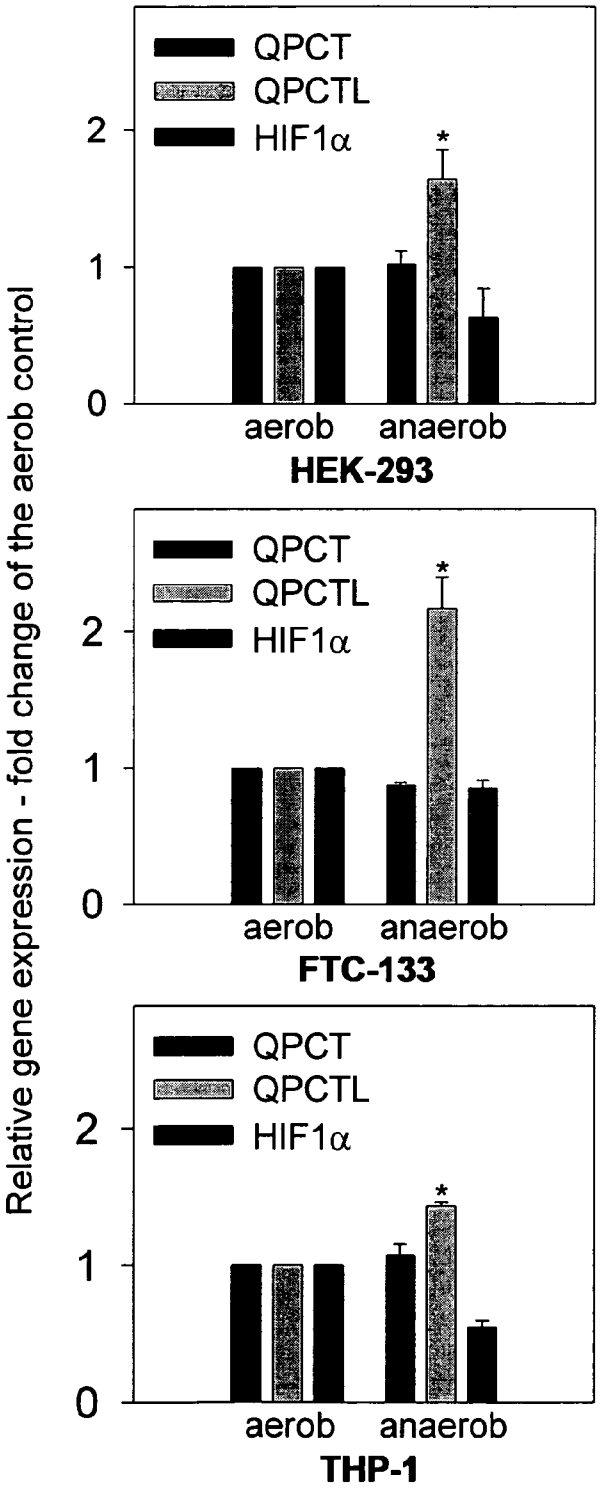


Figure 39


Relative gene expression - fold change of the aerob control
QPCT
QPCTL
HIF1α
aerob
anaerob
HEK-293
FTC-133
THP-1
0
1
2
*