WP1.5. Development of insulin mimetics (Jiráček, Jahn, Konvalinka)

Research aims

- To design and synthetize a series of azides and polyfunctional non-natural α-amino, β-amino, β,γ-diamino, α,β,γ-triamino acid derivatives, and oxidatively modified amino acids.
- To prepare fast, accurate, non-radioactive and sensitive assays for the KD determination of modulators of insulin and insulin receptors interactions and modulators of IGF-1 and IFG-2 and IGF-1 receptor interactions.
- To design and validate a high-throughput method for testing of compounds.
- To develop new insulin mimetics, which will effectively bind to the insulin receptor (IR) and elicit or inhibit the biological effects of insulin.
- To develop new IGF mimetics, which will effectively bind to the IGF-1 receptor (and elicit or inhibit the biological effects of IGFs)

Research plan and methodology

The non-complete structural information about the molecular nature of the insulin-IR interaction and the dynamic complexity of this interaction do not fully allow a straighforward use of computational techniques to predict structures of efficient IR binders. However, we will take advantage of our detailed knowledge of insulin 3D structure and the key IR binding epitopes in the insulin molecule. We believe that molecules mimicking several different non-contiguous binding hotspots at the insulin surface have better chances to be potent insulin agonists than compounds mimicking only one epitope. Moreover, we believe that combinatorial approach for synthesizing and testing thousands of different compounds is the best starting strategy for the discovery of the first binders, which can be further optimized by molecular modelling and structure-function relationship approach.

For this purpose, we have already developed two variants of a versatile tri-orthogonal scaffold with orthogonally protected arms that can be selectively functionalized by different azides[1] (Fig. 2) or aldehydes[2]. Such functionalized scaffolds could cover several separated hotspots of the targeted insulin receptor and thus mimic insulin action. Moreover, this scaffold can be attached to the solid support which enables facile solid-phase generation of libraries of scaffold-based compounds. We have already optimized the synthesis of scaffolds modified by relatively simple organic azides but also with tripeptides. The molecular dynamics of a model scaffold has revealed that the compounds are able to adopt extended conformation with distances between distal carbon atoms as much as ~24 Å; similar to the space occupied by the insulin molecule (20-25 Å in diameter).

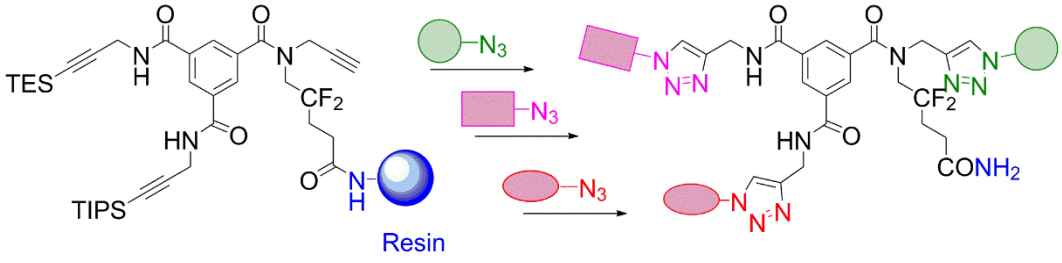

Fig. 2. Recently developed tri-orthogonal scaffold[1,2] for the solid-phase "click" synthesis of insulin mimetics.

(i) We will synthesize a large pool of different azides and a library of new polyfunctional non-natural enantiomerically pure α-amino, β-amino, β,γ-diamino, and α,β,γ-triamino acid derivatives (Fig. 3). The central bicyclic core is accessible in a single step, in which each of the functionalities is orthogonally protected and individually selectively addressable[3]. The resulting amino acid derivatives are conformationally constrained, thus they will actively contribute to secondary or even tertiary structure formation of the peptides, in which they are incorporated.

(ii) The supramolecular structure of these oligopeptides will be determined and characterized. Initially, foldamers of certain lengths will be prepared, whose structure can be determined by advanced NMR techniques or X-ray crystallography. These amino acid derivatives will be incorporated to diverse peptide sequences (Fig. 3). Because of their nature, these peptides are expected to display high stability, since the metabolic machinery of cells will not be able to degrade peptides near the position of the new amino acids, thus they are expected to be valuable for in vivo studies.

(iii) Selected ligands (peptidomimetics with an azido functional group) will be used for the attachment to individual tri-orthogonal scaffold arms (Fig. 2) either synthesizing individual compounds or libraries of compounds using split and mix combinatorial strategies.

In (i-iii), we will extensively use the newly purchased semipreparative HPLC apparatus (Jiráček group) and flash chromatography apparatuses (Jahn group) for the purification of synthesized azides, peptidomimetics and oligopeptides and their precursors.

(iv) Insulin mimetics will be tested for their binding to the insulin receptor isoforms (IR-A or IR-B) or to IGF-1R in membranes of selectively transfected cells and for their ability to induce autophophorylation of tyrosine kinase domains of the respective receptors. We will also focus on the ability of compounds to induce phosphorylation of some key cellular substrates activating metabolic or mitogenic pathways (e.g. IRS-proteins, ERKs, Akt/PKB). These experiments should also reveal agonist or antagonist properties of the selected compounds. Additionally, the interaction of mimetics will be studied by a new, promising approach to visualizing the function of membrane proteins is two-photon polarization microscopy (2PPM), a technique being independently developed by J. Lazar's Group (WP2.7.).

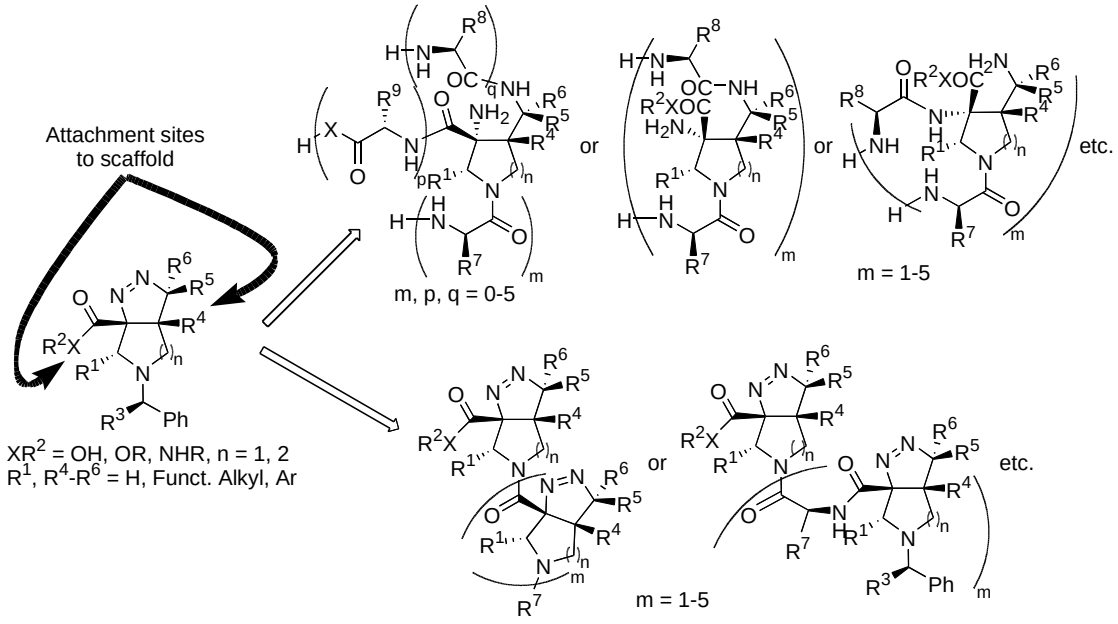

Fig. 3. Targeted non-natural amino acid skeleton with selected peptide structures derived thereof.

(v) We also propose to develop high-throughput screening (HTS) method for screening small molecules resulting from the combinatorial libraries described above. We will prepare a sandwich ELISA-like solid phase assay, based on immuno-PCR (iPCR), which is broadly used for ultrasensitive protein detection, where the protein is first captured by an immobilized antibody and then bound by the second antibody tagged by DNA for detection in quantitative PCR (qPCR) (Fig 4). The assay for insulin/IGF receptors will utilize the widely used anti-FLAG M2 antibody (Sigma), the receptor will be captured from lysate of HEK293 transfected by full receptors tagged at C-terminus by FLAG sequence. The detection probes will consist of the ligand (insulin, IGF-1, IGF-2) covalently linked to 3' end of 55 base long single strand oligonucleotide. The quantity of the bound ligand will be precisely assessed by qPCR. Once the probes will be synthesized, their affinity will be determined in standard assay and will be used to develop the

assay for screening of modulators of receptor-ligand interaction. The developed assay will be then adapted for HTS by automation of the assay workflow using the microtiter plate handling robot, also proposed within the project. We will screen IOCB compound library and identified hits will be used as ligands in tri-orthogonal scaffold as proposed in (iii).

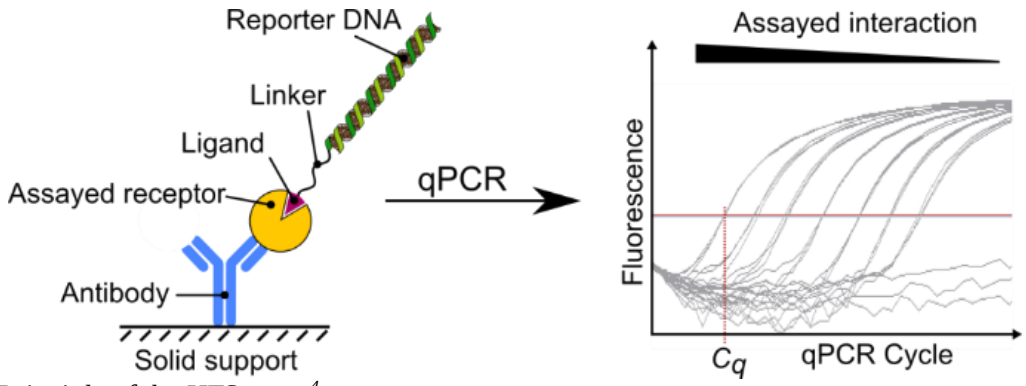

Fig. 4. Principle of the HTS assay[4].

(vi) Moreover, since the newly developed amino acid derivatives will be highly, but orthogonally functionalized containing additional functionality, they are suited to incorporate reporter units, such as fluorescent tags into the peptides to be studied. This will be very convenient for studying the interactions with the IR and IGF-1R receptors.

(vii) Additionally, another line of research is to modify the tri-orthogonal scaffolds with oxidatively modified peptides (Fig. 5). Here especially the glycine or diketopiperazine dipeptide units are expected to be prone to the oxidative introduction of alkoxyamine functionality, making them reactive, but they can be also envisaged to be stable enough to be incorporated into oligopeptides or even proteins. Under physiological conditions, these modified peptides are expected to be stable. But if the pH value drops it can be expected that they will react after binding to the receptor with nearby nucleophilic amino acid side chain residues, such as those in cysteine, lysine, aspartate, glutamate, serine or threonine. This concept maybe extended to light-activated oxidatively modified peptides, which are prone to radical cross-linking. Hence, their binding to the receptors can be mapped.

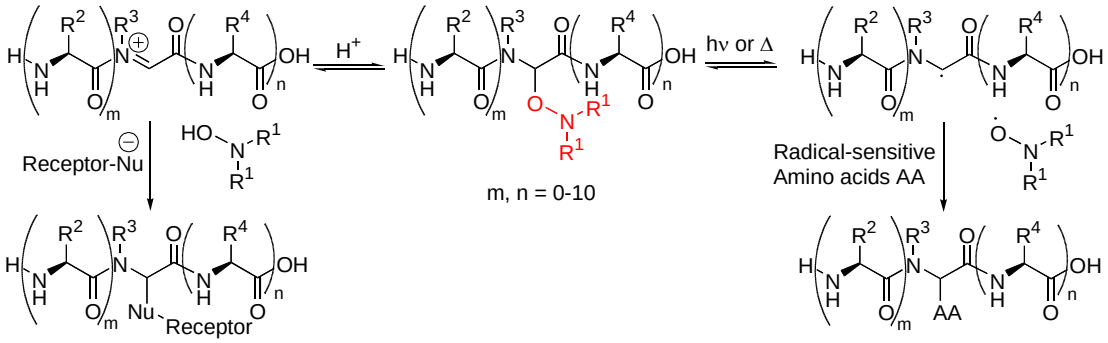

Fig. 5. Oxidatively modified peptides and potential modes of cross-linking to receptors.

[1] Vanek, V.; Picha, J.; Fabre, B.; Budesinsky, M.; Lepsik, M.; Jiracek, J., *Eur. J. Org. Chem.* **2015**, (17), 3689-3701.

[2] Fabre, B.; Picha, J.; Vanek, V.; Budesinsky, M.; Jiracek, J., *Molecules* **2015**, *20* (10), 19310-19329.
[3] Kapras, V.; Pohl, R.; Cisarova, I.; Jahn, U., *Org. Lett.* **2014**, *16* (4), 1088-1091.
[4] Niemeyer, C. M.; Adler, M.; Wacker, R., *Nat. Protoc.* **2007**, 2 (8), 1918-1930

Research schedule

2017-2022

- Design and synthesis of series of azides and polyfunctional non-natural α-amino, β-amino, β,γ-diamino, α,β,γ-triamino acid derivatives, and oxidatively modified amino acids.
- Design and synthesis of peptides and peptidomimetics.
- Structural characterization of amino acid derivaties and peptidomimetics.
- Design and validation of high-throughput method for library testing.
- Design and solid phase synthesis of combinatorial libraries of scaffold-based compounds.

2019-2022

- Testing of biological properties of compounds.
- Structural characterization of active compounds.
- Molecular modelling of the best hits and comparison with the insulin structure bound to IR.
- Optimization of the active structures.
  Mapping or receptor binding sites with selectively labelled/modified compounds.

Publications and patents

Publications (Jimp)

	Jimp
2017	1
2018	2
2019	3
2020	4
2021	5
2022	5
Total	20

Chemistry a European Journal
Journal of Medicinal Chemistry
ACS Chemical Biology
Journal of Biological Chemistry
Biochemistry
Bioconjugate Chemistry
Bioorganic and Medicinal Chemistry
Organic and Biomolecular Chemistry
Journal of Organic Chemistry
ChemBioChem
PlosOne

Patents and patent applications

	Patents (granted)	International patent applications (filed)
2017	0	0
2018	0	0
2019	0	0
2020	0	1
2021	0	1
2022	1	0
Total	1	2

We expect IP protection in the following areas:
High throughput screening method.
Insulin or IGF mimetics.

Cooperation with foreign institutions

- Dr. Andrzej M. Brzozowski from the Structural Biology Laboratory (YSBL) of the University of York in the U.K.,
- Prof. Carol Robinson's team at the University of Oxford in UK