https://doi.org/10.17952/37EPS.2024.P1125

Fine-Tuning the Immune-Stimulatory and Cancer Cell Binding Properties of Immune System Engagers (ISErs)

Hannah Back[a,b], Manuel Felkl[a], Susanne Kremer[c], Claudia Altdorf[c], Ute Steinbusch[c], Christian FW Becker[a]

[a] Institute of Biological Chemistry, Faculty of Chemistry, University of Vienna, Währinger Str. 38, 1090 Vienna, Austria

[b] University of Vienna, Vienna Doctoral School in Chemistry (DoSChem), Währinger Str. 42, 1090 Vienna, Austria

[c] Syntab Therapeutics GmbH, ZBMT, Pauwelstrasse 17, 52074 Aachen, Germany

Email address: hannah.susanne.back@univie.ac.at

1. Introduction

- **Targeted therapies against cancer** have become a key therapeutic approach as distinct surface markers are addressed using specific compounds such as monoclonal antibodies (mAbs) or small molecule drugs.[1,2]

- **Immune system engagers (ISErs)**, a novel class of peptide-based therapeutics, offer potential advantages over mAbs with respect to issues such as poor tumor penetration, prolonged bioavailability, and high production costs. They consist of at least two binders recognizing cancer cell-specific targets and one or more effector moieties recruiting innate immune cells. The binders and effector are linked via a polyethylene glycol chain (PEG), mimicking the epitope length in antibodies.[3-5]

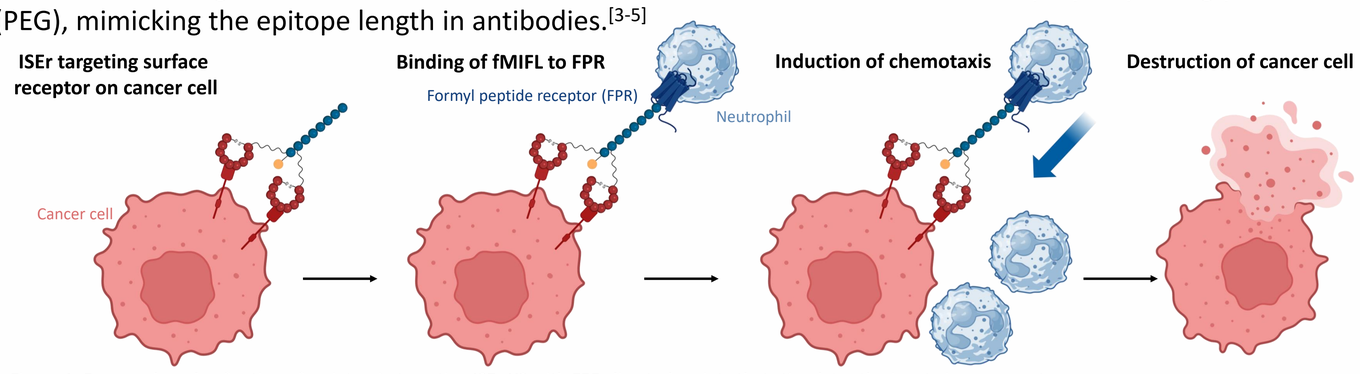


Figure 1: Proposed mode of action of α_3-integrin targeting ISEr-Y9 with FPR-directed moiety that stimulates the innate immune system.

- **Innate immune cell stimulation** is achieved with a chemoattractant, the short and very potent N-formylated peptide (fMIFL), which binds to formyl peptide receptors (FPRs), classified pattern recognition receptors (PRRs) expressed on innate immune cells.[6,7]

2. Motivation

- The goal of this work is the generation of ISEr variants with optimized binding affinities/avidity towards different cancer surface markers and with tailored immune stimulating properties.

- Establishing a **PEG linker library** and assessing the binding avidity via a competitive binding assays.

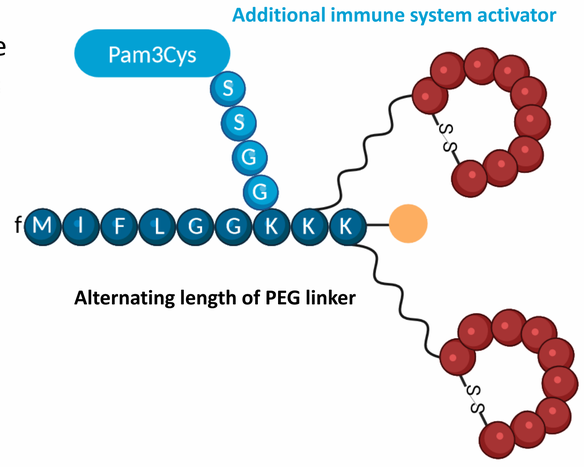


Figure 2: Schematic structure of an ISEr showing moieties that offer potential for possible modification sites.

- Toll-like receptor 2 (TLR2), an integral transmembrane glycoprotein and PRR, is targeted with the TLR2 agonist **Pam3Cys** (N-acyl-S-diacylglycerol cysteine). Binding leads to heterodimerization with TLR1 and stimulation of the MyD88-dependent signaling pathway, which triggers activation of the host defense mechanism via the innate immune system.[8]

3.A Results: ISEr-Y9 with varying PEG linkers

- PEG linkers increase the drug size and therefore circulation time and bioavailability.[9]

- Increased PEG linker lengths prevent the event of proteolysis, however, shorter linkers might improve ligand receptor interaction by limiting conformational freedom.[9]

- The ISEr-Y9 PEG series was successfully synthesized.

- In the competitive binding assay, the ISEr-Y9 PEG series was titrated against 50 nM Biotin-ISEr-Y9 PEG_{27}.
  ISEr-Y9 PEG_{27} showed lowest IC_{50} value in both human and murine breast cancer cell lines, indicating the best binding avidity in comparison to ISEr-Y9 with PEG_3 and PEG_{11} linker, respectively.

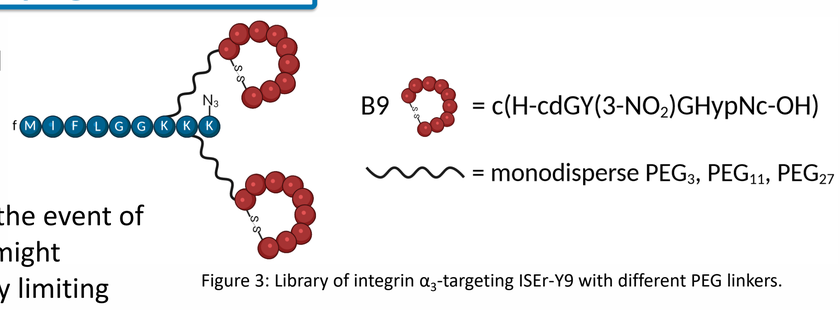
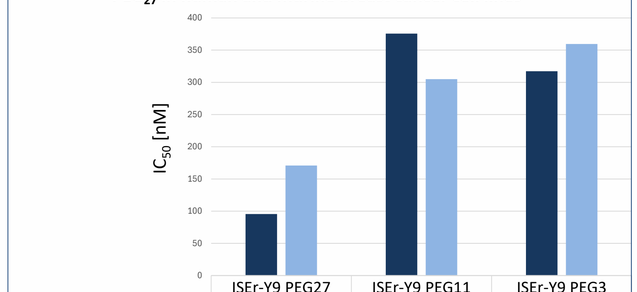


Figure 3: Library of integrin α_3-targeting ISEr-Y9 with different PEG linkers.

Competitive binding assay:
Titration of ISEr-Y9 PEG series against 50 nM Biotin-ISEr-Y9 PEG_{27} in human and murine breast cancer cell lines



	ISEr-Y9 PEG27	ISEr-Y9 PEG11	ISEr-Y9 PEG3
Human MDA-MB231 cells	95.56	375.7	317.3
Murine 4T1 cells	170.8	304.9	359.5

Figure 5: IC_{50} of ISEr-Y9 PEG series in human and murine breast cancer cell lines.

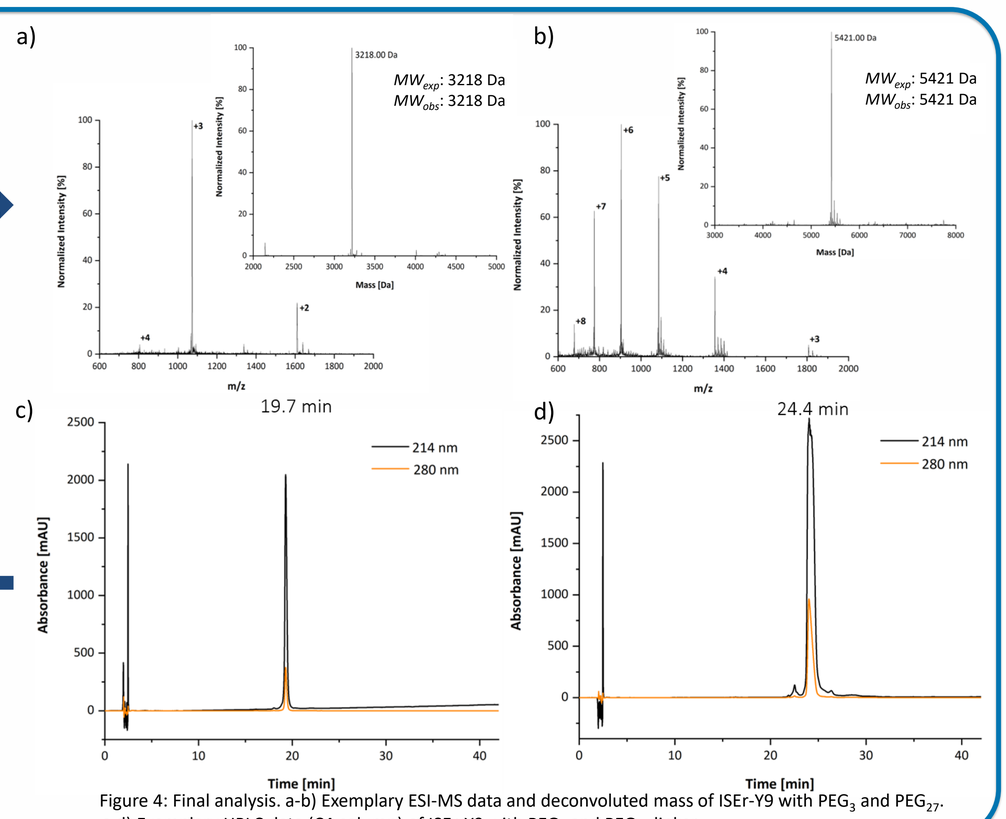


Figure 4: Final analysis. a-b) Exemplary ESI-MS data and deconvoluted mass of ISEr-Y9 with PEG_3 and PEG_{27}. c-d) Exemplary HPLC data (C4 column) of ISEr-Y9 with PEG_3 and PEG_{27} linker.

3.B Results: Palmitoylated ISEr

- Attachment of the amino acid Pam3Cys, which targets TLR2 on immune cells to induce an antimicrobial pathway that can be monitored by cytokine release.

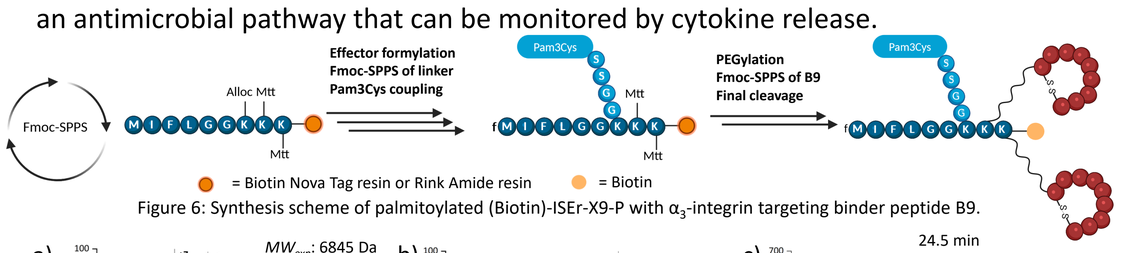


Figure 6: Synthesis scheme of palmitoylated (Biotin)-ISEr-X9-P with α_3-integrin targeting binder peptide B9.

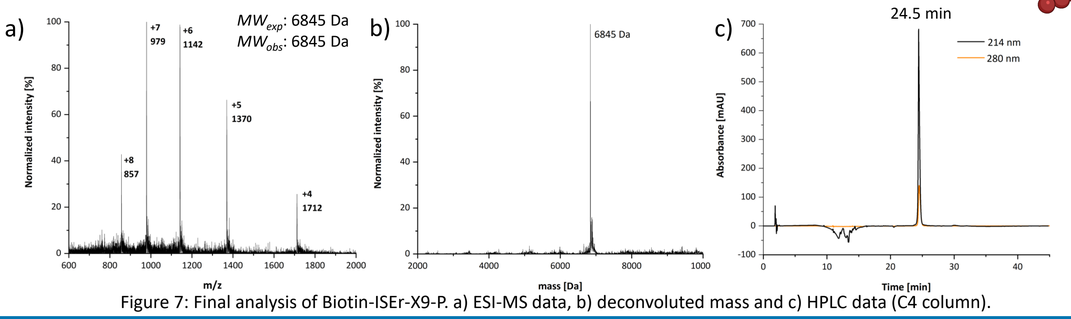


Figure 7: Final analysis of Biotin-ISEr-X9-P. a) ESI-MS data, b) deconvoluted mass and c) HPLC data (C4 column).

- ISEr-X9-P increases TNF-α, IL-6 and IL-8 levels in blood cells, indicating successful TLR2 stimulation.

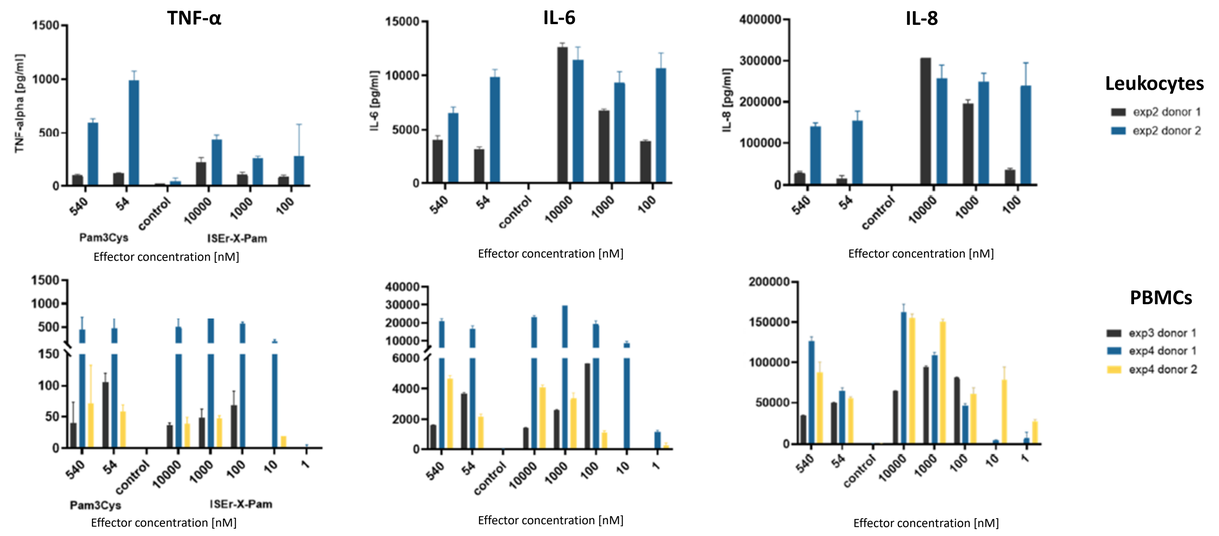


Figure 8: Cytokine release assay. Elevated cytokine levels triggered by ISEr-X9-P (ISEr-X-Pam) in leukocytes and peripheral blood mononuclear cells (PBMCs).

4. Summary & Outlook

- **Results I:** ISEr-Y9 with PEG_{27} linker showed the highest binding avidity on human and murine breast cancer cell lines. Next, the library will be extended with a PEG_{54} variant and evaluated for binding properties compared to previously synthesized PEG variants. Furthermore, this PEG library will be adapted to an ISEr series with an ephrin A2 targeting binder peptide (B59).

- **Results II:** Palmitoylated Biotin-ISEr-X9-P was successfully synthesized. The immunostimulatory effect of palmitoylated ISEr-X9-P on TLR2 was validated in a cytokine release assay and showed increased levels of TNF-α, IL-6 and IL-8. Next, the library will be extended to include a palmitoylated ISEr with an ephrin-A2 targeting binder peptide (B59) and will need to be evaluated for binding avidity and immunostimulatory potential.

5. References

[1] Scott, A. M. *et al*. *Nat Rev Cancer* **12**, 278-287 (2012).
[2] Padma, V. *et al. Biomedicine (Taipei)*, (2015).
[3] Conibear, A. C. *et al*. *Front Chem* **7**, 113 (2019).
[4] Mitra, S. *et al*. *J Genet Eng Biotechnol* **19**, 159 (2021).
[5] Potgens, A. J. G. *et al*. *Biochemistry* **58**, 2642-2652 (2019).
[6] Conibear, A. C. *et al*. *Chembiochem* **19**, 459-469 (2018).
[7] Dalpiaz, A. *et al*. *Eur J Pharmacol* **411**, 327-333 (2001).
[8] Nalla, N. *et al*. *Bioorg Med Chem* **23**, 5846-5855 (2015).
[9] Lim, C. *et al*. *Int J Nanomedicine* **18**,1615-1630 (2023).

Figures were created with BioRender.com