CHAPTER FOUR

Recent Developments in Targeting Neuroinflammation in Disease

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1. INTRODUCTION

Neuroinflammation (NI) is the process by which an organism attempts to remove an injurious stimulus in the central nervous system (CNS) and initiate the healing process to protect the cells and overall function of the brain. NI may be accompanied by increased vascular permeability, invasion of peripheral immune cells, release of inflammatory mediators (cytokines, reactive oxygen species, etc.), and tissue dysfunction.[1] The primary mediators of NI are microglia, the only immune cells residing within the CNS. However, neuroinflammatory responses can also be driven by astrocytes, oligodendrocytes, and neurons, as well as cells of the peripheral immune system such as monocytes/macrophages and T cells.[2] These cell

Annual Reports in Medicinal Chemistry, Volume 47
ISSN 0065-7743
http://dx.doi.org/10.1016/B978-0-12-396492-2.00004-7

types are all capable of releasing various pro- and anti-inflammatory mediators. Typically, inflammation is an acute process whose ultimate goal is resolution and repair of injured cells/tissue. However, when inflammation becomes excessive or prolonged, it becomes pathological and is associated with a variety of diseases.

The case can be made that most CNS disorders, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), autism, epilepsy, HIV-associated neurocognitive disorder, Huntington's disease (HD), multiple sclerosis (MS), neuropathic pain, Parkinson's disease (PD), schizophrenia, stroke, and spinal cord injury (SCI), exhibit NI pathology.[3–12] For some indications, such as MS, the peripheral immune system is the primary cause and driver of disease progression. In other cases, such as SCI, the peripheral immune system is not a cause, but certainly a well-characterized contributor to inflammation. For neurodegenerative disease, NI is typically chronic and may begin a decade or more before the onset of symptoms (e.g., HD[13]). Furthermore, it is well established that modulating NI genetically and pharmacologically can alter the course of disease in animal models of these diseases. There is also convincing evidence, mostly from preclinical models, that microglia play an integral role in the development of neuropathic pain.[14] Since few if any CNS disorders are completely or adequately treated by any single drug, finding an effective therapy that slows the progression of a disease would be an important advancement. Reducing NI may not address the cause of the disease, but it may mitigate or resolve the inflammatory response that drives disease progression. As few drugs on the market specifically target NI, this approach presents a promising alternative, or cooperative, drug discovery strategy for the treatment of many CNS disorders.

Modulating the interaction and communication between microglia, neurons, and cells of the immune system, by targeting ion channels, G-protein-coupled receptors, enzymes, and kinases, for example, may ameliorate NI associated with CNS diseases. Metabotropic and ionotropic neurotransmitter receptors, enzymes such as cyclooxygenase and inducible nitric oxide synthase, antioxidants, and biologics targeting adhesion molecules or anti-inflammatory peptides are well reviewed elsewhere.[15] Kinases such as MAPK, MEK, CDK5, GSK, JNK, and IRAKs are not very specific to a particular pathway, cell type, or biology and are also reviewed elsewhere.[16,17] Therefore, in this review, we focus on examples from several major drug target classes where recent medicinal chemistry advancements have been made, in order to provide a broad overview of the diverse strategies to target NI.

2. ION CHANNELS

2.1. Purinergic receptors and neuroinflammation

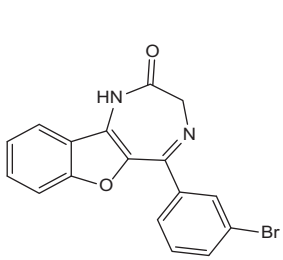
Purinergic receptors are ion channels expressed on various cells in the CNS and immune system and can be classified into two subfamilies: G-protein-coupled metabotropic (P2Y) and ligand-gated ionotropic (P2X) receptors. Although metabotropic purinergic signaling has been implicated in neuroinflammation,[18] the focus here centers on recent discoveries of the P2X receptors, notably $P2X_4$ and $P2X_7$, which are the predominant ligand-gated purinergic receptors expressed on microglia.

2.1.1 $P2X_4$ receptor ($P2X_4R$)

Activation of $P2X_4R$ results in increased intracellular Ca^{2+}, K^+, and Na^+ and subsequent activation of p38-MAPK signaling. One factor released in response to activation of this receptor is BDNF which acts on the TRK-B receptor on lamina I neurons in the dorsal horn of the spinal cord, resulting in their hyperexcitability.[19,20] Blockade of $P2X_4R$ function significantly ameliorates neuropathic pain in preclinical models, a finding that is supported by a variety of genetic and pharmacological studies. This was first demonstrated by using the antagonist of $P2X_{1-4}Rs$ (TNP-ATP) to reverse tactile allodynia in a spinal nerve injury model.[21] In contrast, pyridoxalphosphate-6-azophenyl-2′4′-disulphonic acid, an antagonist of $P2X_{1-3,5,7}Rs$, but not $P2X_4R$ was ineffective at reversing allodynia in rats with nerve injury. Genetic approaches such as knockdown of the $P2X_4R$ using antisense oligonucleotides[21] and genetic ablation of the $P2X_4R$ gene[22] also resulted in a significant reduction in pain behavior. While most data support a role for $P2X_4R$ in modulating neuropathic pain, $P2X_4$ receptors also regulate microglial responses, making them attractive targets for other CNS indications.

There is a scarcity of published $P2X_4R$ chemical matter suggesting potential issues of chemical tractability around this target.[23] However, several chemotypes have been identified with modest potency, including benzofuro-1,4-diazepin-2-one analog **1** ($P2X_4R$ IC_{50} 0.5 μM) and 1-aryl-2-phenoxymethyl-piperazine analogs, exemplified by the SSRI paroxetine ($P2X_4R$ $IC_{50} \sim 3$ μM).[23,24] More recently, diazapinedione derivatives,[25] exemplified by **2** and 4-aryl-2-quinazolinone compounds[26] exemplified by **3** were reported with $P2X_4R$ IC_{50} values from 0.16 μM. The utility of these analogs as tools to probe $P2X_4R$ function *in vivo*

remains unclear since their receptor selectivity, brain penetration, and PK data have not yet been reported.



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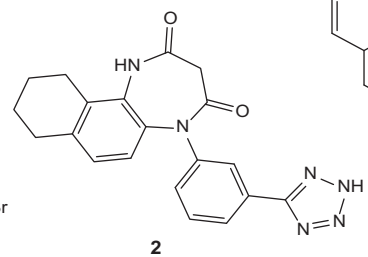

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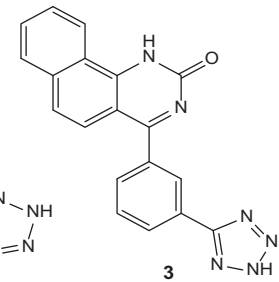

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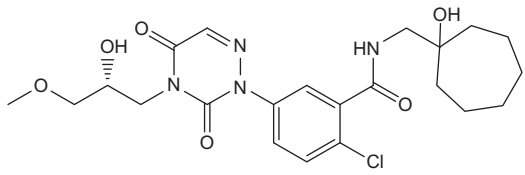
2.1.2 $P2X_7$ receptor ($P2X_7R$)

The $P2X_7R$ is activated by high extracellular concentrations of ATP (>100 μM) that leads to Ca^{2+} influx/K^+ efflux and initiation of a cascade leading to maturation and release of the proinflammatory cytokine IL-1β. In the CNS, $P2X_7R$ is primarily expressed on microglia, oligodendrocytes, and activated astrocytes, though the data supporting neuronal expression are controversial. Elevated receptor expression and function have been associated with various CNS diseases, including depression, AD, MS, epilepsy, ALS, and neuropathic pain.[18] Several compounds have entered clinical trials for peripheral indications, but to date, this target has not been probed clinically for a CNS indication. 1-Hydroxycycloheptyl analog **4** (CE-224,535) is a potent human $P2X_7R$ antagonist that inhibits IL-1β release from lipopolysaccharide (LPS)- and ATP-stimulated human whole blood with IC_{50} and IC_{90} values of 1.0 and 4.7 nM, respectively.[27] This candidate was evaluated in a clinical trial for rheumatoid arthritis, but failed to show significant efficacy, even with trough free plasma concentrations significantly above the IC_{90} in human whole blood. The CNS penetrability of this compound is not reported, and it lacks sufficient rodent $P2X_7R$ potency for testing in preclinical models, a common issue with $P2X_7R$ antagonists due to wide differences in receptor homology across species.



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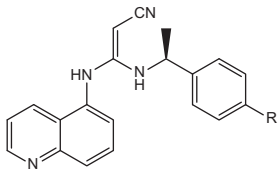
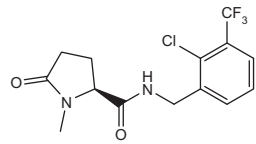
Many of the early $P2X_7R$ antagonists either lack sufficient CNS penetration or rodent $P2X_7R$ antagonist activity to support testing in preclinical *in vivo* NI models. However, there have been recent advances in overcoming these challenges. For instance, 2-cyano-guanidine analog **5** (A-804598) is one of the few selective $P2X_7R$ antagonists with good CNS penetration and similar activity in human, rat, and mouse ($P2X_7R$ IC_{50}: 11, 10, and 9 nM, respectively), thus making it a potential tool compound to probe preclinical rodent models where NI is a component. In addition, tritiation of **5** at the 4-position of the phenyl ring (**6**) provides a radioligand with high specific activity.[28]



5 R = H
6 R = T



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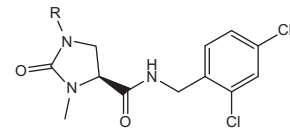


8 R = H
9 R = 3-(2-methyl)pyridine

A series of pyroglutamic acid amides, represented by **7**, with a human pIC_{50} of 8.5 and a rat pIC_{50} of 6.5 were reported recently. While **7** is 100-fold less active in rat, efficacy was still observed in a rat model of Freund's complete adjuvant (FCA)-induced centralized inflammatory knee joint pain and in a chronic constriction injury model of neuropathic pain. Exposure at the minimal effective doses provided free drug fractions in excess of the rat pIC_{50} in both the periphery and the CNS.[29] The importance of CNS activity in the FCA knee joint pain model was supported by a comparative study of 2-oxo-4-imidazolidinecarboxamide analogs **8** and **9** differing in their brain penetration capabilities. Compound **9** which was inactive in the FCA model had superior free plasma exposure and $P2X_7R$ potency, but poor brain penetration. On the other hand, the less potent, brain penetrant $P2X_7R$ antagonist **8** showed efficacy in this knee joint pain model.[30]

In summary, significant progress has been made in identifying tools that offer both sufficient CNS penetration and rodent potency to probe the utility of $P2X_7R$ antagonism for the treatment of disease where NI is present.

3. ENZYMES

3.1. Kynurenine pathway in neuroinflammation

The kynurenine pathway of tryptophan metabolism is an area of growing interest for NI and has received much attention in recent years.[13,31–33] Throughout the body, including the CNS, the primary fate of

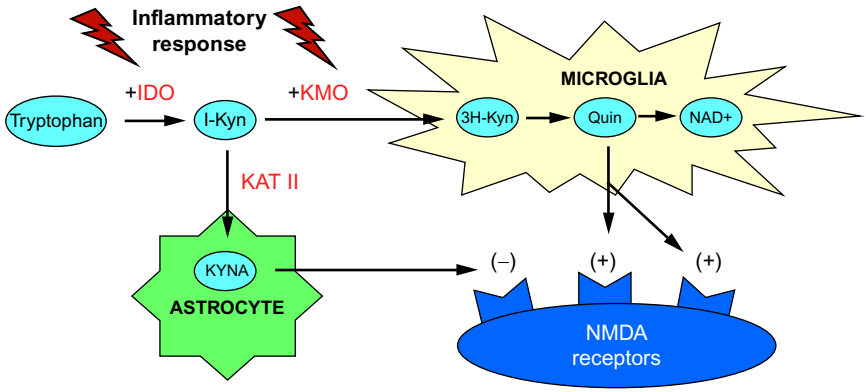


Figure 4.1 Kynurenine pathway. Enzymes regulating kynurenine metabolism in the CNS are reportedly upregulated in response to inflammation. Selective inhibition at points within the kynurenine pathway may be beneficial in treating NI-related conditions. Indolamine 2,3-dioxygenase (IDO), kynurenine 3-monooxygenase, kynurenine aminotransferase II (KAT II).

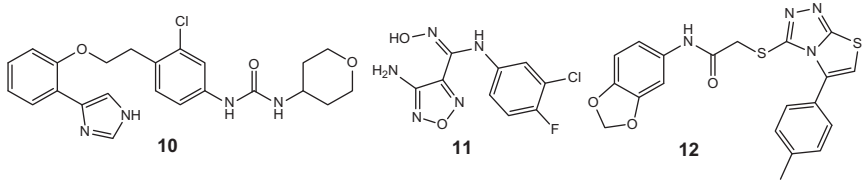
tryptophan is conversion to l-kynurenine. In turn, l-kynurenine is processed into several metabolites including kynurenic acid (KYNA) and quinolinic acid (QUIN) that subsequently activate or inhibit NMDA neurotransmission. KYNA and QUIN are produced in distinct cell types in the brain based on the distribution of the catabolic enzymes (Fig. 4.1).[34] The functional roles of the kynurenine pathway enzymes have been previously reviewed[35] and are currently the target of several drug discovery efforts. Recent evidence suggests that kynurenine metabolic enzymes are activated by inflammatory responses and their products may also impact NI and immune function.[36,37]

3.1.1 Indole 2,3-dioxygenase

IDO (IDO1; INDO) is the first and rate-limiting step in the kynurenine metabolic pathway of tryptophan in the CNS. TNFα and INFγ, elevated in inflamed tissue, stimulate IDO expression which regulates T-helper and T-regulatory cell populations[38] making IDO an attractive target for immune-related therapies. Within the CNS, inflammation-mediated IDO expression increases tryptophan metabolism, thereby depleting its availability for serotonin production.[39] Therefore, IDO inhibitors are speculated to have antidepressant activity for those suffering from chronic CNS inflammatory conditions.

Progress has been achieved in identifying new chemical matter for IDO. Historically, medicinal chemistry efforts have focused on tryptophan analogs, which act in a competitive fashion. More recently, noncompetitive β-carboline inhibitors have been identified.[40] In general, these compounds have IDO activity in the 10–100 μM range. The cocrystal structure of human IDO with the noncompetitive inhibitor phenyl-imidazole was solved recently,[41] and this key achievement has led to the rational design of new chemical motifs, some with improved potency. A recent patent application expands upon the phenyl-imidazoles with a number of compounds, such as **10** in the submicromolar range.[42,43] Several new noncompetitive chemotypes have been reported recently, which broadens the chemical space for additional discovery efforts.[44,45] A series of competitive inhibitors identified from a high-throughput screen, with submicromolar activity, was recently reported.[46] Specifically, lead compound **11** inhibits IDO with an IC_{50} of 67 nM. In a mouse *in vivo* model, **11** reduces plasma kynurenine levels by 60% at free plasma exposures 2.5-fold above the IC_{50}.

Identification of selective analogs has also become more challenging with the discovery of a second enzyme that metabolizes tryptophan into l-kynurenine, IDO2 (INDOL1), discovered in 2007 with 43% homology to IDO1.[47] Recently, a series of thiazolopyrazolo analogs with excellent IDO1 selectivity were described. For example, compound **12** has an IDO1 IC_{50} of 3 μM and is >80-fold selectivity versus IDO2.[48] This selectivity over IDO2 is corroborated by docking studies based on the crystal structure of IDO1 and a homology model of IDO2.

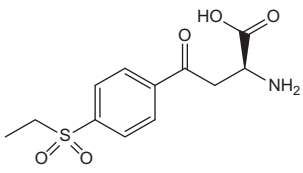


3.1.2 Kynurenine aminotransferase II

The KYNA branch of the kynurenine pathway is regulated by kynurenine aminotransferases (KATs I–IV), and specifically KAT II in the brain.[49] Though often referred to as the "neuroprotective" branch of the kynurenine pathway due to an inhibitory effect on glutamate neurotransmission, KYNA has recently been shown to also act as an endogenous agonist of the aryl hydrocarbon receptor (AhR)[50] suggesting a potential role in inflammation and immune responses. IL-1β-mediated release of IL-6 from astrocytes was

synergistically augmented by KYNA through the activation of AhR. While this field is still in its infancy, these data are intriguing in the context of neuroscience where elevated IL-6 levels are the most commonly associated change in IL expression in inflammation-mediated mood disturbances.[47]

Historically, there has been a paucity of selective and potent tools available to elucidate the role of KYNA in the brain. The discovery of agents such as **13**, *S*-ESBA, was an important advancement in KYNA biology. However, due to its poor CNS penetration, direct injection into the brain is required for *in vivo* studies. While this may be appropriate for measuring effects on glutamate neurotransmission, intrathecal injection compromises NI evaluation since local drug application compromises the blood–brain barrier and is likely to elicit an acute inflammatory reaction. However, with the discovery of the KAT II crystal structure,[51] progress is being made in the development of more potent and brain penetrable agents. In 2010, a second generation KAT II inhibitor, BFF-122 (**14**), was disclosed which covalently binds to the enzyme cofactor pyridoxal phosphate (PLP) in the catalytic pocket creating an irreversible adduct.[52] Though still possessing poor brain penetration, **14** was the first reported compound with submicromolar affinity for the human KAT II enzyme representing an important improvement in potency. More recently, PF-4589989 (**15**) was disclosed as the first low nanomolar affinity brain penetrable KAT II inhibitor.[53,54] Like BFF-122, PF-4859989 reportedly forms a covalent adduct with PLP in the binding pocket of KAT II resulting in inactivation of the enzyme. While irreversible inhibitors create challenges in defining the biological off-rate of their effects, these agents will be critical in understanding both the central and peripheral roles of KYNA production in inflammation and immune biology.



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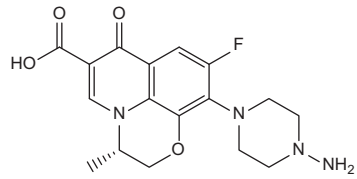

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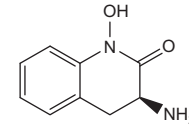

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4. RECEPTORS

4.1. Toll-like receptors

Toll-like receptors (TLRs) play a critical role in the proper coordination of innate and adaptive immune responses to foreign pathogens and injury.[55,56] TLRs are type 1 transmembrane glycoproteins, expressed either on plasma

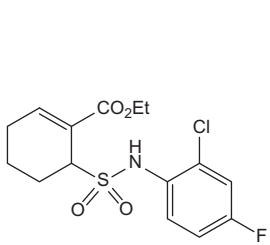
membranes or on intracellular membranes, which form homo- or heterodimers that undergo conformational shifts upon ligand binding. This shift leads to the recruitment of adaptor and signaling molecules which, in turn, induce specific patterns of cytokine and chemokine expression. There are 10 known TLRs in humans, and despite a high degree of structural similarity, each receptor complex recognizes a distinct pathogen (PAMP)- or danger-associated molecular pattern (DAMP). TLRs are traditionally thought of as peripheral immune cell (leukocyte) targets[57–59]; however, a growing body of evidence indicates that TLRs (in particular, TLRs 2, 3, 4, 8, and 9) also play a crucial role within the CNS.[17,60] For example, increased TLR expression and NI have been observed in the brains of AD patients as well as in animal models of AD.[61] Furthermore, TLR-induced leukocyte activity in the periphery can regulate the pathology of certain neurodegenerative CNS disorders in animal models.[62–64] Such findings hint at the possibility of treating specific CNS disorders by peripheral modulation of TLRs,[65] perhaps bypassing the need for CNS penetrant compounds. This section will focus on two representative TLRs of high biological interest, TLR4 (plasma membrane bound) and TLR9 (endolysosomal membrane bound).

4.1.1 TLR4

TLR4 has several known agonist ligands, both pathogen derived as well as endogenous (HSP60, HSP90, beta-amyloid, α-synuclein, fibrinogen, and opioids).[61,66–69] Binding of LPS to MD2 triggers a structural rearrangement that is transduced to TLR4 and its cytosolic toll and interleukin 1 receptor (TIR) like domain resulting in clustering of TLR4 receptors enabling interactions with other signaling proteins.[70] In the CNS, TLR4 is primarily expressed on microglia although expression on astrocytes and neurons has been reported.[71] Clinical and preclinical data implicate TLR4 in a variety of CNS diseases including AD, ALS, epilepsy, MS, neuropathic pain, PD, stroke, and ischemia.[17,60,61,71–76] For stroke prevention and plaque clearance in AD, an agonist would be desirable based on preclinical models, although extreme TLR4 activation could result in sepsis. For treatment of stroke, PD, neuropathic pain, and ALS, an antagonist would be required; however, immunosuppression could be a drawback. While safety issues are a potential concern as a result of modulating the immune system, there is compelling biological rational suggesting that TLR4 modulation could have substantial clinical impact on a variety of diseases.

Several recent reviews highlight the chemical matter for TLR4.[57,77–79] TLR4 antagonists block LPS signaling by a variety of modes including

interfering with TLR4 directly or interfering with the TLR4 and LPS binding proteins interaction.[80] The small molecule irreversible inhibitor **16**, TAK-242, is one of only a few examples of small molecules that interact directly with TLR4 through binding to cys-747 in its intracellular domain. TAK-242 was in phase III clinical development for the treatment of sepsis but was recently discontinued due to lack of efficacy. Eritoran tetrasodium is a lipid A analog in phase 2 clinical trials for sepsis and is administered by IV injection. This compound binds to MD2, thus blocking the interaction of LPS with the TLR4–MD2 complex. Eritoran tetrasodium inhibits LPS-induced TNF-α release in human whole blood with an IC_{50} of 10 nM. In a similar mode of action, small molecule benzylpyrazoles, exemplified by **17** ($EC_{50} = 18.7$ μM), were reported[81] to inhibit LPS-induced nitric oxide production by binding to the MD2 region that interacts with LPS. These analogs may offer a new starting point for medicinal chemistry efforts to provide orally available TLR4 antagonists.



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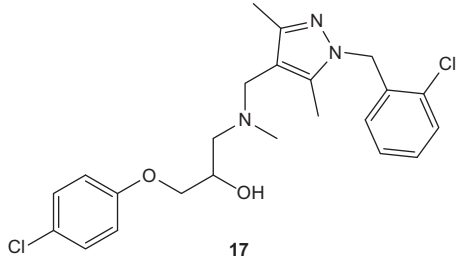
TAK-242



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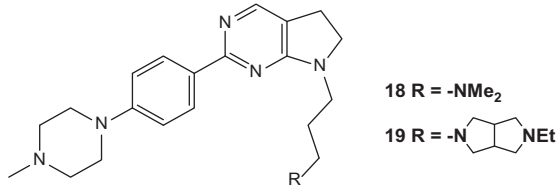
4.1.2 TLR9

While most TLRs are expressed on the cell surface of leukocytes, TLR9 primarily functions within subcellular endolysosomes (digestive organelles)[82–84] where it is ideally situated to detect PAMP ligands from internalized and digested DNA material. TLR9 biology has been implicated in a range of CNS infection, injury, and disease settings including meningitis and herpes,[85] AD,[62,86] MS,[87,88] Guillain–Barré syndrome,[89] and ischemic stroke.[63] Depending on disease context, either TLR9 agonists or antagonists could serve as therapeutic agents.

TLR9 agonists: Short fragments of single-stranded DNA (ssDNA) containing unmethylated cytosine-phospho-guanine (CpG) motifs, which are overrepresented in bacterial and viral, but not mammalian DNA, serve as naturally occurring PAMP ligands for TLR9.[90] Agonist SARs for synthetic

CpG ssDNA sequences and their sugar backbone modifications have been extensively reviewed.[59,90–92] However, recent work has identified novel sequence and structural modifications, as well as novel ssDNA carriers, which improve the pharmacokinetic properties of CpG ssDNA. While most CpG ssDNA has limited secondary or tertiary structure, three-dimensional "origami" CpG ssDNA structures have been described that elicit more robust immunological responses than equivalent amounts of standard CpG ssDNA.[93] In addition, the immunostimulatory effects of CpG ssDNA are enhanced when formulated with novel liposome carriers[94] or carbon nanotubes[95] or boron nitride nanospheres,[96] perhaps due to increased delivery and exposure of CpG ssDNA within the TLR9-expressing endolysosomes.[82] To date, only CpG ssDNA-based TLR9 agonists have been disclosed.

TLR9 antagonists: Both nucleotide and small molecule TLR9 antagonists have been described. For example, novel 4,5-fused pyrimidine derivatives **18** and **19** have been shown to inhibit CpG-induced cytokine expression *in vitro* and *in vivo*.[97] Gold nanoparticles, ideal for medical applications due to their bio-inert and noncytotoxic properties, have also recently been shown to inhibit TLR9-specific signaling in a particle size- and concentration-dependent manner.[98] In addition, ssDNA inhibitors of TLR9 have been previously described.[99]



4.2. Chemokine receptors

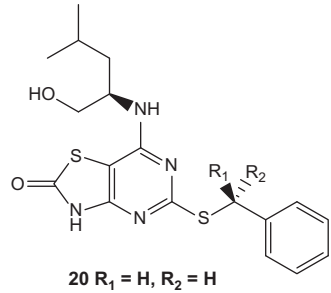
Chemokines have previously been reviewed in Annual Report in Medicinal Chemistry. (ARMC see 30, 209; 35, 191; 39, 117). Chemokines are defined and classified by conserved cysteine residues that form intramolecular disulfide bonds. The number of cysteine pairs as well as the number of amino acids separating the two internal residues determines their class and name. For example, the chemokine ***C***X3***C***L1 has two internal *cysteine residues* separated by 3 amino acids (X3) where L denotes ligand and R denotes receptor CX3C***R***1. In all, there are at least 46 chemokine ligands and at least 18 functional receptors. All chemokines are secreted in relatively large quantities except CXCL6 and

CX3CL1, as they are produced as membrane-bound ligands. Furthermore, there is tremendous promiscuity in the receptor/ligand interactions with most chemokine receptors binding multiple ligands and most chemokine ligands binding to multiple receptors. This biology has been reviewed in detail.[100]

4.2.1 CX3CR1

CX3C*R*1 (*R*eceptor for CX3CL1) is an attractive target because it has a 1:1 binding specificity with its ligand, CX3CL1 (also known as fractalkine). CX3CL1 is the sole member of the class of chemokines whose cysteines are separated by 3 amino acids. While CX3CL1 is produced as a membrane-bound ligand by neurons, constitutive cleavage liberates soluble chemokine that acts as a chemoattractant. Under stress, chemokine cleavage is increased via a different set of inducible proteases.[101,102] In the periphery, CX3CR1 is expressed on T cells, dendritic cells, and a small subpopulation of monocytes,[102] while in the CNS, the receptor is exclusively expressed on microglia. Peripherally, CX3CL1 is expressed on vascular endothelial tissue where it may be involved in leukocyte extravasation into tissues, and in the CNS, the ligand is predominantly expressed on neurons. There is considerable evidence that in models of neuropathic pain CX3CL1 signals neuronal stress to microglia, resulting in microglia-induced enhancement of pain sensation. Additionally, several *in vivo* studies have been published that suggest that altering (increasing or decreasing) CX3CL1 signaling can significantly modulate neurodegeneration and pathology in animal models of ALS, AD, PD, and SCI,[99,103–107] making this a promising target for therapeutic intervention in diseases with NI.

New small molecule-based chemical matter for CX3CR1 has been relatively limited with the exception of a group of published patents on a series of thiazolopyrimidines and thiazolopyrimidones.[108–111] These compounds were identified based on cross-reactivity profiling of CXCR2 antagonists. The reference compound benzylthio analog **20** has a K_i of 54 nM for inhibiting ^{125}I-CX3CL1 binding in membrane preparations from cells expressing CX3CR1 but is nonselective over CXCR2. Addition of an alpha-methyl to the benzyl group, as in **21**, improves the CX3CR1 K_i to 7.8 nM and reduces the K_i for CXCR2 to 1359 nM. The chirality of the alpha-methyl group is important for driving CX3CR1 selectivity as the *R*-diastereomer **22** has similar potency as **21** at CX3CR1, but has a K_i of only 240 nM at CXCR2.



20 R_1 = H, R_2 = H
21 R_1 = H, R_2 = Me
22 R_1 = Me, R_2 = H

5. CONCLUDING REMARKS

In summary, NI is an important factor of many CNS diseases, and alleviating NI is anticipated to reduce disease severity and improve patient outcome in a majority of cases. There are a variety of traditionally druggable targets for NI such as enzymes, receptors, and ion channels. In this review, targets were highlighted where advances in medicinal chemistry had been achieved in the recent past. In terms of future perspectives, a better understanding of inflammation in the brain and the relationship between peripheral and central inflammation is required. Since most currently approved anti-inflammatory drugs target the periphery, it is important to understand the extent to which these compounds affect NI. Even though increased risk of infection is a potential issue for NI targets owing to immunomodulatory effects,[112,113] there is significant opportunity to discover new molecules for the alleviation of NI in CNS diseases.

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