Journal of Peptide Science
*J. Pept. Sci.* 2006; **12**: 515–524
Published online 8 May 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.760
JOURNAL OF PEPTIDE SCIENCE

# Novel monocyclic and bicyclic loop mimetics of brain-derived neurotrophic factor

**JORDAN M. FLETCHER and RICHARD A. HUGHES***
Department of Pharmacology, The University of Melbourne, Victoria 3010, Australia

*Received 18 December 2005; Revised 23 February 2006; Accepted 26 February 2006*

**Abstract:** Brain-derived neurotrophic factor (BDNF) is a protein that promotes the survival of neurons. It is widely thought to possess clinical potential for the treatment of neurodegenerative diseases, and in recent years, has been found to play a role in the pathogenesis of some tumours. BDNF is thought to bind to its cellular receptors trkB and p75$^{NTR}$ primarily by way of solvent-exposed loops on the BDNF dimer. In this paper, we describe our recent progress towards the development of small peptides as mimetics and inhibitors of BDNF. Two classes of peptides were prepared: disulphide-constrained monomeric monocyclic peptides designed to mimic a single solvent-exposed loop; and homo- and heterodimeric bicyclic peptides designed to mimic pairs of loops. Each peptide was examined in cultures of embryonic chick dorsal root ganglion sensory neurons, both alone, and in competition with BDNF. All peptides were found to inhibit BDNF-mediated neuronal survival, while one – a dimeric peptide based on the two loop 4 regions of BDNF – behaved as a partial BDNF-like agonist. The work described in this paper supports the proposed receptor-binding role of loops 1, 2, and 4 of BDNF, and provides valuable steps towards our long-term goal of developing BDNF mimetics and inhibitors for clinical use. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** BDNF; neurotrophin; agonist; antagonist; neurotrophic factor; dimeric; cyclic; disulphide

## INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of neurotrophic factors, along with nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Like all neurotrophins, BDNF has been shown to possess neurotrophic activity in a variety of *in vitro* and *in vivo* paradigms (reviewed in Ref. 1), and plays an important role in maintaining neuronal plasticity (reviewed in Ref. 2).

Because of its neurotrophic activities, in particular its ability to promote neuronal survival and prevent neurodegeneration, BDNF has long been touted as a potential treatment for a variety of neurodegenerative conditions [3]. Indeed, in a number of experimental systems, BDNF has been shown to promote the survival of several populations of neurons that are susceptible in common disease states. For example, BDNF promotes the survival of motoneurons [4,5], which degenerate in amyotrophic lateral sclerosis (ALS) [6]; striatal neurons [7], which are vulnerable in Huntington's Disease [8]; basal forebrain cholinergic neurons [9], which are affected in Alzheimer's disease [10]; and dopaminergic neurons of the substantia nigra [11], which are lost in Parkinson's disease [12]. In addition to its neurotrophic actions, BDNF also promotes the growth and survival of neuroblastoma (reviewed in Ref. 13) – one of the most common extracranial tumours found in children – as well as certain forms of prostate cancer (for review, see Ref. 14). Thus, while BDNF (or indeed, BDNF-like mimetics) may possess clinical potential for the treatment of neurodegenerative diseases, inhibitors of BDNF may be of utility in the treatment of BDNF-dependent malignancies.

At the receptor level, BDNF exerts its biological effects through interaction with two cell surface proteins: trkB and p75$^{NTR}$. TrkB is a member of the trk family of receptor tyrosine kinases (reviewed in Ref. 15), of which there are three members, each with their preferred neurotrophin binding partners: BDNF and NT4/5 bind to trkB; NGF to trk A; and NT-3 primarily to trkC, and to a lesser extent to trkA and trkB (reviewed in Ref. 16). Activation of trk receptors is thought to follow the general scheme established for receptor tyrosine kinases [17]: firstly, neurotrophin binding induces the stepwise homodimerisation of trk receptors [18], which is followed by the activation of receptor tyrosine kinase domains and the induction of a number of second-messenger cascades that ultimately lead to the arrest of programmed cell death (reviewed in Ref. 15). p75$^{NTR}$ on the other hand, is a transmembrane glycoprotein capable of binding all neurotrophins with similar affinity, but with different kinetics [19]. Structurally, p75$^{NTR}$ possesses both an intracellular 'death domain' [20] and a small juxtamembrane sequence known as 'chopper' [21], both of which have been implicated in signalling apoptosis. Physiologically, the actions mediated by p75$^{NTR}$ activation are complicated, and have proven difficult to resolve experimentally. In

* Correspondence to: R. A. Hughes, Department of Pharmacology, The University of Melbourne, Victoria 3010, Australia;
e-mail: rahughes@unimelb.edu.au

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general terms however, p75[NTR] plays two primary roles: the augmentation and enhancement of trk receptor binding [22], and the direct, trk-independent initiation of second-messenger signalling to regulate survival *or* death decisions of neurons (for a review, see Ref. 23).

The neurotrophins are homodimers consisting of two non-covalently linked monomers of approximately 120 amino acids apiece. X-ray crystal structures of NGF [24], NT-3 [25], NT-4/5 [26], and a BDNF/NT-4/5 heterodimer [26] reveal a common fold. Each monomer contains seven $\beta$-strands, six of which are paired to form $\beta$-sheets and which largely constitute the dimer interface. Linking these $\beta$-sheets are three solvent-exposed $\beta$-hairpin loops, loops 1, 2, and 4, and a longer, elongated loop 3. Each monomer is further stabilised by six fully conserved cysteine residues that form three disulphide bridges arranged in a so-called cystine knot motif.

In the early 1990s, a series of site-directed metagenesis studies by Ibáñez and co-workers (reviewed in Ref. 27) suggested that amino acids present on loops 1, 2, and 4 play host to the bulk of residues directly involved in the binding of neurotrophins to their cognate receptors. In later years, the X-ray crystal structure of NGF bound to the Ig-2 domain of trkA confirmed the involvement of loop 1 [28]. Supporting the binding role of loop 2 based residues, our laboratory has previously described conformationally constrained monomeric and dimeric peptides based on the loop 2 region of BDNF, which behave as BDNF inhibitors [29] and partial BDNF-like agonists [30], respectively.

Despite its strong performance in pre-clinical studies, BDNF has unfortunately met with little success in the clinic. For example, in a phase III clinical trial in patients with ALS, BDNF was found to be of no benefit [31]. Several plausible explanations have been put forward for this lack of clinical effect [32,33], at the forefront of which are concerns about the pharmacokinetic properties of BDNF. For example, BDNF has a plasma half-life of less than a minute in the rat [34], and is unable to penetrate the blood–brain barrier to an appreciable extent [35]. To circumvent these shortcomings, researchers are primarily pursuing two different tactics: (i) improving the delivery of BDNF; and (ii) developing small molecule analogues of BDNF with improved pharmacokinetic properties, such as the dimeric loop 2 mimetics previously reported by us [30].

In this work, we have extended our earlier studies and examined all the BDNF receptor-binding loops (1, 2, and 4) as templates for the design of additional peptides. In this way we hoped to generate novel classes of BDNF-like agonists (which may have potential for the treatment of neurodegenerative disease) or antagonists (which may be of use in the treatment of BDNF-dependent malignancies, or as pharmacological tools). In total we generated five monomeric monocyclic and five dimeric bicyclic peptides, and examined them in primary cultures of embryonic chick sensory neurons either as BDNF antagonists, or as BDNF-like agonists.

## MATERIALS AND METHODS

### Materials

The materials used and their sources are as follows: Fmoc protected $\alpha$-L-amino acids, HOBt, HBTU, DIPEA, DMF, piperidine, and TFA: Auspep, Parkville, Vic., Australia; Cys(Trt) and Cys(Acm) pre-loaded 2-chlorotrityl resin: Novabiochem, Darmstadt, Germany; methanol, dichloromethane, acetic anhydride, and L-ascorbic acid: Merck, Kilsyth Vic., Australia; iodine: Merck, Darmstadt, Germany; acetonitrile and $NH_4HCO_3$: BDH Laboratory Supplies, Poole, England; DMSO: FSE, Homebush NSW; Australia; C-18 Rocket and C-18 semi-preparative HPLC columns; Alltech, Baulkham Hills, NSW, Australia; recombinant human BDNF: Research and Diagnostic Systems, Minneapolis, USA; fertilised chicken eggs: Research Poultry, Research, Vic, Australia; trypsin: Worthington, Freehold, NJ, USA; horse serum, penicillin, and streptomycin: CSL Parkville, Vic., Australia; L-15 medium: Gibco BRL, Grand Island, NY, USA; poly-DL-ornithine: Sigma, St Louis, USA; laminin: Collaborative Biomedical Products, Bedford, MA, USA; Nunclon 10 cm-diameter tissue culture dish: Nalge Nunc International (Roskilde, Denmark); 48-well tissue culture plates: Becton Dickinson (Franklin Lakes, NJ, USA); all other reagents: Sigma, Castle Hill, NSW, Australia.

Electrospray ionisation mass spectrometry (ESI-MS) analysis was performed using a Micromass Platform II single quadrupole mass spectrometer.

### Molecular Design

A model of the three-dimensional structure of BDNF (Figure 1) – previously obtained by protein homology modelling [29] – was used as a template for the design of peptides based on the solvent-exposed loops of BDNF. Previous work in our laboratory [29] has shown that pairs of amino acid residues that possess $\beta$-carbon (C$\beta$) atoms between 4.5 and 6.0 Å apart represent suitable locations for the insertion of a cystine cyclising constraint, requiring minimal re-organisation of the remainder of the peptide to maintain the native loop conformation. Using SYBYL molecular modelling software (Version 6.4, Tripos Inc), each receptor-binding loop of BDNF was isolated, distances between C$\beta$ atoms of amino acid residues present in the given loop measured, and suitable positions for cystine cyclising constraints selected. To design peptides that mimic a *pair* of BDNF loops, we used Cys-to-Cys disulphide bonds as tethers to link together two monomeric monocyclic loop mimetics in a manner likely to reflect native orientation of the two loops, as previously described [30]. To find appropriate locations for such disulphide linkers, the BDNF homodimer was visually inspected and the regions identified, in which the receptor-binding loops were in close spatial proximity. Distances between C$\beta$ atoms were then measured to allow the identification of locations suitable for the insertion of a Cys-to-Cys disulphide linker (C$\beta$-to-C$\beta$ distance of 4.5 to 6.0 Å). Using this approach, appropriately protected monomeric monocyclic

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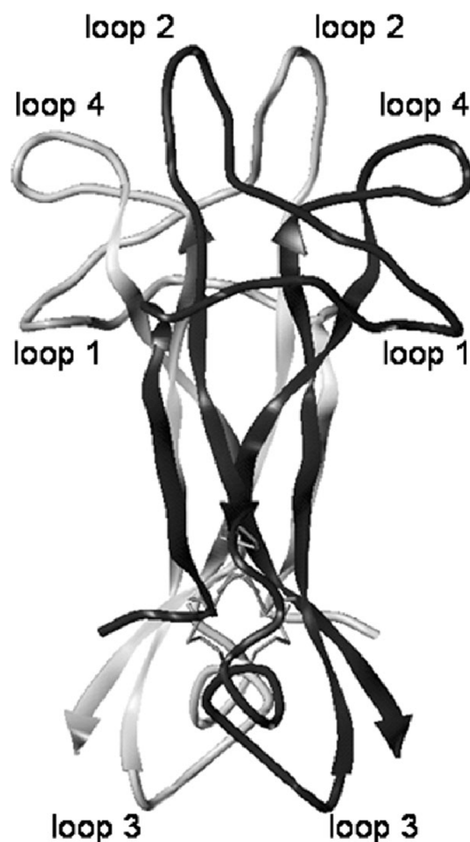


**Figure 1** Three-dimensional structure of BDNF obtained by homology modelling [29]. One chain of the dimer is shown in grey, the other in white. Antiparallel $\beta$-sheets are shown as broad ribbons while $\beta$-hairpins, and all other non-$\beta$-strand regions are shown as tubes. Solvent-exposed loops are labelled. Images were generated using SYBYL molecular modelling software (Version 7.0, Tripos Inc.).

peptides were used as 'building blocks' for the assembly of the dimeric bicyclic peptides as mimetics of pairs of BDNF loops.

### Peptide Synthesis

Linear precursors to cyclic peptides were synthesised manually by standard solid-phase techniques [36] from 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids on Cys(Trt) or Cys(Acm) pre-loaded 2-chlorotrityl resin, as appropriate. The side-chain protecting groups used were: Arg(Pmc), Asp(OtBu), Gln(Trt), Glu(OtBu), Lys(Boc), Ser(tBu), and Thr(tBu), while a mixed Cys(Trt) and Cys (Acm) protection strategy was used for cysteine residues, to later allow the selective formation of cystine cyclising constraints or dimerising linkers from these residues, respectively. Cleavage of peptides from the resin and the removal of side-chain protecting groups (sparing Acm) were achieved by treating the protected, resin-attached peptide with a cocktail of trifluoroacetic acid(TFA)/ethanedithiol(EDT)/water (18 : 1:1 (v/v)) for 90 min. Each Acm-protected linear peptide was then cyclised to the corresponding Acm-protected monomeric monocyclic derivative by treatment with 10% DMSO in an aqueous solution (0.1 M) of $NH_4HCO_3$ [37]. All cyclisations were monitored by RP-HPLC.

### Peptide Dimerisation

Homo- and heterodimeric bicyclic target peptides were prepared by the oxidative dimerisation of Acm-protected monomeric monocyclic peptides in a modification of a method reported by Kamber *et al.* [38]. Reactions were performed under a blanket of nitrogen in a vigorously stirred solution of iodine in deoxygenated methanol (50 mM, 200 µl). Homodimerisation reactions saw 4.0 µmol of monomer dimerised under these conditions, whereas heterodimerisation reactions saw equimolar (2.0 µmol) quantities of two monomeric monocyclic precursors dimerised to yield both the desired heterodimeric bicyclic target compound and potentially two homodimeric bicyclic species as reaction by-products (Scheme 1). All peptides prepared in this study were analysed and purified by reverse-phase HPLC using linear gradients of solution A (0.1% TFA in $H_2O$) and solution B (0.1% TFA in 70% $MeCN/H_2O$).

### Biological Assay

All animal experimental procedures were undertaken in accordance with The University of Melbourne Animal Experimentation Ethics Committee (AEEC Register No. 97152). The biological activities of all peptides were assessed in primary cultures of embryonic chick dorsal root ganglia sensory neurons which were prepared as previously described [29,30]. Peptides were assayed either alone, or in competition with BDNF at 360 pM – the concentration determined experimentally to give ~90% of the maximal neuronal survival effect (data not shown). Positive control wells were treated with BDNF alone, while negative controls contained neither peptide nor BDNF. Each treatment was performed in triplicate. Forty-eight hours after plating, the surviving neurons were counted and data normalised for each treatment between BDNF positive (100%) and negative (0%) controls. Neuronal survival was then expressed as mean $\pm$ SEM of 3–4 independent experiments and compared to either BDNF positive (competition studies) or negative (compounds tested alone) controls by one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni's multiple comparisons test.

## RESULTS

### Molecular Design

To further our understanding of the role of the BDNF solvent-exposed loops in mediating receptor binding, we designed: (i) monomeric monocyclic peptides as mimetics of a *single* solvent-exposed loop (i.e. loops 1, 2, or 4); and (ii) novel dimeric bicyclic analogues as mimetics of a *pair* of solvent-exposed loops. Distances between C$\beta$ carbon atoms were measured, from which suitable positions for the insertion of Cys-to-Cys disulphide constraints and later, dimerising linkers, were identified. The following residues in the BDNF primary sequence were chosen as locations for Cys-to-Cys disulphide conformational constraints: loop 1: $Ala^{30}$–$Val^{38}$; loop 2: $Lys^{43}$–$Lys^{52}$ (as previously reported [29]); loop 4: $Leu^{92}$–$Trp^{102}$.

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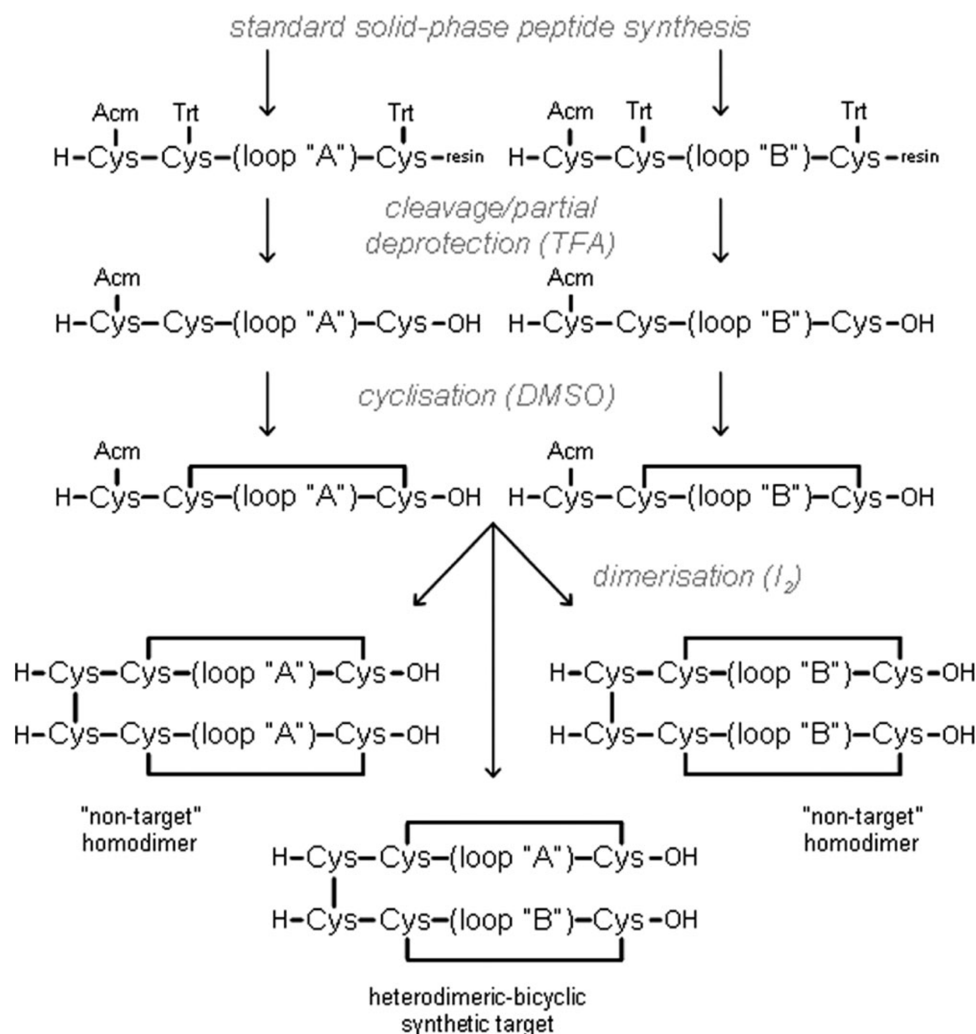


**Scheme 1** Method for the synthesis of monomeric monocyclic, and dimeric bicyclic peptides.

To design peptides that mimic a pair of solvent-exposed loops, the BDNF homodimer was inspected to identify pairs of loops that lay in close proximity to one another. By far the closest pair of these loops were the two loop 2s, mimetics of which we have previously described [30]. Others which lay in close proximity included pairs of loops derived from the *same* chain of the BDNF homodimer (i.e. intrachain loop pairs) such as the pairs loop 1 and loop 2, and loop 2 and loop 4, as well as pairs of loops derived from *different* chains of the BDNF dimer (i.e. interchain loop pairs) such as the two loop 4s. To generate suitable sites for the incorporation of Cys-to-Cys disulphide linkers, it was necessary to consider amino acid residues external to those chosen as the locations for the cyclising constraints of individual loops. By this means, the following pairs of residues from the native BDNF sequence were selected as locations for Cys-to-Cys disulphide linkers: loop 1–loop 2 (*intrachain*): $Val^{40}$–$Leu^{41}$; loop 2–loop 4 (*intrachain*): $Val^{40}$–$Ala^{91}$; loop 4–loop 4 (*interchain*): $Val^{89}$–$Val^{89}$.

Appropriately tethered monomeric monocyclic peptides were used as building blocks for the preparation of dimeric bicyclic peptides. Because of the different locations via which the different loop combinations were linked together, two loop 2, and two loop 4 monomeric monocyclic peptides were needed. For example, one loop 2 mimetic (peptide **L2a**) was required for the preparation of the intrachain loop 1–loop 2 mimetic (**L1–L2a**), and another, with an additional *N*-terminal residue, (**L2b**) was used for the preparation of the loop 2–loop 4 mimetic (**L2b–L4a**). Similarly a loop 4 mimetic with a long *N*-terminal region (**L4b**) was required for the preparation of a loop 4–loop 4 mimetic (**L4b–L4b**). Table 1 shows the structures of the designed monomeric and dimeric peptides, and the BDNF $\beta$-hairpin loops they were designed to mimic.

## Peptide Synthesis

A total of five Acm-protected monomeric monocyclic peptides were prepared that were used for the construction of homo- and heterodimeric bicyclic peptides.

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**Table 1** Structure, Mass Spectrometry Data, and Summary of Peptide Design and Dimer Assembly

| Peptide | Structure | $m/z$ (M + H predicted) | Loop(s) mimicked |
|---|---|---|---|
| | *Monomers* | | |
| **L1** | Ac-CVDMSGGTCTC(Acm)-OH | 1210.4[c] (1187.4) | Loop 1 |
| **L2a** | H-C(Acm)ECVPVSKGQLC-OH | 1335.2 (1334.6) | Loop 2 |
| **L2b** | H-C(Acm)LECVPVSKGQLC-OH | 1448.8 (1447.7) | Loop 2 |
| **L4a** | H-C(Acm)CTMDSKKRIGC-OH | 1414.7 (1413.7) | Loop 4 |
| **L4b** | H-C(Acm)RACTMDSKKRIGC-OH | 1641.9 (1640.8) | Loop 4 |
| | *Dimers* | | |
| **L1–L2a**[a] | Ac-CVDMSGGTCTC-OH / H-CECVPVSKGQLC-OH | 2378.9 (2376.9) | Loops 1 and 2 (intrachain) |
| **L2b–L4a** | H-CLECVPVSKGQLC-OH / H-CCTMDSKKRIGC-OH | 2718.4 (2716.2) | Loops 2 and 4 (intrachain) |
| **L4b–L4b** | H-CRACTMDSKKRIGC-OH / H-CRACTMDSKKRIGC-OH | 3138.8 (3136.4) | Loops 4 and 4 (interchain) |
| | *'Non-target' dimers*[b] | | |
| **L2b–L2b** | H-CLECVPVSKGQLC-OH / H-CLECVPVSKGQLC-OH | 2752.6 (2750.2) | —[d] |
| **L4a–L4a** | H-CCTMDSKKRIGC-OH / H-CCTMDSKKRIGC-OH | 2684.2 (2682.2) | —[d] |

[a] Previously reported [39].
[b] Obtained as reaction by-products during the assembly of dimeric peptides.
[c] $m/z$ consistent with $[M + Na]^+$ ion ($[M + Na]^+$ predicted: 1209.4).
[d] 'Non-target' dimers have no structural correlate with the BDNF template.

Peptides were synthesised using standard Fmoc solid-phase techniques and an orthogonal cysteine protection strategy (see Scheme 1). Linear, Acm-protected peptides were cyclised in the presence of DMSO to yield the monomeric monocyclic loop mimetics, **L1** (a loop 1 analogue), **L2a**, and **L2b** (loop two analogues), and **L4a** and **L4b** (loop 4 analogues) (Table 1).

## Peptide Dimerisation

Dimeric bicyclic peptides were assembled by the $I_2$-mediated oxidative dimerisation of appropriate monomeric monocyclic derivatives (Scheme 1) to give the heterodimeric bicyclic peptides **L1–L2a** and **L2b–L4a**, and the homodimeric bicyclic peptide **L4b–L4b**. In addition, the 'non-target' homodimeric bicyclic peptides **L2b–L2b** and **L4a–L4a** were also obtained as reaction by-products. Despite successfully obtaining all our desired dimeric peptide derivatives, RP-HPLC analysis of the dimerisation reactions showed that they did not proceed cleanly, with several other reaction by-products (in addition to the 'non-target' homodimer peptides – see Scheme 1) also produced (data not shown). The synthesis of peptide **L1–L2a**, via Scheme 1, was particularly troublesome and low-yielding; a large amount of highly insoluble precipitate – an **L1–L1** homodimer – was seen to form concomitantly with the loss of the **L1** starting material with the formation of only a small amount (~2% overall yield) of the desired peptide. Because of this, an alternative regioselective method for the preparation of such heterodimeric bicyclic peptides was developed and used for the preparation of **L1–L2a** (see [39]).

## Biological Analysis

The ability of both the monomeric monocyclic and homo- and heterodimeric bicyclic peptides to affect neuronal survival was examined in primary cultures of sensory neurons, prepared from embryonic day 8 chick dorsal root ganglia. This assay has been

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commonly employed in the biological characterisation of the neurotrophins; BDNF causes a dose-dependent survival of neurons in this system ([30]). All peptides were tested either in competition with BDNF (to examine their effects on BDNF-mediated survival) or alone (to assess their intrinsic neuronal survival activity).

## Monomeric Monocyclic Peptides in Competition with BDNF

When assayed in competition with BDNF, all monomeric monocyclic peptides produced a significant and concentration-dependent inhibition of BDNF-mediated survival (Figure 2(A)). The loop 1 mimetic **L1** appeared to be the least potent compound, in that it gave significant inhibition only at $1 \times 10^{-5}$ M ($30 \pm 6\%$ inhibition, $p < 0.01$, $n = 4$). All other peptides already showed significant inhibition at $10^{-7}$ M. The loop 2 mimetic **L2a**, appeared to be the most potent of the monomeric monocyclic loop mimetics, exhibiting dose-dependent inhibition of BDNF-mediated survival from $1 \times 10^{-9}$ to $1 \times 10^{-5}$ M, with a maximal inhibition of $44 \pm 4\%$ ($p < 0.001$, $n = 4$) at $10^{-5}$ M. The second loop two mimetic **L2b**, which contains an additional Leu residue towards the *N*-terminus, showed markedly reduced effects (maximal inhibition of $28 \pm 7\%$ at $10^{-7}$ M, $p < 0.01$, $n = 4$) compared to **L2a**. The loop 4 mimetics, **L4a** and **L4b**, significantly inhibited BDNF at $10^{-7}$ M, and showed greatest inhibition of $49 \pm 10\%$ ($p < 0.001$, $n = 4$) and $41 \pm 7\%$ ($p < 0.001$, $n = 4$), respectively, at $1 \times 10^{-5}$ M.

## Homo- and Heterodimeric Bicyclic Peptides in Competition with BDNF

Like their monomeric counterparts, the homo- and heterodimeric bicyclic compounds were also found to act as inhibitors of BDNF-mediated neuronal

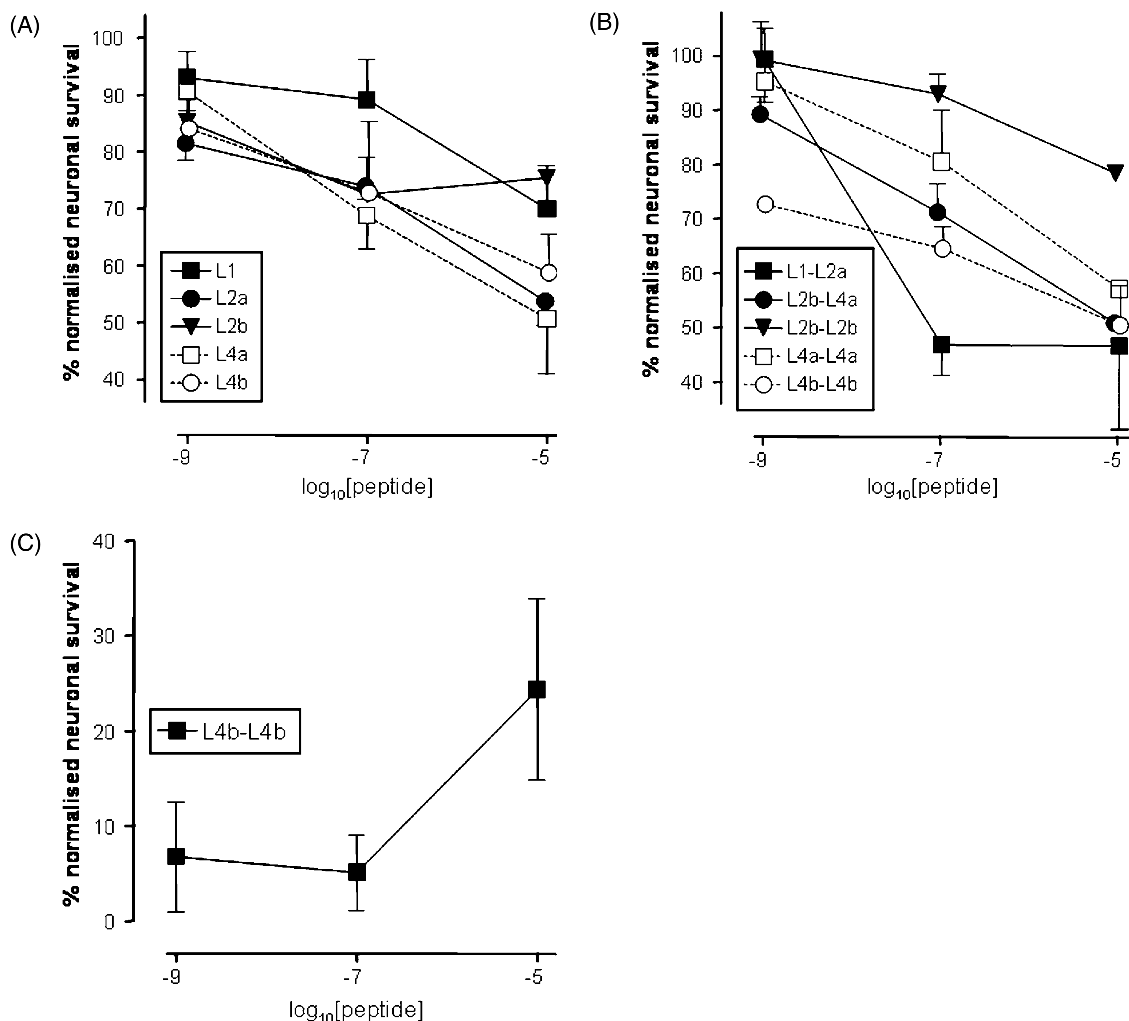


**Figure 2** Effects of BDNF loop mimetics on the survival of cultured embryonic chick dorsal root ganglion sensory neurons. Three sets of curves are shown: (A) monomeric monocyclic peptides **L1**, **L2a**, **L2b**, **L4a**, and **L4b** in competition with BDNF; (B) dimeric bicyclic peptides **L1–L2a**, **L2b–L4a**, **L4b–L4b**, **L2b–L2b**, and **L4a–L4a** in competition with BDNF; (C) homodimeric bicyclic peptide **L4b–L4b** assayed alone (i.e. in the absence of BDNF). Neuronal survival is shown as mean ± SEM from three or four experiments and is normalised between BDNF positive (set to 100%) and negative (0%) controls.

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survival (Figure 2(B)). The heterodimeric bicyclic peptide **L1–L2a** gave statistically significant inhibition of BDNF from $1 \times 10^{-7}$ to $1 \times 10^{-5}$ M, with an inhibition of $54 \pm 6\%$ ($p < 0.001$, $n = 3$) at $1 \times 10^{-5}$ M. It is interesting to note that unlike all other compounds tested, the inhibition caused by **L1–L2a** reaches a clear plateau of around 50% inhibition. The other heterodimeric bicyclic peptide **L2b–L4a** significantly inhibited BDNF-mediated survival at $1 \times 10^{-7}$ M, and gave $49 \pm 1\%$ ($p < 0.001$, $n = 3$) inhibition at $1 \times 10^{-5}$ M, while the homodimeric bicyclic peptide **L4b–L4b** significantly inhibited BDNF-mediated survival at all concentrations tested, with inhibition ranging from $27 \pm 1\%$ ($p < 0.01$, $n = 3$) at $1 \times 10^{-9}$ M, to $50 \pm 7\%$ ($p < 0.01$, $n = 3$) at $1 \times 10^{-5}$ M. The 'non-target' homodimeric peptides **L2b–L2b** and **L4a–L4a** were also found to inhibit BDNF-mediated neuronal survival, although the effects of **L2b–L2b** were modest, with a maximal inhibition at $10^{-5}$ M of only $21 \pm 1\%$ ($p < 0.05$, $n = 3$).

### Intrinsic Neuronal Survival Activity of a Homodimeric Bicyclic Peptide

When assayed alone in cultured sensory neurons, the homodimeric bicyclic peptide **L4b–L4b** was found to significantly promote neuronal survival at $1 \times 10^{-5}$ M ($24 \pm 9\%$ ($p < 0.05$, $n = 4$); Figure 2(C)), suggesting that the compound is acting as a BDNF-like agonist. None of the other peptides prepared in this study showed enhancement (or indeed inhibition) of neuronal survival relative to negative controls (data not shown).

## DISCUSSION

In this study, we used a model of the 3D structure of BDNF as a template to design cyclic peptides as conformationally constrained mimetics of the solvent-exposed loops of the neurotrophic factor BDNF. In total, five Acm-protected monomeric monocyclic peptides were designed and synthesised, each mimicking a single loop of BDNF: **L1** (loop 1 mimetic), **L2a** and **L2b** (loop 2 mimetics), and **L4a** and **L4b** (loop 4 mimetics). These single loop mimetics were subsequently used to construct two heterodimeric bicyclic peptides (**L1–L2a** and **L2b–L4a**) and one homodimeric peptide (**L4b–L4b**) as mimetics of pairs of BDNF loops. The peptides were assayed in cultures of sensory neurons prepared from the dorsal root ganglia of embryonic chicks, and were tested for their ability to (i) inhibit BDNF-mediated neuronal survival; and (ii) intrinsically promote neuronal survival, i.e. to act as BDNF-like agonists. All peptides examined were found to inhibit BDNF-mediated survival, while one compound, the homodimeric bicyclic peptide **L4b–L4b**, was found to also have partial BDNF-like agonist activity, being able to intrinsically promote neuronal survival.

The synthesis of the peptides described in this study ranged from routine (i.e. monocyclic monomeric derivatives) to challenging (i.e. dimeric bicyclic). The method we chose for the synthesis of the homodimeric and heterodimeric bicyclic peptides involved the $I_2$-mediated oxidative coupling of Acm-protected monomeric monocyclic precursor peptides (Scheme 1). We have previously used this method successfully in our laboratory to synthesise homodimeric bicyclic mimetics of loop 2 of BDNF [30], as well as homodimeric and heterodimeric bicyclic mimetics of loops 1, 2, and 3 of vascular endothelial growth factor (Fletcher and Hughes, unpublished observations). When used to synthesise a heterodimeric peptide, the approach would be expected to also give the corresponding homodimers as by-products (as shown in Scheme 1). This matter notwithstanding, we found that this method was not optimal to the synthesis of any of the dimeric peptides (whether hetero or homo) in this study. Although we were able to obtain the heterodimer **L2b–L4a** and the homodimer **L4b–L4b** in roughly equal proportions and in quantities sufficient for biological analysis, the syntheses typically resulted in low to moderate yields and a number of reaction by-products. Attempts to identify these by-products by MS met with little success, as they either gave ions that did not correspond with expected oxidation products, or they failed to ionise. Treatment of loop 4 derivatives with $I_2$ may potentially have been expected to have given rise to oxidation of the Met residue present in these derivatives, but no evidence of appreciable Met(O) was found. In the case of the heterodimer **L1–L2a**, the method described in Scheme 1 gave only a trace amount of the desired heterodimer, with the major product being the highly insoluble **L1–L1** homodimer. To obtain synthetic access to **L1–L2a**, we developed a regioselective approach, which allowed targeted formation of the desired heterodimer by way of a novel Acm to *S*-pyridinyl exchange [39]. Although we did not investigate it, we anticipate that this regioselective synthetic approach could also have been used to improve the synthetic yield and quality of the other homo- and heterodimeric peptides described in this study.

Site-directed mutagenesis studies with BDNF and the other neurotrophins indicate that the solvent-exposed loops of BDNF likely harbour many of the residues involved in the interactions of BDNF with its receptors trkB and p75 (reviewed in Ref. 27). The biological data presented in this paper supports these observations, in that monomeric monocyclic peptides designed to mimic loops 1, 2, or 4 of BDNF inhibit the ability of BDNF to promote neuronal survival in cell culture. In previous studies from our laboratory, we described similar BDNF-inhibiting effects of a range of monomeric monocyclic peptides based on loop 2 of BDNF [29]. The relatively weak inhibitory effects of the two monomeric monocyclic loop 2 peptides in this study (**L2a** and **L2b**) compared to our previous study may be a result

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of the presence of the *N*-terminal extension on **L2a** and **L2b** included for subsequent dimerisation. Taken together, these data are consistent with the hypothesis that conformationally constrained BDNF loop mimetics are competitive antagonists of trkB, which by binding to trkB inhibit the binding of BDNF and thus prevent the dimerisation of trkB required for neuronal survival signalling through this receptor. Furthermore, the similar pattern of inhibition – concentration dependent and reaching a plateau of maximal inhibition of approximately 50% – seen with all the loop mimetics strongly suggests that residues from all three loops of BDNF are contributing to receptor binding. It is interesting to note that in the X-ray crystal structure of NGF complexed with the Ig-2 domain of its receptor trkA, the only loop residues to make contact with the receptor are from loop 1 [28]. The inhibitory action on neuronal survival reported here of the BDNF loop 1 mimetic implies that this interaction is also important in the BDNF/trkB system. However, the inhibitory actions of mimetics of BDNF loops 2 and 4 suggest that these additional BDNF loops also make contact with other trkB domains, such as the leucine-rich repeat [40].

Given that mimetics of each of the individual solvent-exposed loops of BDNF acted as BDNF inhibitors, we reasoned that heterodimeric bicyclic peptides that mimic of pairs of loops should act as more potent inhibitors. To determine this, we synthesised two such peptides **L1–L2a** and **L2b–L4a**, designed as mimetics of loops 1 and 2 and loops 2 and 4, respectively. The heterodimeric bicyclic peptides were designed to mimic pairs of loops on the same BDNF monomer chain. This was possible because the distances between the corresponding interchain loops was greater, and too large to be bridged by the dimerising constraint. In the case of **L1–L2a**, this heterodimerisation did appear to improve antagonist potency, with maximal inhibition (50%) clearly achieved at $10^{-7}$ M, whereas its monomeric monocyclic components **L1** and **L2a** gave only 10 and 18% inhibition at the same concentration. However, the heterodimer **L1–L2a** is no more potent than the best of the monocyclic monomeric loop 2-based BDNF inhibitors that we have described previously, again indicating that the *N*-terminal extension required for dimerisation might be interfering with antagonist action. In contrast to **L1–L2a**, the potency of **L2b–L4a** did not appear to be greater than either of its component monomers. If both the monomeric monocyclic mimetics **L2b** and **L4a** are binding to trkB, then the lack of antagonist improvement shown by the heterodimer **L2b–L4a** suggests that its two loop components (unlike those of **L1–L2a**) are unable to participate together in trkB binding, possibly because of an inability to adopt a conformation to allow such a cooperative interaction. Structural studies of the unbound and receptor bound ligands would be required to determine if this is the case.

The homodimeric bicyclic peptide **L4b–L4b** was found to be a partial BDNF-like agonist, inhibiting BDNF-mediated neuronal survival when assayed in the presence of BDNF, yet able to promote survival itself in the absence of the neurotrophic factor. This is the second distinct class of homodimeric BDNF loop mimetics that we have described with such BDNF-like agonist activity: in previous work, we have shown that dimeric bicyclic mimetics of loop 2 of BDNF are partial BDNF-like agonists [30]. Together, these data suggest that such homodimers are able to dimerise two trkB molecules and thus bring about neuronal survival. **L4b–L4b** is not particularly potent or efficacious in its neuronal survival activity, particularly when compared to the previously described dimeric bicyclic loop 2 mimetics. Indeed, the potency of the dimeric bicyclic loop 2 mimetics was sensitive to the nature and position of the dimerising constraint. Although no exploration of the dimerising linker in **L4b–L4b** was made in this study (other than to devise a linker that was compatible with the homology model of the BDNF dimer), it is noteworthy that the 'non-target' loop 4 homodimer **L4a–L4a** lacked BDNF-like neuronal survival activity. The dimerising linker in **L4a–L4a** was significantly shorter (by four residues) than that of **L4b–L4b**, suggesting that the two cyclic loop 4 mimicking regions in **L4a–L4a** are simply unable to bridge the distance spanned by the two loop 4s in BDNF. Similar reasoning can be used to explain why the intrachain heterodimers described in this study act as pure antagonists and are devoid of neuronal survival activity.

The lack of neuronal survival activity of the 'non-target' loop 2 homodimer **L2b–L2b** is particularly interesting. Our previously described loop 2 homodimers [30] were dimerised via a cystine bridge within the monocyclic loop 2 mimicking sequence, a Glu-to-Lys side-chain-to-side-chain amide link at the *C*-terminus, or both (to give a highly potent tricyclic dimer). In any case, careful consideration was given to creating a dimerising constraint that would be likely to be able to maintain the native relative loop 2 orientation. In the case of **L2b–L2b**, the dimerising disulphide link is at the *N*-terminus, a position that is clearly incompatible with **L2b–L2b** being able to adopt a native dimeric loop 2 conformation. The data support the view that BDNF-like partial agonist activity arises in these compounds because of their ability to dimerise trkB in a manner similar to the native protein. Furthermore, they serve to highlight the value of the structure-based approach in assisting in the design process.

In summary, a rational design approach using BDNF as a structural template has enabled us to prepare several novel BDNF loop mimetics, including BDNF inhibitors and a partial BDNF-like agonist. These compounds – along with the loop 2 based BDNF

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agonists and antagonists described previously by us [29,30] – provide further insight into the nature of the interactions between BDNF and its receptors (particularly trkB) and are an important step towards the development of structural mimetics of BDNF for use as pharmacological tools. In future studies in our laboratory, we will examine ways of enhancing the drug-like properties of these BDNF mimetics, in particular their proteolytic stability (e.g. through *N*-methylation of scissile bonds) and ability to cross cell membranes (e.g. though reductions in size and polarity), with a view to achieving our long-term goal of developing BDNF mimetics suitable for therapeutic use.

## Acknowledgements

This work was funded by the National Health and Medical Research Council of Australia. JMF is the recipient of a Melbourne Research Fellowship.

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