Broad Spectrum Chemokine Inhibitors Related to NR58-3.14.3

David J. Grainger*[,1], Jill Reckless[1] and David J. Fox[1,2]

[1]*Department of Medicine, University of Cambridge, Box 157, Addenbrooke's Hospital, Cambridge, CB2 2QQ, UK*

[2]*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK*

Abstract: The chemokine family consists of more than 50 structurally-related small proteins which signal through type 1 G-protein coupled receptors (GPCRs) to regulate a range of immune functions, with particular focus on regulating leukocyte trafficking. They have been implicated both in normal physiological leukocyte traffic, and in recruitment of leukocytes to sites of pathological inflammation. As a result, chemokine inhibitors may have useful anti-inflammatory therapeutic properties *in vivo*. Compounds with chemokine-inhibitory properties that have been described to date, fall into two broad categories: receptor-specific antagonists which block the action of one or a small number of related chemokines, and broad-spectrum chemokine inhibitors (BSCIs) which block leukocyte migration in response to many, if not all, chemokines simultaneously. Since many chemokines apparently show functional redundancy *in vivo*, the BSCI class are attractive candidates for development as anti-inflammatory therapies. Here, we review the development of BSCIs, with particular focus on the design and characterisation of non-peptide compounds. The key structural requirements for BSCI activity are discussed, together with their implications for the mechanism of BSCI action.

INTRODUCTION

Inappropriate leukocyte recruitment is an important component of a wide range of diseases, both those with an obvious inflammatory component (such as autoimmune disorders [1] or atherosclerosis [2]), and those where role of the leukocytes is more subtle, but nonetheless, central to the pathogenic mechanism (such as osteoporosis [3] or Alzheimer's Disease [4,5]). Consequently, the signalling molecules responsible for regulating leukocyte trafficking have become important subjects for academic research and key targets for the pharmaceutical industry.

The chemokine family plays a central role in this regulation of leukocyte trafficking, acting as chemoattractants (and perhaps in a few cases as chemorepellents) for various leukocyte subsets [6,7]. The structural diversity of the chemokine family (with more than 50 ligands and some 20 different receptors) allows for considerable fine tuning of leukocyte recruitment, allowing both the tissue location and the leukocyte population participating in the inflammatory response to be tightly controlled.

However, in addition to allowing fine control of leukocyte recruitment, the complexity of the chemokine system also embodies considerable functional redundancy, which has been highlighted by the results of gene knockout studies in mice [8]. Consequently, the chemokine inhibitors that have been described to date can be divided into two broad classes: (a) receptor antagonists which are specific for one or a small number of chemokine receptors [9-11] and (b) functional inhibitors that block leukocyte recruitment in response to a broad range of chemokines [12]. These latter agents have been termed broad-spectrum chemokine inhibitors (BSCIs).

It seems likely that the receptor antagonists (which have mostly been identified through conventional screening programmes) will allow subtle control of the inflammatory response, perhaps modulating the composition of the leukocyte population present, but due to the redundancy of the chemokine system, they may not abrogate all of the pathological aspects of an inappropriate inflammatory response. To some extent, this view has been borne out by the limited *in vivo* data which has been reported on these compounds [10,11,13,14].

In marked contrast, BSCIs would be expected to exhibit a powerful anti-inflammatory effect (perhaps similar to that seen with steroids), but the concern must be that broad inhibition of chemokine function (and hence leukocyte trafficking) would lead to undesirable side effects, resulting from at least a partial blockade of normal immune cell function. Again, this expectation has only been partly supported by the published studies of BSCI activity *in vivo*: the best studied BSCI compound, a cyclic D-amino acid containing 13-mer peptide NR58-3.14.3, has the expected wide-ranging anti-inflammatory properties [15], but apparently very few acute or chronic side-effects [12].

Based on the limited *in vivo* data published so far, it is plausible that there will exist therapeutic applications ideally suited to specific receptor antagonists (for example, where relatively subtle changes in the leukocyte addressing are required), and different applications ideally suited to BSCIs (where a more general anti-inflammatory effect is required). The receptor-specific chemokine antagonists have been extensively reviewed previously [9], and elsewhere in this issue. The biological properties of BSCIs, and in particular of NR58-3.14.3, have also been reviewed previously [12]. The purpose of this article is to review the development of BSCIs, with particular focus on the design and characterisation of non-peptide analogues of NR58-3.14.3.

STRUCTURE ACTIVITY RELATIONSHIP AMONG PEPTIDE BSCIs

The first molecule to be described with BSCI properties was a 12-amino acid peptide sequence from the C-terminal

*Address correspondence to this author at the Department of Medicine, University of Cambridge, Box 157, Addenbrooke's Hospital, Cambridge, CB2 2QQ, UK; Tel: 01223 336812; E-mail: djg15@cam.ac.uk

1389-5575/05 $50.00+.00

region of the human chemokine MCP-1. This peptide, termed Peptide 3, inhibited the migration of both freshly prepared human leukocytes and immortal leukocyte cell lines in response to several different chemokines, but did not affect migration induced by other chemoattractants (including fMLP, C5a and TGF-β) [16]. Although Peptide 3 was not very potent (having a half-maximal effect at around 10μM), it was nevertheless able to completely block chemokine-induced leukocyte migration at concentrations above 100μM.

As a first step towards generating an analogue of Peptide 3 which was suitable for use *in vivo*, we described the properties of a cyclic retroinverso analogue [17] of a Peptide 3-related sequence [15] (termed NR58-3.14.3). This compound possessed similar properties to Peptide 3, but was considerably more potent, inhibiting MCP-1 induced leukocyte migration with an ED50 of approximately 5nM [15]. Although NR58-3.14.3 is rapidly excreted *via* the kidneys following intravenous injection (with a plasma half-life of less than 30 minutes [18]), it was nevertheless useful to demonstrate that BSCIs do indeed have anti-inflammatory properties *in vivo*. NR58-3.14.3 has now been shown to reduce leukocyte recruitment (and ameliorate related symptoms) in a wide range of animal models of acute and chronic inflammation, including LPS-induced endotoxemia, LPS-induced dermal inflammation, ovalbumin-induced asthma, atherosclerosis, stroke and lung fibrosis [12,15,19,20].

Having demonstrated the therapeutic potential for BSCIs using NR58-3.14.3, the next step was the development of compounds with BSCI activity, which were suitable for pharmaceutical development. Such molecules would ideally have oral bioavailability, good stability and pharmacokinetic properties, potency *in vivo* and good toxicological profiles, as well as being cheaper to synthesise than the D-amino acid containing peptides such as NR58-3.14.3.

Designing such non-peptide analogues required an understanding of the key structural elements of the large NR58-3.14.3 molecule, which conferred the BSCI properties. We therefore performed an extensive deletion and substitution analysis of the peptide sequence in order to identify the key pharmacophores [21]. This approach identified a tripeptide motif at the C-terminus of the original peptide 3 sequence which retained most of the activity of the original peptide. Furthermore, substitution of these same residues, and in particular, the glutamine at position 12 completely abolished the BSCI activity of the peptide (Fig. **1**).

Based on these findings, we synthesised a small library of compounds, which combined a range of glutamine mimetics with simple hydrophobic tail groups. Surprisingly, one of these compounds, (S)-3-(undec-10-enoylamino)glutarimide possessed potent BSCI activity [21].

N-(ACYLAMINO)GLUTARIMIDES AS BSCIs

(S)-3-(undec-10-enoylamino)glutarimide (also termed NR58,4) inhibited chemokine-induced leukocyte migration with an ED50 of approximately 5nM, but had no effect on

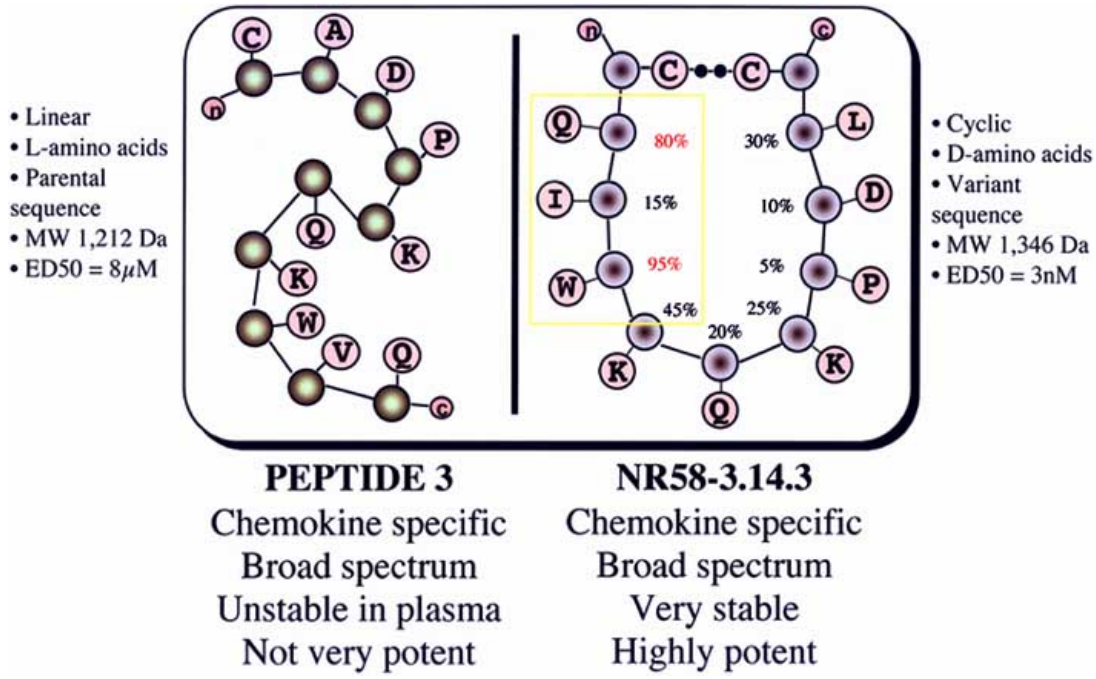


Fig. (1). Development of NR58-3.14.3. The properties of the retroinverso analogue NR58-3.14.3 are compared with the parental Peptide 3 sequence. Green spheres represent amino acid α-carbon centres with L-configuration, while blue spheres represent the D-configuration. The percentage figures assigned to each residue in the NR58-3.14.3 sequence represent the percentage of amino acid substitutions at that position which result in at least a 10-fold decrease in potency as a BSCI, assayed as the ability to inhibit IL-8 induced migration of freshly prepared human neutrophils (data taken from Figure 1 of ref 21). The key pharmacophores identified by this substitution analysis, and confirmed by deletion analysis, are highlighted by the yellow box. Figure reproduced with permission from www.graingerlab.org.

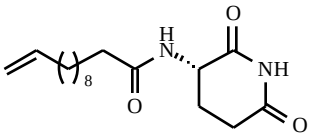
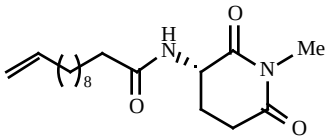
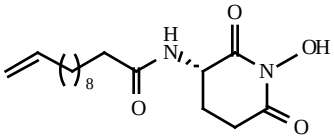
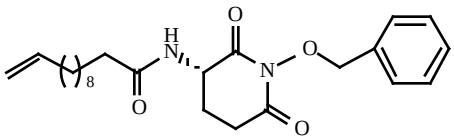
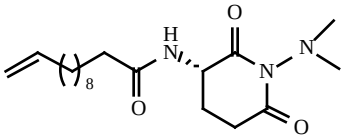
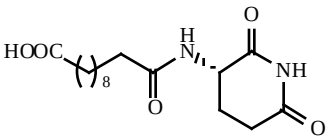
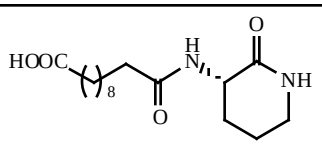
leukocyte migration induced by a range of other chemoattractants (and therefore had biological activity equivalent to NR58-3.14.3) [21]. We synthesised a range of compounds around this lead structure, to determine the structural features which were responsible for the BSCI activity.

Most changes to the aminoglutarimide ring resulted in considerable loss of BSCI activity. For example, substitution at the imide nitrogen (with methyl, hydroxy, O-benzyl or dimethylamino groups) all resulted in at least 5-fold loss of activity (Table **1**). In general, the bulkier substituents reduced the activity to the greatest extent, with the O-benzyl derivative being essentially devoid of BSCI activity. Similarly, deletion of the carbonyl group at position 6 to yield the 6-deoxo analogue reduced BSCI activity 20-fold [21]. To date, the 2-deoxo analogue has not been reported.

The stereochemistry of the ring is also important for BSCI activity. Both the (R)- and (S)-enantiomers of NR58,4 have been synthesised and compared for activity. Fortunately, the hydrogen at the 3-position of the ring is less acidic than in thalidomide, which is the most extensively studied aminoglutarimide. As a result, although thalidomide racemises relatively quickly in aqueous solution [22] (and hence the α-methyl derivatives had to be used to compare the activity of the enantiomers [23]), short-chain N-acyl-3-aminoglutarimides are completely stable in aqueous solution at physiological pH, at least for a period of hours (Fox, D. and Greig, I, personal communication). The biological activity of the (R)-enantiomer of NR58,4 was at least 25-fold lower than the (S)-enantiomer.

The nature of the acyl substituent also has some effect on the BSCI activity of the compounds, although generally, the activity is tolerant of more structural diversity than in the aminoglutarimide ring [21]. The only exception is the amide linker: N-(alkylamino)glutarimides which lack the amide carbonyl group are completely inactive as BSCIs [12]. Changes at the 2-position of the acyl group are also

Table 1. BSCI Activity of Various Analogues of NR58,4

cyclic(CQIWKQPDLC) **NR58-3.14.3**	3nM
 **NR58, 4**	5nM
	33nM
	2,000nM
	80,000nM
	300nM
	66nM
	100nM

The activity shown represents the ED50 for inhibition of migration of the human myelomonocytic cell line THP-1 induced by MCP-1 (25ng/ml) determined as described previously [15]. The activity of the parental peptide [15] and NR58,4 [21] are shown for comparison.

unfavourable, with bulky substituents reducing the BSCI activity considerably (for example, both (S)-3-(2'2'-dimethyldodec-10-enoylamino)glutarimide [12] and (S)-3-(2'-propylpentanoylamino)glutarimide [21] are at least 100-fold less active than NR58,4. Interestingly, this is consistent with the findings from the original Peptide 3 structure:function analysis which demonstrated that substitution of the equivalent carbon in WxQ tripeptides reduced the BSCI activity (see Fig. **2** of ref [21]).

Changes further away from the aminoglutarimide ring are generally well tolerated, and aminoglutarimides with a wide range of peptide and peptide-like groups attached through a glycine linker have been synthesised and shown to have BSCI activity (J. Dong, personal communication). A potentially interesting exception was the compound (S)-3-(9-carboxynonanoyl)-aminoglutarimide: introduction of the charged carboxyl group at the ω-position reduced BSCI activity by more than 10-fold, when assayed using the human myelomonocytic cell line THP-1, but had virtually no effect on biological activity assayed using human neutrophils [21]. This is in stark contrast to the other BSCIs which show no cell type selectivity (Fig. **2**).

Any interpretation of the impact of ω-substitution on the cell type specificity of the BSCI activity is hampered by our general lack of understanding of the molecular mechanism of any of the BSCI compounds (reviewed in depth elsewhere [12]). However, it tentatively suggests that the aminoglutarimides may be acting at subtly different molecular targets (potentially cell surface receptors) in the different leukocyte subsets. Until the molecular targets have been positively identified, however, it will be difficult to confirm or refute this postulate, although ultimately ω-substituted BSCIs may find utility in the treatment of diseases where inhibition of specific leukocyte subset is required.

A wide range of substituted aminoglutarimides have now been synthesised and tested for BSCI activity *in vitro* [12,21]. To our knowledge, however, only one (NR58,4) has been tested for anti-inflammatory activity *in vivo*. The contribution of structural diversity to biological activity *in vivo* is therefore entirely unknown. NR58,4 has potent anti-inflammatory effects in models of acute inflammation *in vivo* (being at least 4-fold more potent than NR58-3.14.3 on a molar basis [21]), but surprisingly was ineffective in models of chronic inflammation where NR58-3.14.3 was known to be effective (such as ovalbumin-induced asthma). Recent stability studies provide a likely explanation for this observation: unlike NR58-3.14.3, we found that NR58,4 was rapidly metabolised in serum at 37ºC, despite being stable in physiological saline solution for many days under the same conditions. The dominant metabolic reaction is apparently due to the action of an imidase present in serum, which opens the aminoglutarimide ring to yield a mixture of N-substituted glutamine and isoglutamine. These metabolites are then further deaminated to eventually yield glutamate. All of the simple N-substituted aminoglutarimides that we have tested are subject to this metabolic reaction, yielding biologically inactive products. It is likely, therefore, that for many *in vivo* applications (particularly where a chronic effect is required), N-(acylamino)glutarimides are unsuitable for use because of the rapid metabolism of the core imide ring.

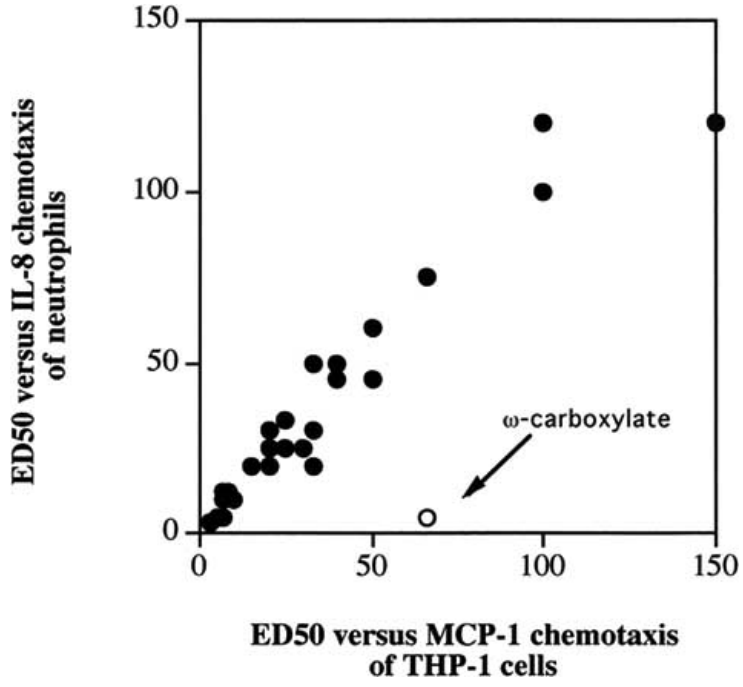


Fig. (2). Most BSCI s show little or no cell type selectivity. The potency of more than 25 different BSCI compounds (including NR58-3.14.3, various (acylamino)-glutarimide, yohimbanic acid derivatives and (acylamino)caprolactams) are shown assayed against MCP-1 (25ng/ml) induced chemotaxis of myelomonocytic THP-1 cells and against IL-8 (100ng/ml) induced chemotaxis of freshly prepared human peripheral blood neutrophils, all as previously described [15]. Of these compounds, only the ω-carboxylate derivative of NR58,4 ([S]-3-(9-carboxynonanoylamino)glutarimide [21]) shows a reproducibly different potency against the two cell types (open circle).

16-SUBSTITUTED DERIVATIVES OF YOHIMBINE

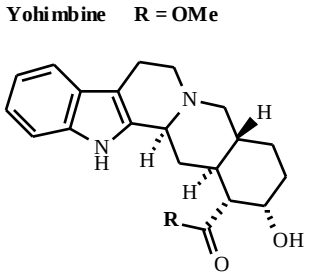
The plant alkaloids yohimbine and raubasine [24], originally purified from the root of *Rauwolfia Serpentina* are well known alpha-adrenoceptor agonists, with nanomolar affinity (Fig. **3**). Crude molecular modelling studies suggested weak structural analogy between yohimbine, and the WxQ motif responsible for the BSCI activity of Peptide 3 and NR58-3.14.3. Yohimbine was therefore tested for chemokine-inhibitory activity, and was found to possess weak BSCI activity (inhibiting MCP-1 induced THP-1 cell migration with an ED50 of 700nM, while having no effect on migration induced by fMLP) [25].

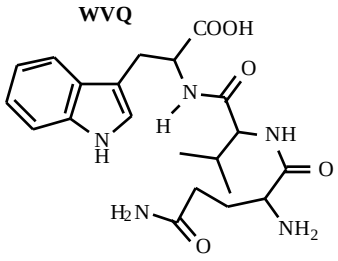
Yohimbine is the methyl ester of yohimbanic acid (which has only weak α-adrenomimetic function and no BSCI activity). We therefore synthesised the primary amide of yohimbanic acid, reasoning that this may more closely recapitulate the structural requirements for BSCI activity, than either the parental acid or methyl ester. Treatment of yohimbine with sodium amide in liquid ammonia yielded yohimban-16-amide in good yield [25], provided that reaction was performed in the dark to prevent the formation of intensely orange pyridinium salts through deprotonation of the C-ring. Yohimban-16-amide possessed considerably more potent BSCI activity than either the acid or methyl ester (Fig. **3**), but was considerably weaker as an α-adrenoceptor agonist than yohimbine. The selectivity for chemokine-inhibition over α-adrenoceptor binding was increased more than 1,000 fold in the primary amide compared with yohimbine. Yohimban-16-amide inhibited leukocyte migration induced by the same range of chemokines that were inhibited by NR58-3.14.3 and NR58,4 suggesting that the mechanism underlying the biological activity we observed was similar. Furthermore, yohimban-16-amide prevented the binding of labelled NR58-3.14.3 to the cell surface, tentatively suggesting they shared a molecular target. It is already clear, however, that this target is not the α-adrenoceptor, since Yohimban-16-amide is markedly more potent as a BSCI than yohimbine, despite having reduced α-adrenoceptor binding capacity.

Only a small number of further yohimban-16-amide analogues have been described (such as the N-methyl and N,N-dimethyl secondary and tertiary amides [25]), all of which are less active as BSCIs than the parental compound. To date, no extensive survey of the properties of yohimban-16-amides as BSCIs has been published outside of the patent literature. The photosensitivity of the free bases (though less so for the acetate salts), together with the residual α-adrenoceptor agonist activity may hinder application of yohimban-16-amide derivatives as BSCIs *in vivo,* although both of these potential disadvantages may be eliminated through screening further analogues.

N-SUBSTITUTED 3-AMINO-ε-CAPROLACTAM DERIVATIVES

Following our recent observations that members of the aminoglutarimide series of BSCIs were rapidly metabolised in serum, we embarked on a project to identify stable variants with acceptable potency as BSCIs. It is plausible that substitution of the imide nitrogen might impair the metabolic ring opening reactions. Consequently, we examined the stability of the N-methyl analogue (S)-3-(undec-10-enoylamino)-N-methyl-glutarimide in serum. Although this analogue underwent enzymatic ring opening with a slower time course than the parental NR58,4, the rate of metabolism was still very significant. Although this N-methyl derivative had acceptable potency as a BSCI *in vitro* (approximately 33nM [21]), other analogues with





R	Ka Versus α2-adrenoceptor	ED50 versus chemotaxis	Selectivity	
OMe	3.8	30	0.13	>10,000 fold improvement
OH	330	1000	0.33	
NH2	770	0.5	1,540	
NHMe	-	75	-	
N(Me)2	-	330	-	

Fig. (3). Yohimbine derivatives are crude structural analogues of WxQ tripeptides and show BSCI activity. The crude structural analogy between a highly constrained confirmation of the tripeptide WVQ (shown with no stereochemical definition) and yohimbine is illustrated. The BSCI activity (versus MCP-1induced migration of THP-1 cells) of various yohimbine analogues is shown in the table, and compared with the α2-adrenoceptor binding of the same compounds (determined by competition of the unlabelled yohimbine analogue for the binding of tritiated RS-79948-197 (0.05μCi per reaction at 1nM) to cell membranes containing α2-adrenoceptors, using 10μM oxymetazoline as the positive control). Consistent with the structural analogy to WxQ tripeptide, replacement of the methyl ester of yohimbine with a primary amide group at the 16-position increased the selectivity for BSCI activity over α2-adrenoceptor binding by more than 10,000 fold.

substituents at the imide nitrogen were considerably less active (Table **1**) and so were not tested for stability. It seems unlikely that substitution of the imide nitrogen is a viable strategy for identifying stable and potent BSCI compounds.

We had already synthesised and tested the 6-deoxo analogue of NR58,4, (S)-3-(undec-10-enoylamino)-tetrahydropyridin-2-one, which contains a lactam, rather than an imide ring. Although this compound was considerably less active as a BSCI (approximately 100nM [21], compared to 5nM for NR58,4), we found that it was almost completely stable in serum at 37ºC. The enzymatic reaction which rapidly opened the imide ring did not metabolise the lactam analogue. We therefore synthesised a range of lactam derivatives to determine whether a structure could be found which retained the stability of the 6-deoxo-NR58,4 but regained the potency of the parental molecule.

Among the analogues we initially synthesised, we found that (S)-3-hexadecanoylamino-ε-caprolactam was the most active, with 5nM potency (comparable to the best analogues in both the aminoglutarimide and yohimban-16-amide series, as well as the cyclic peptide NR58-3.14.3). Consistent with the structure activity relationship in the aminoglutarimide series, we found that BSCI potency increased with increasing alkyl chain length. We also found that the (S)-enantiomer was 10-25 fold more active than the (R)-enantiomer. Both these observations suggest that the aminocaprolactam BSCIs were binding to a similar molecular target to the aminoglutarimide BSCIs, and that the structural features which determined potency in the aminoglutarimide series would also apply in large part to the aminocaprolactam series.

Although (S)-3-hexadecanoylamino-ε-caprolactam is both a potent BSCI *in vitro* and a stable compound *in vivo*, it nevertheless has the disadvantage of very poor solubility in any vehicle useful for administering compounds *in vivo*. For example, it is not soluble at concentrations above about 1mg/ml in DMSO, ethanol, DMA or DMF containing vehicles, although it can be administered in soy oil or as a suspension. While a degree of lipophilicity may impart beneficial pharmacokinetic properties on a structure, the degree of insolubility encountered with (S)-3-hexadecanoylamino-ε-caprolactam severely impairs further investigation of its properties *in vivo*.

We are currently investigating a range of strategies to improve the solubility of aminocaprolactam BSCIs, and early findings are encouraging. (S)-(Z)-3-(octadec-9-enoylamino)-ε-caprolactam (BN 83253), for example, is considerably more soluble in DMSO and other biologically compatible solvents than the parental structure. The introduction of a cis-double bond markedly lowers the melting point of the compound, and presumably favours dissociation of the solid into solution. As with aminoglutarimide series, BSCI activity is tolerant of the introduction of double bonds into the alkyl tail group, and indeed, may actually increase slightly.

The substituted aminocaprolactam BSCIs have only recently been described, and it is still too early to be certain of the properties of these compounds, particularly *in vivo*. Although both (S)-3-hexadecanoylamino-ε-caprolactam and (S)-(Z)-3-(octadec-9-enoylamino)-ε-caprolactam have potent anti-inflammatory activity in a murine acute endotoxemia model, their properties in models of inflammation more relevant to human disease are unknown. Nevertheless, their combination of facile synthesis, stability *in vivo*, good oral bioavailability and potency *in vitro* suggest they are promising candidates for further investigation.

ANTI-INFLAMMATORY EFFECTS OF BSCIs *IN VIVO*

Since chemokines have been implicated in a wide range of diseases with an inflammatory component [12,15,19-21], it is difficult to be certain in which indications BSCIs might be most effective. However, while the cyclic peptide NR58-3.14.3 was not ideally suited for administration in humans, nevertheless it has been very useful for probing the impact of broad-spectrum chemokine inhibition in a variety of animal models of inflammation.

The initial proof-of-principle study was performed in a rodent model of dermal inflammation, in which the chemokine MCP-1 was injected directly into rat skin, resulting in a mononuclear cell rich inflammatory response over 24 hours. Systemic treatment with a single dose of NR58-3.14.3 abolished leukocyte recruitment in response to MCP-1 *in vivo* [15]. This powerful anti-inflammatoryeffect was also seen when bacterial lipopolysaccharide was used as the inflammatory stimulus suggesting that chemokines are responsible for much of the leukocyte recruitment that occurs in response to LPS challenge. Crucially, NR58-3.14.3 was more effective in this model than neutralising antibodies against any individual chemokine [15] and referencestherein, tentatively suggesting that BSCIs may possess more powerful anti-inflammatory activity than the chemokine-receptor specific antagonists.

Much of the systematic characterisation of BSCI function *in vivo* has been performed using a related LPS-induced inflammatory response: TNF-α production 2 hours after a single intraperitoneal injection of LPS in mice [21]. This model has a number of advantages, mostly related to the speed and ease of execution of the experiments. It is important to remember, however, that LPS-induced TNF-α production is a very indirect endpoint that may reflect the systemic anti-inflammatory properties of the administered compound, but that provides no measure of the chemokine-dependency of the activity, or the degree to which local leukocyte recruitment is inhibited. Furthermore, any anti-inflammatory activity in this model depends not only on the BSCI activity of the molecule, but also on its pharmacokinetic properties: a very active molecule introduced *via* an inappropriate route produces no suppression of TNF-α. For example, oral administration of NR58-3.14.3 (which is less than 1% orally bioavailable compared with sub-cutaneous injection) has no effect on TNF-α production in this model. Overall, this model is useful as a preliminary *in vivo* screen, but promising compounds need to be investigated in depth in disease-relevant preclinical models. Past experience, however, indicates that murine sub-lethal endotoxemia is not a useful model of human endotoxic shock.

The fact that NR58-3.14.3 has been reported to have powerful anti-inflammatory activity, and associated benefits, in a wide range of animal models of disease is encouraging, but can hamper selection of an appropriate indication for

development of the more potent non-peptide BSCIs. Factors distinct from the biological properties of the BSCIs, such as the relative ease of performing early stage clinical trials, seem likely to govern this decision.

MOLECULAR MECHANISM OF BSCI ACTIVITY

While the anti-inflammatory activity of BSCIs *in vivo* is now well documented [12,15,19-21], the molecular interactions which lead to chemokine-specific blockade of leukocyte migration remain enigmatic. Previous studies [12] have eliminated a number of plausible sites of action for the compounds: for example, they have been shown to have no effect on the binding of a wide range of chemokines to their receptors, nor to bind allosterically to those chemokine receptors. They do not affect chemokine receptor expression or trafficking to or from the cell surface, nor do they block early signalling events (such as calcium mobilisation), shortly after chemokine receptor activation. Moreover, they do not bind to the chemokine ligands themselves, influence chemokine binding to glycosaminoglycans or even modulate chemokine dimer formation in solution.

Instead, it seems likely that they mediate their BSCI activity by binding to a distinct cell surface receptor, most probably acting as an agonist at this site. Each of the structurally distinct BSCI classes binds to the same extracellular site (demonstrated by cross-competition for binding), and this site is implicated in the bioactivity of the

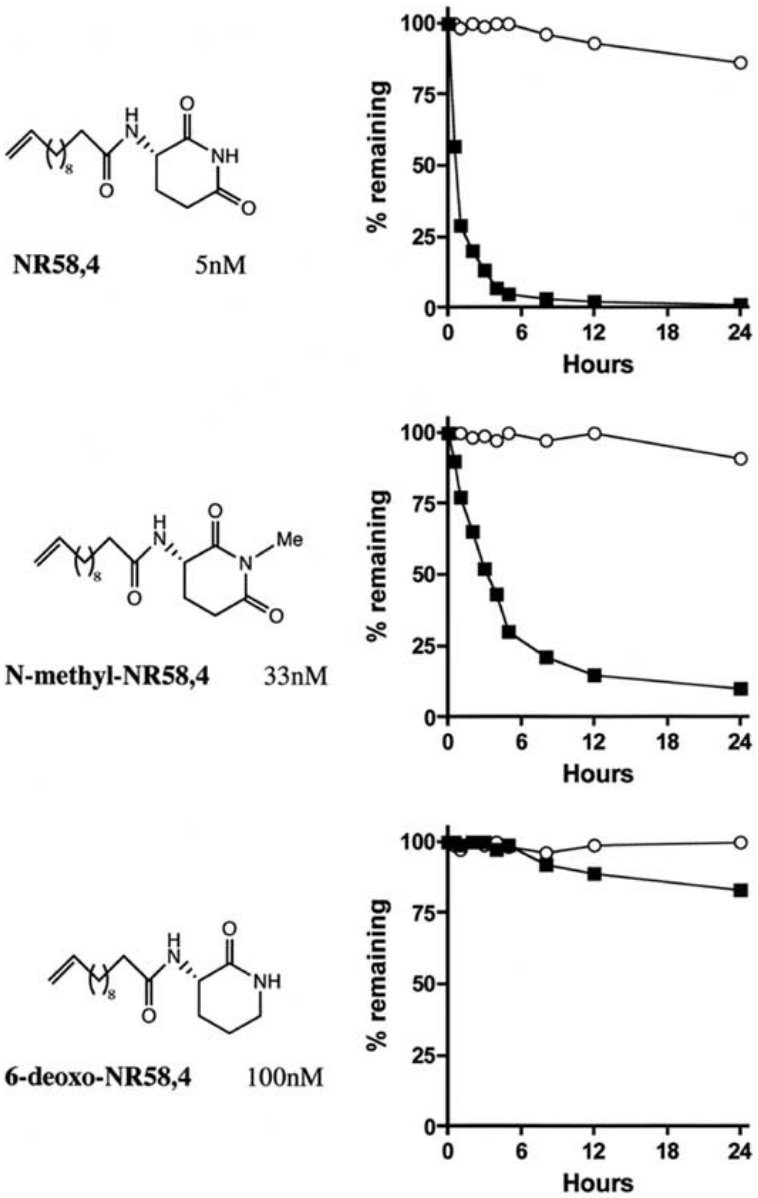


Fig. (4). Enzyme-catalysed metabolism of various (acylamino)-glutarimides. The hydrolysis rates of three different (acylamino)-glutarimides in buffered saline pH 7.4 (PBS; open circles) and in rat serum (filled squares) at 37ºC, determined by reverse phase HPLC analysis, are shown. While all 3 analogues are stable in saline at 37ºC, the parental NR58,4 molecule is rapidly broken down in serum, *via* a ring-opening reaction to yield N-acyl-glutamine and N-acyl-isoglutamine. N-methyl substitution slows this reaction to some degree, while the lactam 6-deoxo analogue is almost completely stable in serum at 37ºC. The rate of hydrolysis of NR58,4 is dramatically slowed by using heat-denatured serum (10 mins at 56ºC; data not shown)

molecules by the correlation between binding affinity at this site and potency of BSCI activity. For example, NR58-3.14.3 binds to this site with ~1,000 fold higher potency than Peptide 3, consistent with their relative potencies as BSCIs in the leukocyte migration assay.

It is plausible, but unproven, that this binding site (which is present on almost all leukocyte populations tested to date) is a GPCR, based on the structural similarity between BSCIs such as yohimban-16-amide and a variety of known GPCR agonists. However, screening for the ability of various BSCIs to block the binding of more than 60 GPCR ligands to their cognate receptors has failed to identify any candidate BSCI receptors (unpublished observations). There may be a number of explanations for this: there are still a large number of GPCR families which have not been screened, and in addition, the BSCI receptor may not be a GPCR; alternatively, the BSCIs may bind to a GPCR at a site distinct from, or only partially overlapping with, the natural ligands such that the receptor would not be revealed in competitive binding studies such as those performed to date. Ultimately, the unambiguous identification of this BSCI binding site may require an expression cloning strategy or the generation of BSCI analogues with photoactivated crosslinkable groups in their structure.

Understanding the molecular mechanism of action of BSCIs will undoubtedly increase the utility of these molecules as tools to unravel the complex intracellular signalling pathways which link cell surface receptor activation with directional locomotion in leukocytes [26]. The fact that BSCIs distinguish powerfully between chemokines and classical chemoattractants like fMLP and C5a, indicates that the intracellular signals generated by these two classes of ligands are likely to be different. Once the molecular mechanism of BSCI activity becomes known, they should be valuable probes of these distinct signalling pathways.

In contrast, the drive towards clinical application of BSCIs seems unlikely to be slowed by the continuing enigma of their molecular target. As long as the compounds continue to demonstrate powerful anti-inflammatory activity at high potency, with few side-effects, it seems certain that a range of clinical applications can be found for such molecules, even if a definitive molecular explanation of their activity remains elusive.

ACKNOWLEDGEMENTS

We are grateful to J.-P. Moreau, Christophe Thurieau, Jesse Dong, Mike Culler, John Taylor and their colleagues at Biomeasure for their support and collaboration on the development of BSCIs. This work is funded by a grant from Ipsen to D.J.G. who is a British Heart Foundation Senior Research Fellow.

REFERENCES

[1] Bebo, B. F. Jr.; Yong, T.; Orr, E. L.; Linthicum, D. S. *J. Neurosci. Res.* **1996**, *45*, 340.
[2] Libby, P. *Nature* **2002**, *420*, 868.
[3] Franceschi, C.; Bonafe, M.; Valensin, S.; Olivieri, F.; De Luca, M.; Ottaviani, E.; De Benedictis, G. *Ann. N. Y. Acad. Sci.* **2000**, *908*, 244.
[4] Rogers, J.; Shen, Y. *Ann. N. Y. Acad. Sci.* **2000**, *924*, 132.
[5] Akiyama, H.; Barger, S.; Barnum, S.; Bradt, B.; Bauer, J.; Cole, G. M.; Cooper, N. R.; Eikelenboom, P.; Emmerling, M.; Fiebich, B. L.; Finch, C. E.; Frautschy, S.; Griffin, W. S.; Hampel, H.; Hull, M.; Landreth, G.; Lue, L.; Mrak, R.; Mackenzie, I. R.; McGeer, P. L.; O'Banion, M. K.; Pachter, J.; Pasinetti, G.; Plata-Salaman, C.; Rogers, J.; Rydel, R.; Shen, Y.; Streit, W.; Strohmeyer, R.; Tooyoma, I.; Van Muiswinkel, F. L.; Veerhuis, R.; Walker, D.; Webster, S.; Wegrzyniak, B.; Wenk, G.; Wyss-Coray, T. *Neurobiol. Aging* **2000**, *21*, 383.
[6] Rollins, B. J. *Blood* **1997**, *90*, 909.
[7] Luster, A. D. *N. Engl. J. Med.* **1998**, *338*, 436.
[8] Gerard, C.; Rollins, B. J. *Nat. Immunol.* **2001**, *2*, 108.
[9] Horuk, R.; Ng, H. P. *Med. Res. Rev.* **2000**, *20*, 155.
[10] Schwarz, M. K.; Wells, T. N. *Nat. Rev. Drug Discov.* **2002**, *1*, 347.
[11] Onuffer, J. J.; Horuk, R. *Trends Pharmacol. Sci.* **2002**, *23*, 459.
[12] Grainger, D. J.; Reckless, J. *Biochem. Pharmacol.* **2003**, *65*, 1027.
[13] Horuk, R.; Clayberger, C.; Krensky, A. M.; Wang, Z.; Grone, H. J.; Weber, C.; Weber, K. S.; Nelson, P. J.; May, K.; Rosser, M.; Dunning, L.; Liang, M.; Buckman, B.; Ghannam, A.; Ng, H. P.; Islam, I.; Bauman, J. G.; Wei, G. P.; Monahan, S.; Xu, W.; Snider, R. M.; Morrissey, M. M.; Hesselgesser, J.; Perez, H. D. *J. Biol. Chem.* **2001**, *276*, 4199.
[14] Anders, H. J.; Vielhauer, V.; Frink, M.; Linde, Y.; Cohen, C. D.; Blattner, S. M.; Kretzler, M.; Strutz, F.; Mack, M.; Grone, H. J.; Onuffer, J.; Horuk, R.; Nelson, P. J.; Schlondorff, D. *J. Clin. Invest.* **2002**, *109*, 251.
[15] Reckless, J.; Tatalick, L. M.; Grainger, D. J. *Immunology* **2001**, *103*, 244.
[16] Reckless, J.; Grainger, D. J. *Biochem. J.* **1999**, *340*, 803.
[17] Jameson, B. A.; McDonnell, J. M.; Marini, J. C.; Korngold, R. *Nature* **1994**, *368*, 744.
[18] Wilbert, S. M.; Engrissei, G.; Yau, E. K.; Grainger, D. J.; Tatalick, L.; Axworthy, D. B. *Anal. Biochem.* **2000**, *278*, 14.
[19] Naidu, B. V.; Farivar, A. S.; Krishnadasan, B.; Woolley, S. M.; Grainger, D. J.; Verrier, E. D.; Mulligan, M. S. *Ann. Thorac. Surg.* **2003**, *75*, 1118.
[20] Beech, J. S.; Reckless, J.; Mosedale, D. E.; Grainger, D. J.; Williams, S. C.; Menon, D. K. *J. Cereb. Blood Flow Metab.* **2001**, *21*, 683.
[21] Fox, D. J.; Reckless, J.; Warren, S. G.; Grainger, D. J. *J. Med. Chem.* **2002**, *45*, 360.
[22] Eriksson, T.; Bjorkman, S.; Roth, B.; Fyge, A.; Hoglund, P. *Chirality* **1998**, *10*, 223.
[23] Wnendt, S.; Finkam, M.; Winter, W.; Ossig, J.; Raabe, G.; Zwingenberger, K. *Chirality* **1996**, *8*, 390.
[24] *The Merck Index*; 12th Edition ed.; Merck & Co,: Whitehouse Station, NJ, **1996**.
[25] Grainger, D. J.; Tatalick, L. *In* Patent US00/00821, **2000**
[26] Heit, B.; Tavener, S.; Raharjo, E.; Kubes, P. *J. Cell Biol.* **2002**, *159*, 91.