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The sneaking ligand approach for cell type-specific modulation of intracellular signalling pathways

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ABSTRACT

Small molecules interfering with intracellular signalling pathways are used in the treatment of multiple diseases including RA. However, small molecules usually affect signalling in most cell types, not only in those which need to be targeted. This general inhibition of signalling pathways causes often adverse effects, which could be avoided by cell type-specific inhibitors. For cell-type specific modulation of signal transduction, we developed the sneaking ligand fusion proteins (SLFPs). SLFPs contain three domains: (1) the binding domain mediating cell type-specific targeting and endocytosis; (2) the endosomal release sequence releasing the effector domain into the cytoplasm; (3) the effector domain modulating signalling. Using our SLFP NF-kappaB inhibitor termed SLC1 we demonstrated that cell-type-specific modulation of intracellular signalling pathways is feasible, that endothelial NF-kappaB activation is critical for arthritis and peritonitis and that SLFPs help to identify disease-relevant pathways in defined cell types. Hence, SLFPs may improve risk-benefit ratios of therapeutic interventions.

1. Introduction

Rheumatoid arthritis (RA) is the most common inflammatory joint disease affecting up to 1% of the world's population [1]. The aetiology is not fully understood, however genetic and environmental factors such as smoking, toxins and certain diets might increase the risk of RA development [2–6].

Dendritic cells, B and T cells, fibroblasts, endothelial cells, monocytes/macrophages and neutrophils contribute to RA pathogenesis and interact in a complex network leading to the production of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNFα, IL-17) and matrix degrading enzymes such as matrix-metalloproteinases (MMPs) [7]. Invasion of inflammatory cells into the joints results in an extensive inflammatory response followed by cartilage and bone destruction [8]. These events severely impact on quality of life of RA patients and even increase mortality, mainly due to increased cardiovascular risk.

Targeted therapies in RA antagonize key pro-inflammatory cytokines such as TNF or their receptors such as the IL-6 receptor [9,10]. Moreover, reducing T cell activation by blocking CD28-CD80/CD86 co-stimulation or depletion of $CD20^+$ B cells are further options to treat RA [11]. Recently, the first small molecules inhibiting the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, tofacitinib and baricitinib, have been approved for the treatment of RA in many countries. Tofacitinib and baricitinib were designed to block the ATP-binding pocket of JAK molecules, thereby preventing STAT signalling events, and eventually RA symptoms and progression [12,13]. Recently, the impact of small molecules targeting other kinases like spleen tyrosine kinase, Bruton's tyrosine kinase and phosphatidylinositol-3 kinase were under investigation and might represent novel therapeutic strategies for inhibiting RA related signalling pathways [14,15].

Nuclear transcription factor kappa B (NF-kappaB) is a masterswitch of the immune and inflammatory response [16,17]. Therefore, research focussed also on the development of NF-kappaB inhibitors. Small molecule inhibitors of IKK2 (IkappaB kinase 2) are able to block phosphorylation of the inhibitor of NF-kappaB proteins (IkappaBs) and, subsequently, inhibit transcriptional induction of pro-inflammatory molecules [18,19]. Such small molecule inhibitors interfering with NF-kappaB activation or intracellular signalling pathways can effectively

Abbreviations: CPPs, cell-permeable proteins; ETA, *Pseudomonas* Exotoxin A; NBP, NEMO-binding peptide; PTDs, protein transduction domains; SLFP, sneaking ligand fusion protein.

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control immune-mediated inflammatory diseases in mice; however, these molecules are taken up by virtually all cells of the organism, not only by those cells which contribute to the inflammatory process. Therefore, this general inhibition of intracellular signalling pathways leads often to serious adverse effects, sometimes precluding therapeutic use in humans as it is the case with IKK2 inhibitors [20].

In this article, we describe multi-modular structured fusion proteins for cell-type specific modulation of intracellular signalling pathways, named sneaking ligand fusion proteins (SLFPs).

2. Methods to deliver cell-membrane impermeable substances into cells

A major hindrance for clinical use of certain pharmacological substances, mainly large natural or synthetic molecules, or gene material may come from their inability to penetrate the plasma membrane. Passive diffusion or natural or artificial mechanisms are routes to cross the lipid bilayer of the plasma membrane or endocytic vesicles in order to reach cytosolic or nuclear compartments [21,22]. A panel of techniques was developed to support drug delivery [23–28]. Some delivery methods such as electroporation and microinjection can be used in vitro only, others such as cargoes with cationic lipids, liposome encapsulation are of limited use due to low cell or organ-specificity, low transfer efficiency, immunogenicity, cellular toxicity or high costs [29].

The development of liposomes, microspheres or nanoparticles for the encapsulation of effector proteins or DNA to transport them to the site of action enormously advanced science [29–32]. Specific cell-targeting could be achieved by immunoliposomes which have whole antibodies, single-chain variable fragments (scFv) or ligands attached to their surfaces and are mainly proposed for use in cancer therapy [33]. However, nanotechnology-based drug delivery systems have also disadvantages that might impede application in vivo. Inefficient rates of release of active substance into the cytoplasm, or low stability limit their therapeutic use [34].

An alternative approach that enables the penetration of molecules into the cytoplasm is to attach specific peptide sequences, also known as protein transduction domains (PTDs), to effector molecules [35]. Researchers identified the basic region (amino acid sequence residues 47–57) of the 86 amino acid human immunodeficiency virus type 1 (HIV-1) TAT protein as a PTD that enter cells in culture and activate transcription [36–38]. Also the PTD deduced from Drosophila homeoprotein antennapedia (Antp) can be used to carry effector proteins across membranes [39–41]. PTD-linked peptides/proteins are also designated as cell-permeable proteins (CPPs). However, the uptake mechanism of these CPPs is not fully understood, whereas passive delivery, endocytosis-mediated or inverted micelle-mediated delivery are under development [38,42,43]. Proteins of large molecular weight and hydrophilic composition impede the uptake into cells [38,43]. Beside these limiting factors, molecules assembled to PTDs might influence homeostasis of the immune system [44].

3. Structure of natural toxins as a model for cell type-specific intracellular targeting

The structure of natural toxins like *Diphteria toxin, Cholera toxin* or *Pseudomonas* Exotoxin A (PE or ETA), represents an appropriate design to target cells and deliver an effector molecule into the cell's interior [45–47]. ETA consists of three domains: (1) the receptor binding domain ETAIa (2) followed by the translocation domain ETAII and (3) the ADP ribosyltransferase domain (ETA1B/III) which exerts cytotoxic effects [48]. Receptor-mediated endocytosis of ETA leads to the formation of early and late endosomes and guides ETA from the Golgi apparatus to the Endoplasmatic Reticulum (ER) in a retrograde manner [49,50]. This Golgi-ER retrograde transport is facilitated through a C′-terminal motif RDEL binding the KDEL-receptor [51]. Once in the ER, ETA is processed by furin resulting in two fragments of ETAI and the ADP ribosyltransferase domain. The ADP ribosyltransferase domain is subsequently transported into the cytosol possibly through the Sec61 translocon and promptly inactivates elongation factor 2 (EF2) by ADP ribosylation which inhibits protein synthesis and kills the cell. However, the intoxication pathway of ETA has not yet been fully elucidated [48,50].

This mode of action is also utilized in immunotoxins which target cells trough specific single-chain variable fragments (scFv) coupled e.g. to the ETAII/ETAIII domain resulting in cell death [52–54].

The three-domain structure of toxins embodies an archetype model for cell-type specific modulation of intracellular signalling pathways and leads us to the development of the "sneaking ligand"- (SL) principle.

4. The "sneaking ligand principle"

SLFPs are composed like ETA of (1) a binding domain, (2) a translocation domain and (3) an effector domain (Fig. 1A).

Fig. 1B illustrates the mode of action of SLFPs that is based on (1) receptor-mediated uptake of SLFPs leading to (2) the formation of early and late endosomes. (3 + 4) Subsequently, a retrograde transport occurs through the Golgi-ER via the KDEL-receptor. In the ER, the SLFPs are proteolytically cleaved at the ETAII domain by furin and the C′-terminal effector domain is released into the cytosol. (5) Specific interaction of the effector domain with its cytosolic or nuclear ligand elicits a blockade of intracellular signalling [55,56].

A main benefit of SLFPs is that the domains are exchangeable; therefore, different cell-types and/or signalling pathways can be easily addressed using a set of modular components.

As for other recombinant proteins or scFvs, prokaryotic expression is a powerful system to produce SLFPs. In general, and to generate the most effective SLFPs, it is important to test several vector/host combinations because the yield of the expressed SLFPs can be strongly variable [57]. The choice of vector, host and codon usage also influences the localisation of proteins expressed in the periplasmatic or cytoplasmatic compartments of E.coli [58,59]. The integration of affinity tags (e.g. His_6-tag or Strep-tagII) into the fusion protein eases purification and detection [60]. Nevertheless, it should be taken into consideration that binding or functionality of the SLFP could be hampered by tags due to steric effects. Therefore, affinity tags have to be assembled at the N′-or C′-terminus of SLFPs and have to be examined for binding and functionality.

5. Applications of sneaking ligand fusion proteins

Among inducible transcription factors involved in the regulation of inflammatory gene expression, NF-kappaB is crucial for the coordinated transcriptional control of various pro-inflammatory mediators including cytokines, chemokines, enzymes and cell adhesion molecules [61–63].

Dysregulated NF-kappaB activation contributes to the pathogenesis of RA [62,93] as well as other pro-inflammatory conditions. Compelling evidence from experimental models of autoimmune diseases supports the concept of NF-kappaB blockade for therapeutic interventions [18,64–67]. However, a systemic interference with the NF-kappaB system influences a variety of homeostatic regulatory pathways, for instance in the liver and the immune system [20]. An ubiquitous pharmacologic suppression of NF-kappaB activity is associated with hazards of severe side effects including profound immunosuppression, liver cell apoptosis and other organ dysfunctions [20,68,69]. Furthermore, NF-kappaB activation is also involved in the resolution of inflammation and may exert positive or negative effects on inflammatory processes depending on the cell type and the disease phase [70,71]. Hence, modulation of gene transcription through NF-kappaB blockade should be restricted to particular cell types contributing to disease pathogenesis in respect to maintain NF-kappaB function in other cells or organs.

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