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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 September 2010 (30.09.2010)

PCT

(10) International Publication Number
WO 2010/108262 A1

(51) International Patent Classification:
A61K 38/48 (2006.01) *A61P 1/18* (2006.01)
A61K 35/39 (2006.01) *A61P 3/10* (2006.01)

(21) International Application Number:
PCT/CA2010/000413

(22) International Filing Date:
25 March 2010 (25.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/163,173 25 March 2009 (25.03.2009) US

(71) Applicant *(for all designated States except US)*: **DIAMEDICA INC.** [CA/CA]; 4-1250 Waverley Street, Winnipeg, Manitoba R3T 6C6 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **WILLIAMS, Mark** [CA/CA]; 192 Parkville Drive, Winnipeg, Manitoba R2M 2J4 (CA). **RICHARDSON , Kevin** [CA/CA]; 606-11 Evergreen Pl, Winnipeg, Manitoba R3L 0E9 (CA).

(74) Agent: **RIDOUT & MAYBEE LLP**; 225 King Street West, 10th Floor, Toronto, Ontario M5V 3M2 (CA).

(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, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

[Continued on next page]

(54) **Title**: TISSUE KALLIKREIN FOR THE TREATMENT OF PANCREATIC β-CELL DYSFUNCTION

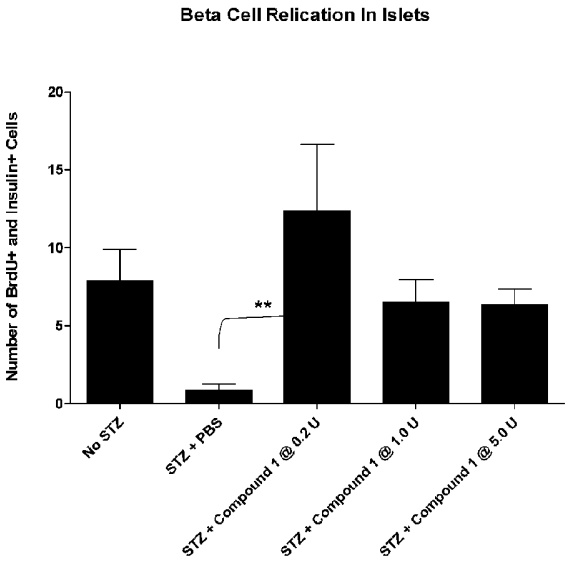

Figure 3

(57) **Abstract**: This invention relates to methods for treating pancreatic islet β-cell dysfunction and the conditions associated with pancreatic islet β-cell dysfunction, including administering a therapeutically effective amount of tissue kallikrein, variants or active fragments thereof.

WO 2010/108262 A1

Published:

— *with international search report (Art. 21(3))*

TISSUE KALLIKREIN FOR THE TREATMENT OF PANCREATIC β-CELL DYSFUNCTION

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of and priority to United States Patent Application Nos. 61/163,173 filed March 25, 2009, under the title TISSUE KALLIKREIN FOR THE TREATMENT OF PANCREATIC β-CELL DYSFUNCTION.

The content of the above patent applications is hereby expressly incorporated by reference into the detailed description hereof.

FIELD OF THE INVENTION

The present invention relates to methods of the treatment of pancreatic β-cell dysfunction and treating pancreatic diseases and conditions associated therewith by the modulation β-cells.

BACKGROUND OF THE INVENTION

Both type I and type II diabetes mellitus are of great concern today. Approximately 24 million people in the United States are affected by the disease (Mueller, *Phys Ther*, 2008, 88(11):1250-3) and the incidence is on the rise around the globe. While type I diabetes can only be treated by insulin injections, type II diabetes may be treated through diet and exercise in some cases. Diet and exercise can even ward off type II diabetes development; however the increasing sedentary lifestyle in many regions of the world is resulting in obesity at epidemic proportions. For those who require it, treatment can be expensive and inconvenient, and may produce several undesirable side effects with the currently available drugs.

β-cells, found in the pancreas, are responsible for the production and release of insulin into the blood stream. They represent the majority of the endocrine cells and form the core of the islets. The pancreatic β-cells secrete insulin in response to increasing glucose levels. Insulin aids in the entry of glucose into the muscle and fat cells. (Ellingsgaard *et al, PNAS*, 2008, 105(35): 13162-7). In individuals with type I diabetes,

the β-cells are attacked by an autoimmune response. The β-cells that remain are insufficient to produce enough insulin to remove the glucose from the blood. They show increased levels of β-cell destruction. For those with type II diabetes, the muscle and liver cells are no longer able to respond to normal blood insulin levels. Therefore they also end up with high blood glucose levels. This can result in β-cell death and loss of β-cell function as compared to healthy individuals. Long standing cases of type I diabetes show ~99% deficiency in β-cell mass while cases of type II diabetes show ~65% deficiency in β-cell mass within the pancreas (Meier, *Diabetologia*, 2008, 51:703-13). Modulation of the levels of healthy β-cells, in particular, increasing the levels or activity of such cells, may therefore serve as an effective therapy to reverse and possibly prevent diabetes.

Modulation of β-cell levels has been attempted through the use of stem cells and organ transplant. These methods are seen to have some drawbacks (Meier, *Diabetologia*, 2008, 51:703-13). Issues regarding stem cell supply and ethics may limit the ability of this therapy. Transplant presents the risks associated with any organ transplant, be it rejection, infection and/or subsequent mortality.

A therapy is therefore desired which can stimulate β-cell production thereby increasing the β-cell mass. This strategy will effectively serve to suppress glucagon secretion (Ellingsgaard *et al, PNAS*, 2008, 105(35): 13162-7) and restore insulin production and secretion to normal levels, resulting in further suppression of glucagon secretion and hepatic glucose production, leading to an improvement in overall peripheral insulin action (Meier, *Diabetologia*, 2008, 51:703-13).

Until the recent work of the present inventors, no one has contemplated the use of tissue kallikrein (KLK1) for the treatment of pancreatic islet β-cell dysfunction and treating diseases and conditions associated therewith by the modulation β-cell mass. KLK1 is a serine protease which cleaves low-molecular-weight kininogen resulting in the release of kallidin (lysl-bradykinin). KLK1 may be formulated to produce a product which can be delivered to modulate β-cell mass without any of the issues associated with the alternate proposed therapies (stem cell usage and pancreatic organ transplant).

SUMMARY OF THE INVENTION

The present invention includes methods of the treatment of pancreatic islet β-cell dysfunction and treating diseases and conditions associated therewith by the modulation β-cell mass comprising administering a therapeutically effective dose of KLKl, variants of KLK1, or active fragments thereof.

In one aspect of the present invention the KLK1 can be a purified/isolated natural form, a synthetic form or a recombinant form.

In another aspect of the present invention the isolated KLK1 can be human KLK1 (SEQ ID NO. 1)

In another aspect of the present invention the isolated KLK1 can be hamadryas baboon KLK1 (SEQ ID NO. 2)

In another aspect of the present invention the isolated KLK1 can be crab eating macaque KLK1 (SEQ ID NO. 3)

In another aspect of the present invention the isolated KLK1 can be cotton top tamarin KLK1 (SEQ ID NO. 4)

In another aspect of the present invention the isolated KLK1 can be dog KLK1 (SEQ ID NO. 5).

In another aspect of the present invention the isolated KLK1 can be sheep KLK1 (SEQ ID NO. 6).

In another aspect of the present invention the isolated KLK1 can be rabbit KLK1 (SEQ ID NO. 7).

In another aspect of the present invention the isolated KLK1 can be bovine KLK1 (SEQ ID NO. 8).

In another aspect of the present invention the isolated KLK1 can be horse KLK1 (SEQ ID NO. 9).

In preferred aspect of the present invention the isolated KLK1 can be pig KLK1 (SEQ ID NO. 10).

In addition, the present invention further provides pharmaceutical compositions and method of treating disease associated with reduced pancreatic islet β-cell functioning and/or reduced pancreatic islet β-cell mass.

In a preferred embodiment the disease or condition associated with reduced pancreatic islet β-cell functioning and/or reduced pancreatic islet β-cell mass is type I or type II diabetes.

In a further aspect of the invention, modulation of β-cell mass can be an increase in the β-cell mass as compared to the diseased state.

In a further aspect of the invention modulation of β-cell mass can be β-cell regeneration.

In the present invention β-cell regeneration refers to the restoration of normal β-cell function by increasing the number of functional β-cells or by fixing impaired β-cell by restoring normal function.

In yet a further aspect of the present invention modulation of β-cell mass can be an increase in β-cell proliferation.

In another aspect of the present invention, KLK1, or a variant or an active fragment thereof, can be administered orally. Oral administration may be an enteral administration, such as a liquid, pill, or capsule to be swallowed.

In a further aspect of the present invention, an oral therapeutic dose can be a maximum dose range of about 1 to about 1000 International Units (IU) per day.

Another aspect of the present invention includes a method as herein described further comprising the use of an additional therapeutic method useful in the modulation β-cell mass or treating diseases or conditions associated therewith. An additional therapeutic method includes, but is not limited to, stem cell transplant and pancreatic organ transplant.

Another aspect of the present invention includes a composition formulated for oral administration comprising about 1 to about 1000 IU of KLK1, or a variant or an active fragment thereof, optionally further comprising a pharmaceutically acceptable excipient, and optionally further comprising an additional therapeutic compound as described above.

DETAILED DESCRIPTION

Definitions

Tissue kallikreins are a family of serine proteases that are primarily noted for their role in controlling hypertension through cleavage of kininogen into lysyl-bradykinin (kallidin) (Yousef et al., *Endocrine Rev*. 2001, 22: 184-204). As there are a large number of known human and animal tissue kallikreins, only one has kininogenase activity, i.e., the ability to release kinin. In humans this enzyme is known as KLK1 or pancreatic/renal kallikrein. The inventors believe that KLK1 appears to be a ubiquitous or multiple target acting enzyme, in addition to its recognized role in hypertension regulation and as such may specifically play an important role in the treatment of pancreatic islet cell dysfunction and treating diseases and conditions associated therewith by the modulation of β-cell mass. As used herein, the term "human tissue kallikrein" or KLK1 is synonymous with the following terms: callicrein, glumorin, padreatin, padutin, kallidinogenase, bradykininogenase, pancreatic kallikrein, onokrein P, dilminal D, depot-Padutin, urokallikrein, or urinary kallikrein. Tissue kallikrein with similar kininogenase activity can also be found in various animals and therefore could be used in the treatment of pancreatic islet cell dysfunction.

A preferred embodiment of the present invention can be human tissue kallikrein precursor polypeptide (kidney/pancreas/salivary gland kallikrein) (KLK1) and has the following sequence (SEQ ID NO:1):

NP_002248 GI:4504875 *Homo sapiens* KLK1_human

1-18 signal peptide
19-24 propeptide
25-262 mature peptide

>gi|4504875|ref|NP_002248.1| kallikrein 1 preproprotein [Homo sapiens]
MWFLVLCLALSLGGTGAAPPIQSRIVGGWECEQHSQPWQAALYHFSTFQCGGIL
VHRQWVLTAAHCISDNYQLWLGRHNLFDDENTAQFVHVSESFPHPGFNMSLLE
NHTRQADEDYSHDLMLLRLTEPADTITDAVKVVELPTEEPEVGSTCLASGWGSIE
PENFSFPDDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDTCVGDSG
GPLMCDGVLQGVTSWGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENS (SEQ ID NO:1)

A further embodiment of the present invention includes hamadryas baboon tissue kallikrein (kidney/pancreas/salivary gland kallikrein) (SEQ ID NO. 2) which has 90% sequence identity to human KLK1 (SEQ ID NO. 1)

Q2877 KLK1_PAPHA

MWFLVLCLALSLGGTGAAPPIQSRIVGGWECSQPWQAALYHFSTFQCGGILVHP
QWVLTAAHCIGDNYQLWLGRHNLFDDEDTAQFVHVSESFPHPCFNMSLLKNHT
RQADEDYSHDLMLLRLTQPAEITDAVQVVELPTQEPEVGSTCLASGWGSIEPENF
SYPDDLQCVDLKILPNDKCAKAHTQKVTEFMLCAGHLEGGKDTCVGDSGGPLT
CDGVLQGVTSWGYIPCGSPNKPAVFVRVLSYVKWIEDTIAENS (SEQ ID NO. 2)

A further embodiment of the present invention includes crab eating macaque tissue kallikrein (kidney/pancreas/salivary gland kallikrein) (SEQ ID NO. 3) which has 90% sequence identity to human KLK1 (SEQ ID NO. 1):

Q07276-1 KLK1_MACFA

MWFLVLCLALSLGGTGRAPPIQSRIVGGWECSQPWQAALYHFSTFQCGGILVHP
QWVLTAAHCISDNYQLWLGRHNLFDDEDTAQFVHVSESFPHPGFNMSLLKNHT
RQADDYSHDLMLLRLTQPAEITDAVQVVELPTQEPEVGSTCLASGWGSIEPENFS
FPDDLQCVDLEILPNDECAKAHTQKVTEFMLCAGHLEGGKDTCVGDSGGPLTCD
GVLQGVTSWGYIPCGSPNKPAVFVKVLSYVKWIEDTIAENS (SEQ ID NO. 3)

A further embodiment of the present invention includes cotton top tamarin tissue kallikrein (kidney/pancreas/salivary gland kallikrein) (SEQ ID NO. 4) which has 82% sequence identity to human KLK1 (SEQ ID NO. 1):

Q9N1Q1_SAGOE

MWFLVLCLALSLGGTGAVPPIQSRIVGGWDCKQHSQPWQAALYHYSTFQCGGV
LVHPQWVLTAAHCISDHYQLWLGRHDLFENEDTAQFVFVSKSFPHPDFNMSLLK
NHTRLPGEDYSHDLMLLQLKQPVQITDAVKVVELPTEGIEVGSTCLASGWGSIKP
EKFSFPDILQCVDLKILPNDECDKAHAQKVTEFMLCAGPLKDGQDTCVGDSGGP
LTCDGVLQGIISWGYIPCGSPNKPSVFVRVLSYVKWIKDTIADNS (SEQ ID NO. 4)

A further embodiment of the present invention includes dog tissue kallikrein (kidney/pancreas/salivary gland kallikrein) (SEQ ID NO. 5) which has 74% sequence identity to human KLK1 (SEQ ID NO. 1):

Q29474_CANFA

MWFLVLCLALSLAGTGAAPPVQSRIIGGWDCTKNSQPWQAALYHYSKFQCGGV
LVHPEWVVTAAHCINDNYQLWLGRYNLFEHEDTAQFVQVRESFPHPEFNLSLLK
NHTRLPEEDYSHDIMLLRLAEPAQITDAVRVLDLPTQEPQVGSTCYASGWGSIEP
DKFIYPDDLQCVDLELLSNDICANAHSQKVTEFMLCAGHLEGGKDTCVGDSGGP
LICDGVLQGITSWGHVPCGSPNMPAVYTKVISHLEWIKETMTANP (SEQ ID NO. 5)

A further embodiment of the present invention includes sheep tissue kallikrein-1 (SEQ ID NO. 6) which has 72% sequence identity to human KLK1 (SEQ ID NO. 1):

A5A2L9_SHEEP

MWFPVLCLALSLAGTGAVPPVQSRIVGGQECEKHSQPWQVAIYHFSTFQCGGVL
VAPQWVLTAAHCKSENYQVWLGRHNLFEDEDTAQFAGVSEDFPNPGFNLSLLE
NHTRQPGEDYSHDLMLLRLQEPVQLTQDVQVLGLPTKEPQLGTTCYASGWGSV
KPDEFSYPDDLQCVDLTLLPNEKCATAHPQEVTDCMLCAGHLEGGKDTCVGDS
GGPLICEGMLQGITSWGHIPCGTPNKPSVYTKVIVYLDWINKTMTDNP (SEQ ID NO. 6)

A further embodiment of the present invention includes rabbit tissue kallikrein-1 (SEQ ID NO. 7) which has 73% sequence identity to human KLK1 (SEQ ID NO. 1):

A5A2M0_RABIT

MWLPVLCLALSLGGTGAAPPLQSRIIGGWVCGKNSQPWQAALYHYSNFQCGGV
LVHPQWVLTAAHCFSDNYQLWLGRHNLFEDEAEAQFIQVSGSFPHPRFNLSLLE
NQTRGPGEDYSHDLMLLKLARPVQLTNAVRVLELPTQEPQVGTSCLASGWGSIT
PIKFTYPDELQCVDLSILANSECDKAHAQMVTECMLCAGHLEGGRDTCVGDSGG
PLVCNNELQGITSWGHVPCGSPNKPAVFTKVLSYVEWIRNTIANNP (SEQ ID NO. 7)

A further embodiment of the present invention includes bovine glandular kallikrein-1 precursor (SEQ ID NO. 8) which has 72% sequence identity to human KLK1 (SEQ ID NO. 1):

Q6H320_BOVIN

MWFPVLCLALSLAGTGAVFPIQSRIVGGQECEKHSQPWQVAIYHFSTFQCGGVL
VAPQWVLTAAHCKSDNYQVWLGRHNLFEDEDTAQFAGVSEDFPNPGFNLSLLE
NHTRHPGEDYSHDLMLLRLQEPVQLTQNVQVLGLPTKEPQLGTTCYASGWGSV

KPDEFSYPDDLQCVDLTLLPNEKCATAHPQEVTEWMLCAGHLEGGKDTCVGDS
GGPLICEGMLQGITSWGHIPCGTPNKPSVYTKVILYLDWINKTMTDNP (SEQ ID
NO. 8)

A further embodiment of the present invention includes horse glandular kallikrein-1 precursor (KLKE1) (SEQ ID NO. 9) which has 70% sequence identity to human KLK1 (SEQ ID NO. 1):

Q6H322_HORSE
MWLPVLCLALSLVGTGAAPPIQSRIIGGWECKNHSKPWQAAVYHYSSFQCGGVL
VDPQWVLTAAHCKGDYYQIWLGRHNLFEDEDTAQFFLVAKSFPHPDFNMSLLE
NNNRLPGEDYSHDLMLLQVEQPDQITVAVQVLALPTQEPVLGSTCYASGWGSIE
PDKFTYPDELRCVDLTLLSNDVCDNAHSQNVTEYMLCAGHLEGGKDTCVGDSG
GPLICDGVFQGVTSWGHIPCGRPNKPAVYTKLIPHVQWIQDTIAANP (SEQ ID
NO. 9)

A preferred embodiment of the present invention includes pig glandular kallikrein-1 precursor (SEQ ID NO.10) which has 67% sequence identity to human KLK1 (SEQ ID NO. 1):

NP_001001911 GI:50054435 *Sus scrofa*

1-17 signal peptide
18-24 propeptide
25-263 mature peptide

>gi|50054435|ref|NP_001001911.1| kallikrein 1 [Sus scrofa]
MWSLVMRLALSLAGTGAAPPIQSRIIGGRECEKDSHPWQVAIYHYSSFQCGGVL
VDPKWVLTAAHCKNDNYQVWLGRHNLFENEVTAQFFGVTADFPHPGFNLSLLK
NHTKADGKDYSHDLMLLRLQSPAKITDAVKVLELPTQEPELGSTCQASGWGSIE
PGPDDFEFPDEIQCVELTLLQNTFCADAHPDKVTESMLCAGYLPGGKDTCMGDS
GGPLICNGMWQGITSWGHTPCGSANKPSIYTKLIFYLDWINDTITENP (SEQ ID
NO. 10)

The term "active fragment" refers to smaller portions of the KLK1 polypeptide that retains the activity of the full-length KLK1 polypeptide, for example, the KLK1 without the signal peptide region, the KLK1 without the signal peptide and without the propeptide regions, and fragments of the KLK1 protein found to have serine protease activity capable of cleaving low-molecular weight kininogen into kallidin.

A "variant" or "mutant" of a starting or reference polypeptide is a polypeptide that 1) has an amino acid sequence different from that of the starting or reference polypeptide and 2) was derived from the starting or reference polypeptide through either natural or

artificial (manmade) mutagenesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. A variant amino acid, in this context, refers to an amino acid different from the amino acid at the corresponding position in a starting or reference polypeptide sequence (such as that of a source antibody or antigen binding fragment). Any combination of deletion, insertion, and substitution may be made to arrive at the final variant or mutant construct, provided that the final construct possesses the desired functional characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Patent No. 5,534,615, expressly incorporated herein by reference. Variants or mutants of a reference polypeptide may, for example, have 95, 90, 85, 82, 80, 75, 74, 72, or 60% identity with said reference polypeptide and may have more, less, or identical activity to the reference polypeptide. Variants may also include sequences added to the reference polypeptide to facilitate purification, to improve metabolic half-life or to make the polypeptide easier to identify, for example, a His tag or a pegylation sequence.

A "wild type" or "reference" sequence or the sequence of a "wild type" or "reference" protein/polypeptide maybe the reference sequence from which variant polypeptides are derived through the introduction of mutations. In general, the "wild type" sequence for a given protein is the sequence that is most common in nature. Similarly, a "wild type" gene sequence is the sequence for that gene which is most commonly found in nature. Mutations may be introduced into a "wild type" gene (and thus the protein it encodes) either through natural processes or through man induced means. The products of such processes are "variant" or "mutant" forms of the original "wild type" protein or gene.

"Percent (%) amino acid sequence identity" with respect to the polypeptides identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of

the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, California.

For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y,

where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference polypeptide-encoding nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively

be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows:

100 times the fraction W/Z,

where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will he appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

The term "amino acid" is used in its broadest sense and is meant to include the naturally occurring L α-amino acids or residues. The commonly used one and three letter abbreviations for naturally occurring amino acids are used herein (Lehninger, A.L., Biochemistry, 2d ed., pp. 71-92, (1975), Worth Publishers, New York). The term also includes all D-amino acids as well as chemically modified amino acids such as amino acid analogs, naturally occurring amino acids that are not usually incorporated into proteins such as Norleucine, and chemically synthesized compounds having properties known in the art to be characteristic of an amino acid. For example, analogs or mimetics of phenylalanine or proline, which allow the same conformational restriction of the peptide compounds as natural Phe or Pro are included within the definition of amino acid. Such analogs and mimetics are referred to herein as "functional equivalents" of an amino acid. Other examples of amino acids are listed by Roberts and Vellaccio, In: The Peptides: Analysis, Synthesis, Biology, Gross and Meiehofer, Eds., Vol. 5 p 341, Academic Press, Inc, N.Y. 1983, which is incorporated herein by reference.

The term "protein" has an amino acid sequence that is longer than a peptide. A "peptide" contains 2 to about 50 amino acid residues. The term "polypeptide" includes proteins and peptides. Examples of proteins include, but are not limited to, antibodies, enzymes, lectins and receptors; lipoproteins and lipopolypeptides; and glycoproteins and glycopolypeptides.

A "fusion protein" and a "fusion polypeptide" refer to a polypeptide having two portions covalently linked together, where each of the portions is a polypeptide having a

different property. The property may be a biological property, such as activity *in vitro* or *in vivo*. The property may also be a simple chemical or physical property, such as binding to a target antigen, catalysis of a reaction, etc. The two portions may be linked directly by a single peptide bond or through a peptide linker containing one or more amino acid residues. Generally, the two portions and the linker will be in reading frame with each other. Preferably, the two portions of the polypeptide are obtained from heterologous or different polypeptides.

The term "therapeutically effective amount" refers to an amount of a composition of this invention effective to "alleviate" or "treat" a disease or disorder in a subject or mammal. Generally, alleviation or treatment of a disease or disorder involves the lessening of one or more symptoms or medical problems associated with the disease or disorder. In some embodiments, it is an amount that can increase β -cell mass as compared to the diseased state.

The terms "treatment" and "treating" refer to inhibiting, alleviating, and healing disease, conditions or symptoms thereof. "Treating" or "treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Treatment can be carried out by administering a therapeutically effective amount of at least one compound of the invention. Parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician.

The term "β-cell regeneration", or "increase in β-cell mass" refers to enhanced β-cell mass in the pancreas as determined by a physician skilled in the art through methods of imaging methods and metabolic assessment.

Imaging methods include, but are not limited to, positron emission spectroscopy, MRI and/or single photon emission computed tomography. Metabolic assessment includes, but is not limited to, the oral glucose tolerance test, the intravenous glucose tolerance test, the intravenous arginine stimulation test and glucose potentiation of arginine-induced insulin secretion. Known mathematic models for assessing β-cell function include, but are not limited to the homeostatic model assessment (HOMA) and continuous infusion of glucose with model assessment (CIGMA). HOMA uses a

structural model of glucose-insulin interaction and assesses β-cell function and insulin resistance from basal glucose and insulin or C-peptide concentrations in a patient (Wallace, 2004). CIGMA evaluates the near steady state glucose/insulin concentration after approximately 1-2 hours, continuous glucose infusion that causes plasma glucose levels similar to postpradial levels (Hermans, 1999)

Methods of Modulating Pancreatic Islet β-Cell Mass

The present invention provides methods for treating pancreatic islet β-cell dysfunction and diseases and conditions associated therewith. One embodiment includes a method of treatment of pancreatic islet cell dysfunction and treating diseases and conditions associated therewith by the modulation β-cell mass in a mammal by administering to the mammal a therapeutically effective amount of KLK1.

Pharmaceutical compositions may be administered orally.

Oral administration includes enteral administration of solution, tablets, sustained release capsules, enteric coated capsules, and syrups.

An "effective amount" or a "therapeutically effective amount" refers to a nontoxic but sufficient amount of drug or agent to provide a desired effect. In a combination therapy, an "effective amount" of one component of the combination is an amount of that compound that is effective to provide a desired effect when used in combination with the other components of the combination. An amount that is "effective" will vary from subject to subject, depending on the age and general condition of an individual, a particular active agent or agents, and the like. An appropriate "effective" amount in any individual case may be determined using routine experimentation.

A therapeutically effective amount of a compound of the invention for treating the above-identified diseases or symptoms thereof can be administered prior to, concurrently with, or after the onset of the disease or symptom. A compound of the invention can be administered concurrently with the onset of the disease or symptom. "Concurrent administration" and "concurrently administering" as used herein includes administering a polypeptide of the invention and another therapeutic agent in admixture, such as, for example, in a pharmaceutical composition or in solution, or separately, such as, for

example, separate pharmaceutical compositions or solutions administered consecutively, simultaneously, or at different times, but not so distant in time such that the compound of the invention and the other therapeutic agent cannot interact and a lower dosage amount of the active ingredient cannot be administered.

Another aspect of the present invention includes a method as herein described further comprising concurrently using an additional therapeutic method useful in the treatment of pancreatic islet cell dysfunction and treating disease and symptoms associated therewith. An additional therapeutic method includes, but is not limited to, an stem cell transplant and pancreatic organ transplant.

"Treatment" and "treating" refer to preventing, inhibiting, and/or alleviating disease or symptoms associated with pancreatic islet cell dysfunction as well as healing disease or symptoms associated with pancreatic islet cell dysfunction affecting mammalian organs and tissues. A composition of the present invention can be administered in a therapeutically effective amount to a patient before, during, and after any above-mentioned condition arises.

The invention will be described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

EXAMPLES

The methods employed herein are similar to those seen in the works of Ellingsgaard (Ellingsgaard *et al, PNAS*, 2008, 105(35): 13162-7).

Example 1: Modulation of Pancreatic Islet Cell Mass

20 Male C57BL/6J mice aged 8 weeks were fed a hypercaloric diet consisting of 58% calories from fat for 8 weeks. Water was provided *ad libitum*. The mice are genetically predisposed to developing type 2 diabetes and are often used for diabetes and obesity studies. 10 mice in the treatment group were injected intramuscularly (2ml/kg) with isolated pig KLK1 having a sequence of SEQ ID NO. 10 at a dosage of 0.375 mg/ml in PBS and daily the control group received a placebo injection of PBS. For the final 14

days the mice were exposed to 1 mg/ml of bromodeoxyuridine (BrdU) in the drinking water, which is used to detect the proliferation of the beta cells.

Animals were sacrificed. The pancreas of each animal was harvested and fixed in paraformaldehyde. Histological analysis was done. The 20 pancreas samples were stained with anti-BrdU, to detect cell proliferation and anti-insulin to detect beta cells.

Results

Data was interpreted manually by a technician. Staining was graded as either weak (1); moderate (2); or strong (3). The percent of the beta cells stained was also recorded. Cells treated with KLK1 showed higher beta cell intensity on average (40% more intensity) and a higher percentage of BrdU in the beta cells (45% more proliferation) as compared to the control cells. Therefore KLK1 is able to trigger β-stem cell proliferation (Table 1. and Table 2.)

Table 1: Effect of PBS on beta cell intensity and BrdU staining

Animal	Beta cell Intensity	% BrdU in Beta Cells
1	2	3%
2	3	5%
3	2	4%
4	3	7%
5	1	5%
6	1	5%
7	2.5	6%
8	3	4%
9	2	8%
10	2	4%
Average	**2.05**	**5.1%**

Table 2: Effect of KLK1 on beta cell intensity and BrdU staining

Case	Beta cell Intensity	% BrdU in Beta Cells
11	2.5	8%
12	3	9%
13	3	7%
14	2.5	8%

15	3	7%
16	3	7%
17	3	8%
18	3	8%
19	2	6%
20	3	6%
Average	**2.80**	**7.4%**

Example 2: Treatment of Streptozotocin treated rats with KLK1

MaleWistar rats 7-9 weeks old (225-275 grams) were used. The animals were fed a standard Purina chow diet. The duration of the study was 28 days but 3 weeks of treatment. The rats were divided into 5 treatment groups: (a) no STZ (n=8), (b) PBS vehicle (n=8), (c) KLK1 0.2U (n=8), (d) KLK1 1U (n=8) and (e) KLK1 5U (n=8). The animals were injected once with 50 mg/kg of streptozotocin (STZ) to cause beta cell death as STZ is a beta cell specific toxin. KLK1 treated animals had BID IP injections on days 7 to 28. All animals had daily IP injections of bromodeoxyuridine (BrdU) (50 mg/kg) on days 7 to 15. BrdU is used for the detection of beta cells.

The animals were sacrificed. Formalin-fixed pancreas samples were embedded in paraffin and sectioned at approximately 5 microns. One serial section was stained with hematoxylin and eosin (H&E) and another serial section underwent an immunohistochemical (IHC) protocol. This included an ant-BRDU antibody conjugated to DAB as a chromagen (brown) and an anti-insulin antibody conjugated to fast red as a chromagen. All slides were examined by a board-certified veterinary pathologist. The following parameters were assessed:

Pancreatic Beta Cell Area:

For each IHC sample 2 typical 4x objective fields were photographed at a fixed pixel density that contained all or mostly all pancreas with representative areas of islets. Using Nikon Elements 3.0 software these images were thresholded for insulin-positive tissue (red tissue) and the number of insulin-positive pixels was recorded. The image was then

thresholded for all pancreatic tissue present (excluding white space and all non-pancreatic tissue such as small intestine or lymph node) and the number of pixels here recorded.

The area of insulin-positive tissue was then reported as the Area of insulin positive tissue / Area of all pancreatic tissue in the image. The 2 such data points for each sample were summed and this data was presented.

Islet Number:

In these same two 4x objective fields per sample the number of islets was counted and recorded.

Pancreatic Beta Cell Replication:

Brown BrdU-positive nuclei that were within insulin-positive cells of islets were counted. The number of such cells in 5 evenly-sized large islets was counted and recorded.

Number of Insulin-Positive Duct Cells:

Five 20x objective fields with large pancreatic ducts were assessed. Any insulin-positive duct cells in these 5 fields were counted and recorded.

Results:

Figure 1 shows Pancreatic Beta Cell Area for the treatment groups. Pancreatic Beta Cell area was reduced in the STZ + PBS group compared to the No STZ group indicating that streptozotocin has successfully induced islet atrophy, as expected.

All three dose groups of Compound 1 (KLK1) notably increase pancreatic beta cell area as compared to the STZ + PBS group.

Figure 2 shows islet number data for the treatment groups. Islet number was notably reduced in the STZ + PBS group compared to the No STZ group indicating that streptozotocin successfully induced islet atrophy, as expected.

All three dose groups of Compound 1 (KLK1) notably increase islet number compared to the STZ + PBS group.

Figure 3 shows data regarding beta cell replication in islets. Beta cell replication is notably reduced in the STZ + PBS group compared to the No STZ group indicating that streptozotocin has successfully hindered beta cell replication in islets, as expected.

All three dose groups of Compound 1 notably increase this parameter compared to the STZ + PBS group, and the 0.2U dose does so in a statistically significant manner (as shown in Figure 3, ** = $p<0.01$).

Figure 4 shows the number of insulin-positive duct cells in the pancreas for all treatment groups. The number of insulin-positive duct cells was slightly reduced in the STZ + PBS group compared to the No STZ group.

All three dose groups of Compound 1 (KLK1) notably increase the number of insulin-positive duct cells in the pancreas, compared to the STZ + PBS group and the No STZ group.

Figure 5 shows representative images of pancreatic islets, for the five treatment groups (No STZ, STZ + PBS, STZ + Compound 1 (KLK1) at 0.2 units, STZ + Compound 1 (KLK1) at 1.0 units, and STZ+ Compound 1 (KLK1) at 5.0 Units. Pancreatic islets were stained bright red via insulin IHC. Images were taken at 40x magnification.

CLAIMS

1. A method of treating pancreatic islet β-cell dysfunction comprising administering a therapeutically effective amount of a purified or isolated KLK1, or a variant or active fragment thereof.
2. The method of claim 1 wherein the purified or isolated KLK1, or variant or active fragment thereof, is a purified and isolated natural KLK1.
3. The method of claim 1 wherein the purified or isolated KLK1, or variant or active fragment thereof, is recombinantly produced.
4. The method of any one of claims 1-3 wherein the purified or isolated KLK1 comprises the amino acid sequence of SEQ ID NO. 1.
5. The method of any one of claims 1-3 wherein the purified or isolated KLK1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, and SEQ ID NO. 10.
6. The method of any one of claims 1-3 wherein the purified or isolated KLK1 comprises a polypeptide having 90% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
7. The method of any one of claims 1-3 wherein the purified or isolated KLK1 comprises a polypeptide having 82% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
8. The method of any one of claims 1-3 wherein the purified or isolated KLK1 comprises a polypeptide having 74% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
9. The method of any one of claims 1-3 wherein the purified or isolated KLK1 comprises a polypeptide having 72% sequence identity to SEQ ID NO. 1 and

serine protease activity capable of cleaving low molecular weight kininogen into kallidin.

10. The method of any one of claims 1-3 wherein the purified or isolated KLK1 comprises a polypeptide having 60% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.

11. The method of claim 3 wherein the purified or isolated KLK1 or variant or active fragment thereof comprises a pegylation sequence.

12. The method of any one of claims 1-11, wherein the pancreatic islet β-cell dysfunction comprises an abnormal β-cell mass.

13. A method of any one of claims 1-12, wherein the purified or isolated KLK1, or a variant or active fragment thereof, is administered orally.

14. A method of any one of claims 1-13, wherein the purified or isolated KLK1, or a variant or active fragment thereof, is administered concurrently with a second therapeutic method useful in treating pancreatic islet cell dysfunction.

15. A method of claim 14, wherein the second therapeutic method is selected from the group consisting of a stem cell transplant, and a pancreatic organ transplant.

16. The method of any one of claims 1-15 whereby the treatment modulates β-cell mass.

17. The method of claim 16 wherein the modulation of β-cell mass is selected from the group consisting of an increase in β-cell mass, β-cell regeneration, a restoring of normal β-cell function by increasing the number of functional β-cells, an increase in β-cell proliferation, and restoring or improving function in impaired β-cells.

18. Use of a purified or isolated KLK1, or a variant or active fragment thereof for the treatment of pancreatic islet β-cell dysfunction.

19. Use of a purified or isolated KLK1, or a variant or active fragment thereof in the preparation of a medicament for the treatment of pancreatic islet β-cell dysfunction.

20. Use of a purified or isolated KLK1, or a variant or active fragment thereof, in the preparation of a medicament for the treatment of pancreatic islet β-cell dysfunction.
21. Use of claim 20 wherein the purified or isolated KLK1, or variant or active fragment thereof, is a purified and isolated natural KLK1.
22. Use of claim 20 wherein the purified or isolated KLK1, or variant or active fragment thereof, is recombinantly produced.
23. Use of claim 20, wherein the purified or isolated KLK1 comprises the amino acid sequence of SEQ ID NO. 1.
24. Use of claim 20, wherein wherein the purified or isolated KLK1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, and SEQ ID NO. 10.
25. Use of claim 20, wherein the purified or isolated KLK1 comprises a polypeptide having 90% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
26. Use of claim 20, wherein the purified or isolated KLK1 comprises a polypeptide having 82% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
27. Use of claim 20, wherein the purified or isolated KLK1 comprises a polypeptide having 74% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
28. Use of claim 20, wherein the purified or isolated KLK1 comprises a polypeptide having 72% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
29. Use of claim 20 wherein the purified or isolated KLK1 comprises a polypeptide having 60% sequence identity to SEQ ID NO. 1 and serine protease activity capable of cleaving low molecular weight kininogen into kallidin.
30. Use of claim 20 wherein the purified or isolated KLK1 or variant or active fragment thereof comprises a pegylation sequence.

31. Use of claim 20 wherein the pancreatic islet β-cell dysfunction comprises an abnormal β-cell mass.
32. Use of claim 20 wherein the purified or isolated KLK1, or a variant or active fragment thereof, is administered orally.

5

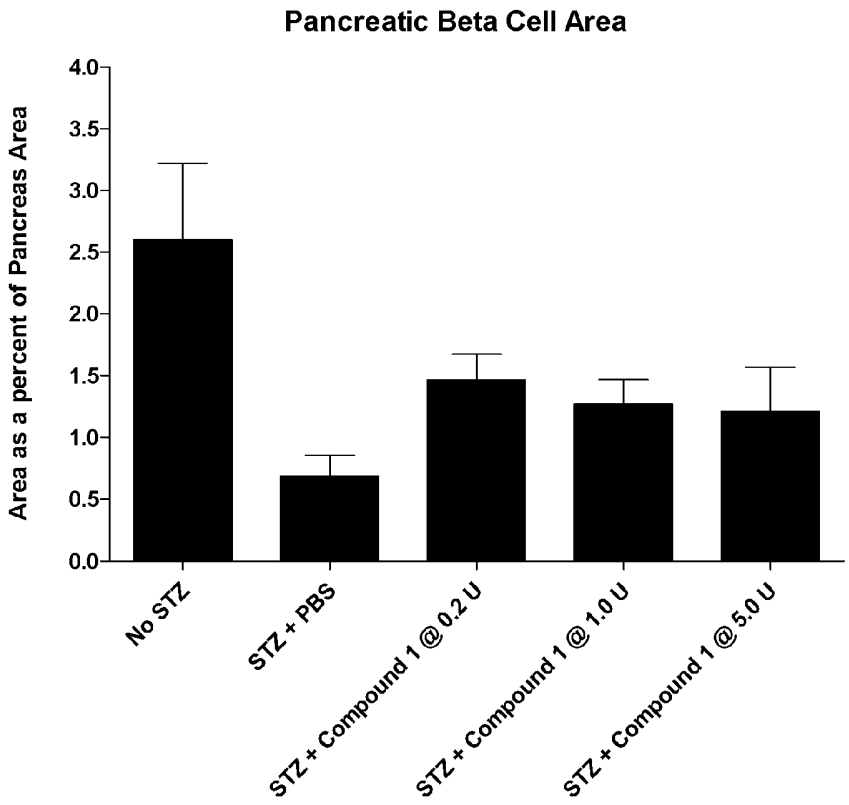
Pancreatic Beta Cell Area
Area as a percent of Pancreas Area
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
No STZ
STZ + PBS
STZ + Compound 1 @ 0.2 U
STZ + Compound 1 @ 1.0 U
STZ + Compound 1 @ 5.0 U

FIGURE 1

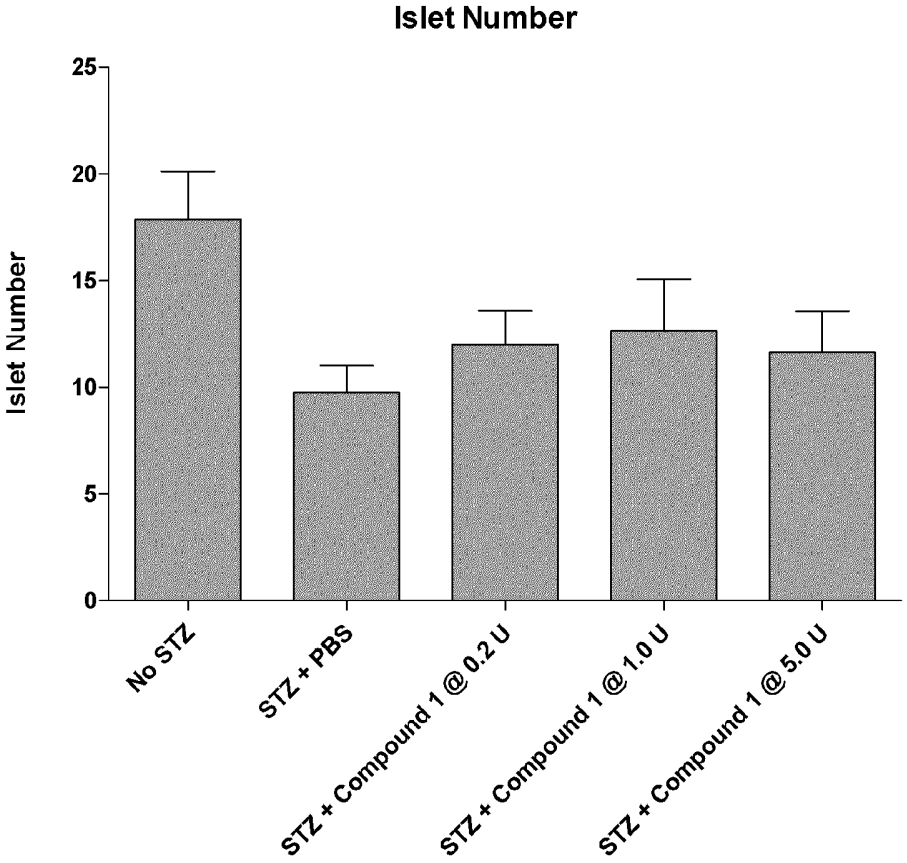
Islet Number
Islet Number
25
20
15
10
5
0
No STZ
STZ + PBS
STZ + Compound 1 @ 0.2 U
STZ + Compound 1 @ 1.0 U
STZ + Compound 1 @ 5.0 U

FIGURE 2

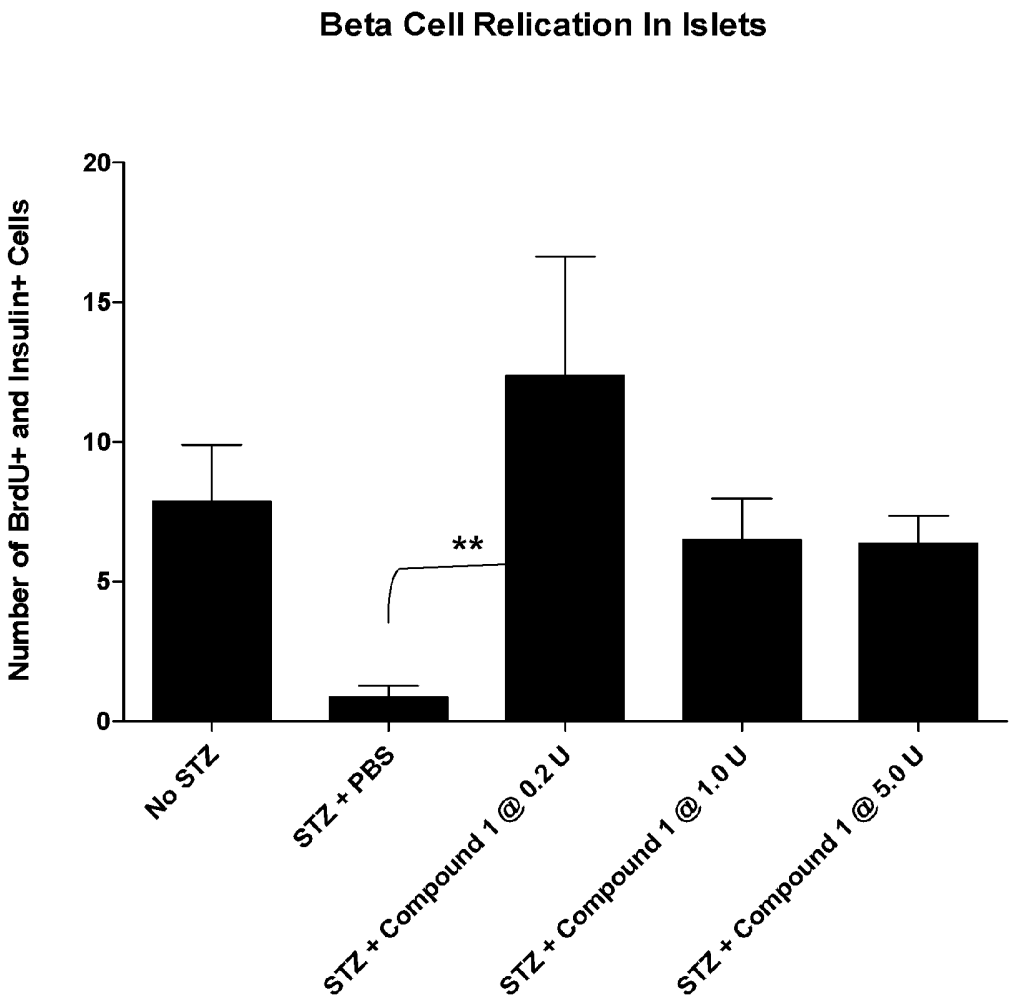
Beta Cell Relication In Islets
Number of BrdU+ and Insulin+ Cells
20
15
10
5
0
**
No STZ
STZ + PBS
STZ + Compound 1 @ 0.2 U
STZ + Compound 1 @ 1.0 U
STZ + Compound 1 @ 5.0 U

Figure 3

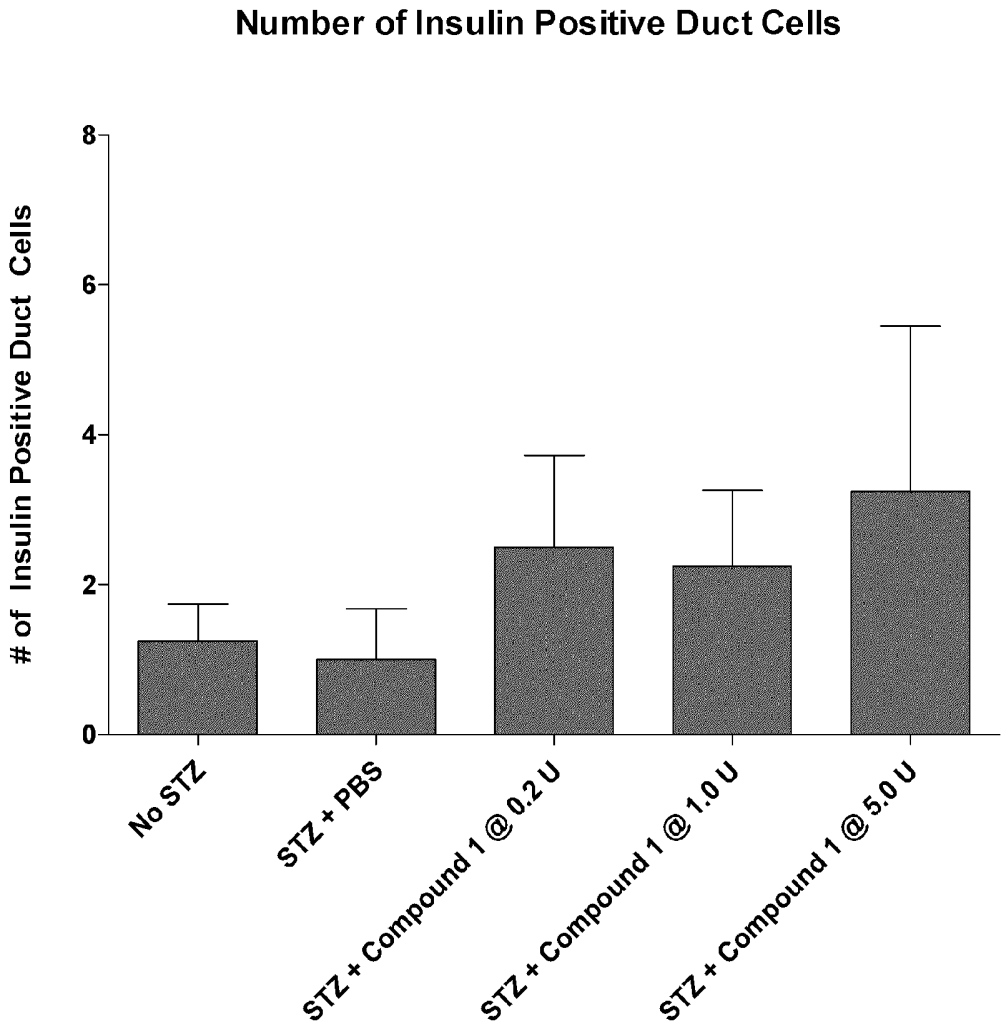
Number of Insulin Positive Duct Cells
# of Insulin Positive Duct Cells
8
6
4
2
0
No STZ
STZ + PBS
STZ + Compound 1 @ 0.2 U
STZ + Compound 1 @ 1.0 U
STZ + Compound 1 @ 5.0 U

Figure 4

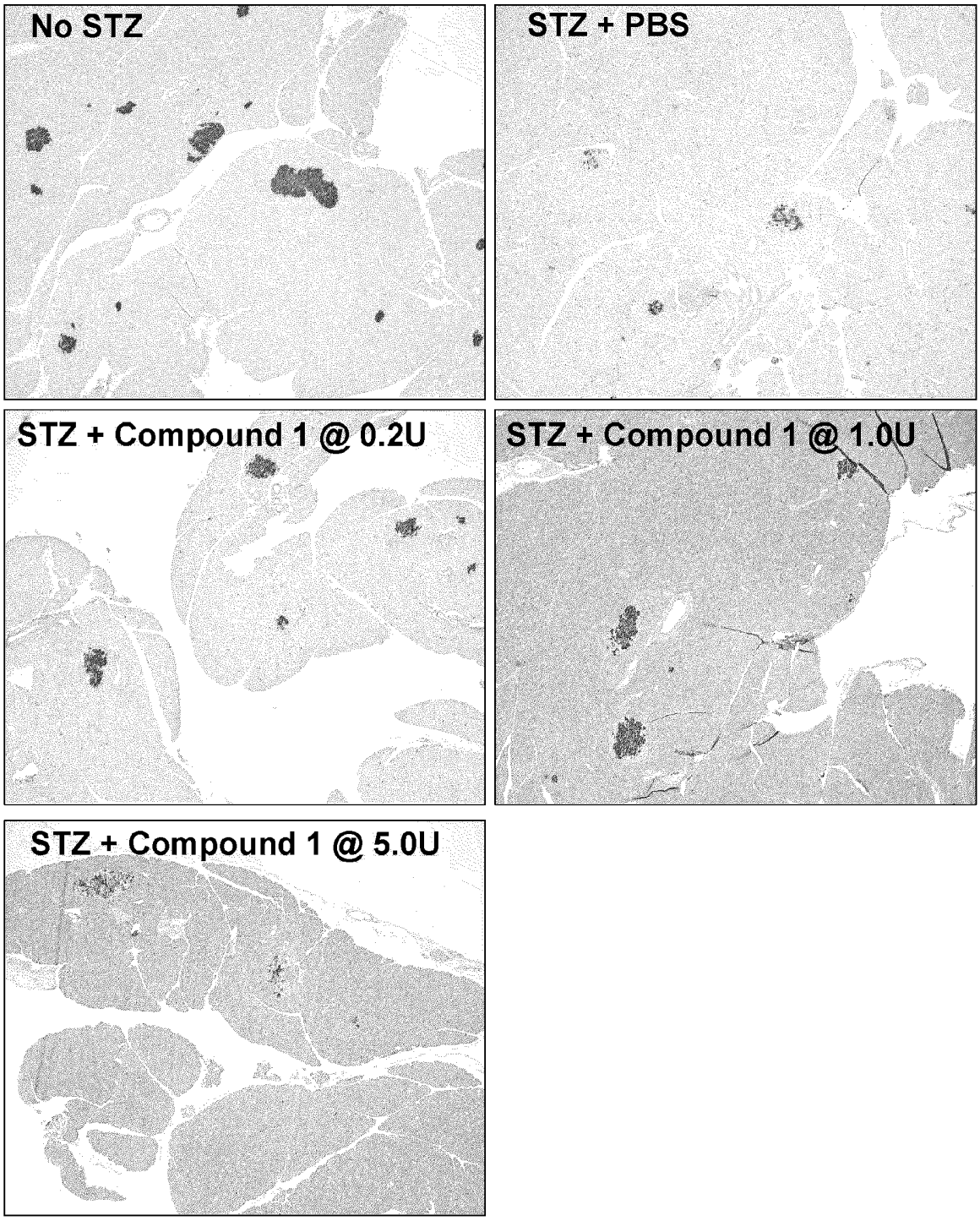
No STZ
STZ + PBS
STZ + Compound 1 @ 0.2U
STZ + Compound 1 @ 1.0U
STZ + Compound 1 @ 5.0U

Figure 5