DESIGN OF POTENT PEPTIDE MIMETICS OF BRAIN- DERIVED NEUROTROPHIC FACTOR*

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SUMMARY

Brain-derived neurotrophic factor (BDNF) has potential for the treatment of human neurodegenerative diseases. However, the general lack of success of neurotrophic factors in clinical trials has led to the suggestion that low molecular weight neurotrophic drugs may be better agents for therapeutic use. Here we describe small, dimeric peptides designed to mimic a pair of solvent-exposed loops important for the binding and activation of the BDNF receptor, trkB. The monomer components that make up the dimers were based on a monocyclic monomeric peptide mimic of a single loop of BDNF (loop 2) that we had previously shown to be an inhibitor of BDNF-mediated neuronal survival (O’Leary, P.D. & Hughes, R.A. (1998) J Neurochem, 70, 1712-1721). Bicyclic dimeric peptides behaved as partial agonists with respect to BDNF, promoting the survival of embryonic chick sensory neurons in culture. We reasoned that the potency and/or efficacy of these compounds might be improved by reducing the conformational flexibility about their dimerizing linker. Thus, we designed a highly conformationally-constrained tricyclic dimeric peptide, and synthesized it using an efficient, quasi one-pot approach. Although still a partial BDNF-like agonist, the tricyclic dimer was particularly potent in promoting neuronal survival in vitro (EC_{50} 11pM). Being greatly reduced in size compared to the parent protein, the peptides described here could serve as useful lead compounds for the development of true neurotrophic drugs, and indicate that the structure-based design approach could be used to obtain potent mimetics of other growth factors that dimerize their receptors.
BDNF\textsuperscript{1} is a member of the neurotrophin family of neurotrophic factors, which includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), (1). In addition to its critical role in helping shape the vertebrate nervous system during development, BDNF is of particular therapeutic interest because of its neurotrophic actions on neuronal populations involved in several neurodegenerative diseases (2) including: sensory neurons, implicated in peripheral sensory neuropathies (3); motor neurons, which degenerate in amyotrophic lateral sclerosis (4); dopaminergic neurons of the substantia nigra, lost in Parkinson’s disease; and cholinergic neurons of the basal forebrain, involved in Alzheimer's disease (5).

BDNF, like the other neurotrophins, produces its effects on neurons through two transmembrane receptors. Binding of a neurotrophin to a specific member of the trk family of receptor tyrosine kinases—NGF binds to trkA, BDNF and NT-4/5 bind to trkB, and NT-3 preferentially binds to trkC—results in the stepwise homodimerization of the receptor, leading to receptor autophosphorylation and the initiation of multiple signal transduction pathways, including those leading to neuronal survival (6). In contrast, the glycoprotein p75 acts as a common low affinity receptor ($K_D \approx 10^{-9}$ M) for all the neurotrophins (7). Unlike the trk family, p75 signals apoptosis via a unique intracellular death domain, Chopper (8), although its precise biological function remains controversial. The high affinity binding sites for BDNF and the other neurotrophins on neurons ($K_D \approx 10^{-11}$ M) likely consist of a combination of the appropriate trk member and p75. The overall response to a neurotrophin therefore depends on the balance of signalling through a trk family member and p75, with the opportunity for modulation and cross talk at multiple levels of the signalling process.
The neurotrophins are homodimers consisting of two monomers each of approximately 120 residues. X-ray crystal structures of NGF (9), NT-3 (10), NT-4/5 and a BDNF/NT-4/5 heterodimer (11) reveal a common fold for the neurotrophins: Each monomer consists of seven β-strands (contributing to three longitudinal antiparallel β-sheets) connected by three solvent-exposed hairpin loops (loop 1, loop 2 and loop 4) and a longer loop (loop 3) and contains three disulfide bridges between six fully-conserved cysteine residues arranged in a cystine-knot motif, characteristic of this growth factor superfamily. However, in contrast to most of the other members of the superfamily, the neurotrophin monomers are arranged in the dimer in a parallel fashion, and are held together solely by non-covalent—largely hydrophobic—interactions.

A number of studies implicate the solvent-exposed loops of BDNF and the other neurotrophins in mediating their biological effects. Site-directed mutagenesis analyses have revealed that the ability to bind and activate trkB can be conferred to NGF by replacing residues in loop 2 with the corresponding residues from BDNF (12). The crystal structure of NGF in complex with one of the Ig domains of trkA shows direct contact between residues in the receptor and loop 1 of NGF (14), although the authors of this paper have since conceded that additional regions of the neurotrophins may well interact with other trk domains (14). In support of the functional role for loop 2, we have reported that conformationally-constrained peptides—of which the monocyclic monomeric peptide 1 (Table 1) was identified as the most effective—designed to mimic a single loop 2 of BDNF, are inhibitors of BDNF-mediated survival of sensory neurons in culture, likely by acting as competitive trkB antagonists (15).

Despite promising preclinical data, clinical trials with recombinant BDNF in patients with amyotrophic lateral sclerosis have proven unsuccessful (16). This failure is likely to be at
least in part due to the unfavourable pharmacokinetics of BDNF: for example, the plasma half life in rats of recombinant BDNF is less than one minute (17). These and similar data from other neurotrophic factors have led to the view that low molecular weight drugs, with more appropriate pharmacokinetic properties than the parent proteins, might prove a more fruitful means of harnessing neurotrophic action for therapeutic use (18).

As a step towards the development of such neurotrophic drugs, we describe here a structure-based approach for the discovery of potent mimetics of BDNF. Given our previous data with monocyclic monomeric BDNF inhibitors (15), we reasoned that appropriately designed dimeric loop 2 mimetics should be able to bring about trkB homodimerization, and thus mimic the actions of BDNF through this receptor. Using this approach, three classes of peptides were designed and synthesized: bicyclic dimeric peptides linked by a disulfide bridge internal to the sequence of monomeric monocyclic peptide 1; a bicyclic dimer linked by an amide bond external to the peptide 1 sequence; and a highly-constrained tricyclic dimeric peptide containing both disulfide and amide dimerizing linkages. The latter compound exhibited extremely high potency—comparable to BDNF—in promoting the survival of chick sensory neurons in culture. The results suggest that this compound is worthy of further preclinical development, and that our structure-based approach may be of general utility in developing potent mimetics of other growth factors and cytokines that dimerize their receptors.
EXPERIMENTAL PROCEDURES

Molecular modelling

Bicyclic dimeric peptides were designed using Sybyl molecular modelling software (version 6.4; Tripos, St Louis, MO) running on an O2 workstation (Silicon Graphics, Mountain View, CA). The loop 2 region from a model of the BDNF dimer—prepared by homology modelling (15)—corresponding to two copies of the following sequence:

\[ \text{Glu}^{40}\text{-Lys}^{41}\text{-Val}^{42}\text{-Pro}^{43}\text{-Val}^{44}\text{-Ser}^{45}\text{-Lys}^{46}\text{-Gly}^{47}\text{-Gln}^{48}\text{-Leu}^{49}\text{-Lys}^{50}\text{-Gln}^{51} \]

was inspected visually to identify positions where the loops were in close proximity to one another. Distances between pairs of \(\alpha\)-carbon atoms were then used to guide the selection of dimerizing linkages between the loops, which were incorporated as appropriate. A cyclizing disulfide bond was introduced into each of the newly linked loops at Lys\(^{41}\) and Lys\(^{50}\) as described previously (15). The residues beyond the terminal residues were deleted, either the zwitterions formed or the termini blocked, and the energy of each compound minimized. The resulting low energy conformation of each bicyclic dimeric peptide was superimposed onto the native BDNF loop 2 region and the degree of fit was quantified as the root mean square (RMS) deviation of the distances between common \(\alpha\)-carbon atoms.

Peptide synthesis

Assembly, cleavage and cyclization of monomeric precursors

Linear peptide precursors were synthesized manually (0.1 mmol scale) from fluorenylmethoxycarbonyl- (Fmoc-) protected amino acids using standard solid phase synthesis methodology, with the following points of note: Peptides for the synthesis of bicyclic disulfide-linked dimers 2-4 were assembled on chlorotrityl resin, preloaded with
Cys residues involved in cyclization were trityl (Trt) protected; the Cys residue needed for the dimerizing linkage was acetamidomethyl (Acm) protected. Peptides required for the preparation of the bicyclic amide-linked dimer 5 and tricyclic dimer 6 were synthesized on Rink amide 4-methylbenzhydrylamine resin, which upon cleavage yields the peptide amide. The C-terminal Glu and Lys residues were incorporated as trifluoroacetic acid- (TFA-) labile O-tbutyl (OtBu) and t-butyloxycarbonyl (Boc) protected derivatives. To improve reaction efficiency, the resins were combined following coupling of Glu(OtBu) and Lys(Boc) residues, and subsequent assembly, cleavage and purification steps carried out on the mixture. Ser and the additional Lys residue were incorporated as the TFA-stable benzylxycarbonyl (Z) and benzyl (Bzl) derivatives. For the tricyclic dimer 6, the Cys residue required for the third cycle was incorporated as the Acm derivative. The N-termini of peptides 5 and 6 were acetylated prior to cleavage. To reduce the likelihood of racemization, the N-terminal Cys residue was coupled to all peptides as the preformed symmetrical anhydride (19). All peptides were cleaved from the resin with TFA/ethanediol/H2O (18:1:1). The crude partially-protected linear peptides were oxidized to the corresponding partially-protected monocyclic monomers by oxidizing the peptide (1mg/ml) in a solution of dimethyl sulfoxide (10%) in 0.1 M aqueous NH4HCO3, pH 8 (20).

**Synthesis of bicyclic disulfide-linked dimers**

Bicyclic disulfide-linked dimers 2-4 were prepared using a modification of the method of Kamber et al. (21) by dissolving the appropriate Acm-protected monocyclic monomeric peptide (8 µmol) in aqueous acetic acid (40 µL, 50 %) containing HCl (10 µL, 1 M), adding I2 (400 µL, 50 mM in 50 % acetic acid) and stirring the mixture at room temperature under N2. Upon completion (typically 6 h), the reaction mixture was quenched with ascorbic acid (10 µL, 1 M) and the desired bicyclic disulfide-linked dimer purified by HPLC.
**Synthesis of partially protected bicyclic amide-linked dimer**

The Bzl/Z-protected bicyclic amide-linked dimer was prepared by dissolving the two Bzl/Z-protected monocyclic monomers (6 µmol each) in dimethylformamide (500 µL) prior to the addition of O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyloxonium hexafluorophosphate (HATU 6.6 µmol) and diisopropylethylamine (DIPEA, 9.9 µmol) and stirring. Upon completion (approximately 6 h), the reaction was diluted 20-fold with 10% acetonitrile and the desired Bzl/Z-protected bicyclic amide-linked dimer purified by HPLC.

**Synthesis of partially-protected tricyclic dimer**

The Z-protected tricyclic dimer was prepared using a combination of the dimerization methods described for disulfide- and amide-linked dimers above. Firstly, the two Acm/Z-protected monocyclic monomers (6 µmol each) were coupled in the presence of HATU/DIPEA. The resultant Acm/Z-protected bicyclic dimer (2.8 µmol) was converted to the Z-protected tricyclic dimer by treatment with I₂.

**HF cleavage**

Remaining Bzl and Z protecting groups were removed from partially protected bicyclic amide-linked dimer and partially protected tricyclic dimer by treating the crude peptides with HF in the presence of m-cresol (10:1) using standard methods.

**Purification and characterization of peptides**

General reaction progress was monitored and peptides purified as appropriate by HPLC using UV detection (220 nm) on analytical (150 x 2.1 mm) or semipreparative (250 x 10 mm) C18 columns. All final peptides were eluted as single peaks following purification. Identity of all peptide intermediates and final products was confirmed by mass
spectrometry, using a Micromass platform II triple quadrupole mass spectrometer with an
electrospray source (carried out at the Victorian College of Pharmacy, Monash University,
Australia). All peptides—intermediates and final products—gave molecular ions with a
mass-to-charge ratio within 0.1% of calculated values.

Sensory neuron cultures

Peptides were assayed in primary cultures of dorsal root ganglion sensory neurons
prepared from 8 day old embryonic chicks as described previously (15). Peptides ($1 \times 10^{-11}
to 1 \times 10^{-4}$ M) were added to cells in triplicate wells 1 h after plating either alone
(concentration-response studies) or with mouse recombinant BDNF ($4 \times 10^{-11}$ M; 
competition studies). Positive control wells contained BDNF only; negative control wells
contained neither BDNF nor peptide. Initial viable neuron numbers were determined by
counting phase-bright cell bodies in 40 microscope fields ($0.25 \times 0.25$ mm, at $200 \times$
magnification) in four randomly-chosen wells. After 48 h incubation, surviving
neurons—phase-bright cells with neurites—were counted.

Data analysis

Cell counts for individual experiments were normalized by setting neuronal survival in the
BDNF-only positive controls (typically 35-40% of the initial number of viable neurons
plated) to 100 %, and survival in negative controls (typically 4-6%) to 0 %. Neuronal
survival was expressed as the mean ± SEM from 4 or 5 different preparations. pEC$_{50}$ and
pIC$_{50}$ values were estimated from logistical sigmoidal curves fit to concentration-response
data. Neuronal survival in the presence of peptides was compared to negative controls
(concentration-response studies) or positive controls (competition studies) by ANOVA
followed by Bonferroni’s multiple comparisons test.
RESULTS

Design of bicyclic dimeric peptides

Examination of the loop 2 region of a model of the three dimensional structure of the BDNF homodimer (Fig.1A) revealed two sites in relatively close proximity: Site 1 (Pro\textsuperscript{43}-Pro\textsuperscript{43}, Val\textsuperscript{44}-Val\textsuperscript{44}, Ser\textsuperscript{45}-Ser\textsuperscript{45}), located within sequence of the monocyclic monomeric peptide 1, and Site 2 (Gln\textsuperscript{51}-Gln\textsuperscript{51}, Tyr\textsuperscript{52}-Tyr\textsuperscript{52}, Phe\textsuperscript{53}-Phe\textsuperscript{53}), located beyond the sequence of peptide 1 (Fig. 1B). The distance between α-carbon atoms of Val\textsuperscript{44}-Val\textsuperscript{44} and Ser\textsuperscript{45}-Ser\textsuperscript{45} fell within the maximal preferred distance of Cys-to-Cys constraint (6.6 Å, determined by conformational analyses). Incorporation of a Cys-to-Cys dimerizing linkage at these two positions yielded the bicyclic dimers 3 and 4 (Table 1), that displayed relatively low RMS deviation values (1.5 Å and 1.4 Å) when superimposed onto the native loop 2 conformation. The distance between the α-carbon atoms of the Pro\textsuperscript{43}-Pro\textsuperscript{43} (11.3 Å) suggested that longer constraints might be more suitable at this position. However, incorporation of Glu-to-Lys or bridged Cys-to-Cys constraints (eg. Cys-to-(CH\textsubscript{2})\textsubscript{5}-to-Cys) yielded peptides that mapped poorly onto the native BDNF conformation (RMS deviations of 3.1 and 2.3 Å), while the shorter Cys-to-Cys constraint (peptide 2; RMS 1.2 Å) was in fact well tolerated, presumably due to favourable local conformation preferences. In site 2, only Gln\textsuperscript{51}-Gln\textsuperscript{51}, situated adjacent to the C-terminal of the monocyclic monomer 1, was investigated as a location for a dimerizing linkage, as the other sites (Tyr\textsuperscript{52}-Tyr\textsuperscript{52} and Phe\textsuperscript{53}-Phe\textsuperscript{53}) were expected to give rise to peptides with inappropriate flexibility. Of the constraints examined in this position, the shorter Glu-to-Lys linkage (peptide 5; RMS 1.7 Å) was better suited to this position than longer constraints (eg. Cys-(CH\textsubscript{2})\textsubscript{7}-Cys, RMS 3.1 Å).

[Fig.1]
Synthesis of bicyclic dimeric peptides

The bicyclic dimers 2-5 were prepared using appropriate orthogonal sidechain protection strategies, to allow the selective formation of cyclizing (i.e. disulfide) and dimerizing (i.e. disulfide or amide) constraints. The disulfide-linked dimers 2-4 were synthesized using TFA-labile Trt protection for Cys required for the formation intramolecular disulfide bonds, and TFA-stable Acm-protection for Cys involved in the dimerizing disulfide bridges. The success of the iodine-mediated removal of Acm and subsequent oxidation to the dimer was found to be dependent on the nature of the iodine scavenger used—best results were obtained following quenching with ascorbic acid solution. Use of other scavengers, eg. sodium thiosulfate, resulted in multiple reaction products.

The amide-linked dimer 5 was synthesized using a combination of tertiarybutyl- (i.e. TFA labile) and benzyl- (i.e. TFA stable) derived protection. Following cyclization of the N- and C- terminally protected monomers, dimerization was effected with the amide bond forming reagent HATU. Other condensation agents and conditions gave rise to multiple and/or incorrect products. The final step in the synthesis of 5 was the removal of the remaining benzyl-derived protecting groups on Ser and Lys by treatment with hydrogen fluoride.

Mass spectral data relating to the bicyclic dimers 2-5 can be found in Table 1.

[Table 1]

Effects of bicyclic dimeric peptides on sensory neuron survival in vitro

The effects of the bicyclic dimers 2-5 on neuronal survival were assessed in primary cultures of embryonic chick dorsal root ganglion sensory neurons, an assay used routinely in the literature to characterize the survival effects of BDNF and the other neurotrophins. When added alone to primary cultures of embryonic chick sensory neurons, the bicyclic
disulfide-linked dimeric peptides 2 and 4, and the bicyclic amide-linked dimeric peptide 5 produced significant and concentration-dependent increases in neuronal survival (Fig 2A). Although peptides 2, 4 and 5 gave a similar maximal survival effect (around 30% of the maximal survival effect of BDNF), peptide 5 was clearly the most potent of the bicyclic dimers (pEC$_{50}$ of 10.0±0.15), being over 100-fold more potent than either 2 or 4. For peptides 2, 4 and 5, the survival effect dropped after reaching a maximum, giving their concentration-response curves a distinct bell-shape. In contrast to the survival promoting effects of 2, 4 and 5, neither the bicyclic disulfide-linked dimer 3 (Fig 2A), nor the monocyclic monomeric precursors to the bicyclic dimers (data not shown) significantly promoted neuronal survival compared to negative controls over the concentration range tested.

[Fig. 2]

To assess the possible partial agonist behaviour of the bicyclic dimeric peptides, the bicyclic disulfide-linked dimeric peptide 4 was added to chick sensory neuron cultures in competition with BDNF. Similarly to its loop 2-based monocyclic monomeric counterpart 1 (15), dimeric bicyclic peptide 4 caused significant and concentration-dependent inhibition of BDNF-mediated neuronal survival (Fig 2B; maximal inhibition of 49 % ± 7 at 1×10$^{-7}$ M; pIC$_{50}$ of 9.43 ± 0.25). Together, these data suggest that bicyclic disulfide linked dimer 4 acts as a partial agonist on the majority of neurons that respond to BDNF, rather than being a full agonist on a subset (30-35%) of BDNF responsive neurons.

**Design of tricyclic dimeric peptides**

We reasoned that the relatively low potency and partial agonist activity of the bicyclic dimers 2-5 may be due, at least in part, to conformational flexibility about the dimerizing
linkages, meaning that the two monomeric units within the peptides are likely to be capable of 360° rotation relative to one another (Fig 3). The bicyclic dimers would likely only behave as trkB agonists when they existed in a conformation able to cause the in-plane dimerization of trkB (i.e. in a roughly parallel conformation). When the relative orientation of the two monomeric units is not favourable to receptor homodimerization, (eg. when the units are antiparallel), the bicyclic dimers would likely behave as antagonists, leading to a reduction in maximum, decrease in slope and rightward shift of the concentration-response curve, as previously shown for monocyclic monomeric peptide acting as competitive trkB antagonists (15).

[Fig. 3]

To explore this hypothesis, and to attempt to obtain a BDNF mimetic with greater potency and efficacy, we designed tricyclic dimeric peptides—effectively hybrids of the disulfide-linked and amide-linked dimers—in which two monocyclic monomeric loop 2 units were linkeded by both an amide and a disulfide linkage (Fig 3). Molecular dynamics simulations of these peptides showed that they possessed greatly reduced conformational freedom compared to their bicyclic counterparts, resulting in significantly greater similarity to the native loop 2 region of BDNF (data not shown). One of these tricyclic peptides, 6, was chosen for synthesis.

**Synthesis of tricyclic dimeric peptide**

The challenging synthesis of the tricyclic dimeric peptide 6 was approached using a combination of the strategies used to synthesize the bicyclic disulfide-linked and amide-linked dimers, allowing complete control to be exercised over the formation of the multiple cyclizing and dimerizing linkages (Fig. 4). Using this approach, the two linear precursors
to peptide 6 were assembled (see Fig. 4, step (a)), cleaved from the resin and partially deprotected (b) and cyclized (c), and the cyclic monomers dimerized (d) as described for the bicyclic amide linked dimer 5. The third cycle was then introduced by treating the bicyclic peptide with iodine (e), bringing about the stepwise removal of the acetamidomethyl protected Cys residues and the subsequent oxidation of the free thiols to the disulfide, as described for bicyclic disulfide-linked dimers 2-4. Treatment of the partially-protected tricyclic dimer with HF (f) yielded the crude desired tricyclic dimeric peptide 6, which was readily purifiable by HPLC.

To maximize the efficiency of this synthetic approach, we carried out steps (a) to (d) (Fig. 4) on the mixture of the two peptide building blocks. To do this, the two portions of resin—one preloaded with Fmoc-Glu(OtBu), the other with Fmoc-Lys(Boc)—were combined, and the subsequent peptide elongation reactions carried out on the resin mixture. The linear protected peptides were cleaved, cyclized and dimerized as a mixture, halving the number both of the reactions and chromatographic purifications. The remaining steps (e) and (f) proceeded as described above yielding a product identical to that obtained following synthesis of discrete compounds. Using this optimized, quasi one-pot approach, it is possible to prepare purified tricyclic dimeric peptide 6 from scratch in under a week.

Effects of tricyclic dimer on sensory neuron survival

When added alone to primary cultures of embryonic chick sensory neurons, the tricyclic dimeric peptide 6 produced significant and concentration-dependent increases in neuronal survival (Fig 2A; maximum survival 35% at 1×10^{-8} M). The tricyclic dimer 6 was
particularly potent, with pEC₅₀ of 10.96±0.32 (corresponding to an EC₅₀ of 11 pM), making it around 10-fold more potent than the bicyclic amide-linked dimer 5, and 1000-fold more potent than the bicyclic disulfide-linked dimer 4. However, like the bicyclic dimers, tricyclic dimer 6 also displayed a bell-shaped concentration-response curve, although the curve width was approximately two-fold greater than either 4 or 5.

In competition with BDNF, the tricyclic dimer 6 caused significant and concentration-dependent inhibition of BDNF-mediated neuronal survival (Fig 2B; maximal inhibition 47%±6 at 1×10⁻⁶ M; pIC₅₀ 10.13±0.08). These values were not statistically different to those of either the bicyclic dimeric peptide 4 or the monocyclic monomeric peptide 1 (ANOVA, Bonferroni's multiple comparison test).
DISCUSSION

This paper describes the design of low molecular weight, conformationally-constrained dimeric peptide mimetics of the neurotrophic factor BDNF. The peptides were based on the monocyclic monomeric peptide 1—a mimetic of a single solvent-exposed loop 2 of BDNF—a compound we had previously shown to behave as a competitive trkB antagonist (15). Using the 3D structure of the BDNF dimer as a template, a series of bicyclic dimeric peptides consisting of pairs of covalently-tethered analogues of peptide 1 were designed and synthesized. Unlike their monomeric counterpart, three of the four bicyclic dimeric peptides acted as BDNF-like agonists, promoting the survival of embryonic chick sensory neurons in vitro, with potency and efficacy influenced by the nature and position of the dimerizing constraint. We reasoned that neuronal survival activity of these bicyclic dimers might be limited by excessive conformation flexibility about the disulfide or amide dimerizing linkage. To test this hypothesis, we subsequently designed and synthesized a tricyclic dimeric peptide, in which the monocyclic monomeric subunits were linked by two dimerizing linkages. Although—like the bicyclic dimers—still a partial BDNF agonist, the tricyclic dimer was ten-fold more potent in its neuronal survival effect than the best of the bicyclic dimers, making it around two-fold less potent than BDNF itself.

There are now several examples in the literature of dimeric peptides able to mimic the effects of larger protein agonists, presumably by being able to bring about receptor dimerization, including: bicyclic peptide agonists of the erythropoietin receptor (22-24) and the thrombopoietin receptor (25), bicyclic peptide antagonists of interleukin-5 (26) and monocyclic dimeric agonists of the cell recognition molecule N-cadherin (27, 28). In the case of erythropoietin agonists, monocyclic peptides discovered through a phage display process could promote erythropoietin receptor homodimerization by virtue of the ability of the peptides to non-covalently self-associate into dimers (23). In this system, potency
could be improved by including a single covalent dimerizing linkage between the peptides (24). It is of interest to note that the 3D structure of a non-covalently dimerized erythropoietin peptide bound to the erythropoietin receptor bears some resemblance to the modelled structure of the tricyclic dimer 6 (data not shown), even though the receptors they activate have no apparent homology. This observation supports the hypothesis that the molecular design approach we describe in this paper—using two appropriately incorporated dimerizing linkages to greatly restrict conformational freedom—could be of general utility in improving the potency of a range of dimeric peptide ligands.

Despite the ability of the bicyclic and tricyclic dimers to promote sensory neuron survival in vitro, they are partial agonists with respect to BDNF, in that they are able to support maximally about 35% of those neurons that would be kept alive by BDNF, and inhibit the neuronal survival effect of BDNF when added in competition. One mechanism for this behaviour is that the compounds are partial agonists of trkB, possibly because they are less efficient than BDNF in bringing about the dimerization of trkB, possibly due to inappropriate conformational flexibility of the dimerizing linkages (although the retention of partial agonism by the highly constrained tricyclic compound would argue against this). Indeed, subtle conformational effects on receptor dimerization have been noted for a number of systems (29, 30) and 3D structural data for the interaction of the neurotrophins with the trks indicate that a symmetrical complex is formed (13, 31). A consequence of reduced dimerization efficiency could be a reduced ability to cause the autophosphorylation of tyrosine residues in trkB. In the cytoplasmic domain of trkB, five tyrosine residues – Y484, Y670, Y674, Y675 and Y785 – have been shown to undergo autophosphorylation upon exposure of cells expressing trkB to BDNF (32, 33). It is feasible that the dimeric peptides do not bring about optimal levels of autophosphorylation at all tyrosines, and/or give a time course of autophosphorylation that differs to that of
BDNF. These differences may cause a reduced activation of downstream signalling pathways and/or activation of specific pathways only, either of which could lead to partial agonism through trkB. Although bulk measurement of autophosphorylation of trkB (eg. 34, 35) would help confirm the hypothesized mechanism of action of the bicyclic dimeric peptides through activation of trkB it is unlikely to be sensitive enough to detect the possible subtle differences in autophosphorylation levels that might lead to partial trkB agonism. A more meaningful investigation might require studies with mutant trkB isoforms lacking individual phosphorylation sites (33), the use of site specific antibodies such as those developed by Segal et al. (36) or the analysis of the specific downstream signally componentry.

An alternative mechanism for the partial agonist behaviour of the dimeric peptides is that they act through trkB only—even as full trkB agonists—and not p75. The bicyclic and tricyclic dimeric peptides all gave bell-shaped concentration-response curves, thus resembling compounds that produce their action through homodimerization of a single receptor, such as growth hormone, and typical growth factors (37, 38). In contrast, BDNF and the other neurotrophins show a more complex concentration response relationship for neuronal survival (39), indicating the involvement of multiple receptors (i.e. a trk member plus p75), although effects known to be elicited through a trk member alone (e.g. neurite outgrowth) do exhibit a simple bell-shaped concentration-response relationship (40). Pharmacodynamic modelling studies we have performed suggest that the dimeric peptides, by acting solely as trkB agonists, may be failing to recruit anti-apoptotic action through p75, giving rise to the reduced maximal survival effect\(^2\). To help elucidate the mechanism of this partial agonistic behaviour, a variety of experimental approaches could be used. Firstly, the binding characteristics of the bicyclic dimeric peptides to trkB and p75 need to be examined. A functional contribution (or lack thereof) of p75 towards the action
of the bicyclic dimeric peptides could be studied using known p75 antagonists, such as blocking antibodies (eg. REX) (41) or by examining the effects of the peptides on downstream targets of p75, such as JNK-p53-Bax, ceramide and NF-κB (42). Similar experiments using known trkB inhibitors, such as K252a (43), could be used to confirm the involvement of trkB.

The tricyclic dimeric peptide described here is the most potent member of a growing class of small molecule neurotrophin mimetics, including a trkA mimetic developed from a small cyclic peptide based on loop 4 of NGF (44), and a series of dimeric β-turn mimetics that act as agonists of trkC (45). To date, the neurotrophins have failed to achieve clinical success in the treatment of neurodegenerative diseases. It remains to be seen whether the currently available neurotrophin mimetics will lead to drugs that effectively harness neurotrophin actions for therapeutic use.

In conclusion, the structure-based drug design approach we describe has yielded potent low-molecular weight peptide mimetics of BDNF with pM potency comparable to that of the native protein. These compounds could serve as a springboard for the development of therapeutically useful BDNF mimetics. Furthermore, given the similarities between them and other growth factor/cytokine receptor dimerizing ligands, the approach may have general utility across a range of ligand receptor systems involving receptor homodimerization.
REFERENCES


FOOTNOTES

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¹The abbreviations used are: BDNF, brain derived neurotrophic factor; NGF, nerve growth factor; neurotrophin 3, NT-3; NT-4/5, neurotrophin-4/5; RMS, root mean square; Fmoc, fluorenylmethoxycarbonyl; Acm, acetamidomethyl; TFA, trifluoroacetic acid; OtBu, O-tertiarybutyl; Boc, tertiarybutyloxycarbonyl; Z, benzyloxycarbonyl; Bzl, benzyl; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA, diisopropylethylamine.

²POL and RAH, manuscript in preparation.
FIGURE LEGENDS

Table 1. Structures, mass spectral data and summary of in vitro neuronal survival effect of compounds described in this study.
* Previously reported (15).
† Maximal survival and pEC$_{50}$ not determined for antagonist.
‡ Survival too low for pEC$_{50}$ to be determined.

Fig. 1. Molecular modelling of dimeric BDNF loop 2 mimetics. Model of the three dimensional structure of the BDNF homodimer derived by homology modelling, showing the positions of the solvent exposed loops 1, 2, 3 and 4 (A). α-Carbon trace of the loop 2 region of the BDNF dimer indicating distances (in angstroms) between residues in adjacent loops (B). The position of the disulfide bond in monocyclic monomeric BDNF inhibitor peptide 1 (Lys$^{41}$ to Lys$^{50}$) is shown as a dashed line. Sites 1 and 2 are areas where the two loops are in closest proximity, within and outside respectively the sequence of peptide 1.

Fig. 2. Effects of dimers 2-6 alone (A) and dimers peptides 4 and 6 (added in combination with BDNF) (B) on survival of embryonic chick dorsal root ganglion sensory neurons in culture. BDNF was used at 4 x 10$^{-11}$ M. Surviving neurons were counted after 48 h in culture. Neuronal survival is expressed as the mean ± SEM from 4 or 5 different preparations after being normalised to survival in BDNF-only positive controls (100 % survival), and negative controls (0 %).

Fig. 3. Rationale for design of dimeric peptides with reduced conformational freedom. The freely-rotating dimerizing linkers in bicyclic disulfide- and amide-linked
dimers were incorporated into a single tricyclic dimer, in which the two cyclic loop mimetics are constrained in a native, BDNF-like conformation.

Fig. 4. Scheme for synthesis of tricyclic dimeric peptide 6.
<table>
<thead>
<tr>
<th>no.</th>
<th>structure</th>
<th>m/z (M+H predicted)</th>
<th>max survival</th>
<th>pEC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H-CVPVSKGQLC-OH</td>
<td>1030.5* (1030.5)</td>
<td>-†</td>
<td>-†</td>
</tr>
<tr>
<td>2</td>
<td>H-CVCVSKGQLC-OH</td>
<td>2072.9 (2070.9)</td>
<td>27%</td>
<td>7.70±0.82</td>
</tr>
<tr>
<td>3</td>
<td>H-CVPCSKGQLC-OH</td>
<td>2067.8 (2066.9)</td>
<td>9%</td>
<td>-‡</td>
</tr>
<tr>
<td>4</td>
<td>H-CVPVCAGQLC-OH</td>
<td>2092.0 (2092.9)</td>
<td>29%</td>
<td>7.84±0.17</td>
</tr>
<tr>
<td>5</td>
<td>Ac-CVPVSKGQLCE-NH$_2$</td>
<td>2382.9 (2382.2)</td>
<td>32%</td>
<td>10.01±0.15</td>
</tr>
<tr>
<td>6</td>
<td>Ac-CVPVCAGQLCE-NH$_2$</td>
<td>2412.0 (2411.1)</td>
<td>35%</td>
<td>10.96±0.32</td>
</tr>
</tbody>
</table>

Table 1
Fig 1
Fig 2
Fig 3
(a) solid phase peptide synthesis

(b) cleavage/partial deprotection (TFA)

(c) cyclization (DMSO)

(d) dimerization (HATU)

(e) tricycle formation (I$_2$

(f) final deprotection (HF)
Design of potent peptide mimetics of brain-derived neurotrophic factor
Paul D. O'Leary and Richard A. Hughes

J. Biol. Chem. published online May 2, 2003

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