British Journal of Pharmacology (1999) 128, 647–652
http://www.stockton-press.co.uk/bjp

Novel strategies for the design of receptor-selective vasopressin analogues: Aib-substitution and retro-inverso transformation

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1 We determined the pharmacological profile of novel backbone-modified peptides designed as protease-resistant, selective analogues of AVP. Binding affinities of peptides were determined at both V_{1A} and V_2 subtypes of vasopressin receptor (VPR). Biological potencies of selected peptides were tested in pressor and antidiuretic bioassays.

2 Substitution of the achiral α-aminoisobutyric acid (Aib) at position 4 or 7 of AVP produced peptides that selectively bound the V_2 VPR. Both [Aib[4]]AVP (140 IU mg^{-1}) and [Aib[7]]AVP (36 IU mg^{-1}) are selective antidiuretic agonists with little or no activity in uterotonic and pressor assays.

3 [Aib[4]] and [Aib[7]] derivatives of the linear V_{1A}-selective antagonist [PhaaDTyr(Et)[2]Arg[6]Tyr(N-H_2)[9]]AVP bound selectively and with high affinity (K_d 0.51 and 4.1 nM respectively) to the V_{1A} VPR. Bioassays confirmed that these peptides were potent antivasopressor agents (pA_2 8.10 and 8.36 respectively).

4 A total retro-inverso strategy was used to prepare protease-resistant mimetics of both AVP and linear V_{1A}-selective antagonists. Cyclic retro-inverso mimetics of AVP did not bind either V_{1A} or V_2 VPRs. In contrast, rationally designed retro-inverso mimetics of linear V_{1A}-selective antagonists selectively bound the V_{1A} VPR.

5 Our findings indicate novel methods to improve the pharmacodynamic and pharmacokinetic parameters of neurohypophysial hormone analogues which could be equally applicable to other peptide-receptor systems.

Keywords: Vasopressin; receptor subtypes; antagonist; α-aminoisobutyric acid; retro-inverso peptide

Abbreviations: Aib, α-aminoisobutyric acid; Benz, benzylamide; K_d, dissociation constant, LVP, [8-lysine]vasopressin; Pa, propionyl; Phaa, phenylactyl; (*R*) indicates a retro-inverso peptide; VPR, vasopressin receptor

Introduction

The mammalian neurohypophysial peptide hormones [8-arginine]vasopressin (AVP) and oxytocin are structurally related cyclic nonapeptides. The phylogenetic history of vasopressin- and oxytocin-like peptides, and the possible co-evolution of peptide : receptor pairs, can be traced to primitive organisms that include hydrozoans (Acher, 1993; Van Kesteren *et al*., 1996). The structure : activity relationships of selective agonists and antagonists of AVP (Lebl *et al*., 1987; Manning *et al*., 1987a), pharmacological studies (Michell *et al*., 1979; Antoni, 1984; Jard *et al*., 1987; Howl & Wheatley, 1995), and the cloning of cDNAs encoding vasopressin receptors (Birnbaumer *et al*., 1992; Morel *et al*., 1992; Sugimoto *et al*., 1994) indicate that mammals express three subtypes of VPR classified as V_{1A}, V_{1B} and V_2. The V_{1A} VPR which mediates the pressor action of AVP and the V_2 VPR which regulates antidiuresis have been studied in most detail and are potential therapeutic targets (László *et al*., 1991; Manning & Sawyer, 1991). Hence, receptor-selective analogues of AVP with enhanced proteolytic stability would have clear advantages over endogenous hormones both as pharmacological tools and therapeutic agents.

A common post-translational modification of the vasopressin/oxytocin family of peptides is a disulphide bond between cysteine residues at positions 1 and 6 generating a 20-membered cyclic ring which was believed to be essential for biological activity. For the design of antagonists at both V_{1A} and V_2 VPRs, the substitution of β,β-dialkylated residues at position-1 of AVP was an important early achievement (reviewed by Manning *et al*., 1987a). More recently, linear (acyclic) analogues of AVP have proven to be high affinity antagonists at both the V_{1A} and V_2 VPR (Manning *et al*., 1987b). These peptides are also suitable precursors for the development of selective hetero bi-functional receptor probes and radioiodinated ligands (Manning *et al*., 1988; Schmidt *et al*., 1991; Howl *et al*., 1993; Howl & Wheatley, 1995). Moreover, the report of linear V_2-selective agonists (Manning *et al*., 1991) finally removed the dogma that a ring structure was essential for agonism at VPRs. Receptor-compatible substitutions at positions 1 and 6 in linear analogues of AVP are, however, highly restricted and there is evidence that residues occupying these positions are interdependent (Manning *et al*., 1990; Howl *et al*., 1994). These observations indicate that linear analogues adopt a similar structure to cyclic peptides when bound to a VPR.

To further determine the contribution of backbone-conformation to ligand : receptor interaction and signalling, we used two complementary strategies to perturb the structure of both cyclic and linear analogues of AVP. Conformational analysis indicates that Aib-containing peptides almost invariably adopt a helical backbone (Karle,

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1996; Balkrishnan *et al*., 1997). Moreover, Aib-substitution has been utilized in the design of protease-resistant receptor ligands and enzyme inhibitors. Hence, we rigorously assessed the pharmacological parameters of cyclic and linear vasopressin analogues modified by Aib-substitution at positions 4, 7 or 9. These substitutions were dictated by extensive structure : activity studies indicating that modifications of residues occupying positions 4, 7 and 9 are compatible with the development of receptor subtype-selective vasopressin analogues (Lebl *et al*., 1987; Manning *et al*., 1987a; Manning & Sawyer, 1991). Our second strategy utilized retro-inverso peptidomimetics of AVP synthesized in reverse sequence from mostly D-amino acids. Theoretical considerations suggest that the side-chain orientation of retro-inverso peptides is equivalent to conventional peptides even though carbonyl and amine groups forming backbone amides are reversed (Chorev & Goodman, 1993). Thus, we also investigated the pharmacological properties of a rationally designed series of retro-inverso homologues of AVP and linear antagonists. Our findings provide data pertinent to the development of V_2-selective agonists and protease-resistant V_{1A}-selective antagonists.

Methods

Peptide synthesis

All peptides were prepared by conventional solid phase methodologies. [Lys^8,Aib^9]vasopressin ([Aib^9]LVP) was synthesized on 4-methylbenzhydrylamine (MBHA) resin using an N-α-tert-butoxycarbonyl (tBoc) protection strategy. Cleavage and deprotection with hydrogen fluoride yielded free nonapeptide, which was oxidized with 0.1 M potassium ferricyanide. Purification by reverse phase high performance liquid chromatography (HPLC, Vydac 218TP510 column) and lyophilization yielded 12.2 mg of pure product. Identity and purity of [Aib^9]LVP was confirmed by a combination of thin layer chromatography and amino acid analysis. Alta Bioscience (University of Birmingham, U.K.) prepared all other analogues of AVP on a 50–100 μmol scale using N-α-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids. Aib was incorporated using a 4 fold excess with equimolar (relative to Aib) 2-(1H-Benzotriazol-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-Hydroxybenzotriazole (HOBT)/Diisopropylethylamine (DIPEA) and a double coupling cycle. Peptide amides were synthesized on Rink amide MBHA resin. Retro-inverso peptides with a C-terminal benzylamido function were prepared on PEG-PS resin derivatized with a base-labile HMBA linker. Aminolysis with benzylamine yielded free benzylamides (Howl & Wheatley, 1998). Acetylation of N-termini used a 5 fold molar excess of acetic anhydride. Cystine bond formation in [Aib^4]AVP and [Aib^7]AVP was achieved by air oxidation in 0.1 M ammonium bicarbonate (Howl & Wheatley, 1993). Peptides were purified to homogeneity using semi-preparative reverse phase HPLC (Vydac C_{18} 218TP510 column; Howl *et al*., 1993; Howl & Wheatley, 1993). A combination of amino acid analysis and analytical HPLC (Phase Separations, Spherisorb C_{18} column 0.4×25 cm; Howl *et al*., 1993; Howl & Wheatley, 1993) indicated that lyophilized peptides were >95% pure. Analysis by matrix assisted laser desorption ionization mass spectroscopy (Kratos) confirmed the predicted mass of each peptide with an accuracy of ± 1. Stock solutions (1–5 mM) of pure peptides were prepared in 0.05% (v v^{-1}) acetic acid.

Ligand binding assays

Cell membranes from bovine kidney medulla (V_2 VPR) and rat liver (V_{1A} VPR) were prepared as previously described (Howl *et al*., 1991a,b; Howl & Wheatley, 1993). Binding assays utilized 0.41–1.07 nM [Phe-3,4,5-^{3}H]AVP (81.0 Ci mmol^{-1}, NEN) as a tracer ligand. Equilibrium binding was achieved by incubation of membranes and ligands at 30°C for 90 min in buffer A (20 mM HEPES, 10 mM $Mg(CH_3CO)_2$, 1 mM EGTA, 0.5 mg ml^{-1} bacitracin, 1 mg ml^{-1} bovine serum albumin, pH 7.4; Howl *et al*., 1991a,b; Howl & Wheatley, 1993). Dissociation constants (K_d) of unlabelled peptides were calculated from IC_{50} values determined by competition binding according to the method of Cheng & Prusoff (1973) with K_d value of AVP at both rat V_{1A} and bovine V_2 VPR equal to 0.68 nM (Howl *et al*., 1991b).

Bioassays

Biological activities of selected analogues were determined by standard assays for neurohypophysial peptides using Wistar rats. Vasopressor tests used phenoxybenzamine treated male rats (Dekanski, 1952). Antidiuretic assays on conscious rats were as previously described (Burn *et al*., 1950; Skopková *et al*., 1981). These tests used synthetic AVP as a standard. Dose-response curves were constructed and activities determined by comparison of threshold doses.

Cyclic peptides

AVP

H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

(*R*)-AVP, X = H; (*R*)-AVP-ac, X = CH_3CO

X-Gly-D-Arg-D-Pro-D-Cys-D-Asn-D-Gln-D-Phe-D-Tyr-D-Cys-NH_2

Linear peptides

[PhaaDTyr(Me)^{2}Val^{4}Arg^6]AVP

C_6H_5-CH_2-CO-D-Tyr(Me)-Phe-Val-Asn-Arg-Pro-Arg-Gly-NH_2

(*R*)-[Benz-Y(Me)^2,R^6,K^9]-ac

CH_3CO-D-Lys-D-Arg-Gly-D-Arg-D-Asn-D-Gln-D-Phe-Tyr(Me)-NH-CH_2-C_6H_5

(*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7]AVP, X = H;
(*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7]AVP-ac, X = CH_3CO

X-D-Arg-Gly-D-Arg-D-Asn-D-Val-D-Phe-Tyr(Me)-NH-CH_2-C_6H_5

(*R*)-desG^9,desP^7[Benz-Y(Me)^2,V^4,R^6]AVP, X = H
(*R*)-desG^9,desP^7[Benz-Y(Me)^2,V^4,R^6]AVP-ac, X = CH_3CO

X-D-Arg-D-Arg-D-Asn-D-Val-D-Phe-Tyr(Me)-NH-CH_2-C_6H_5

Figure 1 Structures of retro-inverso peptides. The structures of (*R*) peptides are compared with representative L-enantiomers AVP and [PhaaDTyr(Me^2)Val^{4}Arg^4]AVP. N- and C-termini of AVP are effectively reversed in the synthesis of (*R*)-AVP. The α-nitrogen of Gly in (*R*)-AVP-ac is acetylated. To preserve side-chain orientation, all linear (*R*) peptides contain Tyr(Me) to mimic the methylated D-Tyr at position-2 of [PhaaDTry(Me)^2 Val^{4}Arg^6]AVP. Phaa, at position-1 of [PhaaDTry(Me)^{2}Val^4 Arg^6]AVP, is mimicked in the synthesis of (*R*) peptides by chemically replacing the carboxylic hydroxyl group of Tyr(Me) with benzylamine.

Detailed descriptions of these methods can be found in Slaninová (1987).

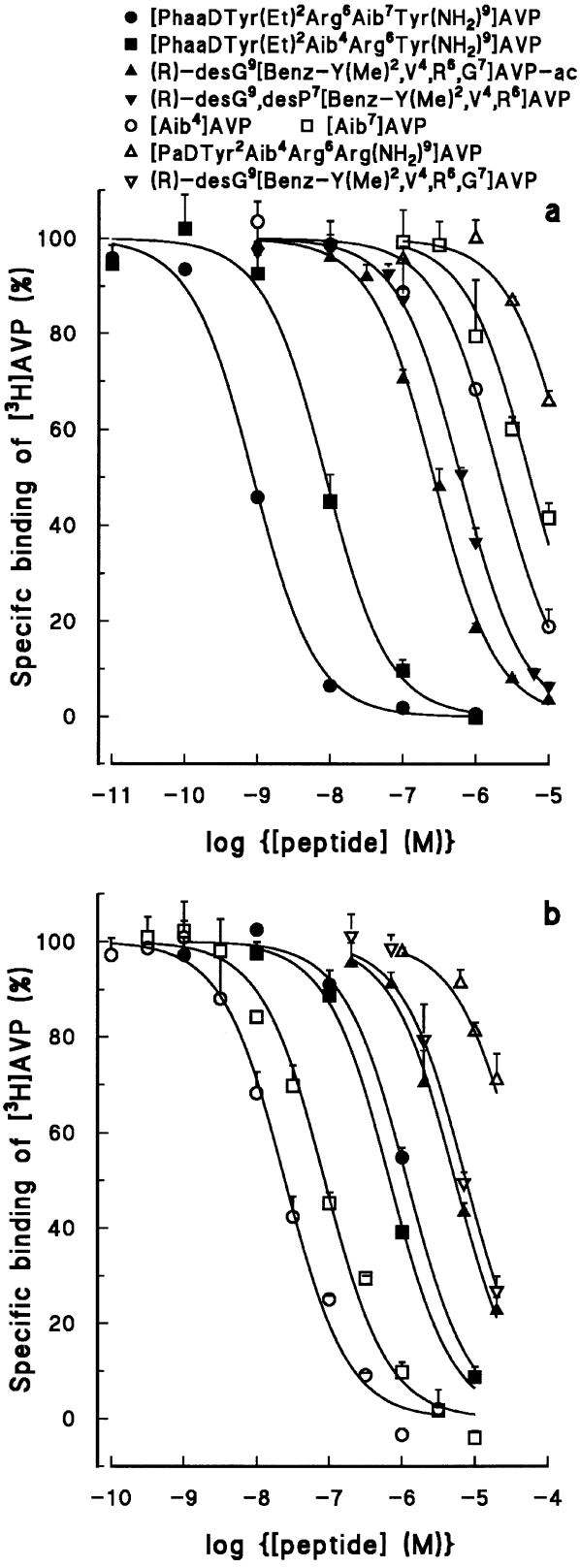

Figure 2 Determination of dissociation constants of vasopressin analogues. Membrane preparations of rat liver (V_{1A} VPR, a) and bovine kidney medulla (V_2 VPR, b) were incubated with 0.41–1.07 nM [^{3}H]AVP and various concentrations of unlabelled peptides. A simple Langmuir binding isotherm was fitted to experimental data using Figure. P. software (Biosoft). Points are means ± s.e.mean of triplicate values from a single representative experiment.

Results

Binding characteristics of Aib-substituted peptides

The binding affinities of Aib-substituted vasopressins and retro-inverso analogues (Figure 1) were determined by competition displacement of [^{3}H]-AVP from the two major subtypes of VPR (Figure 2a,b). These studies revealed that mono-substitution of AVP with Aib at positions 4 and 7 produced analogues which bound the V_2 VPR with affinities of 10.7 and 26.8 nM respectively. Both [Aib^4]AVP and [Aib^7]AVP displayed low affinity for the rat V_{1A} VPR (Table 1). These data indicate that substitution of Gln^4 or Pro^7 by Aib in a cyclic analogue of AVP is more compatible with binding to the V_2 VPR.

Aib-substitution at positions 4 and 7 was also studied in the linear V_{1A}-selective antagonist [PhaaDTyr(Et)^{2}Arg^{6}Tyr(NH$_2$)^9]AVP (Manning *et al.*, 1991). Comparative studies (Table 1) revealed that both [PhaaDTyr(Et)^{2}Aib^{4}Arg^{6}Tyr(NH$_2$)^9]AVP and [PhaaDTyr(Et)^{2}Arg^{6}Aib^{7}Tyr(NH$_2$)^9]AVP were high affinity and selective ligands for the V_{1A} VPR. The mono-substitution of Aib for Pro at position 7 increased selectivity for the V_{1A} VPR 4.5 fold compared to the parent peptide [PhaaDTyr(Et)^{2}Arg^{6}Tyr(NH$_2$)^9]AVP (Table 1). The binding affinities of [PaDTyr^{2}Aib^{4}Arg^{6}Arg(NH$_2$)^9]AVP and [PaDTyr^{2}Val^{4}Arg^{6}Aib^{7}Arg(NH$_2$)^9]AVP, Aib-substituted analogues of the V_2-selective linear agonist [PaDTyr^{2}Val^{4}Arg^{6}Arg(NH$_2$)^9]AVP (Manning *et al.*, 1991), were disappointing. These peptides displayed low affinities ($K_d > 1$ μM) for both the V_{1A} and V_2 VPR (Table 1).

Biological activities of Aib-substituted peptides

The activities of three Aib-substituted cyclic analogues of AVP and LVP are provided in Table 2. Results obtained with [Aib^4]AVP and [Aib^7]AVP corresponded to the V_2-selective characteristics of these peptides indicated by binding analysis (Table 1). [Aib^4]AVP and [Aib^7]AVP displayed activities of 140 IU mg^{-1} and 36 IU mg^{-1} respectively in a rat antidiuretic (V_2 VPR) assay but very low or absent activities in a pressor assay (Table 2). [Aib^9]LVP displayed no activity in pressor assays. Clearly, the substitution of Aib at position 4 or 7 could be utilized in the design of highly V_2-selective agonists.

Data from *in vivo* bioassays (Table 2) confirmed that Aib-substituted linear peptides were potent antivasopressor agents (pA_2 8.10 and 8.36 respectively). These effects of Aib-substitution in a linear antagonist are clearly quite different to those observed in a cyclic agonist.

Table 1 Binding affinities of vasopressin analogues

Peptide	*Binding affinity* (nM) V_{1A}	V_2
AVP	0.68 ± 0.14[a]	0.68 ± 0.09[a]
[Aib^4]AVP	762 ± 221	10.7 ± 3.1
[Aib^7]AVP	3760 ± 950	36.8 ± 4.9
[PhaaDTyr(Et)^{2}Arg^{6}Tyr(NH$_2$)^9]AVP	0.15 ± 0.01[b]	40.8 ± 1.3[b]
[PhaaDTyr(Et)^{2}Aib^{4}Arg^{6}Tyr (NH$_2$)^9]AVP	4.1 ± 0.7	364 ± 59
[PhaaDTyr(Et)^{2}Arg^{6}Aib^{7}Tyr (NH$_2$)^9]AVP	0.51 ± 0.09	628 ± 60
[PaDTyr^{2}Aib^{4}Arg^{6}Arg(NH$_2$)^9]AVP	9360 ± 2,590	> 20,000
[PaDTyr^{2}Val^{4}Arg^{6}Aib^{7}Arg(NH$_2$)^9] AVP	1280 ± 230	2070 ± 680

All values are means ± s.e.mean from at least three independent determinations of K_d as indicated in Figure 1. [a]Value previously reported in Howl *et al.* (1991b), [b]data from Howl & Wheatley, (1996).

Design and characterization of retro-inverso analogues of AVP

The design of the retro-inverso analogues used in this study (Figure 1) was based upon previous observations (Howl & Wheatley, 1996) that (*R*)-[Benz-Y(Me)^2,R^6,K^9]AVP, a linear retro-inverso antagonist, binds weakly but selectively to the V_{1A} VPR (Table 3). Deletion of glycyl and prolyl residues in Val^4-containing peptides is compatible with high affinity binding of conventional linear antagonists (Manning *et al*., 1989). Thus, with the aim of improving binding affinity, we used a similar strategy to shorten the 'carboxyl' terminal of D-Val-containing retro-inverso peptides.

All linear retro-inverso peptides selectively bound the V_{1A} VPR (Table 3). (*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7]AVP, a linear octapeptide in which a glycyl residue replaces the D-prolyl residue in (*R*)-[Benz-Y(Me)^2,R^6,K^9]AVP, displayed the highest affinity for both the rat V_{1A} VPR (K_d = 151 nM) and bovine V_2 VPR (K_d = 1480 nM).

We further investigated the effect of acetylation on the binding parameters of both cyclic and linear retro-inverso AVP analogues. As indicated in Table 3, acetylation of (*R*)-AVP to produce (*R*)-AVP-ac (Figure 2) did not improve binding to either VPR subtype. Similarly, acetylation of (*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7]AVP and (*R*)-desG^9,desP^7[Benz-Y(Me)^2,V^4,R^6]AVP had little effect on binding parameters (Table 3).

Table 2 Biological activities of Aib-substituted vasopressin analogues

Peptide	*Activity* [IU mg^{-1}] *or* pA_2 *Pressor*	*Antidiuretic*
Agonists		
AVP	412	465
[Aib^4]AVP	1.2[a]	140
[Aib^7]AVP	0	36
[Aib^9]LVP	0	N.D.
Linear antagonists		
[PhaaDTyr(Et)^{2}Aib^{4}Arg^{6}Tyr(NH$_2$)^9]AVP	$pA_2 = 8.10 \pm 0.20$	N.D.
[PhaaDTyr(Et)^{2}Arg^{6}Aib^{7}Tyr(NH$_2$)^9]AVP	$pA_2 = 8.36 \pm 0.30$	N.D.

Where indicated, errors are s.e.mean values from 3–5 independent experiments. [a]Response rapidly fades to the basal state. Data for reference compound AVP are taken from Lebl *et al*., 1987.

Table 3 Binding affinities of retro-inverso vasopressin analogues

Peptide	*Binding affinity* (nM) V_{1A}	V_2
(*R*)-AVP	>100,000[a]	>100,000[a,b]
(*R*)-AVP-ac	>100,000	>100,000[d]
(*R*)-[Benz-Y(Me)^2,R^6,K^9]AVP-ac	780 ± 30[a]	3400 ± 1000[a,b]
(*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7]AVP	169 ± 35	2790 ± 610
(*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7]AVP-ac	151 ± 56	1480 ± 500
(*R*)-desG^9,desP^7[Benz-Y(Me)^2,V^4,R^6]AVP	367 ± 95	1500 ± 260
(*R*)-desG^9,desP^7[Benz-Y(Me)^2,V^4,R^6]AVP-ac	211 ± 46	2040 ± 910

All values are means ± s.e.mean from at least three independent determinations of K_d as indicated in Figure 1. [a]Value previously reported in Howl & Wheatley, (1996), [b]data obtained at rat V_2 VPR.

Discussion

The development of receptor-selective analogues of neurohypophysial peptide hormones has facilitated the characterization of receptor subtypes and provided tools to address the diverse roles of these important peptide mediators. Moreover, the recent description of novel hypotensive vasopressin peptides (Chan *et al*., 1998) and the development of chimeric vasopressin analogues (Howl *et al*., 1997) indicate new avenues for structurally modified vasopressin analogues. Thus, modifications to improve receptor-selectivity and/or stability and optimise pharmacodynamic and pharmacokinetic properties could enhance the utility and therapeutic potential of vasopressin/oxytocin analogues.

The achiral amino acid Aib, a helix promoter (Karle, 1996; Balakrishnan *et al*., 1997), has been utilized in the synthesis of receptor-selective analogues of peptide hormones and neuropeptides that include bradykinin (Regoli *et al*., 1990) and opioid peptides (Bryant *et al*., 1997). In addition to modifying the pharmacophore of small peptides, Aib-substitution could also enhance resistance to proteases. Based upon our studies with Aib-substituted oxytocins (Assisomytis *et al*., 1996), we chose to introduce Aib at positions 4, 7 and 9 of vasopressin analogues as these positions have been successfully modified in the design of many selective neurohypophysial hormone analogues (for reviews see Lebl *et al*., 1987; Manning *et al*., 1987a). Whilst inter-specific differences in the pharmacology of vasopressin analogues are possible, our bioassays revealed that both [Aib^4]AVP and [Aib^7]AVP are V_2-selective agonists in the rat. Moreover, these data correlated with their binding affinities in bovine kidney medulla, indicating that [Aib^4]AVP and [Aib^7]AVP do not discriminate between species-specific isoforms of the V_2 receptor in a manner characteristic of some other V_2 receptors ligands (Howl *et al*., 1995).

Surprisingly, and in contrast to results with Aib-substituted cyclic agonists, substitution of Aib at positions 4 and 7 in a linear V_{1A}-selective antagonist was well tolerated by the rat hepatic V_{1A} VPR. Both [PhaaDTyr(Et)^{2}Aib^{4}Arg^{6}Tyr(NH$_2$)^9]AVP and [PhaaDTyr(Et)^{2}Val^{4}Arg^{6}Aib^{7}Tyr(NH$_2$)^9]AVP selectively bound with high affinity to the rat V_{1A} VPR. These results could indicate fundamental differences in the pharmacophore of agonists and antagonists of VPRs. Alternatively, Aib might induce more general structural changes which, in a conformationally-restrained cyclic peptide, are detrimental to VPR receptor binding. Iodination of [PhaaDTyr(Et)^{2}Aib^{4}Arg^{6}Tyr(NH$_2$)^9]AVP and [PhaaDTyr(Et)^{2}Val^{4}Arg^{6}Aib^{7}Tyr(NH$_2$)^9]AVP could provide useful ligands for receptor localization studies (Schmidt *et al*., 1991). Unfortunately, Aib-substituted analogues of [PaDTyr^{2}Arg^{6}Arg(NH$_2$)^9]AVP, a linear V_2-selective agonist (activity = 24 IU mg^{-1}; Manning *et al*., 1991), bound with very low affinity to both the V_{1A} and V_2 VPR.

Though partial retro-inverso transformation is a common pseudopeptidic strategy applicable to the design of many peptide hormones and neuropeptides including vasopressin (Manning *et al*., 1992), the synthesis of such peptides is not routine. Thus, a total retro-inverso strategy using conventional solid phase methodology could be an attractive alternative to the synthesis of protease-resistant analogues of important peptide mediators. Theoretical predictions indicate that the side-chain orientation of retro-inverso peptides can mimic the topology of native peptides (Chorev & Goodman, 1993). As a concept, the retro-inverso strategy is most applicable to small, highly flexible linear peptides. Indeed, structural studies indicate that secondary and tertiary structures of larger peptides or proteins are more difficult to precisely mimic using

a retro-inverso strategy (Sánchez *et al*., 1996). Other potential caveats to the design of retro-inverso mimetics of small peptide hormones include residues with additional chiral centres, structural restraints imposed by proline and the effective reversal of amino- and carboxyl-termini.

Preliminary investigations with analogues of vasopressin, bradykinin and angiotensin II have endorsed the total retro-inverso strategy (Howl & Wheatley, 1996; 1998). Significantly, the modifications described herein have improved the binding affinity of total retro-inverso analogues of V_{1A}-selective vasopressin antagonists. Thus, (*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,-G^7]AVP-ac binds with a 5 fold higher affinity to the V_{1A} VPR than the prototype (*R*)-[Benz-Y(Me)^2,R^6,K^9]AVP-ac (Howl & Wheatley, 1996). The design of (*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7] replaced the potentially problematic D-prolyl residue of (*R*)-[Benz-Y(Me)^2,R^{6}K^9]AVP-ac with glycine. Though the binding affinity of (*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,-G^7]AVP-ac at the V_{1A} VPR remains some four orders of magnitude lower than that achieved by conventional linear antagonists, this peptide retains good V_{1A}/V_2 selectivity and provides a template for further improvement. Detailed structural comparisons of (*R*)-desG^9[Benz-Y(Me)^2,V^4,R^6,G^7]AVP-ac and conventional peptides could provide data pertinent to the improved design of total retro-inverso peptides and also indicate motifs involved in the molecular recognition of receptor proteins.

In summary, the strategies described in this paper are clearly applicable to the design of analogues of vasopressin with enhanced receptor-subtype selectivity. Both Aib-substitution and retro-inverso transformation will also delay peptide degradation *in vivo*. Hence, in addition to providing valuable tools for pharmacological studies, the novel modifications reported herein could improve both the pharmacodynamic and pharmacokinetic parameters of vasopressin analogues to allow their therapeutic potential to be realized (László *et al*., 1991).

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(Received May 21, 1999
Revised July 23, 1999
Accepted July 27, 1999)