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Neuroligin-2-derived peptide-covered polyamidoamine-based (PAMAM) dendrimers enhance pancreatic β-cells' proliferation and functions†

Anna Munder,[a] Yoni Moskovitz,[a] Aviv Meir,[a] Shirin Kahremany,[ab] Laura Levy,[a] Michal Kolitz-Domb,[a] Guy Cohen,[c] Efrat Shtriker,[a] Olga Viskind,[a] Jean-Paul Lellouche,[ad] Hanoch Senderowitz,[a] Steven D. Chessler,[e] Edward E. Korshin,[a] Sharon Ruthstein *[a] and Arie Gruzman *[a]

Pancreatic β-cell membranes and presynaptic areas of neurons contain analogous protein complexes that control the secretion of bioactive molecules. These complexes include the neuroligins (NLs) and their binding partners, the neurexins (NXs). It has been recently reported that both insulin secretion and the proliferation rates of β-cells increase when cells are co-cultured with full-length NL-2 clusters. The pharmacological use of full-length protein is always problematic due to its unfavorable pharmacokinetic properties. Thus, NL-2-derived short peptide was conjugated to the surface of polyamidoamine-based (PAMAM) dendrimers. This nanoscale composite improved β-cell functions in terms of the rate of proliferation, glucose-stimulated insulin secretion (GSIS), and functional maturation. This functionalized dendrimer also protected β-cells under cellular stress conditions. In addition, various novel peptidomimetic scaffolds of NL-2-derived peptide were designed, synthesized, and conjugated to the surface of PAMAM in order to increase the biostability of the conjugates. However, after being covered by peptidomimetics, PAMAM dendrimers were inactive. Thus, the original peptide-based PAMAM dendrimer is a leading compound for continued research that might provide a unique starting point for designing an innovative class of antidiabetic therapeutics that possess a unique mode of action.

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Introduction

Type 2 diabetes mellitus (T2DM) is reaching catastrophic numbers in human populations and causing high morbidity and mortality due to its severe complications.[1–3] One of the most important pathophysiological components in T2DM is the peripheral tissues' insulin resistance, which in turn leads to increased efforts of β-cells to secrete more than physiological amounts of insulin.[4,5] This chronic overstimulation of β-cells occurs because of futile efforts to compensate for the lack of insulin activity. This overstimulation, in turn, causes β-cell dysfunction. At the beginning of the disease, decreased numbers of normal functioning β-cells are usually detected in the pancreas.[6] Thus, preservation or even augmentation of functional pancreatic β-cell mass is one of the main goals of therapeutic development for T2DM.[7,8] In addition, successful transplantation of β-cells/islets is dependent on a sufficient amount of functioning β-cells.[9,10] Thus, recent scientific efforts have focused on identifying new strategies, which will be able to positively influence β-cell function and/or proliferation and thereby prevent or delay the onset of the disease and its late complications.[11–13] Several β-cell mass protective approaches have been recently developed. This includes, for example, the use of H_2S, which protects cells from apoptosis induced by high glucose,[14] targeting mitochondrial DNA,[15] developing compounds that protect cells under ER stress-induced autophagy,[16] and many others.[17,18]

Regarding T1DM, which is also a life-threatening disease, causing severe complications and increased mortality, there is similarly a need for ways to protect β-cell mass.[19–22] In this

[a] Department of Chemistry, Faculty of Exact Sciences, Bar-Ilan University, Ramat-Gan, Israel. E-mail: sharon.ruthstein@biu.ac.il, gruzmaa@biu.ac.il
[b] Department of Pharmacology, Cleveland Center for Membrane and Structural Biology, School of Medicine, Case Western Reserve University, Cleveland, OH, USA
[c] Skin Research Institute, Dead Sea and Arava Research Center, Masada, Israel
[d] Nanomaterials Research Center, Institute of Nanotechnology & Advanced Materials (BINA), Bar-Ilan University, Ramat-Gan, Israel
[e] Division of Endocrinology, Diabetes & Metabolism, Department of Medicine, University of California, Irvine, CA, USA

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autoimmune disease, patients lose the vast majority of their β-cells owing to an aberrant immune attack.[23] The main challenge in T1DM is to protect the remaining β-cells, or, in the ideal case, to even induce β-cell regeneration. To date, only immunosuppressive therapies that begin early after T1DM onset have been able to slightly slow β-cell degeneration and death.[24,25] Together with immunosuppression, autoantigen therapy and agents that can facilitate β-cell regeneration might also be possible drug development options.[26] Because of the huge therapeutic potential in both types of diabetes, the search for novel approaches for preserving or even increasing the β-cell mass has always been very intense.

We recently reported that the HSA-28 peptide, which was derived from NL-2 and covalently attached to the surface of ytterbium(III) cation-doped maghemite nanoparticles (NPs), improved β-cell functionality.[27] The peptide was selected based on the interfacial residues that seem to dominate the interactions between NL-2 and its binding partner neurexin (NX), and which are also completely preserved in the corresponding NL-2 sequence. The rationale to use NL-2 as a basis for developing protective β-cell therapeutics was based on the fact that the secretory/cell contact machinery of the β-cells is similar to that of neurons.[28–31] One of the interacting protein pairs (NLs and NXs) was targeted because of its role in establishing the normal β-cell functioning brought about by β-cell clustering.[28–31] We hypothesized that β-cells treated with NL-2 mimetics might be more functionalized and protected after transplantation.

Indeed, HSA-28-covered maghemite NPs increased proliferation, resistance to oxidative stress, and insulin secretion in both *in vitro* (INS-1E cells) and *ex vivo* (mouse islets) models.[27] However, HSA-28-covered ytterbium(III) maghemite (γ-Fe_2O_3)-based nanoparticles might be not suitable for use in humans, because of genotoxicity[32,33] and neurobehavioral impairments.[34] Herein, our aim was to improve peptide-based cluster functions by using another core platform. Thus, different types of functionalizing NPs were synthesized by conjugation between HSA-28 and the surface amines of a commercially available PAMAM dendrimer. Dendrimers have three-dimensional, highly branched unique polymeric architectures (classically 5000–500 000 g mol^{-1}) and have the benefits of a low polydispersity index, controlled biodegradability, biocompatibility, and the presence of various terminal multivalent functional groups on their surface.[35] Dendrimers are widely used in nanomedicine as potential carriers for various drugs, bioactive molecules, and genes.[36,37] The prepared HSA-28D (HSA-28-coated PAMAM) improved β-cell functions in terms of the rate of proliferation, glucose-stimulated insulin secretion (GSIS), cell maturation, and insulin accumulation. In addition, this functionalized dendrimer protected β-cells under stressful conditions that exist in diabetes: oxidative and ER stress.

Peptides play a variety of important roles in biological systems and are also used as therapeutic agents.[38–42] However, the use of peptides as drugs is limited because of several pharmacokinetic disadvantages (for example, protease-induced degradation).[43–45] Although *in vitro* biological evaluation of HSA-28D showed promising results, we tried to take this project even further; various novel peptidomimetic scaffolds of HSA-28 were designed, synthesized, and conjugated to the surface of PAMAM. Peptidomimetics are compounds whose chemical moieties mimic the 3D structure of natural peptides and retain the ability to bind to biological targets, but without the disadvantages of peptides.[46–49] However, covered by peptidomimetics, PAMAM dendrimers were inactive in the proliferation screening assay. Thus, in this work, synthesis of peptidomimetics was used only for developing new organic scaffolds that might be used for the mimicking of glutamic, phenylalanine, and leucine amino acids containing peptides (as it was in our case). HSA-28-based PAMAM dendrimers are leading compounds for upcoming work that might reveal an innovative class of antidiabetic therapeutics that possess a unique mode of action: increasing β-cell mass and functionality *via* targeting its neuronal secretory and adhesion machinery.

Methods

Materials

PAMAM dendrimer (ethylenediamine core, fifth generation), bovine serum albumin (BSA), glucose oxidase (GO), thapsigargin (Tg), protease inhibitor cocktail, radioimmunoprecipitation assay buffer (RIPA), Rink amide resin, 2,2-diphenylethylamine, benzhydrylamine, phenethylamine, benzylamine, glycinamide hydrochloride, α- and β-naphthaldehyde, 1,2,3,4-tetraisoquinoline, *n*-bromoethylphthalimide, potassium carbonate, potassium iodide, *tert*-butyl acrylate, isobutylamine, triethylamine, 2-iminothiolane, and 3-maleimidopropionic acid were purchased from Sigma-Aldrich (Merck) (Rehovot, Israel). Fetal calf serum (FCS), phosphate buffered saline (PBS), L-glutamine, trypan blue, Roswell Park Memorial Institute medium (RPMI-1640), and antibiotics were purchased from Biological Industries (Beth-Haemek, Israel). Formaldehyde (4% in PBS) was purchased from Bio-Lab (Jerusalem, Israel). Rink amide resin, DIEA, and protected amino acids were obtained from Chem Impex (Wood Dale, IL, USA). β-Mercaptoethanol was purchased from Bio-Rad (Hercules, CA, USA). Alfa Aesar (Ward Hill, MA, USA) supplied *t*-isopropanol-silane. Alexa Fluor 633 Phalloidin was purchased from Life Technologies (Carlsbad, CA, USA). Anti-glucagon, anti-C-peptide, and anti-Pdx1 antibodies were supplied by Abcam (Cambridge, MA, USA). The Mercodia Insulin ELISA kit was purchased from Mercodia (Uppsala, Sweden). All organic solvents were purchased from Carlo Erba Reagents (Val De Reuil, France).

Peptide synthesis

The synthesis was performed as described in the literature.[50] The obtained peptide was coupled with 3-maleimidopropionic acid by the N-termini of the peptide according to the peptide synthesis procedure.

HPLC purification

Preparative HPLC (Young Lin Instruments, Anyang, Korea) was performed on a LUNA C18 preparative column (10 μm, 100 × 30 mm) from Phenomenex, Inc. (Torrance, CA, USA). Compounds were purified using an increasing linear gradient of acetonitrile in DDW at room temperature.

Peptide conjugation onto the surface of PAMAM dendrimer

Two hundred μL of PAMAM dendrimer in methanol solution (which corresponds to 0.01 g of PAMAM dendrimer) was used for preparing each sample. First, the methanol was evaporated under reduced pressure and the dendrimer was dissolved in 0.5 ml of PBS. Then the solution of dendrimer was treated with a 2-fold excess of 2-iminothiolane (from 0.1 M stock solution of 2-iminothiolane in PBS). After having been shaken for 5 min, a 5-fold excess of HSA-28 peptide or mimetics in PBS was added to the solution above, up to 2 ml of the total volume. The reaction mixture was shaken overnight at room temperature.

The coated NPs were purified using a centrifuge ultrafiltration system (3000 rpm, 20 min, 10 °C, 3 times) until the filtrate did not contain any traces of the compound (monitoring by TLC under UV light or under vanillin stain).

Cell line

INS-1E β-cells were courtesy of Prof. Shlomo Sasson (Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem, Israel). Cells were grown and maintained as described by Pasternak *et al.*[51]

Cell counting (trypan blue exclusion assay)

Cells were detached by trypsin and mounted using trypan blue (0.4%). Only uncolored cells were counted (live cells). Cell counting proceeded according to the Abcam (Cambridge, MA, USA) online protocol for work with a hemocytometer.

MTT cell viability assay

Cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT, 2 mg ml^{-1}) in growth medium for 2 h at 37 ° C. The medium was then aspirated, and DMSO was added to solubilize the cells and colored crystals. Absorbance at 570 nm was measured in a SpectraMax M5 spectrophotometer (Sunnyvale, CA, USA).

Induction of oxidative stress

Oxidative stress conditions[52] were induced by supplying glucose oxidase (GO, 50 mU ml^{-1}) to the growing medium of the INS-1E cells. This resulted in an elevated H_2O_2 concentration in the medium (reaching 29.0 ± 9.6 μM after 4 h of incubation). The standard MTT cell viability test was performed after 4 h of incubation.

Induction of ER stress

ER stress conditions were induced by supplying thapsigargin (Tg, 0.75 μM) to the growing medium of the INS-1E cells.[53] The standard MTT test was performed after 24 h of incubation.

Immunofluorescence measurement of C-peptide, glucagon and Pdx1 level

Experiments were conducted in INS-1E cells that were seeded on coverslips in 6-well plates. The cells were incubated with HSA-28D for 72 h (control cells were untreated). Following incubation, the slides were washed three times with prewarmed PBS and fixed with formaldehyde (4% in PBS). Subsequently, the slides were washed three more times with prewarmed PBS. The level of C-peptide was determined by immunocytochemistry using anti-C-peptide antibody according to the manufacturer's protocol. The membranes were stained with Alexa Fluor 633 Phalloidin according to the manufacturer's protocol. The slides were washed three times with prewarmed PBS. Nuclei were stained with DAPI according to the manufacturer's protocol. Fluorescent signals were visualized with a confocal-Zeiss microscope equipped with a 60×/1.4 objective (Oberkochen, Germany). For the glucagon measurement, cells were grown as described above. Slides were incubated with HSA-28D (control cells were untreated) for 72 h. The level of glucagon was determined by immunocytochemistry using anti-glucagon antibody. Thereafter, the slides were fixed with formaldehyde as described above. Cell membranes and nuclei were stained as previously described. Visualization of fluorescent signals proceeded as described above. For Pdx1 level identification, cells were grown as described above. Slides were incubated with HSA-28D for 72 h. The level of PDX1 was determined by immunocytochemistry using anti-PDX1 antibody. Thereafter, the slides were fixed with formaldehyde as described above. Cell membranes and nuclei were stained as previously described. Visualization of fluorescent signals proceeded as described above.

GSIS and insulin content assays

The assays were performed on INS-1E cells as described.[54] Insulin quantification was performed using the Mercodia Insulin ELISA kit according to the manufacturer's protocol.

The pharmacophore design

The pharmacophore was designed based on the structure of HSA-28 peptide and NX-1 using LigandScout 4.0 software.[55]

Synthesis of HSA-28 mimetics

The compounds were obtained by a reaction between commercially available or synthesized *in house* aromatic amines and *tert*-butyl acrylate as described by Ingallinella *et al.*[56] The detailed synthesis procedures are presented in the ESI.†

HSA-28 mimetic conjugation onto the surface of the PAMAM dendrimer

3-Maleimidopropionic acid was coupled to all the synthesized compounds above according to the procedure described by Ruzza *et al.*[57] The coupling reaction was followed by the treatment of all compounds with neat TFA for 1 h as described in the literature. The conjugation of all compounds onto the surface of PAMAM dendrimer was performed as described before.

Estimation of the dendrimer coating by HAS-28D and its peptidomimetics

Polyacryl gel electrophoresis (PAGE). For estimating the percentage of dendrimer coating the gel electrophoresis of the peptide or peptidomimetics conjugated to dendrimers was determined. In addition, as a negative control, we evaluated the electrophoresis of PAMAM that was coated only by 3-maleimidopropionic acid and PAMAM alone. All samples were loaded to glycine using 6% SDS PAGE gel and run against Tris-Tricine-SDS (TTS) running buffer and stained using silver stain.

Gel permeation chromatography (GPC). The molecular weight and polydispersity index (PDI) were determined using gel permeation chromatography (GPC) consisting of a Waters Spectra Series P100 isocratic HPLC pump with an ERMA ERC-7510 refractive index detector and a Rheodyne (Cotati, CA) injection valve with a 20 μL loop (Waters, MA). The samples were eluted with super-pure HPLC water through a linear BioSep SEC-s-3000 column (Phenomenex) at a flow rate of 1 mL min^{-1}. The molecular weight was determined relative to poly(ethylene glycol) standards (Polymer Standards Service-USA, Silver Spring, MD, USA) with a molecular weight range of 100–450 000 Da, human serum albumin (67 kDa, Sigma Aldrich), and bovine plasma fibrinogen (340 kDa, Sigma Aldrich), using Clarity chromatography software.

UV spectroscopy

An Agilent Cary 300 UV-vis spectrophotometer with a slit of 4 nm and a scan speed of 400 nm min^{-1} was used for the measurements. The absorbance measurements were performed at pH 7.4 by keeping the concentration of the samples at 0.175 mM in 1 cm quartz cuvettes. The wavelength ranged from 250 to 500 nm.

Results

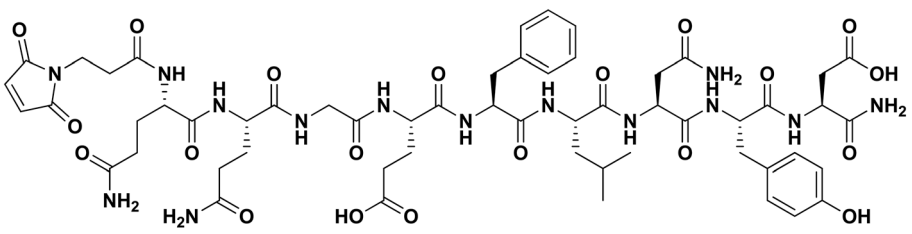
Conjugation of HSA-28 peptide onto the surface of PAMAM and estimation of the percentage of coating

The HSA-28 peptide was synthesized as described in the Methods section with an amide moiety on its C-terminal and a maleimide moiety on its N-terminal (Scheme 1). PAMAM dendrimer (fifth generation) with an ethylene diamine core and 128 amino groups available for reaction was chosen for evaluating the peptide assembling because of its size similarity to previously active maghemite nanoparticles (5.4 nm).[27] The conjugation was performed using a known quick single-step reaction involving maleimido-derivatized HSA-28 peptide (Scheme 2).[58] The coated dendrimer (HSA-28D) was purified by centrifuge ultrafiltration until the filtrate did not show any traces of starting materials (monitoring by TLC, followed by vanillin staining).

For estimating the percentage of dendrimer coating, 6% SDS PAGE was performed in TTS (Fig. 1A). As expected, the coated nanoparticles appeared as a broad band at around 50 kDa, which corresponded to approximately 21 units of peptide that was conjugated to the PAMAM. Based on this information, the calculated percentage of the covering was approximately 16%.

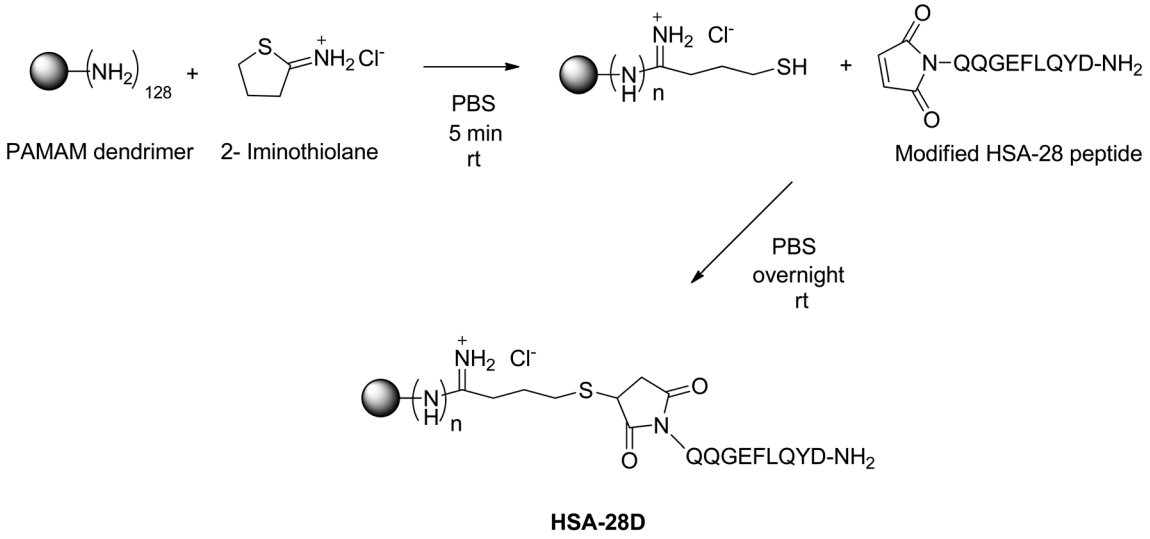
In addition, the GPC analysis of HSA-28D revealed a single peak at 47 kDa, compared to the calibration curve. This peak has a PDI value of 1.0029, calculated by MW/Mn, where MW is the weight's average molecular weight and Mn is the number's average molecular weight. This indicates the purity of HSA-28D, due to the clear homogeneity seen in the GPC analysis. This result is also well correlated with the result provided by the Dendritech (the company that produced the PAMAM dendrimer, 5 generations with an ethylenediamine core) MS results for a specific lot that was used by our team (please see Fig. S1†). The provided spectra indicate that the "naked" PAMAM has a major peak approximately at 26 kDa.

Finally, the UV spectra of both dendrimers, the "naked" and HSA-28D, clearly show a significant difference (Fig. S2†) in the intensity of absorption in the UV range. HSA-28 has two aromatic rings (phenylalanine and tyrosine), which are responsible



Mal-Gln-Gln-Gly-Glu-Phe-Leu-Asn-Tyr-Asp-NH_2

Scheme 1 Chemical structure of modified HSA-28 peptide.



Scheme 2 General procedure for one-step preparation of HSA-28D.

for the absorption in the aromatic wavelength range. Obviously, the "naked" dendrimer does not have aromatic rings; hence, the absorption level in the UV range is very low compared with HSA-28D. Based on the results obtained from all these analytical techniques, the coupling of the peptide to the "naked" dendrimer should be considered as validated.

In vitro evaluation of HSA-28D biological activity

The possible effect of HSA-28D on the rate of cell proliferation was investigated using the INS-1E cell line. Fig. 1B shows that HSA-28D enhanced the rate of cell proliferation (around 40%) compared with untreated cells. The highest stimulatory effect on the proliferation rate was obtained after 72 h of incubation with HSA-28D (the concentration of the conjugated HSA-28D with PAMAM HSA-28 was 3 μg ml^{-1}). Interestingly, the non-coated PAMAM dendrimer exhibited a highly toxic effect toward the cells (data not shown).

The next goal was to determine the possible HSA-28D cell protective effect *in vitro*. To this end, two apoptosis-inducing systems were used: ER and oxidative stress. Both types of stress are involved in diabetes-related β-cell death.[59,60] To mimic ER stress *in vitro*, thapsigargin (Tg) was used.[61] The toxin dramatically elevates the intracellular levels of calcium and depletes ER calcium stores, thereby inducing ER stress. A glucose oxidase (GO)/glucose system was used as an inducer of oxidative stress.[62] We preferred to use this more controllable system to generate H_2O_2 than to directly add it to the medium at a high dose. The system continuously produces constant concentrations of H_2O_2. GO catalyzes the transfer of two oxygen electrons to H_2O_2, using reducing equivalents from the oxidation of glucose to glycolic acid. Glucose is present in the medium at a high concentration (to mimic the hyperglycemic conditions *in vivo*). Hence, the ROS generation never runs out because of the accessibility of the substrate to the enzymatic reaction.

Cells were incubated with HSA-28D and then exposed to Tg or GO. Fig. 1C shows that the compound has excellent cytoprotective characteristics by assisting cells in overcoming both types of stress conditions.

HSA-28D's effect on C-peptide, glucagon, and the PDX levels was investigated afterward. Determining the accumulation of C-peptide in β-cells is a common method for indirectly evaluating insulin production.[63,64] This small peptide links the two chains of insulin and assists in hormone maturation. Direct measurement of insulin *in vitro* can be problematic because, in addition to INS-1E-produced insulin, even the smallest amount of insulin from the growth medium masks the actual results. In contrast, C-peptide in different species is different, and C-peptide in FCS does not cross-react with the C-peptide from rat INS-1E cells. The ratio between C-peptide and insulin levels is 1 : 1 in secretory granules and both are secreted simultaneously.[65] INS-1E cells were treated with HSA-28D. Slides were obtained as described in "Methods" and the plasma membrane and nuclei were denoted by red and blue, respectively. Thereafter, slides were exposed to a primary antibody against C-peptide (green) and were evaluated by confocal microscopy. Cells treated with HSA-28P expressed surprisingly more C-peptide (approximately 6-fold) compared with untreated cells. (Fig. 1D). We normalized the intensity of the C-peptide level by the intensity of the membrane signal. Peptide alone and naked PAMAM did not have any effect on C-peptide accumulation (data not shown).

It is a known phenomenon that an increased proliferation rate of β-cell lines, such as INS-1E, leads to reduced differentiation levels.[66] Such a transformation might be manifested by abnormally elevated levels that are not related to β-cell hormones, for example, the production and secretion of the insulin antagonist, glucagon.[67] The basal levels of glucagon, which is still produced and secreted by INS-1E, might rise because of losing the phenotype; this is characterized by high

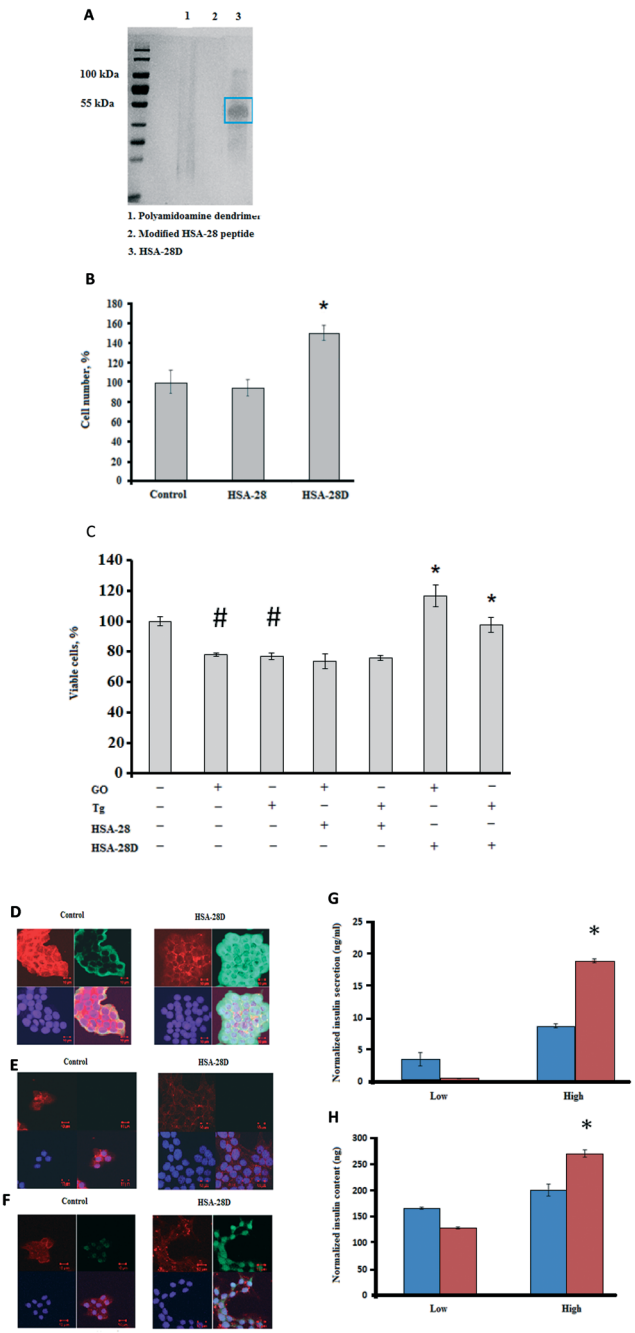


Fig. 1 (A) 6% SDS PAGE of HSA-28D. Samples (purified HSA-28D and loading buffer, 40 μl both) were boiled for 5 min, loaded to SDS PAGE, and run against TTS buffer. Bands were visualized using silver stain. (B) The effect of HSA-28D on the proliferation rate of INS-1E cells. INS-1E cells were seeded in 24-well plates and incubated for 72 h with the medium supplemented with HSA-28D ([HSA-28]-3 μg ml^{-1}). Thereafter, the cells were detached by trypsin, stained using trypan blue, and counted. The results are presented as a percentage compared to non-treated cells. *$p \leq 0.05$, $n = 6$. MEAN ± SE. (C) The effect of HSA-28D on cell viability under oxidative and ER stress conditions. INS-1E cells were incubated for 72 h with medium supplemented with HSA-28D ([HSA-28]-3 μg ml^{-1}). After incubation, 50 mU ml^{-1} glucose oxidase (GO) was added for an additional 1.15 h and 0.113 μg ml^{-1} thapsigargin (Tg) was added for an additional 24 hours. Upon completion of both experiments, a standard MTT assay was performed. The cell viability of both experiments is presented as a percentage compared to non-treated cells. *,#$p < 0.05$, $n = 3$. MEAN ± SE. (D) The effect of HSA-28D on C-peptide intracellular levels. Experiments were conducted on INS-1E cells that were seeded on coverslips in 6-well plates. INS-1E cells were incubated for 72 h with HSA-28D ([HSA-28]-3 μg ml^{-1}). Cells were fixed with formaldehyde, permeabilized, and exposed to anti-C-peptide or anti-glucagon (E) or anti-Pdx1 (F) antibodies, followed by secondary antibody (green). Thereafter, the cell membranes were exposed to Alexa Fluor 633 Phalloidin (red) and the nuclei were stained with DAPI (blue). Fluorescent signals were visualized with a confocal-Zeiss microscope. The experiment was run several times using the triplicate method, $n = 6$. Representative pictures are shown. The effect of HSA-28D on GSIS (G) and insulin content (H) under low and high glucose concentrations. The INS-1E cells (blue columns) were grown in 24-well plates and treated with HSA-28D ([HSA-28]-3 μg ml^{-1}) (red columns). After 72 h, the GSIS was evaluated in the presence of 3.3 mM (low) or 16.7 mM (high) glucose. H. Measurement of insulin content. The ELISA assay was performed for INS-1E lysates. *$p < 0.05$, $n = 3$. MEAN ± SE.

levels of differentiated β-cells.[68] Thus, the extent of intracellular accumulation of glucagon was investigated in INS-1E cells; as shown in Fig. 1E, HSA-28D did not lead to glucagon accumulation.

To further investigate the biological activity of HSA-28D, the possible effect of the compound on Pdx1 (pancreatic and duodenal homeobox 1) production was tested. Pdx1 is a transcription factor needed for pancreatic development and β-cell maturation.[69] Type 2 diabetes is characterized by the presence of a subpopulation of Pdx1-deficient β-cells.[70] The data presented in Fig. 1F clearly show that HSA-28D significantly elevated the basal level of the transcription factor (around 12 times).

Finally, the effect of HSA-28D on glucose-stimulated insulin secretion (GSIS) and insulin content in INS-1E cells was evaluated. HSA-28D significantly (around 2-fold) enhanced GSIS in comparison with non-treated cells (Fig. 1G). The total insulin content was also profoundly elevated upon treatment with HSA-28D in INS-1E cells, which were maintained at high glucose concentrations (Fig. 1H).

Pharmacophore-based design and synthesis of HSA-28 peptide mimetics

Peptides are one of the most abundant biomolecules in the physiology of any organism. However, the pharmacological use of peptides is very restricted because of their substantial problematic pharmacokinetic and pharmacodynamic properties, such as rapid proteolytic degradation, non-specific biological activity due to binding to "off target" macromolecules, low oral and even systemic bioavailability, the intense rate of elimination in the liver, and finally, also the high possibility of activating the immune system. To circumvent these challenging issues, several modern pharmacological approaches are in use: smart oral pharmaceutical formulations, slow release techniques, conjugates with organic molecules (usually polymers) that are targeted to specific tissues, peptides and peptidomimetics.[71–73] Peptidomimetics are small organic compounds that can mimic the peptide arrangement (pharmacophores) by replacing peptide functional groups and amide bonds by very similar structural domains and moieties. The 3D structures of peptides are closely reconstructed by peptidomimetics with the ability to interact and affect the biological targets. In contrast, the pharmacological and pharmacokinetic disadvantages of peptides are not present, due to changes in the covalent structures of these molecules.

Usually, computer modeling is used for designing a set of peptidomimetics, based on the structure of a known biologically active peptide. However, mimicking the structure of a long peptide such as HSA-28 (9 amino acids) was very

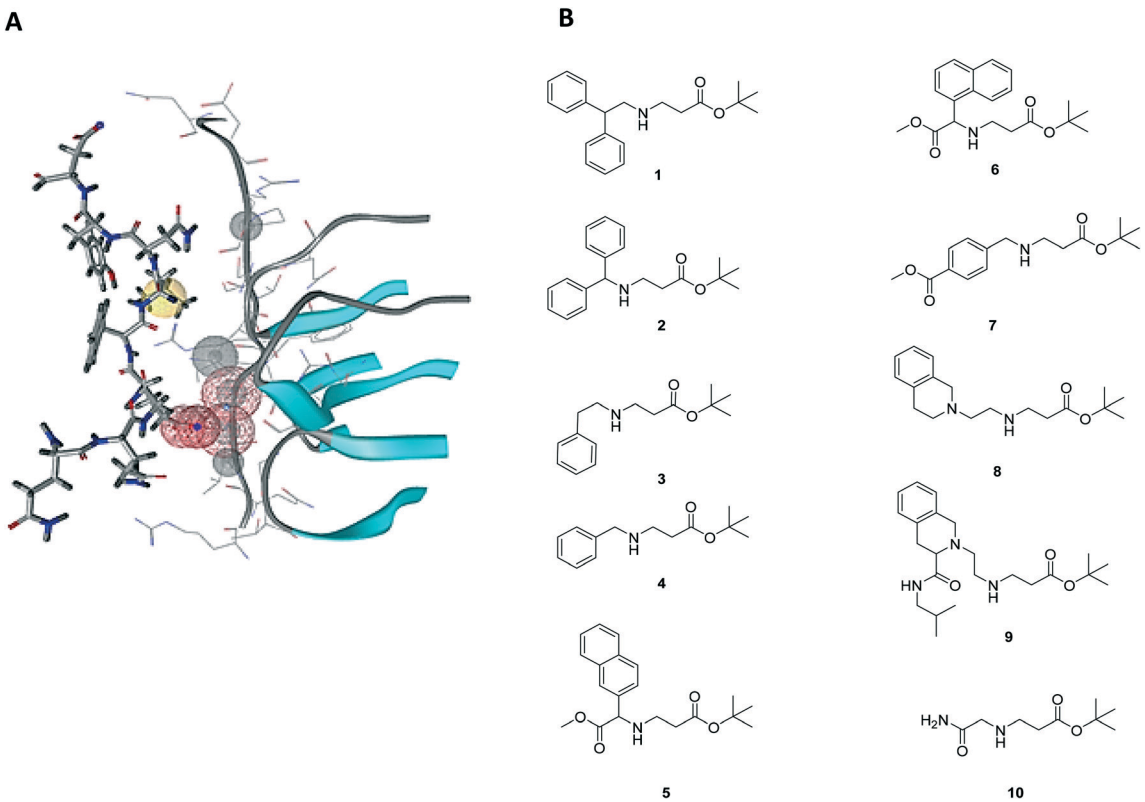


Fig. 2 (A). Pharmacophore design. Structure-based pharmacophore model generated with LigandScout from the structure of HSA-28 and NX-1. Pharmacophore features are color-coded: red represents negative charge features, yellow represents the hydrophobic feature, and gray represents excluded volumes. Excluded volumes are points in space occupied by protein atoms, which represent steric hindrances in the binding site. Such points cannot be occupied by ligand atoms. (B). The structures of the synthesized HSA-28 mimetics.

challenging. Thus, only side chains critical for the binding of glutamic, phenylalanine, and leucine amino acids were mimicked, as shown in Fig. 2A. The pharmacophore model was created, based on the structure of the complex between HSA-28 and NX-1 using LigandScout 4.0 software.[74]

Ten compounds (Fig. 2B) that fitted the pharmacophore model were synthesized: *tert*-butyl 3-((2,2-diphenylethyl)-amino)propanoate (**1**); *tert*-butyl 3-(benzhydrylamino)-propanoate (**2**); *tert*-butyl 3-(phenethylamino)propanoate (**3**); *tert*-butyl 3-(benzylamino)propanoate (**4**); *tert*-butyl 3-((2-methoxy-1-(naphthalen-2-yl)-2-oxoethyl)amino)propanoate (**5**); *tert*-butyl 3-((2-methoxy-1-(naphthalen-1-yl)-2-oxoethyl)amino)-propanoate (**6**); methyl 4-(((3-(*tert*-butoxy)-3-oxopropyl)amino)-methyl)benzoate (**7**); *tert*-butyl 3-((2-(3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)amino)propanoate (**8**); *tert*-butyl 3-((2-(3-(isobutylcarbamoyl)-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)-amino)propanoate (**9**, novel compound); and *tert*-butyl 3-((2-amino-2-oxoethyl)amino)propanoate (**10**).

All compounds were obtained by a reaction between commercially available or synthesized in-house aromatic amines and *tert*-butyl acrylate (all synthetic procedures and analytical characterization studies are described in detail in the ESI†). All the synthesized final compounds were coupled with 3-maleimidopropionic acid and treated with TFA to remove the *tert*-butyl group. The obtained molecules were used for further conjugation to PAMAM NPs. The percentage of NPs used for covering was evaluated similarly to HSA-28D (the results are presented in the ESI,† Fig. S3).

The possible effect of all INS-1E cells being coated by peptidomimetic dendrimers was tested according to their proliferation rate. However, none of the compounds significantly affected the rate of cell proliferation (Fig. S4†).

Discussion

PAMAM dendrimers bring a unique possibility to create tree-like homogenous structures around one central core. The presence of free primary amine groups on the PAMAM surface allows a large spectrum of conjugational reactions. Regarding the attachment of biogenic peptides, it was shown that non-direct conjugation of a peptide to PAMAM *via* a PEG linker or in combination with an additional short linker is the most useful approach.[75–79] Different linkers besides PEG were also used for the biogenic peptide conjugation to PAMAM.[80–84] In addition, direct covering of PAMAM dendrimers by peptide was also reported.[85] However, to our knowledge we are the first to show the successful binding of a peptide to PAMAM using the 2-iminothiolane/malonic anhydride system. Indeed, A. Harada reported the conjugation of PAMAM *via* a 2-iminothiolane linker to polylysine; however, the S–S bond was used for coupling by the authors and not the peptide-3-(alkylthio)pyrrolidine-2,5-dione moiety, as we used.[86] To prove that HSA-28 conjugates with the PAMAM dendrimers to a sufficient extent, we applied a non-traditional method for the conjugation chemistry technique: SDS-PAGE. Based on the positions of the molecular weight markers, the average size (50 kDa) and the covering efficiency (around 16%) of the modified PAMAM dendrimers were identified (Fig. 1A).

It was shown that adult pancreatic β-cells still have a high ability to proliferate *in vivo* and that this self-duplication process is still important together with the generation of new β-cells from stem cells.[87,88] The observed ability of HSA-28D to increase the rate of INS-1E cell proliferation (see Fig. 1B) may be very important; further work is necessary to determine if the proliferative effect seen here with the INS-1 β-cell line would be seen also with primary β-cells. Prior results, however, demonstrate that NL-2 strongly influences the β-cell number *in vivo*.[29] Interestingly, cells treated with the compound were almost 50% more proliferative.

The most important observation here was related to the fact that HSA-28D was able to protect INS-1E cells against oxidative and ER stress, as shown in Fig. 1C. The β-cells are very sensitive towards oxidative stress because of their relatively weak molecular antioxidative defense system.[89] One of the major pathophysiological causes of diabetes is oxidative stress.[90] Lack of effective antioxidant protection triggers β-cell dysfunction in the early stage of diabetes followed by cell apoptosis in the progressive stages of the disease.[91] The combination of a low replication rate of the β-cells and the absence of a sufficient antioxidative response leads to cell death during diabetes.[92] Classical antioxidant therapy was tested in many clinical antidiabetic trials.[93] Unfortunately, direct antioxidative stress protection of β-cells and maintenance of β-cell mass were never achieved.[94] In addition to its own direct toxicity, oxidative stress causes total cellular dysfunction, which appears as cytosolic, mitochondrial, peroxisomal, or endoplasmic reticulum stress.[94] The latter plays a crucial role in diabetic β-cell apoptosis.[95] In general, ER stress is caused by a disproportion between the rate of proper protein folding and misfolding.[96] Misfolded proteins are unable to undergo degradation in proteasome or lysosome and accumulate in the ER lumen. In order to restore ER functionality, cells activate an emergency chain of actions termed unfolded protein response (UPR). There is substantial evidence for the presence of ER stress in diabetic β-cells.[97] Chronic ER stress leads to apoptosis.[98] ER stress was also targeted as a possible site for a pharmacological intervention to protect β-cells under diabetic conditions.[99,100] The use of chemical and more specific pharmaceutical chaperones, prevention of eIF2B inhibition, calcium leak blockers, stimulators of ER folding capacities, and other groups of compounds was proposed.[95] However, using this approach, substantial progress in β-cell protection has not yet been achieved.

HSA-28D displayed a significant protective effect in β-cells that were maintained under both main apoptotic triggers (ER and oxidative stress). In light of the failure of conventional anti-oxidative stress and anti-ER stress therapeutic approaches for diabetes, such a dual effect might play a very important role in the future development of NL-2 mimetic-based β-cells for protective therapy.

It is known that non-covered PAMAM dendrimers (especially cationic ones) are extremely toxic for cells.[101–104] According to our experience, INS-1E cells died at a 34.6 μM concentration of a "naked" PAMAM dendrimer. This observation was in agreement with recently published articles about the toxicity of PAMAMs.[105,106] In addition, it is also known that in general, high concentrations of positively charged polyamines can destroy and damage the plasma membrane and kill animal cells.[107,108] However, by covering PAMAM nanoparticles with peptides (even at 16%), positively charged amines can be eliminated by creating a neutral amide bond. Thus, covered PAMAM (even partially) was not toxic to the cells in biologically active concentrations.

The test compound dramatically increased C-peptide (insulin) accumulation in INS-1E cells (Fig. 1D). Cells incubated with HSA-28D in the presence of 22.5 mM glucose for 72 h enhanced the intracellular insulin content almost 6-fold. These data support our hypothesis that mimicking NL-2/NX interaction between β-cells leads to high levels of maturation of those cells and not only to increased proliferation. The stability of the β-cell phenotype after treatment with HSA-28D was proved by the absence of glucagon in the cytosol (Fig. 1E). Moreover, the high maturation of the treated cells was also indicated by the increased intracellular levels of PDX1. PDX1 is a master transcription factor that tightly regulates pancreatic development, the β-cell proliferation, and most importantly, sufficient structural and functional maturation of β-cells.[109] Normal expression of PDX1 in β-cells blocks glucagon expression and selectively promotes the expression of β-cell-specific genes.[110] It is important to mention that T2DM is characterized not only by apoptosis of the β-cells but also by dematuration of β-cells and their transdifferentiation to different types of cells (usually to α-cells). PDX1-deficient β-cells are found in progressive T2DM.[111] Apoptosis, low rates of proliferation, and transdifferentiation lead to a constant decline in β-cell mass during the progression of T2D and these result in a constant failure to generate enough new β-cells. It was very encouraging that under HSA-28D treatment, PDX1 levels in INS-1E were significantly elevated (Fig. 1F), suggesting that the increased insulin accumulation in treated cells is attributed to increased maturation.

The most important test for the functionality of the observed effects of the test compound on INS-1E was GSIS. As shown in Fig. 1G, three days of administration of HSA-28D led to a significant augmentation of the rate of insulin secretion under physiological conditions (medium glucose level 13 mM). Interestingly, INS-1E cells treated with the test compound slightly decreased basal insulin secretion. These results underscored two very important issues related to the HSA-28D mode of action: the insulin secretion occurred only as a physiological response to elevated glucose levels and not by some non-specific leaking of insulin from damaged plasma membranes. Second, large quantities of insulin that accumulated during three days of exposing the cell to the test compound were able to be secreted physiologically. Measured by ELISA, the cell insulin content was also markedly high in the treated HSA-28D cells (Fig. 1H) and these results were correlated with the results that we obtained by fluorescence microscopy (Fig. 1G). However, the difference was considerably lower than that obtained fluorescently (6-fold in the fluorescence method *versus* 2.1-fold in the colorimetric method), which might be explained by the higher sensitivity of fluorescence detection. Alternatively, the maturation of insulin granules in HSA-28D treated cells might also explain the difference.

To overcome possible low bioavailability and other pharmacokinetically related disadvantages, our next step in the project was to try to develop peptidomimetics based on the HSA-28 structure. Such molecules (conjugated to PAMAM), on the one hand, might mimic the 3D structure of the native peptide, but on the other hand, non-peptide bonds and non-natural amino acid moieties should create more biologically stable molecules with identical or even upgraded activity. Based on the *in silico* pharmacophore model, feasible structures for the organic synthetic compounds were created and fitted to the model. After evaluation of the *in silico* binding score, the first ten molecules were synthesized. We tried to mimic the three most important interactions/natural amino acid moieties in HSA-28, such as the branched lipophilic moiety of leucine, the charged carboxylic acid of glutamic acid, and finally, the phenyl moiety of phenylalanine.

Compound **9** is a novel peptidomimetic obtained in 7-step synthesis with a linear yield of 24%. Such a molecule might be used for general glutamic, phenylalanine, and leucine amino acid mimetics in the development of mimetics absolutely non-relevant to NL-2 peptidomimetics.

A proliferation assay was used for evaluating molecules covered by ten peptidomimetic PAMAMs. Unfortunately, none of the synthesized molecules had a positive effect on the INS-1E proliferation rate (Fig. S4†). This disappointing result can be explained by the fact that the peptide conformations chosen for mimicking the peptide interactions *in silico* might not be reproducible by peptidomimetics when they are conjugated to PAMAM. In addition, it is also possible that for sufficient mimicking of NL-2 interactions with NXs, an entire scaffold of nine amino acids is essential. In this case, the synthesis of a nine amino acid peptidomimetic sequence can be very challenging.

In summary, the ability of the functionalized NPs reported here to prevent β-cells's substantial apoptosis, an increase in their mass, maturation, and most importantly, their ability to secrete insulin in a glucose-dependent manner might be used for developing novel β-cell protective therapeutic agents and β-cell transplantation supporters, especially considering that a PAMAM-based core for such NPs is biocompatible.

Conflicts of interest

The authors declare no conflict of interest.

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