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(54) Title: ADENOSYLHOMOCYSTEINASE BINDING SUBSTANCES FOR MEDICAL USE

(57) Abstract: The invention concerns an adenosylhomocysteinase (AHCY) binding substance for use in the treatment of deficiency of function of AHCY in a human being or an animal, wherein the AHCY binding substance is the cofactor nicotinamide adenine dinucleotide (NAD + /NADH), or an analogue of the cofactor NAD + /NADH, or the substrate adenosine or S-adenosyl-L-homocysteine (SAH), or an analogue of a substrate of AHCY.

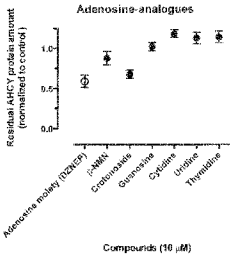

Fig. 10a

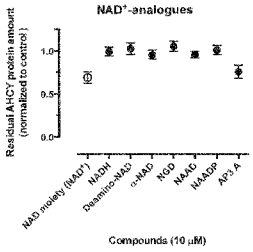

Fig. 10b

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Adenosylhomocysteinase Binding Substance

The invention concerns an adenosylhomocysteinase (AHCY) binding substance. AHCY forms an enzymatical active tetramer. AHCY was formerly named S-adenosylhomocysteine hydrolase (SAHH). Missense mutations in the AHCY gene result in AHCY having a significantly reduced enzymatic activity. Patients with such a reduced enzymatic activity show a severe phenotype such as severe myopathy and hypotonia, developmental delay, impaired myelination and hepatopathy. An effective treatment for the disorder is not available.

From Belužić, R. and Vugrek, O., Rad 508. Medical Sciencies 35(2010), pages 77 to 92 "S-Adenosylhomocysteine Hydrolase (AHCY) Deficiency: A Natural Model System for Methylation Research" it is known that AHCY deficiency is a human methylation disorder. Cellular methylations are catalyzed by substrate-specific methyltransferase enzymes. All these enzymes share the same methyl donor, namely S-adenosylmethionine (SAM). Apart from methylated substrate, SAM-dependent methylation reactions result in formation of S-adenosylhomocysteine (SAH). SAH is hydrolyzed to adenosine and homocysteine by AHCY. While adenosine is further deaminated to inosine, homocysteine is either remethylated to methionine or enters the transsulfuration pathway resulting in formation of cystathionine or, finally, cysteine. Remethylation pathway enzymes methionine synthase and betaine-homocysteine methyltransferase together with methionine-adenosyltransferase and AHCY complete the metabolic cycling of methionine and homocysteine. SAH strongly inhibits many SAM-dependent methyltransferases. SAH hydrolysis is the only source of homocysteine in mammals. Therefore, an impaired AHCY activity would effect a wide variety of cellular processes.

It is speculated that the described mutations either result in a high instability of resulting proteins or in a steric change impairing the substrate or water molecule access or product release. The publication discloses a therapy including restricted

methionine intake with supplementation of phosphatidylcholine, creatine and cysteine. However, this diet alone improved the severe phenotype only gradually.

The doctoral theses "Einfluss des Kofaktors NAD^+/NADH der S-Adenosylhomocystein-Hydrolase auf die Adenosinbindung", Lüdtke Angelika, 2003, Faculty of Medicine of the Eberhard-Karls-Universität Tübingen, Germany, discloses the binding of the NAD^+ molecule in a groove of the NAD^+ binding domain of AHCY via hydrophobic interactions and hydrogen bonds. Furthermore, it is disclosed that the NAD^+ binding domain is located in each monomer near the center of a tetramer formed from AHCY monomers and that these binding domains are interconnected which interconnection supports stability of AHCY.

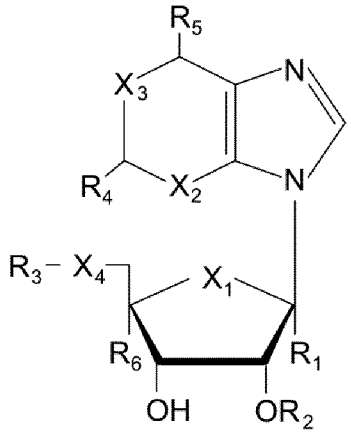
Belužić, R., et al., Biochemical and Biophysical Research Communications 368 (2008), pages 30 to 36, Belužić, R., et al., Biochem. J. (2006) 400, pages 245 to 253 and Barić, I., J. Inherit. Metab. Dis. (2009) 32, pages 459 to 471 disclose R49C, D86G, A89V, E115L and Y143C mutations resulting in an exchange of amino acids in the protein. It is speculated that in case of the A89V mutation structural abnormalities represent one basis for the pathological effects and that the Y143C mutation results in a reduced stability of the tetramer resulting in a disassembly into monomers followed by a loss of enzymatic activity. Belužić, R., et al., Biochem. J. (2006) 400, pages 245 to 253 further discloses that activity of Y143 and E115L is unaffected by addition of exogenous NAD, indicating that conditions used for purification and storage support association of NAD with mutant proteins or that additional NAD is not bound by mutant proteins.

From Muntau, A. C., et al., N. Engl. J. Med., Vol. 347, No. 26, December 26, 2002 tetrahydrobiopterin is known as an alternative treatment for mild phenylketonuria. Tetrahydrobiopterin is a natural cofactor of aromatic amino acid hydroxylases and nitric oxide synthase. It is speculated that mutations in the phenylalanine hydroxylase gene may result in misfolding of the protein and reduced enzyme activity and that tetrahydrobiopterin may act as a chemical chaperone that prevents misfolding.

The problem to be solved by the present invention is to provide a substance for use in the treatment of deficiency of function of AHCY.

The problem is solved by the features of claim 1. Embodiments are disclosed in dependent claims 2 to 8.

The invention provides an AHCY binding substance for use in the treatment of deficiency of function of AHCY in a human being or an animal, in particular a mammal, wherein the AHCY binding substance is the cofactor nicotinamide adenine dinucleotide (NAD^+/NADH), or an analogue of the cofactor NAD^+/NADH, or the substrate S-adenosyl-L-homocysteine (SAH), or an analogue of a substrate of AHCY, or an ion of any of said analogues, in particular an ion of any of said analogues which analogue is not charged, or an oxidized or reduced form of any of said analogues, or a hydrate of said ion or of said oxidized or reduced form or a hydrate of any of said analogues. The AHCY binding substance may be a AHCY binding molecule. The analogue of the cofactor NAD^+/NADH is nicotinamide 1,N^6-ethenoadenine dinucleotide (epsilon-NAD^+), P1-(5'-adenosyl)P3-(5'-adenosyl)triphosphate (AP3 A), nicotinamide 8-bromo-adenine dinucleotide (8-Bromo-AP2), 2'-bromo-ribofuranosyl-nicotinamide diphosphate (2'-Bromo-dAP2), or nicotinamide 8-bromo-adenine dinucleotide (8-Bromo-AP2(Nic)). The analogue of the substrate of AHCY is ilimaquinone, 1-[(2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1*H*-1,2,4-triazole-3-carboxamide (ribavirin), cytidine, uridine, thymidine, or beta-nicotinamid mononucleotide (beta-NMN). It is also possible that the analogue of the cofactor NAD^+/NADH or the analogue of the substrate of AHCY is a compound of following formula (I)



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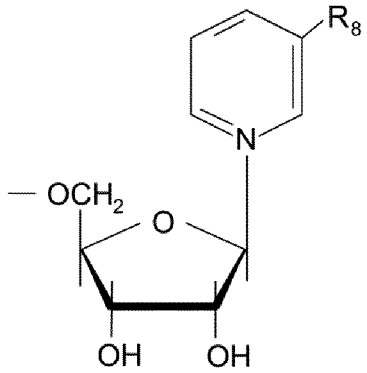
wherein X_1 is CH or O, X_2 is CH, NH or N, X_3 is NH or N, wherein X_3 may be N if X_2 is NH or wherein X_3 may be N if X_2 is NH or CH, X_4 is O or S, R_1 is –H or $–CH_3$, R_2 is –H or $–PO(OH)_2$, R_3 is –H, $–CH_2–CH_2–CHNH_2–COOH$, –POOH–O–POOH–POOH–R_7, or –POOH–O–POO^-–R_7, R_4 is –H, –O, or $–NH_2$, R_5 is –O or $–NH_2$, and R_6 is –H or $–CH_3$, wherein R_7 is



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wherein R_8 is –COOH, $CONH_2$, or $CSNH_2$.

The inventors recognized that all missense mutations in the AHCY gene known at the time of the invention result in a reduced thermal stability and proneness of the protein to aggregate. They further recognized that the mutations neither concern the active center nor the binding sites for the substrate S-adenosyl-L-homocysteine nor for the cofactor NAD^+/NADH. They showed in cultured cells that the conformational instability is accompanied by a loss of function of the AHCY protein.

They further recognized that the protein or the protein tetramer can be stabilized by any AHCY binding substance that binds to the binding site of the cofactor or to the substrate binding site of AHCY. Such substances are known in the art and commercially available.

The AHCY binding substance binding to the cofactor binding site is NAD^+/NADH or an analogue of the cofactor NAD^+ or NADH. An analogue of NAD^+/NADH can be understood as any substance or molecule that binds to an NAD^+/NADH binding site of AHCY without being NAD^+ or NADH. An analogue of a substrate of AHCY can be understood as any substance or molecule that binds to the substrate binding site of AHCY. Usually the analogue of a substrate of AHCY is not a substrate of AHCY or at least not the natural substrate of AHCY.

The observation that activity of mutated AHCY proteins in enzyme kinetics is unaffected by exogenous NAD disclosed in Belužić, R., et al., Biochem. J. (2006) 400, pages 245 to 253 cannot be confirmed by the inventors of the present Invention. As speculated in Belužić, R., et al., Biochem. J. (2006) 400, on page 252, left column the observation may be caused by conditions used for purification and storage of the proteins. It may be, e.g., that the isolated mutated proteins are already saturated with NAD such that added NAD had no further effect.

Regardless of whether it is comprised by the above formula (I) the analogue of the cofactor NAD^+/NADH may be thionicotinamide adenine dinucleotide (S-NAD^+), beta-nicotinamide adenine dinucleotide (beta-NAD^+), nicotinamide hypoxanthine dinucleotide (deamino-NAD^+), nicotinamide guanine dinucleotide, alpha-dihydronicotinamide adenine dinucleotide (alpha-NADH), beta-dihydronicotinamide adenine dinucleotide (beta-NADH), beta-nicotinamide adenine dinucleotide phosphate (beta-$NADP^+$), nicotinamide adenine triphosphate (AP3), ribofuranosyl-nicotinamide diphosphate (dAP2), ribofuranosyl-nicotinamide triphosphate (ApCpp), nicotinamide thymine dinucleotide (TP2), nicotinamide uracil dinucleotide (UP2), nicotinamide cytosine dinucleotide (CP2), nicotinamide

guanine dinucleotide (GP2), P^1-(5'-adenosyl) P^3-[5'-(1-beta D-ribofuranosyl-nicotinamide)] triphosphate (AP3(Nic)), 1,2,4-triazole-3-carboxamide adenine dinucleotide (AP2Ribavirin), P^1-[5'-(2'-deoxy-adenosyl)] P^2-[5'-(1-beta D-ribofuranosyl-nicotinamide)] diphosphate (dAP2(Nic)), P^1-(5'-adenosyl) P^3-[5'-(1-beta D-ribofuranosyl-nicotinamide)] [(alpha, beta)-methyleno]triphosphate (ApCpp(Nic)), nicotinamide thymine dinucleotide (TP2(Nic)), nicotinamide uracil dinucleotide (UP2(Nic)), nicotinamide cytosine dinucleotide (CP2(Nic)), nicotinamide guanine dinucleotide (GP2(Nic)), nicotinamide adenine dinucleotide phosphate ($NADP^+$), alpha-nicotinamide adenine dinucleotide (alpha-NAD), nicotinamide guanine dinucleotide (NGD), nicotinic acid adenine dinucleotide (NAAD), or nicotinic acid adenine dinucleotide phosphate (NAADP). The AHCY binding substance may be an oxidized or reduced form of any of the analogues. An example of the reduced form of the analogue is NADPH if the analogue is $NADP^+$ and an example of the oxidized form of the analogue is beta-NAD^+ if the analogue is beta-NADH.

The ion of any of said analogues may be an ion of a sodium salt.

Regardless of whether it is comprised by the above formula (I) the analogue of the substrate may be 3-deazaneplanocin A (DZNep), adenosine, guanosine, or crotonoside.

All the AHCY binding molecules according to the invention may act as pharmacological chaperones by stabilization of the AHCY monomer or tetramer.

The adenosylhomocysteinase (AHCY) binding substance of the invention can be contained in a pharmaceutical composition comprising a pharmaceutical acceptable carrier for oral application or for application by injection. The carrier can be a liquid such as water or a physiological salt solution. If the carrier is the physiological salt solution the composition may be applied by injection, in particular subcutaneous or intravenous injection. For oral application the carrier can be, e. g., a sugar such as glucose or saccharose. The pharmaceutical

composition may comprise the substance in a concentration of 0.1 to 1000 mg/g or mg/ml, in particular 10 to 500 mg/g or mg/ml, in particular 20 to 350 mg/g or mg/ml. The use can comprise administration of the substance in a dosage of 1 to 50 mg, in particular 5 to 30 mg, in particular 10 to 25 mg, per kg body weight of the human being or animal. The substance may be administered one a day or 2, 3, 4, 5, 6, 7, or 8 times per day.

Embodiments:

Fig. 1a - Fig. 1f	show results obtained by testing of wild-type (wt) protein and mutant proteins together with different concentrations of NAD^+ in Differential Scanning Fluorimetry (DSF),
Fig. 2a - Fig. 2f	show the T_m values resulting from the assays according to Fig. 1a - Fig. 1f,
Fig. 3	shows the T_m values obtained with different concentrations of S-NAD^+,
Fig. 4	shows the results obtained with different concentrations of epsilon-NAD^+,
Fig. 5	shows the results obtained with different concentrations of ribavirin,
Fig. 6	shows the results obtained with different concentrations of DZNep,
Fig. 7	shows the results obtained with different concentrations of ilimaquinone,

Fig. 8a - Fig. 8f show the results of Right Angle Light Scattering (RALS) measurements obtained for wild-type protein and mutated proteins together with different AHCY binding molecules,

Fig. 9a and Fig. 9b show further results obtained by testing of wild-type AHCY protein (Fig. 9a) and mutant AHCY protein D86G (Fig. 9b) with different adenosine- and NAD-analogues in DSF and

Fig. 10a and Fig. 10b show the results obtained by forming cell culture experiments with different adenosine- and NAD-analogues.

The potential of the binding of NAD^+ or an analogue of NAD^+ to the NAD^+/NADH binding site and of the binding of an analogue of a substrate of AHCY to the substrate binding site of AHCY in stabilizing the enzyme and its activity was analyzed by use of Differential Scanning Fluorimetry (DSF). The following substrates were tested in the given concentrations:

	Concentration I	Concentration II	Concentration III
NAD^+	5 µM	11 µM	100 µM
S-NAD^+	5 µM	11 µM	100 µM
epsilon-NAD^+	5 µM	11 µM	100 µM
Ribavirin	1 µM	10 µM	100 µM
DZNep	1 µM	10 µM	100 µM
Ilimaquinone	1 µM	10 µM	100 µM

The following five mutant proteins were selected for testing:

R49C, D86G, Y143C, A89V and Y379C.

Details of these mutations can be taken from the following table:

cDNA	Exon	Amino Acid	Mature Protein	Location of Mutation
c.145 C>T	2	p.Arg49Cys	R49C	beta-sheet 1 catalytic domain
c.257 A>G	3	p.Asp86Gly	D86G	alpha-Helix 3 catalytic domain
c.266 C>T	3	p.Ala89Val	A89V	alpha-Helix 3 catalytic domain
c.428 A>G	4	p.Tyr143Cys	Y143C	alpha-Helix 5 catalytic domain
c.1139 A>G	10	p.Tyr379Cys	Y379C	Connection of alpha-Helix 19 and beta-sheet 15

Co-overexpression of AHCY or mutated AHCY and chaperonins GroESL and purification of AHCY and mutated AHCY

Recombinant pMAL-c2E AHCY expression plasmids (New England Biolabs Inc. USA) were co-transformed with pGroESL encoding the proteins GroES and GroEL in E. coli strain DH5alpha cells (Invitrogen, Thermo Fisher Scientific Inc. USA) and grown in 2YT medium (1% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37 °C. When OD_{600} reached 0.6, IPTG was added/supplemented to a final concentration of 1 mM to induce protein overexpression and 50 µg/ml chloramphenicol and 100 µg/ml ampicillin were added to 2YT medium. Cell culture was continued for an appropriate time, e. g. 22 h, at 28 °C. Bacteria were harvested by centrifugation and lysed by sonification. All mutated enzymes were purified from the soluble fractions of E. coli extracts and protein purification was performed using ÄKTAxpress (GE Healthcare USA) at 4 °C by affinity chromatography, followed by size-exclusion chromatography with a HiLoad 16/60 Superdex 200 column (GE

Healthcare USA). The fractions containing isolated tetrameric fusion proteins were collected. Protein concentrations were determined using the assay of Bradford.

Determination of the thermal stability of AHCY and mutated AHCY

Differential Scanning Fluorimetry (DSF)

Fluorescence measurements were carried out on a 7900 HT fast real-time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc. USA). The data was recorded in Abi Prism SDS software. Samples contained AHCY-Maltose-Binding Protein fusion proteins, HEPES, and SYPRO Orange fluorescence dye (SYPRO® Orange Protein Gel Stain from Invitrogen, Thermo Fisher Scientific Inc. USA) in a 1:1000 dilution), which binds to exposed hydrophobic sites of unfolded proteins. For this dye, the wavelengths used for excitation and emission are 492 nm, respectively 610 nm. The assay was measured in quadruplicates on a 96-well PCR plate. Thermal denaturation was monitored by following the changes in SYPRO Orange fluorescence emission (excitation at 492 nm, emission at 610 nm).

In a first series of experiments measurements were performed in a 25 °C to 75 °C range, wherein temperature raised at a rate of 2 °C/min. Thermal denaturation curves were obtained by the plotting of fluorescence intensities against temperature. Melting temperature (T_m) values as the midpoint of the protein-unfolding transition were calculated using the Boltzmann sigmoidal non-linear regression function in GraphPad Prism (GraphPad Prism 5.0, GraphPad Software Inc. USA). All measurements were corrected for background reduction of SYPRO Orange fluorescence. To analyze the stabilizing effect of different compounds different concentrations of NAD^+, NAD^+ analogues and substrate analogues were added to the samples. Significances between wild-type and mutated apoenzymes and holoenzymes with stabilizing agents were calculated by one-way analysis of variance followed by Dunnett's post test (GraphPad Prism 5.0, GraphPad Software Inc. USA).

In a second series of experiments AHCY wild-type and D86G mutated AHCY were tested with different compounds in concentrations of 10 µM and 50 µM. Results were interpreted as a stabilization of AHCY when the melting temperature T_m during thermal denaturation compared to the value obtained without addition of a compound increased more than the range of fluctuation when no compound was added. The observed range of fluctuation for wild-type AHCY and mutated AHCY was less than 0.2 °C.

Right Angle Light Scattering (RALS)

RALS measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., acquired by Agilent Technologies USA) equipped with a temperature-controlled Peltier multicell holder (Varian Inc., acquired by Agilent Technologies USA). Samples contained AHCY protein (1 mg/ml) with and without different adenosine- and NAD-analogues in a concentration of 10 µM in 20 mM HEPES buffer at pH 7.0 containing 200 mM NaCl. Increase in turbidity at a constant temperature of 37 °C was monitored for 60 minutes (excitation at 330 nm, emission at 335 nm, 5.0 nm slit width). Turbidity curves were illustrated with GraphPad Prism 5.0.

Cell culture

Eukaryotic expression of AHCY and mutated AHCY

At the moment there is no animal model for a deficiency of a function of AHCY. Therefore, a cell culture model for investigation of the effect of several substances on mutated AHCY protein in a living system was developed. The model comprises addition of the substances to be tested to the cell culture medium of eukaryotic cells expressing mutated AHCY proteins, culturing the cells in presence of the substances for 48 hours and determination of the residual AHCY protein in the cells after this period.

For the cell culture experiments the cDNA of human wild-type AHCY gene was subcloned into pEF DEST 51 expression vector (Invitrogen, Thermo Fisher Scientific Inc. USA) encoding a c-terminal V5-tag by using the gateway recombination cloning technology (Gateway Cloning Technology, Invitrogen, Thermo Fisher Scientific Inc. USA). Presence and correct orientation of AHCY in the plasmid was initially checked by restriction endonuclease mapping and later verified by DNA sequencing. 2 µg DNA were transfected into 2 million COS-7 cells via electroporation (Amaxa™ Nucleofector™ Technology, Switzerland). Cells were cultured in RPMI 1640 medium with stable glutamine with 10% fetal bovine serum and 1% Gibco® Antibiotic-Antimycotic 100X (Thermo Fisher Scientific Inc. USA). Cells were passaged into fresh growth medium 24 h after transfection. Test substances were added in a concentration of 10 µM to the growth medium and cells were cultured for 48 hours. COS cells were harvested by trypsinization, washed with PBS and then collected by centrifugation. The amount of residual AHCY in transfected COS-7 cells was detected by immunoblotting. Therefore, cells were treated with lysis buffer (50 mM Tris-HCl, pH = 7.4, 150 mM NaCl, and 2 mM complete protease inhibitor) and lysed by three freeze-and-thaw cycles. 1 µl of each of the resulting total cell lysates was dotted onto a nitrocellulose membrane. The membrane was dried at ambient temperature for 15 minutes and then blocked with 5% milk in 1 x TBS (Tris buffered saline, 50 mM Tris [2-Amino-2-hydroxymethyl-propane-1,3-diol] and 150 mM NaCl). This was followed by 1 h of incubation with the primary antibody, mouse anti-V5 tag (1:5000 dilution, anti-V5 epitope tag antibody R960-25, Thermo Fisher Scientific Inc. USA), and 1 h of incubation with the secondary antibody, anti-mouse HRP (goat anti-mouse IgG (H+L) poly-HRP secondary antibody, Thermo Fisher Scientific Inc. USA). Dot blots were visualized with a substrate (SuperSignal™ West Femto Chemiluminescent Substrate, Thermo Fisher Scientific Inc. USA) and chemiluminescence was monitored with an imaging system (DIANA III chemiluminescence imager, Raytest USA Inc. USA). Resulting dots were quantified by the Protein Array Analyzer for ImageJ (http://image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-ImageJ.htm) using the NIH Image software (https://imagej.nih.gov).

Statistical analysis

Non-linear regression analyses and statistical tests were performed using GraphPad Prism 4.0c (GraphPad Software Inc. USA).

Results

Stabilization against thermal stress

DSF is an excellent means to monitor global thermal unfolding events by probing the accessibility of the fluorescent dye Sypro Orange to hydrophobic groups within a protein. DSF was used in order to assess whether mutated AHCY proteins can be stabilized by various compounds at increasing temperatures. The stabilizing effect was evaluated by comparison of the unfolding transition midpoints (fraction unfolded = 0.5) as an operative measure of protein stability and by graphical analysis of the thermal denaturation curves. The midpoint of thermal unfolding of wild-type AHCY was shifted by an average of 2.27 °C towards higher temperatures by NAD^+ and NAD^+ analogues. The addition of substrate analogues had an even more pronounced effect by showing an average shift of 3.86 °C.

a) First series of experiments

A right shift in the unfolding transition was observed for every substance tested, indicating a stabilizing effect on the protein. Among all compounds analyzed, DZNep which addresses the substrate binding site, showed the highest increase in T_m, stabilizing the unfolding of D86G by 11.18 °C. Comparable effects were observed for all mutated AHCY proteins.

Stabilization by the natural cofactor NAD^+

The individual results can be seen from Figs. 1a to 1f and 2a to 2f and are summarized in the following table:

Mutation	Wt		R49C		D86G		A89V		Y143C		Y379C	
	T_m	*SD*	T_m	*SD*	T_m	*SD*	T_m	*SD*	T_m	*SD*	T_m	*SD*
No NAD^+	51,98	*0,1167*	49,84	*0,0802*	49,55	*0,0876*	50,29	*0,06043*	49,34	*0,07141*	50,01	*0,0354*
NAD^+ 5 µM	52,24	*0,1349*	51,48	*0,1213*	51,88	*0,0384*	51,97	*0,04197*	50,62	*0,04564*	51,31	*0,0465*
NAD^+ 11 µM	53,99	*0,0749*	52,55	*0,1219*	53,51	*0,035*	53,52	*0,16540*	52,36	*0,19150*	52,45	*0,0689*
NAD^+ 100 µM	54,32	*0,2627*	52,75	*0,1726*	54,02	*0,72*	54,59	*0,9564*	52,17	*0,07868*	52,48	*0,0446*

T_m = Temperature at the midpoint of protein unfolding transition

SD = Standard Deviation

The cofactor NAD^+ showed very good effects on the thermal stability of AHCY wild-type and mutated AHCY proteins. For AHCY wild-type, the addition of 100 µM NAD^+ stabilized the unfolding transition by 2.34 °C. Comparable behavior was observed for the mutated AHCY proteins. D86G showed the highest increase in T_m with NAD^+ (ΔT_m = 4.47 °C for 100 µM NAD^+).

For NAD^+, the stabilizing effect in the DSF assay was observed to be concentration-dependant.

Stabilizing effect of the NAD^+ analogues S-NAD^+ and epsilon-NAD^+

S-NAD^+ is a side-chain thione analogue modified in the nicotinamide part of the coenzyme (Li et al.). epsilon-NAD^+ (ε-NAD^+) was initially synthesized by the reaction of chloracetaldehyde with NAD^+ and is modified in the adenine part of the cofactor.

As shown in Fig. 3 S-NAD^+ showed a similar stabilizing pattern as the natural cofactor NAD^+. Again, especially the variant D86G showed improved thermal stability: the midpoint of thermal unfolding was shifted by 6.7 °C (100 µM). In summary, upon addition of S-NAD^+, all mutated AHCY proteins showed improved resistance against thermal stress.

Fig. 4 shows that considerable stabilization was also observed for epsilon-NAD^+ but the substance was proved to be less effective than S-NAD^+. Here, the substance was particularly beneficial for the variant R49C, which showed an increase in T_m of ΔT_m = 4.36 °C.

Stabilizing effect of ribavirin

Ribavirin is a purine nucleoside analogue, which binds to the adenosine binding site of AHCY. It was described to stop viral RNA synthesis and viral mRNA-capping. Ribavirin is used primarily to treat hepatitis C and viral hemorrhagic fevers.

As can be seen from Fig. 5 the addition of ribavirin addressing the substrate binding site showed a good stabilizing effect on AHCY proteins. Both wild-type and mutated

AHCY proteins revealed marked changes in the shape of the denaturation curves. The highest increase in T_m was shown for R49C. Interestingly, best benefit was observed by adding the substance in a concentration of 1 µM.

Stabilizing effect of DZNep

DZNep is a competitive inhibitor addressing the substrate binding site of AHCY. As can be seen from Fig. 6 DZNep showed the strongest effect on the thermal shift of all AHCY proteins. Among all mutated AHCY proteins, D86G showed the highest increase of ΔT_m (13.5 °C at 10 µM DZNep). A shift of > 4.33 °C was observed for all mutated AHCY proteins. It may be presumed that the responsiveness of AHCY mutants to DZNep is based on the tight binding to the catalytic centre of the enzyme which stabilizes its folding state.

Stabilizing effect of ilimaquinone

Ilimaquinone is a natural metabolite (sesquiterpene quinone) shown to have anti-inflammatory, antimicrobial, and antimitotic properties. As an analogue of adenosine it binds to the active site of AHCY.

Fig. 7 shows that upon addition of ilimaquinone, a protecting effect against thermal unfolding was revealed. All mutated AHCY proteins showed a benefit concerning the ΔT_m. In particular, considerable stabilization was detected for the variant A89V, shifting the midpoint of thermal unfolding by 8.9 °C.

In conclusion, all compounds tested enhanced the thermal stability of their targets.

b) Second series of experiments

As shown on the left panels of Fig. 9a and Fig. 9b adenosine, the substrate SAH and the adenosine-analogue DZNEP resulted in a clear stabilization of wild-type and mutated AHCY in a range of 2 to 4 °C. Beta-NMN showed a stabilizing effect in the range of 1 °C for wild-type AHCY and mutated AHCY. Crotonoside showed a stabilizing effect of about 1 °C for wild-type AHCY and mutated AHCY only at a

concentration of 50 µM. Other tested adenosine-analogues shown in Fig. 9a and 9b showed no stabilizing effect in this test system.

The right panels in Figs. 9a and 9b show that all tested NAD^+-analogues had a stabilizing effect on wild-type AHCY and mutated AHCY. NAD^+, NADH and alpha-NAD showed a shift of the melting point of about 3 to 4 °C for wild-type and mutated AHCY at a concentration of 50 µM. At a concentration of 10 µM stabilization for wild-type and mutated AHCY was about or more than 2 °C. Deamino-NAD showed a clear stabilization in the range of 1 to 2 °C for wild-type and mutated AHCY.

Stabilization against protein aggregation

RALS experiments probing the formation of insoluble aggregates were carried out to investigate whether the addition of substances that showed a stabilizing effect in the thermal denaturation assay also prevents early aggregation. For this purpose time-dependant aggregation profiles of wild-type and mutated AHCY proteins were measured at constant temperature of 37 °C.

For evaluation of the data, the slope of the aggregation curve was analyzed by linear regression and statistical significance was determined by one-way analysis of variance using GraphPad Prism (GraphPad Prism 5.0, GraphPad Software Inc. USA).

Figures 8a to 8f show that, particularly for mutated AHCY proteins, the binding of small molecules can reduce protein aggregation. Upon addition of NAD^+, NAD^+ analogues or substrate analogues, all mutated AHCY proteins showed decelerated aggregation.

Cell culture experiments

The aim of the use of the AHCY binding substance in the treatments of deficiency of function of AHCY is a stabilization of the AHCY protein such that a bigger amount of functional protein is available and the loss of function of mutated AHCY is at least partly compensated. An increase of the residual amount of AHCY after 48 hours of

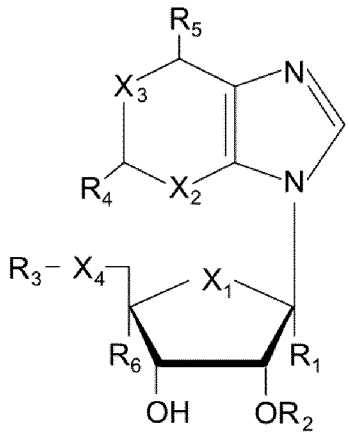
incubation with the substances to be tested showed a stabilizing effect in a living system.

The results shown in Fig. 10a showed that all tested adenosine-analogues resulted in an increased residual amount of AHCY compared to cells incubated with DZNEP. As can be seen in Fig. 10a beta-NMN and Guanosine resulted in an increase of residual AHCY protein by a factor of about 1.5 and Cytidine, Uridine and Thymidine resulted in an increase by a factor of about 2.

As can be seen in Fig. 10b also the tested NAD^+-analogues resulted in an increase in the residual amount of AHCY protein.

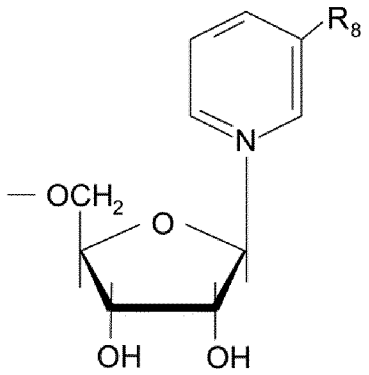
Claims

1. Adenosylhomocysteinase (AHCY) binding substance for use in the treatment of deficiency of function of AHCY in a human being or an animal, wherein the AHCY binding substance is the cofactor nicotinamide adenine dinucleotide (NAD^+/NADH), or an analogue of the cofactor NAD^+/NADH, or the substrate adenosine or S-adenosyl-L-homocysteine (SAH), or an analogue of a substrate of AHCY, or an ion of any of said analogues or an oxidized or reduced form of any of said analogues, or a hydrate of said ion or of said oxidized or reduced form or a hydrate of any of said analogues, wherein the analogue of the cofactor NAD^+/NADH is nicotinamide $1,N^6$-ethenoadenine dinucleotide (epsilon-NAD^+), P1-(5'-adenosyl)P3-(5'-adenosyl)triphosphate (AP3 A), nicotinamide 8-bromo-adenine dinucleotide (8-Bromo-AP2), 2'-bromo-ribofuranosyl-nicotinamide diphosphate (2'-Bromo-dAP2), or nicotinamide 8-bromo-adenine dinucleotide (8-Bromo-AP2(Nic)), or wherein the analogue of the substrate of AHCY is ilimaquinone, 1-[(2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1*H*-1,2,4-triazole-3-carboxamide (ribavirin), cytidine, uridine, thymidine, or beta-nicotinamid mononucleotide (beta-NMN), or wherein the analogue of the cofactor NAD^+/NADH or the analogue of the substrate of AHCY is a compound of following formula (I)



,

wherein X_1 is CH or O, X_2 is CH, NH or N, X_3 is NH or N, X_4 is O or S, R_1 is –H or –CH_3, R_2 is –H or –$PO(OH)_2$, R_3 is –H, –CH_2–CH_2–$CHNH_2$–COOH, –POOH–O–POOH–POOH–R_7, or –POOH–O–POO^-–R_7, R_4 is –H, –O, or –NH_2, R_5 is –O or –NH_2, and R_6 is –H or –CH_3, wherein R_7 is



wherein R_8 is –COOH, $CONH_2$, or $CSNH_2$.

2. The adenosylhomocysteinase (AHCY) binding substance of claim 1 for use according to claim 1, wherein the analogue of the cofactor NAD^+/NADH is thionicotinamide adenine dinucleotide (S-NAD^+), beta-nicotinamide adenine dinucleotide (beta-NAD^+), nicotinamide hypoxanthine dinucleotide (deamino-NAD^+), nicotinamide guanine dinucleotide, alpha-dihydronicotinamide adenine dinucleotide (alpha-NADH), beta-dihydronicotinamide adenine dinucleotide (beta-NADH), beta-nicotinamide adenine dinucleotide phosphate (beta-$NADP^+$), nicotinamide adenine triphosphate (AP3), ribofuranosyl-nicotinamide diphosphate (dAP2), ribofuranosyl-nicotinamide triphosphate (ApCpp), nicotinamide thymine dinucleotide (TP2), nicotinamide uracil dinucleotide (UP2), nicotinamide cytosine dinucleotide (CP2), nicotinamide guanine dinucleotide (GP2), P^1-(5'-adenosyl) P^3-[5'-(1-beta D-ribofuranosyl-nicotinamide)] triphosphate (AP_3(Nic)), 1,2,4-triazole-3-carboxamide adenine dinucleotide (AP_2Ribavirin), P^1-[5'-(2'-deoxy-adenosyl)] P^2-[5'-(1-beta D-ribofuranosyl-nicotinamide)] diphosphate (dAP2(Nic)), P^1-(5'-adenosyl) P^3-[5'-(1-beta D-ribofuranosyl-nicotinamide)] [(alpha, beta)-methyleno]triphosphate (ApCpp(Nic)), nicotinamide thymine dinucleotide (TP2(Nic)), nicotinamide uracil dinucleotide (UP2(Nic)), nicotinamide cytosine dinucleotide (CP2(Nic)), nicotinamide guanine dinucleotide (GP2(Nic)), nicotinamide adenine dinucleotide phosphate ($NADP^+$), alpha-nicotinamide adenine dinucleotide (alpha-NAD), nicotinamide guanine dinucleotide (NGD), nicotinic acid adenine dinucleotide (NAAD), or nicotinic acid adenine dinucleotide phosphate (NAADP).

3. The adenosylhomocysteinase (AHCY) binding substance of claim 2 for use according to claim 1, wherein said ion is an ion of a sodium salt.

4. The adenosylhomocysteinase (AHCY) binding substance of claim 1 for use according to claim 1, wherein the analogue of the substrate is 3-deazaneplanocin A (DZNep), adenosine, guanosine, or crotonoside.

5. The adenosylhomocysteinase (AHCY) binding substance of any of claims 1 to 4 for use according to claim 1, wherein the substance is contained in a pharmaceutical composition comprising a pharmaceutical acceptable carrier for oral application or for application by injection.

6. The adenosylhomocysteinase (AHCY) binding substance of claim 5 for use according to claim 1, wherein the pharmaceutical composition comprises the substance in a concentration of 0.1 to 1000 mg/g or mg/ml, in particular 10 to 500 mg/g or mg/ml.

7. The adenosylhomocysteinase (AHCY) binding substance of any of claims 1 to 6 for use according to claim 1, wherein the use comprises administration of the substance in a dosage of 1 to 50 mg, in particular 5 to 30 mg, per kg body weight of the human being or animal.

8. The adenosylhomocysteinase (AHCY) binding substance of any of claims 1 to 7 for use according to claim 1, wherein X_3 is N if X_2 is NH or wherein X_3 is N if X_2 is NH or CH.

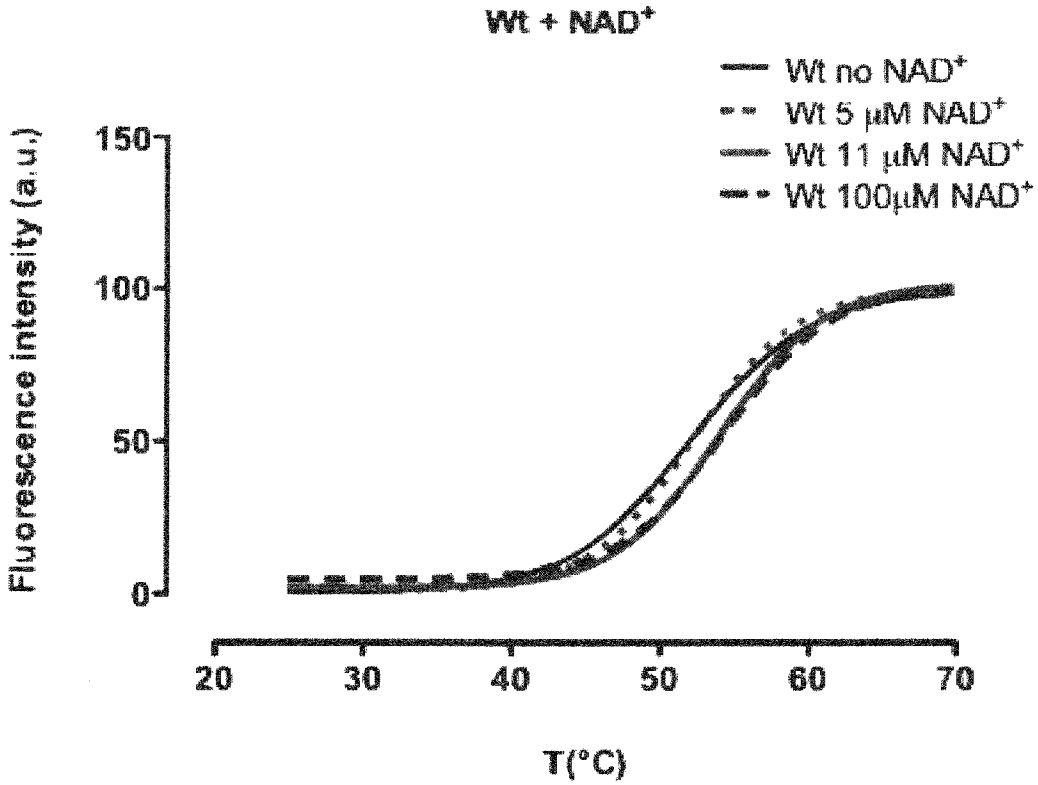

Wt + NAD+
Wt no NAD+
Wt 5 µM NAD+
Wt 11 µM NAD+
Wt 100µM NAD+
Fluorescence intensity (a.u.)
150
100
50
0
20
30
40
50
60
70
T(°C)

Fig. 1a

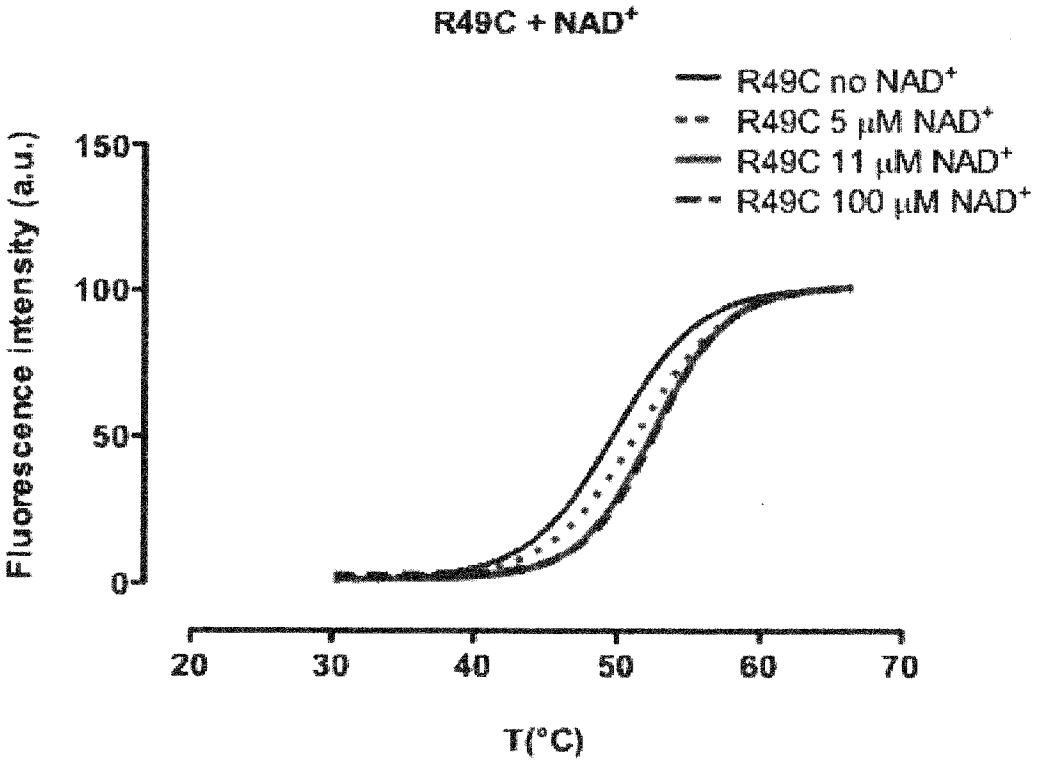

R49C + NAD+
R49C no NAD+
R49C 5 µM NAD+
R49C 11 µM NAD+
R49C 100 µM NAD+
Fluorescence intensity (a.u.)
150
100
50
0
20
30
40
50
60
70
T(°C)

Fig. 1b

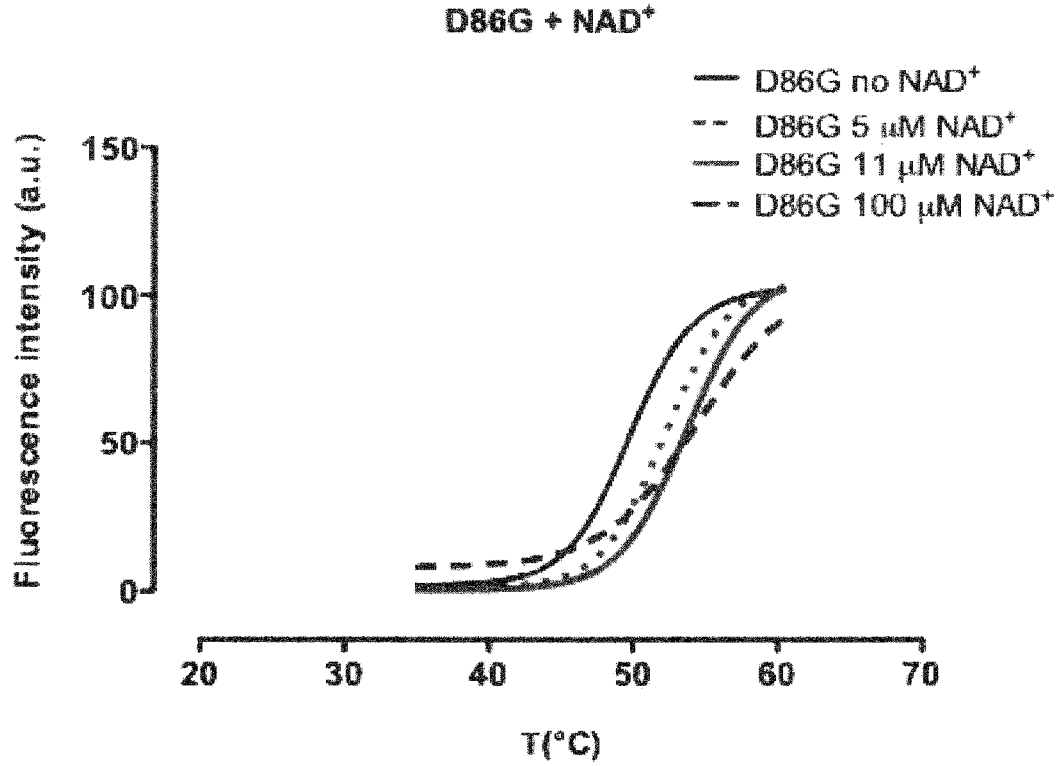

D86G + NAD+
D86G no NAD+
D86G 5 µM NAD+
D86G 11 µM NAD+
D86G 100 µM NAD+
Fluorescence intensity (a.u.)
150
100
50
0
20
30
40
50
60
70
T(°C)

Fig. 1c

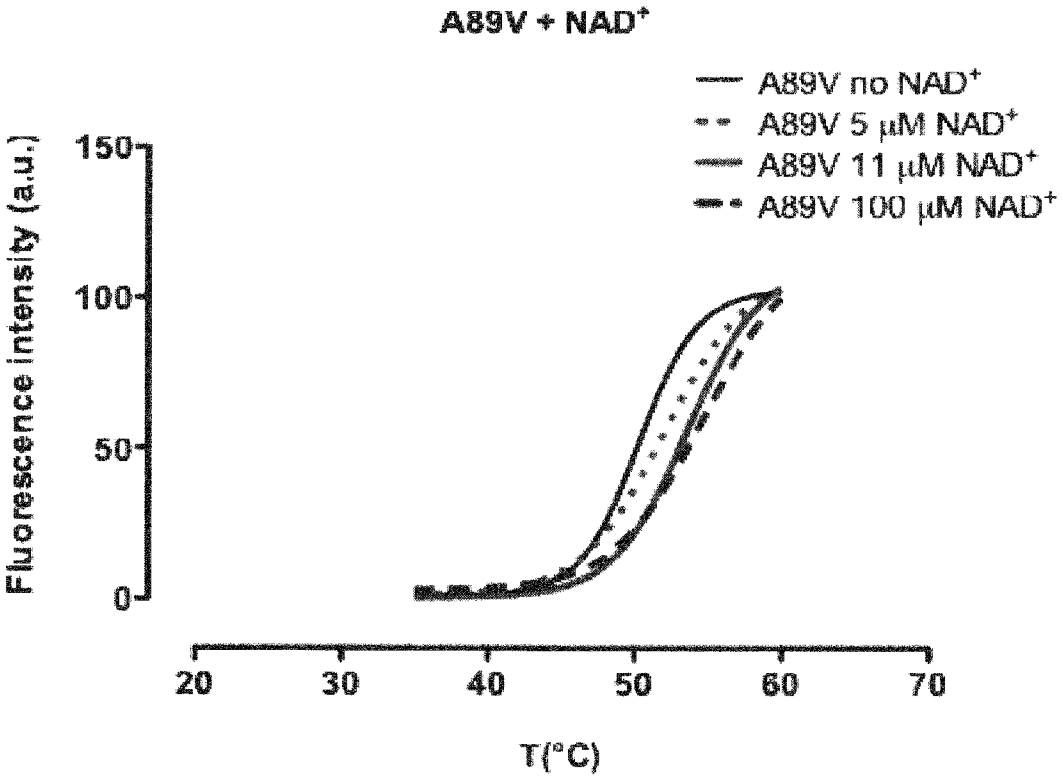

A89V + NAD+
A89V no NAD+
A89V 5 µM NAD+
A89V 11 µM NAD+
A89V 100 µM NAD+
Fluorescence intensity (a.u.)
150
100
50
0
20
30
40
50
60
70
T(°C)

Fig. 1d

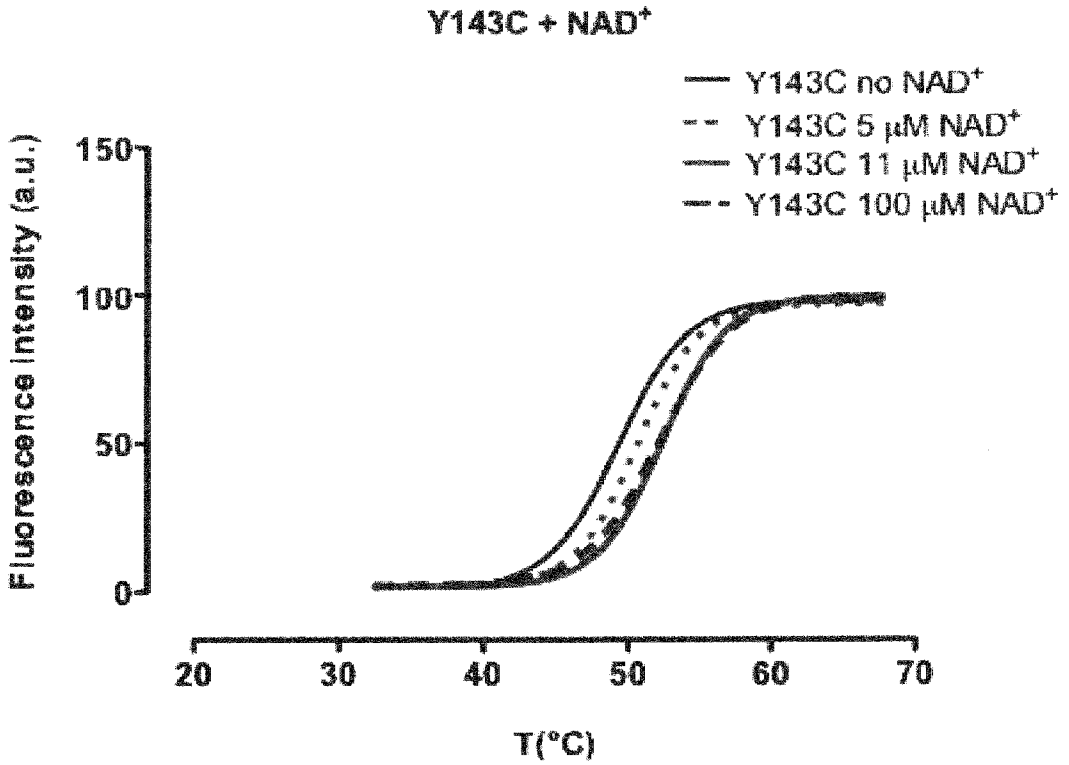

Y143C + NAD+
Y143C no NAD+
Y143C 5 µM NAD+
Y143C 11 µM NAD+
Y143C 100 µM NAD+
Fluorescence intensity (a.u.)
150
100
50
0
20
30
40
50
60
70
T(°C)

Fig. 1e

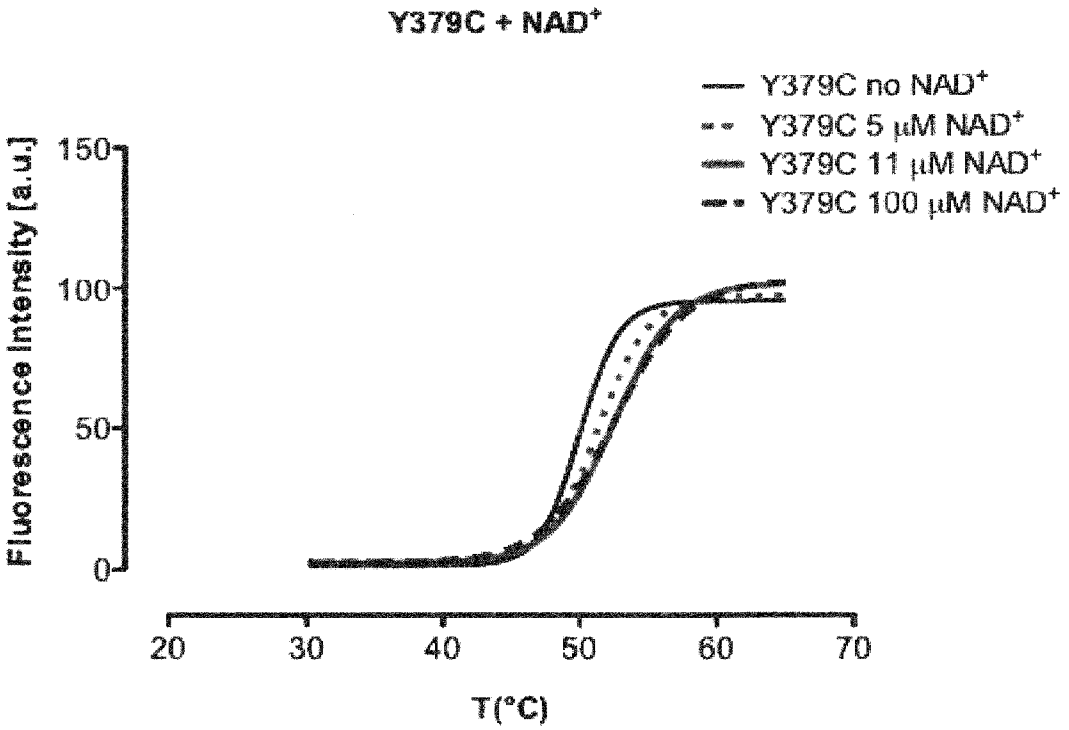

Y379C + NAD+
Y379C no NAD+
Y379C 5 µM NAD+
Y379C 11 µM NAD+
Y379C 100 µM NAD+
Fluorescence Intensity [a.u.]
150
100
50
0
20
30
40
50
60
70
T(°C)

Fig. 1f

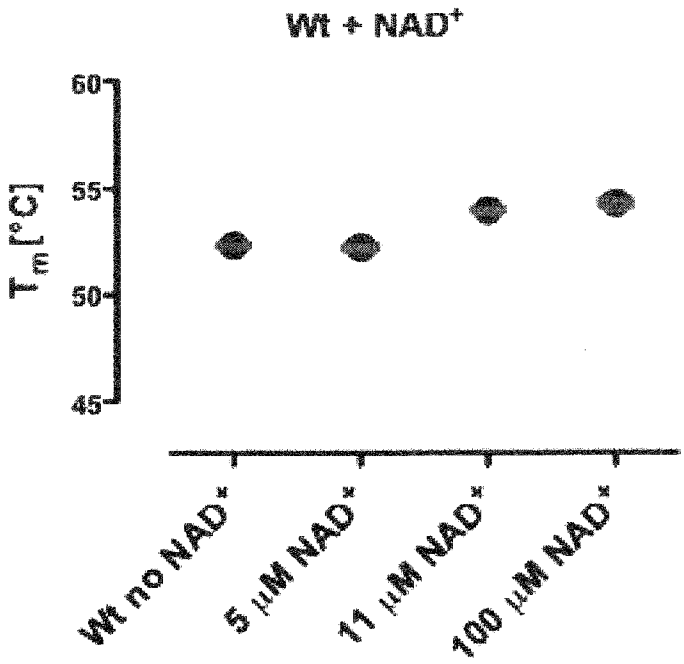

Wt + NAD+
60
55
50
45
Tm [°C]
Wt no NAD+
5 µM NAD+
11 µM NAD+
100 µM NAD+

Fig. 2a

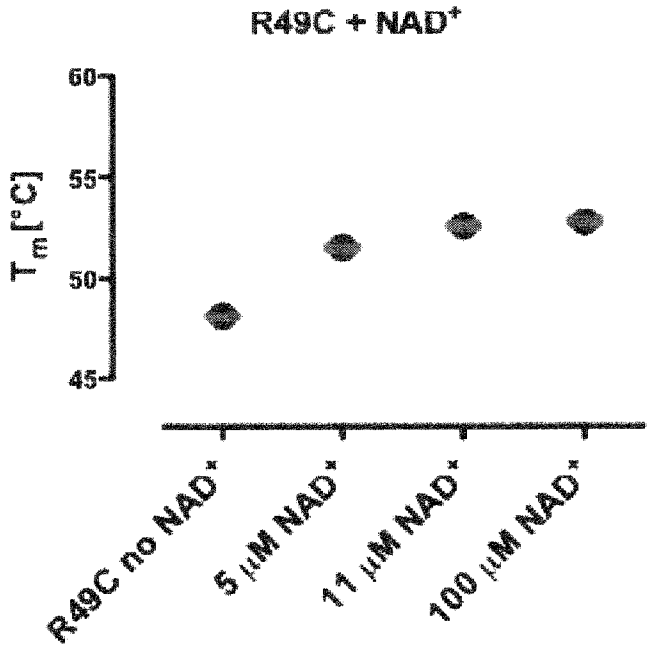

R49C + NAD+
60
55
50
45
Tm [°C]
R49C no NAD+
5 µM NAD+
11 µM NAD+
100 µM NAD+

Fig. 2b

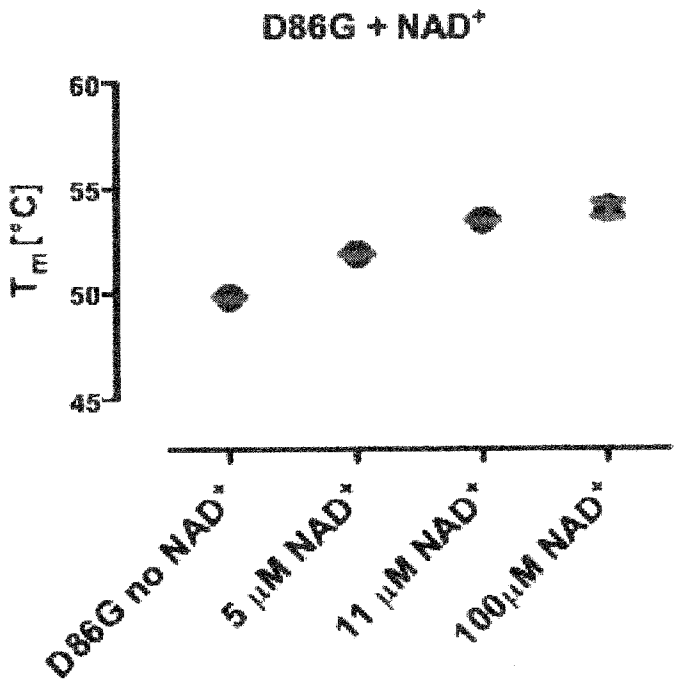

D86G + NAD+
60
55
50
45
Tm [°C]
D86G no NAD+
5 µM NAD+
11 µM NAD+
100µM NAD+

Fig. 2c

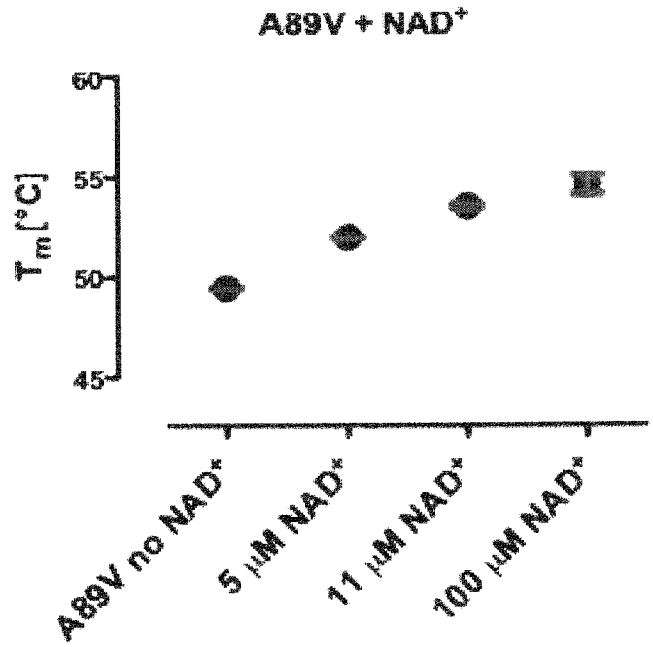

A89V + NAD+
60
55
50
45
Tm [°C]
A89V no NAD+
5 µM NAD+
11 µM NAD+
100 µM NAD+

Fig. 2d

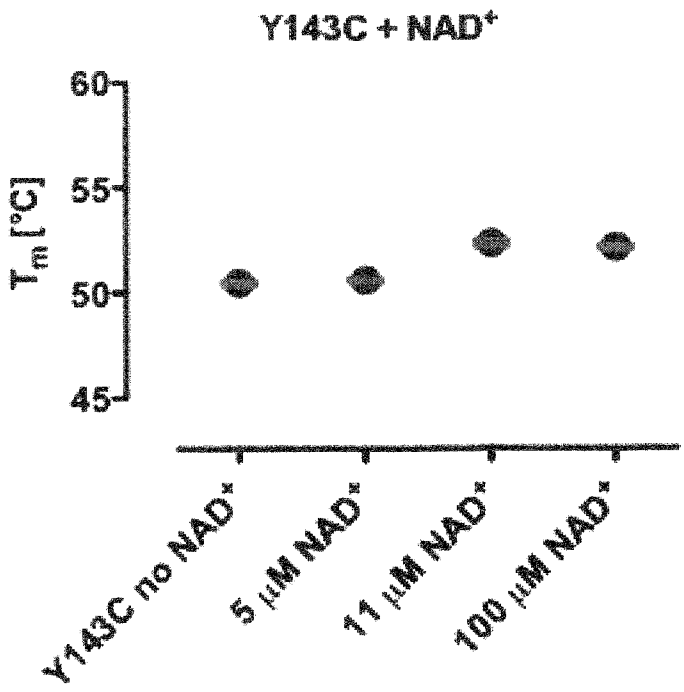

Y143C + NAD+
60
55
50
45
Tm [°C]
Y143C no NAD+
5 µM NAD+
11 µM NAD+
100 µM NAD+

Fig. 2e

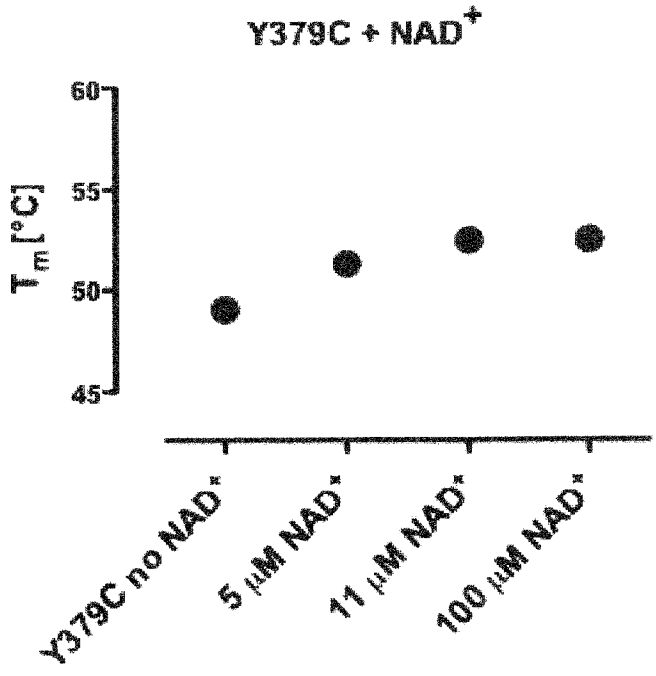

Y379C + NAD+
60
55
50
45
Tm [°C]
Y379C no NAD+
5 µM NAD+
11 µM NAD+
100 µM NAD+

Fig. 2f

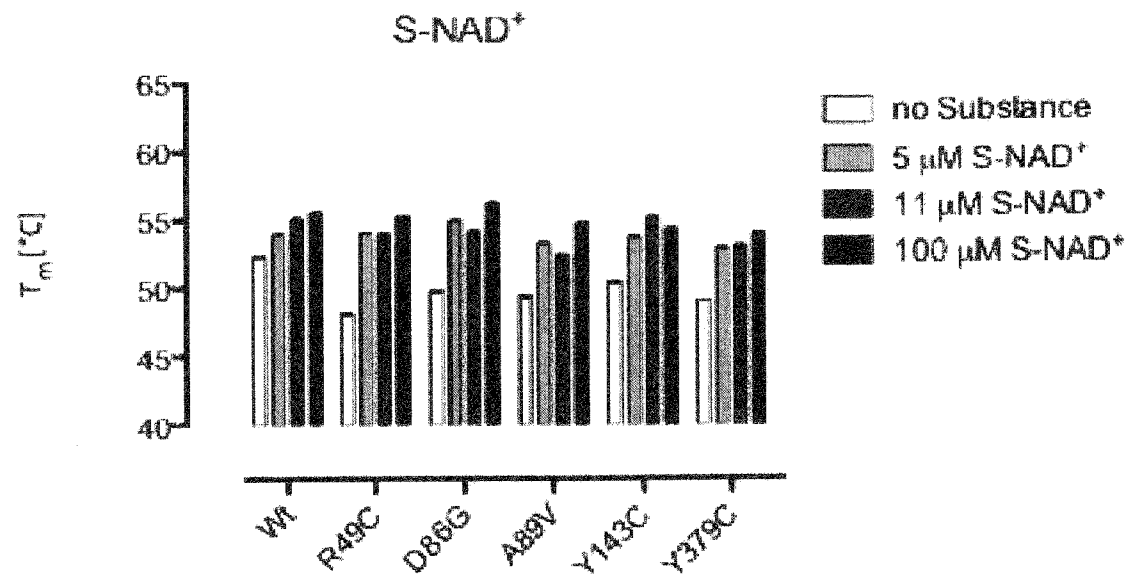

S-NAD+
65
60
55
50
45
40
T_m [°C]
no Substance
5 µM S-NAD+
11 µM S-NAD+
100 µM S-NAD+
Wt
R49C
D86G
A89V
Y143C
Y379C

Fig. 3

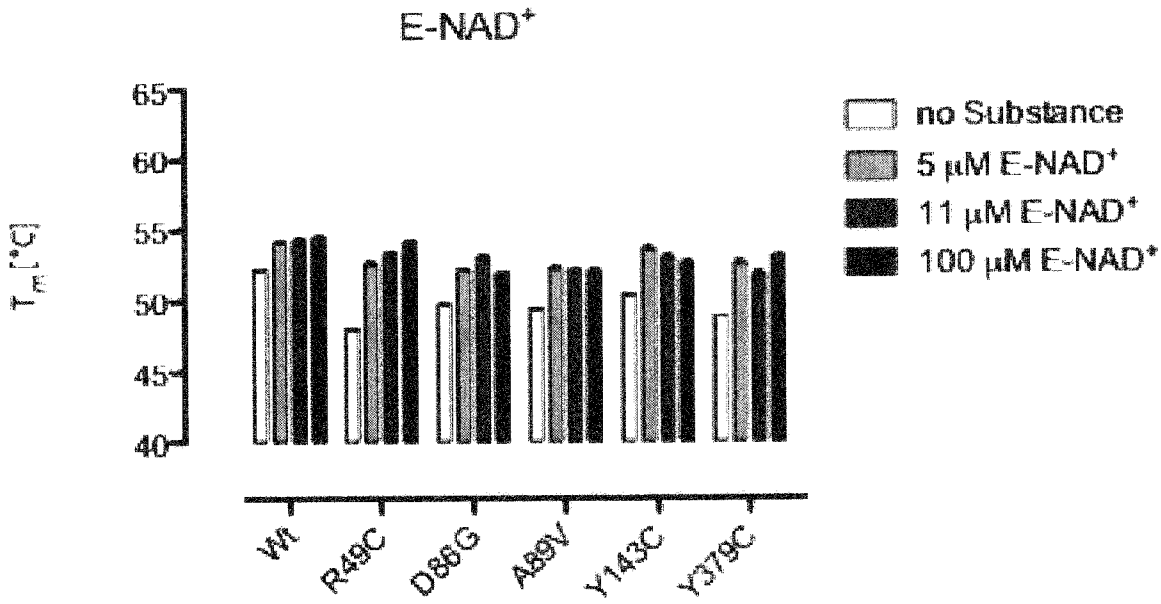

E-NAD+
65
60
55
50
45
40
T_m [°C]
no Substance
5 µM E-NAD+
11 µM E-NAD+
100 µM E-NAD+
Wt
R49C
D86G
A89V
Y143C
Y379C

Fig. 4

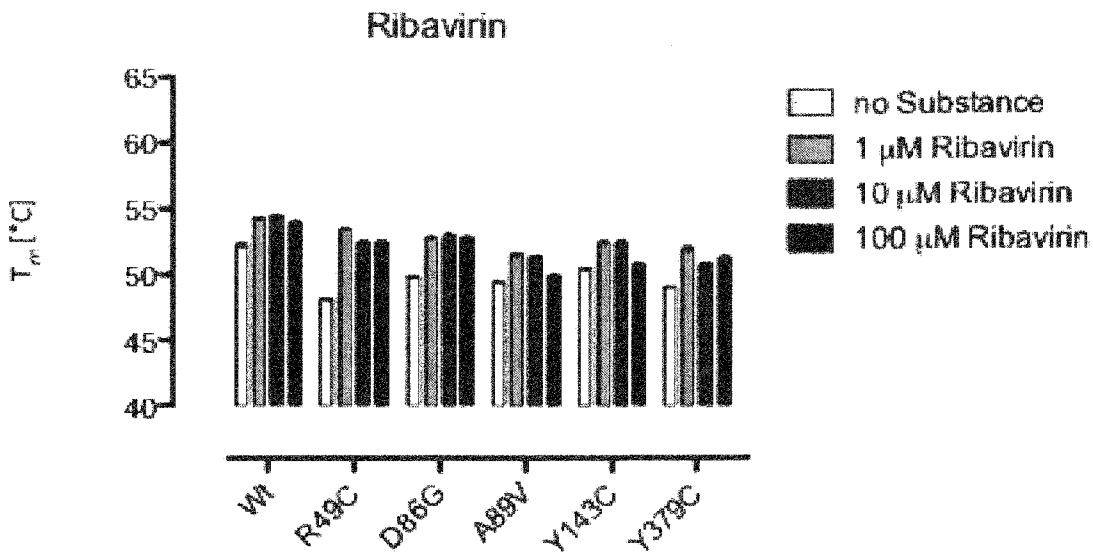

Ribavirin
65
60
55
50
45
40
T_m [°C]
no Substance
1 µM Ribavirin
10 µM Ribavirin
100 µM Ribavirin
WT
R49C
D86G
A89V
Y143C
Y379C

Fig. 5

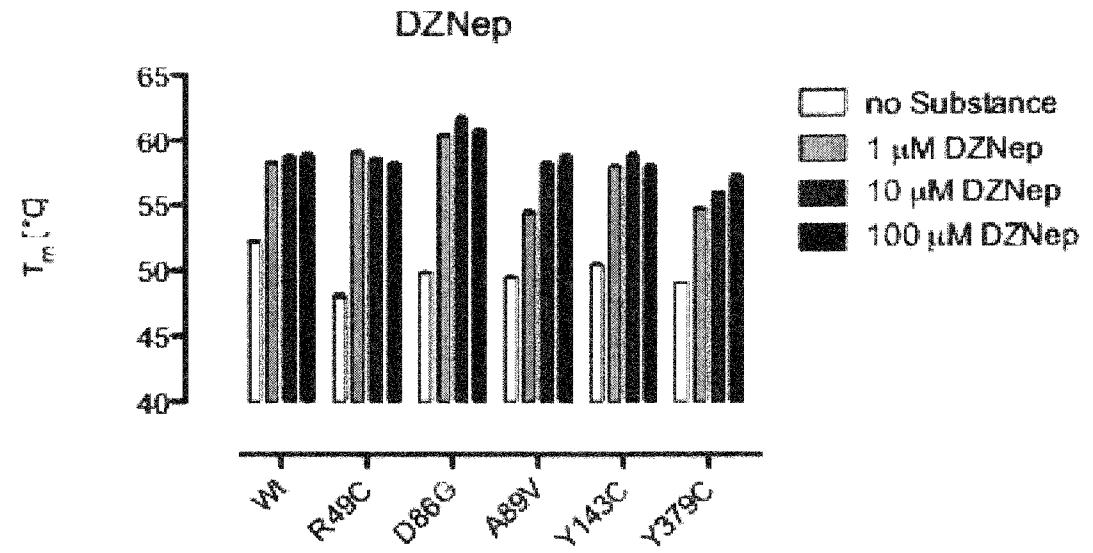

DZNep
65
60
55
50
45
40
T_m [°C]
no Substance
1 µM DZNep
10 µM DZNep
100 µM DZNep
WT
R49C
D86G
A89V
Y143C
Y379C

Fig. 6

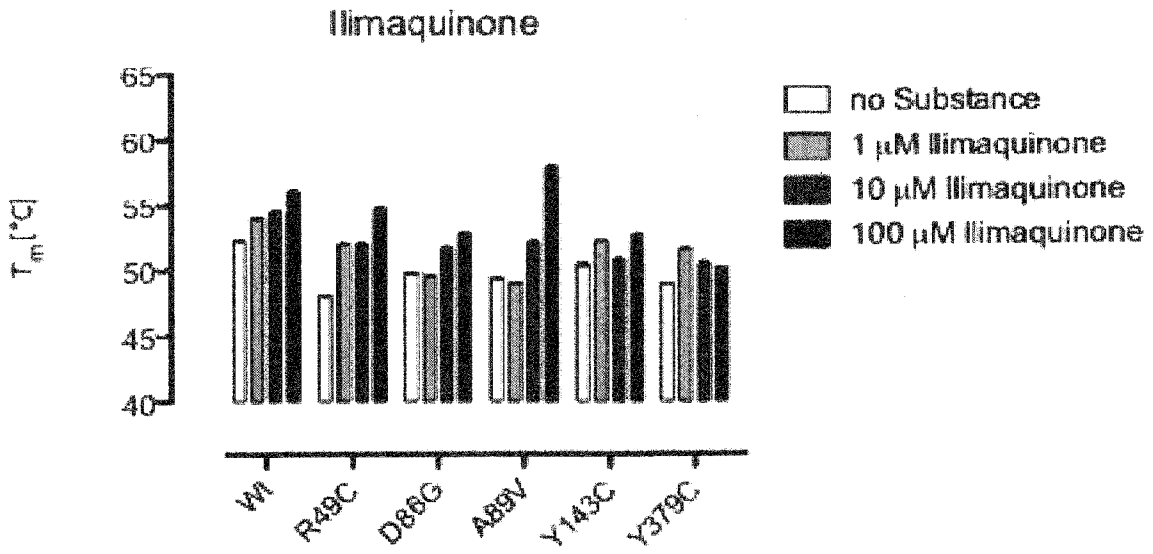

Ilimaquinone
65
60
55
50
45
40
T_m [°C]
no Substance
1 µM Ilimaquinone
10 µM Ilimaquinone
100 µM Ilimaquinone
Wt
R49C
D88G
A89V
Y143C
Y379C

Fig. 7

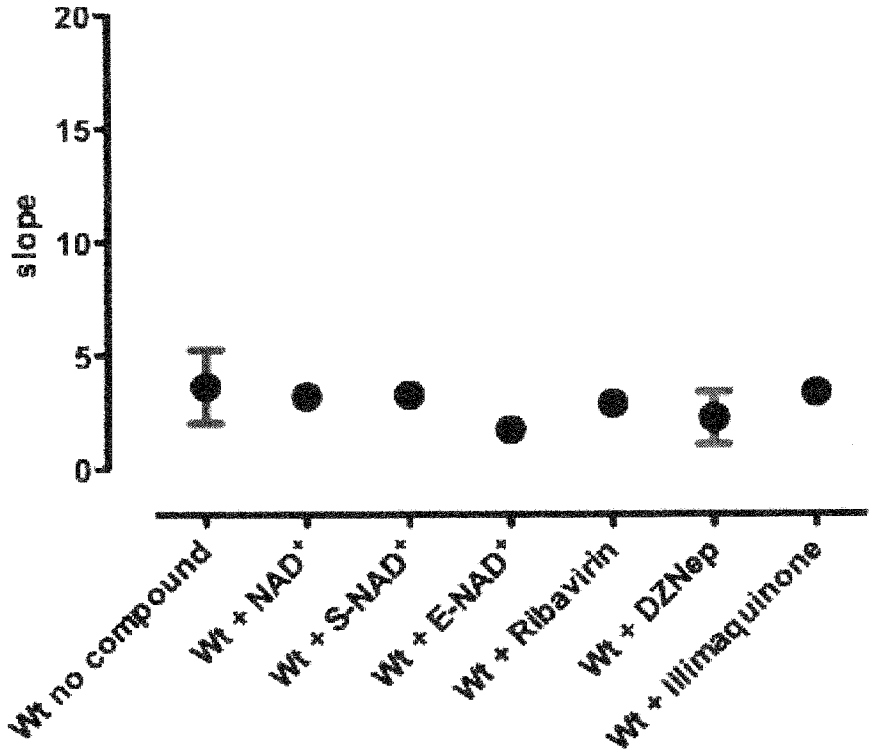

20
15
10
5
0
slope
Wt no compound
Wt + NAD+
Wt + S-NAD+
Wt + E-NAD+
Wt + Ribavirin
Wt + DZNep
Wt + Ilimaquinone

Fig. 8a

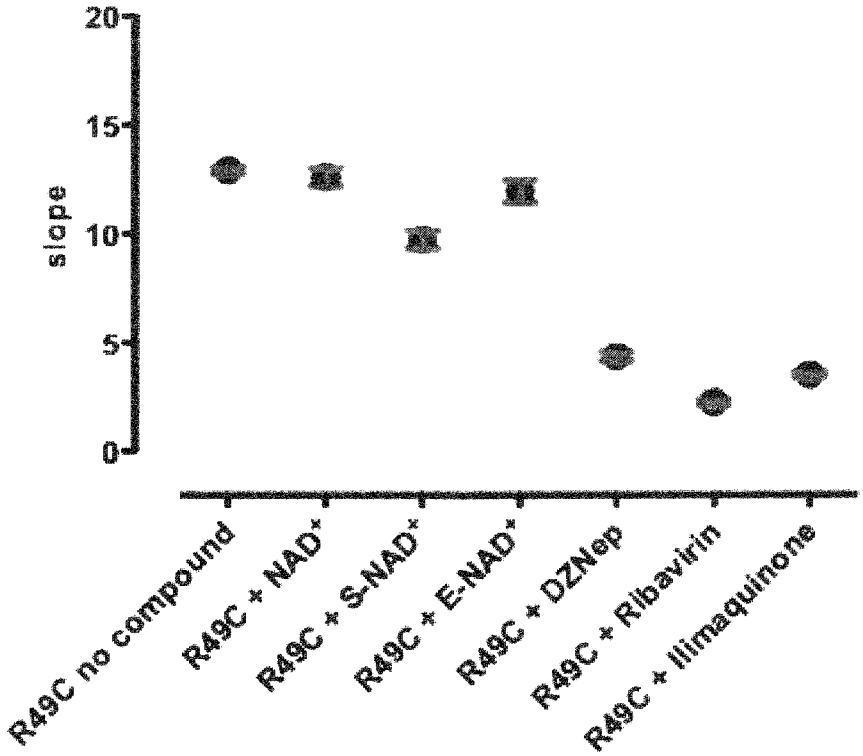

20
15
10
5
0
slope
R49C no compound
R49C + NAD+
R49C + S-NAD+
R49C + E-NAD+
R49C + DZNep
R49C + Ribavirin
R49C + Ilimaquinone

Fig. 8b

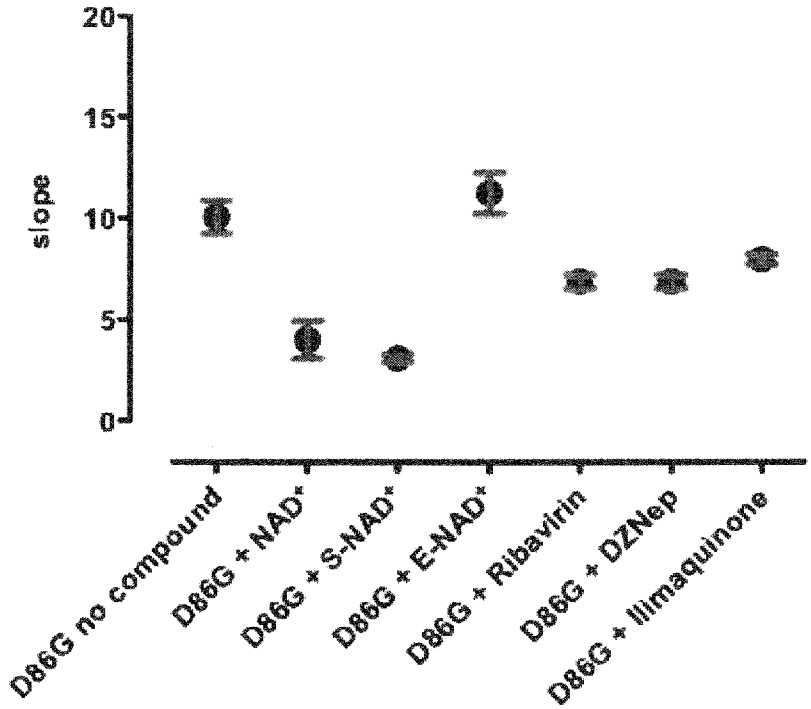

20
15
10
5
0
slope
D86G no compound
D86G + NAD+
D86G + S-NAD+
D86G + E-NAD+
D86G + Ribavirin
D86G + DZNep
D86G + Ilimaquinone

Fig. 8c

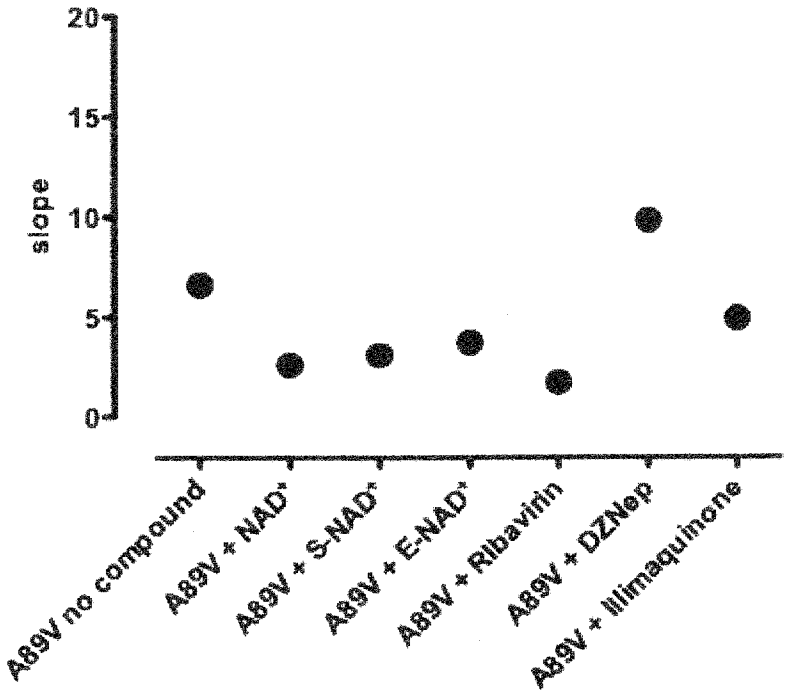

20
15
10
5
0
slope
A89V no compound
A89V + NAD+
A89V + S-NAD+
A89V + E-NAD+
A89V + Ribavirin
A89V + DZNep
A89V + Ilimaquinone

Fig. 8d

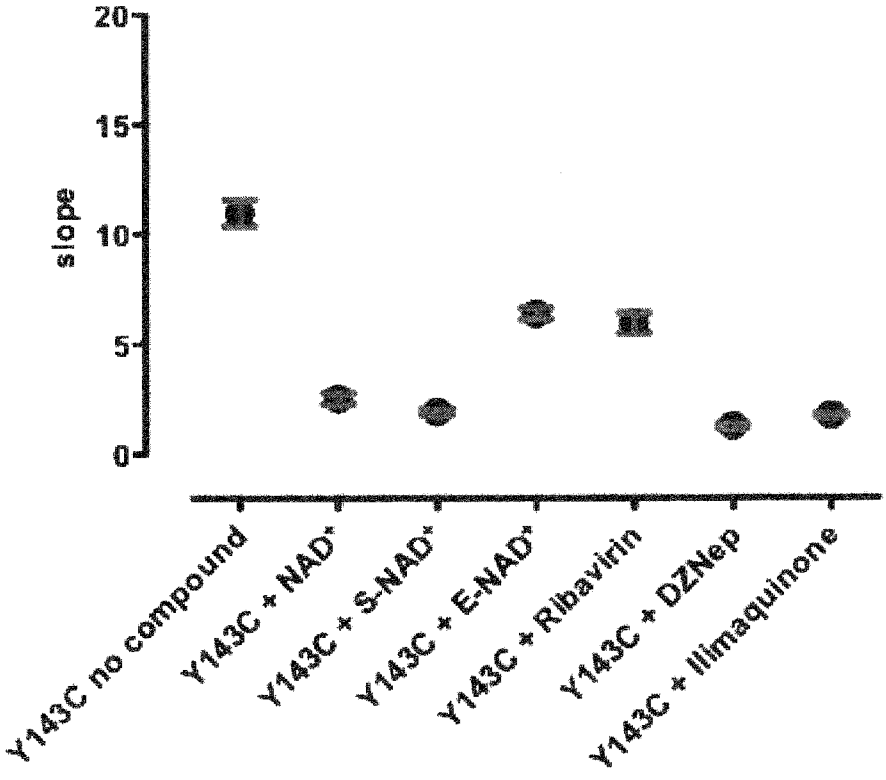

20
15
10
5
0
slope
Y143C no compound
Y143C + NAD+
Y143C + S-NAD+
Y143C + E-NAD+
Y143C + Ribavirin
Y143C + DZNep
Y143C + Ilimaquinone

Fig. 8e

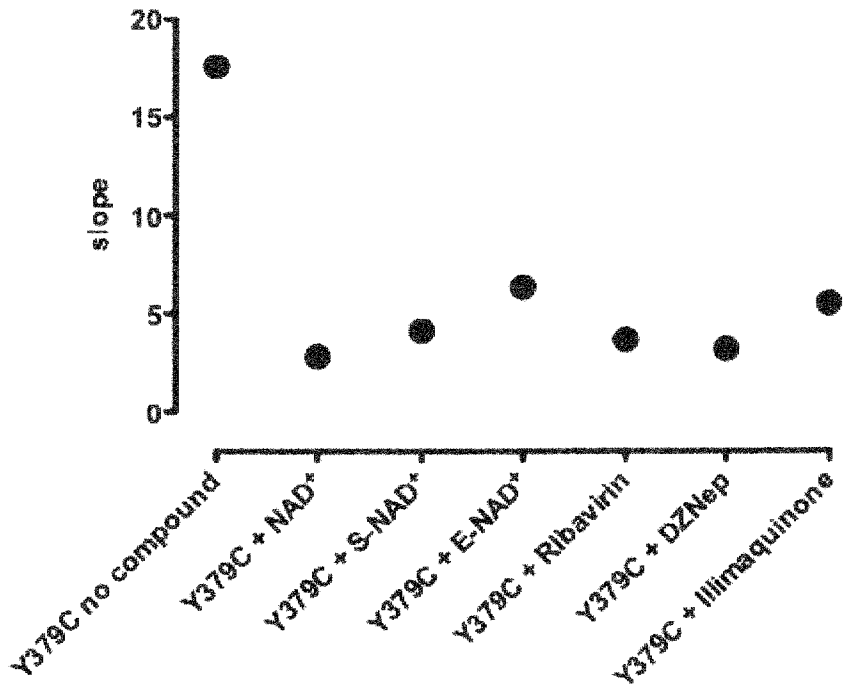

20
15
10
5
0
slope
Y379C no compound
Y379C + NAD+
Y379C + S-NAD+
Y379C + E-NAD+
Y379C + Ribavirin
Y379C + DZNep
Y379C + Ilimaquinone

Fig. 8f

AHCY–Wild-Type

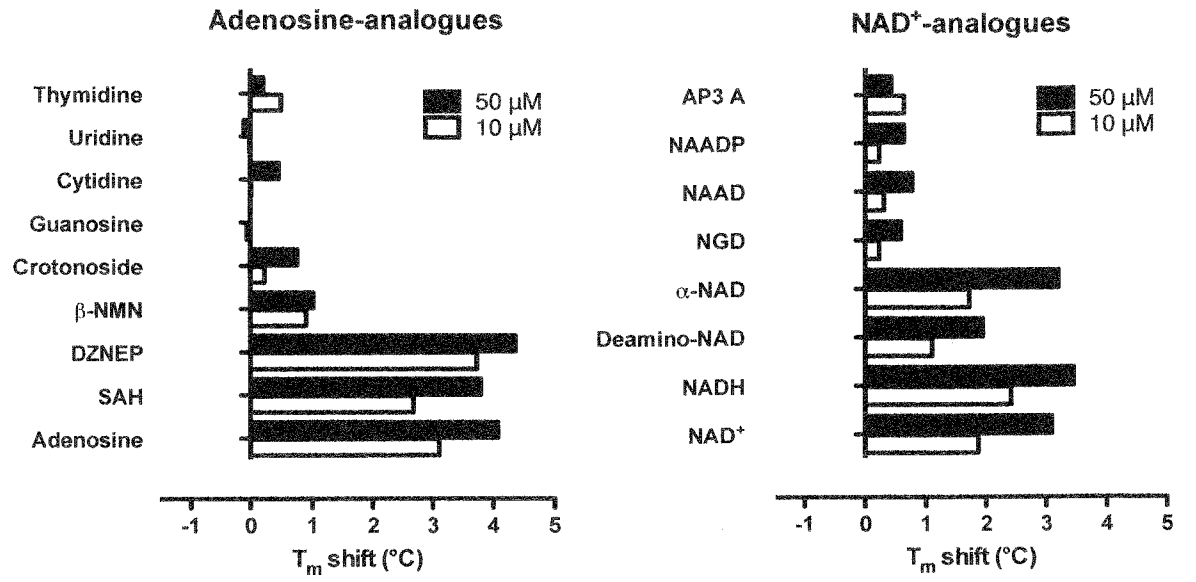


Fig. 9a

AHCY–D86G

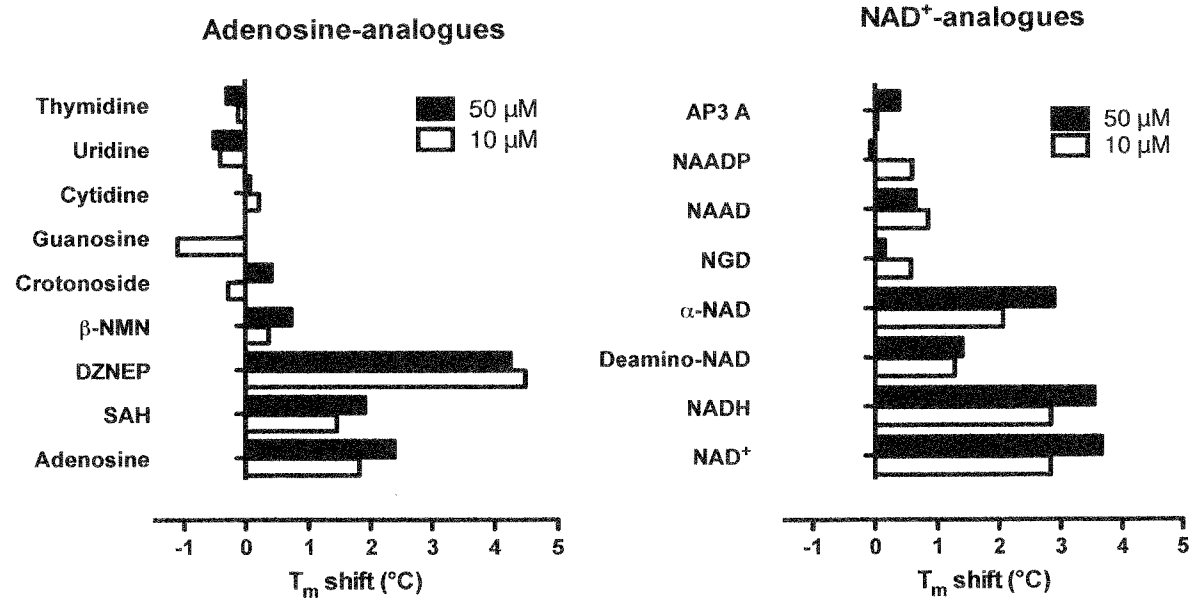


Fig. 9b

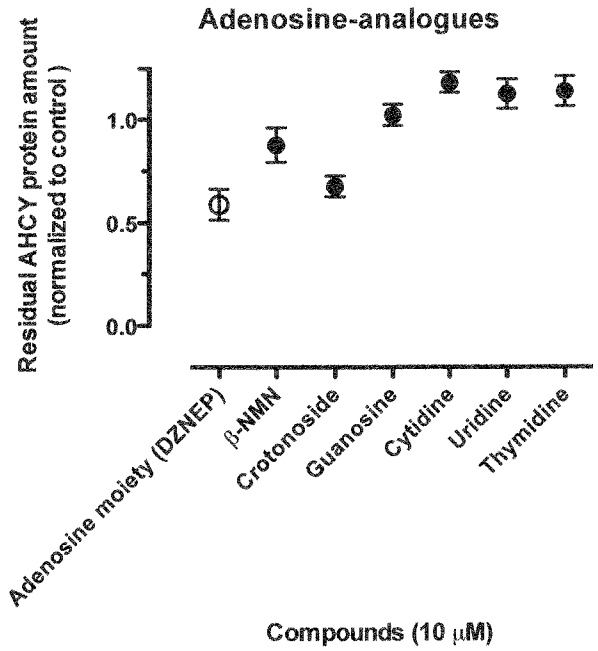


Fig. 10a

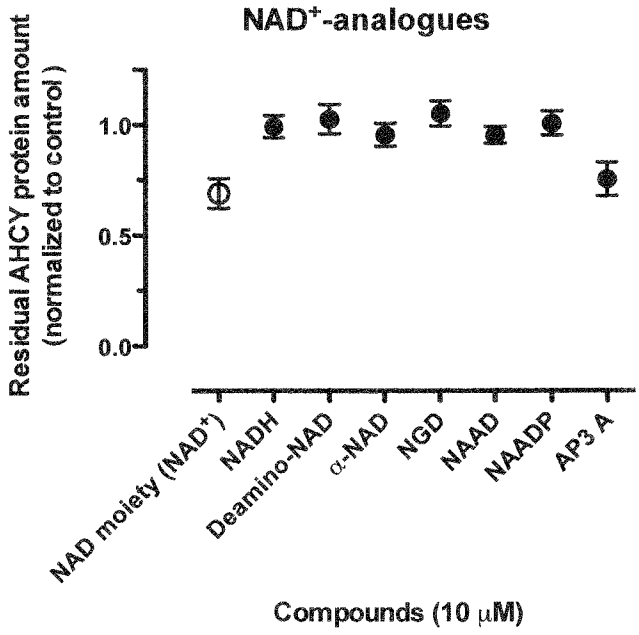


Fig. 10b

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/082636

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/122 A61K31/7056 A61K31/706 A61K31/7068 A61K31/7072
A61K31/7076 A61K31/708 A61K31/7084 A61P43/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2010/052510 A1 (RUDJER BOSKOVIC INST [HR]; CINDRIC MARIO [HR]; KRALJEVIC PAVELIC SANDR) 14 May 2010 (2010-05-14) page 1, line 13 - page 2, line 3 ----- -/--	1-8

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 2017	08/03/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Albrecht, Silke

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROBERT BELUZIC ET AL: "A single mutation at Tyr 143 of human S -adenosylhomocysteine hydrolase renders the enzyme thermosensitive and affects the oxidation state of bound cofactor nicotinamide-adenine dinucleotide", BIOCHEMICAL JOURNAL, vol. 400, no. 2, December 2006 (2006-12), pages 245-253, XP055276845, GB ISSN: 0264-6021, DOI: 10.1042/BJ20060749 cited in the application the whole document, in particular figure 5 and page 252, column 1, paragraph 2	1-8
A	IVO BARIC: "Inherited disorders in the conversion of methionine to homocysteine", JOURNAL OF INHERITED METABOLIC DISEASE, KLUWER ACADEMIC PUBLISHERS, DO, vol. 32, no. 4, 7 July 2009 (2009-07-07), pages 459-471, XP019727835, ISSN: 1573-2665, DOI: 10.1007/S10545-009-1146-4 cited in the application the whole document	1-8
A	BELUZIC R ET AL: "S-Adenosylhomocysteine hydrolase (AdoHcyase) deficiency: Enzymatic capabilities of human AdoHcyase are highly effected by changes to codon 89 and its surrounding residues", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 368, no. 1, 28 March 2008 (2008-03-28), pages 30-36, XP027016408, ISSN: 0006-291X, DOI: 10.1016/J.BBRC.2008.01.042 [retrieved on 2008-01-22] cited in the application the whole document	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/082636

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010052510 A1	14-05-2010	HR P20080563 A2	31-08-2010
		WO 2010052510 A1	14-05-2010