

Selecting a Broad-spectrum Chemokine Inhibitor for use in vivo

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The chemokines are a family of small (8-12kDa) signalling proteins structurally related to IL-8 which play a central role in the regulation of immune function, and in particular in the control of leukocyte trafficking. As a result, inhibition of chemokine signalling is an attractive strategy for developing anti-inflammatory therapies. We have described a series of compounds which inhibit leukocyte migration in response to every member of the chemokine family tested to date. These compounds, termed Broad-Spectrum Chemokine Inhibitors (BSCIs) have promising anti-inflammatory properties in vivo. Here we provide an overview of the four different structural families of BSCIs, with a particular focus on the use of the compounds in vivo. We conclude that NR58-3.14.3 (a cyclic 13-mer peptide composed of D-amino acids) and Olfoxamide (a N-substituted aminocaprolactam) represent the best BSCIs for use in vivo and describe the optimum conditions of achieving chemokine blockade in vivo using these compounds.

KEY WORDS: *Inflammation; chemokines; BSCIs; pharmacokinetics*

The chemokine family of small signalling proteins play a central role in the regulation of the mammalian immune system. With more than 50 ligands and 20 receptors, the chemokine network encodes the requisite information density to tightly control the spatial and temporal trafficking of various leukocyte subsets, and to orchestrate their journey from bone marrow through the circulation to secondary lymphoid organs and ultimately to sites of inflammation.

Inhibition of chemokine signalling is therefore an attractive target for anti-inflammatory drug design. Compounds which block chemokine signals ought, in principle, to interfere with leukocyte recruitment and consequently reduce the side-effects of inappropriate inflammation. In contrast to current drugs (such as NSAIDs) which dominantly target the consequences of leukocyte activation, rather than preventing the inappropriate accumulation of leukocytes in the first place, chemokine inhibitors would be expected to ameliorate the tissue damage (such as fibrosis) which often accompanies inappropriate inflammation.

Unfortunately, the large number of chemokine ligands, often acting in parallel through different chemokine receptors, leads to considerable functional redundancy within the system. Studies of genetically modified mice with homozygous deletions of the chemokine receptors has

demonstrated that loss of a single chemokine signal often has little or no discernible effect on immune function.

As a result, classical receptor antagonists, acting at a single chemokine receptor may be less effective as anti-inflammatory therapies than was once hoped. A range of such compounds have been identified in classical high-throughput screening approaches, and their properties have been reviewed in depth elsewhere [1].

Broad-spectrum Chemokine Inhibitors

In marked contrast, we have described a series of compounds which are capable of blocking leukocyte migration in response to a wide range of chemokines. The first compound with such properties to be described was the dodecapeptide termed Peptide 3 [2], derived from the sequence of the human chemokine MCP-1. Peptide 3 block migration both of freshly prepared peripheral blood leukocytes and of the myelomonocytic cell line THP-1 in response to MCP-1, MIP-1a, RANTES, IL-8 and SDF-1a. Although this peptide was not particularly potent (blocking 50% of the chemokine-induced migration at concentrations around 10 μ M), it was nevertheless powerful: at

Abbreviations used in this paper:

BSCI, Broad-spectrum Chemokine Inhibitors; MCP-1, monocyte chemoattractant protein-1

concentrations of 100 μ M and above chemokine-induced migration was completely abolished. Peptide 3, however, had no effect whatsoever on leukocyte migration induced by a range of non-chemokine chemoattractants, including TGF- β , fMLP and C5a.

Peptide, such as Peptide 3, are not particularly useful for administration *in vivo*, being rapid cleared by the kidneys and also subject to rapid degradation by proteases. However, retroinverso peptide analogs (composed of D-configuration amino acids in the reverse sequence of the original peptide) have more suitable properties *in vivo*, but often fail to retain the biological activity of the parent peptide. In the case of Peptide 3, the cyclic retroinverso analog (termed NR58-3.14.3) was not only active as a chemokine inhibitor, it was almost 1,000 times more potent than the L-amino acid containing peptide [3].

NR58-3.14.3 has been extensively studied both *in vitro* and *in vivo* [4], and it is already clear that it possesses powerful anti-inflammatory properties. Unfortunately, it has a number of disadvantages: in particular, D-amino acid containing peptides are expensive to synthesise. A typical human dose has been estimated to cost up to \$6,000 for a single injection that would have to be given daily.

This has driven a programme to develop non-peptide BSCIs, which retain the ability of NR58-3.14.3. to block leukocyte migration specifically induced by chemokines, but which are considerably cheaper to produce. Such compounds may also improve on the pharmacokinetic properties of NR58-3.14.3, which like all water-soluble peptides is rapidly cleared from the circulation (with a half-life less than 30 minutes) and is not orally bioavailable [5].

An extensive medicinal chemistry programme has identified at least 3 structurally distinct families of non-peptide BSCIs (Figure 1).

The structure:function studies of these compound series have been reviewed extensively elsewhere [6], and the structures shown in Figure 1 represent the most potent members of each structural class which have been described in the public domain.

Selecting a BSCI for use *in vivo*

Each of the 4 families of BSCIs have advantages and disadvantages for use *in vivo* (Table 1). However, the aminoglutaramide family (represented by NR58,4) can be eliminated as a practical choice for all but the shortest duration experiments because of its instability *in vivo*. We have recently demonstrated that NR58,26 (a closely related analog of NR58,4) undergoes a rapid enzyme-catalysed ring opening reaction in serum, yielding N-substituted glutamine and isoglutamine metabolites which are then more slowly deaminated to N-acyl-glutamate [7].

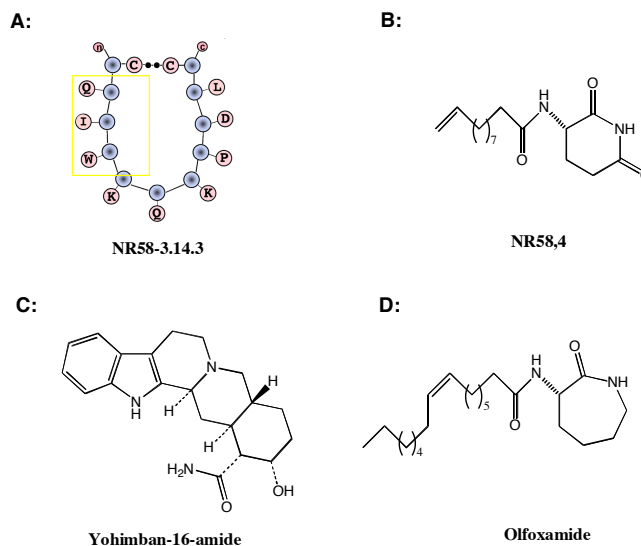


FIGURE 1: Chemical Structures of Representative BSCIs from 4 Different Structural Families. A schematic of the structure of NR58-3.14.3 (A:) is shown as a representative of the peptide BSCIs. The key WxQ pharmacophore is highlighted by the yellow box. NR58,4 (N-undec-10-enoyl)-3-amino glutaramide; B:) is shown as a representative of the N-substituted aminoglutaramides. Yohimban-16-amide (C:) is shown as a representative of the yohimbamide series. Olfoxamide (N-oleoyl- ϵ -aminocapro lactam; D:) is shown as a representative of the Foxamide (aminocapro lactam) series.

Elimination of the carbonyl group at the 6-position yielded the stable analog 6-deoxo-NR58,4 [8], which was subsequently modified to yield Olfoxamide. In marked contrast to the rapid metabolism of the aminoglutaramide ring structure, the aminocapro lactam ring of the Foxamide series is completely stable *in vivo*. Largely as a result of this increased stability, Olfoxamide is an order of magnitude more potent as an anti-inflammatory agent *in vivo* than NR58,4.

The Yohimbamide series, typified by yohimban-16-amide has similar potency *in vivo* to Olfoxamide, but it is considerably more expensive to synthesise and is less readily stored due to the facile photo-catalysed oxidation of the C-ring to yield intensely orange pyridinium salt derivatives on standing in air. Although this problem can be mitigated by storing yohimbamides as the hydrochloride salt rather than as the free base, the small residual α -adrenergic agonist activity makes experiments using these compounds more difficult to interpret than for the Foxamide series which do not apparently cross-react to any other GPCRs. Consequently, except in exceptional circumstances, we recommend the use of either NR58-3.14.3 or Olfoxamide to achieve broad-spectrum chemokine inhibition *in vivo*.

Selecting between these two compounds is more difficult, and depends on the particular experimental design being

Peptide BSCIs NR58-3.14.3	Aminoglutarimides NR58,4	Yohimbamides Yohimban-16-amide	Foxamides Olfoxamide
<p>Advantages: Well established Readily available Low toxicity</p> <p>Disadvantages: Expensive Relatively low potency Poor pharmacokinetics Not orally bioavailable</p>	<p>Advantages: Cheap, facile synthesis Properties published</p> <p>Disadvantages: Unstable in vivo</p>	<p>Advantages: Good pharmacokinetics</p> <p>Disadvantages: α-adrenergic activity Not readily available Free base not very stable</p>	<p>Advantages: Cheap, facile synthesis Good pharmacokinetics High potency Orally bioavailable</p> <p>Disadvantages: Few published properties Not readily available</p>

TABLE 1: Advantages and Disadvantages of Various BSCI Compounds for Use In Vivo.

considered. NR58-3.14.3 has been extensively described in the peer-reviewed scientific literature, and its properties in vivo are relatively well understood. It is also readily commercially available (most custom peptide manufacturers can supply this reagent), although it is very expensive, particularly as a result of its relatively low potency in vivo which requires doses in excess of 10mg/kg/day. Consequently, NR58-3.14.3 is usually the BSCI of choice only for short duration experiments in small animals (particularly mice), at least for the present.

In contrast, Olfoxamide was only synthesised in 2003, and even the first manuscripts describing its properties have yet to be published. Furthermore, without resorting to custom organic synthesis, this reagent is only available under license. However, it is cheap to synthesise and therefore can readily be made in large quantities, and it is relatively potent in vivo reducing the required daily dose. It is also orally bioavailable and has a long plasma residence time facilitating chronic dosing studies, even in larger animals. Thus, as publications describing the properties of Olfoxamide in more detail reach the literature it seems likely this compound will become the BSCI of choice for most, if not all, in vivo experiments, particularly if chronic dosing is required.

NR58-3.14.3

For experiments in models of acute inflammation (such as sub-lethal endotoxemia), NR58-3.14.3 has been routinely delivered as a single bolus dose. It can be administered by intravenous (iv), subcutaneous (sc) or intraperitoneal (ip) routes, as a simple solution in aqueous buffered saline. NR58-3.14.3 is very water soluble, and solutions up to 100mg/ml can be readily prepared. As a result, in most experiments we have used a physiological buffered saline

as the injection vehicle. The pharmacokinetics following iv, sc and ip injection are similar, although peak plasma concentration is achieved immediately after iv dosing, but 20-40 minutes after sc or ip dosing.

Where dose ranging has been performed (for example in the sub-lethal endotoxemia model or in the lung fibrosis model [9]), a maximal anti-inflammatory effect is achieved at doses of 30mg/kg and higher. The dose required for half-maximal effect is approximately 10mg/kg (Figure 2).

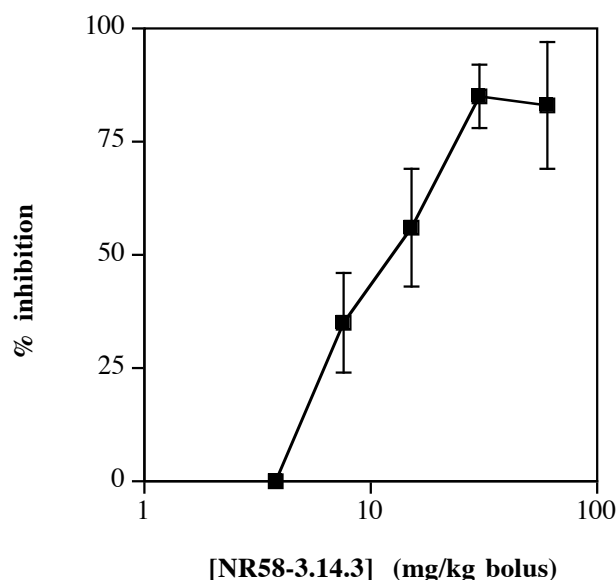


FIGURE 2: Dose-response curve for NR58-3.14.3 in a rodent model of lung fibrosis. NR58-3.14.3 inhibits lung fibrosis [9] in a dose-dependent fashion with half-maximal effect at ~10mg/kg and maximal effect at 30mg/kg bolus, via the sc route. Data courtesy of Dr. M Mulligan (Seattle, Wa., USA). Most experiments using NR58-3.14.3 published to date have administered the drug via the sc route. This allows

the inflammatory challenge to be delivered intraperitoneally, and ensures that there is no local delivery effect which might result if the drug and inflammatory challenge were delivered to the same anatomical site. Thus, we can be confident that NR58-3.14.3 is a systemically acting anti-inflammatory agent. However, in the few instances where local delivery of the drug has been attempted no benefit has been observed over systemic administration.

Although no comprehensive study has been reported, we routinely administer the drug prior to the inflammatory challenge. In the sub-lethal endotoxemia model, where NR58-3.14.3 has been most extensively studied, the maximal anti-inflammatory effect was seen when the drug was administered via the sc route between 30 and 120 minutes prior to the inflammatory challenge (Figure 3).

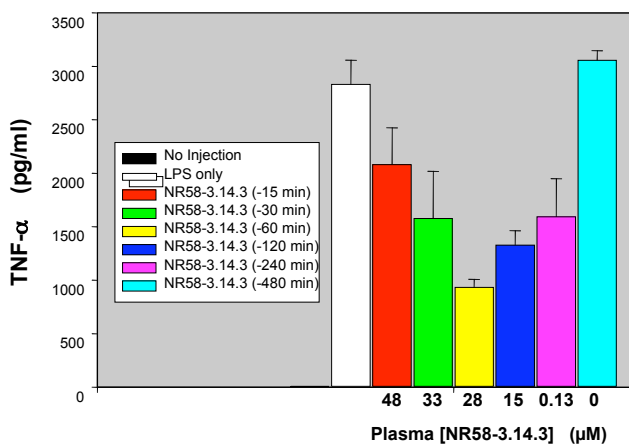


FIGURE 3: Effect of duration of pre-treatment with NR58-3.14.3 on the subsequent anti-inflammatory effect. In the murine sub-lethal endotoxemia assay [8], groups of 6 mice received a standard 30mg/kg bolus dose of NR58-3.14.3 via the sc route at various times (from 15mins to 4 hours) prior to the LPS challenge. Three hours after LPS challenge, the invoked inflammation was estimated using the plasma TNF- α concentration. The anti-inflammatory effect of NR58-3.14.3 was maximal when the drugs was given 60 minutes prior to the inflammatory challenge, even though the plasma drug concentration at the point of LPS challenge was higher at earlier timepoints.

NR58-3.14.3 has also been used in chronic models of inflammation, although there are considerably fewer reported studies where dosing was continued for more than a few days. For periods of up to 14 days, it is practicable to administer the drug by daily subcutaneous injection, although the pharmacokinetics of NR58-3.14.3 are such that drug levels in the plasma would only be detectable for 4-6 hours in each 24 hour period. Nevertheless, daily sc dosing at 30mg/kg/day has proved to have a powerful anti-inflammatory effect in a number of chronic models of

inflammation including lung fibrosis and peritoneal adhesion formation. This tentatively suggests that the biological effects of NR58-3.14.3 persist to some extent even after the drug has been cleared from the system.

An alternative approach for chronic dosing is continuous administration, either via an indwelling catheter (for shorter experiments) or via implantable osmotic minipumps (which have been used successfully for up to 6 months). In the former case, complete suppression of leukocyte recruitment was achieved by administration of a bolus 2mg/kg dose, followed by 0.5mg/kg/hour via a jugular catheter in a rodent model of cerebral ischemia [10]. This suggests that NR58-3.14.3 is more potent when the plasma concentration is maintained by constant infusion, compared with repeated sc injections.

A summary of the recommended dosage regimens for NR58-3.14.3 in both acute and chronic experimental models is presented in Box 1.

Drug: NR58-3.14.3 acetate salt
Mol. Wt: 1359
Vehicle: Buffered saline

Acute

oral: Not orally bioavailable
s.c.: 30mg/kg
i.p.: 30mg/kg
i.v.: 30mg/kg

Chronic

oral: Not orally bioavailable
s.c.: 30mg/kg daily injections
 or 30mg/kg/day by minipump
i.p.: 30mg/kg daily injections
i.v.: 2mg/kg bolus + 0.5mg/kg/hr
 continuous infusion

BOX 1: Recommended dosing regimens for NR58-3.14.3 in vivo. NR58-3.14.3 is soluble in physiological buffered saline vehicle to 100mg/ml. The dose required for maximum efficacy via a range of routes is given.

Olfoxamide

One of the major disadvantages of using Olfoxamide as a BSCI in vivo is the lack of information on effective dosing regimens, compared with NR58-3.14.3. To date, all of the available information on the use of this compound in vivo has come from the sub-lethal LPS-induced endotoxemia model [8], and it is difficult to extrapolate from this acute inflammatory model to more disease-relevant models of chronic inflammation. However, we note that for NR58-3.14.3 the dosing regimens which have anti-inflammatory properties in the acute endotoxemia model are also effective in chronic

models of inflammation, providing encouragement that the conditions established for Olfoxamide may also have broad applicability.

In the experiments performed in the acute sub-lethal endotoxemia model, Olfoxamide has been routinely delivered as a single bolus dose by subcutaneous injection. Unlike NR58-3.14.3, however, the Foxamide series have negligible solubility in aqueous solutions, necessitating the use of an organic solvent vehicle. Although Olfoxamide is considerably more soluble than the canonical Foxamide C16, it is nevertheless necessary to use a vehicle such as DMSO to achieve solutions of 10mg/ml. We routinely use a vehicle of 75% DMSO in physiological saline for sc injections of Olfoxamide.

One of the major advantages of using Olfoxamide, however, is that the Foxamides are considerably more potent than NR58-3.14.3, at least in the sub-lethal endotoxemia model (Figure 4). The dose required to achieve a half-maximal inhibition of TNF- α is approximately two orders of magnitude lower for Olfoxamide (0.2 mg/kg versus 15 mg/kg for NR58-3.14.3). Doses of 2mg/kg and higher result in maximal suppression of TNF-a in this model.

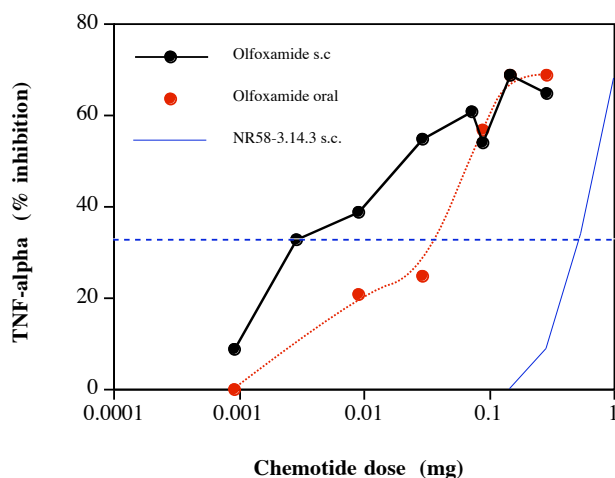


FIGURE 4: Dose-response curve for Olfoxamide in a rodent model of endotoxemia. Olfoxamide inhibits LPS-induced TNF- α production in vivo [8] in a dose-dependent fashion with half-maximal effect at ~0.2mg/kg and maximal effect at 2mg/kg bolus, via the sc route (black line). Olfoxamide is also effective via the oral route (red line) although it is significantly less potent. Data for NR58-3.14.3 via the sc route (blue line) is shown for comparison.

Another major advantage of Olfoxamide and other Foxamides, compared with the peptide BSCIs, is that they exhibit anti-inflammatory activity when dosed via the oral route. The data for Olfoxamide in the sub-lethal endotoxemia model is shown in Figure 4. A single oral administration of Olfoxamide at 3mg/kg maximally

inhibited TNF- α production in this model. While sc injection is a viable route for short term studies in acute models of inflammation, the ability to perform oral dosing considerably simplifies chronic experiments which have previously required implantable osmotic minipumps to perform using NR58-3.14.3. It is worth noting, however, that to date the ability of Olfoxamide to block chemokine-induced leukocyte recruitment in a model of chronic inflammation (whether by the sc or oral route) has not yet been reported.

The nature of the vehicle may be important when comparing sc and oral routes of delivery. The high DMSO content of the vehicle we use for sc injections might artificially elevate the apparent oral bioavailability of a hydrophobic molecule such as Olfoxamide by carrying it through the stomach and into the small intestine. Such an effect might be beneficial in mechanistic studies investigating the impact of broad-spectrum chemokine inhibition on an inflammatory disease process, but it may be unhelpful in drug development studies. Consequently, we routinely perform oral dosing in a low DMSO formulation, consisting of 0.6% DMSO and 1% carboxymethylcellulose in physiological saline. This formulation results in a fine milky microsuspension rather than a solution, but the data in Figure 4 clearly demonstrates that acceptable absorption does occur under these conditions.

Drug : Olfoxamide
Mol. Wt : 392
Vehicle: 75% DMSO in buffered saline
 or 0.6% DMSO / 1% CM-cellulose
 in physiological saline

Acute

oral : 5mg/kg
s.c. : 2mg/kg
i.p. : 2mg/kg suggested
i.v. : 2mg/kg suggested

Chronic

oral : 5mg/kg/day suggested
s.c. : 2mg/kg/day suggested
i.p. : 2mg/kg/day suggested
i.v. : Not recommended

BOX 2: Recommended dosing regimens for Olfoxamide

in vivo. Olfoxamide is soluble in 75% DMSO in physiological buffered saline vehicle to 100mg/ml. Alternatively, a microsuspension can be formed in 0.6% DMSO / 1% CM-cellulose in buffered saline (the drug is first dissolved to 200mg/ml in 100% DMSO, then diluted into CM-cellulose containing saline solution). The dose required for maximum efficacy via a range of routes is given. Where the effect of Olfoxamide delivered by a particular route has not been reported, a suggested dosing regimen is given.

A summary of the recommended dosage regimens for Olfoxamide in both acute and chronic experimental models is presented in Box 2.

Other considerations

An important consideration which can affect the choice of BSCI for experimentation is the cost and availability of the various molecules. All of the BSCIs described to date are the subject of patents (either granted or pending) belonging to the University of Cambridge, UK. However, the structure of the various compounds described here are in the public domain, and it is legal to synthesise these agents for any not-for-profit purposes, such as academic research. None of the BSCIs are available from commercial sources “off-the-shelf”, but the peptide NR58-3.14.3 can be readily synthesised by custom peptide manufacturers. Unfortunately, the D-amino acid composition of the peptide means that the cost of purified NR58-3.14.3 is high. Alternatively, it may be possible to obtain these BSCIs from the patent owner or its current licensee, and the current contact details to request access to the compounds are appended to this document.

Much of the data reported on the use of BSCIs in vivo has come from rodent models (particularly murine sub-lethal endotoxemia, and rat models of dermal and cerebral inflammation). However, we have shown that NR58-3.14.3 is equally effective in vitro against sheep, rabbit and human leukocytes. Consequently, it seems likely that the BSCIs described here will be functional in a broad range of mammalian species in vivo, although this is yet to be formally demonstrated.

To date, there has been no extensive reports of the toxicological characteristics of BSCIs, although it is clear from the efficacy studies reported that both acute and chronic administration of these compounds is relatively non-toxic. The acute LD50 for NR58-3.14.3 in mice is approximately 500mg/kg iv, with death resulting from the compound precipitating out of solution in the lungs. Consequently, NR58-3.14.3 can be considered a very safe compound, at least following a single administration. The no effect level in mice is approximately 100mg/kg by sc injection: at higher doses mild hypoactivity is seen, which increases in severity as the doses rises, ultimately resulting in ptosis and ventral decubitus at very high doses. Consequently, the dosing regimens recommended here to achieve maximal anti-inflammatory activity are not associated with any detectable toxicity. The window between safety and efficacy is considerably wider for the Foxamide compounds, with maximal efficacy at 2mg/kg but still with a no effect level of 100mg/kg. Although no formal toxicity studies have been performed using multiple dosing protocols, we have performed a histological examination of mice following a six month treatment period with NR58-3.14.3 at approximately 30mg/kg/day via implantable osmotic minipumps: no histological

abnormalities were detected. Importantly, we demonstrated that the resting blood leukocyte counts were unaffected by either acute or chronic treatment with NR58-3.14.3, suggesting that the compounds specifically target stimulated leukocyte recruitment during inflammation and leave basal leukocyte trafficking relatively untouched.

Although these compounds were identified as functional inhibitors of chemokine-induced leukocyte migration, it is clear that they do not act as chemokine receptor antagonists. The precise molecular mechanism of action is therefore unknown, although several possibilities have been discussed at length [4,6]. It seems likely that the compounds described here are agonists at a currently unidentified GPCR, and that the signals generated effectively blind the cell to the directional movement cue provided by the chemokine signal. Despite the current lack of detailed knowledge of the molecular mode of action, it is clear that the BSCIs described here are highly selective for chemokines: they do not block leukocyte migration induced by a range of other non-chemokine chemoattractants. Ultimately, their utility as academic tools to probe the physiology of inflammatory disease processes will depend on the elucidation of the molecular mechanism and this aspect of the biology of BSCIs is the subject of intensive study at the present time,

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