a parameter of the correlation. This result implies that a side chain with a large projection surface perpendicular to the C_β–C_γ bond[18] is not favorable to activity. In other words, the catalytic site of renin seems to accommodate linear chains (norvaline) and β-branched chains (valine, cyclopentylglycine) but not γ- nor δ-branched side chains (cyclohexylalanine, phenylalanine). From this point of view, the side chain of histidine should not be favorable.

The model that resulted from multiple regression (Table III) predicts higher inhibitory activity for relatively high δH_C values and lower activity for relatively high σ_I and ν values. Screening of a number of amino acid side chains for which the three constants are available[13] and which have not yet been introduced in position 2 of the analogues suggests that threonine (calculated SCC = 0.7) and to a lesser extent aminobutyric acid (0.6) and *tert*-butylglycine (0.45) should lead to very active compounds while aspartic acid (−0.9) or adamantylalanine (−1.3) should not. Although it cannot be guaranteed that these precise inhibitory activities will be observed, the model provides at least a rational basis for the choice of the next analogues to be synthesized.

Two clear-cut outliers were discovered in the series. For the first one, Tyr(Bzl), the model predicts a higher activity than observed. The chosen structural descriptors do not apparently reflect the properties of this side chain properly. One reason might be the fact that this substituent is the only one in the series that contains two aromatic rings: it is therefore likely that the values of δH_C, σ_I, and ν are insensitive to the C_α-remote benzyl aromatic ring. As a matter of fact, these three constants are identical for Tyr(Bzl) and Phe, and further descriptors are required such as ν_V for a proper distinction of the two side chains; in contrast, a very different behavior may be expected for Ser(Bzl). Of greater significance is the case of the second outlier, histidine, since here the observed activity is much higher than predicted. The result implies that some property of the histidine side chain potentiates the inhibitory effect of its pepstatin derivative significantly more than in other derivatives. This property is likely to be less related to the steric properties of the imidazole ring; rather, it may be accounted for by charge, hydrogen bond donor and acceptor, aromaticity, or tautomerism. Although the structural reason for the outstanding behavior of histidine is unknown, the histidine-2 derivative may obviously serve as a lead compound for the synthesis of more potent inhibitors. Moreover, the presence of a histidine residue in the corresponding site of the natural N-terminal angiotensinogen substrate and also in several highly potent renin inhibitors[24] suggests that the availability of pepstatin derivatives containing analogues of histidine[25] in this position should contribute to the refinement of our quantitative model and serve as a guide for future design purposes.

Although this QSAR study of a bioactive peptide did not focus on selectivity toward aspartyl proteinases, but only on potency toward renin, it is one more example wherein several useful conclusions as to the factors effective in binding and proteolysis could be drawn, which could not have been deduced from simple inspection of the chemical structure.

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Potent Vasopressin Antagonists Modified at the Carboxy-Terminal Tripeptide Tail

Fadia El-Fehail Ali,*† Hsin-Lan Chang,† William F. Huffman,† Grace Heckman,‡ Lewis B. Kinter,§ Edwin F. Weidley,§ Richard Edwards,§ Dulcie Schmidt,‡ Daryl Ashton-Shue,§ and Frans L. Stassen‡

Research and Development Division, Smith Kline & French Laboratories, Swedeland, Pennsylvania 19479.
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In a continuing effort to design more potent renal vasopressin (V_2 receptor) antagonists, we have focused our attention on the carboxy-terminal tripeptide tail (Pro-Arg-Gly-NH_2), a fragment common to both agonists and antagonists. Vasopressin antagonist analogues having a dibasic dipeptide tail, e.g., Arg-Arg-NH_2 or Arg-Lys-NH_2, attached directly to the cyclic hexapeptide ring are potent V_2-receptor antagonists. Similar modification of a representative agonist drastically reduces its potency. We report the synthesis and pharmacological properties of a series of potent V_2-receptor antagonists **3–9** where a combination of D or L dibasic dipeptide has been utilized to replace the common tripeptide fragment. Our results suggest a difference in the way agonists and antagonists bind to vasopressin receptor and further support the difference in the structure–activity relationships of agonists and antagonists. These results provide potentially useful insights for the design of novel V_2-receptor antagonists.

Antagonists of the antidiuretic response to vasopressin can be powerful pharmacological tools and physiological probes and may have clinical potential for the treatment of vasopressin-induced water-retention states of diverse etiologies.[1–4] The design and synthesis of effective antagonists of the vasopressor (V_1 receptor) and antidiuretic (V_2 receptor) responses to vasopressin have been major goals of structure–activity studies on this peptide.[5–10] As part of a continuing effort to design and synthesize potent antagonists of the vasopressin V_2 receptor and to delineate in greater detail the structural requirements for such ac-

† Department of Peptide Chemistry.
‡ Department of Molecular Pharmacology.
§ Department of Pharmacology.

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Table I. Biological Activity of Vasopressin Analogues: Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-X[a]

no.	X	K_{bind},[b] nM	K_i,[c] nM	ED_{300},[d] μg/kg
1	Pro-Arg-NH_2	12.0 ± 1.2 (8)	3.9 ± 0.4 (8)	8.4 ± 0.7 (17)
2	Arg-NH_2[11]	9.1	2.5	58
3	Arg-Arg-NH_2	3.4 ± 0.5 (3)	1.7 ± 0.1 (4)	7.2 (2)
4	Arg-Lys-NH_2	7.0	2.6	9.7
5	Lys-Arg-NH_2	8.8	4.2	9.8
6	Arg-D-Arg-NH_2	5.4	1.7	13.5 ± 2.5 (5)
7	D-Arg-Arg-NH_2	2.7	1.9	14.6 ± 0.4 (4)
8	D-Arg-D-Arg-NH_2	5.2	0.88	11.5 ± 3.3 (4)
9	Arg-Arg-OH	21	7.8	10.6
10	[Mpr[1],Val[4],-Arg[7],desGly[9]]-AVP	>1000[e]		

[a] Abbreviations: Pmp, β-mercapto-β,β-pentamethylenepropionic acid; Mpr, β-mercaptopropionic acid, D-Tyr(Et) is D-tyrosine ethyl ether. All amino acids are in the L configuration unless otherwise noted. [b] K_{bind} is a measure of the affinity of ligand for receptor in porcine renal medullary membrane. It is derived from the equation for competitive inhibition $K_{bind} = IC_{50}/(1 + L/K_D)$[15] where IC_{50} is the concentration of the ligand for 50% inhibition of [^{3}H]-LVP binding, L is the concentration of the ligand, and K_D is the dissociation constant of [^{3}H]LVP. [c] K_i is the inhibition constant for inhibition of 8-lysine-vasopressin (LVP) stimulated adenylate cyclase of pig kidney medullary membranes and is derived from the equation for competitive inhibition as described.[16] Mean of triplicate determination. [d] ED_{300} is the dose (μg/kg) required to decrease urine osmolality in rats from the hydropenic levels (~ 1500 mosm/kg of H_2O) to 300 mosm/kg of H_2O. [e] For comparison, the K_{bind} for arginine-vasopressin (AVP) is 4.2 nM and for the agonist of compound **2**, i.e., [Mpr[1],Val[4],desPro[7],desGly[8]]AVP, is 15.3 μM.

tivity, we have focused our efforts on modification of the carboxy-terminal tripeptide tail (Pro-Arg-Gly-NH_2), a fragment common to agonists and many antagonists. We reported previously that the entire tripeptide tail was not required for vasopressin renal V_2 receptor antagonist activity, although it is required for agonist potency.[11] In fact, potent V_2-receptor antagonists were obtained by deletion of the proline or deletion of both the proline and glycine residues from anagonists.[12] We also have presented evidence that an effective pharmacophore for V_2-receptor antagonists can be presented by a cyclic hexapeptide ring with a basic moiety attached to the ring.[13]

In an attempt to further define the anagonist pharmacophore requirements in the tripeptide tail region, we investigated the effects of dipeptide tails in which both residues were basic by synthesizing compounds **3–9**.[14] To find out if such a modification is compatible with the vasopressin agonist activity, an agonist **10** with a modified dibasic dipeptide tail was also prepared. In this paper, the synthesis and preliminary pharmacological properties in vitro (pig) and in vivo (rat) of these peptides are reported.

Results and Discussion

The results of the bioassays of analogues **3–9** are summarized in Table I. For comparison, the activities of the parent compounds **1** and **2** and the corresponding agonist with the modified dibasic dipeptide tail **10** are shown as well. As can be seen, incorporation of an extra Arg or Lys at the carboxy terminal of **2** resulted in analogues **3** and **4**, which exhibited a high degree of antidiuretic antagonist potency.

To assess the effects of the position of the dissimilar basic amino acid i.e., Arg vs. Lys on the antagonist potency, peptide **5** was prepared. It demonstrated potency similar to that of its congener **4**. These results indicate that a second Arg or Lys, regardless of its position on the carboxy-terminal tail, is well tolerated by the V_2 receptor. A similar modification of an agonist [Mpr-Val[4],Arg[7],des-Gly[9]]AVP resulted in analogue **10**, which exhibited greatly decreased affinity for the porcine V_2 receptor relative to arginine-vasopressin (AVP; K_{bind} for **10** is greater than 1.0 μM and for AVP is 4.2 nM).[17] These results indicate that while such a modification is tolerated by the receptor without loss of antagonist potency, it certainly is detrimental for agonist potency.

Since antagonists with dibasic dipeptide tails display high potency, we prepared analogues **6–8** to evaluate the effects produced by substituting D-amino acid residues in the tail. These analogues were again highly potent antagonists both in vitro and in vivo as well.

To probe the importance of the C-terminal amide group in antagonist potency, analogue **9**, lacking the C-terminal

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amide, was prepared. This molecule again retained a high degree of antagonist potency in vitro and was very potent in vivo as well. This suggests that whether the C-terminus of the antagonist is a free carboxylic acid or a carboxamide, an effective pharmacophore can be presented.

In conclusion, we have shown that substitution of the C-terminal Pro-Arg-Gly-NH_2 by a dibasic dipeptide in vasopressin antagonist and agonist analogues result in potent antagonists but a poor agonist, respectively. Furthermore, these findings coupled with our earlier reported studies[11-13] indicate that certain modifications on the C-terminal tripeptide portion of vasopressin molecule are tolerated for binding of antagonists, but not agonists, to the receptor. Antagonist potency is retained with the proviso that one or more basic groups are attached to a cyclic hexapeptide ring that contains the structural features required for antagonist potency. It should be noted that Manning et al. have recently reported[14] a series of similar modified tail antagonists where the tripeptide tail contains two arginine residues, i.e., Pmp^1,D-Phe^2,Val^4,Pro^7,Arg^8,Arg^9NH_2. These analogues have retained antagonist potency as well. These findings provide new insights that may be applicable to the design of novel V_2-receptor antagonists.

Experimental Section

1. **Peptide Synthesis.** The protected peptide-resin intermediates required for the synthesis of peptides 1–10 were synthesized by the solid-phase method[18] on either benzhydrylamine-resin for the C-terminal amides or chloromethylated resin for the C-terminal acid. Benzhydrylamine-resin (BHA, 1% cross-linked S-DVB, 200–400 mesh, 1.0 mequiv/g) was synthesized according to an improved procedure.[19] Boc-Arg(Tos)-*O*-Bzl-resin (1% cross-linked S-DVB, 0.31 mequiv/g) was obtained from Peninsula Laboratories. Amino acid derivatives were supplied by Peninsula Laboratories or Chemical Dynamics Inc. Boc-D-Tyr(Et) was synthesized according to a published procedure.[20] A detailed description for the synthetic procedure used is exemplified in the synthesis of compound 3. Solvents and reagents were analytical grade, methylene chloride (CH_2Cl_2) was HPLC grade, dimethylformamide (DMF) was filtered from molecular sieves (4 Å) prior to its use, and trifluoroacetic acid (TFA) used for high-performance liquid chromatography (HPLC) was redistilled prior to its use. The yield of pure peptides was not optimized. Greater emphasis was given to obtaining high purity, which resulted in decreased yields. When counter-current distribution (CCD, Craig-Post, 240 transfers)[21] or partition column chromatography (Sephadex G-25) were used for purification, 1-butanol-acetic acid-water (B:A:W, 4:1:5, v/v/v) solvent system was used. Purity and homogeneity of the peptides were routinely checked by HPLC and thin-layer chromatography (TLC). HPLC was performed with 4.5 mm × 25 cm Altex Ultrasphere 5 μ ODS column for analytical work and 10 mm × 25 cm column for preparative work with UV detection at 220 nm. The following solvent system was used for HPLC: acetonitrile–water containing 0.1% TFA (20:80 to 50:50 linear in 20 min in gradient or 45:55 in isocratic runs unless otherwise noted). TLC was on precoated silica gel (0.25 mm; 5 × 20 cm, E. Merck) and developed in one of the following solvent systems: A, 1-butanol–acetic acid–water–ethyl acetate (B:A:W:E, 1:1:1:1, v/v/v/v); B, 1-butanol–acetic acid–water–pyridine (B:A:W:P, 15:3:3:10, v/v/v/v). Loads of 10–20 μg were applied to the plates, and chromatograms were developed to a minimum length of 10 cm. Spraying with clorox (10%) and KI–starch (1%) solutions was used for detection. For amino acid analysis, peptides (1.0 mg) were hydrolyzed for 18 h at 110 °C with concentrated hydrochloric acid–propionic acid (1:1, v/v, 1–2 mL) or with 1–2 mL of 0.2% phenol in concentrated hydrochloric acid in evacuated and sealed ampules. The analyses were performed with a Kontron Liquimat III automatic amino acid analyzer and were satisfactory except for cysteine, which was partially destroyed, and Tyr(OEt), which was partially hydrolyzed to Tyr. A good ratio of Tyr was obtained upon hydrolyzing the peptides with 0.2% phenol in constant boiling hydrochloric acid. FAB mass spectrometry was performed by the Analytical, Physical and Structural Chemistry Department of Smith Kline & French Laboratories on a VG ZAB high-resolution spectrometer. FABMS gave a strong molecular ion peak cluster at $(M + H)^+$ for each peptide as well as $(M - H)^-$ in the negative-ion spectra. Fragmentations of the molecules and the iminium ions of the form $H_2N^+ = CHR$ (where R = an amino acid side chain) were also observed.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Arg-Arg-NH_2 (3). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-Arg(Tos)-BHA-R was prepared in eight cycles of solid-phase peptide synthesis using benzhydrylamine-resin (BHA-R). The first cycle included the loading of Boc-Arg(Tos) (3.0 mmol) activated with dicyclohexylcarbodiimide (DCC, 3.0 mmol) and *N*-hydroxybenzotriazole (HOBT, 6.0 mmol) on 1.0 g of BHA-resin in DMF–CH_2Cl_2 for 2 h to give a loading of 0.61 mmol/g of resin of Boc-Arg(Tos)-BHA-R. This was followed by seven cycles of deprotection with 50% TFA–CH_2Cl_2 (v/v), neutralization with 7% diisopropylethylamine (DIEA–CH_2Cl_2, v/v), and coupling with the next protected amino acids. β-[(4-Methylbenzyl)thio]-β,β-pentamethylenepropionic acid (Pmp-4-MeBzl)[22] was activated with DCC (3.0 mmol) and coupling was facilitated with 4-(dimethylamino)pyridine (DMAP, 3.0 mmol). All coupling steps were carried out in DMF–CH_2Cl_2 solvent mixture for 2–4 h, with either an automated peptide synthesizer (Beckman 990 B) or a manual shaker. Completion of the coupling reactions was monitored by the ninhydrin test.[23] A general protocol used for each coupling on the resin was given previously.[11] The protected peptide-resin intermediate precursor of 3 (2.3 g) in anisole (3 mL) was cleaved and deprotected with anhydrous liquid HF, 30 mL at 0 °C for 1 h, with Teflon Kel-F vacuum line in the hood. The HF was evaporated and the residue was treated with anhydrous ether to remove anisole and byproducts. The crude peptide was extracted from the resin with degassed DMF (50 mL) and acetic acid (HOAc, 40%, 50 mL) into 3.5 L of degassed water. The aqueous diluted disulfhydryl octapeptide was oxidatively cyclized with 0.01 M potassium ferricyanide solution at pH 7.2 until color persisted for 30 min. After the completion of the oxidation reaction, the pH of the solution was adjusted to 4.5 with glacial HOAc. This solution was passed through a weakly acidic cation-exchange resin (Bio-Rex 70, 50–100 mesh, 2.5 × 12 cm column). The column was eluted with pyridine–acetate buffer (pyridine–HOAc–H_2O, 30:4:66, v/v/v). The pyridine acetate was removed by distillation in vacuo, and the residue was lyophilized from 10% HOAc to give 285 mg of partially purified peptide. This was purified on CCD followed by gel filtration on a Sephadex G-15 column (112 mg) using 0.2 M HOAc as an eluent to afford 78 mg of 3.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Arg-Lys-NH_2 (4). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-Lys(Cl-Z)-BHA-R was synthesized from Boc-Lys(Cl-Z)-BHA-R in seven cycles of deprotection, neutralization, and coupling similar to the procedure described for the preparation of 3. Boc-Lys(Cl-Z)-BHA-R was prepared from Boc-Lys(Cl-Z) (3.0 mmol) on BHA-resin (1.0 mmol) with DCC (3.0 mmol) HOBT (6.0 mmol). Anhydrous HF cleavage-deprotection, oxidative cyclization, and purification on

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Bio-Rex 70 gave 702 mg of partially purified peptide. Purification by CCD followed by gel filtration on a Sephadex G-15 column (100 mg) eluted with aqueous HOAc (0.2 M) gave 67 mg of **4**.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Lys-Arg-NH_2 (5). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Lys(Cl-Z)-Arg(Tos)-BHA-R was synthesized from Boc-Arg(Tos)-BHA-R in seven cycles of deprotection, neutralization, and coupling. Anhydrous HF cleavage–deprotection and oxidative cyclization, followed by purification in Bio-Rex 70 gave 710 mg of partially purified peptide. Purification by CCD followed by gel filtration on a Sephadex G-15 column (100 mg) and elution with aqueous 0.2 M HOAc gave 70 mg of **5**.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Arg-D-Arg-NH_2 (6). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-D-Arg(Tos)-BHA-R was synthesized from Boc-D-Arg(Tos)-BHA-R (0.35 mmol/g) in seven cycles of deprotection, neutralization, and coupling. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by purification on Bio-Rex 70 gave 253 mg of partially purified peptide. Purification by CCD followed by gel filtration on a Sephadex G-15 column (110 mg) and elution with 0.2 M HOAC gave 70 mg of **6**.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-D-Arg-Arg-NH_2 (7). The protected peptide-resin intermediate Pmp(4-Bzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-D-Arg(Tos)-Arg(Tos)-BHA-R was synthesized from Boc-Arg(Tos)-BHA (0.48 mmol/g) in seven cycles of deprotection, neutralization, and coupling. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by purification on Bio-Rex 70 gave 371 mg of partially purified **7**. Further purification by CCD followed by gel filtration on a Sephadex G-15 column (106 mg) and elution with 0.2 M HOAc gave 51 mg. Final purification by preparative reverse-phase isocratic (40:60) HPLC (25 mg) gave 15 mg of recovered pure **6**.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-D-Arg-D-Arg-NH_2 (8). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr(Et)-Phe-Val-Asn-Cys(4-MeBzl)-D-Arg(Tos)-D-Arg(Tos)-BHA-R was synthesized from Boc-D-Arg(Tos)-BHA-R (0.62 mmol/g) in seven cycles of deprotection, neutralization, and coupling. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by purification on Bio-Rex 70 gave an oily product. Purification by CCD gave 858 mg of partially purified **8**. Further purification by gel filtration on a Sephadex G-15 column (123 mg) gave 75 mg. Final purification by preparative reverse-phase isocratic (40:60) HPLC (50 mg) gave 12 mg of pure **8**.

Pmp-D-Tyr(Et)-Phe-Val-Asn-Cys-Arg-Arg-OH (9). The protected peptide-resin intermediate Pmp(4-MeBzl)-D-Tyr-(Et)-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-Arg(Tos)-OBzl-R was prepared form Boc-Arg(Tos)-OBzl-R (Peninsula, 0.31 mequiv/g) in seven cycles of deprotection, neutralization, and coupling. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by flash chromatography on a C-18 reverse-phase column with 50% aqueous CH_3CN containing 0.1% TFA as an eluent gave 550 mg of partially purified **9**. Further purification by partition column chromatography (120 mg) followed by preparative reverse-phase isocratic HPLC (40 mg) gave 22.0 mg of pure **9**.

Mpr-Tyr-Phe-Val-Asn-Cys-Arg-Arg-NH_2 (10). The protected peptide-resin intermediate Mpr(4-MeBzl)-Tyr-Phe-Val-Asn-Cys(4-MeBzl)-Arg(Tos)-Arg(Tos)-BHA-R was prepared form Boc-Arg(Tos)-BHA-R (0.46 mmol/g) in seven cycles of deprotection, neutralization, and coupling. Anhydrous HF cleavage–deprotection and oxidative cyclization followed by purification on Bio-Rex 70 gave 650 mg of **10**. Purification by partition column chromatography (165 mg) gave 36 mg of pure **10**.

2. Bioassay Methods. Peptides 1–10 were tested in vitro for vasopressin V_2 receptor affinity and antagonist activity on the pig renal medullary membrane preparations as previously described.[15] Affinity for the receptors was determined by competition with [^{3}H]LVP and is expressed as K_{bind}. The antagonist potency of analogues 1–9 was determined by measuring their ability to inhibit LVP-stimulated adenylate cyclase in the same preparation and is expressed as the inhibition constant K_i. The in vivo evaluation for the antagonists 1–9 was carried out by using the hydropenic rat model as previously described.[24,25] Briefly, groups of male rats were deprived of food and water overnight to establish a stable hydropenic state, characterized by elevated plasma vasopressin levels and new maximal urine concentrating ability (urine osmolality of 1500 mosm/kg of H_2O).[25] On the morning of the study, rats were housed in individual metabolism cages (four rats/cage) and administered (intraperitoneally) vehicle or antagonist. Urine volume and osmolality (Uosm) were determined and dose–response relationships for dilution of urine were developed. Potency was expressed as the effective dose (ED_{300}, μg/kg) required to reduce Uosm from hydropenic levels to plasma osmolality levels (300 mosm/kg of H_2O), level at which the renal-free water clearance switches from a net negative to a net positive value (i.e., water diuresis). Potency values presented represent an average of one to three determinations.

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