New drug targets in inflammation and immunomodulation

Terance Hart, Alan Lamont and David Williams

The identification of novel target molecules for a range of inflammatory diseases remains a significant objective for the pharmaceutical industry. The ultimate goal of this research is the development of new immunomodulatory compounds that may augment or even replace existing treatments. The authors describe the key developments made towards this broad objective for several therapeutic areas in the light of a recent conference covering this topic.

Several recent advances have been made in research of inflammatory diseases, both in terms of understanding the mechanisms of known target molecules and the identification of new ones. This progress was highlighted at the RSC 2nd International Conference on *New Drug Targets in Inflammation and Immunomodulation* held at Glaxo Wellcome (Stevenage, UK) on 15–16 July 1998.

Targeting TNF – a case study

The pathological role that tumour necrosis factor α (TNF-α) plays in several inflammatory conditions has been extensively described; however, the sequence of signalling events that occurs when TNF-α binds to the TNF-R1 receptor has been enigmatic. Mike Rothe's group (Tularik, South San Francisco, CA, USA) has recently shown that TNF-α can promote nuclear translocation of the cytosolic transcription factor NF-κB (nuclear factor-κ binding) – a member of the Rel family of transcription factors, which can then induce the upregulation of several genes, including those encoding pro-inflammatory cytokines, adhesion molecules and proteolytic enzymes. Until recently, the sequence of events that led to NF-κB activation were unknown, but by working upstream of NF-κB, Rothe's group have dissected these events in the signalling pathway linking TNF-R1 activation with the release of NF-κB from its complex with IκB (inhibitor of NF-κB). Furthermore, NF-κB appears to be at a convergent signalling and feedback point for several external pro-inflammatory signals because it is activated under pathological conditions by a variety of stimuli, including interleukin 1 (IL-1) and arachidonic acid metabolites.

There are currently 15 members of the TNF-R superfamily, of which four (DR3, DR4, Fas and TNF-R1) contain the cytosolic death domain. Upon ligand binding, the cytosolic death domain of the receptor recruits two proteins, TRADD (TNF-receptor-associated death domain) and RIP (receptor-interacting protein), and TRADD, in turn, may recruit other molecules [e.g TNF-receptor-associated factor 1 (TRAF1) and TRAF2] to the receptor complex. The relationship between these proximal signalling complexes and downstream activation has been investigated with yeast two-hybrid experiments. By using TRAF2 as bait, NIK [NF-κB-inducing kinase – a mitogen-activated protein-3 kinase (MAP3K) Ser/Thr kinase] has been identified as a potential binding partner. Subsequent employment of NIK as the yeast two-hybrid bait trapped two other serine kinases, IκB kinase α (IKK-α) and IKK-β, the latter being more effective in activating a NF-κB-dependent reporter gene

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construct. It now appears that activation of NF-κB is a direct result of phosphorylation of IκB by a ternary complex containing IKK-α and IKK-β combined with NIK, which suggests that drugs that modulate the activity of the IKK complex are likely to have therapeutic value in inflammatory diseases.

Structure and function of the IKK complex

David Rothwarf and coworkers (University of California at San Diego, CA, USA) have worked on the structure and function of the IKK complex. The complex can exist in two forms (approximately 900 kDa and 300 kDa), both having kinase activity. Structurally, both IKK-α (85 kDa) and IKK-β (87 kDa) contain a protein kinase domain at the N-terminal region and a protein-interaction motif, comprised of a helix-loop-helix (HLH) and a leucine zipper (LZ) motif, at the C-terminal region. Most importantly, LZ mutants of both IKK-α and IKK-β had greatly reduced IκB kinase activity. Using antisense, the team has demonstrated that IKK-α is essential for NF-κB activation by TNF-α, IL-1, TPA (12-*O*-tetradecanoylphorbol-13-acetate) or okadaic acid. The story is still unfolding, because recent research has identified IKK-γ, a helical coil protein as another component of the 900 kDa IKK complex. It has subsequently been shown that both IKK-β and IKK-γ are essential for the activity of the IKK complex.

TNF-α-converting enzyme inhibitors

TNF-α-converting enzyme (TACE) is responsible for the processing of the membrane-bound version of TNF-α into a soluble trimer of 17 kD subunits. Roy Black (Immunex, Seattle, WA, USA) presented a rational for designing inhibitors of the enzyme. The released protein is responsible for the full spectrum of TNF-α-mediated effects, so it is anticipated that small-molecule inhibitors of TACE may have therapeutic utility. The X-ray structure of TACE complexed with a peptidic inhibitor revealed that the S1′ and S3′ pockets could be the main substrate recognition sites.

TACE, otherwise known as ADAM17 (a disintegrin and metalloproteinase), is one of several metalloproteinases, including ADAM10, that are also capable of cleaving 26 kDa TNF-α *in vitro*. Nevertheless, a critical role for TACE in TNF-α processing *in vivo* was proposed by Black based on the finding that T cells and monocytes from TACE −/− mice, release considerably less TNF-α than the corresponding wild-type cells. This enzyme may also be responsible for processing of other cell-bound molecules. For example, phenotypic similarities between TACE −/− mice and transforming growth factor α (TGF-α) −/− mice led to experiments that indicated that TGF-α was a substrate for TACE. Other protein substrates for TACE have not yet been identified, but possible candidates include other 'shed' proteins involved in the immune response, such as colony-stimulating factor 1α receptor (CSF-1R), IL-2Rα, type 2 IL-1R and L-selectin.

The premise that TNF-α represents an important target for drug development programmes was further underlined by George Spencer-Green (Immunex), who presented results obtained from the clinical trials of the Immunex TNF-α antagonist, Enbrel (p75 TNF-R2 linked to the Fc domain of IgG) in rheumatoid arthritis (RA) patients. Significant beneficial effects of the fusion protein were observed in a Phase III clinical trial of 234 severe RA patients in 13 sites. The response rate was based on criteria established by the American College of Rheumatology (Atlanta, GA, USA), and involved a reduction in patients' disease symptoms of 50%. Two doses were examined, with the high dose giving a response rate of 41% at six months compared with 13% for low dose, and 8% for placebo. Further long-term studies have been conducted, in which over a 12 month period, from 106 patients started on the therapy, 89 patients were continuing and 17 had dropped out. Only five of these were due to a lack of efficacy. Overall, the data indicate that Enbrel will represent a major advance in the treatment of this autoimmune disease.

Chemokines and their actions

The relevance of chemokines (chemotactic cytokines, CC) as a target for therapy of a range of inflammatory and infectious diseases has been widely researched. Significant redundancy in chemokine–receptor interactions exist at least *in vitro*, with many chemokines being able to recognize a single receptor, and single chemokines acting as a ligand for multiple receptors. For example, the chemokine receptor CCR5 can recognize the chemokines RANTES, macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, while other receptors, such as CCR1, CCR3 and CCR4, can also utilize RANTES. With such redundancy *in vitro*, how can specificity be achieved *in vivo*? In answer to this question, Amand Proudfoot (Serono Pharmaceutical Research Institute, Geneva, Switzerland) described the several levels of control that have been proposed and which encompass both temporal and spatial elements to produce selectivity of effect. Many laboratories who are working in

this field are trying to unravel this network of fine control mechanisms.

Approaches to receptor antagonist development have been aided by the identification of N-terminally modified versions of RANTES, which exhibit potent inhibitory activity both *in vitro* and *in vivo*. Met-RANTES has been demonstrated to reduce inflammation associated with a model of collagen-induced arthritis and to block MIP-1α-induced footpad swelling in mice. In addition, this antagonist can reduce eosinophil infiltration into the airways of sensitized rats. Modification of the N-terminus of RANTES by inclusion of an aminooxypentane group (AOP-RANTES) produces an antagonist of CCR5 that can potently inhibit HIV-1 infection of macrophages. This effect appears to be related to the ability of the modified chemokine to promote receptor internalization and prevent its subsequent recycling; it thus represents a novel therapeutic concept for agents that prevent HIV infection.

CCR1 antagonists

The development of low molecular weight antagonists of the receptor CCR1 has been undertaken by Richard Horuk's group at Berlex (San Francisco, CA, USA). This receptor was targeted because a convincing role exists for this receptor and its ligands in the induction and maintenance of inflammatory lesions associated with animal models of autoimmune disease. Fibroblast cells stably expressing CCR1 were used to screen in-house compound libraries for inhibition of radiolabelled ligand binding. Several compound families were obtained that gave IC_{50} values of ~500 nM. Although no structures were divulged, the best compound had an IC_{50} value of 40 nM, and demonstrated ~250-fold selectivity against a range of other 7-transmembrane (7-TM) receptors. *In vitro*, this compound can inhibit MIP-1α-induced calcium flux in cells and can prevent MIP-1α- and RANTES-induced T-cell chemotaxis. The lead compounds have also been tested successfully in animal models and are currently undergoing toxicological assessment prior to further development.

Natural chemokine inhibitors

It is often a truism in protein science that what man has achieved through years of dedicated work in the laboratory, nature has already achieved in fundamentally more elegant ways. Such is the case with inhibitors of chemokines, as was discovered by Geoff Smith (University of Oxford, UK). His group has identified a chemokine-binding protein (CKBP) present in vaccinia virus, which, in terms of primary sequence homology, appears unrelated to any of the known chemokine receptors. The protein molecule interacts solely with CC chemokines and has an estimated affinity of 100 pM. Viral CKBP can inhibit CC chemokine-driven calcium flux in eosinophils, but has no effect on an equivalent response stimulated by CXC chemokines in neutrophils. This protein represents one of many strategies employed by the virus to evade the host immune system, and it is a challenge of future research in this field to define which are the key strategies utilized by the virus at critical stages in its life cycle. Furthermore, the intriguing possibility remains that by understanding the key structural features of viral CKBP–chemokine interaction, an alternative strategy for the design of low molecular weight inhibitors of chemokines will be possible.

Chemotaxis

The events that may occur as a consequence of chemokine release – the processes of cell migration from the circulation and extravasation into sites of inflammation – have been investigated by Ann Ager at the National Institute of Medical Research (London, UK). Using a novel *in vitro* culture system that allowed the initial events of lymphocyte binding to high endothelial venules (HEV), it was possible to examine the subsequent migration of lymphocytes through the endothelial layer. Phenotypic differences in lymphocytes that had migrated through the layer were observed, in particular, the shedding of L-selectin and the activation of α_4 integrin adhesion molecules. Attention subsequently focused on the proteolytic activity responsible for the cleavage of the molecule (termed L-sheddase). The cleavage site was defined between residues K283 and S284, and the activity was unaffected by a range of standard serine protease inhibitors. Inhibition, however, was observed using both TIMP-3 and a selective matrix metalloproteinase inhibitor (Ro319790). Both of these inhibitors can also prevent TNF-α release, however, the relationship between L-sheddase and TNF-α convertase remains unknown. Ro319790 (Fig. 1) can prevent transendothelial migration *in vitro* and a preliminary analysis using fluorescently labelled lymphocytes indicates that this compound also prevents migration through the HEV layer *in vivo*. Clearly, further definition of proteases that regulate expression of cell adhesion molecules may yield viable targets for drug discovery in the future.

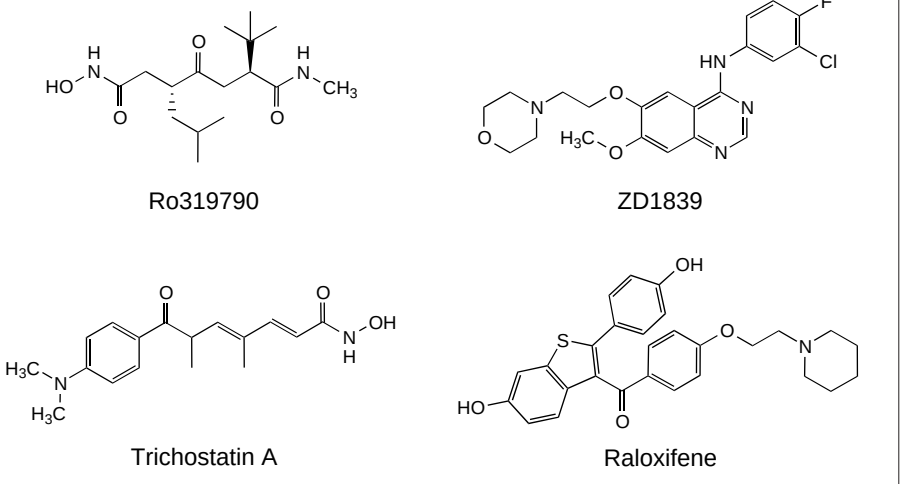

Figure 1. *Considerable research in the pharmaceutical industry has been focused upon the development of new immunomodulatory compounds to augment or replace current therapies, including the four examples shown (see text).*

Intracellular signalling by kinases

There is a diversity of intracellular pathways which exist to transmit signals received at the cell membrane from external stimuli and translate them into an appropriate cellular response. In only the most basic sense are many of these processes understood, yet they represent potentially critical points for therapeutic intervention in many diseases.

A structural insight into the regulation and inhibition of protein kinases has been provided by Michael Eck (Harvard University, Cambridge, MA, USA), who analysed the differences between the active and inactive structures of a variety of protein tyrosine kinases (PTKs), including the insulin and fibroblast growth factor (FGF) receptor protein tyrosine kinases (RPTKs), and the Src-family of kinases (Src, Hck and Lck). The factors affecting the binding modes of a variety of cofactors, substrates and inhibitors were also included in the structural analysis.

It was noted that in the Src-family, the Src-homology 2 (SH2) domain of Src interacts with Tyr527 in the C-terminal tail region to lock the kinase in an inactive mode. The SH3 domain is intimately associated with the linker region between the SH2 and kinase domains, which although not rich in proline, as might be expected for an SH3 binding ligand, nevertheless adopts a polyproline type II helical conformation. Both domains effectively pack onto the back of the kinase domain, clamping it closed and inactive. The interaction of the SH2 and SH3 domains with their respective ligands, however, may be of low to moderate affinity, suggesting that recognition of a cognate high-affinity ligand by either domain may be sufficient to destabilize these intramolecular interactions and promote the transition to an active conformation.

Further structural information on PTKs has been provided by David Williams (Peptide Therapeutics, Cambridge, UK). The structure of the C-terminal Src kinase (CSK) catalytic domain was solved as a co-complex with the general protein kinase inhibitor staurosporine. Staurosporine binds to the CSK by an induced fit mechanism which changes the orientations of conserved magnesium ion-binding residues (Asp332 and Phe333) in the N-terminal kinase lobe. The inhibitor forms only three H-bonds with CSK residues in contrast to the four bonds observed in the crystal structures of staurosporine bound to protein kinase A (PKA) and cyclin-dependent kinase 2 (CDK2). These observations are vital for understanding the exact molecular mechanisms of staurosporine specificity and provide a basis for lead inhibitor optimization.

Protein kinase inhibitor design

A system for rapidly probing the substrate-binding site of a protein kinase (combinatorial approach for selective kinase inhibitor design – CASKAiD™) has been developed by Peptide Therapeutics. This system utilizes mixtures of peptides arranged in a self-deconvoluting 96-well-plate format using phosphorylation assays to probe the peptide substrate-binding site of a protein kinase. Single peptide substrates are unequivocally identified along with sequences that are not phosphorylated by the kinase, providing a unique enzyme fingerprint. The data obtained provides a clear idea of the catalytic site environment, allowing the generation of refined enzyme structure homology models and substrate pharmacophores. These pharmacophoric models are then used to design non-peptide inhibitor molecule libraries for screening. The Syk-family protein tyrosine kinases, Syk and ZAP-70, were used to illustrate the development and application of this approach.

The successful development of a low molecular weight compound for PTK inhibition has been achieved by Andrew Barker (Zeneca, Macclesfield, UK). At the recent conference he presented the SAR surrounding 4-anilino quinazoline ATP-competitive inhibitors of epidermal growth factor (EGF)-RPTK. The initial lead, discovered via random screening possessed a K_i value of 180 nM and was unsubstituted at the 6 and 7 positions. Incorporation of 6,7-dimethoxy groups increased activity 50-fold, but the resulting series of compounds showed poor pharmacokinetics. Halogen substitution in the 3- and 4- positions of the aniline ring retained activity and provided a slightly better pharmacokinetic profile, which was further improved by substituting the 7-methoxy function with a morpholinyl propyloxy group [ZD1839 (Fig. 1), IC_{50} = 23 nM for EGF-RPTK and IC_{50} = 80 nM for an EGF-stimulated cell line]. This molecule reversibly inhibited the growth of EGF-stimulated cells while having no effect on basal growth rates at the same concentrations. Growth rates were unaffected in insulin-stimulated MCF-7 or platelet-derived growth factor (PDGF)-stimulated cells. The compound, which simulates a cellular on–off switch, is considered by Zeneca to be highly selective within the range of kinases that they screened, and ZD1839 is currently in Phase II clinical trials as an anticancer treatment.

Cytokine signalling – JAKs and STATs

John O'Shea's group (National Institutes of Health, Bethesda, MD, USA) has investigated signalling via type 1 and 2 cytokine receptors, and in particular the role of the Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) within these pathways. The importance of these molecules to the maintenance of a functioning immune system is underlined by rare naturally occurring deficiencies in human populations. For example, mutations in JAK3 are associated with the development of severe combined immunodeficiency (SCID) in babies – fatal in the absence of bone marrow transplantation. In addition, mutations in the common γ-chain of cytokine receptors to which JAK3 binds following cytokine administration, also give rise to a SCID phenotype. JAK3 knockout mice have been generated; they show a defect in the production of B cells and have impaired T-cell function. Thus, for cytokines that utilize the common γ-chain (e.g. IL-2, IL-4, IL-7, IL-9, IL-15), the importance of JAK3 in signalling is clear.

No crystal structure is available for any of the JAKs as yet. Therefore, structural information relating to the regulation of JAK catalytic activity is not available. The importance of the pseudokinase [Jak homology 2 (JH2)] domain in regulating the activity of the JH1 domain has, however, been indicated through site-directed mutagenesis studies. One hypothesis suggests that interaction of these two domains is required for maximal activity, raising the intriguing possibility that screening for inhibitors of domain interaction may represent a valid strategy for drug discovery.

By contrast, recent structural information regarding the interaction of STAT homodimers bound to DNA has provided valuable insight into how the activity of these transcription factors is regulated. Although the general model of STAT phosphorylation followed by dimerization and translocation to the nucleus is well accepted, several critical questions remain unanswered. For example, the significance of serine phosphorylation in certain STATs following cytokine stimulation and the identity of the kinases responsible remain unknown. It is these issues that are currently being addressed in many laboratories.

MAPK signal cascades

Moving away from the more proximal membrane events in signalling, the composition of the pathways that constitute the MAPK signalling cascades have been researched by Simon Cook (Babraham Institute, Cambridge, UK). Different systems were utilized to demonstrate the effect of sustained versus transient activation of MAPK in cell-cycle progression and in differentiation. Thus, in the rat neuronal cell line PC12, sustained activation of the extracellular-signal-related kinase (ERK) by nerve growth factor (NGF) resulted in growth arrest and differentiation, while more transient activation by EGF promoted cell proliferation. Similarly, in CCL39 cells, thrombin stimulates sustained activation of p44 MAPK and DNA synthesis, while agonist peptides stimulate only transient activation and no DNA synthesis. Furthermore, there is a direct correlation in these cells between sustained MAPK activation and cyclin D1 expression, providing a link between MAPK and cell-cycle regulation. The duration of MAPK activity therefore will critically influence cellular responses that occur downstream.

Transcriptional regulation within the nucleus

According to Alan Wolffe (National Institutes of Health), acetylation is a critical control process in transcriptional regulation. Histone acetylation can alter the structure of the nucleosome through derivatization of the ε-NH_2 of lysine

on histones. Neutralization of the positive charge may disrupt the ionic interaction between histone lysine and the DNA phosphate backbone, resulting in chromatin unwinding and subsequent transcriptional activation. A variety of transcription factors may form complexes with acetyltransferase enzymes to promote this process. Conversely, transcriptional repressors, including steroid and nuclear receptors, YY1, Mad/Max, Rb and MeCP2 form similar complexes with deacetylases, and these enzymes may cause transcriptional repression by favouring intramolecular chromatin packing. The potential for pharmacologic intervention in the transcriptional process by modulating histone acetylation or deacetylation has been considered. Trichostatin A, a fungal-derived inhibitor of histone deacetylase (Fig. 1), has been used in oncogenic cell lines to promote differentiation, and block transformation. Trichostatin A is currently being investigated as a combination therapy with retinoic acid for the treatment of promyelocytic leukemia.

In the final presentation, a detailed analysis of the conformational differences induced in the structure of estrogen receptor ligand-binding domain (ER-LBD) when complexed either with its endogenous agonist (17β-oestradiol) or with the mixed agonist–antagonist (Raloxifene; Fig. 1). was described by Ashley Pike (University of York, UK). This provided a most important insight for selective drug design of steroid agonists and antagonists.

The ER receptor family possesses a series of common structural motifs including an N-terminal transactivation domain, a central DNA-binding domain and a C-terminal ligand-binding domain (LBD). Binding of the 17β-oestradiol to the ER activates these three regions of the LBD, which are responsible for nuclear localization, receptor dimerization and ligand-dependent transactivation.

It was noteworthy that, although both agonists and antagonists are capable of binding in the same site, because of the great flexiblity of the protein, they induce different conformations in the transactivation domain of the LBD. In the presence of 17β-oestradiol, helix 12 (H12), which contains the AF-2 transcriptional activating function, lies over the ligand-binding cavity. In contrast, the basic side chain of Raloxifene prevents H12 from adopting this position and, instead, rotates 130° to rest in a hydrophobic cleft that is associated with the binding of the LXXLL motif of nuclear receptor coactivators. These findings provide clear evidence to suggest that the antagonistic properties of Raloxifene are primarily based upon its ability to prevent the transcriptionally active AF-2 conformation. In summary, these fascinating results provide the first structural evidence for predicting whether a ligand will be a functional agonist or antagonist on binding to a member of the steroid receptor family.

Concluding remarks

In the two years which have passed between the inaugural RSC meeting and the recent one, significant advances in the fields of inflammation and immunomodulation have been made. The result of these advances has been an increased level of understanding of the biological/ pathological role of many existing target molecules, and in addition, it has helped to define new ones. The impact of genomics and proteomics for target identification is increasing. The challenge facing the pharmaceutical industry for the future is both to be responsive to new technologies and developments and to prioritize its discovery research programmes to maximize the chances of success. How it manages these two potentially conflicting ideals will determine its success into the next millennium.

In short...

IGEN International (Gaithersburg, MD, USA) have recently completed the development of a new technique that can be used to discover potential drugs for disease treatment based on gene regulation.

When used in combination with their ORIGEN technology-based high-throughput screening (HTS) system, it may provide new lead candidates for the development of drugs that have therapeutic value in the treatment of cancer, cardiovascular, inflammatory, autoimmune and infectious diseases.

Techniques currently available for testing compounds that regulate genes are time consuming, do not possess the necessary sensitivity and are not suitable for HTS formats. This has restricted their utility. However, IGEN's new testing format provides a high-throughput, cost-effective solution characterized by increased sensitivity and precision.

IGEN have filed a patent application on this novel format and have previously announced agreements with major pharmaceutical companies in relation to its HTS system. This new test format will no doubt complement the breadth of applications the HTS system offers.