Journal of Peptide Science
J. Peptide Sci. **11**: 161–165 (2005)
Published online 11 October 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.612

JOURNAL OF PEPTIDE SCIENCE

Structure-activity studies on prolactin-releasing peptide (PrRP). Analogues of PrRP-(19–31)-peptide

ROBERT G BOYLE,[a] ROBERT DOWNHAM,[a] TANMOY GANGULY,[b] JOHN HUMPHRIES,[c] JEFF SMITH[b] and STUART TRAVERS[a]*

[a] Millennium Pharmaceuticals Limited, Granta Park, Great Abingdon, Cambridge CB1 6ET, UK
[b] Millennium Pharmaceuticals Inc., 40 Lansdowne Street, Cambridge, MA 02139, USA
[c] Sareum Ltd, 2 Pampisford Park, Cambridge CB2 4EE, UK

Received 8 July 2004; *Revised 28 July 2004*; *Accepted 6 Aug 2004*

Abstract: An investigation of a series of single replacement analogues of PrRP-(19–31)-peptide has shown that good functional activity was retained when Phe[31] was replaced with His(Bzl), Phe(4Cl), Nle, Trp, Cys(Bzl) or Glu(OBzl); when Val[28] or Ile[25] was replaced with Phg; when Gly[24] was replaced with D-Ala, L-Ala, Pro or Sar; when Ser[22] was replaced with Gly and when Ala[21] was replaced with Thr or MeAla. The results confirm that the functionally important residues are located within the carboxyl terminal segment, -Ile-Arg-Pro-Val-Gly-Arg-Phe-NH_2. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: agonist; orphan receptor; oGPCR10/hGR3; prolactin releasing peptide (PrRP)

INTRODUCTION

Prolactin releasing peptides, PrRP20 and PrRP31, have been identified by Hinuma *et al.* [1,2], as ligands of the orphan receptor oGPCR10/hGR3 [3–5]. Expression and distribution studies of PrRP have been reported in locations of the brain [6–9,14], CNS [9] and the periphery [9,10]. The physiological function of PrRP is not yet completely understood. The original hypothesis that its role was associated with its weak prolactin releasing property [1,2,11,12] has now given way to alternative suggestions that it is involved as a regulator of the CNS [13,14] or that it has an influence on hypothalamic hormone secretion, such as CRF and LH/FSH [15,16] or oxytocin [17]. More recently there has been growing evidence that PrRP is involved with food regulation, especially since icv-administered PrRP inhibited food intake and body weight gain in rats without affecting water intake [18,19]. Consequently a good PrRP agonist could provide a useful therapeutic approach to obesity management.

Some SAR data have been reported for PrRP [1,13,20]. It was early recognized that PrRP with a carboxyl terminal acid function was inactive [1]. Roland *et al.* [13] found that PrRP-(25–31)-heptapeptide was the minimal active agonist fragment and used an Ala scan to show the importance of the three Arg residues. Danho *et al.* [20] found that Ac-PrRP-(26–31)-hexapeptide was the smallest agonist sequence, and concluded that the critical amino acids were Arg[26], Pro[27], Val[28], Arg[30] and Phe[31]. Structural studies have indicated a possible conformation for PrRP. D'Ursi *et al.* [21] used NMR, CD and other techniques to investigate PrRP20, i.e. PrRP-(12–31)-peptide. They concluded that the carboxyl terminal region consisted of an amphipathic helix with one side of the helix being hydrophobic because of the presence of the apolar side chains of Ala[21], Ile[25], Val[28] and Phe[31] and with the other side hydrophilic due to the polar side chains of Arg[23], Arg[26] and Arg[30]. The side chains of Asp[14] and Ile[15] also displayed some organized structure at the amino terminus. From NMR studies, Danho *et al.* [20] concluded that PrRP20 existed as an L-shaped molecule in which the *N*-terminal portion appeared to be flexible and exhibited no regular secondary structure, whereas the biologically important carboxyl terminal region adopted a well-defined conformation consisting of one full helical turn preceded by a kink. The helical structure was thought to bring together the side chains of the important residues of Arg[26], Val[28] and Phe[31] to form a hydrophobic cluster with the side chain of the critical Arg[30] pointing opposite this cluster.

This paper reports the results of an investigation to identify the chief features within the human form of PrRP for agonist activity, as these were required to aid non-peptide drug design. Unlike previous studies, this SAR programme was designed around tridecapeptide analogues of PrRP-(19–31)-peptide, on which no publications have yet appeared. The approach used the FLIPR functional assay, guided to some cautious extent by binding results, and the conclusions are drawn from the results of over 80 linear and some cyclic analogues. It was initially assumed that the active site was located within the carboxyl terminal region and that increased binding potency was achieved by amino terminal extension so as to include the Trp[19] and Tyr[20] moieties. The actual SAR study was centred on

* Correspondence to: Stuart Travers, Paradigm Therapeutics Ltd, 214 Cambridge Science Park, Cambridge CB4 0WA, UK;
e-mail: stravers@paradigm-therapeutics.co.uk

positions 21 to 31 with the main emphasis on positions 24 to 31.

RESULTS AND DISCUSSION

Preliminary binding studies on truncated sequences of PrRP-(1–31)-peptide suggested that the fragment, PrRP-(19–31)-peptide **1**, was a good starting point for an analogue programme, as it was a good agonist and retained good binding activity, with a K_i of 4.49 nM. Binding was significantly decreased by further shortening at the amino- or the carboxyl-terminals. The results are given in Table 1. For brevity, most analogues that have little or no agonist activity have been omitted from the Table. In this study, the most potent analogues had a functional EC_{50} of between 0.01 and 0.06 μM and a binding K_i within the range 0.0047–0.042 μM. Less potent agonists having a functional EC_{50} up to about 1 μM had K_i values between 0.0115 and 0.235 μM and those analogues judged to be essentially functionally inactive with EC_{50} values >1 μM had K_i values between 0.0485 and 5.000. No inhibition data are available for these analogues.

H-Trp^{19}-Tyr^{20}-Ala^{21}-Ser^{22}-Arg^{23}-Gly^{24}-Ile^{25}-
Arg^{26}-Pro^{27}-Val^{28}-Gly^{29}-Arg^{30}-Phe^{31}-NH_2

PrRP-(19–31)-peptide **1**

Extensive SAR studies were made on the very important Phe^{31} carboxyl terminal position. Functional activity was maintained when the side chain was modified, as in the His(Bzl) **6**, Phe(4-Cl) **7**, Nle **8**, Trp **9**, Cys(Bzl) **10** and Glu(OBzl) **11** analogues, with EC_{50} values ranging from 0.02 to 0.06 μM. It should be noted that since the coupling of His(Bzl) is notoriously prone to racemization [22], **6** may also contain stereoisomers with the D-configuration at the His(Bzl) residue. In the related analogues, containing Tyr(Bzl) **12**, Gln **13** and Met **14**, the functional EC_{50} potency was reduced to between 0.17 and 0.35 μM. Little, if any, functional activity was shown by other analogues in this position, including those containing Tic, Cha, Leu **17**, Ile, Pro, or the polar residues of Arg, Ser or Glu. Also, the binding results strongly suggested that substitution with Pyr **20** could be tolerated well, but not for analogues in which the phenyl group in the side chain was repositioned, with respect to the backbone, as with Phg **16**, Hph **18** or with D-Phe **19**. Whereas good binding was seen for the benzyl ester Glu(OBzl) **11**, the analogue having the corresponding saturated ester, Glu(OcHx), appeared not to bind. The Phe side chain can be replaced by other CH_2Ar groups, in which the aromatic group can be larger than that in Phe itself, but the spacing of the phenyl group in relation to the backbone is very important. The results suggest that for the side chain

Table 1 Activities for the PrRP-(19–31)-peptide Analogues

Compound number	Replacement	FLIPR EC_{50} μM	Binding K_1 μM
1	None	0.02	0.0053
2	Phe^{31}-NHMe	n/a	0.0044
3	Phe^{31}-OMe	n/a	0.0353
4	Phe^{31}-ol	0.89	0.235
5	Phe^{31}-OH	NF	5.000
6	His(Bzl)^{31}	0.02	0.0047
7	Phe(4Cl)^{31}	0.02	0.0055
8	Nle^{31}	0.05	0.0195
9	Trp^{31}	0.05	0.0245
10	Cys(Bzl)^{31}	0.04	0.025
11	Glu(OBzl)^{31}	0.04	0.042
12	Tyr(Bzl)^{31}	0.23	0.0365
13	Gln^{31}	0.17	0.0635
14	Met^{31}	0.35	0.0955
15	MePhe^{31}	4.95	0.215
16	Phg^{31}	n/a	0.199
17	Leu^{31}	2.06	0.395
18	Hph^{31}	n/a	0.517
19	D-Phe^{31}	n/a	0.887
20	Pyr^{31}	n/a	0.0063[a]
21	Sar^{29}	0.27	0.0215
22	Pro^{29}	0.6	0.0460
23	Ala^{29}	0.24	0.0945
24	MeAla^{29}	2.27	0.3750
25	D-Ala^{29}	NF	5.0000
26	Phg^{28}	0.01	0.0047
27	Achc^{28}	n/a	0.0236
28	D-Val^{28}	n/a	2.6400
29	Hyp(Bzl)^{27}	0.51	0.0345
30	Hyp^{27}	0.51	0.0540
31	MeAla^{27}	0.13	0.0630
32	Ala^{27}	n/a	0.1870
33	Lys^{26}	0.12	0.0220
34	His^{26}	1.18	0.0975
35	Orn^{26}	NF	0.1020
36	Phg^{25}	0.01	0.0082
37	Pro^{25}	1.44	0.0485
38	Ala^{25}	0.13	0.0505
39	MeVal^{25}	0.60	0.1350
40	D-Ala^{24}	0.02	0.0036
41	Ala^{24}	0.01	0.0115
42	Sar^{24}	0.06	0.0120
43	Pro^{24}	0.02	0.0130
44	β-Ala^{24}	0.20	0.0135
45	Pro^{23}	0.62	0.0115
46	Gly^{22}	0.01	0.0078
47	Pro^{22}	0.33	0.0305
48	Thr^{21}	0.02	0.0068
49	MeAla^{21}	0.03	0.0077
50	Pro^{21}	0.12	0.0135

[a] IC_{50} value: standard PrRP-(19–31)-peptide = 0.00658 μM)
NF, non-functional. n/a, not available.

in position 31, generalized here as backbone-linker-Ph, good agonist activity is retained when the linker is CH_2 (Phe) or CH_2-S-CH_2 (Cys(Bzl)), but not in cases where the linker is absent (Phg) or is CH_2CH_2 (Hph). Restrictions on side chain branching are also apparent from results with analogues carrying aliphatic-based side chains, where good activity was retained by the Nle and Glu(OBzl) analogues, and to a limited extent with the Gln and Met analogues, but not for the branched Ile, Leu or Cha analogues. With the single exception of Nle **8**, the most potent analogues possessed an aromatic moiety somewhere in the side chain.

The replacement of Phe^{31} with the *N*-methylated residue, MePhe **15**, eliminated functional activity and significantly decreased binding, strongly suggesting the high importance of this backbone NH, in agreement with an earlier study on a shorter PrRP fragment [20]. A suitably blocked carboxyl terminal also appears to be essential. As expected, the terminal acid **5** was not functionally active [1]. However, the carboxyl terminal primary amide can be replaced by the secondary methylamide **2** with complete retention of excellent binding affinity, suggesting that this may also act as a good agonist. The methyl ester **3** also retained significant binding activity but the analogue having a terminal alcohol **4** only displayed minimal effects. These results emphasize that the terminal Phe^{31} position is extremely important for activity because it provides a suitably designed hydrophobic side chain in the L-configuration, a free backbone NH and a suitably blocked carboxyl terminus. But it can tolerate some variation and can accommodate other groups containing bulkier aromatic moieties, unbranched aliphatic groups and even certain esters, and the terminal primary amide is not essential.

No functional or binding activity was observed when Arg^{30} was replaced by other polar residues, such as Lys, Orn, His, Ser, or Glu, or by Pro. Binding results also indicated that the related replacements with D-Arg, Har or Dab(Gly) would not produce functionally active peptides either. Clearly an Arg residue in position 30 is absolutely essential and provides an appropriately located diffuse positive charge in an L-configured side chain. Earlier studies with shorter analogues have also emphasized the high importance of the side chain [13,20] and also the backbone NH [20] in position 30.

Gly was by far the best residue for position 29. Although the replacement of Gly^{29} by Sar **21**, Pro **22** or Ala **23** gave somewhat marginal functional activity, the corresponding MeAla **24**, D-Ala **25**, β-Ala and Nip (piperidine-3-carboxylic acid) analogues were much less effective. As functional activity is significantly reduced by even very small structural changes, it is feasible that the Gly^{29} residue lies at a functionally important position. This residue could be acting as a spacer residue that requires unhindered flexibility for assisting essential structural folding or, alternatively, that is situated very close to the receptor surface so that on steric grounds substitution cannot be tolerated. It is curious that as agonists, mono-methylation of residue 29 was tolerated on the backbone nitrogen (Sar) and at the α-carbon in the L-configuration (Ala) but not in the D-configuration (D-Ala).

Both functional and binding activity was completely retained when Val^{28} was replaced by Phg **26**. However, functional activity was essentially lost when the Val was replaced by Pro, His or His(Bzl). In our hands the backbone *N*-methylated analogue, [MeVal^{28}]-PrRP(19–31)-peptide, was also non-functional. A previous report [20] indicated that the shorter analogue, [MeVal^{28}]-PrRP(24–31)-peptide, appeared to retain activity. Although the α,α-disubstituted Achc^{28} analogue **27** retained quite good binding, binding was essentially lost when Cha or D-Val **28** was in this position. These results suggest that position 28 is very important in contributing a residue having a reasonably sized hydrophobic side chain in the L-configuration and with branching close to the peptide backbone, and also a free backbone NH.

The peptide that retained the Pro residue in position 27 was by far the most potent. Although reduced functional activity was given by the related, but acyclic, MeAla^{27} analogue **31**, substitution with Hyp(Bzl) **29** or Hyp **30** gave poor functional activity. Little binding was shown by analogues containing the non-*N*-alkylated residues of D-Phe, Phe, Ala **32** or Achc. The results could indicate that a small turn-inducing residue is preferred for this position, especially as the MeAla^{27} analogue **31** appears to be more active than the Ala^{27} analogue **32**.

Replacement of Arg^{26} with Lys **33** retained some functional and binding activity. However, similar substitutions with His **34**, Orn **35**, Gln, Ser, Glu or Pro gave analogues that were functionally ineffective. Danho *et al.* [20] also reported that some functional activity was retained when the polar Arg^{26} residue in PrRP-(24–31)-peptide was substituted by a hydrophobic Phe residue or by Lys, Cit or MeArg. Overall, it is concluded that the Arg side chain in position 26 is important, especially for potency, but that Arg^{26} is less critical than Arg^{30}.

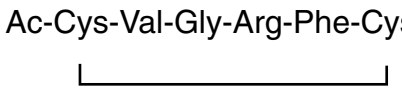
The Ile in position 25 could be replaced by Phg **36** with full retention of both functional and binding activity. The corresponding Ala^{25} analogue **38** also retained some functional activity, but substitution with MeVal **39** or Pro **37** was not very effective. Interestingly, substitution with a methylated isoleucine residue, as in MeIle^{25}-PrRP-(24–31)-peptide, was reported to retain agonist activity [20]. Therefore, it would appear that position 25 is important for contributing a residue having a reasonably sized hydrophobic side chain with branching close to the peptide backbone.

The Gly^{24} residue does not appear to be essential as very good functional activity was maintained by

replacement with structurally different small residues, such as D-Ala **40**, L-Ala **41** and the *N*-alkylated residues of Sar **42** and Pro **43**. Even the β-Ala^{24} analogue **44** retained some activity, suggesting that the additional methylene group intercalated into the backbone at this position did not cause any serious disruption of receptor interaction. The results may indicate that Gly^{24} may simply be acting as a spacer.

Very few analogues were investigated based on changes in positions 21 to 23, which were initially regarded as containing non-essential residues, and the results have largely vindicated this assumption. Positions 21 and 22 also include sites of natural species variation such as Gly^{22} for bovine- and Thr^{21}Gly^{22} for rat-PrRP. Replacement of Arg^{23} by Pro significantly decreased potency. However, complete retention of functional and binding activity was shown on replacing Ser^{22} with Gly **46**, or replacing Ala^{21} with either MeAla **49** or Thr **48**. Functionally, in these positions, the Pro analogues were less active and showed an order of potency as Pro^{21} **50** > Pro^{22} **47** and Pro^{23} **45**.

A cyclic disulfide analogue of the important carboxyl terminal region, essentially a cyclized -VGRF-sequence, **51** did not bind, perhaps because it lacked the essential carboxyl terminal amide grouping of Phe^{31}-NH_2.



Cyclic disulfide VGRF analogue **51**

CONCLUSIONS

From a study of 86 analogues of the tridecapeptide, PrRP-(19–31)-peptide amide, many new sequences were identified as having both excellent functional and binding activities. At this point no conclusions are possible on the contribution of backbone carbonyl functions. The results highlight the role of the functionally important residues that are located within the carboxyl-terminal heptapeptide segment Ile^{25}-Arg^{26}-Pro^{27}-Val^{28}-Gly^{29}-Arg^{30}-Phe^{31}-NH_2. The only essential amino acid residue in PrRP is Arg^{30}, which donates both a critical basic side chain in the L-configuration and is also reported [20] to contribute an essential backbone NH. The carboxyl terminal position occupied by Phe^{31}-NH_2 is very important and provides an essential backbone NH, an essential suitably blocked terminus and an appropriately designed, hydrophobic side chain in the L-configuration. Preliminary results from carboxyl terminal modifications suggest that other capping groups, such as substituted amides or esters, could be functionally active. The Gly^{29} is very important for good functional activity and even slight changes are poorly tolerated. Pro was the best substituent for position 27 and is perhaps required for its turn-promoting property. The Val^{28} residue is relatively selective to substitution but could be replaced by Phg with full retention of functional and binding activity. The results suggest that this position is supplying both a reasonably sized hydrophobic side chain in the L-configuration, having branching close to the peptide backbone, and an important free backbone NH. Although the Ile^{25} position resembles the Val^{28} position in accepting substitution by Phg, it would appear to be a less important position, as substitution with Ala retained considerable functional activity. The results from a limited number of analogues in positions 21 to 23 confirm that these residues are less important. Finally, the current results significantly extend the PrRP SAR data, providing further information on the essential structural contributions provided from the functionally important carboxyl terminal segment, and these should be of interest in future research design, both as biological tools and in therapeutic studies, such as for obesity management.

PEPTIDE SYNTHESIS

The peptides were obtained by contract synthesis using standard Fmoc-solid phase procedures and purified by preparative-HPLC, usually to greater than 95% by HPLC (C-18, Vydac 218TP54, 250 × 4.6 mm) with mass confirmation by MALDI-MS mass spectrometry.

BIOLOGICAL ASSAYS

Calcium Mobilization Assay of HEK293-ASR1 Cells

HEK293-ASR-1 cells were seeded at a density of 60 000 cells per well in black-wall, clear-bottom, poly-D-lysine-coated 96-well plates the day before the experiment. On the day of the experiment, culture medium was replaced with dye loading solution (Hanks Balance Salt Solution, Gibco, with 20 mM Hepes, 2.5 mM probenecid, 1 mg/ml bovine serum albumin and 1% (v/v) fetal bovine serum containing 4 μM FLUO-3). Dye loading was carried out for 1 h at 37 °C. The cells were then washed with assay medium (same as dye loading solution excluding fetal bovine serum and dye), and a fluorescence imager plate reader carried out intracellular calcium measurements in response to compound addition (FLIPR, argon laser, 488 nm, Molecular Devices). The EC_{50} for hPrRP31 was measured as 17 nM in this system.

Eu-(Lys)PrRP31 Displacement Binding Assay of HEK293-ASR1 Cells

HEK293-ASR1 cells were plated at a density 80 000 cells per well on poly-D-lysine coated 96-well plates the day before the experiment. The cells were washed with binding buffer (25 mM Hepes, 0.075 mM EDTA,

11.5 mM KCl, 115 mM NaCl, 6 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4), and then incubated with Eu-(Lys)PrRP31[†] (final assay concentration of 1 nM) and the appropriate concentration of test compound for 2 h at room temperature. The cells were then gently washed seven times with wash buffer (25 mM Hepes, 0.1 mM $CaCl_2$, 115 mM NaCl), 150 µl enhancement solution (Wallac) was added to each well and time resolved fluorescence was measured between 50 and 400 µs with a Victor II Plate Reader at 340 nm excitation and 615 nm emission. Specific binding was calculated by subtracting the non-specific from the total binding and was fitted to a single-site binding model.

Acknowledgement

The authors would like to thank Neil Thompson, Julie Marley and Stan Moore of Bachem UK Ltd for their helpful assistance with peptide syntheses. Thanks are also given to Wei Li of Millennium Pharmaceuticals, Inc. for development of the FLIPR based screening assay.

REFERENCES

1. Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya M, Fukusumi S, Kitada C, Matsuo Y, Asano T, Matsumoto H, Sekiguchi M, Kurokawa T, Nishimura O, Onda H, Fujino M. A prolactin-releasing peptide in brain. *Nature* 1998; **393**: 272–302.
2. Hinuma S, Onda H, Fujino M. The quest for novel bioactive peptides utilizing orphan seven-transmembrane-domain receptors. *J. Mol. Med.* 1999; **77**: 495–504.
3. Marchese A, Heiber M, Nguyen T, Heng HH, Saldivia VR, Cheng R, Murphy PM, Tsui LC, Shi X, Gregor P. Cloning and chromosomal mapping of three novel genes, GPR9, GPR10 and GPR14, encoding receptors related to interleukin 8, neuropeptide Y and somatostatin receptors. *Genomics* 1995; **29**: 335–344.
4. Welch SK, O'Hara BF, Kilduff TS, Heller HC. Sequence and tissue distribution of a candidate G-coupled receptor cloned from rat hypothalamus. *Biochem. Biophys. Res. Commun.* 1995; **209**: 606–613.
5. Langmead CJ, Szekeres PG, Chambers LK, Ratcliffe SJ, Jones DNC, Hirst WD, Price GW, Herdon HJ. Characterisation of the binding of [125I]-human prolactin releasing peptide to GPR10, a novel G protein coupled receptor. *Br. J. Pharmacol.* 2000; **131**: 683–688.
6. Takahashi K, Yoshinoya A, Arihara Z, Murakami O, Totsune K, Sone M, Sasano H, Shibahara S. Regional distribution of immunoreactive prolactin-releasing peptide in the human brain. *Peptides* 2000; **21**: 1551–1555.
7. Fujii R, Fukusumi S, Hosoya M, Kawamata Y, Habata Y, Hinuma S, Sekiguchi M, Kitada C, Kurokawa T, Nishimura O, Onda H, Sumino Y, Fujino M. Tissue distribution of prolactin-releasing peptide and its receptor. *Regul. Pept.* 1999; **83**: 1–10.
8. Marutama M, Marsuboto H, Fujiwara K, Kitada C, Hinuma S, Onda H, Fujino M, Inoue K. Immunocytochemical localization of prolactin-releasing peptide in the rat brain. *Endocrinology* 1999; **140**: 2326–2333.
9. Matsumoto H, Murakami Y, Horikoshi Y, Noguchi J, Habata Y, Kitada C, Hinuma S, Onda H, Fujino M. Distribution and characterisation of immunoreactive prolactin-releasing peptide in rat tissue and plasma. *Biochem. Biophys. Res. Commun.* 1999; **257**: 264–268.
10. Nieminen M-L, Brandt A, Pietita P, Panula P. Expression of mammalian RF-amide peptides neuropeptide FF, prolactin-releasing peptide and the PrRP receptor in the peripheral tissues of the rat. *Peptides* 2000; **21**: 1695–1701.
11. Jarry H, Heuer H, Schomberg L, Bauer K. Prolactin-releasing peptides do not stimulate prolactin release *in vivo*. *Neuroendocrinology* 2000; **71**: 262–267.
12. Matsumoto H, Noguchi J, Horikoshi Y, Yawamata Y, Kitada C, Hinuma HS, Onda H, Nishimura O, Fulino M. Stimulation of prolactin release by prolactin-releasing peptide in rats. *Biochem. Biophys. Res. Commun.* 1999; **259**: 321–324.
13. Roland BL, Sutton SW, Wilson SJ, Luo L, Pyati J, Huvar R, Erlander MG, Lovenberg TW. Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. *Endocrinology* 1999; **140**: 5736–5745.
14. Ibata Y, Iijima N, Kataoka Y, Kakihara K, Tanaka M, Hosoya M, Hinuma S. Morphological survey of prolactin-releasing peptide and its receptor with special reference to their functional roles on the brain. *Neurosci. Res.* 2000; **38**: 223–230.
15. Matsumoto H, Maruyama M, Noguchi J, Horikoshi Y, Fugiwara K, Kitada C, Hinuma S, Onda H, Nishimura O, Inoue K, Fujino M. Stimulation of corticotropin-releasing hormone-mediated adreno-corticotropin secretion by central administration of prolactin-releasing peptide in rats. *Neurosci. Lett.* 2000; **285**: 234–238.
16. Seal LJ, Small CJ, Kirn MS, Stanley SA, Taheri S, Ghatei MA, Bloom SR. Prolactin releasing peptide stimulates luteinizing hormone and follicle stimulating hormone via a hypothalamic mechanism in male rats. *Endocrinology* 2000; **141**: 1909–1912.
17. Maruyama M, Matsumoto H, Fujiwara K, Noguchi J, Kitada C, Hinuma S, Onda H, Nishimura O, Fujino M, Higuchi T, Inoue K. Central administration of prolactin-releasing peptide stimulates oxytocin release in rats. *Neurosci. Lett.* 1999; **276**: 193–196.
18. Lawrence CB, Celsi F, Brennand J, Luckman SM. Alternative role for prolactin-releasing peptide in the regulation of food intake. *Nat. Neurosci.* 2000; **7**: 645–646.
19. Ellacott KL, Lawrence CB, Rothwell NJ, Luckman SM. PRL-releasing peptide interacts with leptin to reduce food intake and body weight. *Endocrinology* 2002; **143**: 368–374.
20. Danho W, Swistok J, Khan W, Truitt T, Kurylko G, Fry D, Greeley D, Sun H, Dvorozniak M, Machie G, Spence C, Goodnow R. Structure-activity relationships and bioactive conformations of prolactin releasing peptides. Ligands for a potential obesity target. *Am. Peptide Symp.* Boston, 2003 (except from poster).
21. D'Ursi AM, Albrizio S, Di Fenza A, Crescenzi O, Carotenuto A, Picone D, Novellino E, Roero P. Structural studies on Hgr3 orphan receptor ligand prolactin-releasing peptide. *J. Med. Chem.* 2002; **45**: 5483–5491.
22. See Jones JH in *Houben-Weyl*, **E22a**: 334–341 and literature there cited.

[†] Eu-(Lys)PrRP31: Human PrRP31 *N*-terminally modified with an additional lysine residue to which europium has been attached.