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Neutralizing endogenous chemokines with small molecules
Principles and potential therapeutic applications

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ABSTRACT

Regulation of cellular responses to external stimuli such as hormones, neurotransmitters, or cytokines is achieved through the control of all steps of the complex cascade starting with synthesis, going through maturation steps, release, distribution, degradation and/or uptake of the signalling molecule interacting with the target protein. One possible way of regulation, referred to as scavenging or neutralization of the ligand, has been increasingly studied, especially for small protein ligands. It shows innovative potential in chemical biology approaches as well as in disease treatment. Neutralization of protein ligands, as for example cytokines or chemokines can lead to the validation of signalling pathways under physiological or pathophysiological conditions, and in certain cases, to the development of therapeutic molecules now used in autoimmune diseases, chronic inflammation and cancer treatment. This review explores the field of ligand neutralization and tries to determine to what extent small chemical molecules could substitute for neutralizing antibodies in therapeutic approaches.

Contents

1. Introduction

Deciphering biological signalling pathways makes use of convergent approaches including direct gene manipulation or downstream information processing intermediates such as messenger RNAs, proteins or signalling small molecules/hormones or their metabolites. Gene manipulation, in particular gene deletion/invalidation, is one of the most widely used approaches to determine the function of a gene and of its products. It presents the major advantage of selectively altering one gene structure or expression so that a given phenotype, when observed, is generally closely associated with the gene of interest and to its products. On the other hand, gene deletion or overexpression can be induced, but not yet in a reversible manner, so that control experiments must be carried out on wild type animals in which developmental or compensatory effects may not have taken place in a comparable manner (Chensue et al., 2001; Auwerx et al., 2004; Brown et al., 2005; Yang et al., 2006). Chemical biology approaches, i.e. methods that use chemical tools to elucidate the function of a protein in a given signalling pathway, and are at the frontier between pharmacology, chemistry and biophysics, are useful too and show complementarity with genetic approaches. They also offer the possibility to transpose small molecule tools into drugs when

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Abbreviations: BSA, Bovine serum albumin; DARC, Duffy antigen receptor for chemokines; GAG, Glycosaminoglycans; GPCR, G-protein-coupled receptor; IL, Interleukin; LPS, Lipopolysaccharide; MS, Multiple sclerosis.

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pathological issues are coming into play during the assessment of the protein function. The advantages and drawbacks of the chemical biology approach are mirror images of the genomic approach. Reversibility of the effect of a molecule can be studied on the same living individual upon cessation of molecule administration. On the other hand, molecules are rarely specific for a given target protein, and the claimed selectivity of a compound generally follows an inverse relationship with the extent of side effects (Wermuth, 2006). Antibodies, and in particular monoclonal antibodies, have arisen as potential substitutes to both the genetic and the chemical biology approaches in the sense that they exhibit quasi-exclusive selectivity for a protein target and that the interruption of treatment leads to reversal of their effects. Antibodies offer in addition the possibility of target interactions, especially large protein–protein interactions, that are difficult to perturb with small molecules. This has led to the exponential development of antibodies or antibody fragments (Chames et al., 2009; Nelson & Reichert, 2009; Wesolowski et al., 2009) for therapeutic purposes. Antibodies are powerful tools in laboratory research because they can be developed much faster than small chemical molecules (see below). They have thus been largely used to validate the involvement of proteins in signal transduction pathways, and as potential target for drug development. On the other hand, antibodies have intrinsic limitations that constrain their use for biological systems exploration. With some exceptions, antibodies and antibody fragments do not cross biological barriers, such as the intestinal or blood brain barriers. The consequence is that antibodies must be injected and most central nervous system proteins will not be reached. Also, antibodies cannot reach intracellular target proteins unless they cycle to the plasma membrane.

For all these reasons, the chemical biology approach using small molecules as tools or drugs remains a useful and valid strategy. In this article, we review examples of small chemical molecules that can be used to neutralize small signalling proteins such as chemokines or cytokines. The reader should appreciate that only a few examples are known to date. The reason for this is that all neutralizing molecule discoveries that are presented here were serendipitous, and specifically designed experimental approaches are only just entering starting blocks. Small molecules are being searched to inhibit protein–protein interactions, with a focus on intracellular compartments and cancer related interactions, or brain function exploration (Berg, 2003; Arkin & Wells, 2004; Arkin, 2005; Wells & McClendon, 2007; Blazer & Neubig, 2009). These will not be reviewed here. We will focus mainly on the family of small signalling proteins, the chemokines, which constitute a well adapted biological system to develop neutralizing small molecules. Examples from other cytokines will be discussed as well.

1.1. Chemokines and chemokine receptors

Chemokines are small secreted chemotactic cytokines endowed with multiple activities. Their main function is chemical attraction of leukocytes, but they also contribute to the regulation of organ development during ontogeny. In inflammation, the chemotactic signal given by chemokines leads to egress of leukocytes from the blood circulation across the walls of small blood vessels. To do this, chemokines that are produced on the site of inflammation cross the endothelial cell wall and remain immobilized on the luminal surface of the endothelium. Circulating leukocytes, depending on their chemokine receptor expression will then be attracted and directed towards the inflamed site along the chemotactic gradient. Chemokines, in addition to attracting cells, contribute to the regulation of gene expression on target cells and help to control cell proliferation and apoptosis, for instance in angiogenesis.

Chemokines are also subdivided into several functional groups depending on whether their expression is constitutive or inducible by inflammatory signals, and also on their capacity to stimulate or inhibit angiogenesis, especially in tumors (Vandercappellen et al., 2008). The CXC chemokines in particular exert angiogenic or angiostatic activities depending on the presence of an ELR (Glu-Leu-Arg) motif in their N-terminal portion (Addison et al., 2000). As important regulators of cell migration, therapeutic intervention of the chemokine system(s) includes infectious diseases, intra-organism alert systems possibly leading to autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis or lupus erythematosus, as well as allergic disorders such as asthma, inflammatory bowel disease, transplant rejection, neuropathies or dermatitis.

More than 50 chemokines are known (Wells et al., 2006). The chemokine structure (Figs. 1 and 3) comprises an N-terminal loop region, three-strand anti-parallel beta-sheets forming the typical core fold of the chemokines and a C-terminal alpha helix which overlays the beta-sheet. CC, CXC and CX3C chemokines comprise in addition two disulfide bridges linking the N-terminal domain with the loop separating sheet 1 and sheet 2 and the N-terminal domain with the end of sheet 3. In order to allow gradients to be formed at the vicinity of the site of release, chemokines bind to extracellular matrix components, i.e. the negatively charged glycosaminoglycans (GAGs), by means of their positively charged amino acids. These positive amino acids form distinct clusters at the surface of the chemokine depending on whether the chemokine belongs to the CC, CXC or CX3C group (Laguri et al., 2008). In the CXC chemokine group, the GAG-binding area is on the side of the protein that does not interact with the receptor (Amara et al., 1999; Santiago et al., 2006; Murphy et al., 2007) and, mutation of the positive amino acids that bind to GAGs does not alter chemokine binding to the receptor (Amara et al., 1999; Proudfoot et al., 2001), and interaction with heparan sulfates does not change the equilibrium binding affinity of the chemokine for its receptor (Valenzuela-Fernandez et al., 2001). In the CC group of chemokines, in contrast, there is significant overlap between receptor binding and GAG-binding areas which, in the case of CCL5 for instance, has influence on receptor subtype-specific interactions (Proudfoot et al., 2001).

Chemokines signal through G proteins coupled to seven transmembrane receptors which are classified according to the chemokines they bind (CXCR, CCR, CX3CR and XCR) (Murphy, 2002). The chemokine receptor family groups twenty G-protein-coupled receptors (GPCRs) and covers extremely diverse physiological responses. As a general rule, structural promiscuity between GPCRs accounts for frequently observed problems of ligand selectivity among subtypes. Reciprocally, GPCR ligands, in particular chemokines, are grouped in small chemical families, so that neutralizing the ligand rather than the receptor may allow good focus on a subset of targeted signalling pathways.

Along with several other signalling proteins (Alcami & Smith, 1992; Colotta et al., 1993; Pitti et al., 1998; Rahaman et al., 2002; Bezerra et al., 2005; Bamias et al., 2008; de Moura et al., 2009; Fili et al., 2009; Funke et al., 2009; Mueller et al., 2009; Scola et al., 2009), chemokines are subject to natural modulation of their concentrations by proteins to which they bind (Fig. 2) without leading to typical signalling (Murphy, 2000; Alcami, 2003; Graham & McKimmie, 2006; Mantovani et al., 2006; Murphy et al., 2007; Graham, 2009; Pruenster et al., 2009). These proteins may be endogenously encoded to modulate chemokine functions or expressed by exogenous sources like pathogens or parasites with the aim of escaping the host immune system (see below). These naturally occurring “scavenger” or “decoy” proteins act as “interceptors” – i.e. intercepting receptors – that neutralize the action of the chemokine. We shall briefly review these systems because they validate the concepts of ligand neutralization, before considering approaches to unnatural neutralization.

2. Natural cytokine and chemokine neutralization

Besides metabolic regulation of hormone or peptide production such as enzymatic degradation, transport (Mortier et al., 2008), a captivating aspect of response regulation is scavenging of ligands by molecules that bind to it and modulate its biological function. This

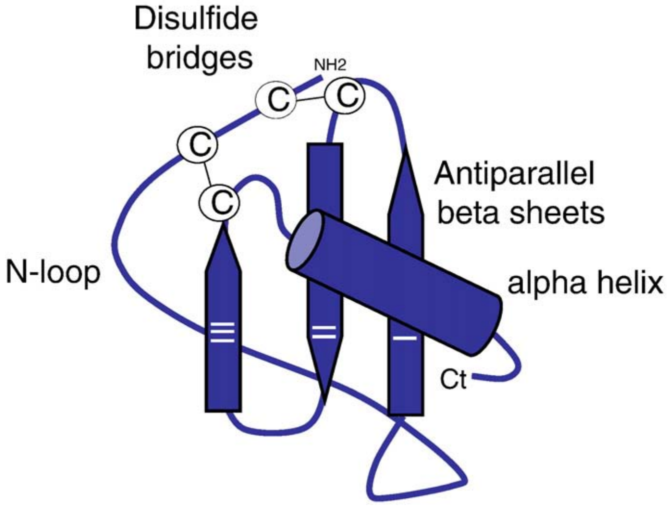

Fig. 1. Folding of chemokines: chemokine adopts a typical structure with 3 anti-parallel β-strands and one carboxy terminal helix. C–C denotes disulfide bridges.

has been illustrated in the past 25 years with the identification of endogenous receptor-like structures that do not lead to conventional signalling in response to small protein ligands but rather seem to contribute to their blockade or elimination (Colotta et al., 1993; Colotta et al., 1995; Bezerra et al., 2005; Mantovani et al., 2006; Mantovani et al., 2007; Thelen & Thelen, 2008; Mantovani et al., 2008; Bamias et al., 2008; Bonecchi et al., 2008b; de Moura et al., 2009; Mueller et al., 2009; Scola et al., 2009). These receptor-like molecules, which can be soluble (Colotta et al., 1993; de Moura et al., 2009; Funke et al., 2009) or membrane-bound (Mantovani et al., 2006; Scola et al., 2009), have been termed “decoy” or “scavenger” proteins. They however serve physiological as well as pathophysiological functions.

Decoy proteins for interleukins IL-1 (Colotta et al., 1993), IL-22 (de Moura et al., 2009), IL-13 (Caput et al., 1996; Rahaman et al., 2002), death ligands TRAIL (Bellail et al., 2009) and CD95L (Pitti et al., 1998), activators of NF-kB-RANK (Simonet et al., 1997; Khosla, 2001) or complement (Cain & Monk, 2002; Scola et al., 2009) generally exhibit ligand selectivity and/or specificity. Those for chemokines (Mantovani et al., 2006; Graham, 2009) display poor ligand selectivity.

2.1. Endogenous chemokine interceptors

There are three, possibly four, endogenous proteins that belong to the structural family of G-protein-coupled receptors, bind chemokines with limited to low selectivity, do not signal toward G-protein-dependent pathways but keep the capacity to internalize and transport the bound chemokine across the plasma membrane. These proteins, DARC, D6, CCX-CKR and possibly CXCR7, act as uptake or re-uptake proteins that trap the ligand, internalize it and direct it towards degradation, possibly also towards transcytosis. These proteins play important roles in inflammation, development, and chemokine-associated diseases such as cancer (Graham & McKimmie, 2006; Mantovani et al., 2006).

2.1.1. Duffy antigen receptor for chemokines

Duffy antigen receptor for chemokines, DARC, binds both CC (CCL-2, -5, -7, -11, and -13) and CXC (CXCL-1, -3, -5, -6, -8, and -11) inflammatory chemokines as well as the homeostatic chemokine CCL14. It is a G-protein-coupled receptor-like protein that lacks the capacity to stimulate G proteins.

DARC is expressed at high levels in the cell membrane of erythrocytes where it was shown to contribute to clearing circulating chemokines (Darbonne et al., 1991). Supporting this role in chemokine clearance, lack of DARC protein is associated with an exaggerated inflammatory response to lipopolysaccharide LPS (Dawson et al., 2000), while overexpression of the protein leads to diminished angiogenesis (Bonecchi et al., 2008a). Further supporting the importance of DARC in

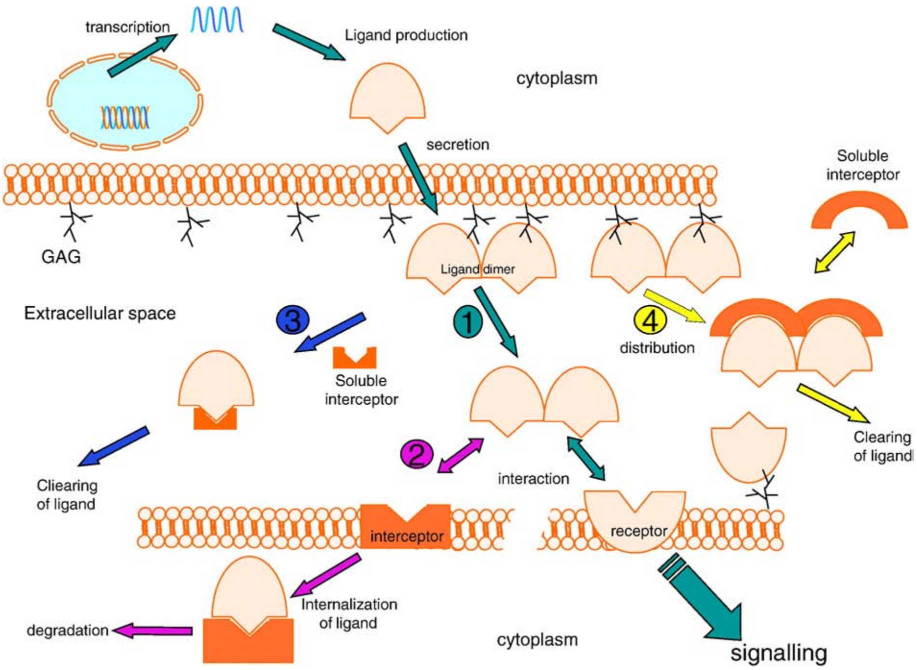

Fig. 2. Examples of different possible routes that can be followed by chemokines/cytokines in the presence of neutralizing macromolecules. Route 1 leads to signalling in the target cell expressing the chemokine/cytokine receptor. Route 2 is used either endogenously or by pathogens. Binding of chemokines takes place without signalling. This event may either lead to degradation of the chemokine or to its transcytosis. Route 3 is used by pathogens that express soluble proteins capable of binding chemokines, generally with moderate affinity and selectivity, and prevents them from normal signalling to the immune system. In Route 4, the neutralizing molecule prevents chemokine binding to glycosaminoglycans. The resulting effect is a collapse of the chemotactic gradient that abolishes leukocyte attraction in the inflamed tissue.

chemokine clearance, circulating CXCL1 is mostly associated with red blood cells in wild type mice, while it is found in the plasma in DARC$^{-/-}$ mice. Accordingly, in a mouse model of acute lung injury, LPS-induced polymorphonuclear leukocyte migration in the alveolar space is elevated two-fold in knock-out animals (Reutershan et al., 2009). The chemokine sequestering function of DARC is also clinically validated as preventing/reducing tumor cell growth, as was demonstrated in breast cancer (Wang et al., 2006b).

DARC is also expressed on endothelial cells, but under normal conditions, this expression is restricted to postcapillary venules (Fra et al., 2003). It may extend to other types of blood vessels (arteries, capillaries) during infection, inflammation or graft rejection (Segerer et al., 2000; Gardner et al., 2006). In endothelial cells, DARC protein serves as a transporter that allows chemokine transcytosis, thus leading to efficient exposure of tissue-derived inflammatory chemokines to the lumen of vessels and subsequent leukocyte recruitment and extravasation (Pruenster et al., 2009).

2.1.2. D6 binds and suppresses inflammatory chemokines

Like DARC, D6 exhibits poor selectivity towards chemokines. It binds 12 different chemokines out of which none belong to the CXC group and 8 are CC pro-inflammatory molecules, implicating D6 as a probable regulator of inflammation. Noteworthy also is the fact that DARC and D6 bind 7 identical chemokines (Bonecchi et al., 2008a). D6 is expressed in lymphatic endothelial cells from non-inflamed skin, gut and lung. Upon inflammation, D6 is expressed in leukocytes, especially in those that invade inflamed tissues (Graham & McKimmie, 2006). In contrast to DARC, D6 does not promote chemokine transcytosis, but rather contributes to their degradation by directing them towards endosomes. D6 is predominantly localized in recycling endosomes capable of trafficking to and from the cell surface in the absence of ligand. In the presence of ligand, D6 can rapidly internalize chemokines; however, D6-internalized chemokines are more effectively retained intracellularly because they more readily dissociate from the receptor during vesicle acidification. These chemokines are then degraded while the receptor recycles to the cell surface (Fra et al., 2003; Galliera et al., 2004; Weber et al., 2004). The most likely physiological role of D6 thus is to clear tissues from remaining chemokines in order to prevent an excessive response, and eventually terminate the inflammatory response (Graham & McKimmie, 2006). In support of this, the lack of D6 expression, in vivo, results in an amplified chemokine-mediated inflammatory response (Jamieson et al., 2005; Martinez de la Torre et al., 2005). D6$^{-/-}$ mice show high levels of inflammatory chemokines in the lymph nodes. By contrast, overexpression of D6 reduces leukocyte responses in inflammation models (Nibbs et al., 2007).

As a result of deregulated expression of chemokine receptors and chemokines in cancer, a role of D6 in carcinogenesis has been proposed (Nibbs et al., 2007; Wu et al., 2008). Due to its capacity to sequester chemokines, D6 protects from tumorigenesis in chemical treatment-evoked skin tumors (Nibbs et al., 2007). In another study, D6 has been reported to reduce intratumor levels of CCL2 and CCL5 chemokines, and consequently to inhibit proliferation and invasion of breast cancer cells in vitro as well as tumorigenesis and metastasis in vivo (Wu et al., 2008).

2.1.3. CCX-CKR binds and suppresses homeostatic chemokines

CCX-CKR is, like DARC and D6, derived from a G-protein-coupled structure, and is devoid of signalling capacity towards G proteins. It is expressed in various organs such as spleen, lymph nodes, heart, kidney, placenta, trachea and brain (Gosling et al., 2000), and in various cell types like T cells, immature dendritic cells, stromal cells, astrocytes (Dorf et al., 2000), ciliated bronchial epithelial cells in pulmonary sarcoidosis (Kriegova et al., 2006), and endothelial cells surrounding cancer cells in tumors (Feng et al., 2009).

CCX-CKR binds the homeostatic chemokines CCL19 and CCL21 which control trafficking of naive T cells, CCL25, and CXCL13 which mediate B cells and helper T cell migration (Gosling et al., 2000; Townson & Nibbs, 2002; Comerford et al., 2006), and directs them towards degradation. In vitro, cells expressing CCX-CKR deplete large quantities of these chemokines (Gosling et al., 2000; Townson & Nibbs, 2002; Comerford et al., 2006). Inflammation promoting signals, such as interleukin 1beta, tumor necrosis factor TNFα or interferon IFN gamma attenuate CCX-CKR mRNA levels, supporting a potential link of this interceptor with inflammation. In vivo, in particular in mice harbouring CCX-CKR transfected xenografts, reduced tumor growth, neovascularization and metastasis are detected (Feng et al., 2009). Also, a clinical study in breast cancer shows the natural level of CCX-CKR expression to correlate with longer survival of the patients (Feng et al., 2009).

2.1.4. Chemokine receptors as temporary interceptors: the debated case of CXCR7

The capacity of G-protein-coupled receptors to endocytose together with their ligands makes it likely that at least some of them behave as interceptors. The expression of CCR5, for example, is up regulated in T cells responding to anti-inflammatory lipids. Such modification represents a mechanism by which chemokines can be trapped and inflammation terminated (Ariel et al., 2006).

The second receptor for CXCL12 and for CXCL11, namely CXCR7, is another example of an atypical receptor. Indeed, although the receptor sequence contains the canonical DRY sequence required for coupling receptors to G proteins and, attempts to detect signalling through G proteins, activation of MAP-kinases or stimulation of PI3-kinase was unsuccessful to date (Balabanian et al., 2005; Burns et al., 2006; Dambly-Chaudiere et al., 2007) with the exception of one report on signalling through Akt (Wang et al., 2008) which may itself result from beta-arrestin recruitment (Kalatskaya et al., 2009; Luker et al., 2009a; Zabel et al., 2009). Whichever the way CXCR7 signals, it appears important during development, and in particular in heart valve formation (Sierro et al., 2007) and for stabilization of cell adhesion after migration towards CXCL12 gradients (Dambly-Chaudiere et al., 2007; Boldajipour et al., 2008). During development of the zebrafish sensory system, formation of the sensory organ, the lateral line, requires long distance migration of primordial germ cells. Dambly-Chaudiere et al. (2007) and Boldajipour et al. (2008) showed that this migration of germ cells involves a chemokinetic response to CXCL12, mediated by CXCR4, that leads to the migration of germ cells. Directionality of the migration is provided by trailing cells that express the second CXCL12 receptor, CXCR7, which prevents backward migration by depleting CXCL12, in the rear of the migrating group of cells. Consistent with this, Mazzinghi et al. reported that human renal progenitor cells use both CXCR4 and CXCR7 receptors for transendothelial migration, but that CXCR7 is a major contributor for cell adhesion to endothelial cells and progenitor cell survival (Mazzinghi et al., 2008). CXCR7-mediated or -enhanced adhesiveness is also clearly established in prostate cancer cells, together with improved cell survival and invasiveness (Wang et al., 2008). In the cases reported above, the contribution of CXCR7 to the physiological responses could be chemokine interception and termination of the subsequent migratory response or at least its modulation. CXCR7 indeed shows significantly higher capacity than CXCR4 to increase cell-association of CXCL12 (Luker et al., 2009b).

Another probable physiological function of CXCR7 is related to its capacity to heterodimerize (Sierro et al., 2007; Levoye et al., 2009), as can be detected by bioluminescence- or fluorescence-energy transfer in heterologous expression systems. In the more recent work by Levoye et al. (2009), CXCR7 is shown to exhibit an apparent paradoxical effect interfering with CXCR4 responses to CXCL12. CXCR7 indeed reduces responses to low CXCL12 concentrations while leaving responses to high concentrations unchanged, as compared to CXCR4 alone. This effect is also detected in isolated human T lymphocytes. The presence of CXCR7 in cells expressing CXCR4 is to render dose–response relationships steeper than expected from the law of mass action. The net result of that interference is a conversion of CXCR4 responses to CXCL12 into an almost all or nothing type of response (Sierro et al., 2007; Levoye et al., 2009) with triggering versus non-triggering CXCL12 doses differing by only three- to five-fold. This physiological effect supports the concept open by the structural demonstrations of CXCR7 heterodimerization. Modelling of the behavior of CXCR7 as a chemokine scavenger could be of interest to discriminate among direct receptor–receptor interactions and the indirect effects of CXCR7 regulating the level of chemokine that would be available to CXCR4.

2.2. Avoidance strategies: examples of neutralizing molecules produced by pathogens

Pathogenic viruses, bacteria or parasites, have set up several strategies to escape host detection and defence systems. They use cytokine or chemokine signalling molecules (receptors and ligands) to infect host cells (Chitnis & Sharma, 2008; Hughes & Nelson, 2009). They also block cytokine signalling by producing antagonists (Damon et al., 1998) which allow to escape alert systems or to redirect them to their own benefit (McFadden et al., 1998; Sozzani et al., 1998; Alcami, 2003; Mantovani et al., 2006; Rosenkilde, 2005; Andreasen & Carbonetti, 2008). The parasite *Leishmania* infecting macrophages, for example, express functional chemokine receptors. These are used as chemoreceptors to promote chemokinesis toward chemokine-producing macrophages. This leads to an efficient *Leishmania* internalization (Roychoudhury et al., 2006) that takes place before an efficacious immune response is set up to clear the pathogen. A second example is taken from the bacterium *Bordetella pertussis*. The pathogen is reported to delay neutrophil recruitment, by slowing down chemokine production by the host (Andreasen & Carbonetti, 2008), through production of *pertussis* toxin the well known inhibitor of Gi protein mediated signalling. Also, as a third illustration, human herpesvirus 6 (HHV-6) produces a chemokine, U83A, that binds to CCR5 to modify its internalization-recycling fate (Catusse et al., 2007; Catusse et al., 2009). Indeed, at variance to other CCR5 chemokines, U83A is a CCR5 agonist that does not drive the receptor towards a clathrin-mediated endocytosis but to a delayed and long lasting caveolin-linked pathway. Combined to the fact that U83A is not recognized by DARC and D6, the viral chemokine thus facilitates clearance of all other CCR5 chemokines which can no longer activate the receptor but remain capable of being trapped by interceptors.

Relevant to the present article are the neutralizing molecules produced by viruses and multicellular pathogens that are used to neutralize the immune response of the host. Several articles and reviews describe the production by viruses, of soluble proteins able to bind chemokines sometimes simultaneously with cytokines such as interferon gamma, interleukine-1β or tumor necrosis factor α (McFadden et al., 1998; Murphy, 2000; Alcami, 2003; Rosenkilde, 2005; Mantovani et al., 2006), all of which are implicated in the host immune response to pathogens. Two major mechanisms of action are depicted: the inhibition of cytokine–cytokine receptor interaction and the inhibition of cytokine–extracellular matrix interaction (McFadden et al., 1998), both of which being associated with improvement of cytokine clearance by elimination and/or degradation. The biologically active scavenging molecule can be a soluble protein, often mimicking the extracellular binding domain of the host cytokine receptor. It may also be a membrane-bound protein, like the decoy receptors mimicking the chemokine receptors which do not contain any soluble portions.

Representatives of soluble proteins that inhibit the interaction between chemokines and glycosaminoglycans (GAGs) from the extracellular matrix are M-T1 and M-T7 produced by the rabbit-infecting *myxoma* virus. M-T7 binds interferon gamma together with chemokines from the CC-, CXC- and C-groups (Lalani et al., 1997). The herpesvirus homodimeric protein M3 and the glycoprotein G also belong to the group of soluble proteins inhibiting chemokine binding to GAGs (van Berkel et al., 2000; Martin et al., 2006). All exhibit original tridimensional structures that do not resemble chemokine receptors. The mechanisms by which they neutralize the immune system may be two-fold. On the one hand, GAGs are well known to contribute to the setting up and maintenance of chemokine gradients close to their sites of production. The inhibition of chemokines binding to GAGs might thus result in chemokine gradient collapses. Altered immune response that could derive from that could be attenuation of signalling intensity or unsuited, or even absence of, leukocyte targeting (Wells et al., 2006). On the other hand, the large size of soluble chemokine binding proteins could hinder the interaction with the chemokine receptor. Thus, although the targeted domain of the chemokine is the GAG-binding domain, the remainder of the large soluble protein might simultaneously prevent interactions with the cognate chemokine receptors. The use of small molecules mimicking the effects of GAG-binding proteins would help to determine the mechanism of action likely to take place.

Another example of soluble proteins produced by parasites is highlighted by recent research developments. Ticks are bloodsucking parasites that transmit the spirochete *Borrelia burgdorferi* responsible for Lyme disease (Hirschfeld et al., 1999; Hajnicka et al., 2001; Guerau-de-Arellano & Huber, 2005; Behera et al., 2006; Vancova et al., 2007; Deruaz et al., 2008). In order to survive, ticks attach and remain feeding on the host for several days–weeks. A particularity of the host–parasite interaction is the absence of an inflammatory response to ticks. This was investigated by several groups who realized that the parasite produces anti-haemostatic, anti-inflammatory and immunomodulatory substances, and secretes them in the host (Waxman et al., 1990; Valenzuela et al., 2000). Anti-chemokine molecules acting against CXCL8 (Hajnicka et al., 2001), CCL2, CCL3, CCL5 and CXCL11 (Vancova et al., 2007) were detected although their identity was not elucidated. Using an expression cloning strategy, the group of Proudfoot identified a family of small proteins, the evasins, that similar to soluble viral chemokine binding proteins, recognize and bind chemokines with various degrees of selectivity, and intercept their signalling to the host immune and anti-inflammatory systems (Frauenschuh et al., 2007; Deruaz et al., 2008). Three identified evasins bind CC (evasins-1 and -4) and CXC (evasin-3) chemokines. The fourth one, evasin-2, is still without a known ligand. The interest in these small proteins resides in their extreme efficacy to delude the immune system, and to their very small size (60–70 amino acids) that inspires searches for chemokine neutralizing motifs with the potential to become drugs.

The second general mode of action of pathogens is reminiscent of intercepting receptors described above. Human and mouse cytomegalovirus, Kaposi-associated herpesvirus and capripoxvirus produce seven transmembrane segment proteins (ORF74, US28, M33, and Q2/3L) which are analogous to G-protein-coupled receptors (Alcami, 2003; Rosenkilde, 2005). These proteins are expressed at the surface of infected cells and act, similarly to DARC or CCX-CKR, as decoy proteins that internalize chemokines and drive them towards degradation.

3. Potential therapeutic interest of soluble decoy proteins

Soon after the discovery of 50 different chemokines, the number of receptors grew to 20 members, all belonging to the G-protein-coupled receptor family for which it should be noted that one given chemokine may activate several receptor subtypes. The chemokine CXCL8 for instance activates two receptors (CXCR1 and CXCR2) and the chemokine CCL5 activates three receptors (CCR1, CCR3, and CCR5). On the other hand, a large number of chemokines may activate a single receptor subtype. This is the case for CXCR2, which is activated by CXCL-1, -2, -3, -5, -6, -7 and -8, for CCR5 that is activated by CCL-3, -4,

Table 1

Chemokine/chemokine receptor	Biological tool	Effect	Reference
CCL1 (I-309) CCR8	Anti-CCL1	Post-operational peritoneal adhesions	Hoshino et al., 2007
CCL2 (MCP-1) CCR2	Anti-CCL2	– Prostate cancer growth inhibition	Loberg et al., 2007; Li et al., 2009a
		– Infectious keratitis	Xue et al., 2007
		– Atherosclerosis	Lutgens et al., 2005
	CCR2 knock out	– Atherosclerosis/multiple sclerosis	Boring et al., 1998; Izikson et al., 2000
	CCL2 knock out	– Age-related macular degeneration/neuroinflammation	Belmadani et al., 2006; Ross et al., 2008
		– Sepsis	Lu et al., 1998
		– Atherosclerosis	Gu et al., 1998
CCL3 (MIP-1a) CCR1/CCR3/ CCR5	Anti-CCL3	– Infectious keratitis	Xue et al., 2007
		– Fever	Soares et al., 2009
		– Sepsis	Takahashi et al., 2002
		– Inflammation in MS	Man et al., 2007
	CCL3$^{-/-}$	– Sepsis	Cook et al., 1995
CCL4	Anti-CCL4	– Lung inflammatory response	Bless et al., 2000
CCL5 (RANTES) CCR5/CCR1/ CCR3	Anti-CCL5	– Autocrine proliferation of Hodgkin lymphoma cell lines	Boring et al., 1998; Izikson et al., 2000; Aldinucci et al., 2008; Levina et al., 2008
	CCL5$^{-/-}$	– Demyelination in MS	
		– Glial activation	Glass et al., 2004
			El-Hage et al., 2008
CCL6 (C10) CCR1	Anti-CCL6	– Lung inflammation and remodeling	Ma et al., 2004
		– Airway allergy and hyperesponsiveness	Hogaboam et al., 1999
		– Phagocytic activity of macrophages	Steinhauser et al., 2000
CCL7 (MCP-3) CCR2	Anti-CCL7	– Airway allergy and hypereosinophilia	Stafford et al., 1997
CCL8 (MCP-2) CCR2/CCR5			
CCL9 (MIP-1g) CCR1	Anti-CCL9	– Osteoclast differentiation	Yang et al., 2006
CCL11 (Eotaxin) CCR3	Anti-CCL11	– Airway allergy/asthma	Ding et al., 2004; Niimi et al., 2007
		– Bronchiolitis	Matthews et al., 2005
	Eotaxin$^{-/-}$	– Acute inflammatory response	Rothenberg et al., 1997
CCL12 (MCP-5) CCR2/CCR5			
CCL13 (MCP-4) CCR2			
CCL14 (HCC-1) CCR1			
CCL15 (HCC-2) CCR1/CCR3			
CCL16 (HCC-4) CCR1/CCR3			
CCL17 (TARC) CCR4	Anti-TARC	– Hypereosinophilia/allergic asthma	de Lavareille et al., 2001; Schnyder-Candrian et al., 2006
		– Pulmonary infections/fibrosis	Belperio et al., 2004; Carpenter and Hogaboam, 2005
		– Lung cancer (?)	Qin et al., 2009
		– Hepatic failure	Yoneyama et al., 1998
		– Skin inflammation	Campbell et al., 1999
CCL18 (PARC) CCR3 (?)	Anti-CCL18	– Rheumatoid arthritis	van der Voort et al., 2005
CCL19 (ELC) CCR7			
CCL20 (MIP-3 alpha) CCR6	Anti-CCL20/anti-CCR6	– Multiple myeloma	Giuliani et al., 2008
	Anti-CCL20	– HPV infection/Langerhans cells migration	Caberg et al., 2009
		– Brain inflammation (MS/EAE)	Ambrosini et al., 2003
CCL21 (SLC) CCR7	Anti-CCL21	– Kidney fibrosis	Sakai et al., 2006; Wada et al., 2007
		– Corneal immunity	Jin et al., 2007b
	CCL21$^{-/-}$ mice	– Thymus development	Liu et al., 2005
CCL22 (MDC) CCR4	Anti-CCL22	– Leukemia cell survival and proliferation	Ghia et al., 2002
		– Eosinophil activation in lung inflammation	Pinho et al., 2003
		– Lung cancer	Qin et al., 2009
CCL23 (MPIF-1) CCR3		– Vascular endothelial cell migration	Son et al., 2006
CCL24 (Eotaxin-2) CCR3		– HIV pathogenicity	Fiorucci et al., 2007
CCL25 (TECK) CCR9	Anti-CCL25	– Intestinal immunity	Feng et al., 2006; Hieshima et al., 2008
CCL26 (Eotaxin-3) CCR3			Cuvelier and Patel, 2001
CCL27 (CTACK) CCR10	Anti-CCL27	Dermatitis/skin disease	Morales et al., 1999; Reiss et al., 2001; Chen et al., 2006
CCL28 (MEC) CCR10	Anti-CCL28	– Intestine and colon immunity	Feng et al., 2006; Hieshima et al., 2008
CXCL1 (Gro alpha) CXCR2	Anti-CXCL1	– Arthritis	Grespan et al., 2008; Lemos et al., 2009
		– Kidney sepsis	Brown et al., 2007
		– Airway inflammation	Issa et al., 2006
CXCL2 (Gro-beta) CXCR2	Anti-CXCL2	– Kidney sepsis	Brown et al., 2007
CXCL3 (Gro gamma) CXCR2			
CXCL4 (PF4) CXCR3b			
CXCL5 (ENA-78) CXCR2	Anti-CXCL5	– Arthritis	Grespan et al., 2008; Smith et al., 2008; Lemos et al., 2009
		– Diabetes	Chavey et al., 2009
		– NSCLC growth/angiogenesis	Pold et al., 2004
CXCL6 (GCP-2)	Anti-GCP-2	– Growth SCLC	Zhu et al., 2006
		– Arthritis	Kelchtermans et al., 2007
CXCL7 (NAP-2)	Anti-NAP-2	– Thrombosis	Amiral et al., 1996; Piccardoni et al., 1996
CXCL8 (IL8) CXCR1/CXCR2	Anti-CXCL8	– Inhibition of NSCLC growth/angiogenesis	Pold et al., 2004
	Anti-CXCR1	– Inhibition of NSCLC proliferation	Zhu et al., 2004
	Anti-CXCL8	– Clearance of apoptotic cells	Iyoda et al., 2005
CXCL9 (Mig) CXCR3	Anti-CXCL9	– Brain immunity and MS	Liu et al., 2001a; Salmaggi et al., 2002
		– Transplant rejection	Belperio et al., 2003; Whiting et al., 2004; Colvin et al., 2005

Table 1 (*continued*)

Chemokine/chemokine receptor	Biological tool	Effect	Reference
CXCL10 (IP-10) CXCR3	Anti-CXCL10	– Axon sprouting and vasculature remodelling following injury	Glaser et al., 2004; Glaser et al., 2006
		– Inflammatory demyelination in MS	Liu et al., 2001; Narumi et al., 2002
		– Coronavirus-induced neurological and liver damage	Walsh et al., 2007
		– Transplant rejection	Belperio et al., 2002
CXCL11 (I-TAC) CXCR3	Anti-CXCL11	– Brain immunity	Rupprecht et al., 2005
CXCL12 (SDF-1alpha) CXCR4	Anti-CXCL12	– Autoimmune disease/lupus erythematosus	Matin et al., 2002; Balabanian et al., 2003; Wang et al., 2009
		– Metastases/tumor proliferation	Muller et al., 2001; Cardones et al., 2003; Orimo et al., 2005; Phillips et al., 2003; Pan et al., 2006; Otsuka and Bebb, 2008
		– Pulmonary hypertension/airway inflammation	Gonzalo et al., 2000; Hachet-Haas et al., 2008; Lukacs et al., 2002; Young et al., 2009
	Anti-CXCR4	– Tumor invasion	Bertolini et al., 2002; Hinton et al., 2008; Li et al., 2009b
		– NSCLC proliferation	
		– Airway inflammation	Otsuka and Bebb, 2008
	CXCL12$^{-/-}$	– Development	Nagasawa et al., 1996
CXCL13 (BCA-1) CXCR5	Anti-CXCL13	– Autoimmunity/myasthenia gravis	Meraouna et al., 2006
		– Arthritis	Zheng et al., 2005
		– Graft rejection	Lee et al., 2006
CXCL14 (BRAK, BMAC)			
CXCL15 (Lungkine)	CXCL15 knock out	– Sepsis	Chen et al., 2001
CXCL16 CXCR6	Anti-CXCL16	– Kidney inflammation	Yang et al., 2008
		– Sepsis	Shimaoka et al., 2003; Xu et al., 2005
		– Arthritis	
		– Graft tolerance	Nanki et al., 2005
			Jiang et al., 2005
	CXCL16$^{-/-}$	– Atherosclerosis	Aslanian and Charo, 2006
CX3CL1 (fractalkine) CX3CR1	Anti-CX3CL1	– Graft tolerance	Ueha et al., 2007
		– Autoimmune disease	Suzuki et al., 2005
		– Atherosclerosis	Schulz et al., 2007
	XC3CL1 knock out	– No phenotype	Cook et al., 2001
XCL1 (lymphotactin) XCR1	Overexpression	– Cancer immunotherapy	Wang et al., 2002
		– Anti-infection immunotherapy	Yue et al., 2009

NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; MS: multiple sclerosis; EAE: experimental autoimmune encephalomyelitis.

-5, -6, -8, -12, as well as for many other receptors (CXCR3, CCR1, CCR2, CCR3 ….) (reviewed in Wells et al., 2006). The question then arose as to which receptor and which chemokine should be targeted to decipher physiological signalling pathways and predict therapeutic approaches for disease treatment. Many research groups could help solve this problem by showing that despite chemokines and cytokines' cooperation to increase inflammatory responses, knock out or neutralization of one chemokine or chemokine receptor will induce significant attenuation of inflammation (see Table 1). Several chemokine gene disruptions result in a clear effect, as for instance knock out of CCL3 that reduces the inflammatory response to viruses such as influenza A and cytomegalovirus (Salazar-Mather et al., 1998). Similarly, the knock-out approach indicates the importance of CCL2 and its receptor in chemoattraction of neural progenitors to inflamed neural sites (Belmadani et al., 2006), that of CCL5 in glial cell activation (El-Hage et al., 2008), of CCL21 in thymus development (Liu et al., 2005) or that of CXCL12 in haematopoiesis (Nagasawa et al., 1996). Very convincing results are also obtained by using proteins or antibodies neutralizing the chemokine ligand. The Lucas and Mc Fadden groups have exploited the neutralizing effect of the *myxoma* virus M-T7 soluble protein to reduce post-operative responses in murine models of tissue engrafting (Liu et al., 2000, 2004; Bedard et al., 2003). They show that intravenous injection of M-T7 protein, that binds all types of chemokines (see Section 2.2) in rats after angioplasty-induced injury diminishes atherosclerosis and restenosis (Liu et al., 2000). This is in good agreement with the phenotype of CCL2$^{-/-}$ mice (Gosling et al., 1999), and with the reported prevention of renal allograft rejection (Bedard et al., 2003) or reduction of aortic allograft vasculopathy through inhibition of chemokine-mediated responses (Liu et al., 2004).

Finally, the newly identified evasin proteins from ticks also display potent anti-inflammatory properties in vivo in animal models (Deruaz et al., 2008). Evasin-1, which binds CCL3 and CCL4, significantly attenuates recruitment of pro-inflammatory cells in phorbol ester-inflamed skin of D6$^{-/-}$ mice and fibrosis in bleomycin-induced lung injury. Evasin-3 recognizes CXCL8 and its mouse homolog KC, as well as CXCL11. It inhibits neutrophil chemotaxis in vitro, as well as neutrophil recruitment to the peritoneal cavity in mice in a model of BSA-induced arthritis.

4. Validation of chemokines in signalling pathways and pathology: importance of anti-chemokine antibodies

Chemokines are directly implicated in many physiological processes including surveillance of organism integrity, elimination of damaged cells and tissues or host defence against pathogens. To this end, they recruit the most adapted cell types on the site where intervention is needed, and promote a controlled reaction generally associated with limited inflammation. Under abnormal conditions, the inflammatory response escapes control, thus leading to pathological states such as inflammatory bowel disease, multiple sclerosis, and probably Alzheimer's disease, among others. In this case, abnormally elevated levels of chemokines, or overexpression of their receptors, lead to permanent recruitment of immune cells and to tissue damage. In a comparable manner, abnormal recognition of antigen initiates autoimmune diseases (myasthenia gravis, lupus erythematosus, Type I diabetes, rheumatoid arthritis…), where abnormally elevated levels of chemokines are detected (Matin et al., 2002; Kong et al., 2009; Wang et al., 2009). The cytokine and chemokine systems are also used by cancer cells to promote cell proliferation, tumor survival and neovascularization, or to establish metastases at distant but non-random places (Vandercappellen et al., 2008). Chemokines also contribute to tissue development (Nagasawa et al., 1996; Mahabaleshwar et al., 2008; Raz & Mahabaleshwar, 2009) by forming gradients of morphogens for migrating cells.

Table 1 summarizes all efforts made to investigate the role of the various chemokines, using approaches targeting the chemokine as directly as possible. The two major approaches, namely gene disruption and anti-chemokine antibodies, do generally lead to convergent observations, although the same phenomenon has only rarely been studied using the two approaches.

Deletion of one of the CCL3 and CCL5 receptors, the CCR5 receptor (CCR5 Δ32 allele found in humans) is associated with protection from HIV infection in humans (Samson et al., 1996; Kindberg et al., 2008; Lim et al., 2008) while deletion of a second receptor, the CCR1 receptor, results in protection from an excessive response to systemic inflammation in mouse models (Gerard et al., 1997). Deletion of the CCR1 and CCR5 ligand, CCL3 (MIP-1alpha), results in weaker inflammatory responses to viral pathogens (Cook et al., 1995), and deletion of CCL5 (RANTES) to a reduced glial cell inflammatory response (El-Hage et al., 2008). Therefore, the absence of perfect matching between chemokines and their receptors is a cause of difficulties encountered when signalling pathways are to be traced, and molecules targeting the function of one receptor do not systematically match the effects of molecules targeting the ligand (Horuk, 2009).

Rather than knocking out chemokine or chemokine receptor genes, neutralizing antibodies, which are rapidly obtained, have been very useful in particular for chemokines. Chemokines are small proteins with a highly stable structure (Fig. 3), which renders them amenable to the development of neutralizing antibodies (Table 1).

In the case of CCL5 for instance, neutralizing antibodies allow the functional role of this chemokine in autocrine proliferation of leukemia cells (Boring et al., 1998; Izikson et al., 2000; Aldinucci et al., 2008) to be demonstrated as well as neuroinflammation in models of multiple sclerosis (Glass et al., 2004). It is interesting to note that although CCL5 binds to the same subset of chemokine receptors as CCL3, the anti-CCL5 neutralizing effect is specific because CCL3 is expressed in other cell types.

Anti-CCL1 antibodies have been used to demonstrate the contribution of CCL1/CCR8 autocrine activation of peritoneal macrophages in the formation of peritoneal adhesion, which constitutes complications in visceral surgery and inflammation (Hoshino et al., 2007).

Also antibodies to CXCL8, CCL2 or CCL5 block the antiapoptotic and proliferative effects of the corresponding tumor-derived cell lines obtained from lung, melanoma, breast ovarian or leukemia cancers (Levina et al., 2008).

Besides their functions in the immune system, the role of chemokines in cancer initiation and progression as well as in tumor survival has been confirmed with neutralizing antibody strategies. Hence, antibodies against chemokines or chemokine receptors block tumor growth and/or migration as well as invasiveness. Various examples may be given, like i) anti-CXCL12/CXCR4 antibodies in ovarian and breast cancer (Muller et al., 2001; Scotton et al., 2002; Kwong et al., 2009), ii) anti-CXCL1 and anti-CXCL2/CXCR2 in lung cancer (Wang et al., 2006a), iii) anti-CXCL13/CXCR5 in cell lines from pancreatic or colon cancers (Meijer et al., 2006), iv) anti-CCL2/CCR2 and -CCL5/CCR5 (Vaday et al., 2006), and -CCL11/CCR2 in ovarian cancer (Levina et al., 2009), and v) anti-CCL21/CCR7 in thyroid tumor cells (Sancho et al., 2006).

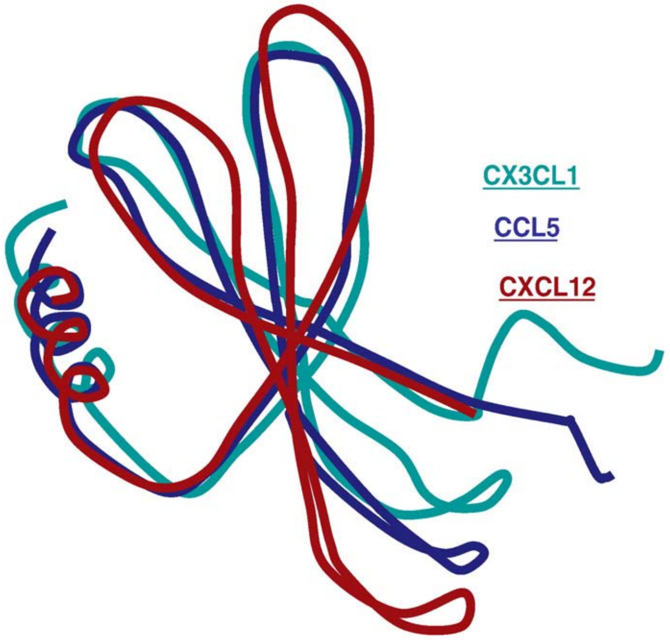

Fig. 3. Superimposition of peptide backbones from CC, CXC and CX3C chemokine groups shows that they have a canonical three dimensional structure.

5. Neutralizing cytokines and chemokines with small chemical compounds

The study of protein–protein interactions is important to understand major regulatory pathways, especially in the intracellular compartment, which is not reached by neutralizing antibodies. The difficulty associated with the study of protein–protein interactions is not only that most of the time, interacting partners are both intracellular, and thus not easily accessible for biophysical or pharmacological manipulations, but also that protein–protein interactions generally involve contact areas that are much larger than small molecules. These contact areas, in addition, are quite featureless in terms of the number of attachment points that can be exploited by medicinal chemists to develop small molecules with high affinity. It follows that small molecules at most bind with modest affinities and frequently hardly compete efficiently to inhibit the interaction between two proteins. Still, favourable cases exist in which neutralization of a protein function can be obtained with a small molecule (Arkin & Wells, 2004; Arkin, 2005; Arkin & Moasser, 2008; Blazer & Neubig, 2009). When the approach works, questions related to the mode of action of the small molecule must be addressed, in order to generalize the principles and extend the approach to other specific cases. The different mechanisms of ligand neutralization are numerous and diverse. In terms of chemical biology, the aim thus being to develop small chemical molecules blocking the function of the protein ligand, we shall not discuss molecules that inhibit synthesis, maturation or release of the protein ligand, nor molecules that modulate its catabolism, already reviewed elsewhere (Foxwell et al., 2003; Vergote et al., 2006; Mortier et al., 2008). Rather we will focus on small organic molecules that bind to the protein ligand and prevent its signalling.

Four main modes of action are encountered (Fig. 4):

i) The small molecule competitively binds to the same site as the receptor;
ii) The small molecule alters the quaternary structure of the protein ligand;
iii) The protein ligand undergoes structural changes that regulate its activity: the small molecule alters tertiary structure of the protein ligand;
iv) The small molecule interferes with ligand bioavailability.

These different modes of chemokine/cytokine neutralization will now be illustrated, and the methods to identify them discussed. These mechanisms of action have been validated, (Berg, 2003; Arkin & Wells, 2004; Arkin, 2005; Blazer & Neubig, 2009) and may be extended to chemokine neutralization.

5.1. The small molecule competitively binds to the same site as the receptor

Based upon structure–function relationship studies, many examples of peptides mimicking receptor domains and acting as inhibitors are available. In the family of chemokines and their receptors, the importance of the extracellular parts of the chemokine receptor (Zoffmann et al., 2002; Duma et al., 2007), and in particular of its amino-terminal domain, for ligand–receptor interactions has been extensively documented (Blanpain et al., 1999; Gayle et al., 1993;

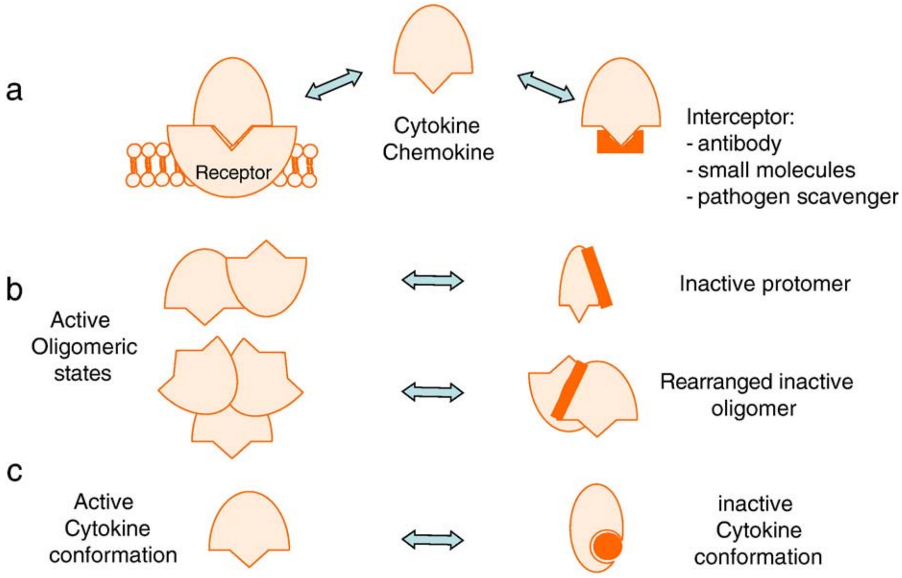

Fig. 4. Illustration of the different modes of action of neutralizing molecules. **a**) The small molecule competitively binds to the same site as the receptor. **b**) The small chemical molecule alters the quaternary structure of the protein ligand. **c**) The protein ligand undergoes structural changes that regulate its activity: the small molecule alters tertiary structure of the protein ligand.

Monteclaro & Charo, 1996, 1997; Pease et al., 1998; Ye et al., 2000; Bannert et al., 2001; Fong et al., 2002; Rajagopalan & Rajarathnam, 2004; Prado et al., 2007; Veldkamp et al., 2008). This has led to the identification of peptide fragments capable of binding to the chemokine in a manner thought to mimic the mode of interaction of the chemokine with the receptor. Peptide fragments corresponding to the 1–35 N-terminal residues from CCR3 (Mayer & Stone, 2000; Ye et al., 2000), 1–40 from CXCR1 (Clubb et al., 1994) or 2–19 from CX3CR1 (Mizoue et al., 1999; Kokkoli et al., 2005) interact with CCL24, CXCL8 and CX3CL1, respectively, with millimolar to micromolar affinities. In all three examples, the interaction area of the chemokine is located in the helical turn of the N-loop and the β1–β2 and β2–β3 hairpin domains, in regions of greatest flexibility and structural variability of the chemokine (Mizoue et al., 1999). These peptides have not been further used to investigate the in vitro or in vivo functions of chemokines. Their discovery however suggested that neutralizing antibodies-based approaches (Table 1) are not the only possible tools with which to inhibit chemokine functions, and paved the way to find high affinity peptides capable of neutralizing chemokines. One approach starts from natural peptides targeting chemokines as illustrated by the work on evasins (Deruaz et al., 2008) described above. Although peptidic in nature, evasins are not immunogenic, at least when secreted by feeding ticks. They are thus expected to represent valuable scaffolds to study, to make analogs and to use as non-peptidic drugs. Another approach consists in identifying new chemokine binding molecules from collections of peptides and peptidomimetics (Burger & Peled, 2009). Such peptides or peptidomimetics may either exhibit selectivity towards a single chemokine or, in contrast, poorly discriminate among chemokines such as CXCL9, CCL2, CXCL8, CXCL12 or CCL11 (Peled, A., Eizenberg, O. Vaizel-Ohayon, D. US patent 7488717). The anti-CXCL12 peptide, BKT 140, identified by surface plasmon resonance and ELISA, is currently in clinical phase I for neutropenia and anemia.

Chemokine neutralizing ligands may also not be peptidic at all. A screening campaign of an academic library of small molecules (Boeglin et al., 2007; Hibert, 2009) was designed, using a fluorescence resonance energy transfer assay (Vollmer et al., 1999; Valenzuela-Fernandez et al., 2001) in order to identify inhibitors of CXCL12–CXCR4 interactions. A chalcone molecule (4′-phenyl, 3-methoxy, 4-hydroxy chalcone) was found to be very effective ($K_i = 50$ nM) at inhibiting CXCL12 binding to CXCR4 and CXCR7, and signalling through CXCR4, including chemotaxis in vitro and in vivo (Hachet-Haas et al., 2008). The molecule however was unable to block cell fusion in an in vitro model (Chanel et al., 2002) of HIV entry. The proposed model of chalcone binding to the chemokine rather than to its receptor could be demonstrated using tryptophan fluorescence and microcalorimetry. This was reminiscent of earlier studies describing a natural derivative of chalcones, the flavone baicalin, isolated from *Scutellaria baicalensis*, which binds to the chemokines CXCL8, CXCL12, CCL4 and CCL8, although affinities were about four orders of magnitude lower (Li et al., 2000). The compound 4′-phenyl, 3-methoxy, 4-hydroxy chalcone, modestly inhibits signalling through CXCL8, a chemokine from the same structural subgroup as CXCL12, but is not active on CCL5, at least not in the micromolar concentration range. Finally, the chalcone compound shows efficacy, in vivo, in a mouse model of allergic hypereosinophilic airway inflammation where it is as powerful as neutralizing antibodies to either CXCL12 or CXCR4 (Hachet-Haas et al., 2008). Although the structure of the chemokine–chalcone has not been solved, molecular modeling and preliminary NMR data (C. Veldkamp, Milwaukee University, personal communication) support the idea that the chalcone binds to the same chemokine area as do chemokine receptor-derived peptides, i.e. in the groove delineated by the N-loop hairpin and β-strands 2 and 3 of the chemokine (Fig. 5). Yet, whether chalcone perturbs the state of chemokine oligomerization remains an open question. Other chalcone molecules are reported to act as inhibitors of allergic inflammatory diseases (Meng et al., 2007). The question as to whether the chalcone backbone acts as a chemical platform for biologically active molecules is open, and the mechanism of action of the molecules in vivo might rely on multiple interactions with different target proteins.

The example of the IL-2 neutralizing small molecules, SP4206 and SP4160, illustrates that flexible regions of small protein ligands can be targeted by high affinity molecules binding at protein–protein interfaces that are poorly druggable, but can adjust their structure to accommodate the ligand (Arkin & Wells, 2004; Thanos et al., 2006). The IL-2 receptor mediates T-helper cell maturation and is a drug target for transplant rejection (Waldmann & O'Shea, 1998; Berard et al., 1999) and autoimmune diseases (Schippling & Martin, 2008). Its ligand, interleukin-2 is a 15 kDa four helix bundle protein that promotes T cell growth. In a drug design program aiming at mimicking the IL-2 part that binds to the IL-2R, a peptidomimetic molecule, Ro26-4550, was discovered (Tilley et al., 1997). The molecule could inhibit IL-2 binding to its receptor with micromolar affinity, and careful

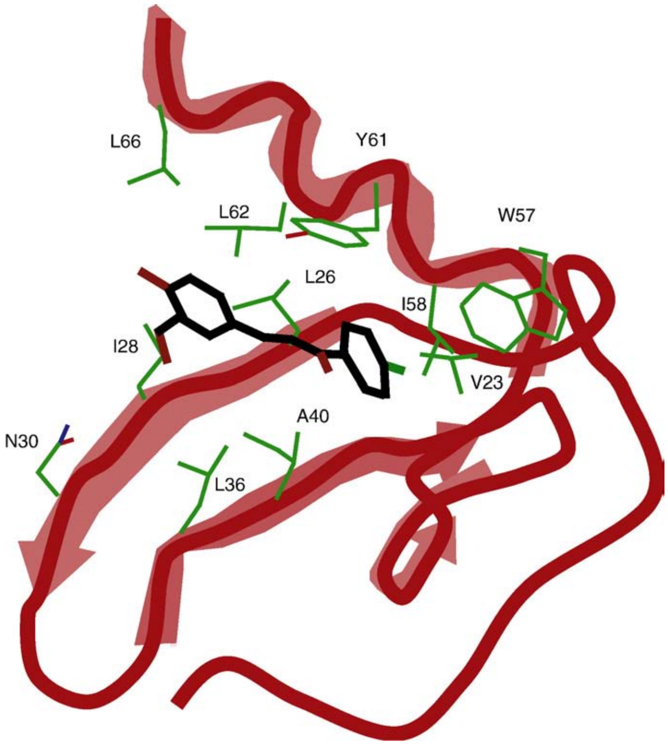

Fig. 5. Proposed model for the interaction between CXCL12 and neutralizing chalcone molecule 4. Redrawn from Hachet-Haas et al. (2008).

characterization of the mode of action led to the identification of the interleukin-2 itself being the receptor for Ro26-4550, not the IL-2R receptor protein (Emerson et al., 2003). Detailed structural analysis of Ro26-4550 interaction with IL-2, in particular using X-ray crystallography and NMR (Arkin et al., 2003) described in detail the binding area. The small chemical compound binds to a pocket that does not pre-exist as such in its absence. In other words, the binding area is the complex result of the adaptation of the local protein folds around the chemical. This particular case illustrates the difficulty to use the structure of the "empty" binding site, a priori, to predict Ro26-4550 structure or binding. However, optimization of the compounds, using X-ray crystallography of complexes, fragment-based approaches and tethering techniques, allowed the affinity of the IL-2 binding molecules to be lower to a few tens of nanomolar (Raimundo et al., 2004; Thanos et al., 2006).

Chemokines are present in the brain in glial cells as well as in neurons (Rostene et al., 2007). Concordant with their known role in the periphery, chemokines are up regulated during inflammation, and contribute to brain immunity and neuroprotection (Cartier et al., 2005; Glass et al., 2005; Madrigal et al., 2009; Omari et al., 2009). They are also involved in progenitor cell migration towards brain tumors (Magge et al., 2009), and participate in brain development and neuronal cell differentiation (Zou et al., 1998; Park et al., 2009). They are even distributed in neurons as neuromodulators are, and modify release of neurotransmitters or neuropeptides (Rostene et al., 2007). These findings, together with the development of pharmacological tools for chemokine receptors offers the possibility to better study chemokine signalling in the brain. Not only agonists or antagonists of the receptors, but also small neutralizing molecules directed against chemokines could be extremely valuable, in particular if they cross the blood brain barrier (BBB) to reach their target cytokines. One example is the conversion of the chemokine CXCL12, an agonist of CXCR4 and ligand of CXCR7, into an antagonist of CXCR3 (Vergote et al., 2006). The chalcone molecule mentioned above is probably a good pharmacological agent to study neuronal toxicity associated with CXCL12 catabolism.

Another application for potential neutralizing ligands is exemplified by the effect of anti-CCL5 antibodies. They are administered to fight the leukocyte infiltration that takes place in the brain as a result of virus-driven CCL5 production in mice. This infiltration is followed by destruction of myelin and appearance of neurological impairment (Glass et al., 2004). Antibodies to CCL5 significantly decrease macrophage accumulation in the brain and demyelinization. CCL5 neutraligands should thus prove useful to investigate the incidence of CCL5 in multiple sclerosis.

5.2. The small molecule alters quaternary structure of the protein ligand

Chemokines show a natural propensity to form dimers or oligomers depending on medium composition (Veldkamp et al., 2005), biochemical environment in particular glycosaminoglycans (Crown et al., 2006), or interaction with chemokine receptors (Veldkamp et al., 2008). Homodimerization/oligomerization is well established for CC chemokines such as CCL2, CCL4, CCL5 and CCL14 (Blain et al., 2007; Jin et al., 2007a; Proudfoot et al., 2003), and heterodimerization is also experimentally supported for instance in CCL2–CCL13, CCL2–CCL11 or CCL8–CCL13 heterodimers (Crown et al., 2006). Up to now, the functional consequences of these homologous or heterologous interactions have not been well understood, but the oligomerization process may be interesting to interfere with when attempting to neutralize chemokine actions (Fig. 4). Indeed, CC chemokine dimers do not correspond to the quaternary structure that interacts with the receptor, because residues critical for receptor binding are buried in the dimer (Jin et al., 2007a), and mutations can be made to abolish dimerization (Proudfoot et al., 2003; Jin et al., 2007a). In the case of CCL2, it could be demonstrated that certain responses, in particular leukocyte attraction in vivo, were abolished. This selective suppression of certain responses which leaves other responses unaffected is reminiscent of previously described multiple active states of G-protein-coupled receptors (Palanche et al., 2001) that can be differentially activated by distinct agonists, or modulated by allosteric effectors (Maillet et al., 2007). If prevention of dimer formation is per se sufficient to block chemoattraction in vivo, then the mechanism of action of the interceptor M3 (from herpesvirus) is probably exquisitely optimized. Decoy receptor M3 (vCKBP3) binds two monomers of CCL2 per M3 homodimer (Alexander et al., 2002). Thus, even though M3 recognizes and binds the GAG-binding domain of CCL2, its aptitude to "dissolve" dimers is thus likely to represent a mechanism of inhibition of CCL2 signalling in vivo (Handel et al., 2008).

In the case of CXC chemokines, dimerization also takes place spontaneously, but the dimer structure differs from that of CC chemokine dimers in that residues important for receptor interaction are not buried in the dimer. CXC chemokines can thus interact with their receptors either as monomers or as dimers/oligomers. At variance with what happens when CC chemokines interact with their receptors, it was demonstrated that a CXC chemokine receptor may itself be responsible for chemokine dimerization (Veldkamp et al., 2008). Tyrosine sulfation of the N-terminal domain of CXCR4 on tyrosines 7 and 12 is indeed a key determinant of receptor-mediated dimer formation since sulfated tyrosine 7 interacts with a CXCL12 monomer, while sulfated tyrosine 12 interacts with the second monomer. Two lines of evidence support the importance of CXCL12 dimerization in signalling: i) amino acids from CXCL12 are involved both in the interaction with heparan sulfates and with CXCR4; heparan sulfates can thus negatively affect CXCL12-evoked chemotaxis in vitro (Murphy et al., 2007), and ii) tethering CXCL12 monomers to obtain permanent CXCL12 dimers results in a partial loss of function of CXCL12. The permanent dimer indeed shows unaltered capacity to promote intracellular calcium elevation in CXCR4 expressing cells, but is no longer capable of triggering in vitro chemotaxis (Veldkamp et al., 2008). Although these results are not yet totally interpreted in terms of structure–function relationships, modulation of chemokine oligomerization represents a promising way to change cellular responses with possible important consequences in vivo. The unsaturated heparin disaccharide used to perturb CXCL12 dimer structure (Murphy et al., 2007) is a plausible starting chemical platform to exploit. Virtual and experimental screening of collections of molecules on chemokine dimers could lead to the discovery of neutralizing molecules, the mechanism of action of which would be prevention of dimer formation. The critical step in this kind of project would be the definition of primary and secondary assays allowing qualitative and quantitative description of the new compound effects.

TNFα is produced in response to pathogens through toll-like receptor activation, and promotes expression of many immune system effectors, including cytokines and chemokines (Balkwill, 2009) that will recruit leukocytes to the site of inflammation. If production of TNFα is excessive, chronic inflammation can develop as in rheumatoid arthritis, Crohn's disease, severe asthma or psoriasis, all diseases in which a prominent role of TNFα has been demonstrated. Anti-inflammatory therapies have been developed, based on inhibition of either the production of TNFα or the neutralization of TNFα itself (Foxwell et al., 2003). The neutralizing monoclonal antibodies, etanercept, infliximab and adalimumab have led to successful treatment in rheumatoid arthritis, and show that neutralizing antibodies can prove valuable not only in acute, but also in chronic human diseases. Yet, two problems remain after several runs of antibody optimization for human use: the mode of administration associated with dosing difficulties, and the elevated cost of treatment have motivated the search for alternative therapeutic tools. In the search for small molecules from collections of combinatorial fragments capable of inhibiting TNFα binding to its TNF-R1 receptor, He et al. (2005) discovered a small molecule inhibitor exhibiting a micromolar affinity constant. When trying to identify by X-ray crystallography the binding site of the small molecule on the large trimeric structure of TNFα, the authors realized that soaking TNFα crystals led to their destruction. Crystallization of the complex was then obtained. It revealed that the mode of action of the small molecule antagonist of TNFα is to dissociate its trimeric quaternary structure into inactive inhibitor-bound dimers with a stoichiometry of one molecule per dimer (He et al., 2005; Berg, 2006). This illustrates one of the modes of action of small molecule inhibitors that, following description of CCL2 monomer failure to signal (Handel et al., 2008), could be applied to the family of chemokine proteins, using detection of quaternary structure as a primary screen.

5.3. The protein ligand undergoes tertiary structural changes that regulate its activity and the small molecule prevents the active conformer

Soluble proteins may undergo three dimensional structural changes, either spontaneously, in a regulated manner (Monod et al., 1965) or as a mechanism of pathogenesis (Dobson, 1999). Lymphotactin is a chemokine with unusual properties. First, in contrast to most chemokines its structure is stabilized by a single disulfide bridge. Second, it has the singular property of existing as two unrelated protein folds (Kuloglu et al., 2002; Tuinstra et al., 2008). One of the two protein folds resembles the canonical chemokine structure with 3 anti-parallel β-strands and one carboxy terminal helix. This conformation binds to and activates its receptor XCR1 but does not interact with glycosaminoglycans. The second protein fold exhibits 4 anti-parallel β-strands but has no helix (Tuinstra et al., 2008). This second structure binds glycosaminoglycans with high affinity but is unable to activate the XCR1 receptor. As this example is unique to date in the field of proteins and protein ligands, its generality is questionable. It is an extreme case of the general field of change in protein conformational equilibrium, which has been tackled by many different laboratories on many different regulatory proteins to select new pharmacological tools and active drugs. Ligands that would stabilize the glycosaminoglycan binding state could reveal neutralizing molecules capable of modulating acute allograft rejection response (Wang et al., 1998) or attenuate inflammatory bowel disease (Boismenu et al., 1996; Middel et al., 2001), as two examples of mucosal immunity in which lymphotactin is involved.

5.4. The small molecule interferes with ligand bioavailability

Chemokines activate G-protein-coupled receptors to recruit leukocytes during organogenesis, immunosurveillance, and inflammation. An important component of this process is the formation of a chemotactic gradient by immobilization of chemokines on the extracellular matrix of cells, in particular on glycosaminoglycans. Analysis of the role played by glycosaminoglycans has been carried out using mutants of CC (Proudfoot et al., 2003) or CXC/XCL (Peterson et al., 2004; Sadir et al., 2004; Johnson et al., 2004; Ali et al., 2005; Jin et al., 2007a) chemokines devoid of key residues known to bind to negatively charged sugar moieties. Suppression of GAG interactions for CCL2, CCL4, CCL5, CCL7 or XCL1 chemokines was found to abolish leukocyte recruitment in vivo, when injected intraperitoneally, although in vitro chemotaxis was not altered.

The importance of these interactions has been further highlighted by showing that GAG-binding mutants of chemokines can block the action of wild type chemokines in normal animals as well as in murine models of diseases (Johnson et al., 2004; Ali et al., 2005; Braunersreuther et al., 2008). Pathogens also target GAG-binding domains of chemokines to prevent their effects. As mentioned above (Section 2.1, avoidance strategies), the poorly selective M-T1 and M-T7 proteins from *myxoma* virus, the M3 protein from herpesvirus or glycoprotein E163 from *ectromelia* virus neutralizes chemokines upon binding to their GAG-binding domain. The likely consequence of this is that high local concentrations of chemokines giving rise to a “Velcro effect” are reduced, and gradients are disrupted. Therefore, leukocyte attraction no longer takes place. Although no small chemical molecule targeting the GAG-binding domain of chemokines has been described up to now, this portion of the protein is validated for chemical biology approaches. Noteworthy are the disaccharides that were used to solve the structure of CCL5 forming complexes with heparin-derived sugars (Shaw et al., 2004) that could be used as starting blocks for drug design. Interestingly too is the small molecule surfen, that was first used as an excipient in drug formulas before being also identified as a heparin neutralizing molecule (Hunter & Hill, 1961) with antibacterial and trypanocidal activity, which could be used to lower excessive heparan sulfate-involving interactions (Schuksz et al., 2008).

6. Concluding remarks and perspectives

Many articles now report that either genetic manipulations of receptors and chemokines, or development of pharmacological agents leads to selective alterations of a subset of responses out of a series of possible responses. One consequence is that, depending on the desired properties of the molecule to be developed for research or disease treatment, preference for a ligand of receptor or a ligand of ligand will need to be validated experimentally. Indeed, chemokines activate members of the family of G-protein-coupled receptors (GPCRs) known to exhibit a significant level (around 10%) of spontaneous isomerization towards active conformations (Lefkowitz et al., 1993a,b; Leurs et al., 2000; Palanche et al., 2001; Alewijnse et al., 2000; Claeysen et al., 2000; Lecat et al., 2002). Spontaneous activity of GPCRs has well established physiological roles (Adan, 2006; Arrang et al., 2007) like control of neurotransmitter (Threlfell et al., 2008) and hormone (Ben-Shlomo et al., 2009) release in relation with higher order behaviors (Fioravanti et al., 2008), or regulation of apoptosis (Lau et al., 2009). Spontaneous activity of chemokine receptors also exists. For herpesvirus-encoded receptors this activity is linked to transforming effects (Burger et al., 1999; Holst et al., 2001). Ligands of GPCRs almost never behave as neutral molecules, and most antagonists are either weak partial agonists (that increase the level of receptor activity) or are so called "inverse agonists" capable of diminishing the level of spontaneous activity of the receptor. Small molecules neutralizing ligands may complement the tool palette of pharmacologists since they are expected to leave the spontaneous activity of the receptors unchanged.

There are four preferred ways to identify small molecules modulating protein–protein interactions, ELISA assays, fluorescence resonance energy transfer (FRET) assays, fluorescence anisotropy (Berg, 2003) and surface plasmon resonance. We have designed a general strategy in order to find fluorescent probes that bind to a soluble protein. This fluorescence anisotropy strategy involves four steps:

1) Synthesis of a library of fluorescent compounds using known chemical scaffolds that exhibit low specificity. The molecules are organized around generic GPCR-preferring chemical scaffolds, are derivatized with charged, hydrophilic and hydrophobic moieties and bear a lissamine fluorophore at the end of a spacing arm,
2) Screening of the library of fluorescent molecules by fluorescence anisotropy measurements in order to successfully fish one fluorescent probe at least. This technique allows to set up a "mix and read" assay that readily pinpoints the probe that interacts with the protein,
3) Characterization of the binding properties of the probe in order to fulfil the desired requirements, i.e. neutralize binding of the ligand to its receptor, or inhibit oligomerization, or inhibit binding to GAGs, and select the desired and optimized screening assay,
4) Screening of libraries of unlabelled and drug-like molecules to identify high affinity molecules to use for chemical biology purposes.

The generalization of such kinds of drug discovery approaches in particular in university laboratories, is highly desirable. The increasing amount of screening data will then lead to large scale chemoinformatics and bioinformatics including data from transcriptional analyses and proteomics (Schadt et al., 2009; Weill & Rognan, 2009), development of complex network modelling as is the case in systems biology in order to establish training sets for interaction network prediction, and to use these models to predict molecule toxicity and metabolism, bioavailability and patterns of biological activity.

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