Mechanistic Investigation into Complementary (Antisense) Peptide Mini-Receptor Inhibitors of Cytokine Interleukin-1**

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Sense peptides are coded for by the nucleotide sequence (read 5′ →3′) of the sense (positive) strand of DNA. Conversely, a complementary peptide is coded for by the nucleotide sequence (read 5′ →3′) of the complementary or antisense (negative) strand of DNA. In many instances, sense and corresponding complementary peptides have been observed to interact specifically. In order to study this process in more detail, longer, shorter and mutant variants of our original complementary peptide, VITFFSL, were synthesised and analysed for binding to and inhibition of cytokine human interleukin-1β (IL-1β) in vitro. The behaviour of all peptides studied is discussed in terms of the Mekler – Idlis (M-I) pair theory, a theory that accounts for specific sense – complementary peptide interactions in terms of through-space interactions between corresponding pairs of amino acid residues (M-I pairs)] specified by the genetic code and its complement.

KEYWORDS:

amino acids · complementary peptides · inhibitors · interleukin-1 · molecular recognition

Introduction

Two antiparallel 2′-deoxypolynucleotide chains make up double-helical DNA. Traditionally, one of these chains, the sense (positive) strand, carries the coding information necessary for proteins and peptides whilst the complementary chain, or antisense (negative) strand, provides the means of propagating that coding information. However, recent evidence suggests that the complementary strand may also be harnessed for transcription to provide coding information as well.[1] In addition there is now growing evidence that peptides coded for by sense and complementary strands of DNA are able to interact specifically in a way comparable to the specific interaction between sense and complementary strands of DNA.[2] Applications of this phenomenon now include the development of anti-idiotypic antibodies for protection against autoimmune disorders,[3] the identification of novel receptors,[4] affinity column purification of proteins[5] and also the design of novel inhibitors of protein – protein interactions.[6–8]

By definition, a sense peptide is one whose sequence is coded for by the nucleotide sequence (read 5′ →3′) of the sense strand of DNA (or more precisely by codons in mRNA whose nucleodide sequence contains the same coding information as the sense strand of DNA). Conversely, the complementary peptide is coded for the nucleotide sequence (read 5′ →3′) of the complementary strand of DNA (or more strictly by codons in complementary mRNA with the same nucleotide sequence information as the complementary strand of DNA). There are two main schools of thought as to how specific interactions between sense and complementary peptides are possible. The first arose from an original observation by Blalock and Smith,[9] that the hydropathic character (measured on the Kyte – Doolittle scale[10]) of an amino acid residue is related to the identity of the middle letter of the mRNA codon from which it is translated. When uridine (U) is the middle nucleoside, the codon always codes for a hydrophobic residue, whilst with adenosine (A), the codon codes for a hydrophilic residue. By contrast codons with either cytidine (C) or guanosine (G) as the middle nucleosides generally code for residues with similar hydrophilic/hydrophobic characteristics. A and U (equivalent to thymidine (T) in DNA) are complementary nucleosides according to Watson – Crick base pairing rules, as are G and C. Consequently, sense and complementary strands of DNA must always code for peptide sequences that are opposite in hydropathic profile to each other. Blalock went on to suggest that a given sense and complementary peptide pair should

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[**] *Antisense Peptide Chemistry, Part 3. Part 2: J. R. Heal, S. Bino, K. P. Ray, G. Christie, A. D. Miller, J. G. Raynes, Mol. Immunol.* **1999**, *36*, 1141 – 1148.

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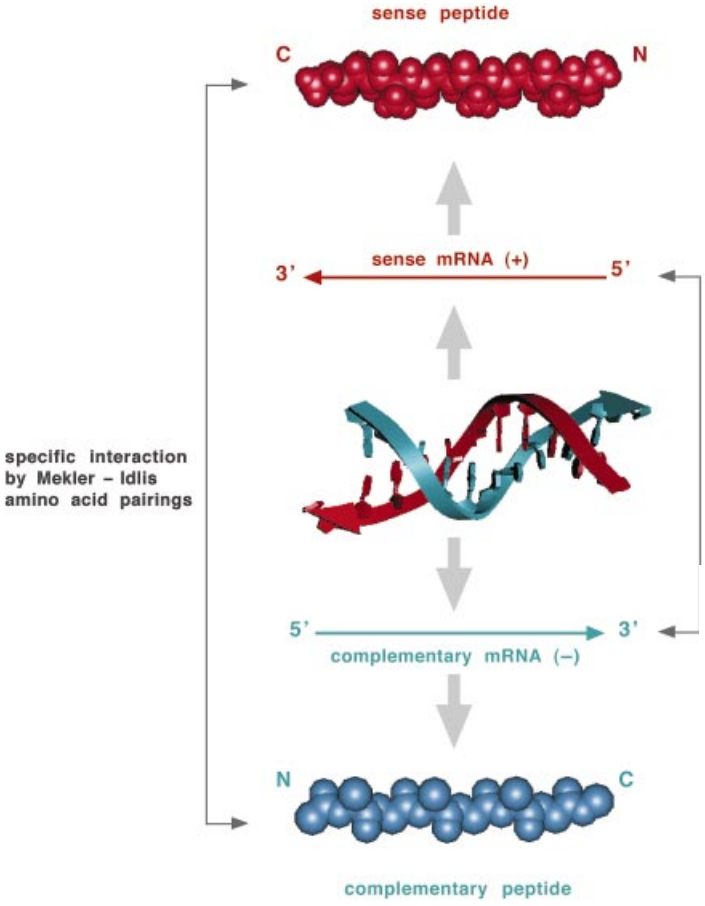

Figure 1. *Diagram to illustrate sense and complementary peptide interactions.*

therefore have mutually complementary shapes (secondary and tertiary structures), owing to the "inverse forces" operating within each peptide because of their opposite hydropathic profiles, and they should, hence, be able to interact specifically.[11] This has been called the molecular recognition theory (MRT).[11, 12]

The second school of thought has arisen from a suggestion of Mekler,[13] that specific interactions between sense and complementary peptides should be mediated by specific through-space, pair-wise interactions between amino acid residues (Figure 1). According to this suggestion, the side chain of each codon-directed amino acid in a sense peptide should be capable of making a specific pair-wise interaction with the side chain of the corresponding complementary codon-directed residue in the complementary peptide. In effect, Mekler was suggesting that the genetic code and its complement are able to specify through-space interactions between pairs of amino acid residues. Mekler and Idlis[14] identified all of the possible putative interacting pairs of amino acid residues and segregated them into three discrete nonoverlapping groups (Figure 2). In many cases, each given amino acid residue has been partnered by more than one possible amino acid residue. This situation arises because the genetic code is itself degenerate. Therefore, any one amino acid residue may be coded for by up to six different codons matched by the same number of complementary codons that in turn may code for up to four alternative matching amino acid residues (Table 1). In effect, these Mekler–Idlis (M-I) pairs appear to represent a protein–peptide equivalent of the Watson–Crick base pairs found in DNA.[2] This theory of Mekler and Idlis that the specific interaction between a given sense and complementary peptide is mediated by M-I pairs (Figure 2) has been called the M-I pair theory.[2]

In our previous work, we developed the concept of antisense or complementary peptide mini-receptor inhibitors.[7] Complementary peptide **1** with a C-terminal amide

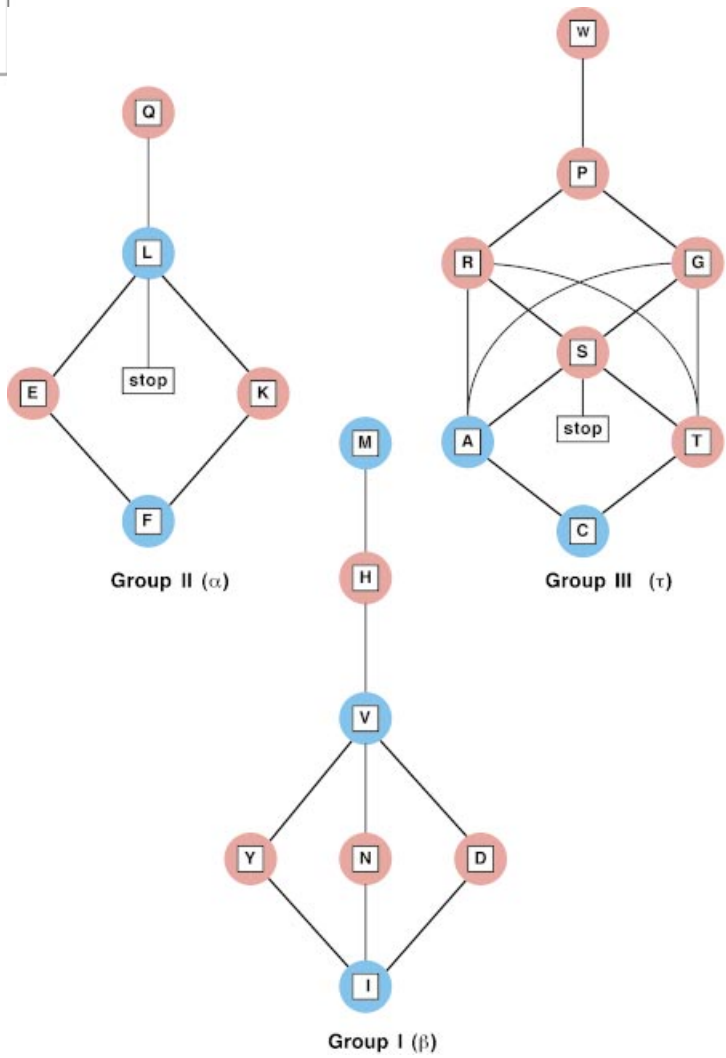

Figure 2. *Schematic representation of Mekler–Idlis (M-I) amino acid pairs. Solid lines connect pairs of amino acid residues related by the M-I pair theory. Nonpolar residues are shaded blue, polar residues are shaded red. "Stop" indicates a stop codon. The single letter amino acid residue abbreviations used are: A Ala, R Arg, D Asp, N Asn, C Cys, E Glu, K Lys, M Met, F Phe, P Pro, S Ser, Q Gln, G Gly, H His, I Ile, L Leu, T Thr, W Trp, Y Tyr, V Val. Adapted from the paper by Mekler and Idlis combined with features from the work of Zull and Smith.[14, 30]*

Table 1. *Table to show the genetic derivation of Mekler – Idlis (M-I) amino acid pairs.*

Amino acid	Codon 5′ →3′[a]	Complementary codon 5′ →3′[a]	Complementary amino acid	Amino acid	Codon 5′ →3′[a]	Complementary codon 5′ →3′[a]	Complementary amino acid
Ala (A)	GCA	UGC	Cys (C)	Ser (S)	UCA	UGA	stop
	GCG	CGC	Arg (R)		UCC	GGA	Gly (G)
	GCC	GGC	Gly (G)		UCG	CGA	Arg (R)
	GCU	AGC	Ser (S)		UCU	AGA	Arg (R)
					AGC	GCU	Ala (A)
					AGU	ACU	Thr (T)
Arg (R)	CGG	CCG	Pro (P)	Gln (Q)	CAA	UUG	Leu (L)
	CGA	UCG	Ser (S)		CAG	CUG	Leu (L)
	CGC	GCG	Ala (A)				
	CGU	ACG	Thr (T)				
	AGG	CCU	Pro (P)				
	AGA	UCU	Ser (S)				
Asp (D)	GAC	GUC	Val (V)	Gly (G)	GGA	UCC	Ser (S)
	GAU	AUC	Ile (I)		GGC	GCC	Ala (A)
					GGU	ACC	Thr (T)
					GGG	CCC	Pro (P)
Asn (N)	AAC	GUU	Val (V)	His (H)	CAC	GUG	Val (V)
	AAU	AUU	Ile (I)		CAU	AUG	Met (M)
Cys (C)	UGU	ACA	Thr (T)	Ile (I)	AUA	UAU	Tyr (Y)
	UGC	GCA	Ala (A)		AUC	GAU	Asp (D)
					AUU	AAU	Asn (N)
Glu (E)	GAA	UUC	Phe (F)	Leu (L)	CUG	CAG	Gln (Q)
	GAG	CUC	Leu (L)		CUC	GAG	Glu (E)
					CUU	AAG	Lys (K)
					UUG	CAA	Gln (Q)
					UUA	UAA	stop
					CUA	UAG	stop
Lys (K)	AAA	UUU	Phe (F)	Thr (T)	ACA	UGU	Cys (C)
	AAG	CUU	Leu (L)		ACG	CGU	Arg (R)
					ACC	GGU	Gly (G)
					ACU	AGU	Ser (S)
Met (M)	AUG	CAU	His (H)	Trp (W)	UGG	CCA	Pro (P)
Phe (F)	UUU	AAA	Lys (K)	Tyr (Y)	UAC	GUA	Val (V)
	UUC	GAA	Glu (E)		UAU	AUA	Ile (I)
Pro (P)	CCA	UGG	Trp (W)	Val (V)	GUA	UAC	Tyr (Y)
	CCC	GGG	Gly (G)		GUG	CAC	His (H)
	CCU	AGG	Arg (R)		GUC	GAC	Asp (D)
	CCG	CGG	Arg (R)		GUU	AAC	Asn (N)

[a] All complementary codons are read in the 5′ →3′ direction to derive the identities of possible interacting M-I partner residues (complementary amino acid residues) in a complementary peptide.

(sequence: *N*-VITFFSL-NH_2) was designed to interact with a key β-bulge surface loop (Boraschi loop; loop sequence: *N*-QGEESND-*C*; residues 48 – 54 (mature protein sequence)) of human interleukin-1β (IL-1β). The sense mRNA sequence of the loop was identified, the complementary mRNA sequence deduced and the sequence of complementary peptide **1** determined by using Table 1 (Figure 3). By interacting with the loop, synthesised **1** was able to inhibit IL-1β-mediated biological responses by sterically blocking the interaction between IL-1β (and also IL-1α) and the interleukin Type 1 receptor (IL-1R). Not only was specific inhibition clearly seen in vitro, but the interaction between IL-1β and the peptide was studied in detail with appropriate controls, which left little doubt that **1** was indeed interacting specifically with the Boraschi loop as expected. Subsequently, we were able to demonstrate the utility of peptide **1** in a separate, independent in vitro biological assay.[8] In this paper, we describe the next stage of our work with this IL-1 system, designed to explore and understand better the interactions between mini-receptor inhibitor peptides and the Boraschi loop of IL-1.

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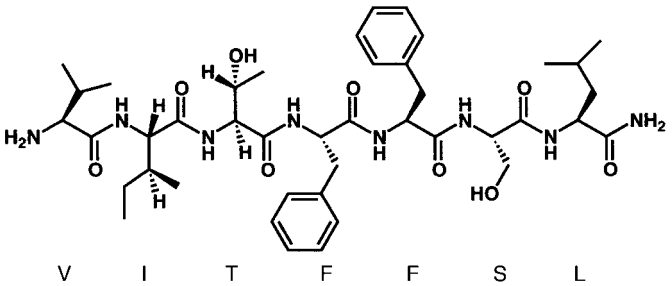

V I T F F S L **1**

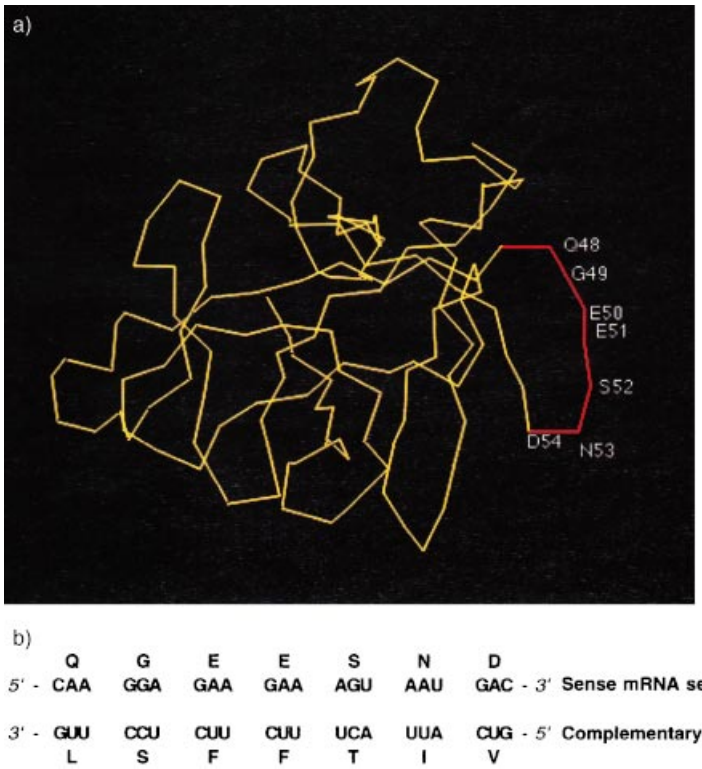

Figure 3. *a) The α-carbon backbone trace deriving from the X-ray crystal structure coordinates of IL-1β, also showing the β-bulge Boraschi loop structure of IL-1β (red). The main amino acid residues of the Boraschi loop have been labelled. b) Illustration of sense mRNA nucleotide sequence for the IL-1β Boraschi loop and the complementary mRNA nucleotide sequence to show the origin of the peptide sequence of complementary peptide* ***1****.*

Results

Resonant mirror biosensor analysis

In our original work, peptide **1** was shown to bind specifically to the human IL-1β Boraschi loop region with a dissociation constant K_d of 10.2 μM,[7] by means of a resonant mirror biosensor. Many control experiments were conducted to prove the specificity of the interaction. However, these previous data were acquired by using high levels of protein immobilised on carboxymethyl dextran (CMD) cuvettes by means of the standard *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (NHS/EDC) procedure, which gave maximal immobilisation responses of >2000 arc s. Typically, more accurate estimations of kinetic parameters are reported to be obtained with lower levels of immobilised proteins corresponding to maximal immobilisation responses in the range 100 – 1000 arc s.[15] However, in this study we quickly found that smaller quantities of IL-1β were difficult to immobilise reproducibly on CMD cuvettes. Therefore, we elected to try to immobilise human IL-1β on aminosilane cuvettes instead, using glutaraldehyde as a coupling agent. This proved successful and sufficient IL-1β was immobilised routinely to give maximal immobilisation responses in the 500 – 1000 arc s range, which corresponds to a modest concentration of immobilised protein (0.39 – 0.40 μM; 1.33 – 1.35 ng mm^{-2}) suitable for accurate binding studies.[15] All binding experiments were then conducted at an experimental temperature of 25 °C (unless otherwise stated), instead of 37 °C as described in our previous studies,[7] to minimise the disparity between the internal temperature of the biosensor and the temperatures of the sample peptide solutions (20 – 200 μM) prior to injection.

The interaction between peptide **1** and aminosilane immobilised IL-1β was studied, followed by the same sets of controls described previously (results of controls not shown).[7] In this way, we were able to demonstrate that peptide **1** was still interacting specifically with IL-1β in spite of the changes to cuvette and immobilisation conditions (control proteins included human interleukin-1 receptor antagonist (IL-1ra), tumor necrosis factor-α (TNFα), interleukin-8 (IL-8), interleukin-18 (IL-18) and bovine serum albumin (BSA)). A typical set of experimental binding data, obtained whilst studying the interaction between peptide **1** and IL-1β, is shown (Figure 4 a, b). Kinetic data and binding constants were then determined from such experimental data with software supplied by Affinity Sensors (FASTfit program; see Table 2).

Identical experiments, including all the above controls, were performed with all of the other longer, shorter and mutant variants of peptide **1**. A complete set of kinetic data and binding constants are shown for the interactions between aminosilane-immobilised IL-1β and each of these peptides in turn (Tables 2 and 3). In all cases, these variant peptides either bound specifically to IL-1β and no other protein or else failed to bind to any protein at all. Hence, those variants competent to bind to IL-1β were apparently doing so specifically and by an equivalent mechanism to the original peptide **1**. These results are all the more impressive given the fact that IL-1ra and IL-18 belong to the same three-dimensional structural family as IL-1β, although IL-1ra actually lacks a Boraschi loop like structure.[7]

Further evidence for the loop specificity of the complementary peptides was provided through competition experiments with other peptides. The association of peptide **1** with IL-1β was inhibited by the presence of a peptide VQGEESNDK, which comprises the Boraschi loop sequence. By contrast, a second peptide, GQEDVNEKS, that contained the same amino acid residues as the Boraschi loop peptide but in a different sequence order did not inhibit the association (Figure 4 c). The clear implication is that the Boraschi loop peptide was inhibiting the interaction of **1** with IL-1β by competing with the IL-1β Boraschi loop region for binding to **1**. The reordered Boraschi loop peptide was unable to do this.

The forces involved in driving the association of complementary peptide **1** with the Boraschi loop were investigated by studying the effects of ionic strength and temperature upon the

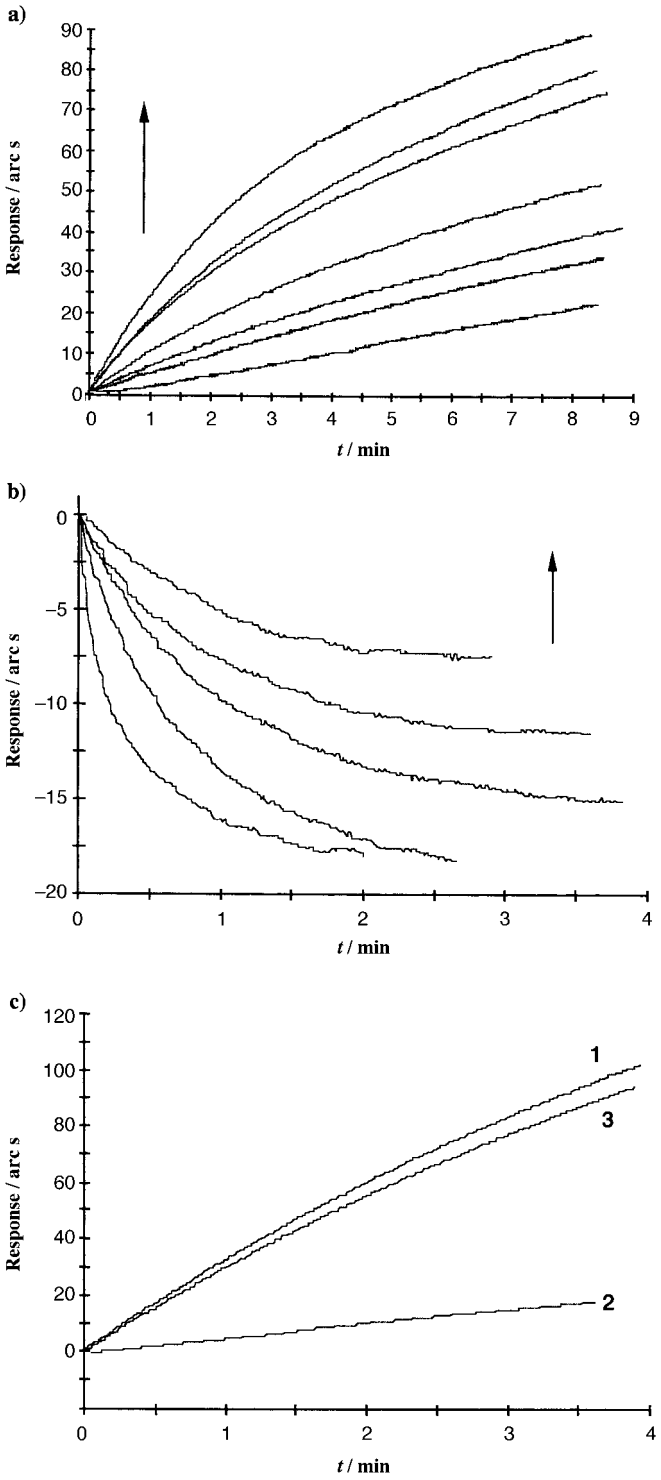

*Figure 4. Resonant mirror biosensor profiles obtained from the interaction of complementary peptide **1** with immobilised IL-1β (surface concentration: 1.33 ng mm^{-2}, 0.39 μM) in phosphate-buffered saline containing Tween-20 (PBS-T; pH 7.4) at 25 °C. a) Association data recorded with increasing concentrations of **1** (from lower to upper trace (in the direction of the arrow): 10, 20, 30, 50, 80, 100, 200 μM). b) Dissociation data recorded with increasing concentrations of **1** (from lower to upper trace (in the direction of the arrow): 10, 20, 40, 60, 100 μM). c) The results of a competition experiment performed with peptide **1** (100 μM) and immobilised IL-1β (surface concentration: 1.35 ng mm^{-2}, 0.40 μM) in PBS-T (pH 7.4) at 25 °C. Association profile 1 was obtained with **1** alone, profile 2 in the presence of the Boraschi loop peptide VQGEESNDK (100 μM) and profile 3 in the presence of a reordered Boraschi loop peptide GQEDVNEKS (100 μM).*

***Table 2.** Summary of binding data determined from resonant mirror biosensor analysis.*

Peptide[a]	k_{ass} [M^{-1}s^{-1}]	$10^4 \times k_{diss}$ [s^{-1}]	K_d [μM]	ESAP inhibition [%][b]
TFFS **2**	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]
VITFFS **3**	23.4 ± 1.3	9.1 ± 1.3	38 ± 9	30 ± 5
VITFFSL **1**	97.1 ± 8.0	4.3 ± 0.4	4.5 ± 2.3	85 ± 5
VITFFSLY **4**	22.4 ± 1.5	1.9 ± 0.7	8.7 ± 3.6	65 ± 5
FVITFFSLY **5**	31.0 ± 1.6	5.2 ± 0.3	17.0 ± 1.7	n.d.[c]
VFITSFL **6**[d]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]
VSTFFFYLI **7**[d]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]
D-VITFFSL **8**	9.3 ± 1.9	16.1 ± 8.5	140 ± 71	n.d.[c]

[a] Results were obtained by analysing (with IAsys software) data from the interaction of the indicated peptides with IL-1β immobilised on aminosilane cuvettes. (See the Experimental Section for details.) [b] The IL-1β concentration was 64 pg mL^{-1}; the peptides were at a concentration of 10 μg mL^{-1}. [c] n.d. = not determined because the binding was too weak. [d] Peptides **6** and **7** are controls; **6** is a reordered version of **1** and **7** is a reordered version of **5**.

***Table 3.** Summary of a second set of binding data determined from resonant mirror biosensor analysis.*

Peptide[a]	k_{ass} [M^{-1}s^{-1}]	$10^4 \times k_{diss}$ [s^{-1}]	K_d [μM]	ESAP inhibition [%][b]
group I				
IITFFSL **9**	16.8 ± 0.7	3.5 ± 0.5	21 ± 4	30 ± 5
VVTFFSL **10**	10.1 ± 1.1	1.9 ± 0.6	20 ± 8	n.d.[c]
group II				
VITLLSL **11**	202 ± 18	164 ± 43	80 ± 6	n.d.[c]
group III				
VIGFFSL **12**	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]
VIAFFSL **13**	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]
VIRFFSL **14**	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]
VITFFPL **15**	352 ± 41	125 ± 21	35.3 ± 1.9	35 ± 5
VITFFTL **16**	8.1 ± 0.2	65.1 ± 8.4	806 ± 12	n.d.[c]
VITFFAL **17**	334 ± 13	102 ± 14	31.0 ± 5.1	40 ± 5
FVVGLLTVK **18**	105 ± 8	34 ± 9.0	32.4 ± 3.2	38 ± 5[d]

[a] Results were obtained by analysing (with IAsys software) data from the interaction of the indicated peptides with IL-1β immobilised on aminosilane cuvettes. (See the Experimental Section for details.) [b] The IL-1β concentration was 64 pg mL^{-1}; the peptides were at a concentration of 50 μg mL^{-1}. [c] n.d. = not determined because the binding was too weak. [d] Peptide at a concentration of 10 μg mL^{-1}.

interaction. The effect of the former was investigated by studying the interaction of **1** with IL-1β in the presence and absence of NaCl at concentrations of 0, 250 and 500 mM, with fixed temperature (25 °C). In the presence of 250 and 500 mM NaCl, the value of the dissociation constant K_d was reduced to approximately half the value obtained in the absence of added NaCl (Table 2). Such a reduction in the value of K_d with increasing salt concentration is one hallmark of the hydrophobic effect.[16] The likely involvement of hydrophobic interactions was further reinforced by studying the interaction of **1** with IL-1β at four different temperatures (4, 15, 25 and 35 °C) and processing the association constant, K_a, data by means of a van't Hoff plot in order to extract thermodynamic data.[17] The resulting van't Hoff plot is illustrated with the calculated thermodynamic parameters inset (Figure 5). These thermodynamic parameters were derived

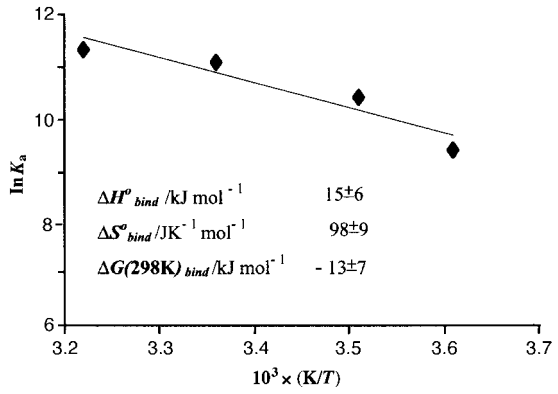

Figure 5. *Van't Hoff plot to illustrate the interaction of peptide **1** with immobilised IL-1β (surface concentration: 1.33 ng mm^{-2}, 0.39 μM) under standard conditions but at different temperatures. Binding data were plotted and processed according to Equation (1) to give the illustrated linear plot. The resulting thermodynamic data are given in the insert.*

with Equations (1) and (2), where ΔH°_{bind} is the standard enthalpy for peptide binding (under the conditions of pH, ionic strength, temperature (25 °C) and fixed IL-1β concentration used in the binding assays), ΔS°_{bind} is the standard entropy, $\Delta G(T)_{bind}$ is the temperature dependent free energy change of binding and R is the molar gas constant (8.314 J mol^{-1} K^{-1}).

$$\ln K_a = -\frac{\Delta H^{\circ}_{bind}}{RT} + \frac{\Delta S^{\circ}_{bind}}{R} \quad (1)$$

$$\Delta G(T)_{bind} = \Delta H^{\circ}_{bind} - T\Delta S^{\circ}_{bind} \quad (2)$$

Binding of **1** to IL-1β is clearly an endothermic process made favorable by entropy. This thermodynamic signature may be attributed to a mixed mode of interaction that combines both hydrophobic and electrostatic interaction forces.[16] The involvement of hydrophobic interactions was further reinforced by the appearance of a slight curvature to the van't Hoff plot implying that the change in heat capacity upon binding, $\Delta C_{p,bind}$, may be slightly negative. This is also a clear signature for hydrophobic interactions.[18]

Interleukin-1 biological assays

E-selectin alkaline phosphatase (ESAP) cell IL-1 assays are very sensitive and were carried out to demonstrate that binding of complementary peptides to IL-1β was not only structurally interesting but also of biological relevance. The assay has been described previously,[8] and needs no further discussion here except to say that dose–response relationships were determined for all the peptides. The ability of peptides to inhibit effects induced by human IL-1β (64 pg mL^{-1}) was evaluated up to a maximum peptide concentration of 50 μg mL^{-1} (Tables 2 and 3). In almost all cases, there is a clear correlation between the ability of a peptide to bind to IL-1β and the extent to which that peptide was able to inhibit the biological activity of IL-1β in vitro.

Discussion

The nature of the interactions between sense and complementary peptides has been studied sporadically over the years. The strength of sense–complementary peptide interactions has been shown to increase with length; longer pairs of sequences usually display a higher mutual affinity than shorter pairs.[19] There is also significant experimental evidence to suggest that sense–complementary peptide interactions are conformationally degenerate, multilocalised/multisite and made up of clusters of stabilising, noncovalent contacts between specific amino acid residues.[19–21] Yet others have noted the deleterious consequences for binding strength of mutations in either a sense peptide or complementary peptide, further underlining the importance of specific amino acid contacts in sustaining sense–complementary peptide interactions.[21, 22] Such observations are very much consistent with the M-I pair theory of interaction. The validity of these pairs is discussed in detail elsewhere in a review.[2] In this study, the interaction of complementary peptide **1** with IL-1β appears to be mediated by a combination of both hydrophobic and electrostatic forces of interaction. Such a mixed mode of interaction is also consistent with the M-I pair theory.

For historic reasons, the M-I pair theory appears to have been largely overlooked in favour of the MRT. However, in our opinion, the M-I pair theory provides a much more satisfactory mechanistic framework with which to interpret the variations in IL-1β binding and in vitro inhibition profiles of complementary peptide **1** and variants. In the X-ray crystal structure of human IL-1β,[23] the Boraschi loop consists of seven contiguous residues (QGEESND) in an extended β-strand-like conformation. The flanking amino acid residues are looped back into the core of the protein. Therefore, complementary peptide **1** could conceivably interact with the loop in an extended β-strand-like conformation so as to maximise interresidue contacts (Figure 6).[2]

Our results are consistent with this picture. In comparing the efficacy of complementary peptides longer and shorter then **1** (Table 2), none were more effective than **1**. Complementary peptide **3** is six residues in length and was able to bind specifically to IL-1β, but binding was an order of magnitude more weak than with **1**. Moreover, peptide **3** was a worse in vitro inhibitor. Peptide **2** is four residues in length and did not bind to or inhibit IL-1β. This decrease in affinity with decreasing length is consistent with the mode of interaction, based upon multilocalised/multisite amino acid side chain contacts between specific amino acid residues, as illustrated in Figure 6.[19–21] Such a mode of interaction would be expected to increase in strength as the length of the interacting peptide segment and the number of interacting amino acid residues increase.[19] Therefore in the absence of other factors, the eight-residue peptide **4** and the nine-residue peptide **5** might have been expected to bind to IL-1β more tightly than **1**. However, the terminal residues of **4** and **5** were probably not in a position to make proper contact with their respective M-I partner residues given the conformation of the Boraschi loop and the sterically congested locations of the flanking amino acid residues (Figure 3a), which lead to a loss in binding affinity. In spite of this, peptide **4** was still found to be an IL-1β inhibitor in vitro.

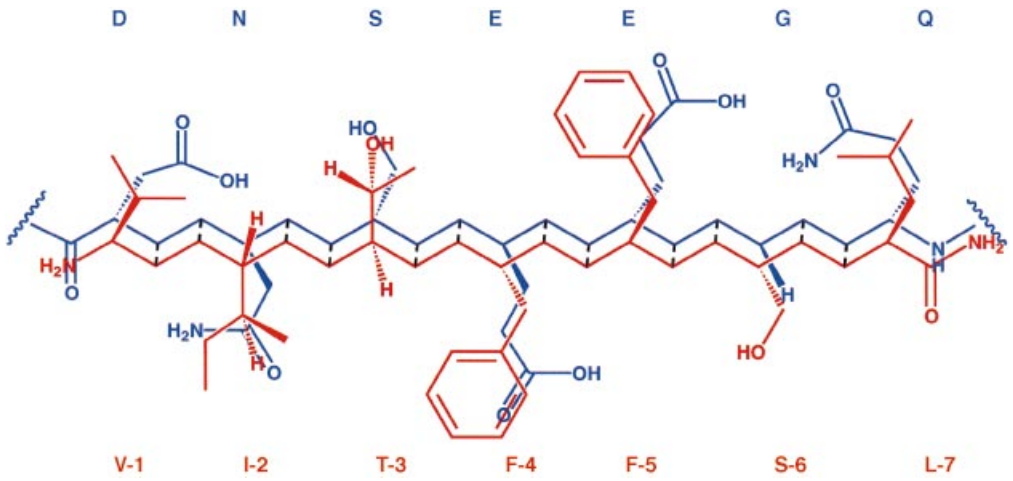

Figure 6. *Diagram to illustrate how complementary peptide **1** (in red) may be aligning with the Boraschi loop sequence (in blue) in order for the side chains of Mekler – Idlis amino acid residue partners to be in close proximity to each other. Both peptide chains are in extended β-strand conformations and, when arranged alongside each other, form the equivalent of an antiparallel β-sheet structure. Peptide links are omitted for clarity but black-dashed lines indicate where hydrogen bonds may originate from these links to create the putative β-sheet structure.*

The importance of correct partner-residue contacts to sustain the interaction is clearly suggested by studies with peptides **6** and **7**. Peptide **6** is a reordered version of **1** and **7** is a reordered version of **5**. The peptide compositions of **6** and **7** are the same as **1** and **5** respectively, but the sequence orders are significantly different. In both cases, **6** and **7** neither bound to nor inhibited IL-1β in vitro (Table 2). On the basis of the M-I pairs, it is reasonable to suggest that reordering the sequences of complementary peptides **1** and **5** abolished peptide binding to the loop because correct M-I pairs were no longer able to make proper through-space contact with each other. Finally, results obtained with peptide **8**, an all-D configuration version of **1**, provide some confirmation of the importance of side-chain contacts in mediating the interaction between complementary peptides and the Boraschi loop (Table 2). Peptide **8** showed a measurable binding affinity for IL-1β, although it was some two orders of magnitude lower than **1**. Results like these have been observed previously,[20] and are consistent with a mode of interaction driven by M-I partner side-chain interactions, but one weakened by the necessity for some stereochemical distortions in order for the side chains of amino acid residues with opposite absolute configurations to make contact with each other. Unfortunately, the affinity of **8** was too low for inhibition of IL-1β in vitro.

Data presented from studies with variants of **1** containing single or double amino acid "mutations" are also consistent with a mode of interaction based upon M-I pairs and begin to suggest something about the relative importance of each of the pairs (Table 3). Previous "mutation studies" performed on complementary peptides of the *S*-peptide of ribonuclease and others have indicated the disruptive effects on binding of amino acid residue substitutions ("mutations").[21, 22] In our case, the selected mutations were made with reference to the peptide sequence of the Boraschi loop and a consideration of the possible codons that might code for that same peptide sequence. All possible codons for each amino acid residue at each position in the Boraschi loop were identified, complementary codons deduced and alternative M-I partner residues suggested (see Table 1 and Figure 2). Mutant complementary peptides were then synthesised to cover the range of possible M-I partner variations at each residue position of peptide **1** in turn. The results are grouped according to whether the "mutations" were within M-I groups I, II or III (Table 3). No one complementary peptide mutant surpassed peptide **1** for binding to and inhibiting IL-1β in vitro, which suggests that the gene sequence of the Boraschi loop is optimal for complementary peptide association (Table 3). Group I mutations (V1I **9**, I2V **10**) resulted in approximately fivefold loss in binding affinity, group II mutations (F4L:F5L **11**) in approximately one order of magnitude loss in affinity. The most interesting effects were observed with group III mutations (T3G **12**, T3A **13**, T3R **14**, S6P **15**, S6T **16**, S6A **17**). Position 6 mutations reduced binding by at least an order of magnitude with a corresponding reduction in the inhibition of IL-1β in vitro. By contrast, all position 3 mutatations abolished binding and any inhibition effects. Therefore, of all the possible mutations, those within the M-I group III amino acid residue pairs (Figure 2) appeared to have the most significant negative effects upon the strength of the binding interaction between complementary peptides and the Boraschi loop. Zhao et al. have very recently used high-performance affinity chromatography to demonstrate that similar mutations within M-I group III pairs can also have a significant impact upon binding interactions involving other sense and complementary peptide systems.[24] Their data corroborates our observations, which demonstrates how variable sense – complementary peptide affinities may be even with the simplest of sequence variations.

Assuming that complementary peptide **1** and other mutant peptides interact with the loop in the manner illustrated (Figure 6), we can account for some of the consequences of mutations in the following way. According to our current understanding about the M-I pairs,[2] V – D, I – N, and L – Q pairs probably associate by hydrophobic interactions between the

side chains of nonpolar residues and the hydrocarbon chains supporting the polar functional groups of partner residues. The E – F pair should be able to benefit from specific π-face hydrogen bonding, similarly the S – T pair should be able to associate by specific hydrogen bond formation. Arguably, the loss of the latter hydrogen bond forming potential when threonine is replaced could explain the completely negative effects of group III mutations at position 3 (T3G **12**, T3A **13** and T3R **14**) on binding and in vitro biological efficacy. In a similar way, the negative effect of group II mutations (F4L:F5L **11**) could be explained by the loss of π-face hydrogen bonding potential when phenylalanine is replaced by leucine. Other mutation effects are less readily accounted for, however the relatively mild effects of group I mutations (V1I **9**, I2V **10**) do suggest that nonpolar amino acid residues such as valine, isoleucine and leucine may be interchangeable in the context of sense – complementary peptide interactions.

As mentioned in the introduction, the MRT model of sense – complementary peptide interactions suggests that peptides are driven to interact specifically by virtue of having opposite hydropathic profiles with respect to each other.[11] We ourselves have discussed sense – complementary peptide interactions with respect to this concept previously.[7, 8] In addition, Sisto reported using the MRT concept to devise a peptide inhibitor (peptide **18**, Table 3) of IL-1β in vitro by analysing for peptide sequences with the highest possible hydropathic complementarity to amino acid residues 47 – 55 of IL-1β, which embrace the Boraschi loop sequence.[25] This was apparently achieved using computer programs developed by Fassina and co-workers.[5d, 20, 26] We ourselves prepared the Sisto peptide and are able to confirm that this peptide both bound to IL-1β and inhibited biological activity in vitro (Table 3). Moreover, the same controls were performed as for complementary peptide **1** with the same results, which suggests that **18** was also binding specifically to the Boraschi loop. At first sight the sequence of **18** looks completely unrelated to that of **1** and offers a direct challenge to the M-I pair theory. However, upon closer inspection, the general principles of the M-I pair theory appear to remain intact. Peptide **18** was designed to complement residues 47 – 55 (mature protein sequence) of human IL-1β and peptide **1** to complement residues 48 – 54. Therefore, neglecting the two terminal amino acid residues of **18**, the core sequence is VVGLLTV. In fact, each amino acid residue at every position of this core sequence except the last belongs both to the same M-I group as the corresponding residue in **1** and is also a recognised alternative (see Figure 2; Tables 1 and 3). Therefore, peptides **1** and **18** are almost exactly equivalent in terms of the M-I pair theory.

There are other examples that may be interpreted in a similar way to the Sisto peptide. For instance, Fassina et al.[20] have described a peptide $C.G._{\cdot RAF}$ that has the highest possible hydropathic complementarity to the *c-raf* protein (residues 356 – 375), according to their computer program. Although obviously different in sequence to the complementary peptide AS_{RAF} derived from the genetic code, each residue of $C.G._{\cdot RAF}$ belongs, once again, to the same M-I group as the corresponding residue in AS_{RAF} and is also a recognised alternative in terms of allowed M-I group pairings.

However, things are not always as clear-cut as this. In some cases, peptide sequences derived on the basis of complementary hydropathy instead of the genetic code may not fit the M-I pair theory in such an ideal way. For example, we described a peptide with the sequence LITVLNI picked out by our own computer program (FINDH) from the sequence of human IL-1R on the basis of hydropathic complementarity to the Boraschi loop.[8] This peptide was found to bind to IL-1β an order of magnitude more weakly than the original complementary peptide **1** ($K_d = 43.1 \pm 6.1$ μM) and proved to be an inhibitor in vitro, but it does not appear to fit the M-I pair theory. However, the M-I pair theory may encompass such a peptide provided we allow leucine, isoleucine and valine to be stereoelectronically equivalent to each other on the basis of their closely related side-chain structures. This seems all the more reasonable in view of the results of the mutation studies described above. In other words, M-I pair theory may still apply if we regard leucine, isoleucine and valine as surrogates of each other in M-I groups I and II. Hence, we would suggest that hydropathic complementarity per se and, by implication, the MRT should not be regarded as being responsible for the specific interactions between sense and complementary peptides. Instead the concept provides an alternative more empirical understanding of M-I pair theory.

The X-ray crystal structure of the complex between human IL-1β and the extracellular domain of IL-1R is known and an examination of the local environment of the Boraschi loop in the complex is very interesting in the light of the foregoing discussion.[27] The loop is not in contact with a contiguous complementary peptide segment equivalent to complementary peptide **1**. Instead, loop residues are partnered by a discontinous array of IL-1R amino acid residues, many members of which are correct M-I pair partners. For instance, N – V and E – F pairs are found. Surrogate M-I pairs such as Q – I are also evident. In other words, M-I pairs appear to represent a significant and important part of the interface between IL-1β and IL-1R. Hence, peptide **1** appears to be an impressively close surrogate of the receptor Boraschi loop binding region and certainly deserves the description complementary (antisense) peptide mini-receptor inhibitor that was conferred by us previously.[7] Furthermore, this discussion appears to suggest that M-I pair theory not only provides a framework with which to understand the specific interaction between sense and complementary peptides but also could represent an important new theory for protein – protein and protein – peptide molecular recognition in nature in general.

Conclusions

A variety of complementary peptides were designed specific to the Boraschi loop of IL-1β. The original complementary peptide **1** was shown in this system to bind specifically to the loop and was found to be the most effective at binding to and inhibiting IL-1β in vitro. The behaviour of this and the other peptides studied was explained in terms of the M-I pair theory (Figure 2). There is a suggestion that leucine, isoleucine and valine may act as surrogates of each other within the M-I groups I and II, and

that M-I group III pairs could have an important role in modulating the interaction between sense and complementary peptides.

Experimental Section

General: The X-ray crystal structure coordinates of IL-1β were obtained from the Brookhaven Protein Data Bank.[23] Unless otherwise indicated, all materials and chemicals were obtained from Sigma – Aldrich (UK). MilliQ water was used throughout ($>10\,M\Omega\,cm$ resistivity). Recombinant mature human IL-1β for the in vitro assays was a gift of Dr. K. Ray (Glaxo Smith Kline, Stevenage, UK). Peptides were synthesised by standard methods on a Schimadzu PSSM-8 multipeptide synthesiser or on an Advanced Chemtech 348 Omega multiple peptide synthesiser with N-terminal 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids (Novabiochem, UK) and a Rink Amide MBHA solid-phase resin (Novabiochem, UK). Following deprotection and cleavage from the resin, peptides were desalted by gel filtration (2×28cm, P2 biogel (Bio-Rad, UK)) eluting with 0.1% aqueous trifluoroacetic acid (TFA). Final purification was effected by reversed-phase high-pressure liquid chromatography (HPLC; Vydac C18 column (Hichrom, UK) with a Gilson HPLC system) eluting with a linear gradient of acetonitrile in 0.1% aqueous TFA. Following freeze drying, peptides were either stored at $-20\,^{\circ}C$ under N_2 in anhydrous conditions, or else stored as stock solutions ($10\,mg\,mL^{-1}$) in dimethylsulfoxide (DMSO) under N_2 in anhydrous conditions. The identity of all peptides was confirmed before use by quantitative amino acid analysis and by positive- or negative-ion fast atom bombardment mass spectrometry (FAB-MS) as appropriate. Purity was judged to be $>95\%$ by reversed-phase HPLC.

Resonant mirror biosensor analysis: Binding analyses were performed on an IAsys plus biosensor (Affinity Sensors, UK). Human IL-1β or appropriate control proteins (IL-1ra, IL-8, IL-18, TNFα, BSA, or blank surface) were immobilised on aminosilane cuvettes over a period of 30 min in 10 mM sodium phosphate buffer at pH 7.7 by using a standard glutaraldehyde coupling protocol. Before use, aliquots of each peptide stock solution in DMSO were diluted into phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 (PBS-T) to create dilution series ranging in concentration from 10 – 200 μM (final peptide concentrations). Each dilution series was maintained at 25 °C and used within a few hours of preparation. Kinetic measurements were obtained to characterise the interactions between these various concentrations of complementary peptides (10 – 200 μM) and immobilised protein (approximately $1-2\,ng\,mm^{-2}$). Each set of measurements involving a given peptide and a given immobilised protein was carried out at 25 °C in PBS-T buffer (unless otherwise stated). For each measurement, the association and dissociation phases were typically 250 s. After the dissociation phase a 2 min washing step (with 10 mM HCl) was used to regenerate the cuvette before the next measurement was obtained. Where necessary, derivatised cuvettes were stored overnight at 4 °C with a covering of Parafilm. Kinetic data and binding constants were determined from the biosensor data by using software supplied by Affinity Sensors (FASTfit). Where appropriate, data were fit with a biphasic function so as to minimise errors, according to standard procedures and equations.[15, 28]

Interleukin-1 assay: The ESAP-1 cell assay has been described in detail by Ray et al.[29] In brief, 25×10^4 ESAP cells mL^{-1} were added to 96 well microtitre plate wells and incubated overnight. Human IL-1β ($64\,pg\,mL^{-1}$) was incubated with peptide at various concentrations ($0-50\,\mu g\,mL^{-1}$) for 30 min at 25 °C and added to the wells for 18 h. Supernatants from test wells and a standard curve (IL-1β; 0 – $2000\,pg\,mL^{-1}$) were assayed for enzyme activity following treatment at 66 °C for 30 min to inactivate endogenous enzyme. Enzyme activity was determined by adding supernatant (25 μL) to the assay buffer (200 μL) and incubating for 60 min at 37 °C. The assay buffer was 1 M diethanolamine (pH 9.6), 0.28 M NaCl, 0.5 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate. The assay is unable to detect IL-1β-induced effects at a concentration $<8\,pg\,mL^{-1}$.

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