Design and synthesis of heterofunctional V_{1a}-selective vasopressin receptor ligands with lysine at position 9

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ABSTRACT

A peptide analogue of [8-arginine]vasopressin (AVP) with Lys substituted for Gly at position 9 ([d(CH_{2})_{3}Tyr(Me)^{2}LysNH_{2}]AVP; ALVP) has been synthesized as a precursor for the production of heterofunctional vasopressin receptor ligands. Three heterofunctional ligands have been prepared by attaching biotin to a photoreactive cross-linker capable of iodination, either alone or in combination, at the ε-amino group of Lys at position 9 in ALVP. The binding characteristics of these novel ligands have been determined at the V_{1a} and V_{2} vasopressin receptors by employing membrane preparations of rat liver and kidney respectively. All of the analogues synthesized during the course of this study bound selectively, and with high affinity, to the V_{1a} vasopressin receptor subtype. Our results demonstrate that the strategies described in this paper provide a convenient means of synthesizing heterofunctional vasopressin receptor ligands with preservation of subtype-specific, high affinity binding characteristics. These parameters establish the potential value of the analogues as probes for investigating V_{1a} receptor structure and function.

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INTRODUCTION

Receptors for the neurohypophyseal peptide hormone [8-arginine]vasopressin (AVP) were originally classified as V_{1} and V_{2}. These subtypes exhibit different pharmacological profiles and V_{1} receptors couple to phosphoinositidase C whereas V_{2} receptors stimulate adenylyl cyclase (Michell et al. 1979). Subsequently, the availability of synthetic analogues of AVP with defined binding characteristics facilitated further subclassification of AVP isoreceptors as V_{1a}, V_{1b} and V_{2} (Antoni, 1984; Baertschi & Friedli, 1985; Jard et al. 1986). The development of synthetic analogues has also proved invaluable for investigating the physiological and pathophysiological roles of AVP in mammals (Manning et al. 1987a; László et al. 1991).

It is known that deletion of the C-terminal glycine or glycaminide residue at position 9 drastically reduces the agonist potency of AVP at the V_{1a} vasopressin receptor (du Vigneaud et al. 1953; Manning et al. 1984). However, the recent synthesis of a vast number of structural analogues, particularly by Manning et al. (1984, 1987b) and Sawyer et al. (1988), has resulted in a range of high affinity ligands which can tolerate the substitution or deletion of Gly or Gly(NH_{2}) at position 9 with little change in antagonist potency.

The availability of heterofunctional ligands (i.e. analogues which recognize V_{1a} receptors selectively and with high affinity, whilst incorporating additional functional moieties) would be a great asset to vasopressin receptor research. Potential uses of these ligands include receptor localization, irreversible ligands and the bases of affinity matrices. This study was to assess the utility of position 9 in the design and synthesis of heterofunctional ligands for the V_{1a} receptor. In this paper we describe the synthesis of a precursor peptide which offers a convenient site of attachment for several functional moieties individually or in consort. Moreover, we demonstrate that the heterofunctional ligands produced using this strategy retain high affinity, and pharmacological selectivity, for the V_{1a} vasopressin receptor subtype.

MATERIALS AND METHODS

Materials

[Phe-3,4,5-^3H]AVP (53.6 and 67.7 Ci/mmol) and the labelled V_{1a}-selective vasopressin antagonist
Preparation and purification of synthetic vasopressin analogues

\[ (d(CH_2)_5)Tyr(Me)^2LysNH_2^9/AVP \text{ (ALVP)} \]

The linear peptide \( d(CH_2)_5^1-Tyr(Me)^2-Phe^3-Gln^4-Asn^5-Cys^6-Pro^7-Arg^8-Lys(NH_2)^9 \) was prepared by solid-phase synthesis. Intramolecular disulfide bond formation was achieved by air oxidation and the cyclized peptide purified by semi-preparative scale reverse-phase C_{18} high-performance liquid chromatography (HPLC) as previously described (Howl et al. 1991b).

\[ (d(CH_2)_5)Tyr(Me)^2Lys(Ne-biotinamidocaproamide)NH_2^9/AVP \text{ (AL(Btn/ASA)VP)} \]

This peptide was synthesised by reacting ALVP with a tenfold molar excess of biotinamidocaproate NHS ester in dimethylformamide (DMF). AL(Btn)VP was purified by HPLC using a Lichrospher C_{8} reverse-phase column (Merck, Darmstadt, Germany).

\[ (d(CH_2)_5)Tyr(Me)^2Lys(Ne-azidosalicylamide)NH_2^9/AVP \text{ (AL(ASA)VP)} \]

ALVP was reacted with a tenfold molar excess of NHS-ASA in DMF and incubated in the dark for 72 h at 50 °C. This procedure resulted in a 95–100% conversion of ALVP to the product AL(ASA)VP. This peptide was purified using semi-preparative scale reverse-phase HPLC, employing a linear 30 min gradient of 20–80% acetonitrile/0-1% trifluoroacetic acid on a Varian C_{18} reverse-phase column (Technicol, Stockport, Cheshire, U.K.), with absorbance being monitored at 220 nm. Pure AL(ASA)VP was collected and lyophilized, and found to be stable for at least 2 months if stored as a powder, or as a 1 mM solution in 0-25% acetic acid, at −20 °C in the dark.

\[ ([d(CH_2)_5]Tyr(Me)^2Lys(Ne-biocytinyl(Na-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionamide))NH_2^9/AVP \text{ (AL(Btn/ASA/VP)} \]

Biocytin (25 mg) was incubated with SASD (12 mg) in 100 mM borate buffer, pH 8-4, for 2 days at room temperature. The product, Ne-biocytinyl-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionamide (BASA), was separated by semi-preparative reverse-phase HPLC and lyophilized. The molecular mass of BASA (696-9 Da) was confirmed by fast atom bombardment (FAB) mass spectroscopy. The presence of biotin was determined using the method of McCormick & Roth (1970), and an intact disulfide bond and azido group were shown to be present by Fourier transfer infra-red (FTIR) spectroscopy.

BASA was mixed with ALVP in equimolar proportions in DMF and stirred overnight with a molar excess of N,N'-dicyclohexylcarbodiimide and N-hydroxybenzotriazole. Peptides were precipitated with ether and dried. AL(Btn/ASA)VP was extracted with 50% MeCN, purified by reverse-phase HPLC, and lyophilized. FTIR mass spectroscopy confirmed the presence of an intact azido group in AL(Btn/ASA)VP, and an intact disulfide bond which could be cleaved by treatment with 1 mM dithiothreitol. The stability of this peptide was equivalent to that of AL(ASA)VP if stored under similar conditions.

To confirm the purity of the synthetic ligands, all peptides were analysed by HPLC using a Spherisorb C_{18} reverse-phase column (30 × 0-4 cm; Phase Sep, Deeside, Clwyd, U.K.).

Membrane preparations and ligand binding assays

Partially purified plasma membrane preparations of rat liver and rat kidney medulla were prepared as previously described (Howl et al. 1991b). Competition binding assays to determine the dissociation binding constants \( K_d \) of synthetic peptides were performed using \([^3H][d(CH_2)_5]Tyr(Me)^2AVP\) as the tracer ligand for the rat liver \( V_{1a} \) receptor subtype, and \([^3H]AVP\) as the tracer ligand for the kidney medulla \( V_2 \) receptor subtype (Howl et al. 1991b). Membranes (100 μg total protein) in 500 μl buffer containing 20 mM Hepes, 10 mM Mg(CH_2COO)_2, 1 mM EGTA and bovine serum albumin (1 mg/ml) were incubated with \(^3\text{H}\)-labelled tracer ligand and various concentrations of unlabelled competing ligand for 90 min, which allowed equilibrium to be established. Membranes were pelleted and washed
to separate bound from free ligand. Membrane pellets were dissolved in 50 μl tissue solubilizer (Soluene-350; Packard, Downers Grove, IL, U.S.A.) and 1 ml scintillation fluid (HiSafe 3; Pharmacia Biosystems Ltd, Milton Keynes, Bucks, U.K.) was added. Radioactivity was determined as described previously (Howl et al. 1991a), with an efficiency of >40%. The concentration of unlabelled peptide which produced 50% inhibition of the specific binding of the [3H]-labelled tracer ligand (IC50) was determined from competition binding experiments. To determine the Kd of each synthetic peptide, IC50 values were corrected for occupancy of [3H]-labelled tracer ligand according to the method of Cheng & Prusoff (1973), using experimentally determined values for the Kd of [3H][d(CH2)5 Tyr(Me)]AVP (0.28 nM) and that of [3H]AVP (1.31 nM).

RESULTS AND DISCUSSION

Previous studies have investigated the effect of substitution or deletion of the C-terminal Gly or Gly(NH2) residue in the design of antagonists of AVP (Manning et al. 1984, 1987b; Sawyer et al. 1988) and oxytocin (Elands et al. 1987; Manning et al. 1989). These studies demonstrated that the nature of the amino acid residue at position 9 is not critical in determining the affinity of synthetic AVP or oxytocin antagonists. Subsequently, it was established that a dibasic tail domain is sufficient for high affinity binding (Ali et al. 1987). These findings were extended by Callahan et al. (1989) who demonstrated, using a series of AVP antagonists, that the entire tripeptide tail (Pro-Arg-GlyNH2) can be replaced by a simple alkylamine or (aminoalkyl)guanidine to produce ligands which retain high affinity binding for the V2 vasopressin receptor. In fact, it was suggested that all that is required for the retention of high affinity vasopressin antagonism is an intact hexapeptide ring with a positive charge extended an optimum distance from it (Callahan et al. 1989). Thus, in designing heterofunctional ligands for the V1a receptor in this study, we took three factors into consideration. First, that the design of our ligands should be based upon a high affinity V1a-selective ligand with Arg at position 8, thereby incorporating a basic function in the tail domain. Secondly, derivatization should be confined to position 9 as this would be least likely to perturb the binding of the synthetic peptides. Thirdly, in order to expedite the production of a range of heterofunctional ligands, we required a precursor peptide which would enable us to utilize the selective reaction of NHS esters.

FIGURE 1. Structural formulae of the heterofunctional analogues of ALVP. AVP, [8-arginine]vasopressin; ALVP, [d(CH2)5Tyr(Me)]LysNH2]AVP. The full names of these peptides are: AL(Btn)VP, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-O-methyltyrosine, 8-arginine, 9-lysinamide (Nε-biotinamidocaproamide)]vasopressin; AL(ASA)VP, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-O-methyltyrosine, 8-arginine, 9-lysinamide(Nε-azidosalicylamide)]vasopressin; AL(Btn/(ASA)VP, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-O-methyltyrosine, 8-arginine, 9-lysinamide (Nε-biocytinyl(Nε-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionamide)]vasopressin.
The synthetic peptide [d(CH$_2$)$_5$Tyr(Me)$_2$]AVP exhibits a very high affinity for V$_{1a}$ receptors and is one of the most V$_{1a}$-selective antagonists reported to date (Kruszynski et al. 1980). We synthesized an analogue of this peptide with LysNH$_2$ replacing the GlyNH$_2$ at position 9, i.e. [d(CH$_2$)$_5$Tyr(Me)$_2$]Lys(Ne-biotinamidocaproamide)NH$_2$]AVP (ALBP; Fig. 1). When the affinities of ALVP for the V$_{1a}$ receptor and the V$_2$ receptor were determined by competition ligand binding (Fig. 2a and b respectively), it was found to exhibit a subnanomolar affinity for V$_{1a}$ receptors and a subtype selectivity comparable with its GlyNH$_2$ analog (Table 1). Incorporating a single primary amino group in the design of ALVP enabled us to direct additional functional moieties to a defined position in the peptide by exploiting the highly selective reaction of NHS esters with this class of amines. The use of a tenfold excess of NHS ester over peptide was optimal and produced very efficient conversion of ALVP to the appropriate derivatized product. The high yield of stable products, together with the use of semi-preparative reverse-phase HPLC peptide purification, ensured the reproducible production of homogeneous heterofunctional analogues. Three different analogues incorporating different modifications were synthesized from ALVP (Fig. 1). The structure and purity of the individual analogues were confirmed by a combination of FAB mass spectroscopy, FTIR spectroscopy and analytical HPLC (Table 1).

The binding of the ligands to rat liver V$_{1a}$ receptor (Fig. 2a) and to rat kidney medulla V$_2$ receptor (Fig. 2b) was determined. A comparison of the $K_d$ values (Table 1) calculated from the $IC_{50}$ values demonstrated that all of the analogues synthesized in this study were selective for the V$_{1a}$ receptor. Although the affinities of the three heterofunctional ligands for V$_{1a}$ receptors were similar (Table 1), the degree of subtype selectivity exhibited was markedly influenced by the modifications at Lys$.^9$. Thus, the V$_{1a}$/V$_2$ selectivities of AL(Btn)VP and AL(ASA)VP were 202 and 15 respectively, even though the peptide moiety was identical in both cases (Fig. 1).

Boku et al. (1989) reported that when the C-terminal glycynamide of vasotocin was substituted with iodotyrosine or p-azidophenylalanine there was little effect on its hydro-osmotic activity. However, biotinylphenylalanine at position 9 resulted in a 100-fold decrease in potency (Boku et al. 1989). The authors hypothesized that this was due to the steric hindrance caused by the biotin or its linker. The findings with vasotocin differ from the observations in this investigation, as biotin and the linker in AL(Btn)VP and AL(Btn/ASA)VP were not very detrimental to high affinity binding to the V$_{1a}$ receptor, producing a 6-15-fold increase in $K_d$ (Table 1).

### Table 1

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**Selectivity**

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<td>AL(Btn/ASA)VP</td>
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<td>2.30±0.21</td>
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**Selectivity**

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<th>Selectivity</th>
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<td>1-31±0.14</td>
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<td>[d(CH$_2$)$_5$Tyr(Me)$_2$]AVP</td>
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<td>ALVP</td>
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**Selectivity**

**Ligand abbreviations:** AVP, [8-arginine]vasopressin; ALVP, [d(CH$_2$)$_5$Tyr(Me)$_2$]Lys(Ne-biotinamidocaproamide)NH$_2$]AVP; AL(Btn)VP, [d(CH$_2$)$_5$Tyr(Me)$_2$]Lys(Ne-biotinamidocaproamide)NH$_2$]AVP; AL(ASA)VP, [d(CH$_2$)$_5$Tyr(Me)$_2$]Lys(Ne-azidosalicylamine)NH$_2$]AVP; AL(Btn/ASA)VP, [d(CH$_2$)$_5$Tyr(Me)$_2$]Lys(Ne-biotinyl-NH$_2$-(p-azidosalicylamido)ethyl-1,3-dithiopropionamide)]NH$_2$]AVP.

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FIGURE 2. Determination of the dissociation binding constants of ALVP and heterofunctional analogues. Membrane preparations of (a) rat liver and (b) kidney medulla were incubated with 0.08–0.20 nM [3H][d(CH2)5Tyr(Me)2]AVP and 0.44–0.69 nM [3H]AVP respectively and various concentrations of unlabelled peptides: ●, AVP; ○, [d(CH2)5Tyr(Me)2]AVP; ▲, ALVP; □, AL(Btn)VP; △, AL(ASA)VP; ◻, AL(Btn/ASA)VP. Points are means ± S.E.M. (n = 3) from a single typical experiment. Values for the specific binding of 3H-labelled tracer ligand in the presence of unlabelled peptide are expressed as percentages of the specific binding in the absence of competing ligand. In these experiments, the total binding of [3H][d(CH2)5Tyr(Me)2]AVP to 100 μg liver membranes was within the range of 1050–2250 d.p.m. and total [3H]AVP binding to kidney membranes was within the range of 850–1550 d.p.m. Non-specific binding varied slightly between preparations and was also dependent upon the concentration of free 3H-labelled ligand in each experiment. In both liver and kidney membranes, levels of non-specific binding were within the range of 10–30% of the total binding. Curves are normalized and represent the best fit of a theoretical simple Langmuir isotherm to the experimental data. Where not shown, error bars are within the symbol. Ligand abbreviations: AVP, [8-arginine]vasopressin; ALVP, [d(CH2)5Tyr(Me)2LysNH22]AVP; AL(Btn)VP, [d(CH2)5Tyr(Me)2Lys(NH2-biotinamidocaproamide)NH22]AVP; AL(ASA)VP, [d(CH2)5Tyr(Me)2Lys(NH2-azidosalicylamide)NH22]AVP; AL(Btn/ASA)VP, [d(CH2)5Tyr(Me)2Lys(NH2-biocytinyl(2-azidosalicylamido)ethyl-1,3'-dithiopropionamide))NH22]AVP.
The smaller modification to ALVP incorporated when synthesizing AL(ASA)VP resulted in a 12-fold decrease in affinity for V1a receptors. This is in contrast to findings with the related V1a antagonist, [d(CH2)5MeAla]AVP. Incorporation of azidobenzoyl into position 8 or 9 of [d(CH2)5MeAla]AVP increased the affinity for V1a receptors tenfold (Farenholz et al. 1986). In synthesizing AL(Btn/ASA)VP, the Ly9 modification increased the molecular weight of the parent peptide by 56%. Furthermore, the bulky moieties were accommodated on a branched linker, which might have been anticipated to increase stearic hindrance. However, the sixfold increase in Kd resulting from these gross structural modifications indicates that not only is position 9 of V1a receptor antagonists not critical for binding, but that this residue projects away from the receptor’s ligand-binding pocket.

In addition to demonstrating the extreme versatility of position 9 of ALVP for derivatization, these studies have also provided very selective heterofunctional probes for studying the V1a receptor protein. AL(ASA)VP is capable of radioiodination and 125I-labelled AL(ASA)VP is currently being assessed as an irreversible label for V1a receptors. By incorporating a crosslinker capable of iodination, photoactivation and thiol-cleavage, together with biotin, into the design of AL(Btn/ASA)VP, we have synthesized a ligand which clearly offers potential for the subsequent purification of the V1a receptor protein utilizing immobilized avidin. Such a strategy is now being developed.

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REFERENCES


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