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Development of proteolytically stable N-methylated peptide inhibitors of aggregation of the amylin peptide implicated in type 2 diabetes

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Abstract

Islet amyloid polypeptide, also known as amylin, is the main component of the amyloid deposits present in approximately 90% of people with type 2 diabetes mellitus (T2DM). In this disease, amylin aggregates into multimeric β-pleated sheet structures which cause damage to pancreatic islet β-cells. Inhibitors of early-stage amylin aggregation could therefore provide a disease-modifying treatment for T2DM. In this study, overlapping peptides were designed to target the 'binding' region (RLANFLVHSS, residues 11-20) of human amylin, and their effects on amyloid fibril formation were determined by Thioflavin-T assay. The first generation peptides showed less than 50% inhibition of aggregation, but a second generation peptide (H_2N-RGANFLVHGR-$CONH_2$) showed strong inhibitory effects on amylin aggregation, and this was confirmed by negative stain electron microscopy. Cytotoxicity studies revealed that this peptide protected human pancreatic 1.4E7 (ECACC 10070102) insulin-secreting cells from the toxic effects of human amylin. Unlike the retro-inverso version of this peptide, which stimulated aggregation, two N-methylated peptides (H_2N-RGAmNFmLVmHGR-$CONH_2$ and H_2N-RGANmFLmVHmR-$CONH_2$) gave very clear dose-dependent inhibition of fibril formation. These two peptides were also stable against a range of different proteolytic enzymes, and in human plasma. These N-methylated peptides could provide a novel treatment for slowing progression of T2DM.

Keywords: diabetes, amylin, islet amyloid polypeptide, IAPP, N-methylated peptide, retro-inverso peptide, amyloid.

Introduction

Amyloid is a generic term for pathological protein deposits that accumulate in many different organs and tissues when protein molecules in a predominantly β-pleated sheet conformation self-associate, mainly by hydrogen bonds, to form long and unbranching 8-10 nm diameter fibrils [1, 2]. More than 30 different proteins are known to form these fibrils in a wide variety of diseases in humans, including various forms of systemic and inherited amyloidosis [3-5], Alzheimer's disease (AD) [6] and other neurodegenerative diseases [7], and type 2 diabetes mellitus (T2DM) [8-10]. One of the most prevalent of these diseases (along with AD) is T2DM, where the amyloid deposits are found in the islets of Langerhans of the pancreas, and are composed of islet amyloid polypeptide (IAPP), also known as amylin [10]. Amylin is a 37 amino acid peptide belonging to the calcitonin family, members of which have a disulphide bridge between Cys residues 2 and 7, as well as an amidated carboxyl terminus [10, 11]. Amyloid deposits have been reported in around 90% of cases of T2DM [12, 13] and amylin aggregation has been strongly linked with the development of islet β-cell failure in this disease [13, 14]. Early studies demonstrated the toxicity of human amylin to cultured islet cells, through induction of membrane damage, Ca^{2+} ion influx, and apoptosis, and suggested that this toxicity resides in the amyloid fibrils themselves [15-18]. However, as is the case with other amyloids, more recent studies have indicated that smaller 'soluble oligomers' could be the most toxic form of this molecule [19-21].

Currently, approximately 425 million people globally have diabetes and this figure is expected to rise to 642 million by the year 2040 [22]. Moreover, total deaths from diabetes have been predicted to increase by 50% in the next 10 years [23]. Diabetes leads to a number of secondary complications including blindness, heart disease, kidney failure and stroke. A healthy diet, weight control, and exercise, are all necessary for management of T2DM [24, 25]. In addition to these lifestyle changes, a number of drug treatment options are available, including insulin therapy. However, these drugs do not provide a cure for diabetes, or prevent secondary complications. There is, therefore, a great need for more research to develop new and potentially more effective treatment options for diabetes.

Compounds that inhibit the self-assembly of amylin are a potential therapeutic target for limiting damage to pancreatic islet cells in T2DM, and this would be expected to slow progression of this disease (i.e. have a disease-modifying effect). The objective of this study was to develop novel peptide-based inhibitors of amylin aggregation that impede the spontaneous assembly of amylin into oligomers and fibrils *in vitro*. In general, it has been challenging to find suitable drug-like therapeutic agents that inhibit the aggregation of amyloid proteins. However, small organic molecules, peptides, peptidomimetics and nanoparticles have all been developed for this purpose. In the case of AD, where this type of therapy is most advanced, a number of inhibitors of aggregation of the Aβ peptide found in senile plaques, including small molecules and peptides, have been developed over the years, but none of these compounds have been successful yet in human clinical trials [26, 27]. This is partly due to the fact that inhibition of amyloid aggregation involves blocking the interactions between protein monomers, and protein-protein interactions are recognized as difficult therapeutic targets [28, 29]. Generally, regions for protein-protein interactions are 1500–3000 Å in size [30, 31], while the region for protein-small molecule interactions is only 300–1000 Å [32, 33]. Therefore, small molecules are not able to build adequate steric interruptions to inhibit protein aggregation [34]. These challenges make it difficult to develop potent and selective small molecule inhibitors of amyloid aggregation.

An alternative strategy for inhibition of amyloid aggregation is the use of peptide-based inhibitors. Peptide-based inhibitors directed against specific amyloid sub-regions represent the first generation of amyloid-based therapeutics, which can then be developed further into more drug-like molecules, and this could be a promising avenue for development of a new disease-modifying therapy for T2DM. In the case of amylin, previous studies along these lines have focused almost exclusively on the primary 'amyloidogenic' region of the peptide (amino acid residues 22-28, with sequence NFGAILS), which is the main region involved in protein misfolding into the toxic β-sheet conformational structure [35, 36]. These peptide inhibitors are designed to act as 'β-sheet breakers' and are typically

compounds that consist of this amyloidogenic motif in combination with a β-sheet breaker element. The latter can be comprised, for example, of methylated amino acids, or prolines [37, 38]. However, these 'β-breaker' peptides do not completely inhibit fibril formation and their inhibitory effects are often seen only at high concentrations, when the peptides are present in molar excess compared to amylin [39-41]. In contrast, the peptide inhibitors described in this report are designed to interact with amylin at the 11-20 'binding' region (RLANFLVHSS), peptide derivatives from which show maximum binding to full-length human amylin [42]. Many peptides face the challenge of insolubility in aqueous solution and/or susceptibility to proteolytic degradation. To improve the solubility of the peptides described here, and to limit their self-aggregation, arginine-glycine residues (RG-GR) were placed at both N- and C-termini (Figure 1). This approach differs from the 'β-sheet blockers' presented in other studies [43-45] and this rationale follows previous successful research where a peptide inhibitor (OR2) with the sequence H_2N-RGKLVFFGR-$CONH_2$ was found to inhibit Aβ oligomer and fibril formation [46]. A proteolytically stable retro-inverso version of this peptide (RI-OR2), with sequence reversal and substitution of L-amino acids with D-amino acids, was then developed [47]. The next step was to attach a 'TAT' transit sequence (Trans-Activator of Transcription from HIV) to RI-OR2 to allow it to penetrate into cells, and cross the blood-brain barrier [48]. In a final iteration, RI-OR2-TAT was attached to nanoliposomes to produce a highly potent multivalent inhibitor [49, 50]. Here, the first steps of a similar strategy are described for inhibition of aggregation of the amylin peptide in T2DM.

Materials and Methods

Peptides

Full length human amylin (1-37 amide) was purchased from American Peptide Company, California, USA. Structures of the new peptides designed for this study are presented in Table 1. Seven peptide inhibitors (IO1-IO7) derived from the 11-20 binding region of amylin (RLANFLVHSS), together with IO8 (the combined amino acid sequences of IO4 and IO5), and NFGAILS (H_2N-RGNFGAILSGR-$CONH_2$, from the primary amyloidogenic region), were purchased from ChinaPeptide Company, Shanghai, China. RI-IO8 (retro-inverso IO8), and two N-methylated peptides (N1-IO8, N2-IO8), were synthesised by Cambridge Peptides, Birmingham, UK. The effects of two previously published inhibitors were also assessed. The first of these is the hexapeptide H_2N-NF(N-Me)GA(N-Me)IL-COOH (abbreviated here to NMeG24 NMeI26) which is a modification of the amylin 22-27 fragment (NFGAIL), with an N-methylation of the amide bonds at G24 and I26 [51], and was purchased from Anaspec EGT Group, California, USA. The second of these peptides, with amino acid sequence H_2N-ANFLVH-COOH [52], was synthesised by ChinaPeptide Company. All peptides were analysed for purity (see Table 1) and had their mass confirmed by HPLC-MS (Supplementary Figure 1).

Determination of peptide aggregation by thioflavin-T assay

Amylin was 'deseeded' in trifluoroacetic acid (TFA), followed by 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), to remove any preformed aggregates prior to these experiments. ThT assays were carried out in 384-well clear-bottomed microtiter plates (NUNC) by incubating the amylin peptide (25 µM) in the presence of ThT (15 µM) in 10 mM phosphate-buffered saline (PBS), pH 7.4, at 30°C. The inhibitors, when present, were at varying molar ratios relative to amylin, with the total volume of solution in each well set at 60 µl. The plates were shaken every 10 mins, and the fluorescence was then read (λex = 442 nm, and λem = 483 nm) in a BioTek Synergy 2 plate reader. Triplicate readings were taken for each condition, with each experiment being repeated three times.

Cell toxicity experiments

Human pancreatic 1.4E7 (ECACC 10070102) insulin-secreting cells were obtained from Public Health England Culture Collection. 1.4E7 is a hybrid cell line formed by the electrofusion of a primary culture of human pancreatic islets with PANC-1, a human pancreatic ductal carcinoma cell line (ECACC 87092802). These cells were routinely grown in RPMI-1640 medium with L-Glutamine (Gibco Life Technologies), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Monolayers of cells were grown in 75 cm^3 flasks with incubation at 37°C, 5% CO_2. Cell viability was assessed using the Promega CellTiter 96 aqueous one solution cell proliferation (MTS) assay. A confluent layer of cells was detached using trypsin, washed, and then suspended and replated, at 250,000 cells/ml, in culture medium. After 24 hrs, the medium was replaced with fresh medium containing amylin (10 or 20 µM), with the required concentration of peptide inhibitor, with replicates of 6 wells. After incubation for 24 hrs, 20 µl of CellTiter 96 aqueous one solution reagent was added to each well and the plate was incubated for a further 3 hrs. Absorbance at 490 nm was determined using a Wallac Victor^2 1420 multilabel counter (PerkinElmer). Each experiment was repeated at least twice.

Determination of peptide stability

Reverse-phase high performance liquid chromatography (RP-HPLC) was used to determine the stability of the peptide inhibitors in plasma, and in the presence of the stated proteolytic enzymes. A C18 column (Phenomenex, 250 x 4 mm) was used for these experiments, with elution by a gradient of acetonitrile, containing 0.01% trifluoro acetic acid (TFA). A sample of human plasma was obtained, with ethical approval (Oldham Ethics Committee), from Prof. David Mann (University of Manchester). Each peptide (5 µl of 100 µM peptide) was added to 95 µl of 50% plasma. To assess the stability of peptides in the presence of proteolytic enzymes, 2 µl of enzyme (1 mg/ml) was added to 98 µL of peptide (100 µM) in PBS. After incubation, the samples were injected onto the RP-HPLC column and eluted with a linear gradient of 0-60% acetonitrile, with continuous monitoring of absorbance (λ = 220nm).

Transmission Electron Microscopy

Solutions of amylin (25 µM), and amylin in the presence of inhibitors at varying concentrations, were incubated in PBS at room temperature for 48 hrs, with continuous orbital shaking, and a 5 µl sample was applied to a carbon-coated formvar grid. After 3 mins, the liquid was adsorbed by filter paper, then 5 µl of 2% aqueous phosphotungstic acid (PTA) (adjusted to pH 7.3 using 1N NaOH) was applied, and left for 1 min. Excess liquid was removed, and the grid was allowed to dry overnight before observation under a Jeol JEM-1010 electron microscope. Five fields were photographed at random for each sample, after first examining the grids for uniformity.

Statistical analysis

Data for ThT and cell toxicity assays are expressed as mean ± standard error of mean (SEM), for one representative experiment. Statistical analysis was performed using a two-tailed Student's *t* test. Anova and confidence interval (CI) analysis ($P < 0.05$ + 95% CI) was used to compare mean values.

Results

The aggregation of human amylin at 25 μM in the presence of varying concentrations of peptides IO1-IO7 was monitored by ThT assay. Figure 1 shows typical examples of ThT aggregation curves, demonstrating the effects of one of these peptides (IO4) on fibril formation. Figure 2 presents data for percentage aggregation of amylin when incubated in the presence of different concentrations of each peptide, as determined by ThT fluorescence after 48 hrs incubation (corresponding to the level of the final plateau phase of fibril formation). Surprisingly, IO1 (H_2N-RGRLANFLVHSSGR-$CONH_2$), which spans the entire binding region of amylin, gave no significant inhibition. Lower concentrations (0.6 and 2 μM) of all of the peptides IO1-IO7 appeared to stimulate fibril formation, and no peptide inhibited aggregation to <50% of the non-treated control. The most convincing inhibition of amylin aggregation was obtained with IO4 and IO5, and particularly with I05 (H_2N-RGNFLVHGR-$CONH_2$) which inhibited at all concentrations ≥12.5 μM, and so another inhibitor, IO8 (H_2N-RGANFLVHGR-$CONH_2$), was designed by combining the sequences of these two peptides. In order to protect IO8 from proteolysis, a retro-inverso version (RI-IO8, Ac-rGhvlfnaGr-$CONH_2$) was also made, by reversing the peptide sequence and replacing the L-amino acids with D-amino acids. IO8 displayed pronounced inhibitory effects on amylin aggregation at all concentrations ≥1 μM (i.e. down to 1:25 molar ratio of inhibitor to amylin), with 100 μM IO8 decreasing ThT fluorescence to levels comparable with a buffer only control (Figure 3A). In contrast, RI-IO8 showed no inhibitory effects on amylin aggregation, but appeared to enhance fibril formation at all concentrations ≤50 μM (Figure 3A). In addition to retro-inversion, another method to improve the physiochemical properties of IO8 is through N-methylation, and so the next step was to carry out ThT assays with two different N-methylated peptides, N1-IO8 and N2-IO8. Both of these peptides displayed a clear and almost identical dose-dependent inhibition of amylin aggregation (Figure 3B). Results for I08 and related peptides from three independent experiments, each carried out in triplicate, are presented in Supplementary Figure 2. Inhibitor IO8 was then compared with peptide 'NFGAILS' (H_2N-RGNFGAILSGR-$CONH_2$) which was derived from the amyloidogenic region of human amylin. NFGAILS enhanced amylin aggregation at all concentrations ≥25 μM. The effects of

NMeG24 NMeI26 [51] and ANFLVH [52], which are inhibitors reported in the literature to reduce amylin fibril formation, were also assessed. ANFLVH did not dissolve in aqueous solution, and NMeG24 NMeI26 showed no inhibitory effects (Figure 3D).

Figure 4 focusses on the early stages of amylin (25 μM) aggregation in the presence of varying concentrations (0.1 – 100 μM) of N1-IO8 (Figure 4A) and N2-IO8 (Figure 4B). It can be seen that increasing concentrations of these inhibitors were found to progressively reduce the steepness of the curve during the fibril growth phase, indicating a reduction in rate of fibril growth. There was also a progressive decrease in the level of the final plateau phase, indicating a reduction in amount of fibrils formed (it has been demonstrated previously that ThT fluorescence correlates linearly with amyloid fibril concentration [54]). The ThT curves, both in the absence and presence of inhibitors, showed virtually no 'lag' phase, and so any effects of the inhibitors on the initial nucleation steps are not clearly defined.

TEM was used to monitor the effects of IO8, RI-IO8, N1-IO8 and N2-IO8 peptides on amylin aggregation, with samples being negatively stained by 2% phosphotungstic acid (PTA). Amylin (at 25 μM) was incubated with 100 μM, 50 μM, 25 μM, 5 μM and 0 μM (non-inhibited control) of each of these peptides. Figure 5A shows the typical dense meshwork of amyloid fibrils that was observed after 48 hrs incubation of amylin alone. With addition of 100 μM, 50 μM or 25 μM of IO8 (i.e. 4:1, 2:1 and 1:1 molar ratios of IO8 to amylin), no fibrils were observed (illustrated for 25μM IO8 in Figure 5B). At 5 μM IO8 (1:5 molar ratio of IO8 to amylin) some fibrils were observed, but at a lower density than those seen in the amylin control. On addition of 100 μM, 50 μM, 25 μM or 5 μM RI-IO8 to 25 μM amylin, very dense fibrillar aggregates of amylin were observed (illustrated for 25 μM RI-IO8 in Figure 5C). In the presence of 100 μM, 50 μM or 25 μM of either N1-IO8 or N2-IO8, no fibrils were seen after 48 hrs incubation (illustrated for 25 μM N1-IO8 and N2-IO8 in Figures 5D, 5E). At 5 μM of N1-IO8, a few fibrils were observed, but no fibrils were seen with 5 μM of N2-IO8. None of the peptides tested

showed any tendency to form oligomers or fibrils when incubated alone (Figure 5F, 5G, 5H, 5I). These TEM results support the ThT data and confirm that IO8, N1-IO8 and N2-IO8 are effective inhibitors of amylin aggregation, whereas RI-IO8 has no inhibitory effect, and may even stimulate fibril formation.

The stability of the most promising inhibitory peptides (IO8, N1-IO8 and N2-IO8) towards individual proteolytic enzymes (see Supplemetary Figure 3), and in human plasma, was assessed by RP-HPLC. The data are summarised in Table 2, with examples of RP-HPLC traces of peptides in plasma presented in Figure 6. IO8 was highly susceptible to the effects of trypsin and chymotrypsin, which rapidly degraded this peptide, but it was also degraded by cathepsin G, elastase, thrombin, kallikrein, plasmin, and factor X. Not surprisingly, therefore, IO8 was very unstable in human plasma (Figure 6A). In contrast, both N1-IO8 and N2-IO8 were stable for up to 24 hrs incubation in plasma (Figure 6B and 6C), and, unlike IO8, remained intact after 3 hrs incubation with each of the individual proteolytic enzymes, although some degradation was noted after 24 hrs incubation (see Table 2).

The toxic effect of human amylin (20 µM and 10 µM) on human pancreatic 1.4E7 cells was determined by MTS assay, in the presence and absence of 1:1 and 1:4 molar ratios (inhibitor:amylin) of the IO8, N1-IO8 and N2-IO8 peptides (for results see Figure 7). Amylin at 20 µM was cytotoxic to PANC-1 cells and consistently reduced cell viability to ~40% of untreated control cells, whereas at 10 µM amylin the results were more variable and cell viability was reduced to 60-90%. All of these inhibitor peptides, at both molar ratios, rescued the cells from the toxic effect of 20 µM amylin and 10 µM amylin. None of the inhibitors alone, at concentrations of up 20 µM, had any effect on cell viability. Cell toxicity results for I08 and related peptides from three independent experiments, each carried out in triplicate, are presented in Supplementary Figure 4.

Discussion

T2DM is the most widespread endocrine disorder [53], and is characterised by a reduction in β-cell mass, insulin resistance, and the presence of amyloid deposits in the pancreatic islets, the main component being amylin [55]. The 22-28 (NFGAILS) segment of amylin is regarded as the most highly amyloidogenic region of this peptide, and will itself assemble into amyloid fibrils [56, 39]. However, residues 8-20 [57], 14-20 [58], and 30-37 [59] have also been reported to form β-sheet fibrils. Although several amyloidogenic regions of human amylin have been proposed, this study was concerned with developing peptide inhibitors from the 'binding' region of human amylin, corresponding to amino acid residues 11-20 (RLANFLVHSS), and on studying their impact on the fibrillogenesis of full-length human amylin. This region is thought to be involved in the initial interactions between two misfolded amylin molecules, after which they begin to aggregate [42]. Thus, preventing this interaction should impede aggregation. This strategy, to target the binding region, has been successfully applied to development of inhibitors of Aβ aggregation as a potential disease-modifying treatment for AD [47-50].

Seven potential inhibitor peptides were derived from this binding region, and investigated for their ability to influence amylin fibril formation, based on the ThT fluorescence assay [60]. Peptides IO2, IO3, IO4, IO5 and IO7 (see Table 1) showed some inhibitory effects, but IO4 and IO5 gave the most promising results, and were considered for further investigation. These two amino acid sequences were combined to give IO8 (amino acid sequence: H_2N-RGANFLVHGR-$CONH_2$). Retro-inverso peptides often retain bioactivity and are stable to proteolysis [61, 47], and so the retro-inverso version of IO8 (RI-IO8: Ac-rGhvlfnaGr-$CONH_2$) was derived by sequence reversal and D-amino acid substitution. The IO8 peptide showed a strong inhibitory effect on amylin aggregation, and, unlike peptides IO1-IO7, did not stimulate amylin aggregation at low concentrations. However, RI-IO8 had no inhibitory effect on amylin aggregation, except at a 4:1 molar ratio RI-IO8 to amylin, where the peptide reduced amylin aggregation to only 77% of a non-inhibited control (Figure 3). At lower concentrations, RI-IO8 actually stimulated amylin aggregation. This finding was unexpected and suggests that RI-IO8 does not interact

in the same way as IO8 to full-length human amylin. Congo red binding experiments have also confirmed the inhibitory effect of IO8, and the stimulatory effect of RI-IO8, on amylin aggregation (data not shown). This finding was further supported by TEM studies, where IO8 abolished and RI-IO8 increased amylin fibril formation (Figure 5). This result with RI-IO8 is contrary to a previous study, where the retro-inverso peptide RI-OR2, developed against β-amyloid (Aβ) aggregation, was shown to inhibit amyloid fibril formation, and also rescue cells from the toxic effects of Aβ, as well as being highly resistant to proteolysis [47].

Since RI-IO8 did not inhibit amylin aggregation, N-methylation was considered as an alternative means to improve its stability and pharmacokinetic properties. It is not surprising that IO8 was rapidly degraded in plasma, and in the presence of proteolytic enzymes, because L-peptides are quickly metabolized in this way [62]. IO8 would be cleaved after amino acid 5 (Phe) by high specificity chymotrypsin, and after amino acids 5, 6 and 8 (Phe, Leu, His) by low specificity chymotrypsin, while trypsin will cleave after position 1 (Arg). N-methylation has been shown to improve the pharmacokinetic properties of peptides, by protecting them from proteolysis [63]. Also, N-methylation of alternate amino acid residues gives one face of the peptide molecule that is not available for H-bonding, which impedes amyloid fibril formation [64]. N-methylated derivatives of Aβ(25–35) have been reported to impede the aggregation of fibrils and prevent Aβ cytotoxicity, and N-methylated analogues of amylin do not form fibrils [18, 65]. Here, IO8 was stabilised against proteolytic degradation through N-methylation of alternate amino acid residues, to give N1-IO8 (H_2N-R-G-Am-N-Fm-L-Vm-H-G-R-$CONH_2$) and N2-IO8 (H_2N-R-G-A-Nm-F-Lm-V-Hm-G-R-$CONH_2$). ThT and TEM data showed that both NI-IO8 and N2-IO8 are excellent and highly convincing inhibitors of amylin aggregation (Figures 3-5) and are relatively stable against proteolytic degradation (Figure 6).

New inhibitors of amylin aggregation are desired, as many of the reported inhibitors only work when present in molar excess over amylin [65-67]. For example, a study on peptide fragments corresponding

to human amylin residues 20-25 (SNNFGA) and 24-29 (GAILSS) showed an inhibitory effect on β-sheet transition and amyloid aggregation at 10:1 and 20:1 molar ratios of peptide to amylin, and the GAILSS peptide had no significant effect on amylin-induced cytotoxicity [41]. The inadequacy of some previously published inhibitors is also highlighted by the comparison of the effects of IO8, ANFLVH [52] and NMeG24 NMeI26 [51] on amylin aggregation in this report. ANFLVH has the same amino acid sequence as IO8, but without the flanking cationic arginine residues together with their glycine spacers. Although ANFLVH was reported to inhibit amylin fibril formation *in vitro* and to protect against amylin cytotoxicity, the latter effect was only observed at 10-fold and 20-fold molar excess concentrations of the peptide [52]. In contrast, the IO8 peptide inhibits amylin aggregation at much lower concentrations than this, and, given the inability to dissolve ANFLVH in aqueous solution, is clearly much more soluble. Moreover, IO8, N1-IO8 and N2-IO8 were seen to rescue human pancreatic 1.4E7 cells from the toxic effects of amylin at a 1:4 molar ratio of these peptides to amylin. In contrast to a previous report [51], no evidence of inhibition of aggregation was observed upon addition of NMeG24 NMeI26, at reasonable concentrations, to amylin. In fact, NMeG24 NMeI26 was seen to promote fibril formation (Figure 3). This discrepancy could be due to the fact that NMeG24 NMeI26 was reported to inhibit IAPP aggregation when added before nucleation [68] but the aggregation system reported here lacks any lag-phase (see Figure 4), and so nucleation may be too rapid for this inhibitor to be effective. Two human amylin-derived peptides, with sequences NFGAIL and SNNFGAILSS, were unable to inhibit fibrillation of human amylin [69]. Another study has indicated that NFGAIL causes an immediate shift of amylin to the β-sheet conformation, suggesting that this peptide promotes fibril formation [41]. These results, together with the lack of effect of the H_2N-RGNFGAILSGR-$CONH_2$ peptide in this study (Figure 3), justify the decision to focus on the RLANFLVHSS (residues 11-20) amylin binding region.

As noted above, modified full-length amylin with N-methylation at positions 24 and 26 has been shown to impede amylin aggregation and its associated cytotoxicity [65]. In addition, a human amylin

derived peptide marketed as pramlintide, with proline substitutions at positions 25, 28 and 29, has undergone clinical trials [70-73] where it was administered, alongside insulin, for management of T2DM. This combination of drugs was able to maintain near-normal glycaemic levels, but pramlintide peptide does not appear to have been assessed as an inhibitor of human amylin aggregation. The short peptides described in this report would be much easier and less expensive to synthesize than these full-length human amylin analogues, and would, potentially, be less immunogenic. N1-IO8 and N2-IO8 in particular appear to be potent and stable aggregation inhibitors that are suitable for further development and testing in human amylin transgenic rodent models as potential disease-modifying agents for T2DM. However, the effects of these inhibitors on oligomer formation are not clear from the data presented here, and will need to be examined in further studies. Also, it is emerging that a significant component of amylin toxicity is mediated by inflammation [74], and so the ability of these inhibitors to attenuate amylin-mediated macrophage activation and associated β-cell dysfunction will also need to be determined.

Table 1

Design of peptide inhibitors employed for this study.

Peptide inhibitor ID	Sequence	Purity %
IO1	H_2N-R G **R L A N F L V H S S** G R-$CONH_2$	95%
IO2	H_2N-R G **R L A N F** G R-$CONH_2$	95%
IO3	H_2N-R G **L A N F L** G R-$CONH_2$	93%
IO4	H_2N-R G **A N F L V** G R-$CONH_2$	95%
IO5	H_2N-R G **N F L V H** G R-$CONH_2$	95%
IO6	H_2N-R G **F L V H S** G R-$CONH_2$	96%
IO7	H_2N-R G **L V H S S** G R-$CONH_2$	95%
IO8	H_2N-R G **A N F L V H** G R-$CONH_2$	95%
RI-IO8	Ac-r G **h v l f n a** G r-$CONH_2$	96%
N1-IO8	H_2N-R G **Am N Fm L Vm H** G R-$CONH_2$	86%
N2-IO8	H_2N-R G **A Nm F Lm V Hm** G R-$CONH_2$	96%
NFGAILS	H_2N-R G **N F G A I L S** G R-$CONH_2$	98%

The amino acid sequence of human amylin shows the binding site for amylin self-association [42] and the main amyloidogenic region [35]. All of the short peptide inhibitors are designed to interact with the binding region of full-length amylin, except for the last peptide. The arginine-glycine flanking residues (RG-GR) impede peptide self-aggregation. In the retro-inverted peptide (RI-IO8), D-amino acids are in lower case. N-methylated peptide residues are indicated by lower case 'm'.

Table 2

Stability of peptide-inhibitors to proteolysis

	IO8	**RI-IO8**	**N1-IO8**	**N2-IO8**
Blood plasma	X	√	√	√
Trypsin	X	√	√*	√*
Chymotrypsin	X	√	√	√
Cathepsin G	X	√	√	√
Elastase	X	√	√	√
Thrombin	X	√	√*	√
Kallikrein	X	√	√*	√*
Plasmin	X	√	√	√
Factor X	X	√	√	√

√ = stable, X = degraded, after 3 hrs incubation with the enzyme. *Some degradation seen after 24 hrs incubation. HPLC traces for IO8, N1-IO8 and N2-IO8 are given in Supplementary Figure 3.

Figure Legends

Figure 1.

Example of ThT fluorescence curves for human amylin in the presence of different concentrations of an inhibitor (IO4). Data are means for a single experiment carried out in triplicate, with readings taken every 10 mins. Amylin alone (at 25 µM) displays a characteristic increase in fluorescence corresponding to the 'sigmoidal' and 'plateau' phases of amyloid fibril formation, while the addition of the inhibitor, at varying concentrations, has a dose-dependent effect on fibril formation. The buffer control ('Control') contained neither amylin nor inhibitor. RFU = relative fluorescence units.

Figure 2.

ThT data showing effects of IO1, IO2, IO3, IO4, IO5, IO6 and IO7 peptides on amylin aggregation, after 48 hrs incubation. All peptides were assayed at 0.6 µM, 2.5 µM, 5 µM, 12.5 µM, 25 µM, 50 µM and 100 µM concentrations in the presence of 25 µM amylin. Results are means +/- SEM, n = 3, for a single experiment. Student's t-test was used to establish significance at *P<0.05, **P<0.01, or ***P<0.001, compared to 100% control (amylin alone).

Figure 3.

ThT data showing the effects of IO8 and related peptides, as well NFGAILS and NMeG24 NMeI26, on amylin aggregation, after 48 hrs incubation. (A) IO8 and RI-IO8. (B) N1-IO8 and N2-IO8. (C) NFGAILS (H_2N-RGNFGAILSGR-$CONH_2$). (D) NMeG24 NMeI26. All peptides were assayed at 0.6 µM, 2.5 µM, 5 µM, 12.5 µM, 25 µM, 50 µM and 100 µM in the presence of 25 µM amylin. Results show means +/- SEM, n = 3, for a single experiment. See Supplementary Figure 2 for the data from 3 independent experiments. Note the clear dose-dependent effects of the two N-methylated peptides (N1-IO8 and N2-IO8).

Figure 4.

ThT fluorescence curves for the first 10 hrs of incubation of amylin in the presence of different concentrations of (A) N1-IO8 and (B) N2-IO8. Data are means for a single experiment carried out in triplicate, with readings taken every 10 mins. Amylin alone (at 25 µM) displays a characteristic increase in fluorescence corresponding to the 'sigmoidal' and 'plateau' phases of amyloid fibril formation (top curve in both cases). In both (A) and (B), the stepwise decrease in the final level of ThT fluorescence in the 10 curves underneath is due to addition of the inhibitors at concentrations of 0.1, 0.3, 0.6, 1, 2.5, 5, 12.5, 25, 50 and 100 µM. RFU = relative fluorescence units.

Figure 5.

Negative stain EM images of amylin incubated in the presence and absence of inhibitors. (A) Sample of amylin (25 µM) incubated for 48 hrs in PBS at room temperature and stained with phosphotungstic acid (2% w/v). (B) Amylin (25 µM) + IO8 (25 µM). (C) Amylin (25 µM) + RI-IO8 (25 µM); (D) Amylin (25 µM) + N1-IO8 (25 µM); (E) Amylin (25 µM) + N2-IO8 (25 µM); (F) IO8 alone (100 µM); (G) RI-IO8 alone (100 µM); (H) N1-IO8 alone (100 µM); (I) N1-IO8 alone (100 µM). Scale bar = 100 nm.

Figure 6.

Reverse-phase HPLC traces showing stability of peptide inhibitors in human plasma. Column (A) IO8; column (B) N1-IO8; column (C) N2-IO8. For each column, the top trace shows elution of the peptide standard without plasma; the middle trace is for 0 hrs incubation in plasma; and the lower trace is after 48 hrs incubation in plasma. Note that IO8 is degraded, whereas N1-IO8 and N2-IO8 are more stable.

Figure 7.

Cytotoxic effect of amylin on human pancreatic 1.4E7 insulin-secreting cells in the presence or absence of inhibitor peptides. (A) Cells were incubated for 24 hrs with 20 µM or 10 µM human amylin in RPMI-1640 medium, with or without IO8 inhibitor, and viability was measured using the MTS assay. (B) Results for N1-IO8. (C) Results for N2-IO8. In all cases, results show mean +/- SEM, n = 6. ANOVA followed by Student's t-test established significance at *P<0.05, **P<0.01, ***P<0.001.

Author Contributions

D.A. conceived of the study and wrote the manuscript. I.O. carried out all of the experiments, data collection and statistical analyses. M.T. designed some of the peptides and N.J.F. was responsible for the electron microscopy. All authors contributed to design of the study and gave final approval for publication.

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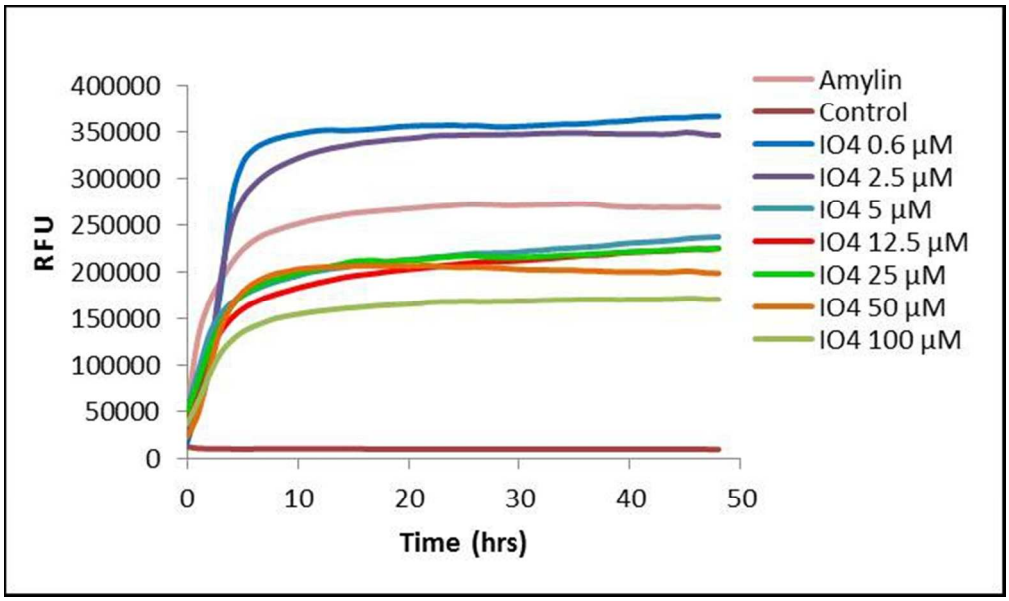

Figure 1. Example of ThT fluorescence curves for human amylin in the presence of different concentrations of an inhibitor (IO4).

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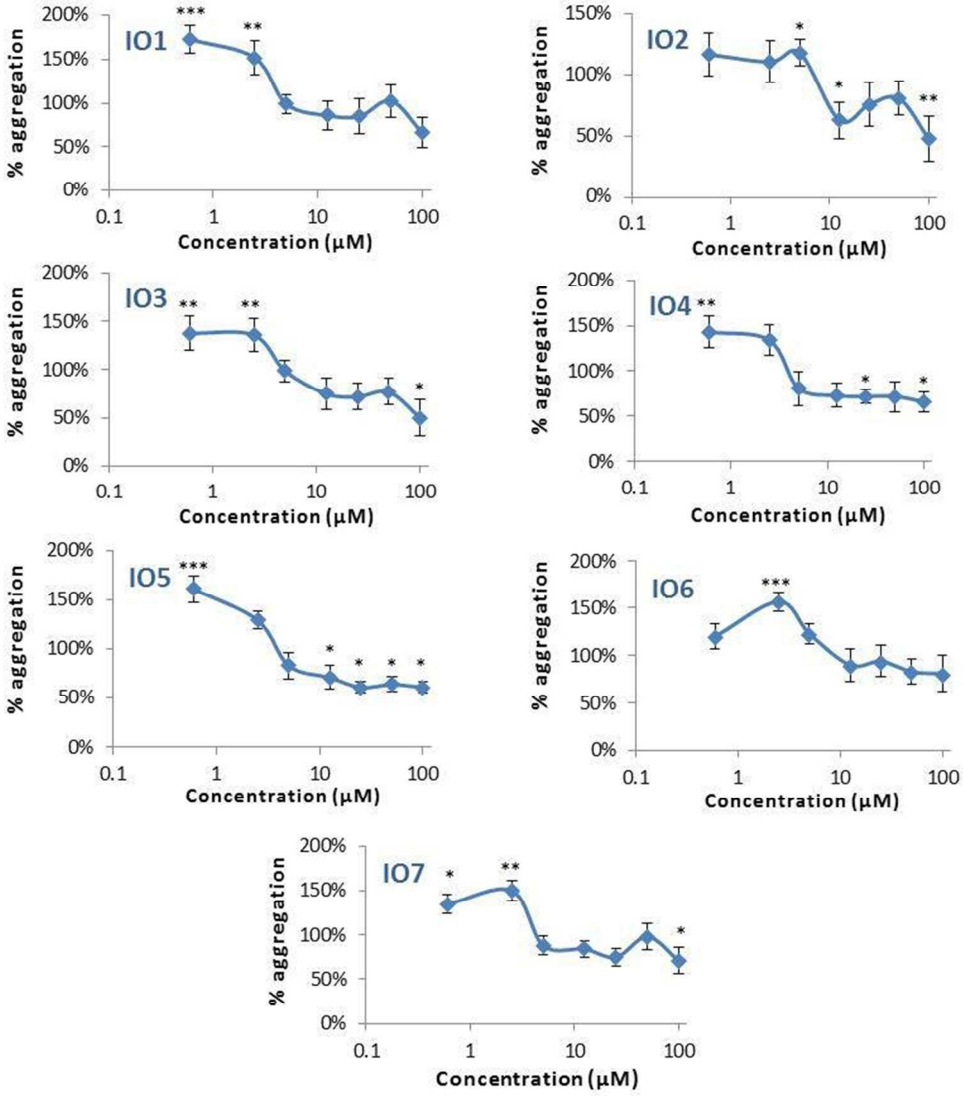

Figure 2. ThT data showing effects of IO1, IO2, IO3, IO4, IO5, IO6 and IO7 peptides on amylin aggregation, after 48 hrs incubation.

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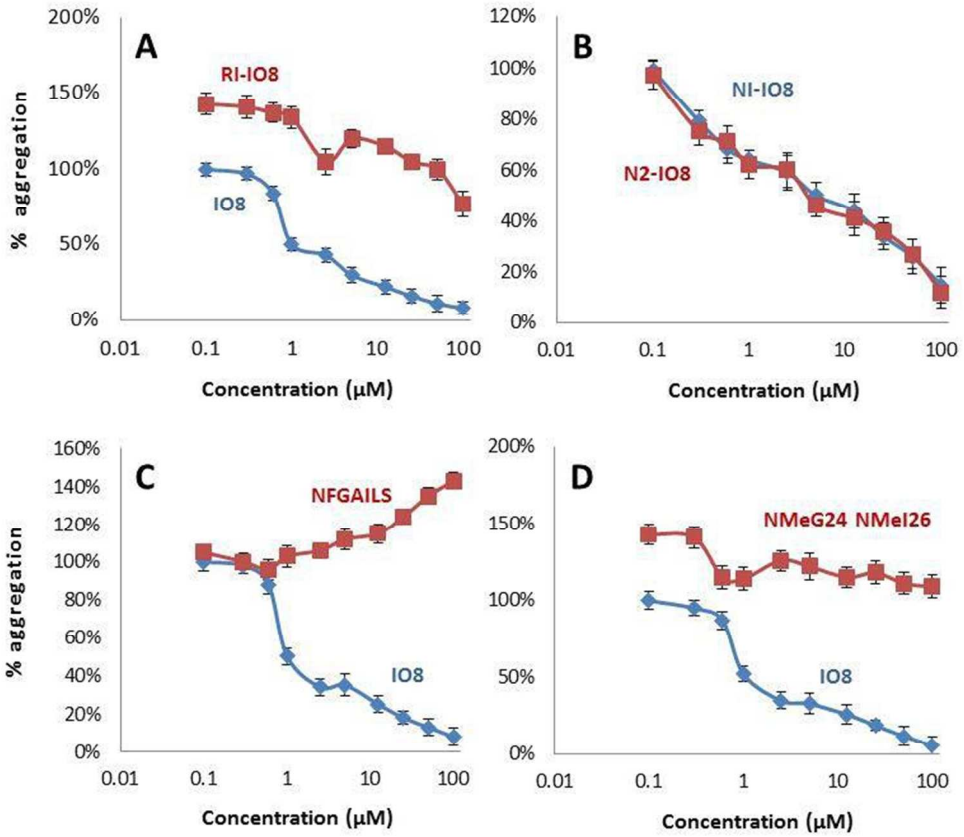

Figure 3. ThT data showing the effects of IO8 and related peptides, as well NFGAILS and NMeG24 NMeI26, on amylin aggregation, after 48 hrs incubation.

166x142mm (120 x 120 DPI)

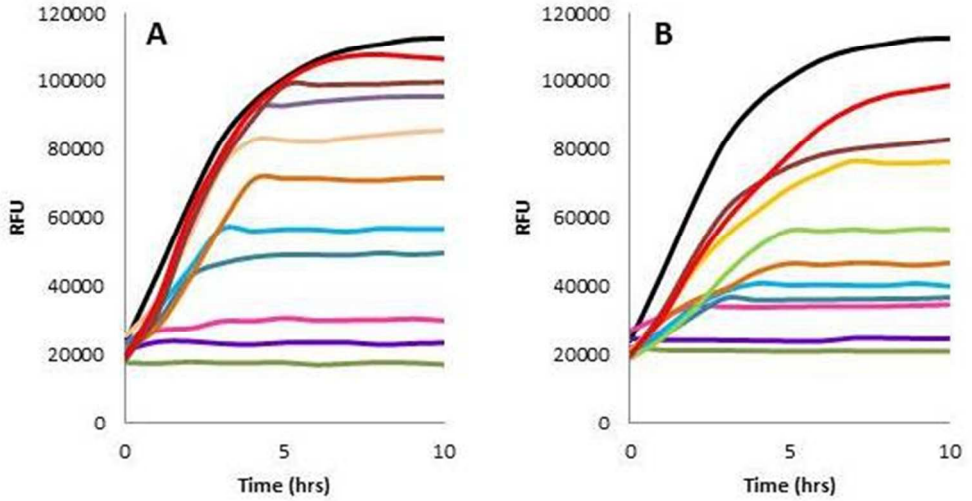

Figure 4. ThT fluorescence curves for the first 10 hrs of incubation of amylin in the presence of different concentrations of (A) N1-IO8 and (B) N2-IO8.

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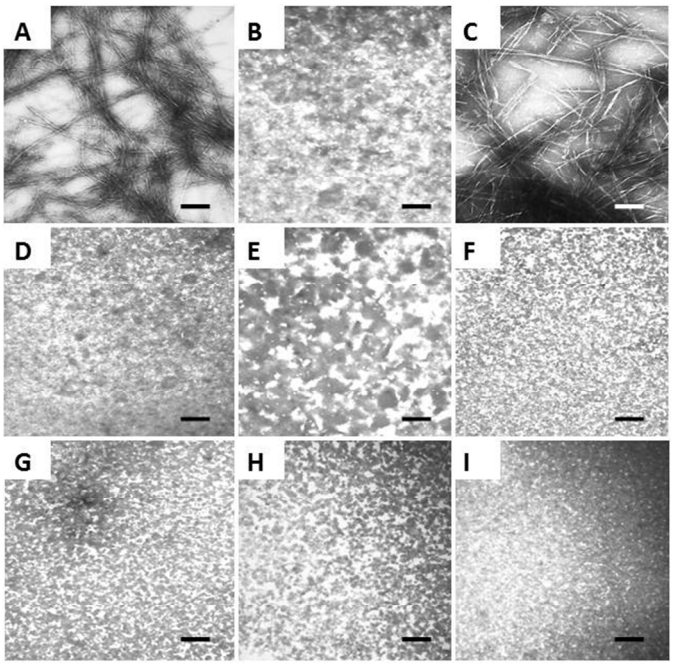

Figure 5. Negative stain EM images of amylin incubated in the presence and absence of inhibitors.

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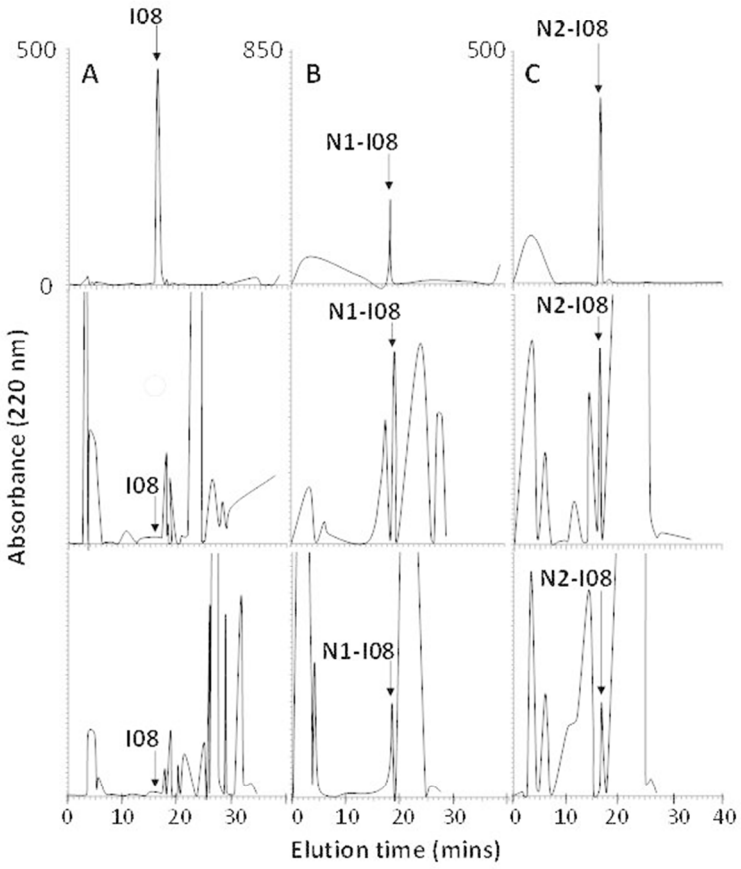

Figure 6. Reverse-phase HPLC traces showing stability of peptide inhibitors in human plasma.

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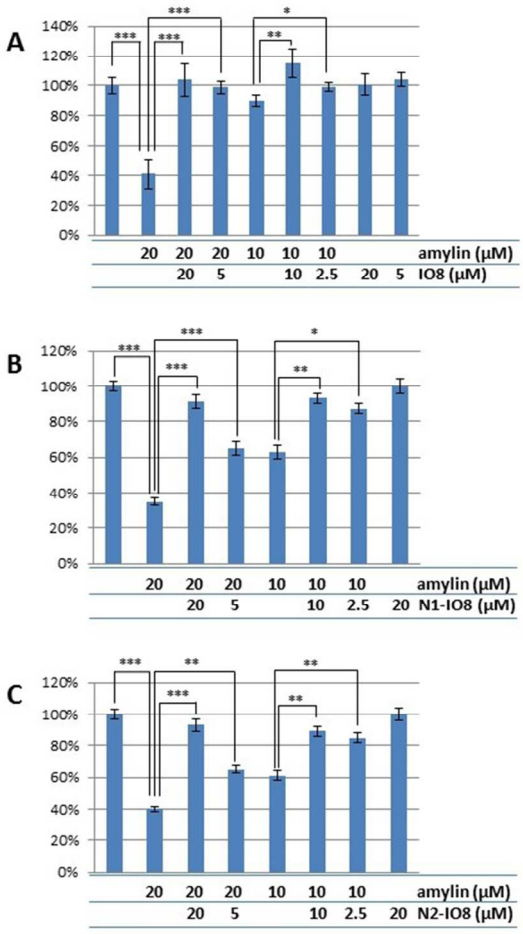

Figure 7. Cytotoxic effect of amylin on human pancreatic 1.4E7 insulin-secreting cells in the presence or absence of inhibitor peptides.

190x254mm (96 x 96 DPI)