A novel screening assay for insulin receptor

Jana Staňurová[1], Kateřina Čermáková[1,2], Lenka Žáková[1], Pavel Šácha[1], Jiří Jiráček[1*], Jan Konvalinka[1,3*]

[1] Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo náměstí 2, 166 10 Prague 6, Czech Republic

[2] First Faculty of Medicine, Charles University, Kateřinská 1660, 121 08 Prague 2, Czech Republic

[3] Faculty of Science, Charles University, Hlavova 8, 128 43 Prague 2, Czech Republic

* Corresponding authors

Abstract

Insulin is a key hormone in glucose homeostasis. Its lack causes severe health complications and has to be compensated by regular administration of insulin. Despite intense long-lasting research, a more stable substitute has yet to be discovered to alleviate patients' issues. Here we report the development of a novel assay for screening potential insulin analogues based on the recently published method DIANA. Our assay meets the need for a fast, non-radioactive method as a sensitive alternative to the commonly used radioactive immunoassay.

Keywords

Insulin, insulin receptor, DNA-linked inhibitor antibody assay, inhibitor

Introduction

Insulin regulates the uptake of glucose from the blood. Upon detection of elevated glucose levels, secretion of insulin from the pancreas is quickly initiated. Signalling via the hormone leads to the relocation of glucose transporter to the plasmatic membrane of target cells, i.e. muscle cells, facilitating the uptake of glucose by these cells. The full function of this signalling pathway is crucial for glucose homeostasis and metabolism, which is tightly regulated by the interplay of different hormones.

Diabetes mellitus type I is caused by the autoimmune destruction of β-cells in pancreas. As a result, glucose homeostasis is disrupted leading to the many symptoms occurring in diabetes mellitus type I patients such as retinopathy, proteinuria caused by nephropathy, neuropathy and pathologies of the cardiovascular system. Lack of treatment may lead to blindness, open

wounds and even coma and death. Subcutaneous insulin administration compensates for the loss of insulin production, so that glucose levels in blood remain under control, preferably at the physiological level of healthy individuals. However, the need for daily medication deteriorates the quality of the patients' lives substantially. An insulin substitute that would be more stable and/or would not require regular injections would dramatically improve this burden. We therefore aimed to set up a new method that would allow us to screen a large number of compounds at a time.

We employed DNA-linked antibody inhibitor assay (DIANA), a technique recently developed in our laboratory, to perform compound screening. DIANA is a sandwich-based assay that relies on the interaction of a selected target protein with its ligand. The ligand is usually a small molecule known to interact with the protein of interest. It is conjugated to a DNA-oligonucleotide forming the DIANA detection probe. Quantification is executed via RT-qPCR. In the competitive setting of the method, the probe is mixed with tested compounds, which leads to a decrease in the amount of bound probe (Figure 1). The K_i value is then determined based on the compound concentration in a single well. DIANA is a fast method only requiring small amounts of sample, it is cost-effective, sensitive and easily scalable[1]. As opposed to insulin receptor radioligand binding assay (IR-A) utilised to measure insulin concentration in samples routinely, there is no need for radioactive labelling in DIANA. Also, the method is not cell-based, thus minimising biological variability in the results. So far, our laboratory successfully developed DIANA for screening of GCPII, CAIX, neuraminidase, FAP and CD73 inhibitors[2,3,4].

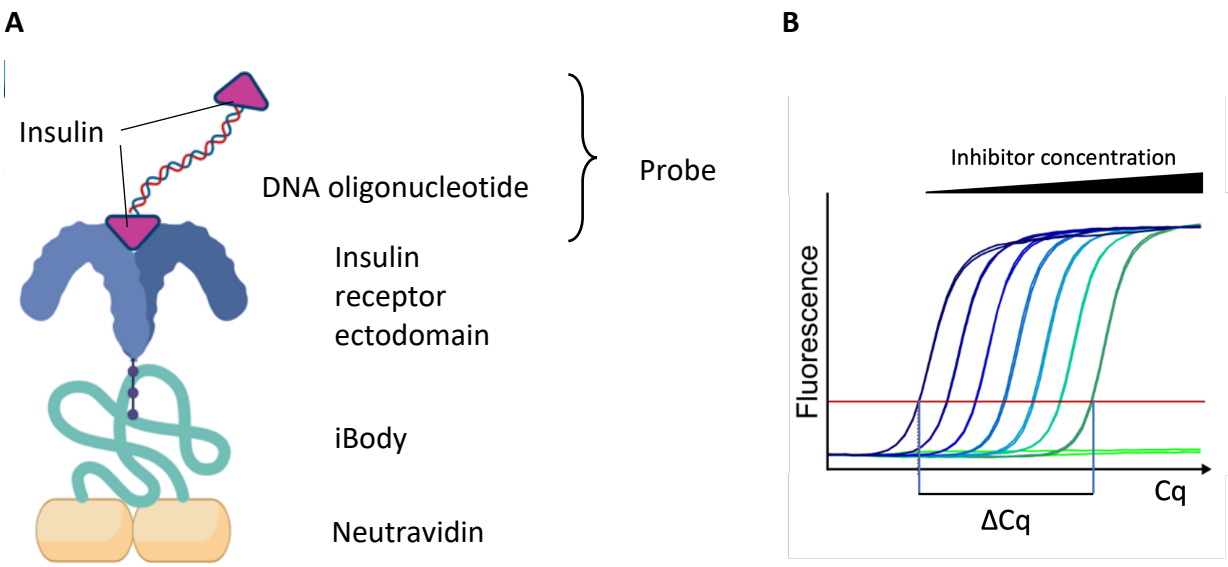

Figure 1: Schematic representation of DIANA. **A.** iBody facilitates a stable binding of the insulin receptor ectodomain to a neutravidin-coated surface via His-tag. Detection probe comprising two insulin moieties attached to one DNA-oligonucleotide binds to the insulin receptor via its insulin molecule. Created with BioRender **B.** The amount of detection probe bound to the IR is measured by qPCR. Higher concentrations of an inhibitor lead to a more pronounced displacement of the detection probe resulting in a higher measured Cq (threshold cycle). ΔCq represents the difference between an uninhibited reaction (left) and a fully inhibited reaction (right).

Methods

DIANA probe preparation and characterisation

DIANA probe is a conjugate of a small molecule inhibitor with a DNA-oligonucleotide. The modification of an inhibitor is a key step in the preparation of DIANA probe. Modification of insulin by an azide group allows conjugation with an alkyne-modified DNA-oligonucleotide facilitated by CuAAC. Insulin was added in 3-fold excess (100 μM) to DNA-oligonucleotide (33 μM) and the mixture with 5 mM aminoguanidine, 5 mM BTTP, 2.5 mM $CuSO_4$ and 20 mM natrium ascorbate was incubated at 37 °C for three hours. The product was purified on a filter column (Amicon, Merck) followed by LC-MS analysis.

The probe was characterised by determining linear range and Kd value. Linear range was assessed as a serial dilution of insulin receptor (0.1-1500 pg/well) detected with 100 pM DIANA probe (Figure S1). K_d value was measured as 1500 pg/well of insulin receptor detected with probe dilution series (40 fM – 20 μM) and calculated according to (Navrátil *et al.* 2017) (Figure S1). The affinity of the probe to insulin receptor is similar to the affinity of insulin alone with K_d = 1.1 ± 0.2 nM (Figure S1). Binding capacity of insulin was not impaired post modification.

The determination of Kd is a fundamental assessment in the evaluation of interactions between two molecules in binding assays[5]. In the context of DIANA, it serves as a crucial parameter for determining the Ki values of inhibitors in a single experiment[1]. As a basic parameter, Kd is influenced by factors such as assay setup, assay type, buffer composition, pH, and assay sensitivity, meaning its value can vary across different methods[5,6]

DIANA optimisation and validation for screening

To ensure successful measurement of inhibitors, the DIANA experimental conditions were optimised. Factors such as blocker type, detergent type, their concentrations, and incubation times all impact key experiment parameters like dynamic range, reproducibility, accuracy, and equilibrium state.

For optimisation, we tested various buffer compositions with different concentrations of casein and detergents (Tween-20 and Pluronic F-127). The conditions for inhibitor screening were chosen based on the highest signal-to-background ratio and assay sensitivity. After selecting the appropriate buffer, we proceeded with optimising the concentrations of protein and probe. The optimal insulin receptor/probe concentration ratio was determined by the highest dCq, which is the difference between the Cq value at a high concentration of insulin and the Cq value of wells without insulin. The final optimal conditions for competitive DIANA were 0.01% Tween-20, 700 pg insulin receptor per well, 50 pM bivalent DIANA detection probe, and one-hour incubation with inhibitors.

Given that most compounds in libraries are dissolved in DMSO, we also examined the impact of DMSO on our assay. As many targets tested in HTS assays are sensitive to higher DMSO concentrations, we focused on conditions where most compounds would remain soluble to avoid false-negative results from precipitates. We selected the highest DMSO concentration that did not significantly reduce the assay's dynamic range or affect Ki values compared to DMSO-free experiments. Therefore, a 10% DMSO concentration was chosen for inhibitor screening, including HTS.

DIANA

DIANA in a competitive setting was developed to determine the compounds' inhibition potency against insulin receptor. First, concentrations of iBody, insulin receptor and probe were optimised as well as their incubation buffers, times, and temperatures. Next, DMSO concentration for inhibitor testing was measured. The total of 10 % DMSO was chosen for the assay.

Working volume in each well was 5 μl, except for casein. Neutravidin was immobilised on polypropylene well plate surface (5 μl, 12 ng/μl) in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated for 1 hour. Blocking by 100 μl 1.1 % casein (20 μl in a 384-well format) solution in TBS overnight followed. Next, iBody decorated with TrisNTA and biotin was applied

to the plate (5 μl, 30 nM) in TBST' (20 mM Tris-HCl, 150 mM Nacl, 0.1 % Tween-20, pH 7.5) and incubated for 1 hour. Then, insulin receptor was added to the plate (5μl, 700 pg/well) in TBST' for 1 hour. Afterwards, detection probe was applied to the plate with or without tested compounds (5 μl, 50 pM) in HEPEST'C (100 mM HEPES, 400 mM NaCl, 0.1 % Tween-20, pH 7.5) complemented with casein. All incubations were carried out at room temperature in the dark. After each step, the plate was washed by automated plate washer (BlueWasher, BlueCatBio) with TBST (20 mM Tris-HCl, 150 mM Nacl, 0.05 % Tween-20, pH 7.5). Finally, master mix (5μl, LightCycler® 480 SYBR Green I Master, Roche) was added. RT-qPCR ensued, quantification analysis was performed, and K_i values were calculated as described in (Navrátil *et al.* 2017).

Inhibitor testing

Protocols were performed in an automated HEPA-filtered workcell. The instruments used included Agilent Bravo Automated Liquid Handling Platform for neutravidin coating, and iBody, insulin receptor and probe addition; Formulatrix Mantis® Microfluidic Liquid Dispenser for casein and qPCR master mix application; Beckman Coulter Echo 650 Acoustic Liquid Handler for transferring library compounds; BlueCatBio BlueWasher for plate washing; and Roche LightCycler 480 II for RT-qPCR runs.

K_i of an inhibitor can be determined from a single-well experiment (Navrátil *et al.* 2017). In case of insulin receptor, we prepared dilution series of each promising inhibitor and calculated the Ki values from a point within the dynamic range of the assay and evaluated them statistically as a mean of assessed K_i ± SD.

HTS screening

Inhibitor screening was performed to determine Ki values using the DIANA method, as previously described[1], with minor modifications outlined in the following method. A bivalent DIANA detection probe was used for this purpose.

A 384-well qPCR plate was coated with neutravidin (5 µl/well at 12 ng/µl) in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.5) and incubated for 60 minutes. Blocker solution (1.1% casein in TBS) was then added at 20 µl per well and incubated overnight. The following day, after washing the plate, insulin receptor was applied (5 µl per well at 700 pg/well in TBST) and incubated for 60 minutes. After another wash, a mixture of 10 µM tested compounds and the bivalent DIANA detection probe was added (5 µl per well) in HEPES buffer containing 0.05%

Tween-20 and 0.0055% casein. This probe-inhibitor mixture was incubated for one hour. After a final wash, qPCR mix was added for probe detection.

Screening of two libraries in a high-throughput format was performed. The OTAVA_alpha_helix_peptidomimetic_library contains 1408 compounds. The assay window of the HTS experiment was 7.3 cycles. The IOCB library contains 10560 compounds. The assay window in this experiment was 8.2 cycles.

IR binding assay

The radioligand binding assay with the human insulin receptor in IM-9 cells was conducted following the method outlined by Morcavallo et al.[7] This assay is based on the competition between unlabelled insulin and radiolabelled insulin for binding to the insulin receptor in living cells. Initially, the standard binding curve for recombinant human insulin to the human insulin receptor in IM-9 cells was determined. In brief, 2.0 x 10^6/ml IM-9 cells were incubated with increasing concentrations of human insulin and a constant concentration of human [125I]monoiodotyrosyl-A14-insulin for 2.5 hours at 15 °C in HEPES binding buffer (100 mM HEPES, 100 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 1 mM EDTA, 10 mM glucose, 15 mM NaOAc, 1 % BSA, pH 7.6), with a total volume of 500 µl. After incubation, 2 x 200 µl samples were centrifuged at 13,000 x g for 10 minutes. Radioactive pellets were counted using a Wizard 1470 Automatic **γ** Counter (PerkinElmer Life Sciences). The binding data were analysed using GraphPad Prism 8 with a non-linear regression method and a one-site fitting program, accounting for potential depletion of the free ligand, which allowed the determination of the dissociation constant for the unlabelled ligand. The dissociation constant of human [125I]monoiodotyrosyl-A14-insulin was set at 0.3 nM.

Determination of insulin concentration in samples by IR binding assay

The insulin concentrations in supernatant samples collected from stimulated MIN6 cells and in extracts from whole cells were measured using the same radioligand binding assay as described above. The appropriately diluted (at least 10x) sample with an unknown insulin concentration was allowed to compete with [125I]monoiodotyrosyl-A14-insulin for binding to the insulin receptor in IM-9 cells, and the bound radioactivity was measured. Each sample was analysed in at least two biological replicates, with each replicate tested in duplicate. The binding curve of human insulin was then used as a calibration curve to correlate the bound

radioactivity to the respective insulin concentration. A separate calibration curve for human insulin was generated for each new series of samples with unknown insulin concentrations. In other words, a fresh binding curve for human insulin on IM-9 cells was determined for each day of measurements.

Results

HTS

We performed high-throughput screening of two libraries – the IOCB library comprising 10560 compounds and the OTAVA_alpha_helix_peptidomimetic_library containing 1408 compounds. The latter library did not yield any hits worth pursuing, since their K_i was over 100 µM. The former library yielded several hits, all of which were insulin derivatives. It is not a surprising outcome that we were not able to obtain any novel insulin mimetics since both libraries are rather small. Especially the IOCB library was established for different targets than the insulin receptor. It is a collection of various compounds synthesised at the IOCB and contains many nucleoside derivatives, which reflects the history of IOCB in developing inhibitors of this nature.

Hit verification by manual DIANA

We verified the hits by re-testing their serial dilution in manually performed DIANA setting and we were able to confirm all of them with an adjustment to the K_i values. This is due to the fact that the substances are measured at 100 µM concentration in the screen, which is likely to provide overestimated values, especially for good inhibitors, since the reaction satiation already occurs at much lower concentrations. Working with a serial dilution yields more precise base for K^i calculations.

Hit validation by IR-A

Utilising IR binding assay, we assessed K_i values of compounds verified as hits in manual DIANA post HTS following the above-described procedure. In this orthogonal assay, we validated all of the compounds as true insulin receptor binders as well. The K_i values we obtained in our measurements were generally lower than those acquired by DIANA experiments, but for rare exceptions. IR-A is regarded as a very sensitive method. Differences in the values may be due

to the distinct setup of both assays, as well as the conditions, in which the measurements are performed, such as buffers, temperatures etc.

Table 1. Comparison of K_i values determined by IR-A and DIANA.

Code	**K_I (nM) IR-A**	**K_i (nM) DIANA**
Compound 1	0,27	0,74
Compound 2	0,16	0,77
Compound 3	1,05	5,29
Compound 4	0,53	2,17
Compound 5	1	3,79
Compound 6	1,07	4,23
Compound 7	0,25	1,78
Compound 8	3	11,91
Compound 9	2,1	14,48
Compound 10	8,6	71,92
Compound 11	3,8	10,17
Compound 12	3,8	25,75
Compound 13	8,3	41,68
Compound 14	1,8	10,56
Compound 15	84	93,66
Compound 16	33	29,21
Compound 17	50	112,99
Compound 18	500	148,15
Compound 19	1250	73,27
Compound 20	4,0	8,05
Compound 21	0,5	5,89

Discussion

Although structurally new hits were not identified for the insulin receptor, insulin DIANA is a valuable tool for screening. It a fast, sensitive method with very good reproducibility, non-radioactive and easily scalable to a HTS format. Moreover, cells are not necessary to perform the measurement, which reduced variability in the results. Another noteworthy merit of the method is the low consumption of material making the assay cost-effective in addition. With DIANA, we are able to reproduce results from IR-A to a good extent.

In general, the Ki values determined by DIANA were higher than those assessed by IR-A. This may be caused partly by different condition but also partly by the setups themselves. In IR-A, buffer is added first, then the tested compounds, only then does the [125I]monoiodotyrosyl-A14-insulin follow. In contrast, in DIANA, a mixture of the compounds with the detection probe is added directly to the insulin receptor. In some DIANA setups, it does not make a difference whether the inhibitors or the detection probe are added in the first place or whether they are applied as a mixture. However, in insulin DIANA, the order of the steps does matter, despite the non-covalent nature of the detection probe. We tested both settings in the detection phase, adding inhibitors first to the insulin receptor, or the probe and compared the results to the common procedure we employ, that is, premixed inhibitors with the probe. in case inhibitors were applied first, we obtained lower K_i values for the inhibitors than with the mixture. In concert with that, the calculated K_i values were higher when the probe was added first than when the mixture was used. These effects most likely occur as a consequence of the unusual proportion of the detection probe. insulin as a ligand for a detection probe is considerably larger than other ligands we commonly conjugate with the DNA oligonucleotide to synthesise a detection probe.

Concerning sensitivity, both methods are mostly comparable as can be demonstrated by the measurement of insulin K_i. IR binding assay uses radioactive materials, which require special handling, storage, and disposal procedures. As opposed to that, DIANA does not utilise radioactive substances, so it is easier and safer to handle. IR binding assay is a relatively time-consuming method due to the need for radioactive counting. DIANA on the other hand is a fast assay that may be upscaled easily requiring the same amount of time. IR binding assay is a more costly method of the two as it utilises radioactive material that itself is more expensive also concerning its disposal, and it is necessary to include calibration curves for each new day

of measurement, meaning additional costs. DIANA in comparison is the more cost-effective alternative requiring only non-radioactive material and no extra consumption for calibration curves. Only internal controls are necessary for interplate accuracy assessments. Moreover, HTS laboratories are often not authorised for work with radioactive substances, which poses a risk of contamination of the whole platform. Concerning quantification, DIANA is again the more reliable method because IR binding assay relies on measuring radiation, which may pose issues with background noise and decay over time. This issue is addressed by employing calibration curves as mentioned above, but the quantification in DIANA certainly is the one more straightforward and stable.

In summary, we propose DIANA as an excellent alternative to the IR binding assay with distinct merits over the latter method.

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