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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 February 2022 (24.02.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/036411 A1

(51) International Patent Classification:
C07K 7/08 (2006.01) *A61K 38/10* (2006.01)
C07K 14/16 (2006.01) *A61P 9/10* (2006.01)
A61K 38/00 (2006.01)

(21) International Application Number:
PCT/AU2021/050928

(22) International Filing Date:
20 August 2021 (20.08.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2020902986 21 August 2020 (21.08.2020) AU

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2022/036411 A1

(54) Title: PEPTIDES FOR USE IN THE TREATMENT OR PREVENTION OF MYOCARDIAL DAMAGE

(57) Abstract: The present invention relates to peptides which bind the L-type Ca2+ channel. The present invention also relates to methods to treat, prevent or ameliorate the effects of myocardial damage. The present invention also relates to pharmaceutical compositions comprising said peptides.

PEPTIDES FOR USE IN THE TREATMENT OR PREVENTION OF MYOCARDIAL DAMAGE

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TECHNICAL FIELD

[0001] The present invention relates to peptides which bind the L-type Ca^{2+} channel and use thereof to prevent and treat myocardial damage, including cardiac hypertrophy. The invention provides methods to treat, prevent or ameliorate the effects of myocardial damage, by administration of peptides and therapeutic compositions comprising peptides.

BACKGROUND ART

[0002] The following discussion of the background art is intended to facilitate an understanding of the present invention only. The discussion is not an acknowledgement or admission that any of the material referred to is or was part of the common general knowledge as at the priority date of the application.

[0003] Pathological cardiac hypertrophy (thickening of the heart muscle) may arise in a human or another animal as a response to stress; disease such as hypertension; heart muscle injury including myocardial infarction; neurohormones; or pollution causing hypoxia due to atmospheric carbon monoxide.

[0004] Familial hypertrophic cardiomyopathy (HCM) is an inherited heart condition characterised by cardiac hypertrophy that affects an estimated 1 in 200 people worldwide (*J. Am. Coll. Cardiol.* 65 (12) (2015) 1249–1254). It is the leading cause of sudden cardiac death in people under 40 years of age. Drug therapy and surgical interventions such as septal myectomy are used to manage symptoms in patients suffering from HCM.

[0005] Certain genetic mutations are associated with the development of HCM. Cardiac troponin (cTn) is a sarcomeric protein complex that consists of three subunits (cTnT, cTnI and cTnC), and plays a critical role in regulating cardiac contraction and relaxation. The entire cTn complex is anchored to tropomyosin via TnT. TnI regulates contraction in response to changes in intracellular calcium. During the relaxed state, TnI inhibits actin-myosin interaction. When calcium binds to TnC, TnI undergoes a conformational change that allows actin-myosin interaction, and as a result, contraction.

[0006] Mutations in the cTnI gene TNNI3 account for approximately 3-5% of genotyped families with HCM. Human HCM causing cTnI mutation Gly203Ser is characterized by apical and ventricular hypertrophy, and in some cases supraventricular and ventricular arrhythmias. In addition, HCM is characterized by myocyte remodelling, myofibril disarray and altered energy metabolism. Mutations in the MYH7 gene, specifically the alphaMHCArg403Ser mutation, is also known to account for approximately 40% of HCM cases.

[0007] Drug therapy used to manage the symptoms of people with HCM may include calcium channel antagonists, β-blockers, calcium channel blockers, amiodarone (Pacerone) or disopyramide (Norpace). Diltiazem, a calcium channel blocker, is used to treat symptoms of HCM such as angina, and heart arrhythmias. However, these drugs can cause negative inotropic (contractile) effects and a decrease in blood pressure leading to heart failure.

[0008] Antiarrhythmic drug therapy includes amiodarone, disopyramide, angiotensin receptor blockers, propafenone, angiotensin-converting enzyme (ACE) inhibitors and perhexilline. These drugs are ineffective in the prevention of arrhythmias. While surgical insertion of an implantable cardioverter defibrillator therapy can prevent sudden death, the socioeconomic and psychological cost to the patient is substantial.

[0009] While these treatments can assist in managing the symptoms of cardiac hypertrophy and HCM, no pharmacological treatment currently exists that can reverse or prevent the development of cardiac hypertrophy in patients.

[0010] Cardiac hypertrophy also presents in patients that do not suffer from HCM. Patients that present with cardiac hypertrophy as a result of complete or partial occlusion of a coronary artery are commonly treated with reperfusion therapy, for example using thrombolytic therapy, percutaneous coronary intervention (PCI), or bypass surgery. However, following reperfusion therapy, when blood supply returns to cardiac tissue after a period of ischemia, reperfusion injury can result. The absence of nutrients and oxygen from blood during the period of ischemia causes a condition in which restoration of circulation results in inflammation and oxidative damage. This occurs as a result of the increase in oxidative stress rather than restoration of normal function.

L-Type Calcium Channels

[0011] The long- or L-type Ca^{2+} channel is the main route for calcium influx into cardiac myocytes producing the invaluable muscle contraction for the pumping heart. Increases in intracellular calcium and oxidative stress are involved in the pathophysiology of cardiac hypertrophy with increased influx through the L-type Ca^{2+} channel or over-expression of the alpha subunit of the channel inducing the hypertrophy.

[0012] It is understood that the primary structure of the pore-forming L-type Ca^{2+} channel alpha-1 (α_1) subunit is composed of 4 homologous repeating motifs (I–IV), each of which consists of 6

putative transmembrane segments (S1–S6) (Figure 1). Cytoplasmic loops between the transmembrane segments are named according to the motifs they link. There are also $\alpha_{2,}$ δ and γ subunits which are extracellular subunits linked to the alpha subunit via a disulphide bridge, and the L-type Ca^{2+} channel beta-2 (β_2) subunit which is entirely intracellular. The structure of the β_2 subunit may be expressed as any of four beta subunit isoforms (β_1–β_4). All isoforms are hydrophilic, nonglycosylated, and intracellular with no membrane-spanning region. The β_2 isoform is tightly bound to a highly conserved motif in the cytoplasmic linker between repeats I and II of all cloned high voltage–activated α_1 subunit isoforms, called the alpha-interaction domain (AID).

[0013] The L-type Ca^{2+} channels also play an important role in regulating mitochondrial function, and this involves both calcium-dependent and calcium-independent mechanisms. Activation of the L-type Ca^{2+} channel with voltage-clamp of the plasma membrane or with DHP receptor agonist BayK(-) is sufficient to increase intracellular and mitochondrial calcium, NADH production, superoxide production and metabolic activity in wt cardiac myocytes, in a calcium-dependent manner. Activation of the L-type Ca^{2+} channel also causes an increase in mitochondrial membrane potential (Ψm), in a calcium-independent manner. This response is attenuated in the presence of F-actin depolymerizing agents, indicating that the response is in part dependent on an interaction between the L-type Ca^{2+} channel and mitochondria, via F-actin. The L-type Ca^{2+} channel influences mitochondrial function through a structural-functional communication between the L-type Ca^{2+} channel and mitochondria via the cytoskeletal network, following conformational changes in L-type Ca^{2+} channel that occur on a beat-to-beat basis.

[0014] Patients with HCM exhibit altered communication between the L-type Ca^{2+} channel and mitochondria and altered metabolic activity. Specifically, cTnI-G203S myocytes exhibit a faster L-type Ca^{2+} channel inactivation rate, and increased Ψm and mitochondrial metabolic activity (consistent with the human condition) in response to activation of the L-type Ca^{2+} channel. These alterations also occur in myocytes isolated from hearts of cTnI-G203S mice that have not yet developed the cardiomyopathy, indicating that alterations in the L-type Ca^{2+} channel kinetics and metabolic activity precede development of the cardiomyopathy.

[0015] The alpha subunit of the channel has been the target of a number of therapies which aim to protect the cardiac muscle during reperfusion and calcium overload. Examples of these therapies include monoclonal antibodies to the alpha subunit; Ca2+ channel antagonists such as the Dihydropyridines, the Benzothiazepines and the Phenylalkylamines. Although Ca2+ channel blockers bind specifically to regions of the α1C subunit of the L-type Ca2+ channel, these drugs have been found to have limited success because they can induce myocardial depression and heart failure.

AID-Peptide

[0016] In WO/2013/113060, the inventors made the observation that restricting the movement of the β_2 subunit of the L-type Ca^{2+} channel with a peptide derived against AID prevented interaction of β_2 and α_1 subunits. The inventors hypothesised in WO/2013/113060 that the β-interaction domain (BID) interacts with the AID through a conserved hydrophobic cleft, termed the alpha-binding pocket (ABP). The I–II loop of the α_1 subunit contains an endoplasmic reticulum retention signal that restricts cell surface expression. The β_2 subunit reverses the inhibition imposed by the retention signal and is able to modulate the biophysical properties of the L-type Ca^{2+} channels α_1 subunit, producing a leftward shift of the current-voltage relationship, which is consistent with the involvement of the S4 region of the α_1 subunit voltage-sensor region.

[0017] As set out in WO/2013/113060, the inventors developed a peptide of sequence QQLEEDLKGYLDWITQAE (SEQ ID NO: 1), (AID peptide) that interacts with the BID of a L-type Ca^{2+} channel β_2 subunit in a heart muscle cell, thereby preventing interaction of that β_2 subunit with the AID of an L-type Ca^{2+} channel α_1 subunit. The peptide-bound β_2 subunit is unable to reverse the inhibition on the α_1 subunit imposed by the endoplasmic reticulum retention signal which restricts cell surface expression of the α_1 subunit. This results in a restriction on the activation of the L-type Ca^{2+} channel and decreases mitochondrial energy consumption.

[0018] However, there remains a need for the development of additional peptides with improved potency in disrupting the interaction of the β_2 subunit with the AID of an L-type Ca^{2+} channel in order to effectively treat or prevent cardiac hypertrophy.

SUMMARY OF INVENTION

[0019] Inventors have found that the activity of the AID peptide is dependent on the composition of the peptide. In particular, the inventors have identified that limited substitution of the third and seventh amino acid in the AID peptide sequence has an unexpected impact on the disruptive capacity that this peptide creates in the interaction of the β_2 subunit with the AID of an L-type Ca^{2+}.

[0020] Accordingly, in a first aspect, the invention provides a peptide comprising the amino acid sequence:

$QQX_1EEDX_2KGYLDWITQAE$ (SEQ ID NO: 2)

wherein X_1 is an amino acid selected from the group comprising: Q, E or R; and
wherein X_2 is an amino acid selected from the group comprising: L or E

or a variant thereof. Preferably, the peptide does not consist of SEQ ID NO: 1.

[0021] Preferably, the invention provides a peptide comprising the amino acid sequence:

$QQX_1EEDX_2KGYLDWITQAE$

wherein X_1 is an amino acid selected from the group comprising: Q, E or R; and

wherein X_2 is an amino acid selected from the group comprising: L or E;

or a variant thereof, wherein the peptide does not consist of SEQ ID NO: 1.

[0022] Preferably, the peptide binds to the BID of a L-type Ca^{2+} channel β_2 subunit. More preferably, the peptide binds to the BID of a human L-type Ca^{2+} channel β_2 subunit.

[0023] Preferably, the invention provides a peptide comprising any one of the amino acid sequences of SEQ ID NO: 4 – 7 or a variant thereof, wherein the peptide does not consist of SEQ ID NO: 1. Preferably, the peptide binds to the BID of a L-type Ca^{2+} channel β_2 subunit. More preferably, the peptide binds to the BID of a human L-type Ca^{2+} channel β_2 subunit.

[0024] In a second aspect, the invention provides a peptide comprising: a peptide portion comprising the amino acid sequence of SEQ ID NO: 2; and a peptide portion comprising the amino acid sequence of:

RKKRRQRRRZaa (SEQ ID NO: 3)

wherein Zaa is a 6-amino hexanoic acid

or a variant thereof. Preferably, the peptide binds to the BID of a L-type Ca^{2+} channel β_2 subunit. More preferably, the peptide binds to the BID of a human L-type Ca^{2+} channel β_2 subunit.

[0025] Preferably, the peptide of the invention comprises an amino acid sequence selected from the group of at least 75%; at least 80%; at least 85%; at least 90%; at least 95%; at least 96%; at least 97%; at least 98%; and at least 99% sequence homology to a peptide comprising SEQ ID NO: 2. More preferably, the peptide of the invention comprises an amino acid sequence comprising one or more conservative amino acid substitutions to SEQ ID NO: 2 selected from the suitable amino acid substitutions set out in Table 3.

[0026] In a third aspect, the invention provides a method for modulating movement of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject, comprising administering to the subject a peptide of the invention.

[0027] In a fourth aspect, the invention provides a method for modulating binding of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject, comprising administering to the subject a peptide of the invention.

[0028] Preferably, the peptide prevents interaction between the beta subunit of the L-type Ca^{2+} channel and an alpha subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject and therefore activation of the channel.

[0029] In a fifth aspect, the invention provides a method for modulating a L-type Ca^{2+} channel in a cardiac cell of a subject, comprising the step of administering to the subject a peptide of the invention.

[0030] In a sixth aspect, the invention provides a method for treating, preventing or reducing myocardial damage and/or oxidative stress in the heart of a subject, comprising the step of administering to the subject a peptide of the invention.

[0031] Preferably the myocardial damage comprises cardiac hypertrophy. Preferably, cardiac hypertrophy and/or oxidative stress is reduced. More preferably, intracellular Ca^{2+} levels in a cardiac cell in the heart of the subject are slightly reduced or substantially maintained in a cardiac cell in the heart of the subject. The subject is preferably a mammal and is more preferably a human. Most preferably, the subject suffers from hypertrophic cardiomyopathy.

[0032] In a seventh aspect, the invention provides a method for treating or preventing cardiac hypertrophy in a subject comprising the step of administering to the subject a peptide of the invention. Preferably, the subject suffers from hypertrophic cardiomyopathy.

[0033] In an eighth aspect, the invention provides a use of a peptide of the invention for modulating movement of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell in the heart of a subject.

[0034] In a ninth aspect, the invention provides a use of a peptide of the invention for modulating a L-type Ca^{2+} channel in a cardiac cell of a subject.

[0035] In a tenth aspect, the invention provides a use of a peptide of the invention for treating, preventing or reducing myocardial damage and/or oxidative stress in the heart of a subject. Preferably, the use is during and/or following reperfusion.

[0036] In an eleventh aspect, the invention provides a polynucleotide encoding a peptide of the invention described herein.

[0037] In a twelfth aspect, the invention provides the use of the peptide of the invention for the manufacture of a medicament to treat, prevent, or ameliorate myocardial damage and/or oxidative stress in the heart of a subject. Preferably, the myocardial damage is cardiac hypertrophy.

[0038] In a thirteenth aspect, the invention provides a pharmaceutical, prophylactic or therapeutic composition comprising the peptide of the invention; and one or more pharmaceutically acceptable carriers and/or diluents.

[0039] In a fourteenth aspect, the invention provides a method for slowing the progression of myocardial fibrosis in a subject comprising the step of administering to the subject a peptide of the invention.

[0040] In a fifteenth aspect, the invention provides the use of a peptide of the invention for slowing the progression of myocardial fibrosis in a subject

[0041] In a sixteenth aspect, the invention provides a kit to treat, prevent or ameliorate the effects of myocardial damage and/or oxidative stress in the heart of a subject, wherein the kit comprises at least a peptide of the invention, packaged in a suitable container, together with instructions for its use.

[0042] In a seventeenth aspect, the invention provides a use of a peptide of the invention for modulating binding to a L-type Ca^{2+} channel alpha-interacting domain in a cardiac cell of a subject.

[0043] In an eighteenth aspect, the invention provides the use of the peptide of the invention for the manufacture of a medicament to treat, prevent, or ameliorate reperfusion injury in the heart of a subject. .

[0044] Further features of the present invention are more fully described in the following description of several non-limiting embodiments thereof. This description is included solely for the purposes of exemplifying the present invention. It should not be understood as a restriction on the broad summary, disclosure or description of the invention as set out above.

BRIEF DESCRIPTION OF THE FIGURES

[0045] The description will be made with reference to the accompanying drawings.

[0046] Figure 1 presents an illustration of the primary structure of the pore-forming L-type Ca^{2+} channel alpha-1 (α_1) subunit which is composed of 4 homologous repeating motifs (I–IV), each of which consists of 6 putative transmembrane segments (S1–S6). $\alpha_2\delta$ consists of a transmembrane protein (δ) and extracellular α_2 protein linked via a disulfide bond (S-S). β_2 is an intracellular protein bound to the linker between motifs 1 and 2 of α_{1C} via the α-interacting domain (AID).

[0047] Figure 2 presents the results of a competitive binding assay demonstrating mean binding affinity ($K_{0.5}$) of each mutant peptide, the full-length AID peptide and diltiazem for the β subunit.

[0048] Figure 3 illustrates the effect of mutant peptides on oxidative stress responses in wild type cardiac myocytes.

[0049] Figure 4 presents the effect of mutant peptides on flavoprotein oxidation, a measure of metabolic activity and oxygen consumption in wt myocytes.

[0050] Figure 5 presents the effect of mutant peptides on flavoprotein oxidation, a measure of metabolic activity and oxygen consumption in myocytes isolated from cTnI-G203S mutant mouse hearts that are hypertrophic.

[0051] Figure 6 presents the effect of mutant peptides on the release of CK and LDH (indication of necrosis), and oxidative stress measured as the ratio of reduced glutathione to oxidised glutathione (GSH:GSSG) in hearts exposed to no-flow ischemia for 20 min *ex vivo*.

[0052] Figure 7 presents a table of sequences referenced in this application.

[0053] Figure 8 presents the echocardiographic parameters of cTnI-G203S mice administered with 10 μM AID(S)-TAT or AID-TAT.

[0054] Figure 9 presents the results of a competitive binding assay demonstrating mean binding affinity ($K_{0.5}$) of each mutant peptide, the full-length AID peptide and diltiazem for the β subunit following exposure to 0, 10 nM, 100 nM, 1 μM, 10 μM and 100 μM of peptide.

[0055] Figure 10 illustrates that the *in vitro* exposure of *cTnI.2* (wt) and *cTnI-G203S* (mutant) cardiomyocytes to variant peptides are more efficacious at restoring metabolic activity than original AID-TAT assessed as changes in flavoprotein oxidation.

[0056] Figure 11 illustrates that the *in vitro* exposure of *cTnI.2* (wt) and *cTnI-G203S* (mutant) cardiomyocytes to variant peptides are more efficacious at restoring metabolic activity than original AID-TAT assessed as changes in JC-1 fluorescence.

[0057] Figure 12 illustrates that the *in vivo* treatment of *cTnI-G203S* (mutant) mice with AID-TAT variant peptides does not alter blood pressure.

[0058] Figure 13 illustrates that the *in vivo* treatment of *cTnI-G203S* mice with AID-TAT variant peptides are not toxic.

[0059] Figure 14 illustrates that the *in vivo* treatment of *cTnI-G203S* mice with AID-TAT variant peptides slows the progression of fibrosis.

[0060] Figure 15 illustrates the echocardiographic parameters of mice exposed to 10 μM AID(S), 10 μM AID-TAT, 5 μM AID_{P7}-TAT, 5 μM AID_{P14}-TAT, 5 μM AID_{P15}-TAT or 5 μM AID_{P16}-TAT.

[0061] Figure 16 illustrates that the *in vivo* treatment of *cTnI-G203S* mice with AID-TAT variant peptides slows the progression of hypertrophy.

SEQUENCE LISTING

[0062] Table 1 below sets out a list of sequences referred to in this specification:

SEQ ID NO	Name	Sequence
SEQ ID NO: 1	AID Peptide	QQLEEDLKGYLDWITQAE
SEQ ID NO: 2	Variant AID Peptide	$QQX_1EEDX_2KGYLDWITQAE$

SEQ ID NO	Name	Sequence
SEQ ID NO: 3	TAT Peptide	RKKRRQRRRZaa
SEQ ID NO: 4	Variant P7	QQQEEDLKGYLDWITQAE
SEQ ID NO: 5	Variant P14	QQEEEDLKGYLDWITQAE
SEQ ID NO: 6	Variant P15	QQEEEDEKGYLDWITQAE
SEQ ID NO: 7	Variant P16	QQREEDLKGYLDWITQAE
SEQ ID NO: 8	Nuclear Localisation Signal	GRKKR
SEQ ID NO: 9	Protein transduction domain	YGRKKRRQRRR
SEQ ID NO: 10	AID[S] Peptide	QKILGEWDLAQYTDQELE
SEQ ID NO: 11	Variant P1	DLKGYLDWITQAE
SEQ ID NO: 12	Variant P4	LEEDLKGYLDWITQAE
SEQ ID NO: 13	Variant P6	KGYLDWITQAE
SEQ ID NO: 14	Variant P10	DLKGYLDWITQA
SEQ ID NO: 15	Variant P11	DLKGYLDWITQ
SEQ ID NO: 16	Variant P12	DLKGYLDWIT
SEQ ID NO: 17	Variant P13	QQLEEDLKGFLDAATQAE

DESCRIPTION OF EMBODIMENTS

[0063] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the

steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0064] The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

[0065] The entire disclosures of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference. No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

[0066] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0067] Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

[0068] The invention described herein may include one or more range of values (for example, size, displacement and field strength etc.). A range of values will be understood to include all values within the range, including the values defining the range, and values adjacent to the range which lead to the same or substantially the same outcome as the values immediately adjacent to that value which defines the boundary to the range.

[0069] Features of the invention will now be discussed with reference to the following non-limiting description and examples.

Variant AID Peptide

[0070] The present invention provides a peptide comprising the amino acid sequence:

$QQX_1EEDX_2KGYLDWITQAE$ (SEQ ID NO: 2)

wherein X_1 is an amino acid selected from the group comprising: Q, E or R; and

wherein X_2 is an amino acid selected from the group comprising: L or E

[0071] Preferably the peptide binds to the BID of a L-type Ca^{2+} channel β_2 subunit. More preferably, the peptide binds to the BID of a human L-type Ca^{2+} channel β_2 subunit. Most

preferably, the peptide binds to the BID of a L-type Ca^{2+} channel β_2 subunit with greater affinity compared with SEQ ID NO: 1.

[0072] The present invention provides an isolated peptide comprising variants to the highly conserved AID motif of the human L-type Ca^{2+} channel α_1 subunit (SEQ ID NO:1).

[0073] Surprisingly, the inventors have identified a number of variants to specific amino acids in the original AID-peptide that improve the binding affinity of the peptide to BID of a L-type Ca^{2+} channel β_2 subunit.

[0074] In an aspect, the improvements in binding affinity result in improved functionality of the peptide *in vitro* and/or *in vivo*. In an aspect, the peptides of the invention can further slow the inactivation rate of the L-type Ca^{2+} channel in comparison to the original-AID peptide. In another aspect, the peptides of the invention can be administered at a lower dose to the subject in comparison to the original-AID peptide to produce the same effect *in vitro* or *in vivo*.

[0075] The inventors have identified specific variations at positions X_1 and X_2 are effective at increasing the binding affinity of the peptide to BID in comparison with the original-AID peptide. For example, the original AID-peptide has hydrophobic amino acid leucine at position X_1, but in some embodiments, variations at position X_1 to a negative (e.g. glutamic acid), positive (e.g. arginine) or polar uncharged (e.g. glutamine) amino acids, each increase binding affinity of the peptide to BID in comparison with the original-AID peptide.

[0076] Binding affinity of the peptides of the invention to the BID can be measured by a number of techniques that are well known in the art, including SDS-PAGE assays.

[0077] Preferably, the peptide is provided in a pharmaceutically acceptable form.

[0078] Preferably the peptide is not QQLEEDLKGYLDWITQAE (SEQ ID NO: 1).

[0079] Preferably the peptide is not toxic. Most preferably, the peptide does not exhibit kidney toxicity and/or liver toxicity. Kidney toxicity can be measured by methods known in the art, including by assessing urea and creatinine concentrations using the Quantichrom Urea assay kit (BioAssay Systems, Hayward, CA) and Quantichrom Creatinine assay kit (BioAssay Systems, Hayward, CA), respectively. Liver toxicity can be measured by methods known in the art including by assessing alanine transaminase (ALT) and aspartate transaminase (AST) concentrations using the Alanine Transaminase assay kit (BioAssay Systems, Hayward, CA) and Aspartate Transaminase assay kit (BioAssay Systems, Hayward, CA), respectively.

[0080] The present invention further and more preferably provides a peptide comprising the amino acid sequence of SEQ ID NO: 4 – 7 as set out in the table below:

Table 2:

SEQ ID NO	Peptide	Amino Acid Sequence
SEQ ID NO: 4	Variant P7	QQQEEDLKGYLDWITQAE
SEQ ID NO: 5	Variant P14	QQEEEDLKGYLDWITQAE
SEQ ID NO: 6	Variant P15	QQEEEDEKGYLDWITQAE
SEQ ID NO: 7	Variant P16	QQREEDLKGYLDWITQAE

[0081] A peptide of the present invention may be recombinant, natural or synthetic. A peptide of the invention may be mixed with diluents, adjuvants or carriers (including nanoparticles) that will not interfere with the intended purpose of the peptide. A peptide of the invention may also be in a substantially purified form, in which case it will generally comprise the peptide in a preparation in which at least 90%, 95%, 98% or 99% of the protein in the preparation is a peptide of the invention. The term 'peptide' as used herein may be used interchangeably with the term 'polypeptide' as referring to a chain of at least two amino acid monomers.

[0082] In some embodiments, the peptides of the invention comprise one or more repeats of a peptide portion comprising SEQ ID NO:2 or a variant thereof.

Variants

[0083] The present invention further includes variants of; (1) the amino acid sequence of SEQ ID NO: 2; or (2) the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3. Preferably, the variant binds to the BID of a L-type Ca^{2+} channel β_2 subunit. More preferably, the peptide binds to the BID of a human L-type Ca^{2+} channel β_2 subunit. Most preferably, the variant binds with a higher affinity to the BID of a L-type Ca^{2+} channel β_2 subunit compared with SEQ ID NO: 1.

[0084] Preferably the variant has an amino acid sequence homology to (1) the amino acid sequence of SEQ ID NO: 2; or (2) the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3 selected from the group consisting of: at least 75% sequence homology; at least 80%; at least 85%; at least 90%; at least 95%; at least 96%; at least 97%; at least 98%; and at least 99%. Preferably, the variant is not QQLEEDLKGYLDWITQAE.

[0085] The term "% sequence homology ", as used here, may for example be calculated as follows. The query sequence is aligned to the target sequence using the CLUSTAL W algorithm (Thompson et al, Nucleic Acids Research, 22: 4673-4680 (1994)). A comparison is made over the window corresponding to one of the aligned sequences, for example the shortest. The window may in some instances be defined by the target sequence. In other instances, the window may be defined by the query sequence. The amino acid residues at each position are compared, and

the percentage of positions in the query sequence that have identical correspondences in the target sequence is reported as % sequence homology.

[0086] Variants of (1) the amino acid sequence of SEQ ID NO: 2; and (2) the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3 include a polypeptide substantially homologous to (1) the amino acid sequence of SEQ ID NO: 2; or (2) the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3 but which has an amino acid sequence different from that of (1) the amino acid sequence of SEQ ID NO: 2; or (2) the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3 sequence because one or more amino acids have been chemically modified or substituted by amino acids analogs. Preferably, any changes to the sequence to create a variant of (1) the amino acid sequence of SEQ ID NO: 2; or (2) the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3 can also include, in addition to amino acid substitutions, amino acid deletions and/or amino acid additions.

[0087] Amino acid substitutions are preferably conservative amino acid substitutions known to those skilled in the art. For example, the person skilled in the art may perform an amino acid substitution by selecting an amino acid from within the same class of amino acid that is shared with the specific amino acid that is identified for substitution. Examples of suitable amino acid substitutions are presented in Table 3 below.

Table 3

Amino acids	Examples of conservative substitutions
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln
Asp (D)	Glu
Cys (C)	Ser, Ala
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Pro, Ala
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe, Norleucine
Leu (L)	Ile, Val, Met, Ala, Phe, Norleucine
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Ile, Phe
Phe (F)	Leu, Val, Ile, Ala, Tyr
Pro (P)	Ala, Gly
Ser (S)	Thr, Ala, Cys
Trp (W)	Phe, Tyr

Amino acids	Examples of conservative substitutions
Thr (T)	Ser
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile Met, Leu, Phe, Ala, Norleucine

[0088] Peptide variants of the present invention also include fusion to further peptides, for example, where an additional peptide sequence is fused to a peptide of the invention to aid in extraction and purification. Examples of additional fusion peptide partners include glutathione-S-transferase (GST), hexahistidine, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the additional peptide partner and the peptide of the invention to allow removal of additional peptide sequences. Preferably the additional peptide will not hinder binding of the peptide of the invention to the BID of a L-type Ca^{2+} channel β_2 subunit.

AID-TAT Fusion Peptide

[0089] In a preferred form, the invention further provides a peptide comprising:

a peptide portion comprising the amino acid sequence of SEQ ID NO: 2; and

a peptide portion comprising the amino acid sequence:

RKKRRQRRRZaa (SEQ ID NO: 3)

wherein Zaa is 6-amino hexanoic acid

or a variant thereof.

[0090] The peptide portion of the peptide of the invention comprising the amino acid sequence of SEQ ID NO: 3 encodes a TAT peptide. Trans-activating transcriptional activator (TAT) from Human Immunodeficiency Virus 1 is a cell-penetrating peptide which is known in the art to deliver attached molecules such as peptides into cells. Thus, not wishing to be bound by any particular mechanism, it is believed the TAT peptide portion in the peptide of the invention facilitates transport of the peptide into cardiac cells via endocytosis or by direct translocation across the plasma membrane. The nuclear localisation signal found within the domain, GRKKR (SEQ ID NO: 8), mediates further translocation of TAT into the cell nucleus. The biological role of this domain and exact mechanism of transfer is currently unknown. The amino acid sequence of the protein transduction domain is YGRKKRRQRRR (SEQ ID NO: 9).

[0091] However, the peptide of the invention may comprise other or additional peptide portions which assist or facilitate in the transport of the peptide into cardiac or other cells, or provide some

other benefit, for example, amongst others, identifying the location of a peptide of the invention within a cell.

[0092] In some embodiments, the peptides of the invention comprise one or more repeats of a peptide portion comprising SEQ ID NO:2 and a peptide portion comprising SEQ ID NO: 3 or a variant thereof.

Polynucleotides

[0093] The present invention also provides an isolated polynucleotide encoding a peptide of the present invention as described herein including peptides comprising SEQ ID No: 2 - 7. It will be understood by a skilled person that due to the degeneracy of the amino acid code, numerous different polynucleotides can encode the same peptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the peptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any particular host organism in which the polypeptides of the invention are to be expressed.

[0094] Polynucleotides of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand. They may also be polynucleotides that include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the in vivo activity or life span of polynucleotides of the invention.

[0095] Where the polynucleotide of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the polynucleotide is single-stranded, it is to be understood that the complementary sequence of that polynucleotide is also included within the scope of the present invention.

[0096] Reference to "isolated" polynucleotide(s) means a polynucleotide, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution.

[0097] Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated peptides of the present invention further include such molecules produced synthetically.

[0098] As indicated above, the polynucleotides of the present invention that encode a peptide of the present invention include, but are not limited to, those peptides encoded by the amino acid sequences of SEQ ID No: 2 and 4-7. Rather the polynucleotides of the present invention may comprise the coding sequence for the peptides and additional sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the peptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals, for example ribosome binding and stability of mRNA; an additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Preferably, the additional sequence comprises the peptide encoded by the amino acid sequences of SEQ ID No: 3. Polynucleotides according to the present invention also include those encoding a peptide lacking the N terminal methionine.

[0099] The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode variants of the peptides of the present invention.

[00100] Such variants include those produced by nucleotide substitutions, deletions or additions that may involve one or more nucleotides. Non-naturally occurring variants may be produced using mutagenesis techniques known to those in the art. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the encoded peptide. Also especially preferred in this regard are conservative substitutions.

[00101] It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a peptide of the invention having one or more properties of the full polypeptide such as being able to interact with the BID of a L-type Ca^{2+} channel β_2 subunit. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

Vectors and Host Cells

[00102] A nucleic acid molecule encoding the amino acid sequence of a peptide of the invention may be inserted into an appropriate expression vector using standard ligation techniques. The vector is typically selected to be functional in the particular host cell employed (*i.e.*, the vector is compatible with the host cell machinery such that amplification of the nucleic acid molecule and/or expression of the nucleic acid molecule can occur). A nucleic acid molecule encoding the amino acid sequence of a peptide of the invention may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems), and/or eukaryotic host cells. Selection of the host cell will depend in part on whether the peptide is to be post-translationally modified (*e.g.*, glycosylated and/or phosphorylated). If so, yeast, insect, or mammalian host cells are preferable.

[00103] Typically, expression vectors used in any of the host cells will contain sequences for plasmid maintenance and for cloning and expression of exogenous nucleotide sequences. Such sequences, collectively referred to as "flanking sequences" in certain embodiments, will typically include one or more of the following nucleotide sequences: a promoter, one or more enhancer sequences, an origin of replication, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a sequence encoding a leader sequence for secretion of the peptide, a ribosome binding site, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the peptide to be expressed, and a selectable marker element.

[00104] Preferred vectors for practicing this invention are those that are compatible with bacterial, insect, and mammalian host cells. Such vectors include, *inter alia*, pCRII, pCR3, and pcDNA3.1 (Invitrogen Company, Carlsbad, CA), pBSII (Stratagene Company, La Jolla, CA), pET15 (Novagen, Madison, WI), pGEX (Pharmacia Biotech, Piscataway, NJ), pEGFP-N2 (Clontech, Palo Alto, CA), pETL (BlueBacII; Invitrogen), pDSR-alpha (PCT Publication No. WO 90/14363) and pFastBacDual (Gibco/BRL, Grand Island, NY).

[00105] Additional suitable vectors include, but are not limited to, cosmids, plasmids or modified viruses, but it will be appreciated that the vector system must be compatible with the selected host cell. Such vectors include, but are not limited to, plasmids such as Bluescript® plasmid derivatives (a high copy number ColE1-based phagemid, Stratagene Cloning Systems Inc., La Jolla CA), PCR cloning plasmids designed for cloning Taq-Taq-amplified PCR products (*e.g.*, TOPO™ TA Cloning® Kit, PCR2.1® plasmid derivatives, Invitrogen, Carlsbad, CA), and mammalian, yeast, or virus vectors such as a baculovirus expression system (pBacPAK plasmid derivatives, Clontech, Palo Alto, CA).

[00106] After the vector has been constructed and a polynucleotide molecule encoding a peptide of the invention has been inserted into the proper site of the vector, the completed vector

may be inserted into a suitable host cell for amplification and/or fusion protein expression. The transformation of an expression vector for a peptide of the invention into a selected host cell may be accomplished by well-known methods including transfection, infection, calcium chloride, electroporation, microinjection, lipofection or the DEAE-dextran method or other known techniques. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook *et al.*, *supra*.

[00107] Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a yeast cell, an insect cell or a vertebrate cell). The host cell, when cultured under appropriate conditions, synthesizes the peptide that can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). The selection of an appropriate host cell will depend upon various factors, such as desired expression levels, peptide modifications that are desirable or necessary for activity, such activity (such as glycosylation or phosphorylation), and ease of folding into a biologically active molecule.

[00108] A number of suitable host cells are known in the art and many are available from the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209. Examples include, but are not limited to, mammalian cells, such as Chinese hamster ovary cells (CHO) (ATCC No. CCL61); CHO DHFR-cells (Urlaub *et al., Proc. Natl. Acad. Sci. USA,* 97:4216-4220 (1980)); human embryonic kidney (HEK) 293 or 293T cells (ATCC No. CRL1573); or 3T3 cells (ATCC No. CCL92). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and screening, product production and purification are known in the art. Other suitable mammalian cell lines are the monkey COS-1 (ATCC No. CRL1650) and COS-7cell lines (ATCC No. CRL1651) cell lines, and the CV-1 cell line (ATCC No. CCL70). Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include, but are not limited to, mouse neuroblastoma N2A cells, HeLa, mouse L-L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines, which are available from the ATCC. Each of these cell lines is known by and available to those skilled in the art of protein expression.

[00109] Similarly, useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (*e.g.*, HB101, (ATCC No. 33694) DH5α, DH10, and MC1061 (ATCC No. 53338)) are well-well known as host cells in the field of biotechnology.

Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

[00110] Many strains of yeast cells known to those skilled in the art are also available as host cells for the expression of peptides of the present invention. Preferred yeast cells include, for example, *Saccharomyces cerivisae* and *Pichia pastoris*.

[00111] Additionally, where desired, insect cell systems may be utilized in the methods of the present invention. Such systems are described for example in Kitts *et al., Biotechniques*, 14:810-817 (1993); Lucklow, *Curr. Opin. Biotechnol.*, 4:564-572 (1993); and Lucklow *et al.* (*J.al., J. Virol.,* 67:4566-4579 (1993). Preferred insect cells are Sf-9 and Hi5 (Invitrogen, Carlsbad, CA).

[00112] One may also use transgenic animals to express glycosylated peptides of the invention. For example, one may use a transgenic milk-producing animal (a cow or goat, for example) and obtain the present glycosylated peptide in the animal milk. One may also use plants to produce peptides of the invention. However, in general, the glycosylation occurring in plants is different from that produced in mammalian cells, and may result in a glycosylated product which is not suitable for human therapeutic use.

Therapeutic Compositions

[00113] Therapeutic compositions are within the scope of the present invention. Peptides of the invention can be combined with various components to produce compositions of the invention. Such compositions can comprise a therapeutically effective amount of a peptide or nucleotide of the invention in admixture with a pharmaceutically or physiologically acceptable formulation agent selected for suitability with the mode of administration. Pharmaceutical compositions may also comprise a therapeutically effective amount of one or more peptide of the invention in admixture with a pharmaceutically or physiologically acceptable formulation agent selected for suitability with the mode of administration. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. See, e.g., Remington's Pharmaceutical Sciences, 19th Ed. (1995, Mack Publishing Co., Easton, Pa.) which is herein incorporated by reference.

[00114] The pharmaceutical composition can contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, colour, isotonicity, odour, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulphite or sodium hydrogen-sulphite); buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates or other organic acids); bulking agents (such as mannitol or glycine); chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin), fillers; monosaccharides, disaccharides; and other carbohydrates (such as glucose, mannose, or dextrins); proteins (such as serum albumin, gelatin or immunoglobulins); colouring, flavouring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate 80, triton, tromethamine, lecithin, cholesterol, tyloxapol); stability enhancing agents (sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides, preferably sodium or potassium chloride), delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants.

[00115] The optimal pharmaceutical composition will be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format, and desired dosage. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the peptide of the invention. The preferred form of the pharmaceutical composition depends on the intended mode of administration and therapeutic application. Pharmaceutical compositions prepared according to the invention may be administered by any means that leads to the peptides of the invention coming in contact with a causative agent of a disease or disorder as herein described including cardiac hypertrophy or oxidative stress.

[00116] The primary vehicle or carrier in a pharmaceutical composition may be either aqueous or non-aqueous in nature. For example, a suitable vehicle or carrier may be water for injection, physiological saline solution, solution or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. Other exemplary pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefor. In

one embodiment of the present invention, pharmaceutical compositions may be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents in the form of a lyophilized cake or an aqueous solution. Further, the peptide product may be formulated as a lyophilizate using appropriate excipients such as sucrose.

[00117] The pharmaceutical compositions can be capable of parenteral delivery. Alternatively, the compositions may be capable of delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

[00118] The formulation components are present in concentrations that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

[00119] When parenteral administration is contemplated, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising the desired peptide of the invention in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which the active agent is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid, acid or polyglycolic acid), or beads or liposomes, that provides for the controlled or sustained release of the product which may then be delivered as a depot injection. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

[00120] It is also contemplated that certain formulations may be administered orally. In one embodiment of the present invention, peptides of the present invention that are administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized, and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the active agent. Diluents, flavourings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

[00121] Another pharmaceutical composition may involve an effective quantity of a peptide of the invention in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit dose form. Suitable excipients include, but are not limited to, inert diluents, such

as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

[00122] Additional pharmaceutical compositions will be evident to those skilled in the art, including formulations involving a peptide of the invention in sustained- or controlled-delivery formulations. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. See for example, PCT Application No. PCT/US93/00829 that describes the controlled release of porous polymeric microparticles for the delivery of pharmaceutical compositions. Additional examples of sustained-sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, for example, films, or microcapsules. Sustained release matrices may include polyesters, hydrogels, polylactides, copolymers of L-glutamic acid and gamma ethyl-L-glutamate, ethylene vinyl acetate or poly-D(-)-3-hydroxybutyric acid. Sustained-release compositions may also include liposomes, which can be prepared by any of several methods known in the art.

[00123] The pharmaceutical composition to be used for *in vivo* administration typically must be sterile. This may be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using these methods may be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration may be stored in lyophilized form or in a solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

[00124] Once the pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations may be stored either in a ready-to-use form or in a form (*e.g.*, lyophilized) requiring reconstitution prior to administration.

[00125] The effective amount of the active agent in the pharmaceutical composition to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will thus vary depending, in part, upon the molecule delivered, the indication for which the active agent is being used, the route of administration, and the size (body weight, body surface or organ size) and condition (the age and general health) of the patient. Accordingly, the clinician may titre the dosage and modify the route of administration to obtain the optimal therapeutic effect. A typical dosage may range from about 0.1 μg/kg to up to about 100 mg/kg or more, depending on the factors mentioned above. In other embodiments, the dosage may range from 0.1 μg/kg up to about 100 mg/kg; or 1 μg/kg up to about 100 mg/kg; or 5 μg/kg up to about 100 mg/kg.

[00126] The frequency of dosing will depend upon the pharmacokinetic parameters of the active agent and the formulation used. Typically, a clinician will administer the composition until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages may be ascertained through use of appropriate dose-response data.

[00127] The peptide or pharmaceutical composition comprising the peptide can be administered to the subject in a range of treatment regimens. For example, the peptide or pharmaceutical composition can be administered hourly, three times daily, twice daily, once daily, once every two days, once every three days, once weekly, once every two weeks, once monthly, once every two months, once every six months, and once yearly. The appropriate regimen can be determined by the person skilled in the art based on the nature of the condition to be treated. For example, the peptide or pharmaceutical composition comprising the peptide can be administered three times per week.

[00128] The route of administration of the pharmaceutical composition is in accord with known methods, *e.g.*, orally, through injection by intravenous, intracoronary, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, intra-ocular, intraarterial, intraportal, or intralesional routes; by sustained release systems or by implants. Where desired, the compositions may be administered by bolus injection or continuously by infusion, or by implantation device.

[00129] Alternatively or additionally, the composition may be administered locally via implantation of a membrane, sponge or another appropriate material on to which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device may be implanted into any suitable tissue or organ, and delivery of the desired molecule may be via diffusion, timed-release bolus, or continuous administration.

[00130] In some cases, it may be desirable to use the pharmaceutical compositions herein in an *ex vivo* manner. In such instances, cells, tissues, or organs that have been removed from the patient are exposed to the pharmaceutical compositions after which the cells, tissues and/or organs are subsequently implanted back into the patient.

[00131] In another form, nanoparticles may be employed as carriers for delivery of peptides of the invention. For example, the nanoparticles may be spherical polymeric nanoparticles. Nanoparticles have been shown to overcome some limitations of conventional therapeutic delivery such as nonspecific biodistribution and targeting, and lack of water solubility, amongst

others. Thus, nanoparticles may be used for delivering peptides of the invention to cardiac cells for treatment of a patient with the peptides.

[00132] In an aspect, the peptides of the invention are delivered through the use of dendronized polymers. Preferably, the peptides of the invention are delivered through the use of linear dendronized polymers (denpols). Most preferably, the peptides of the invention are complexed with linear deondronized polymers to form polymer-peptide nanoparticles. The polymer-peptide nanoparticles can be delivered intracellularly.

[00133] The routes of administration described herein are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient.

Uses and Methods for Peptides of the Invention

[00134] The invention provides a use of a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3, or variants thereof; for modulating binding to a L-type Ca^{2+} channel alpha-interacting domain. This preferably occurs intracellularly in a cardiac cell.

[00135] The invention provides a use of a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 3, or variants thereof; for modulating movement of a L-type Ca^{2+} channel β_2 subunit in a cardiac cell such as a myocyte in the heart of a subject.

[00136] In this respect, the invention also provides a method for modulating movement of a L-type Ca^{2+} channel β_2 subunit in a cardiac cell such as a myocyte in the heart of a subject, comprising administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or SEQ ID NO: 2 and SEQ ID NO: 3, or variants thereof.

[00137] Binding of the peptide to the alpha-interacting domain can prevent movement of the β_2 subunit during activation and inactivation of the L-type Ca^{2+} channel. Since the β_2 subunit is proposed to facilitate inactivation of the alpha subunit, this can result in a delay in inactivation of the current.

[00138] A subject that can be treated with a peptide of the invention will include humans as well as other mammals and animals.

[00139] In an aspect, the invention provides the use of a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof; for modulating a L-type Ca^{2+} channel in a cardiac cell such as a myocyte in the heart of a subject.

[00140] In this respect, the invention also provides a method for modulating a L-type Ca^{2+} channel in a cardiac cell such as a myocyte in the heart of a subject, comprising the step of administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof.

[00141] In another aspect, the invention provides the use of a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof; for preventing or reducing myocardial damage and/or oxidative stress in the heart of a subject.

[00142] In this respect, the invention also provides a method for preventing or reducing myocardial damage and/or oxidative stress in the heart of a subject, comprising the step of administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof.

[00143] The myocardial damage may include cardiac hypertrophy. Preferably, cardiac hypertrophy is reduced or prevented but intracellular Ca^{2+} levels are reduced or substantially maintained in the heart of the subject. Substantially maintained indicates Ca^{2+} levels which are the same or close to what is observed normally in the subject such as before cardiac hypertrophy.

[00144] The binding of a peptide of the invention to a L-type Ca^{2+} channel results in a decrease of mitochondrial oxygen consumption or metabolism. As the peptide immobilizes the β_2 subunit, rather than the pore-forming α1C subunit, this can occur without substantially altering calcium influx. Since this can occur at a concentration that does not alter calcium influx (1μM), it may be a preferable agent to use in treating or preventing cardiac hypertrophy because any agent that decreases calcium influx may decrease contractility.

[00145] Thus, the invention provides uses and methods for the peptides of the invention as a treatment to prevent or reduce damage and/or oxidative stress in myocardial cells in a subject by modulating L-type Ca^{2+} channel activity.

[00146] The peptides of the invention may be administered to treat or prevent cardiac hypertrophy in patients that have developed or are at risk of developing cardiac hypertrophy. The peptides of the invention can also be administered to reduce damage and/or oxidative stress to ischemic myocardial cells following a myocardial infarction in a subject during and after reperfusion therapy. The peptides may be administered before, after or during reperfusion.

[00147] In this respect, the invention also provides a use of a peptide comprising: the amino acid sequence of SEQ ID NO: 2; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO:

3; in the preparation of a medicament for treating, preventing or ameliorating myocardial damage to a patient.

[00148] The invention also provides a use of a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof; in the manufacture of a medicament for treating, preventing or ameliorating myocardial damage to a patient, and/or oxidative stress in myocardial cells.

[00149] It may be preferable to administer the peptides of the invention in combination with other therapeutic agents that are useful for treating cardiac hypertrophy or HCM in a subject, or other agents which assist in reducing myocardial damage and/or oxidative stress. Such combinations could use conjugates comprising the peptides or the therapy could be concomitant or involve the sequential administration of the agents. Examples of such therapeutic agents may include, as some non-limiting examples, Antioxidants such as N-acetylcysteine, reduced glutathione, TAT-conjugated catalase or TAT-conjugated superoxide dismutase.

[00150] The peptides may be administered via a variety of methods, for example, as a therapeutic depending on the particular circumstances and as deemed appropriate by a medical practitioner.

[00151] In one non-limiting example, a peptide of the invention may be administered via the coronary arteries by a cardiologist/physician at the time of angiography or angioplasty in a hospital after admission with chest pain and diagnosis of coronary occlusion (myocardial infarction).

[00152] In another non-limiting example, a peptide of the invention may be administered to HCM patients prior to the development of cardiomyopathy. The peptide of the invention may be administered via intraperitoneal injection or via the coronary arteries to patients at risk of developing cardiomyopathy. Prevention of the development of cardiomyopathy may be measured by a decrease in intraventricular septal thickness or posterior wall thickness, and increase in left ventricular end diastolic dimension on echocardiography. The peptide of the invention may be administered about 3 times a week, on the basis that the half-life of the peptides of the invention in the body is likely to be approximately 3 to 4 days, consistent with the turnover rate of Cav1.2 channel protein (as described in Catalucci et al., *The Journal of Cell Biology* 184, 923-933 (2009)).

[00153] The effect of the administered therapeutic composition can be monitored by standard diagnostic procedures.

[00154] For example, effectiveness of the peptide may be monitored by echocardiography (ultrasound analysis of cardiac function) in one example. Size of damage could be assessed by release of muscle enzymes into the blood and by changes on electrocardiography (ECG).

Methods of Treatment

[00155] In yet another aspect, the present invention provides a method for modulating movement of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell in the heart of a subject, comprising administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3.

[00156] The present invention further provides a method for modulating binding of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell in the heart of a subject, comprising administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3. Preferably, the peptide prevents interaction between the beta subunit of the L-type Ca2+ channel and an alpha subunit of the L-type Ca2+ channel in a cardiac cell of a subject.

[00157] The present invention further provides a method for modulating a L-type Ca^{2+} channel in a cardiac cell in the heart of a subject, comprising the step of administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or the amino acid sequence of SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof.

[00158] The present invention further provides a method for reducing myocardial damage and/or oxidative stress in the heart of a subject, comprising the step of administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or a peptide comprising the amino acid SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof.

[00159] The method of the invention may reduce cardiac hypertrophy but substantially maintain intracellular Ca^{2+} levels in the heart of the subject.

[00160] Patients suffering from HCM present with elevated Ψm and mitochondrial metabolic activity, and a faster L-type Ca^{2+} channel inactivation rate, even prior to the development of cardiomyopathy. Accordingly, the administration of the peptides of the invention, which can increase L-type Ca^{2+} channel inactivation rate and decrease mitochondrial metabolic activity, can be used to prevent the development of cardiac hypertrophy in HCM patients.

[00161] Preferably, the method of treatment or use does not cause vasodilatory effects or negative inotropic effects as can occur with treatment with calcium channel antagonists.

[00162] In an aspect, the invention provides a method of preventing cardiac hypertrophy in the heart of a subject, comprising the step of administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or a peptide comprising the amino acid SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof. Preferably, the subject suffers from HCM.

[00163] In a further aspect, the invention provides a method or use for slowing the progression of myocardial fibrosis in a subject, comprising administering to the subject a peptide comprising: the amino acid sequence of SEQ ID NO: 2, or variants thereof; or a peptide comprising the amino acid SEQ ID NO: 2, and SEQ ID NO: 3, or variants thereof.

[00164] In a further aspect, the invention provides a method or use for modulating binding of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject, comprising administering to the subject a peptide of this invention, wherein the method is more effective at modulating the binding of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject compared to a method of administering QQLEEDLKGYLDWITQAE (SEQ ID NO:1).

[00165] In a further aspect, the invention provides a method or use for modulating a L-type Ca^{2+} channel in a cardiac cell of a subject, comprising the step of administering to the subject a peptide of this invention, wherein the method is more effective at modulating the L-type Ca^{2+} channel in a cardiac cell of a subject compared to a method of administering QQLEEDLKGYLDWITQAE (SEQ ID NO:1).

[00166] In a further aspect, the invention provides a method or use for reducing myocardial damage and/or oxidative stress in the heart of a subject, comprising the step of administering to the subject a peptide of this invention, wherein the method is more effective at reducing the myocardial damage and/or oxidative stress in the heart of a subject compared to a method of administering QQLEEDLKGYLDWITQAE (SEQ ID NO:1).

[00167] As used herein the term “subject” generally includes mammals such as: humans; farm animals such as sheep, goats, pigs, cows, horses, llamas; companion animals such as dogs and cats; primates; birds, such as chickens, geese and ducks; fish; and reptiles. The subject is preferably human.

[00168] Effective doses of the compositions of the present invention, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, and other medications administered. Treatment dosages need to be titrated to optimize safety and efficacy.

[00169] The following Examples are to be construed as merely illustrative and not limitative of the remainder of the disclosure in any way whatsoever. These Examples are included solely for the purposes of exemplifying the present invention. They should not be understood as a restriction on the broad summary, disclosure or description of the invention as set out above. Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. In the foregoing and in the following

examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

EXAMPLES

Example 1 - Synthesis of AID-Peptides

[00170] A peptide corresponding to the α1C-β2a interaction domain (AID) within the cytoplasmic I-II linker of the cardiac α1C subunit was synthesized by using the amino acid sequence, QQLEEDLKGYLDWITQAE (SEQ ID NO:1). A scrambled (inactive) control peptide (AID[S]) was also synthesized with the sequence, QKILGEWDLAQYTDQELE (SEQ ID NO: 10). Where required, a cell-penetrating TAT sequence was tethered to AID or AID(S) via 6-aminohexanoic acid (6-Ahx) (RKKRRQRRR) (SEQ ID NO: 3), to yield AID-TAT and AID(S)-TAT peptides.

[00171] Variant AID-peptides were also synthesised as listed in Table 4. Based on the original AID-TAT sequence, variant sequences were generated by truncating the amino end and/or carboxyl end of the original sequence or by inducing point mutations in amino acids.

Table 4: Variant AID-peptides.

Peptide	Amino Acid Sequence	SEQ ID NO
Variant P1	DLKGYLDWITQAE	SEQ ID NO: 11
Variant P4	LEEDLKGYLDWITQAE	SEQ ID NO: 12
Variant P6	KGYLDWITQAE	SEQ ID NO: 13
Variant P7	QQQEEDLKGYLDWITQAE	SEQ ID NO: 4
Variant P10	DLKGYLDWITQA	SEQ ID NO: 14
Variant P11	DLKGYLDWITQ	SEQ ID NO: 15
Variant P12	DLKGYLDWIT	SEQ ID NO: 16
Variant P13	QQLEEDLKGFLDAATQAE	SEQ ID NO: 17
Variant P14	QQEEEDLKGYLDWITQAE	SEQ ID NO: 5
Variant P15	QQEEEDEKGYLDWITQAE	SEQ ID NO: 6
Variant P16	QQREEDLKGYLDWITQAE	SEQ ID NO: 7

Example 2 – Binding Assay – NHS Magnetic Beads

[00172] The affinity of each of the variant peptides to the β_2 subunit was investigated through binding competition assays.

[00173] PureProteome™ NHS FlexiBind Magnetic Beads (Cat. #LSKMAGN04, Merck) were used following manufacturer recommendations. Aminohexanoic acid linked AID peptide

was bound to the NHS flexibind magnetic beads at a concentration of more than 2mg/mL to obtain saturation of the beads with peptide. Beads were first primed with an equilibration buffer (1mM HCL) to prepare them for binding. The Ahx-AID peptide was dissolved in wash/coupling buffer (PBS, pH 7.4) and added to the beads. The beads-AID peptide mixture was left to mix uniformly on a multiplate shaker at a speed of 300rpm at room temperature for 2 hours. To reduce non-specific binding the beads were incubated in Quench Buffer (100 mM Tris-HCl, 150 mM NaCl, pH 8.0) at room temperature for 1 hour. After washing the cardiac lysate was added to increasing concentrations of the variant peptide to allow for competitive binding with the AID bound beads in the concentrations showed in Table 5.

Table 5: Sample preparation for pre-binding the AID/variant peptide to the β_2 subunit in whole heart lysate at varying concentrations.

Final Peptide Concentration	Stock Peptide Concentration (µM)	Volume of stock peptide required in 200 µL (µL)	Volume of β_2 Lysate sample required in 200 µL (µL)	Volume of *Wash/Coupling buffer* required in 200 µL (µL)
1nM	1	2	100	98
30 nM	1	6	100	94
100 nM	1	20	100	80
1 µM	10	20	100	80
10 µM	100	20	100	80

[00174] The AID-peptide bound beads were split into 5 aliquots for the 5 samples prepared in Table 5. The whole heart lysate added to each of the peptide-bound beads to allow competitive binding for 2 hours at room temperature.

[00175] Upon completion, the conjugate was washed in coupling buffer, to remove unbound fraction. SDS sample buffer was added to the beads, with the tubes then heated for 5 minutes at 95°C. The eluted sample stored at -20°C to be run through sodium dodecyl sulfate – polyacrymalide gel electrophoresis (SDS-PAGE) to determine the binding affinity of each of the variants.

Example 3 – Binding Assay – Streptavidin Beads

[00176] The use of streptavidin dynabeads and biotinylated peptide was developed to improve the binding affinity assays. The peptide was initially biotinylated using EZ-link amine-PEG11-biotin (Spacer arm: 53.2 Å, Thermofisher Scientific , Cat. #26136) which was dissolved in 0.1M MES buffer ((2-N-morpholino)-ethanesulfonic acid) at a concentration of 1mM. Just prior to the reaction, 0.1M EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride) was dissolved in MES buffer. In order to achieve complete biotinylation of the AID peptide, a 50x molar

excess of biotin to peptide was required, with the total volumes of each component required shown in Table 6.

Table 6 - Volumes of each component required to achieve biotinylation of the AID peptide enough for one complete assay.

	Amount/reaction (nM)	Stock conc (mM)	Volume/ reaction (µl)	For dose-response curve (conc range: 0 nM -100 µM = 6 reactions) for each version of variant AID
AID	0.02	0.1	0.2	1.2 µl
MES		100	9.8	58.8 µl
amine-PEG_{11}-biotin	1	1	1	6 µl
EDC	0.2	0.1	2	12 µl
Total			13	78 µl

[00177] Each 78µL aliquot of the biotinylated peptide shown in Table 4 was enough for 6 reactions, covering the range of concentrations in which the heart lysate was again prepared with varying concentrations of the variant AID peptides according to Table 7.

Table 7. Sample preparation for pre-binding the AID/variant peptide to the β_2 subunit in whole heart lysate at varying concentrations.

Final Peptide Concentration	Stock Peptide Concentration (µM)	Volume of stock peptide (µl)	Volume of heart lysate (µl)	PBS (µl)	Total (µl)
0	**-**	-	15.4	34.6	50
10 nM	**0.1**	5	15.4	29.6	50
100 nM	**1**	5	15.4	29.6	50
1 µM	**10**	5	15.4	29.6	50
10 µM	**100**	5	15.4	29.6	50
100µM	**1000**	5	15.4	29.6	50

[00178] The 78µL aliquot of biotinylated AID-peptide was mixed with streptavidin dynabeads (1 mg Dynabeads per every 200 pmol biotinylated peptide) for half an hour at room temperature. The heart lysate (100 □g) was incubated with the variant peptides at room temperature for 2 hours. After removing all unbound peptide from the beads, they were mixed with the pre-bound heart lysate for 2 hours at room temperature. The unbound fraction was removed. The conjugate was heated at 95°C for 5 minutes, with the sample buffer then eluted off and stored at -20°C for SDS-PAGE analysis.

Example 4 – Binding Affinity Analysis using SDS-PAGE

[00179] SDS-PAGE was used to analyse all samples resulted from the binding affinity assays. 10 well Mini-Protean TGX Stain-Free Gels (Biorad) were loaded with 25µL of each samples. A molecular weight ladder (Precision Protein Western C) was also loaded alongside the samples to be able to identify the presence of the β2 subunit (68kDa). Gels were run in a Biorad electrophoresis tank powered by the PowerPac 3000 at constant current of 30mA for 35 minutes. Upon completion, the gels were placed on nitrocellulose membrane to be transferred on the TransBlot Turbo for 3 minutes. The membranes were placed in blocking buffer (5% BSA) for 1 hour, before overnight incubation with Rabbit Anti-Cavβ2 polyclonal primary antibody (Alomone Labs #ACC-105) at 4°C. The membrane was washed three times for 10 minutes each with TBST before incubation with Goat Anti-Rabbit IgG (H+L)-HRP Conjugate secondary antibody (Abcam #ab97080, preabsorbed) in 5% BSA at room temperature for 1 hour. To visualize the protein bands Luminata Crescendo, Western HRP Substrate (Merck Millipore) and ChemiDoc imager (Biorad) with ImageLab software. Densitometric analysis performed using ImageJ software to quantify the intensity of the bands on the membrane corresponding to the Cavβ2.

[00180] The average $K_{0.5}$ was calculated for each variant. These values are set out in Figure 2, and the table below. Variants P7, P14, P15 and P16 demonstrated a greater binding affinity (lower $K_{0.5}$) for the target β2 subunit than the original AID peptide.

Example 5 – Isolation of adult mouse cardiac myocytes

[00181] Adult male C57BL/6J mice were anesthetized with pentobarbitone sodium (240 mg/kg) via intraperitoneal injection prior to excision of the heart. Ventricular myocytes were isolated. Mouse hearts were cannulated onto a Langendorff apparatus via the aorta and perfused retrogradely with Krebs-Henseleit Buffer (KHB) containing (in mM): 120 NaCl, 25 $NaHCO_3$, 4.8 KCl, 2.2 $MgSO_4$, 1.2 NaH_2PO_4 and 11 glucose (pH = 7.35 with O2/CO2 at 37°C) for 4 min at 37°C. Hearts were then perfused with KHB supplemented with 2.4 mg/ml collagenase B for 3 min, then 8 min perfusion in the presence of 40 µM calcium. Following perfusion, aorta and atria were removed, and ventricles gently teased apart and triturated to dissociate myocytes into suspension. Myocyte suspension was centrifuged at 500 RPM for 3 min, supernatant discarded, and myocytes resuspended in calcium free HEPES-Buffered Solution (HBS) containing (in mM): 140 NaCl, 5.4 KCl, 0.5 MgCl2, 5.5 HEPES, 11 glucose (pH = 7.4 at 37°C) in the presence or absence of 3 mM EGTA (for 0 mM calcium JC-1 experiments). For calcium containing (uptake) experiments, calcium was titrated back to achieve a final extracellular concentration of 2.0 mM.

Example 6 – In vitro assessment of cardiac myocyte function

[00182] All in vitro studies were performed in freshly isolated myocytes at 37°C. Fluorescence was recorded using a Hamamatsu Orca ER digital camera attached to an inverted

Nikon TE2000-U microscope. Following a 20 min incubation with 0.5-1μM AID(S)-TAT, AID-TAT, peptide 6 or peptide 7, fluorescent signal of individual myocytes was quantified using Metamorph 6.3 to measure signal intensity of manually traced cell regions. An equivalent region not containing cells was used for background and was subtracted.

Assessment of intracellular calcium levels

[00183] Intracellular calcium was monitored in cardiac myocytes using the fluorescent indicator Fura-2 AM (Fura-2, 1 μM, ex 340/380 nm, em 510 nm, Molecular Probes). Oxidative stress was induced by applying a non-necrotic/non-apoptotic concentration of H_2O_2 (30 μm) to the myocytes for 5 min followed by 10U/ml catalase. Fluorescence at 340/380nm excitation and 510nm emission were measured at 1 minute intervals with an exposure of 50ms before and after exposure to 30μM H2O2 (5min) and 10U/ml catalase (5min). Ratiometric 340/380nm signal was quantified and reported as a percentage from the baseline pre-treatment average.

[00184] The results are presented in Figure 3(a). Figure 3(a) demonstrates the effects of the peptides on an increase in intracellular calcium induced by a hydrogen peroxide insult (H_2O_2). Only P7 altered the increase in Fura 2 following H_2O_2. The mutant P7 was more effective at decreasing H_2O_2 induced increases in intracellular Ca^{2+} (Fura 2) and superoxide production (DHE) than AID-TAT and had a similar effect on mitochondrial membrane potential (JC-1) as AID-TAT.

Assessment of intracellular superoxide levels

[00185] Superoxide generation was assessed in cardiac myocytes using the fluorescent indicator dihydroethidium (DHE, 5 μM, 515-560 nm ex filter, 590 long pass em, Merck). Fluorescent images were taken at 1 min intervals with 200 ms exposure before and after exposure to 30μM H2O2 (5min) and 10U/ml catalase (5min). Fluorescence was reported as percentage change in the slope of the signal measured at 20-40 min (treatment) over 1-10 min (pre-treatment).

[00186] The results are presented in Figure 3(b). Peptide 7 reduced the generation of superoxide in a statistically significant manner compared with peptide 6, AID-TAT, or AID(S)-TAT.

In vitro assessment of mitochondrial membrane potential

[00187] Mitochondrial membrane potential (Ψm) was monitored in cardiac myocytes by assessing alterations in 5,5′,6,6′-tetrachloro- 1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide fluorescence (JC-1: 200 nM, ex 480 nm, em 580/535 nm, Molecular Probes). Myocytes were incubated in calcium-free HBS (0 mM calcium, supplemented with 3 mM EGTA) for at least 3 h prior to measuring changes in Ψ_m. Fluorescent images were taken every 2 min before and after exposure to 30μM H_2O_2 (5min) and 10U/ml catalase (5min) (exposure = 50 ms). Ratiometric

580/535 nm fluorescent signal was quantified and reported as a percentage from the baseline pre-treatment average. 40 mM NaCN was added at the end of each experiment to collapse Ψ_m, confirming that the JC-1 signal was indicative of Ψ_m.

[00188] The results are presented in Figure 3(c). Only AID-TAT and peptide 7 reduced the mitochondrial membrane potential.

In vitro assessment of mitochondrial flavoprotein oxidation

[00189] Autofluorescence was used to measure flavoprotein oxidation in cardiac myocytes based on previously described methods (480 nm ex, 535 nm em). Fluorescent images were taken at 1 min intervals with 1s exposure before and after exposure to L-type Ca2+ channel agonist BayK(-). Fluorescence was reported as a percentage from the baseline pre-treatment average.

[00190] The results from mutant peptides (P6-TAT, P7-TAT, P14-TAT and P15-TAT) and AID-TAT peptide are presented in Figure 4 in wildtype cardiac myocytes and in Figure 5 in myocytes from HCM hearts. The results show that P7, P14 and P15 presented a statistically significant reduction in flavoprotein oxidation in comparison with AID-TAT peptide in HCM hearts, and that P7 presented a statistically significant reduction in flavoprotein oxidation in comparison with AID-TAT peptide in wild type hearts.

Example 7 – Assessment of myocardial ischemia-reperfusion injury

[00191] Adult male guinea pigs were anesthetized with pentobarbitone sodium (240 mg/kg) via intraperitoneal injection prior to excision of the heart. Guinea pig hearts were cannulated onto a Langendorff apparatus via the aorta and perfused retrogradely with Ca^{2+} containing KHB comprising (in mM): 120 NaCl, 25 $NaHCO_3$, 4.8 KCl, 2.2 $MgSO_4$, 1.2 NaH_2PO_4, 11 glucose and 1.5 $CaCl_2$ (pH = 7.35 with O_2/CO_2 at 37°C) at 37°C for 30 min at a rate of 7 mL/min, followed by 30 min no-flow ischemia, then 30 min reperfusion as previously described.

[00192] Perfusates were collected 20 and 25 min pre-ischemia and 20 and 30 min following reperfusion. A single dose of 0.5-10μM AID(S)-TAT, AID-TAT, peptide 6 or peptide 7 was added to Ca^{2+}-containing KHB solution just prior to reperfusion. Creatine Kinase (CK) and Lactate Dehydrogenase (LDH) activity was measured in perfusates collected pre- and post-ischemia, and post-ischemia values normalized to pre-ischemia values.

[00193] For each sample, 10 μL of perfusate was added to 100 μL CK enzyme reagent (CK NAC-activated diagnostic kit, Randox Laboratories) and the rate of increase in absorbance recorded over 15 min at 30°C using a spectrophotometer (PowerWave XS, BioTek, 340 nm). CK activity was calculated according to the equation:

$$CK\ Activity\ (U/L) = 4127 \times \left(\frac{\Delta A_{340nm}}{min}\right)$$

[00194] The results are presented in Figure 6(a). CK activity was reduced compared to the AID(S)-TAT peptide in the AID-TAT, P7-TAT (1µM) and P7-TAT (0.5µM) samples. CK activity was reduced in both P7-TAT (1µM) and P7-TAT (0.5µM) compared to the P6-TAT peptide. P7-TAT (0.5µM) was at least as effective as AID-TAT (1µM).

[00195] To determine LDH activity, 150 µL of each sample was mixed with 50 µL of LDH reagent (50mM imidazole buffer, 375 µM NADH, pyruvate and 0.05% BSA, pH = 7) and the rate of decrease in absorbance recorded over 15 min at 25°C using a spectrophotometer (PowerWave XS, BioTek, 340 nm). LDH activity was calculated according to the equation:

$$LDH\ activity\ (U/mL) = \frac{\frac{\Delta A_{340nm}}{min}(Test) - \frac{\Delta A_{340nm}}{min}(Blank)\ \text{x}\ 3\ \text{x}\ (\text{dilution factor})}{6.22\ \text{x}\ 0.15}$$

[00196] The results are presented in Figure 6(b). LDH was reduced compared to the AID(S)-TAT peptide in the AID-TAT, P7-TAT (1µM) and P7-TAT (0.5µM) samples. P7-TAT (0.5 µM) reduced LDH at least as well as AID-TAT (1 µM).

[00197] To determine GSH:GSSG, whole heart tissue was immediately homogenized following ischemia-reperfusion protocol, and GSH and GSSG levels assessed using a GSH/GSSG ratio detection assay kit as per manufacturer's instructions (abcam, ab205811).[8] Total glutathione and GSH were measured using a FLUOstar OPTIMA (BMG Labtech, ex 485-12 nm, em 520 nm). GSSG was calculated by subtracting GSH from total glutathione.

[00198] The results are presented in Figure 6(c). AIDS-TAT samples at 1µM did not show a statistically significant increase in the GSH:GSSG ratio, but AID-TAT samples at 10µM did show a statistically significant increase in the GSH:GSSG ratio. P7-TAT samples at 1µM and at 0.5µM resulted in statistically significant increase in the GSH:GSSG ratio, comparable to the AID-TAT samples at 10µM.

[00199] The reduction in CK activity, LDH activity and increase in GSH:GSSG ratio each represent a reduction in oxidative stress. The results demonstrate that the administration of P7-TAT reduced oxidative stress with reference to each of these markers. Further, P7-TAT was more effective than AID-TAT at reducing oxidative stress, with either superior results at the same concentration, or similar results with lower dosages of P7-TAT in comparison to AID-TAT.

Example 8 – Prophylactic administration of AID-TAT to cTnIG203S mice

[00200] 10µM AID-TAT was administered to cTnIG203S mutant mice by intraperitoneal injection 3 times per week prior to the development of hypertrophic cardiomyopathy, for 5 weeks. The peptide was dissolved in phosphate buffered saline solution (PBS), and the total

amount of the 10μM AID-TAT administered over 5 weeks was 0.9mg. Administration prevented the development of the hypertrophy as evidenced by a decrease in intraventricular septal thickness and increase in left ventricular end diastolic dimension on echocardiography. Fractional shortening also improved. The results are presented in Figure 8. The AID-TAT peptide was administered 3 times per week because it was hypothesised that the bound AID-TAT peptide lasts 3-4 days, consistently with the turnover rate of the Cav1.2 channel protein.

Example 9 - In vitro binding to the $I_{Ca\text{-}L}$ β_2 subunit demonstrates increased binding affinity of AID_{P7}, AID_{P14}, AID_{P15} and AID_{P16}

[00201] In this experiment, a binding affinity assay was utilized to examine the binding efficacy of AID peptide variants presented in Table 8, compared to the original AID peptide.

Table 8

Peptide	Amino acid sequence
Original AID-TAT (full-length active)	QQLEEDLKGYLDWITQAE
AID(S)-TAT (scrambled inactive)	QKILGEWDLAQYTDQELE
AID_{P1}-TAT Mutant	~~QQLEE~~DLKGYLDWITQAE
AID_{P4}-TAT Mutant	~~QQ~~LEEDLKGYLDWITQAE
AID_{P6}-TAT Mutant	~~QQLEEDL~~KGYLDWITQAE
AID_{P7}-TAT Mutant	QQ**Q**EEDLKGYLDWITQAE
AID_{P10}-TAT Mutant	~~QQLEE~~DLKGYLDWITQA~~E~~
AID_{P11}-TAT Mutant	~~QQLEE~~DLKGYLDWITQ~~AE~~
AID_{P12}-TAT Mutant	~~QQLEE~~DLKGYLDWIT~~QAE~~
AID_{P13}-TAT Mutant	QQLEEDLKG**F**LD**AA**TQAE
AID_{P14}-TAT Mutant	QQ**E**EEDLKGYLDWITQAE
AID_{P15}-TAT Mutant	QQ**E**EED**E**KGYLDWITQAE
AID_{P16}-TAT Mutant	QQ**R**EEDLKGYLDWITQAE

[00202] *Biotinylation of original AID (no TAT):* For each reaction (a dose-response curve consisted of 6 reactions), 0.02 nM AID-TAT was incubated in biotinylation solution (0.1M MES buffer containing 1 nM amine-PEG_{11}-biotin and 0.2nM EDC) for 2 hrs at room temperature with agitation (400rpm). Affinity beads (Dynabeads M-280 Streptavidin, ThermoFisher Scientific) were then incubated with biotinylated AID peptide for 30 min at room temperature with agitation (400rpm). Afterwards, biotinylated AID-coated affinity beads were washed, initially in 0.1% BSA in PBS and then three times in PBS for 3 min.

[00203] *Pre-binding AID (no TAT) mutant peptide of interest to Cavβ2 subunit:* AID mutant peptides were serially diluted (0, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM) and incubated with 100 μg of Cavβ2 subunit from cardiac homogenate and cytoplasmic protein preparation for 2 hrs at room temperature with agitation (400rpm) as previously described (Haase H, Podzuweit T, Lutsch G, Hohaus A, Kostka S, Lindschau C, et al. Signaling from beta-adrenoceptor to L-type calcium channel: identification of a novel cardiac protein kinase A target possessing similarities to AHNAK. *FASEB J.* 1999;13(15):2161-72; Hohaus A, Poteser M, Romanin C, Klugbauer N, Hofmann F, Morano I, et al. Modulation of the smooth-muscle L-type Ca2+ channel alpha1 subunit (alpha1C-b) by the beta2a subunit: a peptide which inhibits binding of beta to the I-II linker of alpha1 induces functional uncoupling. *Biochem J.* 2000;348 Pt 3:657-65; Haase H, Striessnig J, Holtzhauer M, Vetter R, Glossmann H. A rapid procedure for the purification of cardiac 1,4-dihydropyridine receptors from porcine heart. *Eur J Pharmacol.* 1991;207(1):51-9).

[00204] *Competitive binding of AID-affinity beads and Cavβ2 subunit:* Cavβ2-AID mutant peptide dilutions were then incubated with biotinylated AID-coated affinity beads for 2 hrs at room temperature with gentle agitation (400rpm). Samples were placed on a magnetic stand to allow affinity beads to migrate to the magnet. Supernatant was removed and treated with 1x sample buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100mM DTT, 0.002% Bromophenol Blue) for 5 min at 95°C. Resulting protein was analysed by immunoblot.

[00205] Using a dose-response curve, decreased K0.5 values were found for AID_{P7}-TAT, AID_{P14}, AID_{P15} and AID_{P16} compared to AID peptide indicating a stronger binding affinity for these variants to the AID region (Figure 9). In Figure 9, n indicates number of technical replicates. P values shown compared with AID(S)-TAT as determined by the Mann-Whitney t-test. Further *in vitro* and *in vivo* experiments focussed on these 4 peptides.

Example 10 - In vitro exposure of cTnI-G203S cardiomyocytes to AID-TAT variants are more efficacious in restoring ψ_m and flavoprotein oxidation

[00206] In this experiment *wt* myocytes were pre-exposed to 0.1, 0.5 or 1 μM for 20 min and assessed changes in flavoprotein oxidation and ψ_m.

[00207] *Measurement of mitochondrial membrane potential:* The fluorescent indicator 5,5', 6,6'-tetrachloro1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was used to measure mitochondrial membrane potential (ψ_m)as previously described (Viola HM, Arthur PG, Hool LC. Transient exposure to hydrogen peroxide causes an increase in mitochondria-derived superoxide as a result of sustained alteration in L-type Ca2+ channel function in the absence of apoptosis in ventricular myocytes. *Circ Res.* 2007;100(7):1036-44). The fluorescence signal was measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Images were acquired at 2 min intervals with 50ms exposure. Metamorph 6.3

(Version 7.10.3) was used to quantify the signal by manually tracing myocytes. An equivalent region not containing cells was used as background and was subtracted. The 580 nm/535 nm ratiometric fluorescence values recorded over 6 min before and 4 min after addition of drugs were averaged and alterations in fluorescent ratios reported as the percentage increase from the basal average. To confirm that the signal was indicative of ψ_m, 40 mM NaCN was added at the end of each experiment to collapse ψ_m. In addition, individual 580 and 535 signals were assessed in each experiment to determine whether the fluorescent indicator was accurately measuring ψ_m. For calcium-free experiments, cells were exposed to calcium-free HBS (supplemented with 3 mM EGTA and 200 nM JC-1) for at least 3 h prior to measuring changes in ψ_m.

[00208] *Measurement of mitochondrial flavoprotein oxidation:* Flavoprotein autofluorescence was used to measure flavoprotein oxidation based on previously described methods (Yaniv Y, Juhaszova M, Lyashkov AE, Spurgeon HA, Sollott SJ, Lakatta EG. Ca2+-regulated-cAMP/PKA signaling in cardiac pacemaker cells links ATP supply to demand. *J Mol Cell Cardiol.* 2011;51(5):740-8). Fluorescence at excitation 480 nm and emission 535 nm was measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Fluorescent images were acquired at 1 min intervals with 200ms exposure. Metamorph 6.3 (Version 7.10.3) was used to quantify the signal by manually tracing myocytes. An equivalent region not containing cells was used as background and was subtracted. Fluorescence values recorded over 5 min before and 5 min after addition of drugs were averaged and alterations in fluorescent ratios reported as the percentage increase from the basal average. FCCP (50 µM) and NaCN (40 mM) was added at the end of each experiment to achieve a respective maximal and minimal fluorescence value indicative of maximal and minimal flavoprotein oxidation.

[00209] The $I_{ca\text{-}L}$ agonist BayK(-) was used to activate the channel and assess mitochondrial function. Consistent with previous findings, in the presence of 10 µM inactive scrambled peptide (AID(S)-TAT), BayK(-) induced an increase in flavoprotein oxidation that was comparable to the response elicited with application of BayK(-) alone (Figure 10A-C) (Viola HM, Shah AA, Johnstone VPA, Cserne Szappanos H, Hodson MP, Hool LC. Characterization and validation of a preventative therapy for hypertrophic cardiomyopathy in a murine model of the disease. *Proc Natl Acad Sci U S A.* 2020;117(37):23113-24). Additionally, exposure of *wt* myocytes to 1 µM AID-TAT peptide was found to significantly attenuate the BayK(-) response (Figure 10A). At 1 µM, peptide variants AID_{P7}-TAT, AID_{P14}-TAT and AID_{P15}-TAT were just as effective at attenuating the response in *wt* myocytes as the original AID-TAT (Figure10A). At a concentration of 0.5 µM, the original AID-TAT did not attenuate the response to BayK(-), whereas AID_{P7}-TAT, AID_{P14} and AID_{P15} were effective ($P < 0.05$). At a concentration of 0.1 µM, neither the original AID-TAT peptide nor any of the AID-TAT peptide variants were effective (Figure 10B-C). In *cTnI-G203S* myocytes, while 0.5 µM AID-TAT did not attenuate the response, AID_{P7}-TAT and

AID_{P14}-TAT significantly attenuated increases in flavoprotein oxidation resulting from BayK(-) exposure ($P < 0.01$ and $P < 0.05$ respectively) (Figure 10D). Figure 10 presents the mean +/- SEM of flavoprotein fluorescence from *cTnI.2 wt* and *cTnI-G203S* mutant myocytes before and after exposure to 10 µM BayK(-) in the presence of 1 µM AID(S)-TAT, and increasing concentrations of variant peptides as indicated. N = no. of animals, n = no. of cardiomyocytes. P values shown compared with AID(S)-TAT as determined by the Kruskal-Wallis test.

[00210] Based on results obtained from *in vitro* assessment of flavoprotein oxidation, the effect of 0.5 µM AID-TAT variants on changes in mitochondrial membrane potential (ψ_m) was further explored. Experiments were performed using JC-1 fluorescence under calcium-free conditions as previously described (Viola H, Johnstone V, Cserne Szappanos H, Richman T, Tsoutsman T, Filipovska A, et al. The L-type Ca(2+) channel facilitates abnormal metabolic activity in the cTnI-G203S mouse model of hypertrophic cardiomyopathy. J Physiol. 2016;594(14):4051-70; Viola HM, Shah AA, Johnstone VPA, Cserne Szappanos H, Hodson MP, Hool LC. Characterization and validation of a preventative therapy for hypertrophic cardiomyopathy in a murine model of the disease. Proc Natl Acad Sci U S A. 2020;117(37):23113-24). Consistent with previous studies, application of BayK(-) induced an increase in ψ_m in the presence of 10 µM AID(S)-TAT, that was comparable to BayK(-) alone, in both *wt* and *cTnI-G203S* myocytes (Figure 11A-B). In both *wt* and *cTnI-G203S* myocytes, exposure to 0.5 µM AID-TAT was found not to be effective at attenuating the response to BayK(-), however, 0.5 µM AID_{P7}-TAT, AID_{P14}-TAT and AID_{P15}-TAT significantly attenuated the response (Figure 11A-B). These data indicate that on an *in vitro* level, AID_{P7}-TAT and AID_{P14}-TAT are the most effective in normalising mitochondrial metabolic activity in *cTnI-G203S* myocytes. Figure 11 presents the mean +/- SE of JC-1 fluorescence from *cTnI.2 wt* and *cTnI-G203S* mutant myocytes before and after exposure to 10 µM BayK(-) in the presence of 1 µM AID(S)-TAT, and 0.5 µM of variant peptides as indicated. N = no. of animals, n = no. of cardiomyocytes. P values shown compared with AID(S)-TAT as determined by the Kruskal-Wallis test.

Example 11 - In vivo treatment of cTnI-G203S mice with AID-TAT variants does not alter blood pressure

Methods

[00211] Twenty to thirty week-old male mice expressing the human cTnI gene encoding the human disease-causing *cTnI-G203S* were generated and used for the *in vivo* studies. By way of background, the mice develop hallmark features of HCM between 20 and 25 weeks (Viola H, Johnstone V, Cserne Szappanos H, Richman T, Tsoutsman T, Filipovska A, et al. The L-type Ca(2+) channel facilitates abnormal metabolic activity in the cTnI-G203S mouse model of hypertrophic cardiomyopathy. *J Physiol.* 2016;594(14):4051-70; Viola HM, Shah AA, Johnstone VPA, Cserne Szappanos H, Hodson MP, Hool LC. Characterization and validation of a

preventative therapy for hypertrophic cardiomyopathy in a murine model of the disease. *Proc Natl Acad Sci U S A.* 2020;117(37):23113-24; Tsoutsman T, Chung J, Doolan A, Nguyen L, Williams IA, Tu E, et al. Molecular insights from a novel cardiac troponin I mouse model of familial hypertrophic cardiomyopathy. *J Mol Cell Cardiol.* 2006;41(4):623-32). Age-matched male mice expressing the normal human troponin I gene were used as controls and indicated as wild-type (*wt*). For *in vivo* studies, 20-week old *cTnI-G203S* mice were treated with 10 µM AID(S)-TAT (2 mg/kg) or 5 µM AID-TAT mutant peptides via intraperitoneal injection, 3 times per week for a total duration of 5 weeks. The peptide was dissolved in phosphate buffered saline solution (PBS), and the total amount of the 10µM AID-TAT mutant peptides administered over 5 weeks was 0.9mg. The total amount of the 5µM AID-TAT mutant peptides administered over 5 weeks was 0.45mg Male mice were utilized to eliminate potential differences in responses due to gender. The number of mice used is indicated by N.

[00212] Blood pressure was assessed prior to commencing the treatment regime, 1 hr after initial injection (acute) and following completion of the 5-week treatment regime. Blood pressure measurements of mice were obtained via the CODA non-invasive blood pressure system (Tail-Cuff Method, Kent Scientific Corporation). Conscious mice were placed into an appropriate size restrainer based on body weight and pre-set parameters to assess blood pressure (SOFTWARE details from Henrietta) were run for a total of 15 cycles (the first 5 cycles are undertaken for acclimatisation). An average of 10 measurements was used.

Results

[00213] Systolic and diastolic blood pressures were recorded at 3 time points, including 1 hour before the first dose, 1 hr after the first dose (acute responses), and at the end of the 5-week treatment regime (chronic responses). No significant alterations in systolic or diastolic blood pressures were observed in mice treated with any of the AID-TAT variants at any time point (Figure 12). These data indicate that the peptides do not cause vasodilatory effects or negative inotropic effects as can occur with treatment with calcium channel antagonists (eg Diltiazem). Figure 12 presents the (A-B) mean ± SEM of blood pressure measurements from *wt* and *cTnI-G203S* mice treated with 10 µM AID(S)-TAT, 5 µM AID_{P7}-TAT, 5 µM AID_{P14}-TAT, 5 µM AID_{P15}-TAT or 5 µM AID_{P16}-TAT (3x/wk/5wk). Measurements were taken before start of treatment (Pre), 1 hr after initial injection (Post 1 hr inj.) or after 5-week treatment regime (Post) as indicated. *N* = number of mice as indicated. P = ns as determined by the Kruskal-Wallis test.

Example 12 – *In vitro* toxicity

[00214] The fluorescent indicator propidium iodide (P4170; Sigma Aldrich) was used to assess AID-TAT mutant peptide toxicity *in vitro* in cardiac myocytes. Fluorescence at excitation 480 nm and emission 580 nm was measured on a Hamamatsu Orca ER digital camera attached

to an inverted Nikon TE2000-U microscope. Fluorescent images were acquired after initial 20 min pre-incubation with 1μM AID_{P7}-TAT (P7-TAT), AID_{P14}-TAT (P14-TAT), AID_{P15}-TAT (P15-TAT), or AID_{P16}-TAT (P16-TAT), and after 5 min incubation with the dye, using 50 ms exposure. No evidence of toxicity was observed.

Example 13 - In vivo treatment of cTnI-G203S mice with AID-TAT variants is not toxic

In vivo toxicity parameters

[00215] Mice were treated with 5μM AID(S)-TAT, AID_{P7}-TAT (P7-TAT), AID_{P14}-TAT (P14-TAT) or AID_{P15}-TAT (P15-TAT) or AID_{P16}-TAT 3x/wk/5wk (equalling 15 doses). The peptide was dissolved in phosphate buffered saline solution (PBS), and the total amount of the 5μM AID-TAT mutant peptides administered over 5 weeks was 0.45mg. Body weight was recorded prior to administration of each peptide dose. A 5-10% reduction in weight may indicate toxicity and warrants additional monitoring as recommended by The Animal Ethics Committee of the University of Western Australia in accordance with the Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes (NHMRC 2008). Following completion of the treatment regime, mice were anesthetised and terminal blood collected. Blood was left to coagulate in a Lithium-Heparin tube at room temperature for 20 min. Plasma was separated using centrifugation at 3000 g for 10 min at 4°C. To measure kidney toxicity, urea and creatinine concentrations were assessed using Quantichrom Urea assay kit (BioAssay Systems, Hayward, CA) and Quantichrom Creatinine assay kit (BioAssay Systems, Hayward, CA), respectively. To measure liver toxicity, alanine transaminase (ALT) and aspartate transaminase (AST) concentrations were measured using Alanine Transaminase assay kit (BioAssay Systems, Hayward, CA) and Aspartate Transaminase assay kit (BioAssay Systems, Hayward, CA), respectively. Assays were performed as per manufacturer's instructions, using a spectrophotometer (CLARIOstar, BMG LABTECH).

Results

[00216] Following completion of the 5-week *in vivo* treatment regime, terminal plasma was collected and assessed for kidney and liver toxicity. No significant alterations in urea, or alanine transaminase (ALT) were observed compared to plasma assessed from AID(S)-TAT-treated *wt* and *cTnI-G203S* mice, indicating treatment with AID-TAT variants does not cause kidney or liver toxicity (Figure 13A-B). Additionally, no significant reduction in body weight was observed over the duration of all treatment protocols (Figure 13C). Overall, these data indicate that *in vivo* treatment of *cTnI-G203S* mice with AID-TAT variants is not toxic. Figure 13 presents the mean ± SEM of urea (A) and ALT (B) concentrations from plasma from *wt* and *cTnI-G203S* mice treated with 10 μM AID(S)-TAT, 5μM AID_{P7}-TAT or 5 μM AID_{P14}-TAT or AID_{P15}-TAT or AID_{P16}-TAT (3x/wk/5wk) as indicated. *N* = number of mice. P = ns as determined by ANOVA and Kruskal-Wallis tests (A-B). (C) Mean ± SEM of body weight recorded from *cTnI-G203S* mice treated with

5 μM AID(S)-TAT, 5 μM AID_{P7}-TAT, 5 μM AID_{P14}-TAT, 5 μM AID_{P15}-TAT or 5 μM AID_{P16}-TAT (3x/wk/5wk, equalling 15 doses in total) as indicated. *N* = number of mice as indicated. Red line denotes 5% weight loss threshold.

Example 14 - *In vivo* treatment of pre-cardiomyopathic cTnI-G203S mice with AID-TAT variants slows the progression of fibrosis

Methods

[00217] Following completion of treatment regimes, mouse hearts were extracted and prepared for Masson's Trichchrome staining based on previously described methods with minor modifications (Van De Vlekkert D, Machado E, d'Azzo A. Analysis of Generalized Fibrosis in Mouse Tissue Sections with Masson's Trichrome Staining. *Bio Protoc.* 2020;10(10):e3629). Hearts were washed in PBS and fixed overnight in 10% buffered formalin saline (FSAL-5L; Hurst Scientific, AUS). After fixation, hearts were washed 3 x 10min in PBS and incubated overnight in 30% sucrose solution (in PBS). After overnight incubation, hearts were then coated in Tissue-Tek OCT compound (IA018; ProSciTech, AUS) and embedded into 4cm-deep cylindrical alfoil moulds containing ~2ml OCT. The moulds were lowered into an Isopentane-filled tube in a liquid nitrogen-containing canister to allow slow freezing of the heart tissue. Tissue samples were stored at -80°C until cryosectioning.

[00218] Hearts were cut into 7 uM sections and collected on warm (RT) subbed Superfrost slides and stored at -80°C until staining.

[00219] To achieve thermal equilibration prior to Masson's trichrome staining, slides were transferred from -80°C to -20°C overnight. After overnight incubation, slides were transferred from -20°C to RT for 1 hr before 1 x 2min wash in running tap H20 to remove OCT and secondary fixation in Bouin's solution overnight. Slides were washed 1-3 x 5min in running H_20 for complete removal of Bouin's solution. Slides were stained using the following steps: Celestine Blue (5 min), running tap H_20 (2min), Harris Hemotoxylin (5 min), running tap H_20 (2min), distilled H_20 (2 min), Ponceau-Fuchsin (10 mins), distilled H_20 (20 sec), Phosphomolybdic acid (4 min), Aniline Blue (1 min) and 1% acetic acid (1 min). Slides were then blot dried with filter paper and dehydration, clearing and mounting steps were done as previously outlined (Van De Vlekkert D, Machado E, d'Azzo A. Analysis of Generalized Fibrosis in Mouse Tissue Sections with Masson's Trichrome Staining. Bio Protoc. 2020;10(10):e3629).

[00220] To observe the levels of cardiac fibrosis in mice, a minimum of 15 sections of microscopic fields for each animal were selected randomly for histological analysis. Images of heart sections were obtained using a Nikon upright light microscope (DS-Qi2, Japan) at 20x magnification and analysed using ImageJ (NIH). The ratio of blue area to the total myocardium

area in Masson's trichrome-stained sections was used to analyse collagen accumulation. Two researchers blindly performed analyses of histological features.

Results

[00221] It is well-established that myocardial fibrosis is a characteristic feature of HCM (O'Connell TD, Rodrigo MC, Simpson PC. Isolation and culture of adult mouse cardiac myocytes. *Methods Mol Biol.* 2007;357:271-96). Myocardial fibrosis is the collagen-rich extracellular matrix that is significantly increased in hypertrophic hearts (Haase H, Striessnig J, Holtzhauer M, Vetter R, Glossmann H. A rapid procedure for the purification of cardiac 1,4-dihydropyridine receptors from porcine heart. *Eur J Pharmacol.* 1991;207(1):51-9). Increased collagen accumulation and fibrosis is maladaptive and is associated with impaired cardiac relaxation, thereby increasing the risk of heart failure (Tang H, Viola HM, Filipovska A, Hool LC. Ca(v)1.2 calcium channel is glutathionylated during oxidative stress in guinea pig and ischemic human heart. *Free Radic Biol Med.* 2011;51(8):1501-11).

[00222] Here we assessed the efficacy of treating 20-week-old pre-hypertrophic *cTnI-G203S* mice with 5 μM AID-TAT variant peptides (3x/wk/5wk) on the development and progression of fibrosis. The peptide was dissolved in phosphate buffered saline solution (PBS), and the total amount of the 5μM AID-TAT variant peptides administered over 5 weeks was 0.45mg. Following the 5-week treatment regime, murine hearts were extracted, fixed, and embedded in OCT for cryosectioning. The collagen deposition in cardiac tissue sections was visualised using Masson's Trichrome staining indicating muscle tissue (stained red), nuclei (stained purple/black) and collagen (stained blue). Images were acquired using a Nikon Upright Microscope and collagen volume fraction (%) was determined using ImageJ colour thresholding. Consistent with the development of HCM, we found that left ventricular posterior wall collagen volume fraction (CVF, %) was significantly increased in *cTnI-G203S* mice treated with 10 μM AID(S)-TAT versus *wt* myocytes (Figure 14). This was significantly decreased, in *cTnI-G203S* mice treated with 5 μM AID_{P7}-TAT (P7-TAT) (Figure 14). Figure 14 presents: (A) Representative sections of left ventricular cardiac tissue. Scale bar = 50 μM. (B) LV collagen volume fraction (%) from sectioned hearts of 25 week old *wt* and 25 week old *cTnI-G203S* mice following treatment with 10 μM AID(S)-TAT, 5 μM AID_{P7}-TAT or 5 μM AID_{P14}-TAT (3x/wk/5wk). *N* = number of mice as indicated. Values reported as mean ± SEM; P values compared with *cTnI-G203S* AID(S)-TAT as determined by the Kruskal-Wallis test.

Example 15 - *In vivo* treatment of pre-cardiomyopathic cTnI-G203S mice with AID peptide variants prevents the development of hypertrophic cardiomyopathy

Methods

[00223] Echocardiographic studies to measure left ventricular function were performed on mice under light methoxyflurane anaesthesia with the use of an i13L probe on a Vivid™ 7 IQ ultrasound system (GE Healthcare, Little Chalfont, UK) as previously described (Viola H, Johnstone V, Cserne Szappanos H, Richman T, Tsoutsman T, Filipovska A, et al. The L-type Ca(2+) channel facilitates abnormal metabolic activity in the cTnI-G203S mouse model of hypertrophic cardiomyopathy. *J Physiol.* 2016;594(14):4051-70; Viola HM, Shah AA, Johnstone VPA, Cserne Szappanos H, Hodson MP, Hool LC. Characterization and validation of a preventative therapy for hypertrophic cardiomyopathy in a murine model of the disease. *Proc Natl Acad Sci U S A.* 2020;117(37):23113-24; Viola HM, Johnstone VPA, Cserne Szappanos H, Richman TR, Tsoutsman T, Filipovska A, et al. The Role of the L-Type Ca(2+) Channel in Altered Metabolic Activity in a Murine Model of Hypertrophic Cardiomyopathy. *JACC Basic Transl Sci.* 2016;1(1-2):61-72). Each *N* represents the average of quantitative measurements from *wt* or *cTnI-G203S* mice for each treatment group.

Results

[00224] Echocardiographic assessment of *cTnI-G203S* mice reveal the development of HCM characteristics from approximately 21 weeks of age (Viola H, Johnstone V, Cserne Szappanos H, Richman T, Tsoutsman T, Filipovska A, et al. The L-type Ca(2+) channel facilitates abnormal metabolic activity in the cTnI-G203S mouse model of hypertrophic cardiomyopathy. *J Physiol.* 2016;594(14):4051-70; Viola HM, Shah AA, Johnstone VPA, Cserne Szappanos H, Hodson MP, Hool LC. Characterization and validation of a preventative therapy for hypertrophic cardiomyopathy in a murine model of the disease. *Proc Natl Acad Sci U S A.* 2020;117(37):23113-24). Consistent with previous findings, 20-week-old *cTnI-G203S* mice treated with 10 µM AID(S)-TAT until 25-weeks of age (10 µM, 3x/wk/5wk) (peptide was dissolved in phosphate buffered saline solution (PBS), and the total amount of the 10µM AID-TAT mutant peptides administered over 5 weeks was 0.9mg), developed a significant decrease in left-ventricular end diameter (LVEDd and LVEDs), and a significant increase in fractional shortening (FS), ejection fraction (EF), left-ventricular posterior wall thickness (LVPWd and LVPWs) and interventricular septum (IVDs), compared to *wt* age-matched mice treated with AID(S)-TAT (Figure 15). Additionally, age-matched*cTnI-G203S* mice treated with 10 µM AID-TAT demonstrated restored FS, EF, IVSd, LVEDd, LVEDs and LVPWd versus AID(S)-TAT treated *cTnI-G203S* mice (Figure 15). Figure 15 presents: LVEDd, left ventricular end diameter during diastole; LVEDs, left ventricular end diameter during systole; FS, fractional shortening; EF, ejection fraction; LVPWd, left ventricular posterior wall in diastole; LVPWs, left ventricular posterior wall in systole; IVDd,

intraventricular septum in diastole; IVDs, intraventricular septum in systole; HR, heart rate. *N* = number of mice. Values reported as mean ± SEM; P values compared with *cTnI-G203S* AID(S)-TAT as determined by the Kruskal-Wallis test.

[00225] The efficacy of treating 20-week-old pre-hypertrophic *cTnI-G203S* mice with 5 µM AID-TAT variants was examined using the same treatment protocol (3x/wk/5wk). We performed echocardiography on mice before and after the 5-week treatment to assess cardiac morphology and function. Treatment of *cTnI-G203S* mice with 5 µM AID_{P7}-TAT (P7-TAT) effectively restored FS, EF, IVSs, LVPWd and LVPWs versus AID(S)-TAT, in a similar manner to 10 µM AID-TAT (Figure 15). A trend toward increased LVEDd and LVEDs, and decreased IVDS was observed (Figure 15). Interestingly, 25-week-old *cTnI-G203S* mice treated with 5 µM AID_{P7}-TAT (SEQ ID 4 - TAT) demonstrate a significant decrease in LVPWs and IVSs that was not achieved with 10 µM AID-TAT (Figure 15). Treatment of *cTnI-G203S* mice with 5 µM AID_{P14}-TAT (P14-TAT) effectively restored all echocardiographic parameters (except LVEDd). This included some that were not altered by 10 µM AID-TAT (FS, EF, IVSd, IVSs, LVEDs, LVPWd and LVPWs). A trend toward increased LVEDd was observed. In other cohorts, 5 µM AID_{P15}-TAT (P15-TAT) effectively restored FS, EF, IVSd, IVSs and LVPWs, while treatment with 5 µM AID_{P16}-TAT (P16-TAT) demonstrated restored IVSd and IVSs only.

[00226] The effect of treatment of the mice with peptides on the development of hypertrophy assessed as changes in heart weight to body weight ratio was also examined. We found that treatment with 5 µM AID_{P7}-TAT (P7-TAT) or 5 µM AID_{P14}-TAT (P14-TAT) significantly decreased heart weight/body weight indicating a regression in the hypertrophy (Figure 16). Figure 16 presents the mean ± SEM of heart weight to body weight ratio measurements from *wt* and *cTnI-G203S* mice treated with 10 µM AID(S)-TAT, 10 µM AID-TAT, 5 µM AID_{P7}-TAT, 5 µM AID_{P14}-TAT, 5 µM AID_{P15}-TAT or 5 µM AID_{P16}-TAT. *n* = number of mice as indicated. *P < 0.05, **P <0.01 compared with *cTnI-G203S* AID(S)-TAT as determined by Kruskal-Wallis tests.

[00227] Overall, this data indicates that treatment prior to the development of hypertrophy with the peptides of the invention prevents the development of hypertrophy, regresses fibrosis and improves cardiac function in *cTnI-G203S* mice. The variant peptides are more efficacious than the original AID-TAT in the prevention of hypertrophy.

CLAIMS:

1. A peptide comprising the amino acid sequence:

$QQX_1EEDX_2KGYLDWITQAE$

wherein X_1 is an amino acid selected from the group comprising: Q, E or R; and

wherein X_2 is an amino acid selected from the group comprising: L or E;

or a variant thereof, wherein the peptide does not consist of SEQ ID NO: 1.

2. A peptide comprising any one of the amino acid sequences of SEQ ID NO: 4 – 7 or a variant thereof, wherein the peptide does not consist of SEQ ID NO: 1.

3. A peptide comprising:

a peptide portion comprising the amino acid sequence of claim 1 or claim 2; and

a peptide portion comprising the amino acid sequence:

RKKRRQRRRZaa

wherein Zaa is a 6-amino hexanoic acid

or a variant thereof.

4. A peptide according to claims 1 to 2 wherein the peptide comprises an amino acid sequence selected from the group of: at least 75%; at least 80%; at least 85%; at least 90%; at least 95%; at least 96%; at least 97%; at least 98%; and at least 99% sequence homology to a peptide comprising SEQ ID NO: 2.

5. A peptide according to claim 4 wherein the peptide comprises an amino acid sequence comprising one or more conservative amino acid substitutions to SEQ ID NO: 2 selected from the suitable amino acid substitutions set out in Table 3.

6. A method for modulating movement of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject, comprising administering to the subject a peptide according to any one of claims 1 to 5.

7. A method for modulating binding of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject, comprising administering to the subject a peptide according to any one of claims 1 to 5.

8. A method for modulating a L-type Ca^{2+} channel in a cardiac cell of a subject, comprising the step of administering to the subject a peptide according to any one of claims 1 to 5.

9. A method for treating, preventing or reducing myocardial damage and/or oxidative stress in the heart of a subject, comprising the step of administering to the subject a peptide according to any one of claims 1 to 5.

10. The method according to claim 9, wherein the myocardial damage comprises cardiac hypertrophy.

11. The method according to claim 9, wherein intracellular Ca^{2+} levels are reduced or substantially maintained in a cardiac cell in the heart of the subject.

12. A method for treating or preventing cardiac hypertrophy in a subject comprising the step of administering to the subject a peptide according to any one of claims 1 to 5.

13. A method for slowing the progression of myocardial fibrosis in a subject comprising the step of administering to the subject a peptide according to any one of claims 1 to 5.

14. Use of a peptide according to any one of claims 1 to 5 for modulating movement of a beta subunit of the L-type Ca^{2+} channel in a cardiac cell of a subject.

15. Use of a peptide according to any one of claims 1 to 5 for modulating binding to a L-type Ca^{2+} channel alpha-interacting domain in a cardiac cell in the heart of a subject.

16. Use of a peptide according to any one of claims 1 to 5 for modulating a L-type Ca^{2+} channel in a cardiac cell of a subject.

17. Use of a peptide according to any one of claims 1 to 5 for treating, preventing or reducing myocardial damage and/or oxidative stress in the heart of a subject during and/or following reperfusion.

18. Use of a peptide according to any one of claims 1 to 5 for slowing the progression of myocardial fibrosis in a subject.

19. A polynucleotide encoding a peptide according to any one of claims 1 to 5.

20. Use of a peptide according to any of claims 1 to 5 in the manufacture of a medicament for the treatment of reperfusion injury in the heart of a subject.

21. Use of a peptide according to any of claims 1 to 5 in the manufacture of a medicament treat, prevent or ameliorate myocardial damage and/or oxidative stress in the heart of a subject.

22. A pharmaceutical, prophylactic or therapeutic composition comprising: a peptide of any of claims 1 to 5, and one or more pharmaceutically acceptably carriers and/or diluents.

23. A kit to treat, prevent or ameliorate the effects of myocardial damage and/or oxidative stress in the heart of a subject, wherein the kit comprises at least a peptide according to any one of claims 1 to 5, packaged in a suitable container, together with instructions for its use.

5

Figure 1

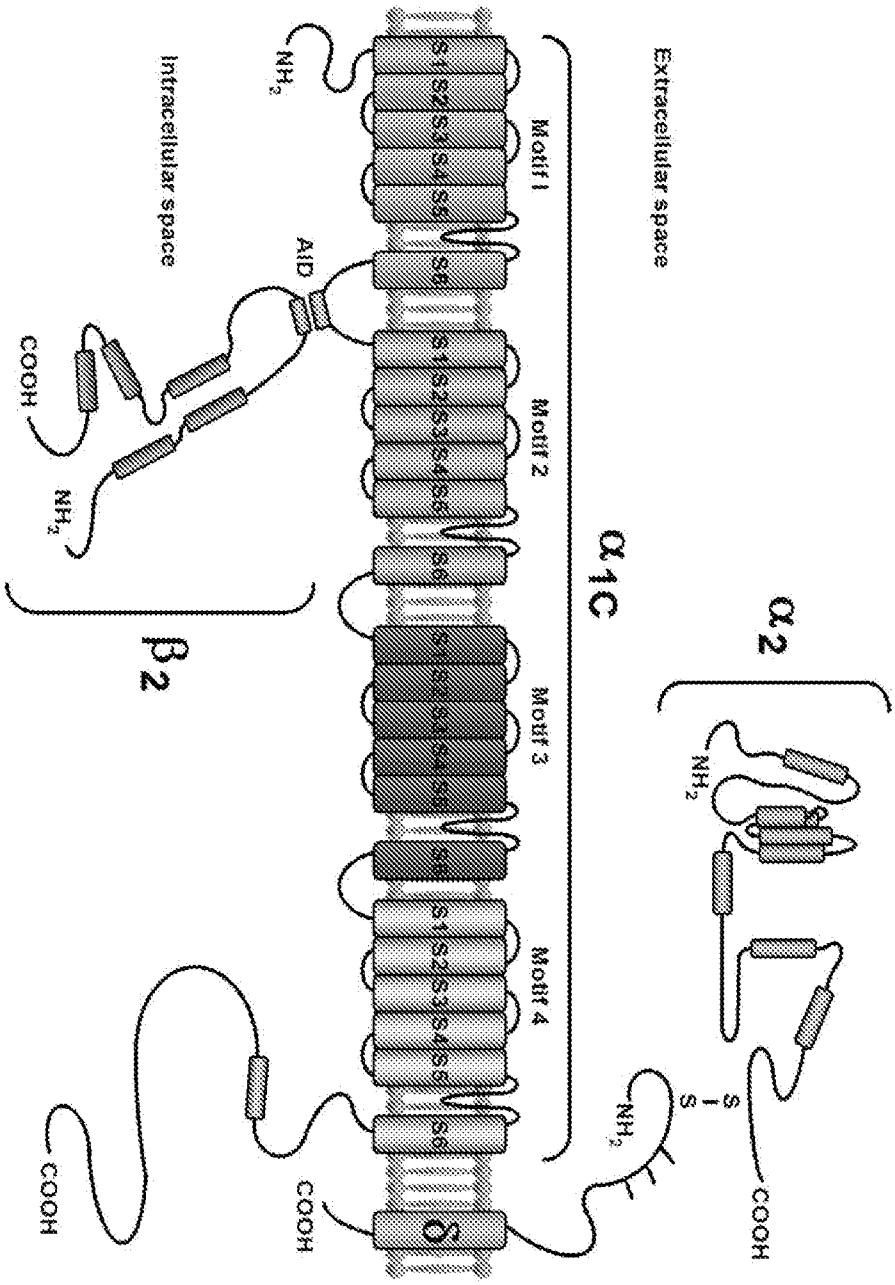

Intracellular space
Extracellular space
AID
COOH
NH2
Motif 1
Motif 2
Motif 3
Motif 4
α1C
β2
α2
S
S

Figure 2

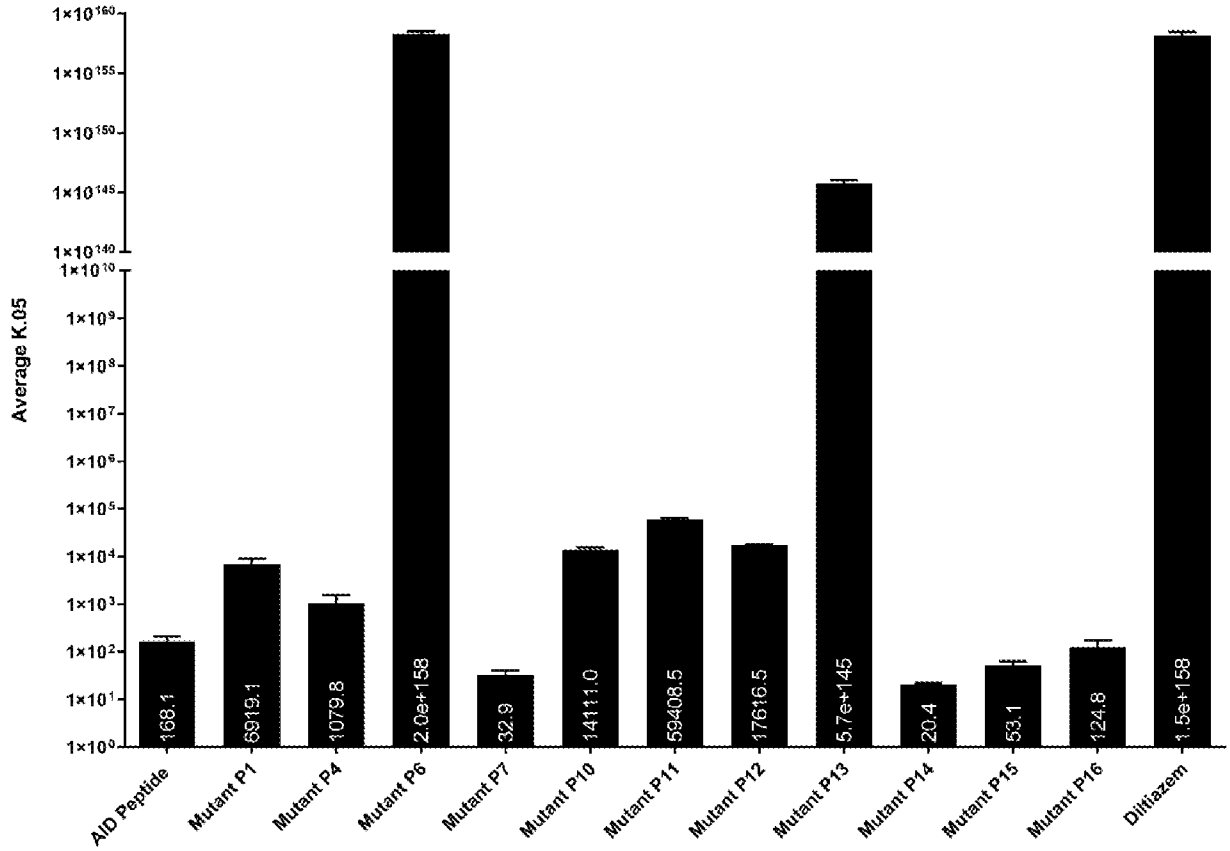

Average K.05
1×10^160
1×10^155
1×10^150
1×10^145
1×10^140
1×10^10
1×10^9
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
1×10^2
1×10^1
1×10^0
168.1
6919.1
1079.8
2.0e+158
32.9
14111.0
59408.5
17616.5
5.7e+145
20.4
53.1
124.8
1.5e+158
AID Peptide
Mutant P1
Mutant P4
Mutant P6
Mutant P7
Mutant P10
Mutant P11
Mutant P12
Mutant P13
Mutant P14
Mutant P15
Mutant P16
Diltiazem

Figure 3(a)

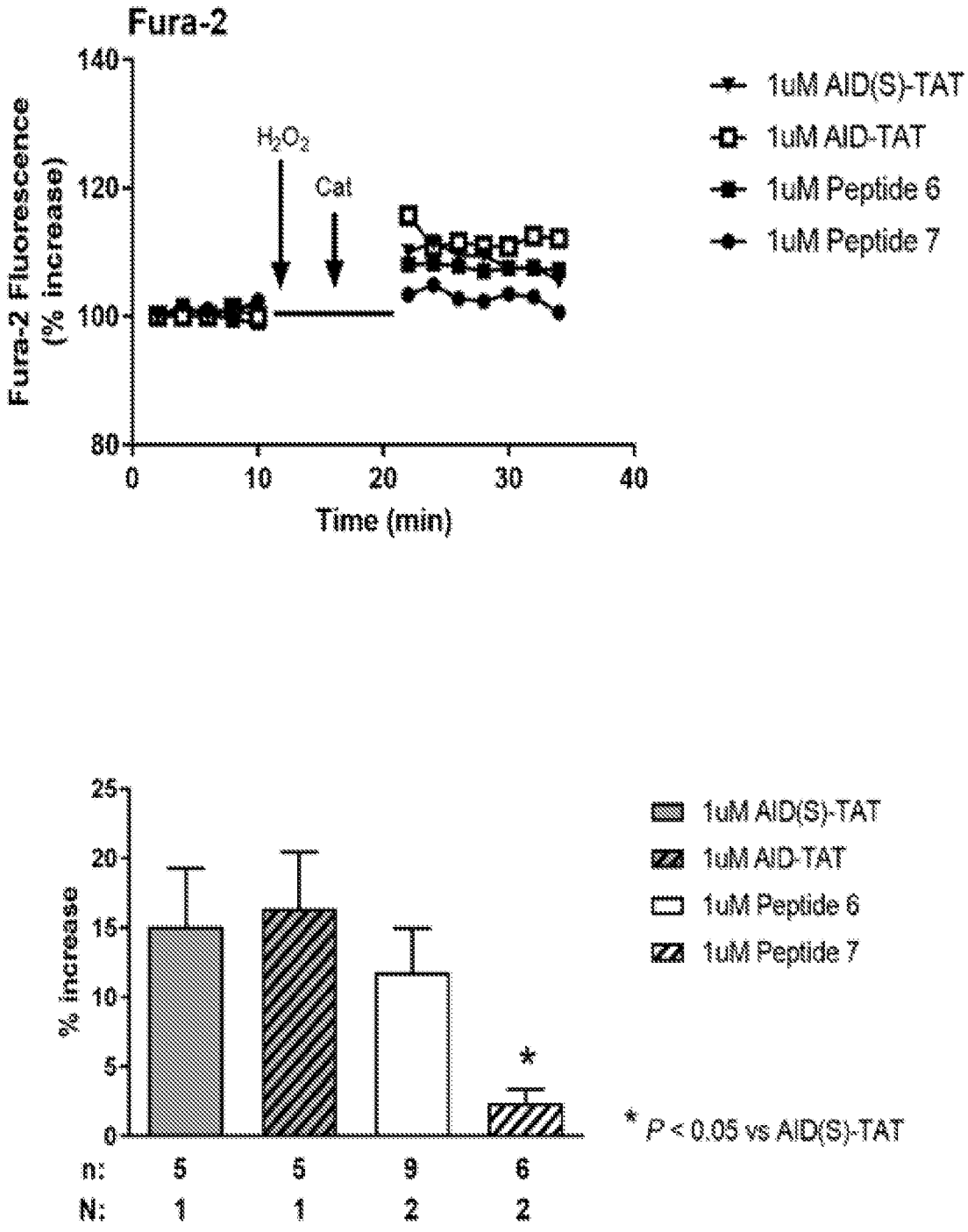

Fura-2
Fura-2 Fluorescence (% increase)
140
120
100
80
0
10
20
30
40
Time (min)
H_2O_2
Cat
1uM AID(S)-TAT
1uM AID-TAT
1uM Peptide 6
1uM Peptide 7
% increase
25
20
15
10
5
0
n: 5 5 9 6
N: 1 1 2 2
*
* P < 0.05 vs AID(S)-TAT

Figure 3(b)

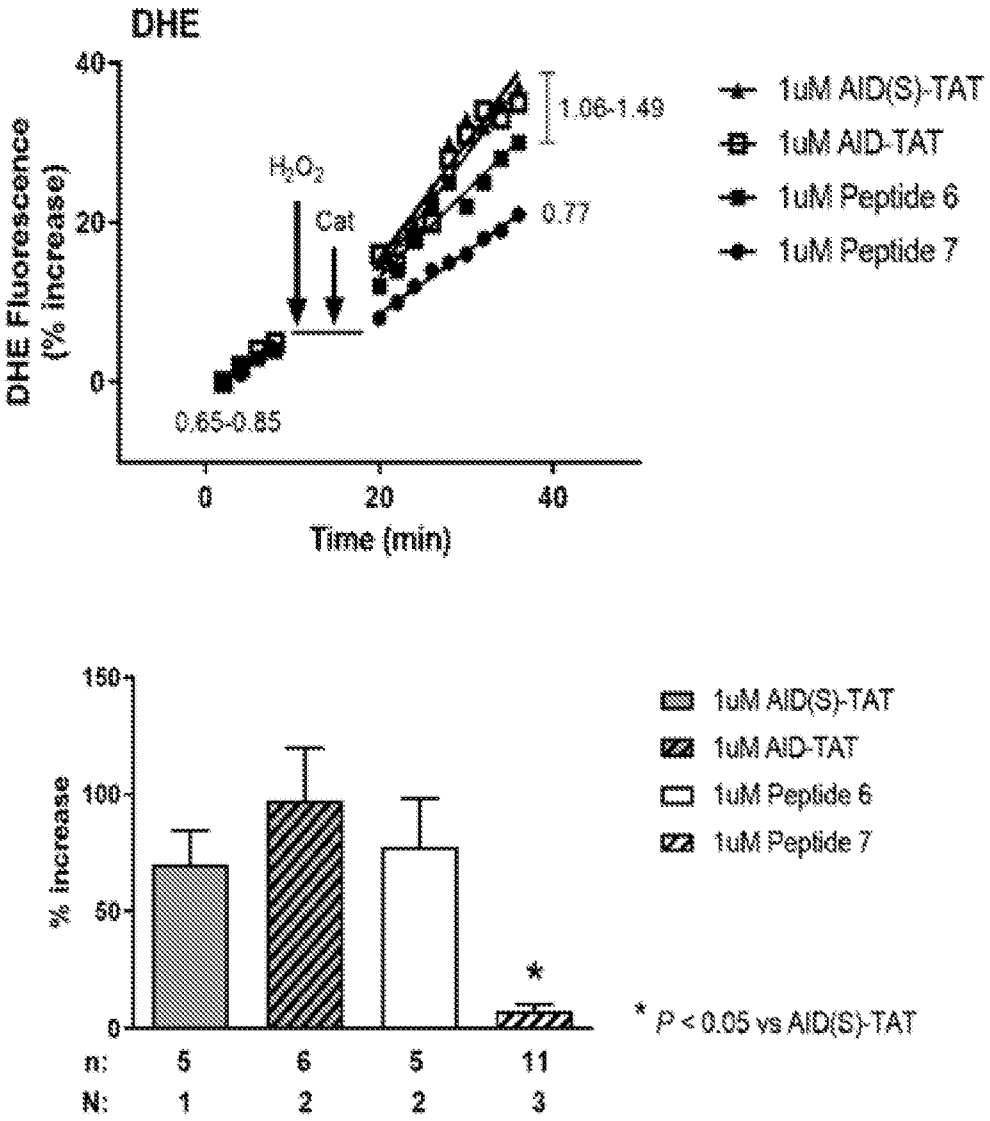
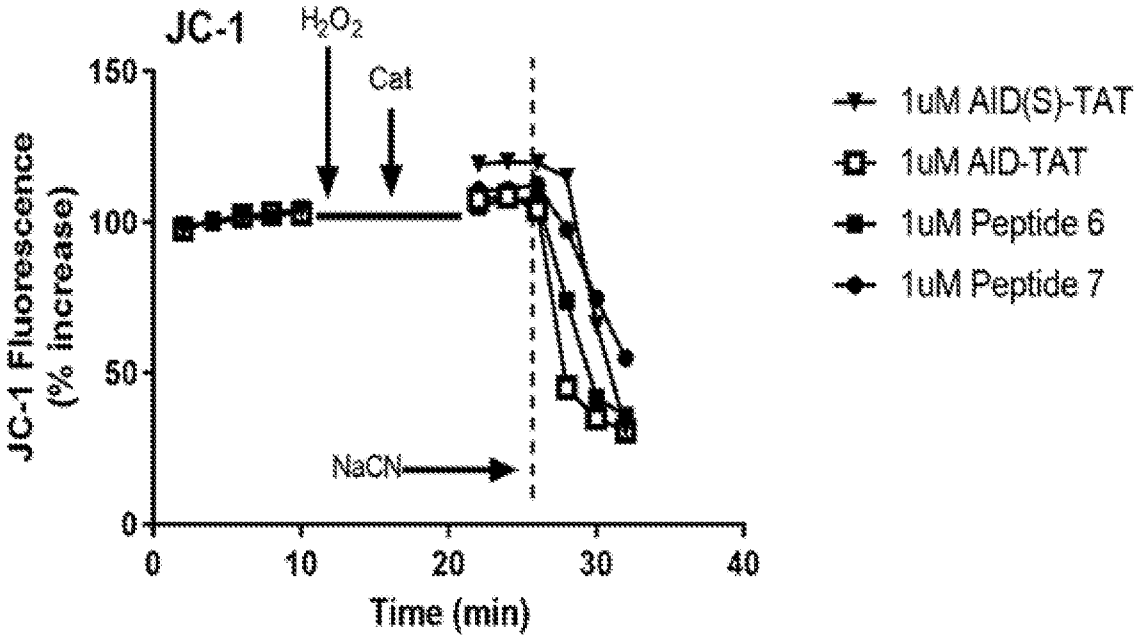

DHE
DHE Fluorescence (% increase)
40
20
0
1.06-1.49
0.77
H_2O_2
Cat
0.65-0.85
0
20
40
Time (min)
1uM AID(S)-TAT
1uM AID-TAT
1uM Peptide 6
1uM Peptide 7
% increase
150
100
50
0
*
n: 5 6 5 11
N: 1 2 2 3
* P < 0.05 vs AID(S)-TAT

Figure 3(c)


JC-1
H_2O_2
Cat
NaCN
JC-1 Fluorescence (% increase)
Time (min)
0
50
100
150
0
10
20
30
40
1uM AID(S)-TAT
1uM AID-TAT
1uM Peptide 6
1uM Peptide 7

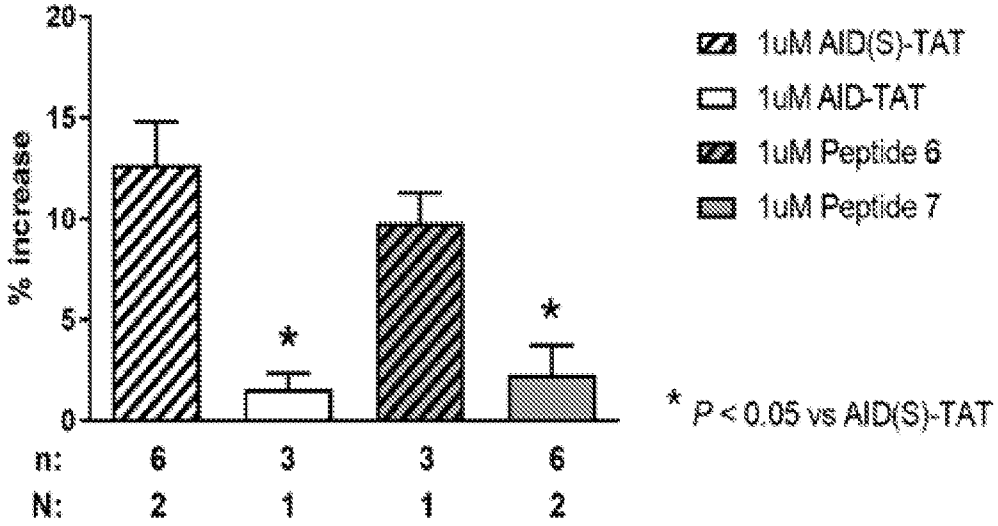

% increase
0
5
10
15
20
n: 6 3 3 6
N: 2 1 1 2
*
*
1uM AID(S)-TAT
1uM AID-TAT
1uM Peptide 6
1uM Peptide 7
* P < 0.05 vs AID(S)-TAT

Figure 4

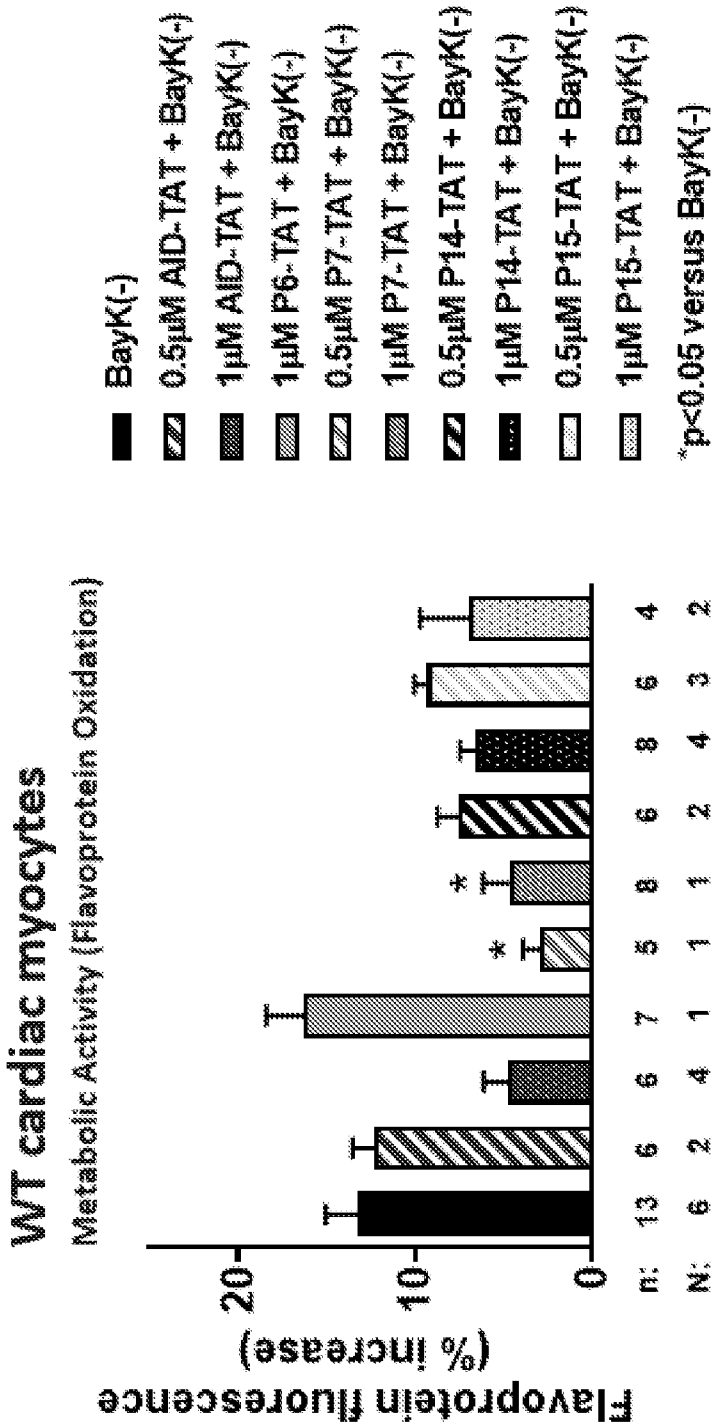

WT cardiac myocytes
Metabolic Activity (Flavoprotein Oxidation)
Flavoprotein fluorescence
(% increase)
20
10
0
n: 13 6 6 4 7 1 5 1 8 1 6 2 8 4 6 3 4 2
N: 6 2 6 6 1 1 1 1 2 1 1 1 1 1 1 1 1 1
*
*
BayK(-)
0.5μM AID-TAT + BayK(-)
1μM AID-TAT + BayK(-)
1μM P6-TAT + BayK(-)
0.5μM P7-TAT + BayK(-)
1μM P7-TAT + BayK(-)
0.5μM P14-TAT + BayK(-)
1μM P14-TAT + BayK(-)
0.5μM P15-TAT + BayK(-)
1μM P15-TAT + BayK(-)
*p<0.05 versus BayK(-)

Figure 5

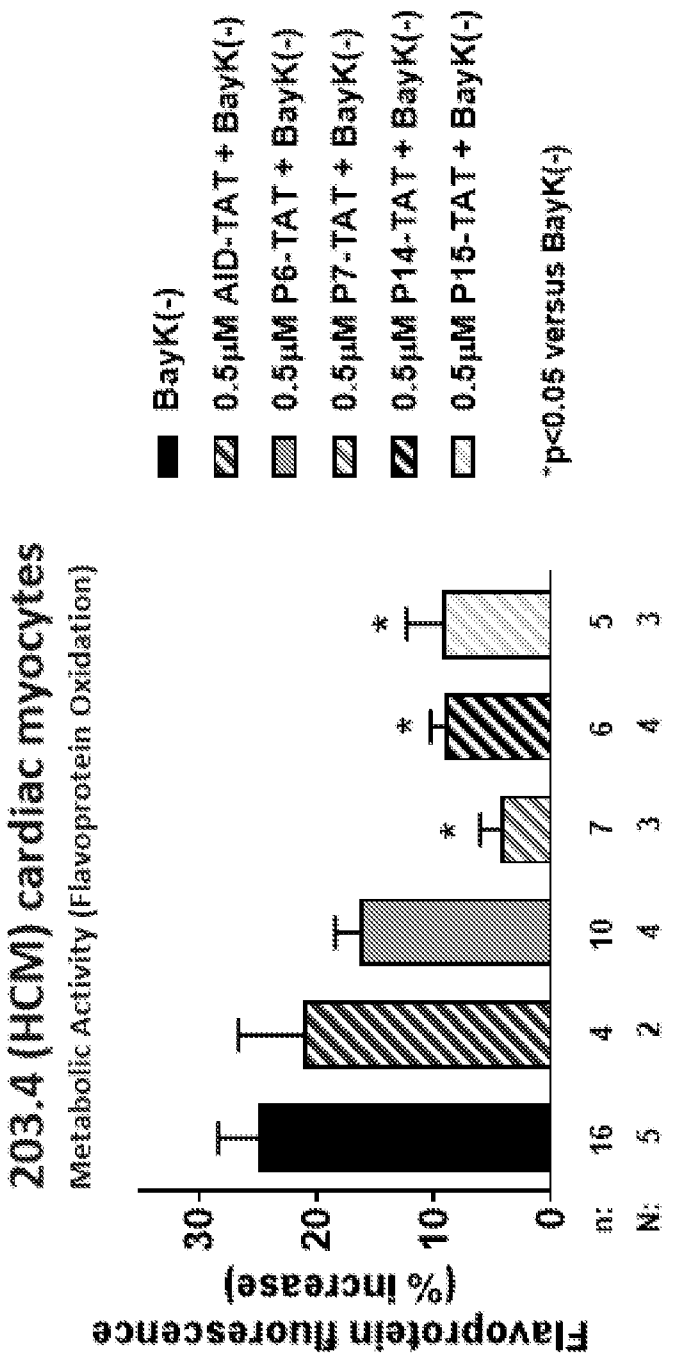

203.4 (HCM) cardiac myocytes
Metabolic Activity (Flavoprotein Oxidation)
Flavoprotein fluorescence
(% increase)
30
20
10
0
n: 16 4 10 7 6 5
N: 5 2 4 3 4 3
*
*
*
BayK(-)
0.5µM AID-TAT + BayK(-)
0.5µM P6-TAT + BayK(-)
0.5µM P7-TAT + BayK(-)
0.5µM P14-TAT + BayK(-)
0.5µM P15-TAT + BayK(-)
*p<0.05 versus BayK(-)

Figure 6(a)

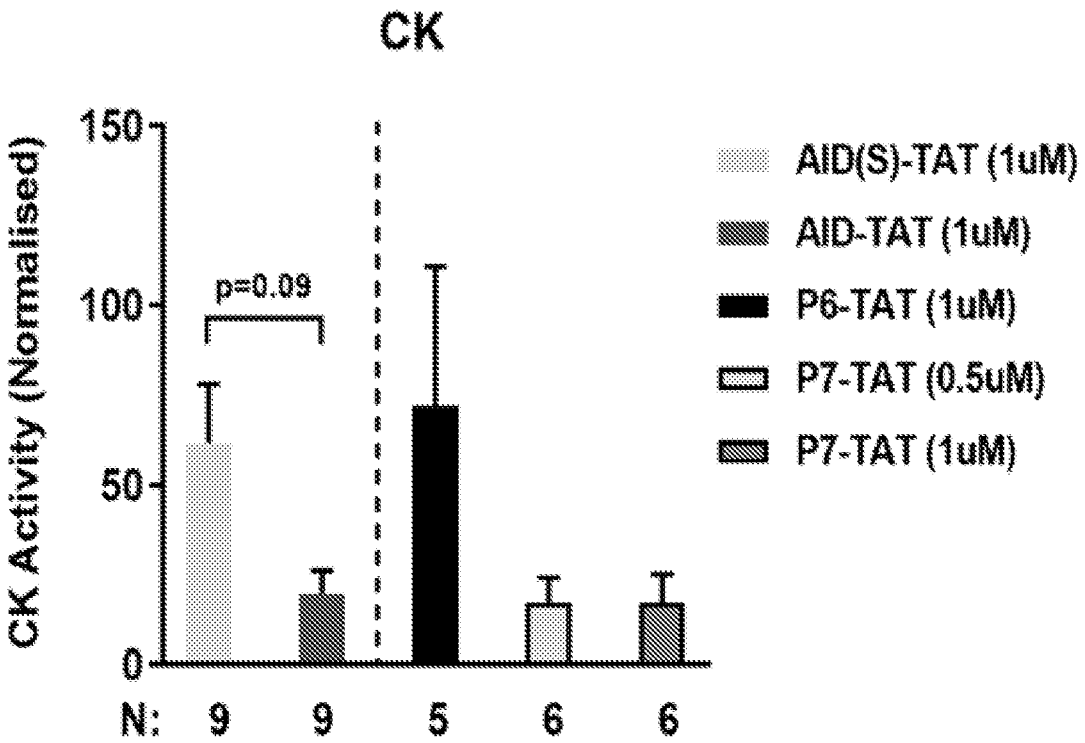

CK
CK Activity (Normalised)
150
100
50
0
p=0.09
N: 9 9 5 6 6
AID(S)-TAT (1uM)
AID-TAT (1uM)
P6-TAT (1uM)
P7-TAT (0.5uM)
P7-TAT (1uM)

Figure 6(b)

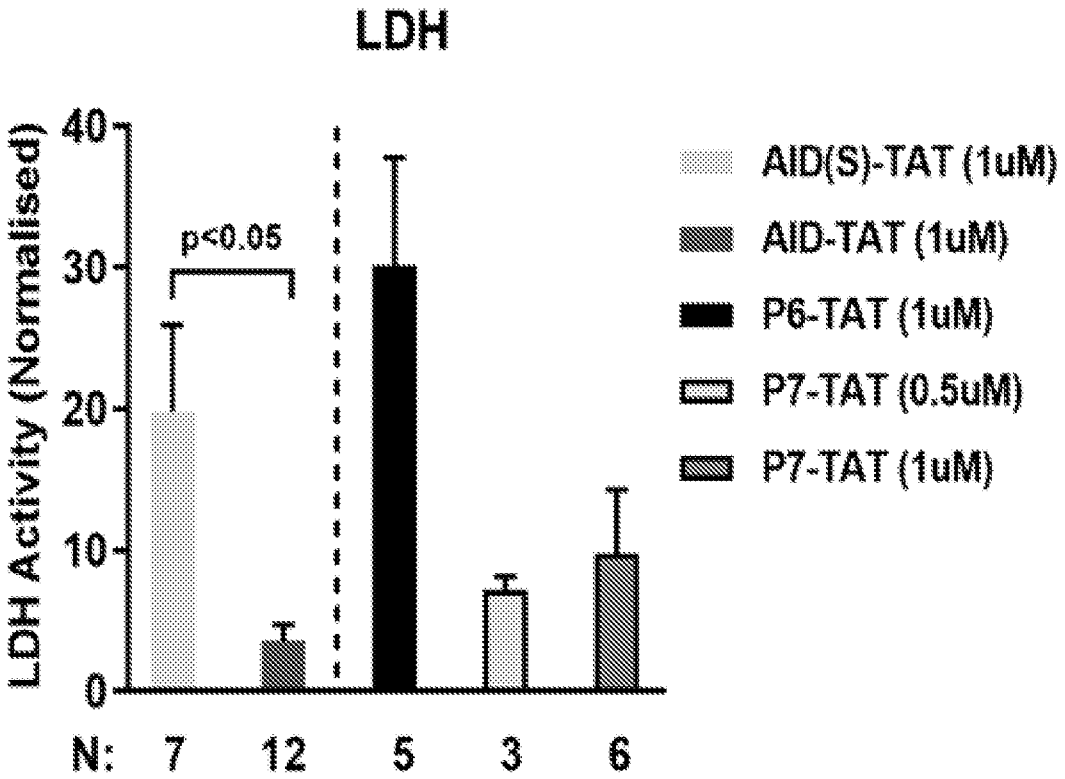

LDH
LDH Activity (Normalised)
40
30
20
10
0
p<0.05
N: 7 12 5 3 6
AID(S)-TAT (1uM)
AID-TAT (1uM)
P6-TAT (1uM)
P7-TAT (0.5uM)
P7-TAT (1uM)

Figure 6(c)

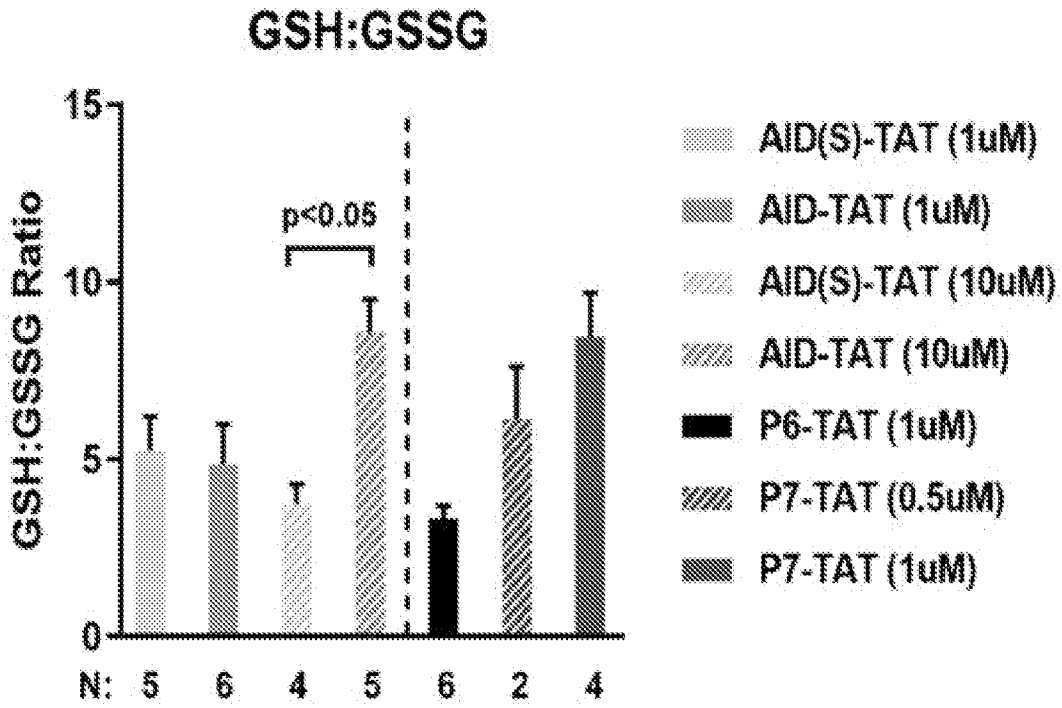

GSH:GSSG
GSH:GSSG Ratio
15
10
5
0
p<0.05
N: 5 6 4 5 6 2 4
AID(S)-TAT (1uM)
AID-TAT (1uM)
AID(S)-TAT (10uM)
AID-TAT (10uM)
P6-TAT (1uM)
P7-TAT (0.5uM)
P7-TAT (1uM)

Figure 7

Sequence IDs

SEQ ID NO	Sequence	Description
SEQ ID NO: 1	QQLEEDLKGYLDWITQAE	AID Peptide
SEQ ID NO: 2	QQX_1EEDX_2KGYLDWITQAE	Variant AID Peptide of the invention
SEQ ID NO: 3	RKKRRQRRRZaa	TAT Peptide
SEQ ID NO: 4	QQQEEDLKGYLDWITQAE	Variant P7
SEQ ID NO: 5	QQEEEDLKGYLDWITQAE	Variant P14
SEQ ID NO: 6	QQEEEDEKGYLDWITQAE	Variant P15
SEQ ID NO: 7	QQREEDLKGYLDWITQAE	Variant P16
SEQ ID NO: 8	GRKKR	Nuclear localisation signal of TAT
SEQ ID NO: 9	YGRKKRRQRRR	Protein transduction domain of TAT
SEQ ID NO: 10	QKILGEWDLAQYTDQELE	AID scrambled peptide
SEQ ID NO: 11	DLKGYLDWITQAE	Variant P1
SEQ ID NO: 12	LEEDLKGYLDWITQAE	Variant P4
SEQ ID NO: 13	KGYLDWITQAE	Variant P6
SEQ ID NO: 14	DLKGYLDWITQA	Variant P10
SEQ ID NO: 15	DLKGYLDWITQ	Variant P11
SEQ ID NO: 16	DLKGYLDWIT	Variant P12
SEQ ID NO: 17	QQLEEDLKGFLDAATQAE	Variant P13

Figure 8

Echocardiographic parameters of cTnI-G203S mice administered 10 µM AID(S)-TAT or AID-TAT.

LVEDD (mm)	LVESD (mm)	FS (%)	LVDPW (mm)	LVSPW (mm)	IVDS (mm)	IVSS (mm)	HW:BW (mg/g)	HR (bpm)
PRE HCM TREATMENT (from 20 weeks)								
25 week old *wt*, AID(S)-TAT (n=4)								
3.56	2.31	35.08	1.26	1.42	0.76	0.86	4.33	450
± 0.12	± 0.06	± 1.11	± 0.08	± 0.05	± 0.01	± 0.01	± 0.07	± 37
25 week old *cTnI-G203S*, AID(S)-TAT (n=7)								
3.08*	1.62*	47.39*	1.31	1.43	0.85*	0.96*	5.14*	450
± 0.06	± 0.05	± 0.81	± 0.03	± 0.04	± 0.01	± 0.02	± 0.10	± 22
25 week old *cTnI-G203S*, AID-TAT (n=6)								
3.33†	2.06†	38.26†	1.23	1.37	0.73†	0.82†	4.70†	452
± 0.06	± 0.08	± 1.61	± 0.03	± 0.03	± 0.00	± 0.01	± 0.17	± 26
POST HCM TREATMENT (from 30 weeks)								
35 week old *cTnI-G203S*, AID-TAT (n=5)								
3.18	1.67	48.48	1.26	1.43	0.95	1.05	5.81	450
± 0.07	± 0.05	± 1.07	± 0.03	± 0.06	± 0.03	± 0.02	± 0.21	± 19

LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; FS, fractional shortening; LVDPW, left ventricular posterior wall in diastole; LVSPW, left ventricular posterior wall in systole; IVDS, intraventricular septum in diastole; IVSS, intraventricular septum in systole. Values reported as mean ± SEM; *$P < 0.05$ compared to *wt* AID(S)-TAT, †$P < 0.05$ compared with *cTnI-G203S* AID(S)-TAT as determined by Kruskal-Wallis tests.

Figure 9

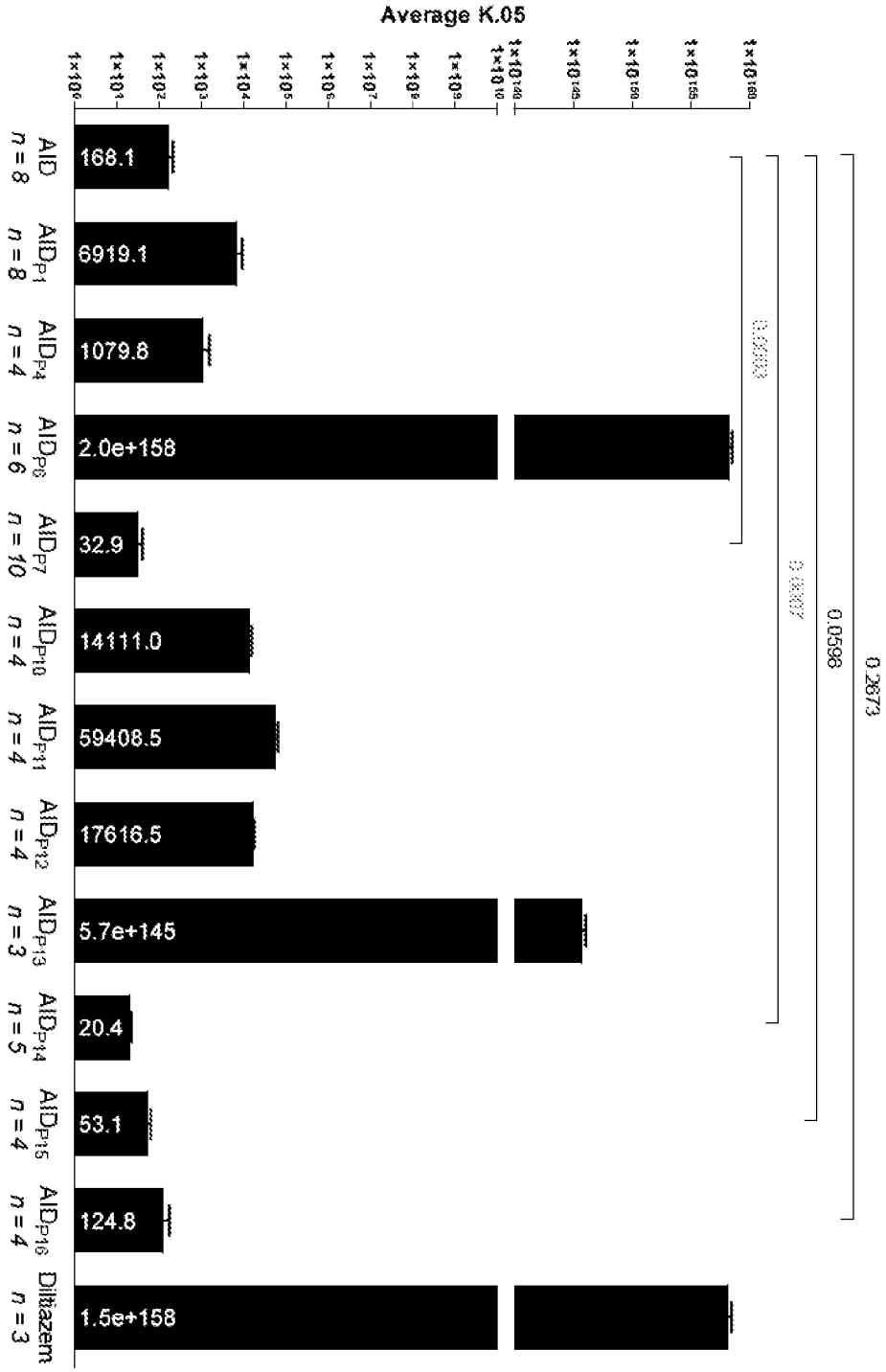

Average K.05
AID n = 8
AID$_{P1}$ n = 8
AID$_{P4}$ n = 4
AID$_{P6}$ n = 6
AID$_{P7}$ n = 10
AID$_{P10}$ n = 4
AID$_{P11}$ n = 4
AID$_{P12}$ n = 4
AID$_{P13}$ n = 3
AID$_{P14}$ n = 5
AID$_{P15}$ n = 4
AID$_{P16}$ n = 4
Diltiazem n = 3
168.1
6919.1
1079.8
2.0e+158
32.9
14111.0
59408.5
17616.5
5.7e+145
20.4
53.1
124.8
1.5e+158
0.0598
0.2673

Figure 10A

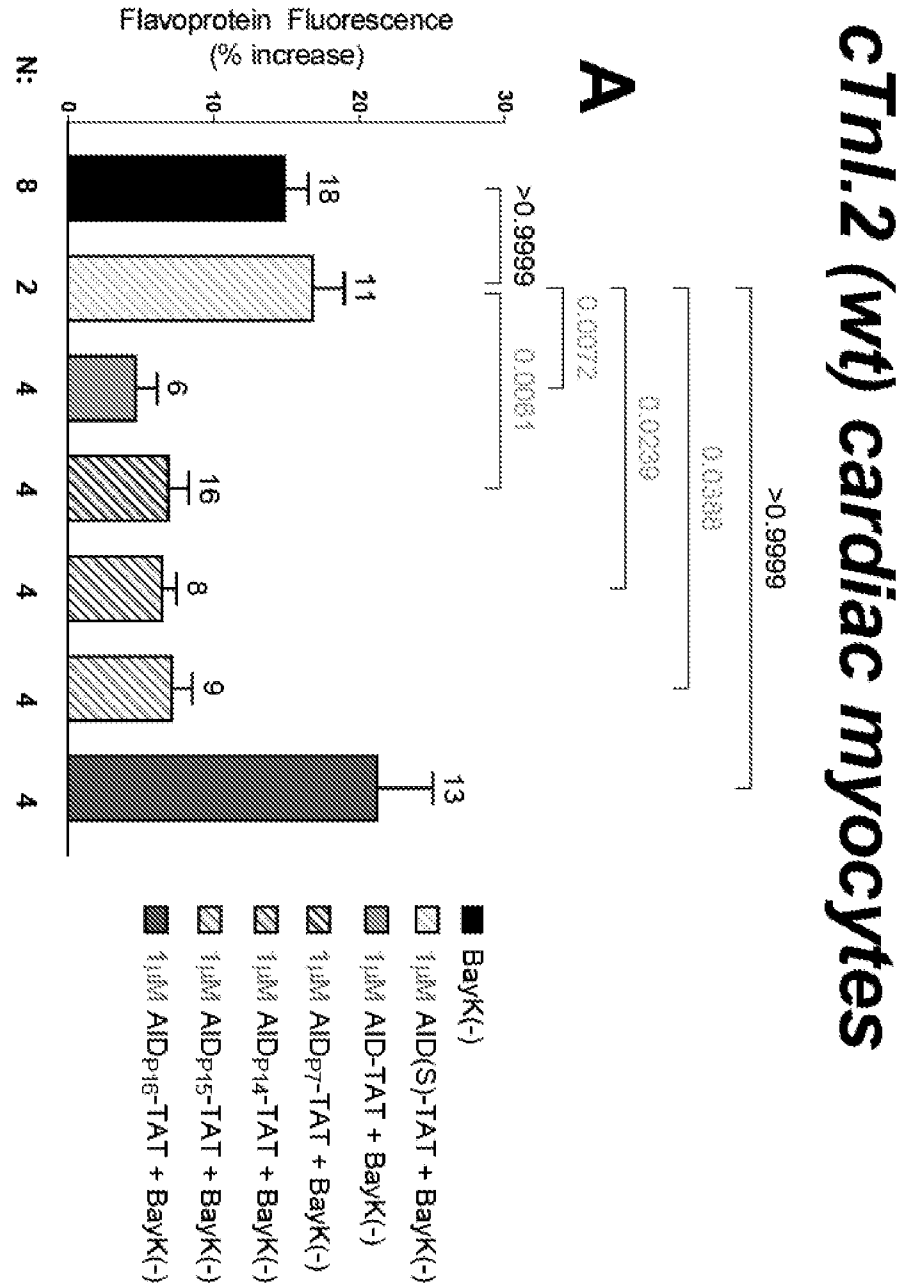

cTnI.2 (wt) cardiac myocytes
A
Flavoprotein Fluorescence
(% increase)
0
10
20
30
N:
8
2
4
4
4
4
4
18
11
6
16
8
9
13
>0.9999
0.0072
0.0001
>0.9999
0.0239
0.0338
BayK(-)
1μM AID(S)-TAT + BayK(-)
1μM AID-TAT + BayK(-)
1μM AID$_{P7}$-TAT + BayK(-)
1μM AID$_{P14}$-TAT + BayK(-)
1μM AID$_{P15}$-TAT + BayK(-)
1μM AID$_{P16}$-TAT + BayK(-)

Figure 10B

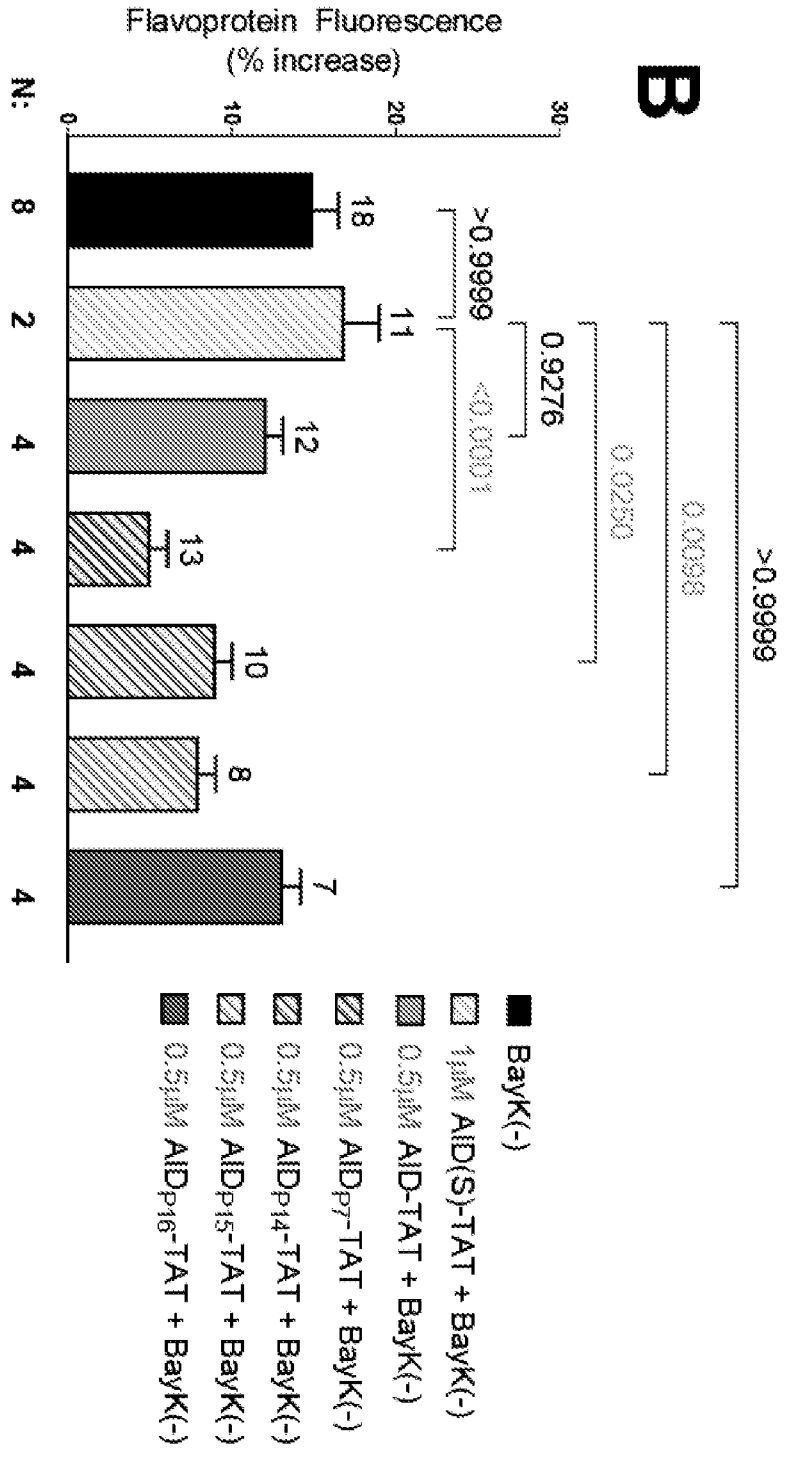

B
Flavoprotein Fluorescence
(% increase)
0
10
20
30
N:
8
2
4
4
4
4
4
18
11
12
13
10
8
7
>0.9999
0.9276
<0.0001
0.0250
0.0098
>0.9999
BayK(-)
1μM AID(S)-TAT + BayK(-)
0.5μM AID-TAT + BayK(-)
0.5μM AID$_{P7}$-TAT + BayK(-)
0.5μM AID$_{P14}$-TAT + BayK(-)
0.5μM AID$_{P15}$-TAT + BayK(-)
0.5μM AID$_{P16}$-TAT + BayK(-)

Figure 10C

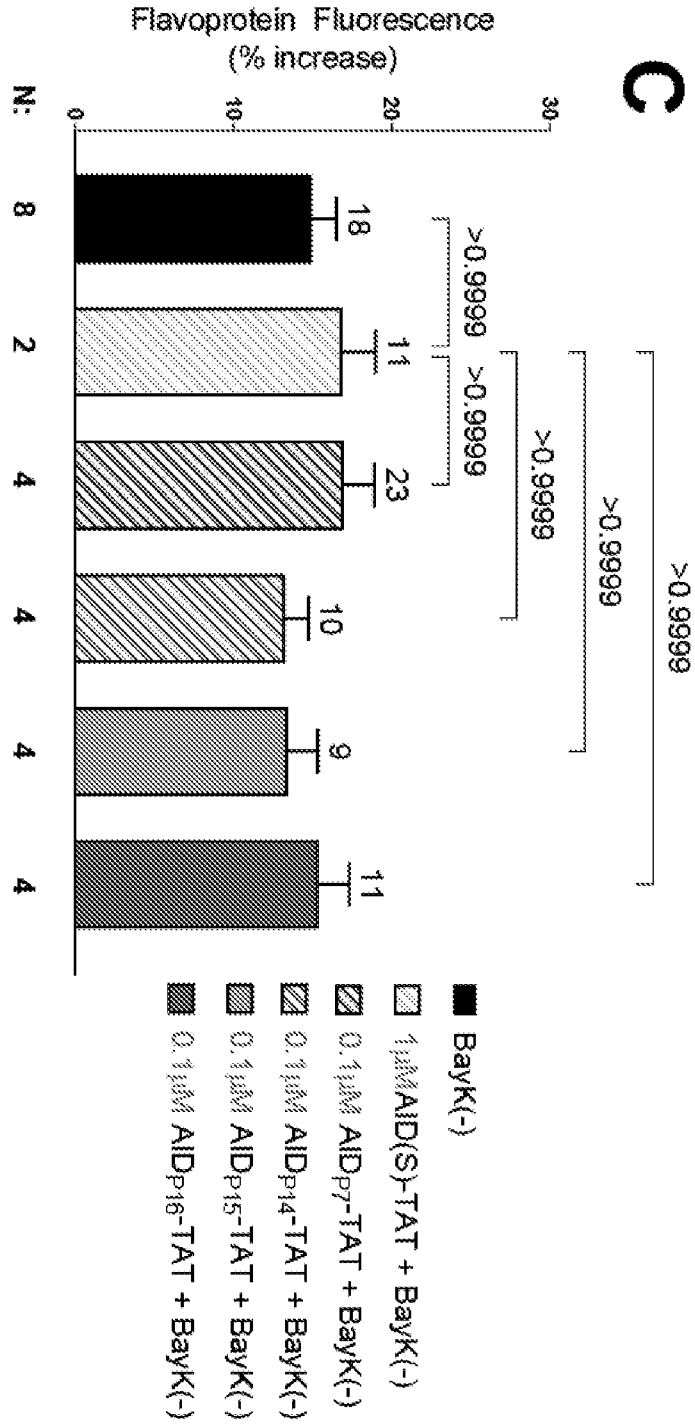

C
Flavoprotein Fluorescence
(% increase)
0
10
20
30
N:
8
2
4
4
4
4
18
11
23
10
9
11
>0.9999
>0.9999
>0.9999
>0.9999
>0.9999
BayK(-)
1μM AID(S)-TAT + BayK(-)
0.1μM AID$_{P7}$-TAT + BayK(-)
0.1μM AID$_{P14}$-TAT + BayK(-)
0.1μM AID$_{P15}$-TAT + BayK(-)
0.1μM AID$_{P16}$-TAT + BayK(-)

Figure 10D

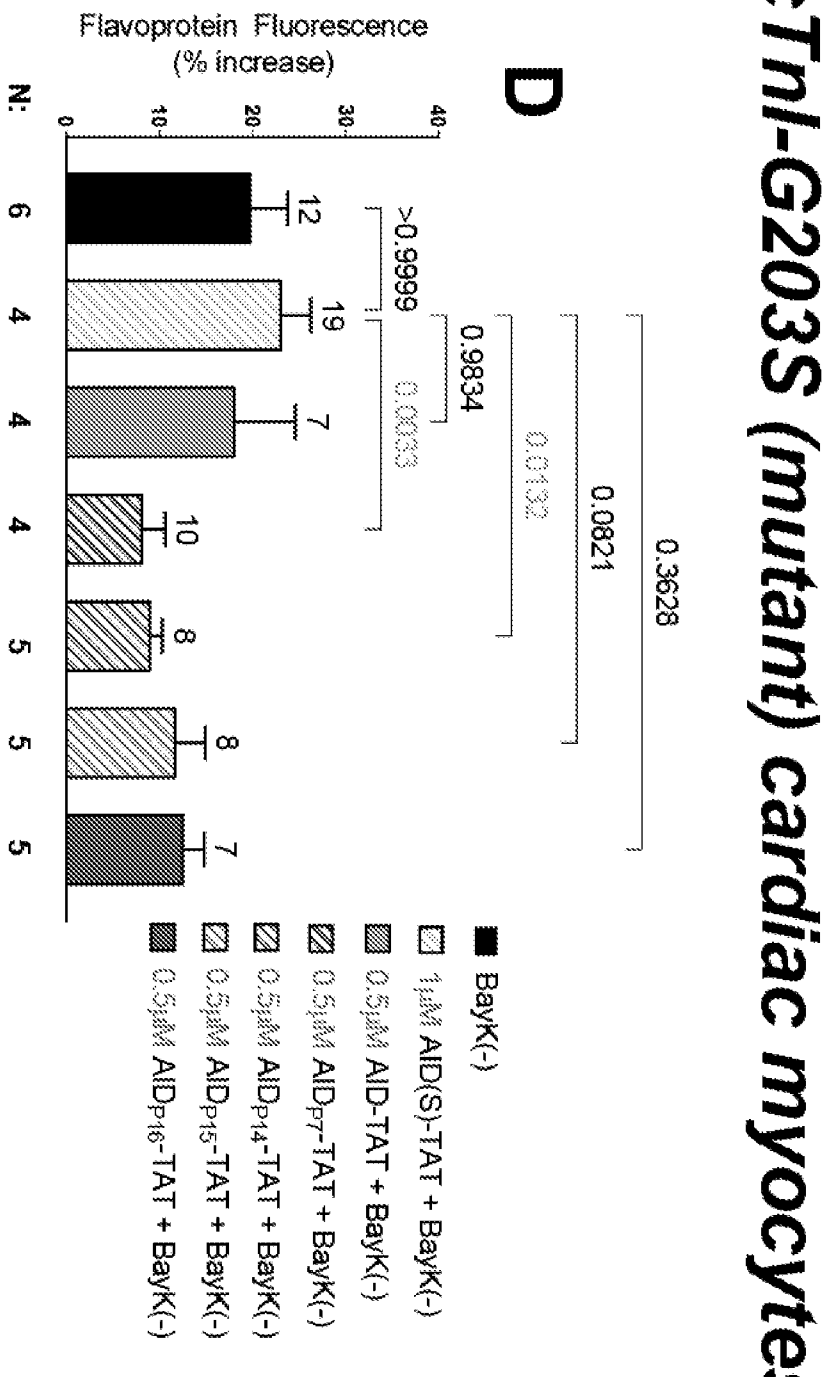

cTnI-G203S (mutant) cardiac myocytes
D
Flavoprotein Fluorescence
(% increase)
0
10
20
30
40
N:
6
4
4
4
5
5
5
12
19
7
10
8
8
7
>0.9999
0.0033
0.9834
0.0132
0.0821
0.3628
BayK(-)
1μM AID(S)-TAT + BayK(-)
0.5μM AID-TAT + BayK(-)
0.5μM AID$_{P7}$-TAT + BayK(-)
0.5μM AID$_{P14}$-TAT + BayK(-)
0.5μM AID$_{P15}$-TAT + BayK(-)
0.5μM AID$_{P16}$-TAT + BayK(-)

Figure 11A

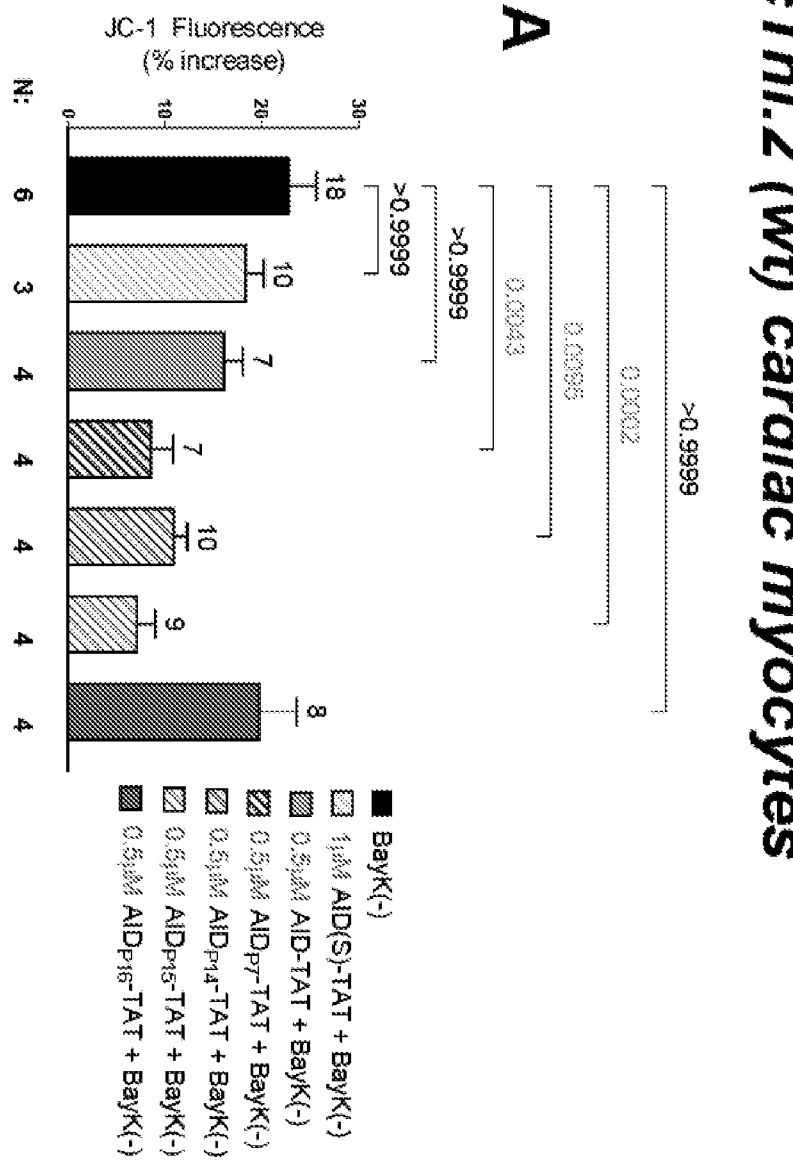

cTnI.2 (wt) cardiac myocytes
A
JC-1 Fluorescence
(% increase)
N:
6
3
4
4
4
4
4
18
10
7
7
10
9
8
<0.9999
<0.9999
0.0043
0.0035
0.0002
<0.9999
BayK(-)
1μM AID(S)-TAT + BayK(-)
0.5μM AID-TAT + BayK(-)
0.5μM AID$_{P17}$-TAT + BayK(-)
0.5μM AID$_{P14}$-TAT + BayK(-)
0.5μM AID$_{P15}$-TAT + BayK(-)
0.5μM AID$_{P16}$-TAT + BayK(-)

Figure 11B

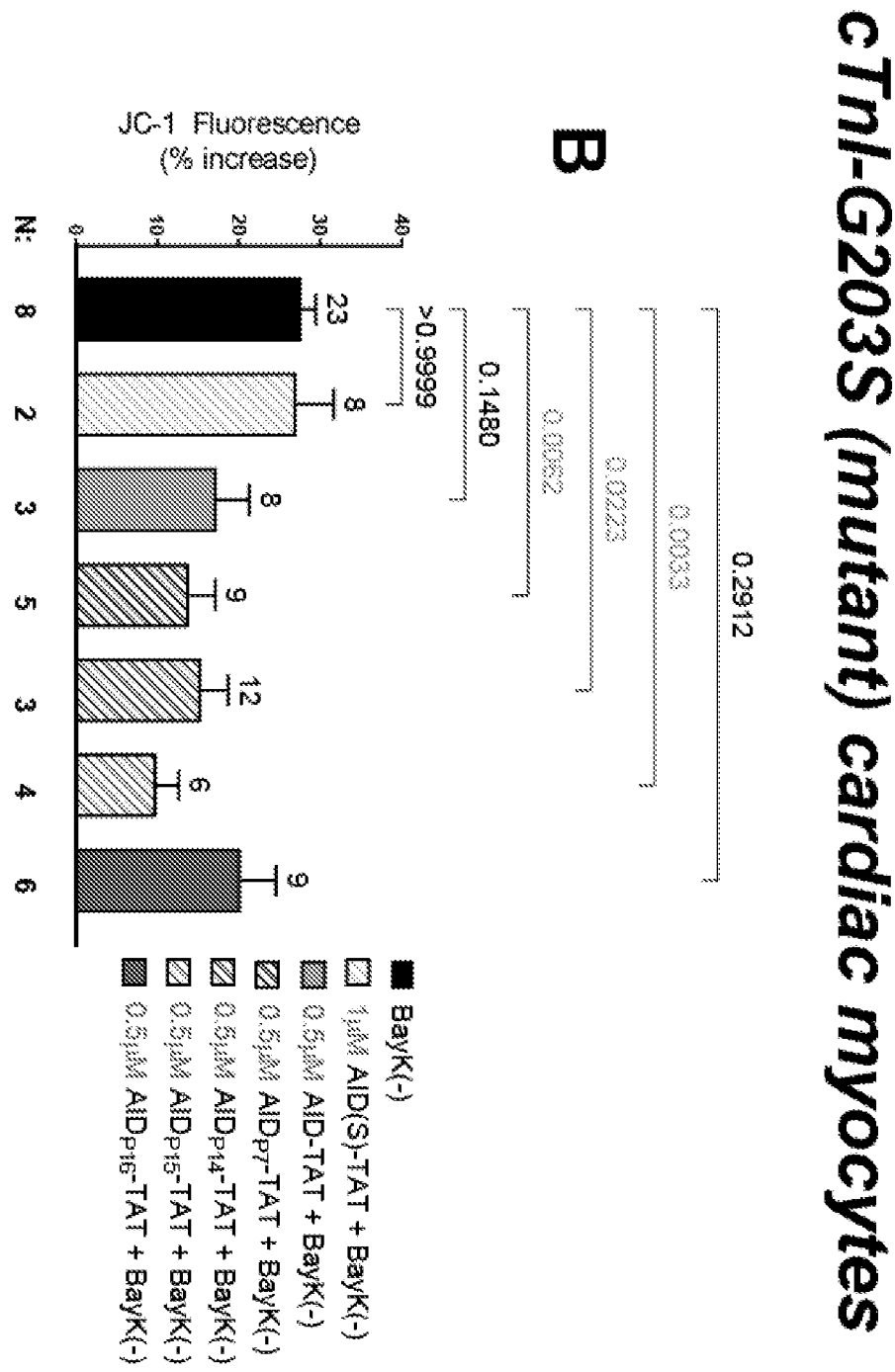

cTnI-G203S (mutant) cardiac myocytes
B
JC-1 Fluorescence
(% increase)
0
10
20
30
40
N:
8
2
3
5
3
4
6
23
8
8
9
12
6
9
0.2912
0.0033
0.0223
0.0052
0.1480
>0.9999
BayK(-)
1μM AID(S)-TAT + BayK(-)
0.5μM AID-TAT + BayK(-)
0.5μM AID$_{P7}$-TAT + BayK(-)
0.5μM AID$_{P14}$-TAT + BayK(-)
0.5μM AID$_{P15}$-TAT + BayK(-)
0.5μM AID$_{P16}$-TAT + BayK(-)

Figure 12A

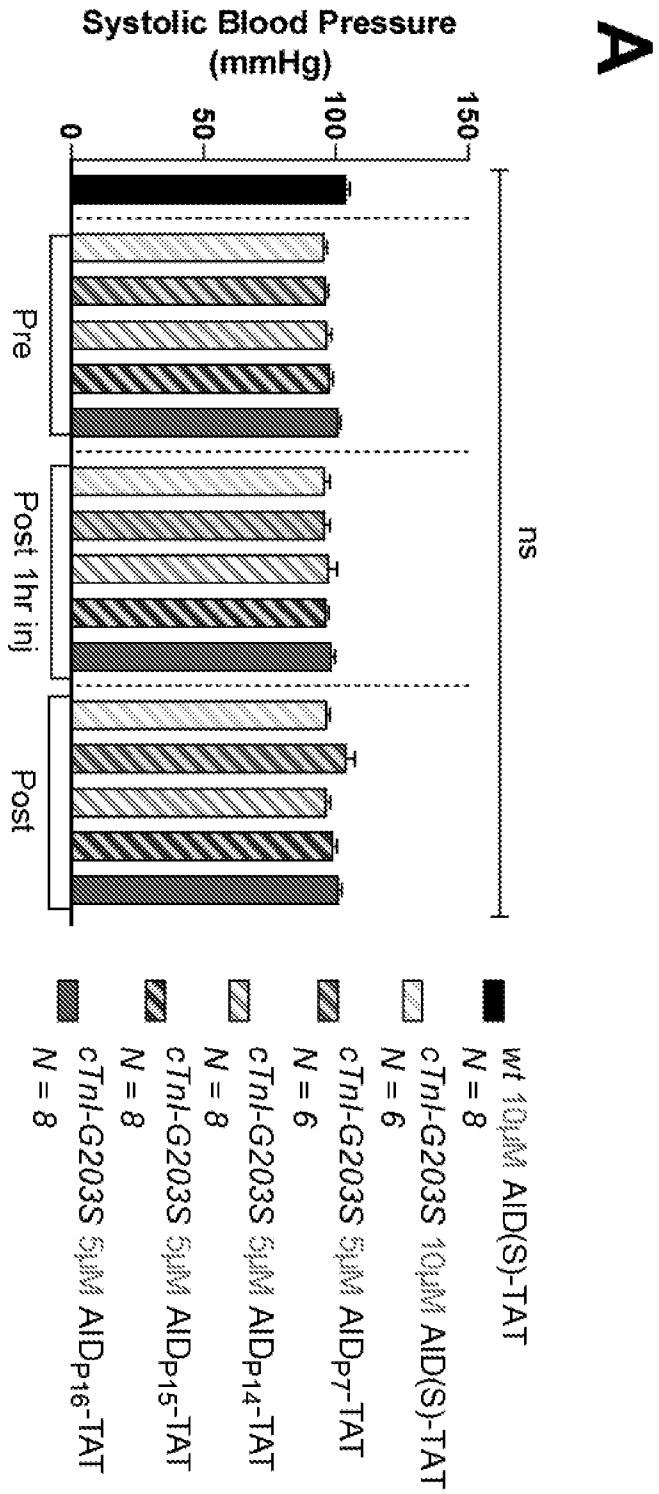

A
Systolic Blood Pressure
(mmHg)
0
50
100
150
Pre
Post 1hr inj
Post
ns
wt 10μM AID(S)-TAT
N = 8
cTnI-G203S 10μM AID(S)-TAT
N = 6
cTnI-G203S 5μM AIDP7-TAT
N = 6
cTnI-G203S 5μM AIDP14-TAT
N = 8
cTnI-G203S 5μM AIDP15-TAT
N = 8
cTnI-G203S 5μM AIDP16-TAT
N = 8

Figure 12B

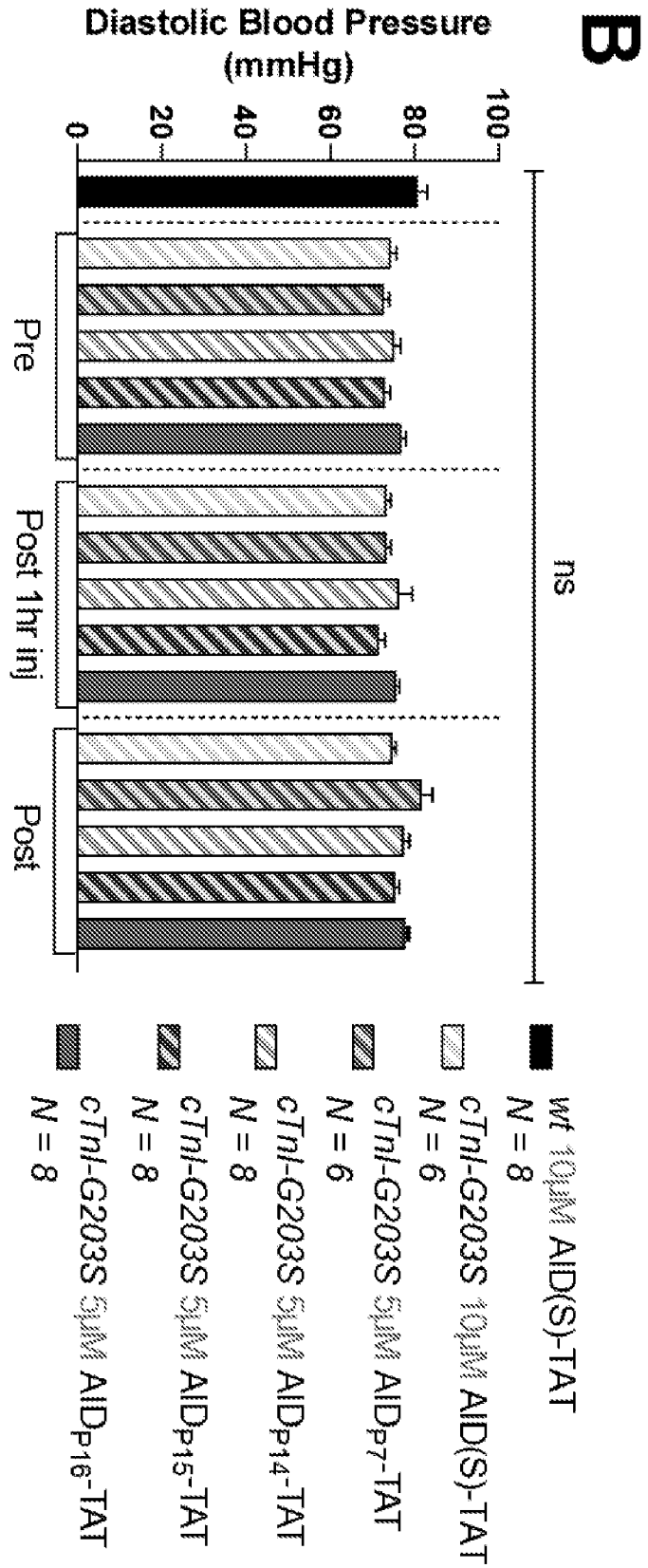

B
Diastolic Blood Pressure
(mmHg)
0
20
40
60
80
100
Pre
Post 1hr inj
Post
ns
wt 10μM AID(S)-TAT
N = 8
cTnI-G203S 10μM AID(S)-TAT
N = 6
cTnI-G203S 5μM AIDP7-TAT
N = 6
cTnI-G203S 5μM AIDP14-TAT
N = 8
cTnI-G203S 5μM AIDP15-TAT
N = 8
cTnI-G203S 5μM AIDP16-TAT
N = 8

Figure 13A

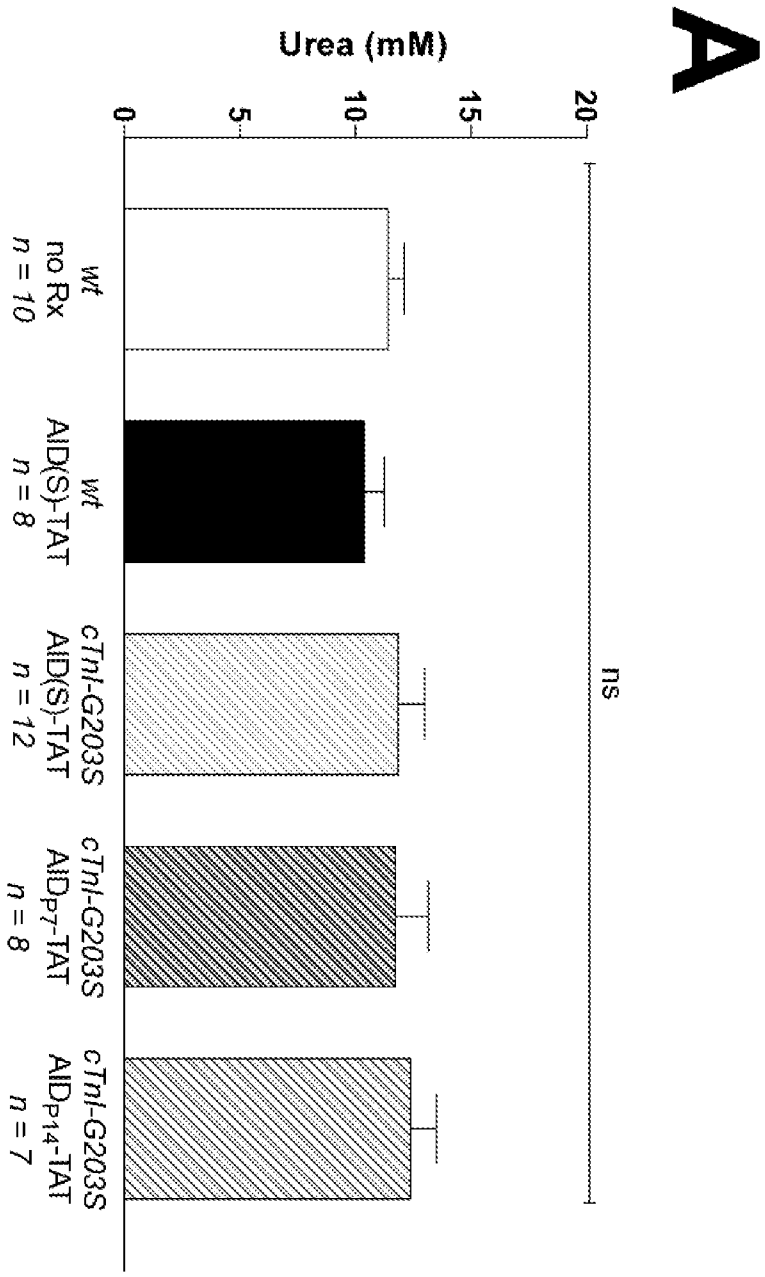

A
Urea (mM)
0
5
10
15
20
wt
no Rx
n = 10
wt
AID(S)-TAT
n = 8
cTnI-G203S
AID(S)-TAT
n = 12
cTnI-G203S
AID$_{P7}$-TAT
n = 8
cTnI-G203S
AID$_{P14}$-TAT
n = 7
ns

Figure 13B

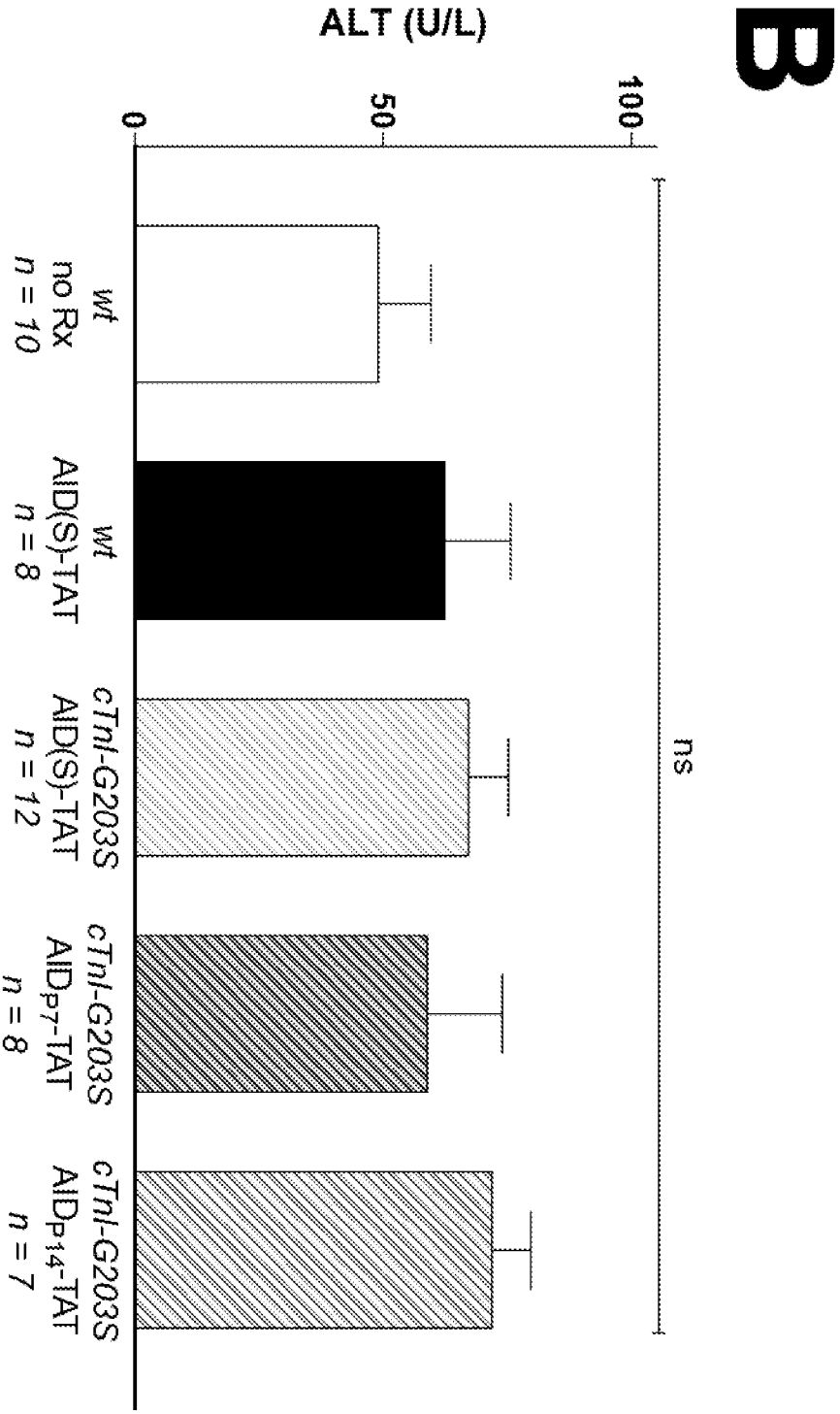

B
ALT (U/L)
0
50
100
ns
wt
no Rx
n = 10
wt
AID(S)-TAT
n = 8
cTnI-G203S
AID(S)-TAT
n = 12
cTnI-G203S
AIDP7-TAT
n = 8
cTnI-G203S
AIDP14-TAT
n = 7

Figure 13C

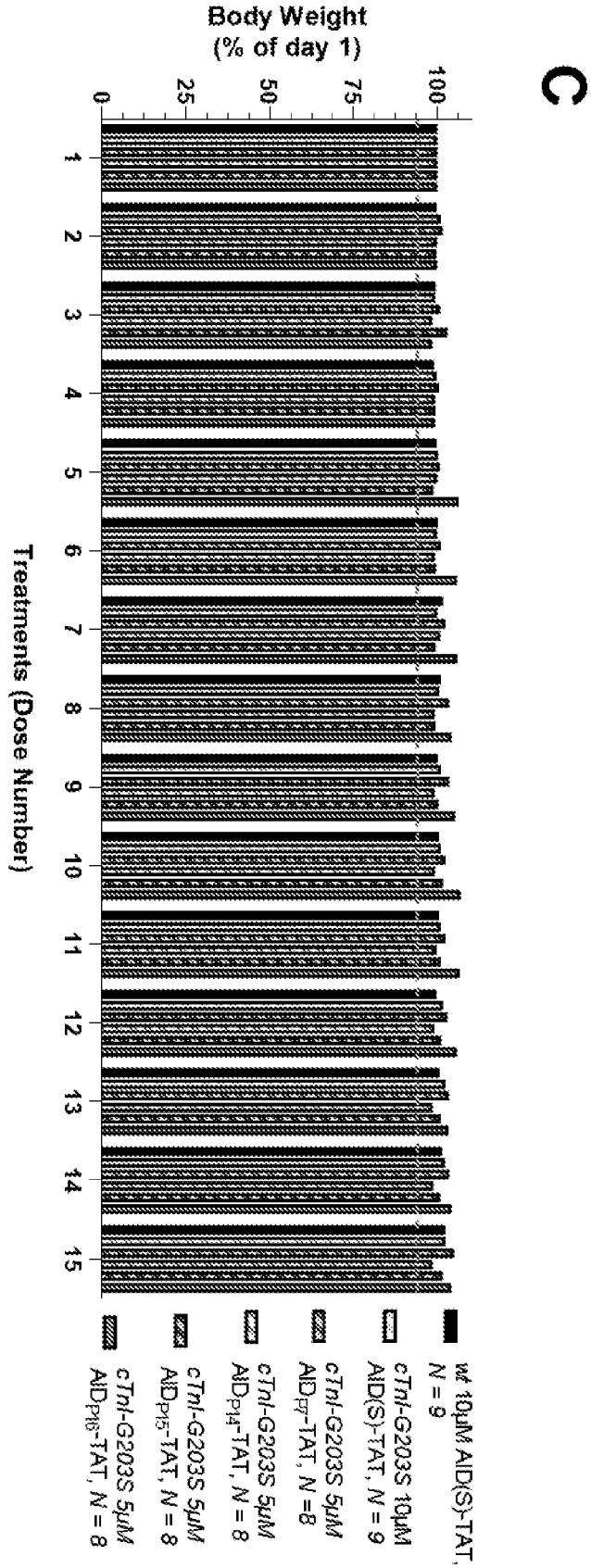

C
Body Weight
(% of day 1)
0
25
50
75
100
Treatments (Dose Number)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
wt 10μM AID(S)-TAT, N = 9
cTnI-G203S 10μM AID(S)-TAT, N = 9
cTnI-G203S 5μM AID$_{PP}$-TAT, N =8
cTnI-G203S 5μM AID$_{PI4}$-TAT, N = 8
cTnI-G203S 5μM AID$_{PI5}$-TAT, N = 8
cTnI-G203S 5μM AID$_{PI6}$-TAT, N = 8

Figure 14A

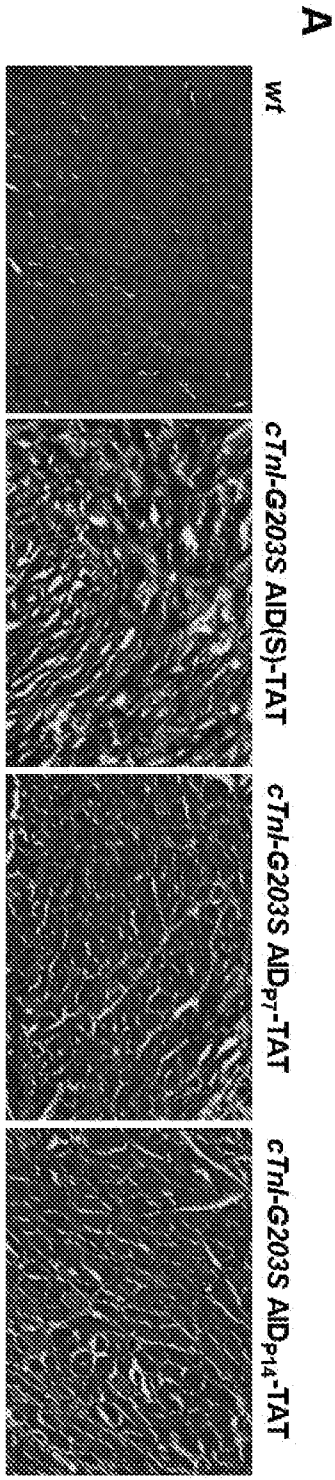

A
wt
cTnI-G203S AID(S)-TAT
cTnI-G203S AID$_{P7}$-TAT
cTnI-G203S AID$_{P14}$-TAT

Figure 14B

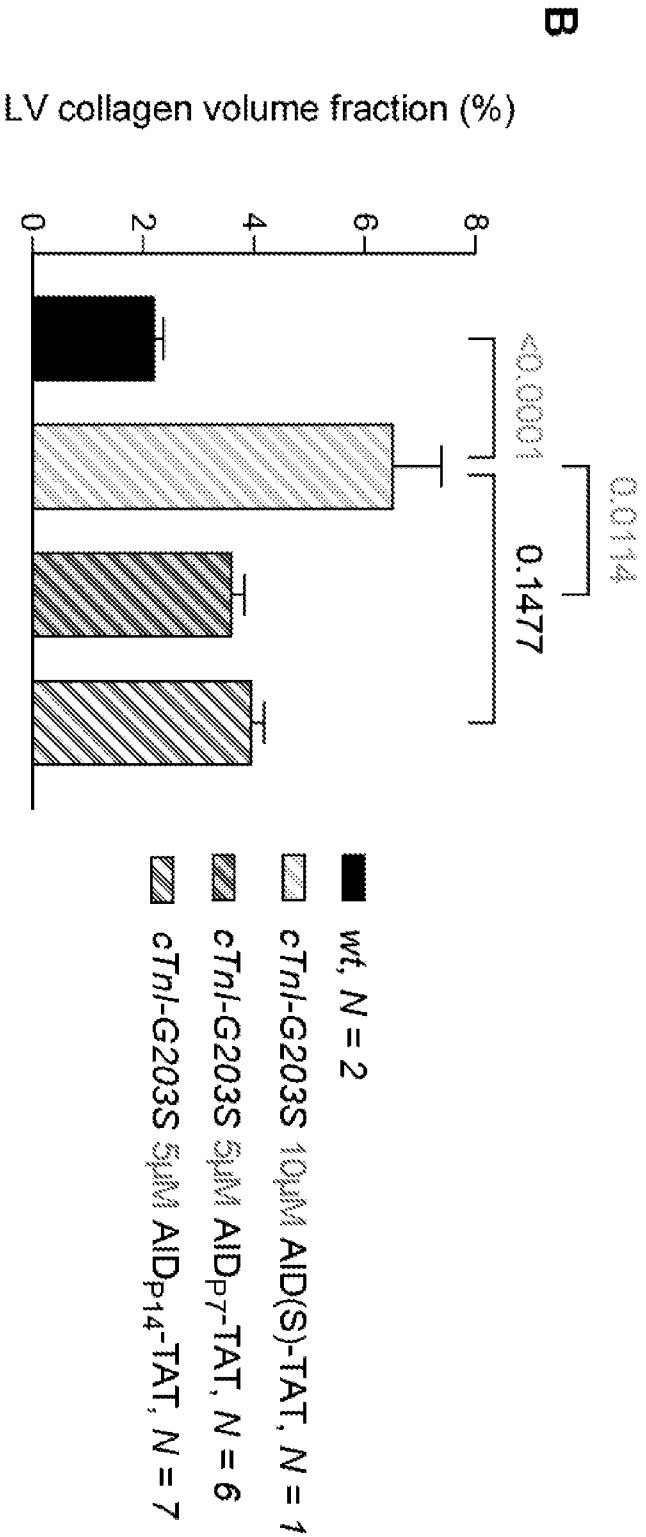

B
LV collagen volume fraction (%)
0
2
4
6
8
<0.0001
0.1477
0.0114
wt, N = 2
cTnI-G203S 10μM AID(S)-TAT, N = 1
cTnI-G203S 5μM AID$_{P7}$-TAT, N = 6
cTnI-G203S 5μM AID$_{P14}$-TAT, N = 7

Figure 15

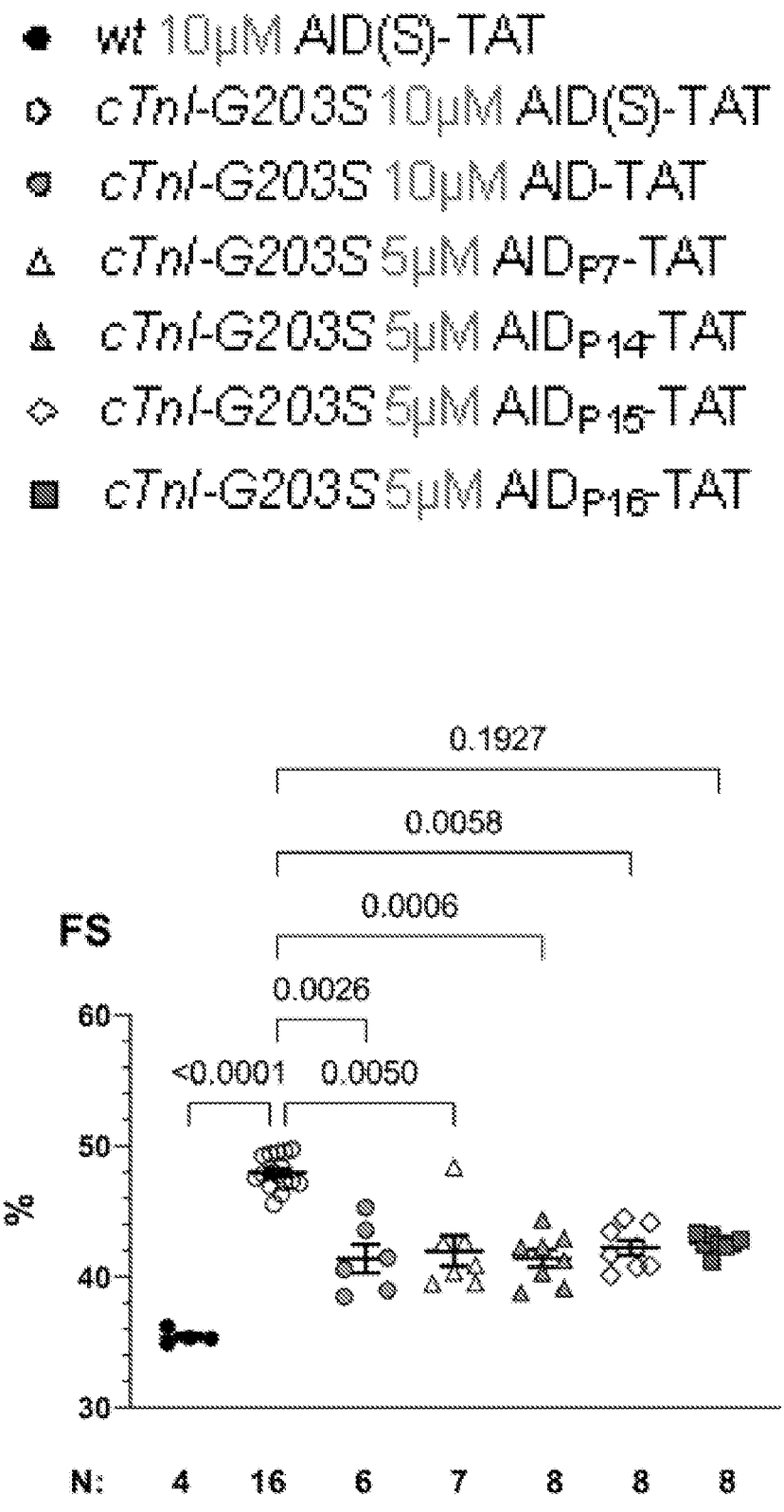

wt 10μM AID(S)-TAT
cTnI-G203S 10μM AID(S)-TAT
cTnI-G203S 10μM AID-TAT
cTnI-G203S 5μM AID$_{P7}$-TAT
cTnI-G203S 5μM AID$_{P14}$-TAT
cTnI-G203S 5μM AID$_{P15}$-TAT
cTnI-G203S 5μM AID$_{P16}$-TAT
0.1927
0.0058
0.0006
0.0026
<0.0001
0.0050
FS
%
60
50
40
30
N: 4 16 6 7 8 8 8

Figure 15 Continued

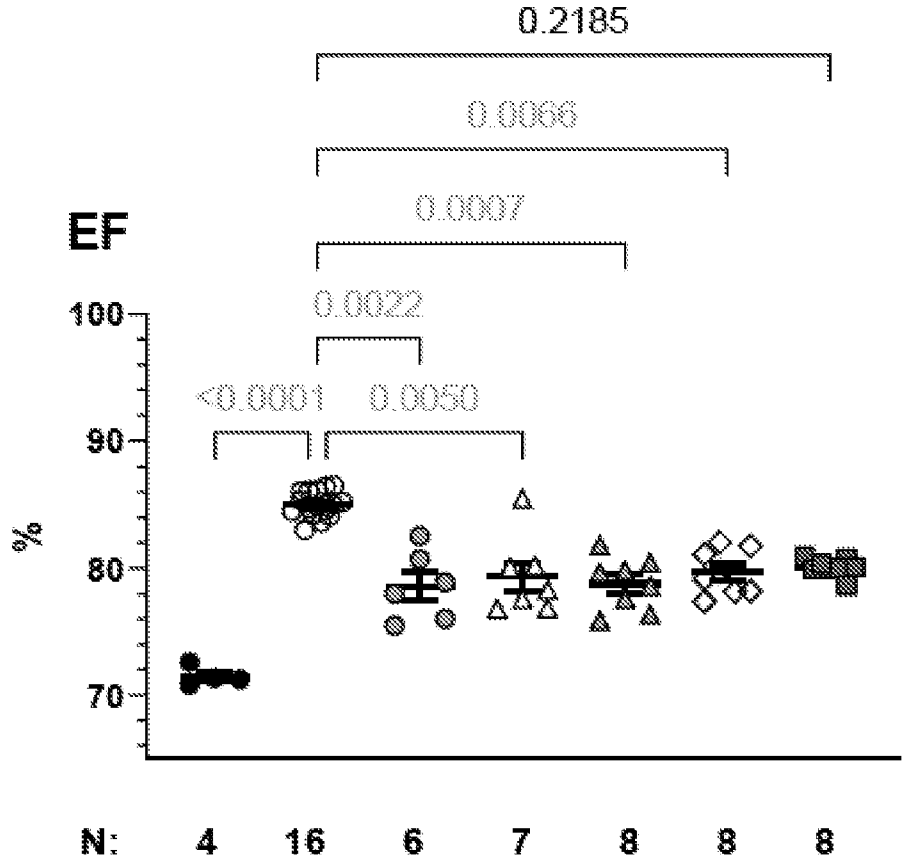


Figure 15 Continued

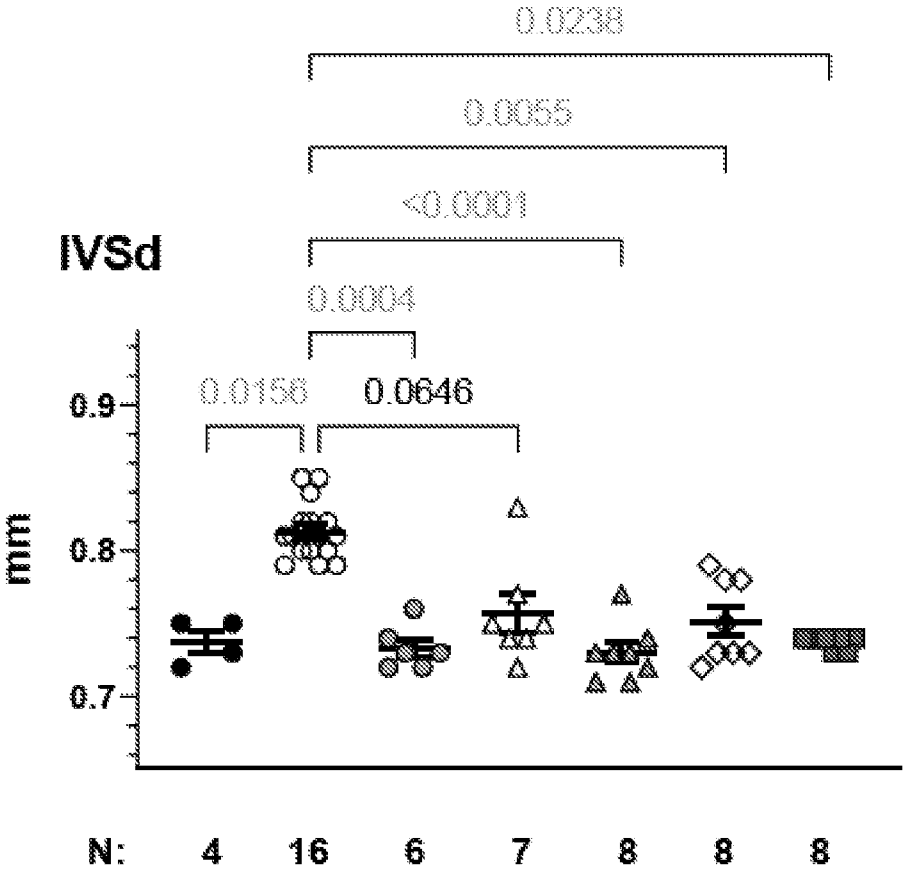


Figure 15 Continued

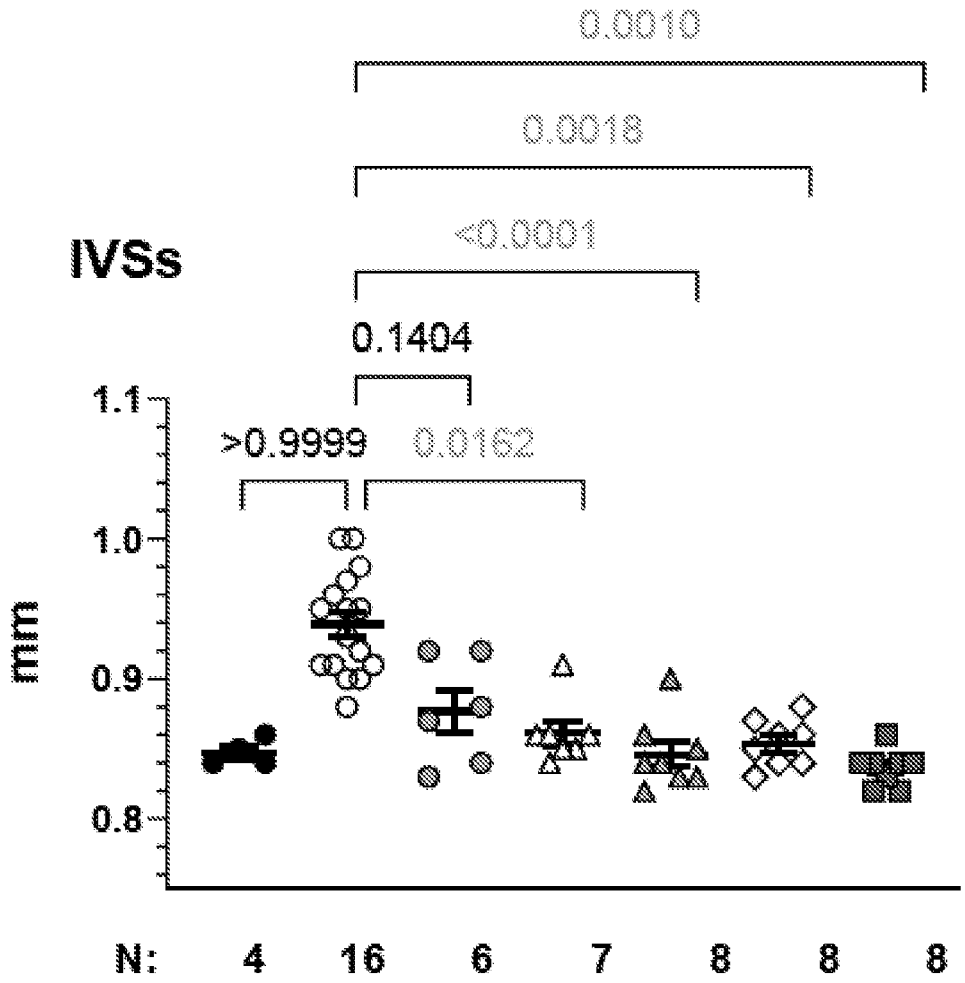


Figure 15 Continued

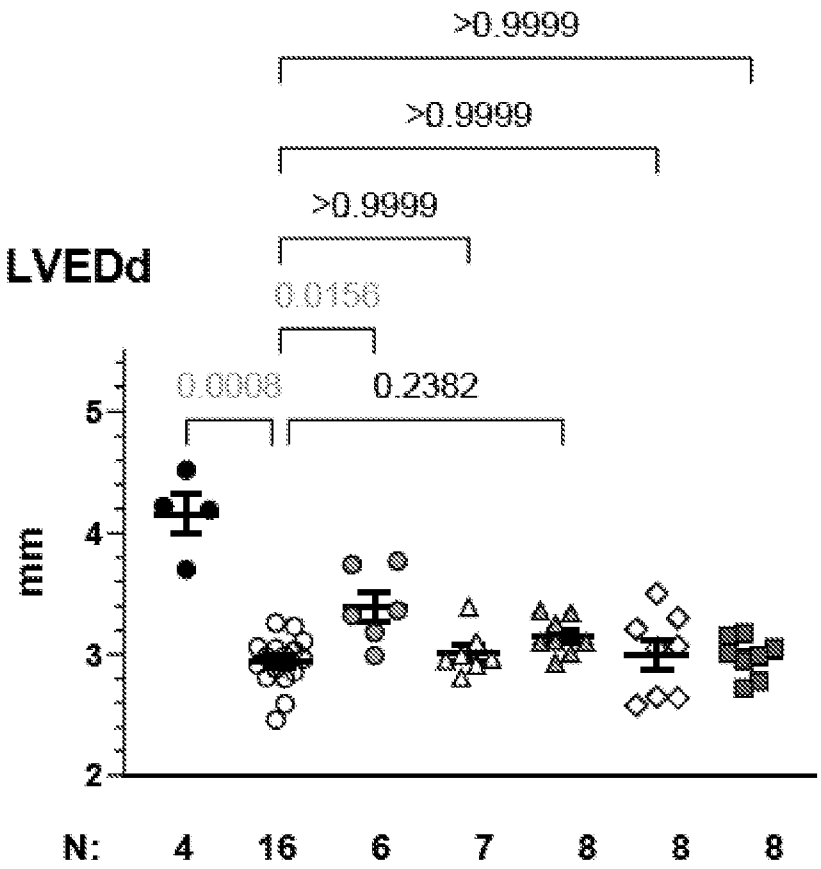


Figure 15 Continued

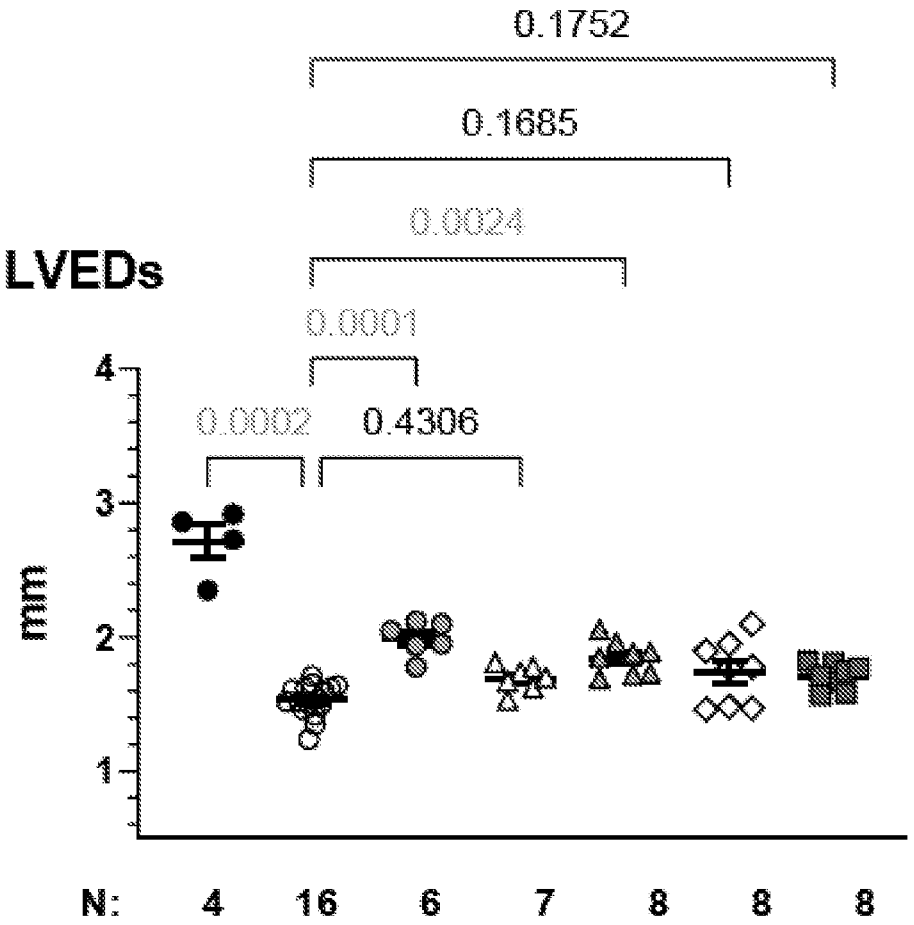


Figure 15 Continued

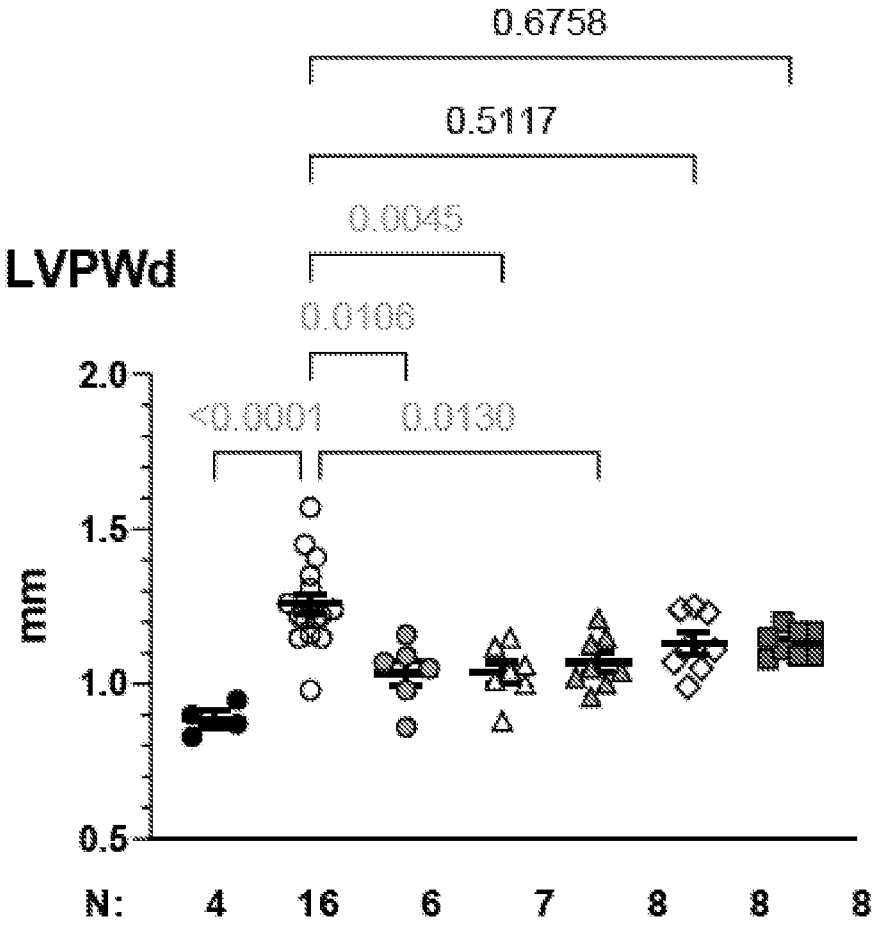


Figure 15 Continued

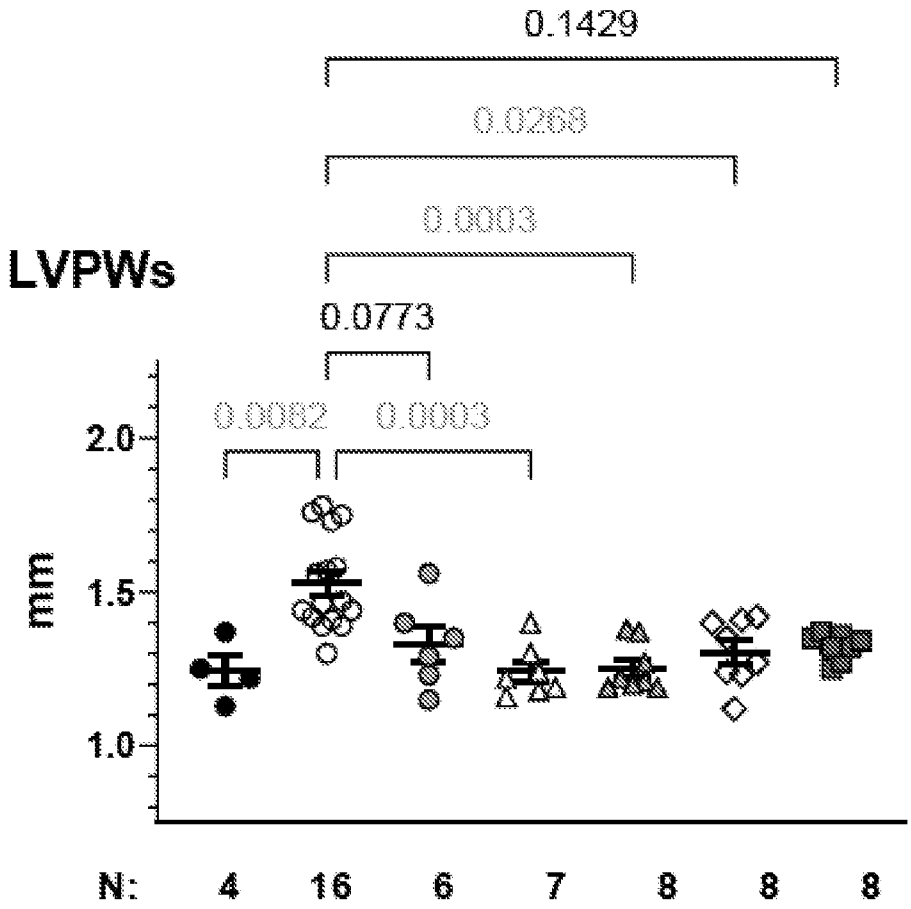


Figure 15 Continued

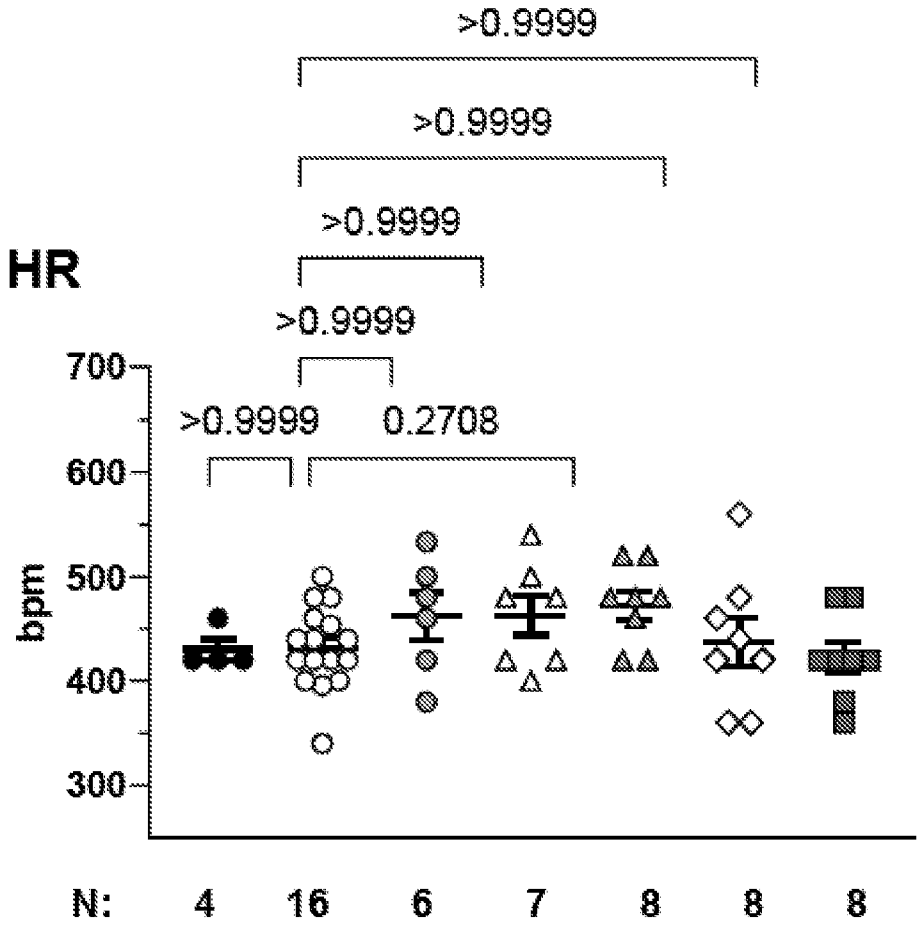
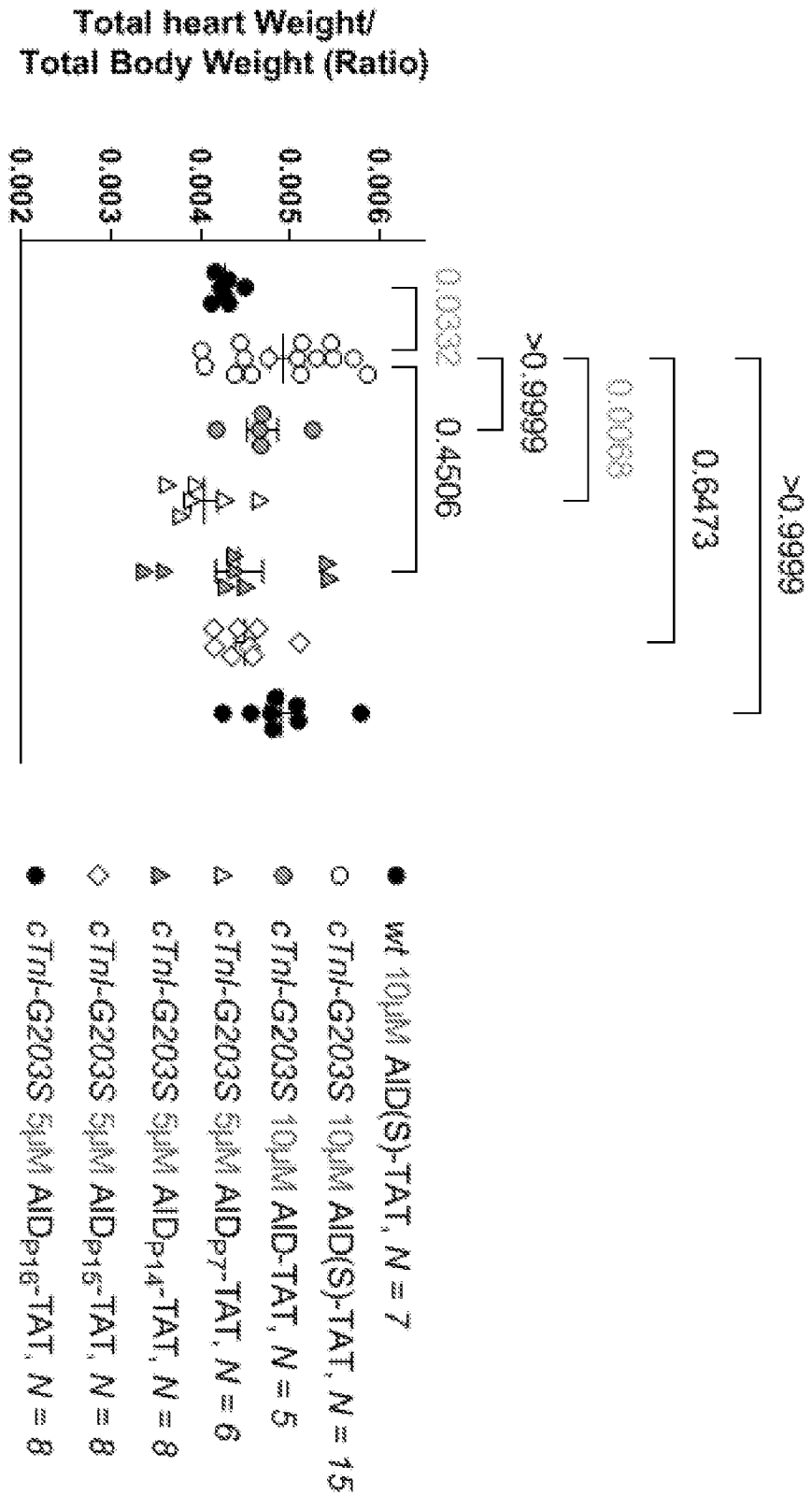


Figure 15


Total heart Weight/
Total Body Weight (Ratio)
0.002
0.003
0.004
0.005
0.006
0.0032
0.4506
>0.9999
0.0003
0.6473
>0.9999
wt 10μM AID(S)-TAT, N = 7
cTnI-G203S 10μM AID(S)-TAT, N = 15
cTnI-G203S 10μM AID-TAT, N = 5
cTnI-G203S 5μM AID$_{P7}$-TAT, N = 6
cTnI-G203S 5μM AID$_{P14}$-TAT, N = 8
cTnI-G203S 5μM AID$_{P15}$-TAT, N = 8
cTnI-G203S 5μM AID$_{P16}$-TAT, N = 8

SEQUENCE LISTING

<110> University of Western Australia

<120> PEPTIDES FOR USE IN THE TREATMENT OR PREVENTION OF MYOCARDIAL DAMAGE

<130> 289086

<160> 17

<170> PatentIn version 3.5

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<212> PRT
<213> Unknown

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1 5 10 15

Ala Glu

<210> 2
<211> 18
<212> PRT
<213> Unknown

<220>
<223> Variant AID Peptide

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Ala Glu

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<211> 9
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<210> 4
<211> 18
<212> PRT
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Ala Glu

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<212> PRT
<213> Unknown

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Ala Glu

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Ala Glu

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Ala Glu

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<220>
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1 5

<210> 9

<211> 11
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1 5 10

<210> 10
<211> 18
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Leu Glu

<210> 11
<211> 13
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1 5 10

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<210> 13
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<210> 14
<211> 12
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<213> Unknown

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<210> 15
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<210> 16
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Ala Glu