A Tetrnanectin-Based Platform for Protein Engineering

A protein engineering platform based on the human protein, tetrnanectin, is being used to derive a library of potential therapeutic proteins which can be trimerised to provide greatly improved successors to existing products.

By Hans Christian Thøgersen and Johanna Holldack at Borean Pharma

Hans Christian Thøgersen (MSc, PhD) is Chief Scientific Officer (CSO) and Founder of Borean Pharma, and Associate Professor at the University of Aarhus (Denmark). He has 25 years’ research experience in molecular biology and, for more than a decade, led the Danish Biotechnology Programme for protein engineering as Project Coordinator and a member of its steering committee. He was awarded the Hallas-Møller Senior Research Fellowship (Novo Foundation) as well as several international research grants, including one from the international Human-Frontiers-of-Science-Programme. During his postdoctoral career at Cambridge, Dr Thøgersen was among the first to establish protein-engineering research at the MRC Laboratory of Molecular Biology and the first to introduce this research area to Denmark, co-funding the Aarhus University Laboratory of Gene Expression. Consequently, he holds several patents and patent applications in this field. Dr Thøgersen holds Masters Degrees in chemistry and physics, and a PhD in molecular biology from the University of Aarhus.

Johanna Holldack is Chief Executive Officer (CEO) of Borean Pharma. She has extensive international management experience in product development including project and quality management, preclinical, clinical and regulatory affairs and process development. Her previous posts included Chief Operations Officer at MediGene AG (Munich, Germany) and Vice President, Global Vaccines and Therapeutics, at Chiron Corp (Emeryville, USA). Dr Holldack studied medicine at the Georg-August-University (Göttingen, Germany). She then spent more than a decade as a Research Associate and Assistant Professor in paediatrics, paediatric oncology and haematology at the Universities of Essen and Freiburg in Germany, and also at the Harvard Medical School in the US, where she was awarded the research fellowship of Deutsche Krebshilfe.

Monoclonal antibodies are now established therapeutic options for a range of indications. But they are expensive to produce and manufacture, and are not always easy to re-engineer to perfection. There is therefore a need for cheaper and easier-to-produce alternatives with equivalent or greater efficacy. In this article, we review a protein engineering platform developed at Borean Pharma that has the potential to deliver whole new classes of antibody analogues that can be manufactured cheaply and easily using *E. coli*.

Protein pharmaceuticals – and in particular antibodies – are now established as therapeutic options superior to small molecule drugs for a wide range of indications including transplant rejection, inflammatory diseases like rheumatoid arthritis and inflammatory bowel disease, infectious diseases and cancer.

However, many of these biological therapeutics come with their own limitations and, as first-generation proteins and antibodies, there is always room for improvement in efficacy, specificity and inherent immunogenicity. The search is now on for the next generation of protein pharmaceuticals. With this aim in mind, the Borean protein engineering platform is being used for the discovery of antibody analogues with the potential to provide greatly improved successors to existing protein pharmaceuticals and antibody therapeutics.

**TETRANECTIN PROTEIN ENGINEERING PLATFORM**

At Borean Pharma, we have identified a member of the family of human C-type lectin-like proteins – the homotrimeric plasma and tissue protein, tetrnanectin (see Box and Figure 1, see page 28) – as a protein engineering platform that can provide superior antibody analogue products that address most of the challenges inherent in conventional antibody technology. Tetrnanectin is a human plasma and tissue protein, and may be involved in fibrinolysis and proteolysis during tissue remodeling, although its precise biological function is not known.
What is known is that the protein family to which it belongs has the potential to bind to a wide variety of protein and carbohydrate targets.

There are two elements to the Borean Pharma protein engineering platform: first, a C-type lectin domain (CTLD) library of proteins; and second, trimerisation, a process whereby existing protein products are re-engineered into a trimeric format to achieve enhanced properties such as improved activity or prolonged circulation half-life.

CTLD LIBRARY

The C-terminal binding domain of tetranectin contains five loops that can be reconfigured in different arrangements so that the ligand binding domain can consist of a vast number of different possible amino acid sequences. Borean Pharma has cloned a library of more than $10^{11}$ individual CTLD-proteins with diversified binding sites. The diversity of the libraries results in a tremendous versatility with respect to the breadth of targets and indications for which highly specific binders can be successfully identified.

Several rounds of panning, followed by stringent washing, result in an enrichment of clones whose trimerised CTLDs typically bind with an affinity in the submicromolar range. In subsequent rounds of maturation, such initial mother clones are readily optimised for affinity to a subnanomolar level.

The libraries can, in effect, be regarded as an artificial immune system comprising numerous variants of antibody-analogues containing specific binders to virtually any target. This CTLD library is being used as a resource to identify promising lead candidates to improve on existing protein pharmaceuticals.

TRIMERISATION

A single protein (or monomer) can have its effectiveness and half-life greatly enhanced through being joined to other monomers of the same protein in polymeric formation. Trimerisation is the process by which monomers are bundled together in sets of threes.

Borean accomplishes this through anchoring the proprietary tetranectin trimerisation domain, derived from the tetranectin molecule, to each monomer (Figure 2a and b, see page 30). This additional domain binds to two other domains anchored to further monomers. In this way, any therapeutic protein can be trimerised. The coiled trimerisation module of tetranectin is remarkably stable, and its derivatives have been shown to be heat-stable at temperatures of up to 70-80°C.

This heat stability has significant implications for developing second-generation pharmaceutical compounds with a shelf-life at least as long as that of the original monomer product, and further ensures that the novel trimerised product does not exchange sub-components with the tetranectin already circulating in the blood. Additionally, trimerisation can be used to improve plasma half-life and overall efficacy for existing protein drugs or new protein drug candidates.

Trimerising proteins greatly increases their effective affinity; that is, it greatly increases their availability for binding to a ligand. It has been shown that dimerisation (two monomers in complex as in an antibody molecule) can provide up to a 100-fold increase in avidity over the monomer, and Borean’s data shows that a 1,000-
fold avidity gain over the monomer can be achieved by trimerisation.

It appears that this increase in avidity is possible in, for example, a case where only one monomer component at a time of the trimerised molecule can bind to a specific receptor; there will always be two further monomer components in very close proximity that can substitute and then substitute again *ad infinitum*, leading to much greater and more prolonged stimulation of the receptor.

Borean uses this trimerisation technology to increase the avidity of CTLD-derived candidates with enhanced therapeutic profiles when compared with approved protein therapeutics. In this way, antibody analogues can be developed with considerably enhanced efficacy and half-life and can consequently have the potential to be administered less often and in lower doses, with all the derived cost and safety benefits.

**LOW COST SCALEABLE MANUFACTURE**

One of the biggest issues with antibody therapeutics is the cost and difficulty of scaling up to manufacture. The trimerised proteins derived from the tetranectin platform can be manufactured in *E. coli*. Proteins expressed in *E. coli* are often harvested as denatured proteins in inclusion bodies, and need to be refolded to their native conformation before they can exert their biological activity. At Borean Pharma, we have developed a methodology for the cost-efficient refolding of *E. coli* expressed proteins.

**A DISCOVERY ENGINE**

The tetranectin platform is being used to discover and develop antibody analogues for a range of indications. Particular attention has been given to validated targets where antibody or protein pharmaceuticals have already been approved. This will ensure that development times are shorter, while the markets for these products are already established. The ease of manufacture, enhanced efficacy and longer half-life that would be expected from successful protein pharmaceuticals developed from the platform should ensure a competitive advantage in the marketplace.

**VALIDATION OF THE TETRANECTIN PLATFORM**

Two of the most successful protein therapeutics of recent years have been the Tumour Necrosis Factor alpha (TNFα) antagonists, Enbrel (etanercept) and Remicade (infliximab). These two products have revolutionised the treatment of rheumatoid arthritis; however, both are difficult to manufacture and therefore expensive. As a validated target for rheumatoid arthritis, TNF was an obvious choice for investigation as a potential target for CTLD-derived leads, not least to provide benchmarking against well-established products.

A novel class of human C-type lectin-derived TNF antagonist derived from the CTLD library and comparable with Enbrel and Remicade – is currently being developed. The TNF antagonists developed so far have reached their present level of efficacy through a sequence of carefully managed *in vitro* evolution steps. A first candidate molecule of high

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**Figures 2a and b:** The trimerisation unit of human tetranectin is a fully self-contained structural unit

It retains its structure and stability whether placed in its natural context of tetranectin, or fused at either or both ends to new protein molecules that in turn will also remain unperturbed, except that they are now assembled in clusters of threes (or sixes).

2a:

Tetranectin

Trimerisation unit

CTLD binding sites

2b:

<table>
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Stable and functional trivalent and hexavalent molecules obtained by fusion to the N- or C-terminus of the tetranectin trimerisation unit
specificity, but moderate affinity, was taken through consecutive steps of affinity and binding kinetics maturation. In this way, molecules with competitive inhibition profiles could be obtained in only three maturation rounds.

The CTLD-derived TNF antagonists have been analysed for specificity in ELISA-based assays; binding affinity and binding parameters have been determined using Biacore; and the blocking of TNF receptor interaction (receptor signalling) has been assessed in a cell assay using 929L cells. To date, they have been found to be comparable with Remicade and Enbrel in terms of binding parameters (KD, Koff and Kon), in terms of specificity and in terms of biological activity, as assessed in cell assays (including the standard L929 TNF inhibition cell assay), inhibition of TNF-stimulated pro-inflammatory cytokine production in human umbilical cord cells, and TNF-induced apoptosis in a human macrophage cell line.

Preliminary animal studies indicate that the basic biosafety, pharmacokinetics and tissue distribution profile of the C-type lectin-derived products are favourable. In addition, a recent proof-of-concept study showed dose-dependent suppression of arthritis progression in TG 197 transgenic mice, expressing human TNF.

CONCLUSION

Borean Pharma has developed a powerful protein-engineering platform using the human protein tetranectin to derive a vast library of potential therapeutic proteins that can be trimerised to further enhance their therapeutic properties. These lead candidates would act as antibody analogues but – unlike therapeutic antibodies – they can be simply and inexpensively produced using E. coli. This platform has been validated through the in-house development of an antibody analogue TNF antagonist that has established proof-of-concept in an animal mode of rheumatoid arthritis – providing evidence that the Borean CTLD products can successfully compete with the very best antibody products currently established.

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