

Inhibitors of NEMO, a crucial protein regulating the IKK complex of the NF- κ B pathway

Les inhibiteurs de NEMO, une protéine clé dans la régulation du complexe IKK de la voie de signalisation NF- κ B

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Along with its well documented role in the regulation of genes involved in inflammation, the NF- κ B pathway is now recognized as an important player in the multifactorial game leading to cancer through its role in the control of apoptosis. Thus inhibition of the pathway could have an anti-oncogenic effect through a pro-apoptotic mechanism. The difficulty, however, is that the NF- κ B dependent genes are involved in many biological processes and it may be severely deleterious to the cells to inhibit completely their expression.

A crucial element in the NF- κ B pathway is the IKK complex to which many stimulating signals converge. The IKK complex has been described is composed of the two protein kinases IKK α and IKK β and of the protein NEMO (NF- κ B Essential Modulator, also called IKK γ), a non-catalytic scaffolding protein necessary for the stimulus dependent activation of the complex. Upon activation, the latter phosphorylates the I κ B inhibitor and allows its subsequent destruction by the proteasome. The most abundant NF- κ B transcription factor, p50/p65 which was sequestered by I κ B in the cytosol, can then enter the nucleus and participate to the regulation of the expression of its target genes. Thus, inhibitors of NEMO function targetting the IKK kinases are potential pro-apoptotic agents which could lead to anti-cancer drugs.

By a combination of *in vitro* and *in cellulo* experiments, we have shown that NEMO forms oligomers (trimers) and that this oligomeric structure is necessary for its function. We have defined the NEMO « minimal oligomerisation domain » comprised in its C-terminal half, which is composed of two coiled-coils named CC2 and LZ. We have established a molecular model for this CC2-LZ domain in which the coiled-coils form a pseudo-six helix bundle similar to that of the GP41 ectodomain of the AIDS

virus. In order to find specific inhibitors of NEMO, we then designed peptides on the basis of this model, that could alter the oligomeric structure of the NEMO and inhibit its function by competing with the interfaces between CC2 and LZ helices within their trimeric arrangement. The peptides were added a cell permeable sequence and their penetration into cultured cells was followed by FACS. Their effect was analysed in cells after stimulation with LPS using β -galactosidase as a reporter gene of the NF- κ B pathway, and *in vitro* for their capacity to bind to the CC2-LZ domain using fluorescence polarisation. Both CC2 and LZ peptides were good inhibitors with IC₅₀ of 22 and 3 μ M respectively, much lower than previously reported peptides designed on the basis of competing their interaction with the kinase. Most interestingly, these peptide are specific of the NF- κ B pathway since they do not affect the MAP kinase and other signal transduction pathway. In addition, they do not impair the basal level of overall activity of the NF- κ B pathway but rather affect the stimulation by extracellular signals. Thus, targetting NEMO to inhibit the pathway may turn to be an important advantage as compared to inhibiting directly the kinase activity itself, because it may elicit less unspecific side effects.

In a further step, we have characterized the mechanism of action of our peptides *in cellulo*. For this, NEMO-GFP and NEMO-Flag constructs were cotransfected in human 293T cells and the potential protein complexes containing oligomers were adsorbed on anti-Flag beads after lysis of the cells. After elution washing of the beads and their elution with a Flag-peptide, reading-outs of GFP fuorescence allowed to probe the oligomeric structure of NEMO *in cellulo*, and to demonstrate that the inhibitory effect of CC2 and LZ peptides was due to their ability to dissociate NEMO trimers.

In order to improve protein-protein interaction inhibitors against the minimal oligomerisation domain, we designed ankyrin and peptide binders using a directed evolution method. Ankyrins constitute an attractive class of stable and small repeat proteins that provide variable and modular binding surfaces to a target protein (Binz et al., 2004 Nature Biotechnology, 22, 575-582). We used the ribosome display method to select ankyrins binding to the NEMO minimal oligomerisation domain from a naive ankyrin library. After four rounds of selection, several ankyrins with affinity in the low nanomolar range were isolated. When expressed in 293T cells, the selected ankyrins strongly inhibit TNF α -mediated NF- κ B activation while having no effect on the basal activity. Controls with naive ankyrin or null plasmid were without effect. Furthermore,

we could show that this NF- κ B inhibition occurs through a specific interaction between ankyrin binders and the endogenous NEMO, resulting in the IKK inhibition. Our findings indicate that selecting high-affinity binders from peptide or chemical libraries against the minimal oligomerization domain of NEMO can be a promising strategy to search for specific NF- κ B inhibitors.

The « ribosome display » directed evolution method was also used to select LZ peptides with higher affinity against the CC2-LZ domain. A library of ca. 5.2×10^{12} peptides was built from the coiled-coil scaffold of LZ by randomizing residues in the hydrophobic interface as well as in the interchain ionic interactions. Like ankyrins, several peptamers with affinity in the low nanomolar range were isolated after four rounds of selection. Once fused to a cell-permeable sequence, they will be tested for their ability to inhibit the activation of the NF- κ B pathway in our cellular models described before and for their ability to induce apoptosis in cancer cells. Our binders might also be used for structural studies for both NEMO and the minimal oligomerization domain.

Bibliography :

1. Agou, F., Ye, F., Goffinont, S., Courtois, G. Yamaoka, S., Israël, A. and Véron, M. (2002) NEMO trimerizes through its coiled-coil C-terminal domain. J. Biol. Chem., 277 : 17464-17475.
2. Agou, F., Courtois, G., Baleux, F., Coïc, Y.-M., Traincard, F., Israël, A. and Véron, M. (2004) Inhibition of NF- κ B activation by peptides targeting NEMO oligomerization. J. Biol. Chem., 279 : 54248-54257
3. Agou, F., Traincard, F., Vinolo, E., Courtois, G., Yamaoka, S., Israël, A., Véron, M. (2004) The trimerization domain of NEMO is comprised of the interacting C-terminal CC2 and LZ coiled-coil domains, J. Biol. Chem., 279 : 27861-27869.
4. Wyler, E., Kaminska M., Véron, M. and Agou, F. Blocking NF- κ B activation by engineering proteins targeted to the minimal oligomerization domain of NEMO, (in preparation)