

From FKBP12 to IMPDH: Ten Years of Immunology Targets at Vertex

Mark Murcko, Vertex Pharmaceuticals Inc., Cambridge, Massachusetts, USA

The evolution of research on some different autoimmune and inflammatory targets (like psoriasis, rheumatoid arthritis, asthma, hepatitis C, or organ-transplant rejection) has been described. The used approach for the design of new inhibitors was based on FLBP12, the target for FK506. NMR and crystal structures were solved in 1991 opening the way for designing more potent compounds. This led to the discovery of a ternary complex formed by calcineurin, FKBP12, and FK506, where calcineurin, a Ca^{2+} - and calmodulin-dependent phosphatase, is involved in the immunosuppressive action of FK506. Calcineurin is a potential component of TCR and IgE receptor signaling pathways involved in transcription and exocytosis. In 1994, the research team abandoned this project, as FK506 seemed to induce nephro- and neurotoxicities, and as clinical studies were suggesting that the properties were not dramatically improved compared to cyclosporin.

Another project focused on p38 mitogen-activated protein (MAP) kinase inhibitors. Blocking p38 has been shown to reduce levels of the proinflammatory cytokines IL-1 β and TNF α , acting as a cytokine suppression factor. It may therefore have applications in the treatment of inflammatory diseases, such as asthma, Crohn's disease and rheumatoid arthritis. A phase-I clinical trial for the VX-745 inhibitor has already started.

A new potent inhibitor (VX-740) has also been developed for IL-1 β -converting enzyme (ICE) as a treatment for rheumatoid arthritis (RA) and other inflammatory diseases. It is now being tested in phase-I clinical trials. VX-740 has been shown to block the production of IL-1 β and IFN γ , and to reduce experimentally induced joint inflammation in animals without significant toxicity.

Another project deals with inosine-5'-monophosphate dehydrogenase (IMPDH). IMPDH catalyses the NAD-dependent oxidation of IMP to XMP which is the rate-limiting reaction of guanosine nucleotide (GMP) *de novo* biosynthesis. As proliferating B- and T-lymphocytes are dependent on this pathway, and not on the salvage pathway, the inhibition of IMPDH is of great therapeutic potential.

Solving the three-dimensional structure in 1996 [1][2] in complex with mycophenolic acid (MPA, from the first generation of inhibitors developed by Hoffmann-La Roche) allowed advanced structure-based drug design of new inhibitors for IMPDH with high potency and tolerability. This research led to the development of VX-497, which seems to be very promising for immunosuppressive therapy. This inhibitor shows potential for treatment of psoriasis, an autoimmune disease of the skin, rheumatoid arthritis, inflammatory bowel disease (IBD), atopic dermatitis, steroid-dependent asthma, organ

transplantation, systemic lupus erythematosus (SLE) or hepatitis-C virus (HCV). VX-497 has a good tolerability when compared to other IMPDH inhibitors like MPA, mizoribine, or ribavirin showing significant side-effects. VX-497 shows IC_{50} values at about 100 nM and is suitable for oral administration. It is active *in vivo* against collagen-induced arthritis in mouse, plaque-forming cell assay in mouse, heterotopic heart transplant in rat, skin transplant in mouse or graft vs. host disease in mouse. Preclinical pharmacology establishes antiviral and immunosuppressive potential whereas toxicology testing demonstrates good safety and tolerability. VX-497 has been tested in a single-dose escalation study in healthy volunteers and is now being investigated in phase-II clinical trials for HCV and psoriasis.

[1] M.D. Sintchak, M.A. Fleming, O. Futer, S.A. Raybuck, S.B. Chambers, P.R. Caron, M.A. Murcko, K.P. Wilson, *Cell* **1996**, *85*, 921–930.

[2] M.A. Fleming, S.P. Chambers, P.R. Connelly, E. Mimmegern, T. Fox, F.J. Bruzzese, S.T. Hoe, J.R. Fulghum, D.J. Livingston, C.M. Stuver, M.D. Sintchak, K.P. Wilson, J.A. Thomson, *Biochemistry* **1996**, *35* (22), 6990–6997.

Regulation of Intracellular Signalling and Transcription by Induced Proximity Using Synthetic Ligands

Isabella Graef, Stanford University, Medical School, Stanford, CA, USA

In signal transduction, molecular interaction results in the transfer of information along specific pathways. Strategies to control signalling pathways came from the insight that ligand-induced protein dimerization (or induced proximity) can initiate or regulate the information transfer. There

are several examples where induced proximity is involved in signalling of mammalian cells, such as the dimerization of membrane receptors (receptors for growth factors and cytokines), phosphorylation-induced association of proteins (ZAP70, JAK, STATS) and the nuclear translocation

of proteins. The strategy for regulating protein interactions by induced proximity can be simplified in a restricted diffusion model. The chemical inducer of dimerization is a small dimeric lipophilic ligand that permeates the cell membrane. In the cytoplasm, the ligand brings together

er two chimeric molecules, each of them characterized by a specific binding site for the lipophilic ligand. The ligand induces the association of the two proteins and might activate biological responses. Characteristics of intracellular dimerization agents should include absence of toxicity and immunogenicity.

They should be able to pass the membrane (lipophilic) and their interaction with the specific hydrophobic binding pocket of the target proteins should be imparted. Examples of ligands that fulfill these characteristics are FK506 and rapamycin, two broadly used immunosuppressants. FK506 binds to FKBP12 (immunophilin, FK-binding protein) resulting in an inhibitory complex that inactivates calcineurin and blocks NF-AT nuclear translocation and, therefore, NF-AT-induced transcription. Rapamycin also binds to FKBP12 but can simultaneously act as a ligand for FRAP (FKBP-rapamycin-associated protein, a regulator of the G1 phase of the cell cycle) and therefore induces heterodimerization between FKBP12 and FRAP.

Ligand-activated regulation of induced proximity offers new opportunities in biological and medicinal intervention, and this strategy has been used to elucidate different steps in signalling pathways. One of the most important advantages of such inducible systems is the possibility to turn-on and turn-off intracellular signals over a short period of time. Further elucidation of T-cell signalling represented one of the first examples where this system made a major contribution. FK1012 (dimer of FK506) was used to regulate signalling of the T-cell receptor. The CD3 ζ chain becomes phosphorylated by the T-cell-receptor engagement and signal molecules like ZAP70 become recruited to the ζ chain and start signal transduction in T cells. Constructs of the CD3 ζ chain and FKBP12 were made, and addition of FK1012 to the constructs initiated signalling by dimerization of the ζ chain and leads to signal transduction. Transient membrane recruitment of ZAP70 by FK1012 resulted in rapid phosphorylation of ZAP70 and activation of the ras/MAPK and the Ca²⁺/calcineurin pathways. SH2 domains of ZAP70 are not required for signalling when the kinase is artificially recruited to the membrane, indicating that the SH2 domains function solely in recruitment and not in kinase activation. SH2 domains can be fully replaced by FK1012-induced membrane recruitment of ZAP70. Using additional synthetic ligands and their binding proteins that recruit ZAP70 equally well but orient it at the cell

membrane in different ways, a specific presentation of ZAP70 to its downstream targets was defined. In contrast to FK1012A and FK1012Z which efficiently induced signalling, rapamycin was unable to conduct signalling. Consequently, the induction of signalling requires a specific configuration of the kinase at the membrane.

Another way to use proximity-inducing ligands is to induce programmed cell death. An FKBP chimera, containing the FAS cytoplasmic domain targeted to the plasma membrane, was able to induce cell death reflected by the activity of a constitutive promoter in response to FK1012. Transgenic mice that express the conditional FAS receptor in thymocytes were used to explore FAS-mediated cell-death pathways during the positive and negative selection in the thymus. The addition of FK1012 to these transgenic animals demonstrated that sensitivity to FAS-mediated apoptosis is restricted to CD4+CD8+ thymocytes. Thus, a population of aberrant cells can be deleted by the FKBP/FK1012 technique.

Dimeric ligands were also shown to elucidate mechanisms in transcriptional control. Maintaining the transcription of endogenous genes *in vivo* requires the continuous presence of the activation domain both in yeast and human cells. The simplest way to activate transcription by induced proximity is to bring together a DNA-binding domain with an activation domain both containing FKBP12 by addition of FK1012. In contrast, transcription can be rapidly switched off by addition of the monomer drug FK506.

This transcriptional switch system has been successfully used in gene therapy to approach erythropoietin (EPO, erythrocytic growth factor) expression in animal models (mice and non-human primates). Two chimeric, human-derived proteins were expressed, which in turn were reconstituted by rapamycin into an active transcription factor complex. Two adeno-associated virus vectors, one expressing the transcription factor chimeras and the other containing the epo gene under the control of the transcription-factor dimers, were used. The results showed that the administration of rapamycin can regulate EPO expression *in vivo*.

In addition to the activation of protein interactions by induced proximity, inactivation of proteins can be achieved with the use of dimeric drugs through mislocalization. A nuclear protein of interest can be dimerized to a chimeric protein with a nuclear export sequence (NES). Dimerization is mediated by rapamycin that binds

to FRB fused to a NES and, on the other hand, to FKBP fused to a nuclear protein such as a transcription factor. Rapamycin permeates the nuclear membrane and induces the dimerization of both fusion-proteins in the nucleus, resulting in rapid translocation to the cytoplasm. In the case of a transcription factor, addition of rapamycin has been shown to rapidly turn off transcription.

Induced proximity has been shown to control mechanisms in cellular signalling. The approach using chimeric proteins containing binding sites for dimeric ligands has been shown to specifically activate signalling pathways allowing elucidation of molecular mechanisms in cell signalling.

- G.R. Crabtree, S.L. Schreiber, *TIBS* **1996**, *21*, 418-22.
- I.A. Graef, L.J. Holsinger, S. Diver, S.L. Schreiber, G.R. Crabtree, *EMBO J.* **1996**, *16*, 5618-5628.
- D.M. Spencer, P.J. Belshaw, L. Chen., S.N. Ho, F. Randazzo, G.R. Crabtree, S.L. Schreiber, *Curr. Biol.* **1996**, *6*, 839-47.
- S.N. Ho, S.R. Biggar, D.M. Spencer, S.L. Schreiber, G.R. Crabtree, *Nature* **1996**, *382*, 822-26.
- X. Ye, V.M. Rivera, P. Zoltick, F. Cerasoli, Jr., M.A. Schrell, G. Gao, J.V. Hughes, M.N. Gilman, J.M. Wilson, *Science* **1999**, *283*, 88-91.
- J.D. Klemm, C.R. Beals, G.R. Crabtree, *Curr. Biol.* **1997**, *7*, 638-44.