LIGAND-RECEPTOR INTERACTIONS OF GONADOTROPIN-RELEASING HORMONE ANTAGONISTS

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Submitted in fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

in the

DEPARTMENT OF CHEMICAL PATHOLOGY
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF CAPE TOWN
MARCH, 1999

ABSTRACT

The interactions of selected GnRH agonists and antagonists with the GnRH receptor were investigated using site directed mutagenesis and photoaffinity crosslinking of the receptor with a labelled GnRH analog.

With the aim of identifying residues involved in ligand binding, the relative affinities of five peptide and one non-peptide GnRH antagonists towards three closely related mammalian GnRH receptors (mouse, sheep and human) were determined using cells transiently expressing GnRH receptors. All of the peptide antagonists [Ac-3-Pro^{1}, D-4-F-Phe^{2}, 2-D-Nal^{3}, D-Ipr-Lys^{6}, Ipr-Lys^{8}]GnRH, [2-Ac-D-Nal^{1}, D-Me-4-Cl-Phe, 3-D-Pal^{3}, D-Arg^{6}, D-AlaNH$_2$^{10}]GnRH, [2-Ac-D-Nal^{1}, D-4-Cl-Phe^{2}, 3-D-Pal^{3}, Nic-Lys^{5}, D-Nic-Lys^{6}, Ipr-Lys^{8}, D-AlaNH$_2$^{10}]GnRH, [Ac-D-Nal^{1}, D-4-Cl-Phe^{2}, D-Pal^{3}, D-Lys(3-pAc)^{6}, Ipr-Lys^{8}, D-AlaNH$_2$^{10}]GnRH and [2-Ac-D-Nal^{1}, D-4-Cl-Phe^{2}, 3-D-Pal^{3}, 1-Me-Pal^{5}, D-Trp^{6}, D-AlaNH$_2$^{10}]GnRH were found to have a higher potency for the inhibition of GnRH stimulated inositol phosphate by the mouse and sheep receptors than by human GnRH receptor. The greatest differences in affinity were exhibited by the antagonist, [2-Ac-D-Nal^{1}, D-Me-4-Cl-Phe, 3-D-Pal^{3}, D-Arg^{6}, D-AlaNH$_2$^{10}]GnRH. This antagonist showed an affinity difference of 54 fold between mouse and human receptors and 68 fold between sheep and human receptors. These results suggest that mutation of residues which differ between human and sheep receptors could be used to identify specific residue(s) involved in the binding of this peptide antagonist. This line of investigation was not pursued further, however, in view of more promising possibilities with non-peptide antagonists.

*FE 101170, a non-peptide GnRH antagonist was found to have 300-fold higher affinity for the human receptor than the sheep receptor. The regions of the receptor responsible for the differential affinity were explored using point-mutated and human/sheep chimeric GnRH receptors. These receptors had one or more residue of the human GnRH receptor replaced by the corresponding sheep residues. Because of the small size and hydrophobic nature of the antagonist, emphasis was made on TM residues. hF227L and L78V mutant receptors showed no change in agonist or antagonist (FE 101177) affinity. The substitution of the lone charged residue in TM4 Arg^{179} by Gly caused a small (4 fold) decrease in affinity to FE 101177. Multiple substitution of the TM4 residues (V160I, 165L, V169I) and of the TM1 residues (*A50T, T51I, A54T*) did not produce any significant changes in agonist or antagonist affinity. The D302N mutant receptor, which lacks the charged Asp residue at position 302, showed a minimal change in its affinity to an agonist and about 12 fold decrease in*

affinity for FE 101177.

More extensive alterations of the receptor were made by constructing receptor chimeras. The substitution of the extracellular N-terminus of the human receptor by the corresponding sheep receptor residues did not cause any change in agonist or antagonist binding. However, when all the sheep residues up to the extracelluar end of TM2 were incorporated, a selective decrease in the affinity for FE 101177 by of 20 fold was observed. Extending the sheep amino acid contribution to the extracellular end of TM4 resulted in a further decrease of the affinity by 40 fold. The results suggest that the interaction of FE 101170 with the GnRH receptor involves a number of residues and that the binding are not fully accounted for by TM domains alone. In addition the results show that the interactions are not purely additive, and that co-operative effects are involved in binding of this ligand.

The interaction of the GnRH receptor with peptide antagonists was also investigated by photoaffinity crosslinking of the GnRH receptor. 10 photoreactive GnRH analogues containing either 4-azidobenzoic acid or 4-azidosalicylic acid attached to the ε-amino group of lysine or the β-amino group of diaminopropionic acid were synthesised and characterized. Of these, [N-Azidobenzoyl-Ac-D-Lys^{1}, D-4-Cl-Phe^{2}, D-Trp^{3}, D-Arg^{6}, D-AlaNH$_2$^{10}]GnRH, named PAnt-1, was found to be a high affinity (Ki = 3.8 ± 0.04 nM) antagonist possessing a high crosslinking efficiency of > 70%. The peptide was shown to crosslink to the GnRH receptor in a specific and functionally relevant manner.

[Ac-D-Nal^{1}, N-Azidobenzoyl-D-Dpr^{2}, D-Trp^{3}, D-Arg^{6}]GnRH and [Ac-D-Nal^{1}, N-Azidosalisy-D-Dpr^{2}, D-Trp^{3}, D-Arg^{6}]GnRH were found to have a moderate affinity. However photolabelling of the GnRH receptor using their iodinated analogs could not be demonstrated. [Ac-D-4-Cl-Phe^{1}, Ac-D-4-Cl-Phe^{2}, D-Trp^{3}, N-Azidobenzoyl-D-Lys^{6}, D-AlaNH$_2$^{10}]GnRH had high affinity for the GnRH receptor in the absence of UV induced photolysis. Crosslinking of this peptide with the GnRH was demonstrated.

The agonist [D-Ala^{6}, N-Azidobenzoyl-Orn^{8}]GnRH was found to have an affinity and potency comparable to that of mammalian GnRH. However, it had a very low efficiency of crosslinking as sustained release of inositol phosphate after crosslinking with the GnRH receptor could not be demonstrated. In addition, photolabelling of the receptor could not be demonstrated with either [^{125}I-D-Tyr^{5}, D-Ala^{6}, N-Azidobenzoyl-Orn^{8}]GnRH or the related [^{125}I-D-Tyr^{5}, D-Ala^{6}, N-Azidosalicyl-Orn^{8}]GnRH.

The photoreactive analogs [N-azidobenzoyl-L-Dpr^2, D-Ala^6]GnRH, [N-azidobenzoyl-L-Drp^3, D-Ala^6]GnRH and [N-azidosalisyl-Dpr^3, D-Ala^6]GnRH, with modifications at residues 2 and 3 residue were found to have low affinity.

Wild type human, mouse and sheep GnRH receptors, as well as a panel of mutant GnRH receptors, were used in peptide mapping studies to define the attachment site of the radioiodinated photoreactive analog PAnt-1. Photoaffinitiy crosslinking of the mouse receptor with PAnt-1 followed by deglycosylation and digestion with endo proteinase Glu-C yielded a 13 kDa labelled fragment. The mouse E8Q and E90Q mutants yielded larger fragments, indicating the labelled fragment in the mouse receptor consists of residues Gln^9-Glu^{90}. Glu-C digestion of human and sheep receptors crosslinked with PAnt-1 peptide gave a labelled band of approximately 8 kDa, consistent with cleavage at residues Glu^{68} in both species and at residues Glu^8 (human) and Glu^{11} (sheep). Digestion of the human N19E mutant GnRH receptor yielded a very small labelled fragment indicating that the attachment site of the photoreactive antagonist to the human GnRH receptor lies between residues 12 and 18 in the extracellular N-terminus.

Comparison of Glu-C cleavage products from PAnt-1 crosslinked receptors under reducing and non-reducing conditions directly demonstrated the presence of a disulphide bridge Cys^{114}-Cys^{196}. In addition the results suggested that the crosslinking site was Cys^{14} in the extracellular N-terminal domain. These findings suggest that related peptide antagonists bind to the GnRH receptor with their N-terminus in the vicinity of the disufide bridge near the second extracelluar loop.

In Summary, molecular determinants of the binding of peptide and non-peptide antagonists to the GnRH receptor have been investigated in the present thesis. The major findings are:

(1) The identification of a peptide GnRH antagonist with differential affinity between human and sheep GnRH receptors, which could be used in future studies to map its binding site.

(2) The identification of a set of residues which contribute to the differential affinity of the non-peptide GnRH antagonist FE 101177 towards sheep and human receptors.

(3) The synthesis and chracterization of PAnt-1, a novel photoreactive peptide GnRH antagonist with high affinity and crosslinking efficiency

(4) The localization of the attachment site of PAnt-1 to a 7-residue segment of the N-terminal domain of the receptor, with Cys^{14} the putative crosslinking site.

DEDICATION

This thesis is dedicated to the fond memories of "Yitbe-Soul" (Dr Yetbarek Elias, 1965-1999) a good friend and an exceptional human being.

ACKNOWLEDGEMENTS

I am greatly indebted to my supervisors, Professors James S. Davidson and Robert P. Millar for their guidance and, financial support through out the project. My thanks also goes to the Department of Chemical Pathology, UCT for allowing me to do the work and to all the members of the Molecular Reproductive Endocrinology Research Unit who made my stay in Cape Town quite enjoyable. I would specially like to thank Bernhard Fromme and Ian Wakefield who apart from being responsible for my newly acquired taste for "braai", "potjie" and Castle lager, many times went out of their way to make sure that I know I am among friends. I also wish to express my heartfelt appreciation and thanks to Mickey and Fre for being my family in Cape Town. Finally my deepest gratitude goes to my wife Selam, my son Zos and my mother Hirut Worku, with out whose love support and encouragement none of this would have been possible.

LIST OF PUBLICATIONS

Publications based on the work described in this thesis and on related works are listed below.

1. Davidson, J.S., Assefa, D., Pawson, A.J., Davis, P., Hapgood, J., Becker, I., Flanagan, C.A, Roeske, R. and Millar, R.P. (1997). Irreversible Activation of the Gonadotropin-Releasing Hormone receptor by photoaffinity cross-linking: Localisation of attachement site to Cys residue in N-terminal segment. Biochemistry **36**: 12881-12889.

2. Forrest-Owen, W., Willars, G.B., Nahorski, S.R., Assefa, D., Davidson, J.S., Hislop, J. and McArdle C.A (1998). The lack of rapid homologus gonadotrophin-releasing hormone (GnRH) receptor desensitization in αT3-1 cells is not due to GnRH receptor reserve or phosphatidylinositol 4,5 bis-phosphate pool size. Molecular and Cellular Endocrinology (In press).

3. Assefa, D., Pawson, A.J., McArdle, C.A., Millar, R.P., Flanagan, C.A., Roeske, R. and Davidson, J.S. (1999). A Gondotopin releasing hormone peptide antagonist with photoreactive group at positon 1 cross-links to the N-terminal domain of the GnRH receptor: implication for antagonist binding (Submitted).

4. Millar, R.P., Assefa, D., Ott, T. Pawson, A.J., Troskie, B., Wakefield I., and Katz, A. (1999). GnRH and GnRH analogs: Structure, actions and clinical applications. Hormone Frontier in Gynecology **5**: 77-83.

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ABBREVIATIONS LIST

2-D-Nal	β-(2-naphthyl)-D-alanine
3-D-Pal	β-(3-pyridyl)-D-alanine
AA	Arachidonic acid
Ac	Acetyl
Atz	Aminotriazolyl
cAMP	Cyclic adenosine 3' 5' monophosphate
DAG	Diacylglycerol
DHP	Dihydropyridine
Dpr	Diaminopropionic acid
EC	Extracellular
FSH	Follicle stimulating hormone
G-proteins	GTP-binding proteins
GPCR	G-protein coupled receptor
GnRH	Gonadotropin-releasing hormone
hGnRH receptor	Human gonadotropin-releasing hormone receptor
IC	Intracellular
IP	Inositol phosphate
IP_3	Inositol trisphosphate
Ipr-Lys	Isopropyl lysin
kDa	Kilo Dalton
LH	Luteinizing hormone
MAPK	Mitogen activated protein kinase
Me-	Methyl
mGnRH	Mammalian gonadotropin-releasing hormone
mGnRH receptor	Mouse Gonadotropin-releasing hormone receptor
NEM	N-ethyl maleimide
Nal	Naphthyl-D-alanine
NMDA	N-methyl-D-Aspartate
Nic-Lys	Nicotyl lysin
PCR	Polymerase chain reaction
PKA	Protein kinase A

PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
TM	Transmembrane
SDS	Sodium dodecylsulfate
sGnRH receptor	Sheep gonadotropin-releasing hormone receptor

Nomenclature for Mutations

Mutations are designated by the original residue followed by the amino acid to which it was mutated. For example $Glu^{301}Gln$ (E301Q) refers to mutation of Glu^{301} to Gln^{301}.

Table (i)

Designation of photoreactive peptides used in this thesis

PAnt-1: [N-Azidobenzoyl-Ac-D-Lys^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-$AlaNH_2{}^{10}$]GnRH

PAnt-1a: [N-Azidosalicyl-Ac-D-Lys^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-$AlaNH_2{}^{10}$]GnRH

PAnt-2: [Ac-D-4-Cl-Phe^1, Ac-D-4-Cl-Phe^2, D-Trp^3, N-Azidobenzoyl- D-Lys^6, D-$AlaNH_2{}^{10}$]GnRH

PAnt-3: [Ac-D-Nal^1, N-Azidobenzoyl-D-Dpr^2, D-Trp^3, D-Arg^6]GnRH

PAnt-3a: [Ac-D-Nal^1, N-Azidosalicyl-D-Dpr^2, D-Trp^3, D-Arg^6]GnRH

PAg-1: [N-Azidobenzoyl-D-Lys^6] GnRH

PAg-2: [D-Ala^6, N-Azidobenzoyl-Orn^8]GnRH

PAg-2a: [D-Ala^6, N-Azidosalicyl-Orn^8]GnRH

PAg-3: [N-Azidobenzoyl-L-Dpr^2, D-Ala^6]GnRH

PAg-4: [N-Azidobenzoyl-L-Dpr^3, D-Ala^6]GnRH

PAg-4a: [N-Azidosalicyl-L-Dpr^3, D-Ala^6]GnRH

Designation of receptor chimeras

In the receptor chimera designation, the letters S and H refer to sheep and human GnRH receptors respectively, and the combination of the letters indicate the type of chimera. The subscript refers to the number of N-terminal amino acid residues contributed by the receptor denoted by the preceding letter. E.g. $S_{32}H$ (N-terminal 32 residues from sheep and the rest from human receptor cDNAs).

CHAPTER ONE

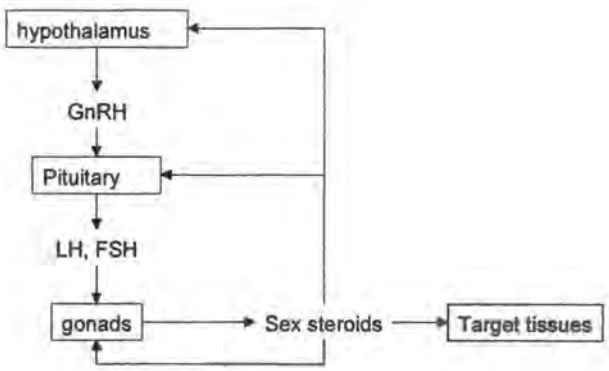
REVIEW OF LITERATURE

1.1 Introduction

1.1.1 Gonadotropin-releasing hormone and its receptor

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in the regulation of reproduction. It is a hypothalamic decapeptide, derived by enzymatic processing from a larger precursor, and is released into the hypophysial portal circulation to be transported to the anterior pituitary where it binds to GnRH receptors on the surface of gonadotrope cells. GnRH receptor activation triggers the release of the two anterior pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Figure 1.1.1). GnRH is secreted by hypothalamic neurones in a pulsatile manner, and each pulse of GnRH is amplified by the pituitary into a pulse of LH and FSH (Nakin and Troen, 1971; Midgley and Jaffe, 1971). A complex set of negative and positive feedback loops of the sex steroids, GnRH and inhibin (McLachan et al. 1989) modulate gonadotropin secretion.

Figure 1.1.1 The hypothalamo-pituitary-gonadal axis



Though the function of GnRH as regulator of LH and FSH release from the pituitary is well understood, it is becoming clear that the peptide plays a variety of other roles, including a neurotransmitter in the central and sympathetic nervous systems, a paracrine regulator in the gonads and placenta, and as an autocrine regulator in tumour cells (King and Millar, 1995, 1997). The evidence for this include (a) the presence of multiple forms of GnRH in the brains of most vertebrate species, (b) expression of GnRH in extra-hypothalamic regions of the nervous system as well as in non-neural tissue, (c) stimulation of the release of other pituitary hormones by GnRH (d) presence of GnRH receptors in extra-pituitary tissues, and (e) effects of GnRH on cells in extra-pituitary tissues.

Neurotransmitter roles of GnRH

Specific binding of GnRH has been shown in several areas of the brain including hippocampus, lateral septal nucleus, anterior cingulate cortex, subiculum, and entorhinal cortex (Stojilkovic and Catt, 1995). The neurotransmitter and neuromodulator role of GnRH is attributed to the chicken GnRH II form which is more prevalent in extra-hypothalamic brain areas and probably in the peripheral nervous system (King and Millar, 1997). For instance, in dissociated bullfrog sympathetic neurones, chicken II GnRH was able to inhibit the M-current at potencies 1000 times that of mammalian, chicken I and lamprey GnRH, suggesting that this peptide may play an endogenous neurotransmitter role mediating the late, slow excitatory post synaptic potential (John, 1987; Troskie *et al.,* 1997a). However, in several vertebrates both chicken GnRH II and the other species-specific GnRH are released in the pituitary and regulate hormone secretion (King and Millar, 1995).

A model of the organisation of GnRH secreting neurones has been proposed in which GnRH neurones have at least two embryonic origins (Muske, 1993). These are the olfactory placode, which gives rise to the terminal nerve-septum-preoptic system, and a second, non-placodal structure, which gives rise to the posterior systems. The two embryonically distinct populations of cells express different molecular forms of GnRH. The posterior neurones express chicken GnRH II which, is an early-evolved form of the peptide while the terminal nerve septum-preoptic neurones express a different

form of GnRH that may vary depending on the species.

Actions of GnRH in peripheral tissues

GnRH is produced in several non-neuronal tissues including the placenta, pancreas, prostate and cells of the immune system (Hsueh and Schaeffer, 1985). Several tumour cell types including breast and pancreatic carcinomas have also been reported to secrete GnRH (Harris *et al.,* 1991; Szende *et al.,* 1991).

GnRH receptors have been described in extra-pituitary tissue including rat ovary (Pieper *et al.,* 1981), testis (Sharpe and Fraser, 1980), placenta (Hsueh and Schaeffer, 1985) and breast tumour cells (Eidne *et al.,* 1985, 1987). The nucleotide sequence of the GnRH receptor expressed in ovarian and breast tissue was shown to be identical to that found in the pituitary (Kakar *et al.*, 1994). However, the functional characteristics of GnRH action in these tissues may be markedly different from that in the pituitary for instance the gonadal actions of GnRH are predominantly inhibitory in nature (Hsueh and Schaeffer, 1985).

1.1.2 GnRH structural variants and their species and tissue distribution

GnRH was first isolated from porcine hypothalamus as the physiologic regulator of gonadotropin release (Matsuo *et al.,* 1971). It was initially considered that there was a single species of the peptide. In 1979 however, other variants from the brains of non-mammalian vertebrates were demonstrated (King and Millar, 1979). In addition, differences in localisation and distinctive distribution of GnRH neurones have been recognised among different species (Halasz *et al.,* 1989). The mammalian GnRH, which was initially isolated from pig hypothalamus, is structurally conserved in the human, sheep, rat and mouse. To date, at least 12 different structural variants of GnRH from mammalian and non-mammalian vertebrates (for reviews see King and Millar, 1995, 1997; Powell, *et al.,* 1996a & b; Sealfon *et al.,* 1997; Jimenez-Linan, *et al.,* 1997) as well as protochordates (Sherwood, 1995, Powell *et al.,* 1996b; Craig *et al.,* 1997,) have been described (Figure 1.1.2). A gene coding for a new form of GnRH ([Trp^8]GnRH) has also been identified in the frog *Rana dubowski* (Kwon, H., University of Korea, unpublished). The peptides are named after the animal from

which they were isolated. In most species, two or more forms of GnRH are present. Chicken GnRH II is the most universally present form and has been conserved through 500 million years of evolution.

Figure 1.1.2 Structures of naturally-occurring GnRH forms: Number at the top of the column indicates position of the residue. The green areas indicate the highly conserved residues.

GnRH	1	2	3	4	5	6	7	8	9	10
Mammalian	PGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH_2
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH_2
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2
Sea Bream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH_2
Guinea Pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH_2
Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH_2
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH_2
Tunicate I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH_2
Tunicate II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH_2

The study of the overall organisation of the GnRH genes of different species suggests that the genes are derived from a common ancestral gene. The gene has apparently undergone a duplication to give rise to an additional protein coding region from which a second form of GnRH has evolved. In most species, two or more forms of GnRH are expressed. The amino acid sequence of GnRH is highly conserved at positions 1, 2, 3 and 4 at the N-terminus and at positions 9 and 10 of the C-terminus. Position 8 is the most variable among the different species followed by positions, 5 and 7 (Figure 1.1.2). The sixth position is conserved in higher vertebrates. In addition to this variation at the gene level, mammalian hydroxyproline GnRH, which is a possible

product of post translational modification, has also been described in rat hypothalamus and frog brain (Gautron *et. al.*, 1992 a & b) and has been shown to have a low gonadotropin releasing activity in mammals (Gautronn *et al.*, 1992) and in birds (R.P Millar and J.A. King, unpublished).

The specificity of the different forms of GnRH within a species may be achieved by anatomic exclusion or inclusion of the peptide in relation to the target tissue. Thus, hypothalamic GnRH is transported via the portal system to the anterior pituitary while gonadal and placental GnRHs are secreted by specific cells to affect adjacent cells in a paracrine fashion. The actions of GnRH subtypes functioning as neurotransmitters or as autocrine regulators would also be locally circumscribed. Although the presence of variant forms of GnRH in a single species suggests the presence of subtypes of the GnRH receptor, a second subtype of the receptor in the same species has not been described. Recently however, evidences for the existance of multiple subtypes of GnRH receptors in vertebrates has been presented (Illing *et al.*, 1999).

Each of the vertebrate GnRH forms is capable of stimulating gonadotropin release in all vertebrate species at sufficient concentration. However, the ligand specificity of the pituitary GnRH receptors differ among species. The mouse GnRH receptor (mGnRH receptor), has evolved a high specificity for mammalian GnRH and binds other forms of GnRH poorly, with the exception of Chicken II GnRH (Millar and King, 1983; Millar *et al.*, 1989). On the other hand, pituitary GnRH receptors of non-mammalian vertebrates are more "promiscuous" while those in sympathetic ganglia are highly specific for chicken GnRH II (Millar and King, 1983; Millar *et al.*, 1986, 1989; Licht *et al.*, 1987; Habibi, *et al,*. 1992).

Differences in the pharmacology of GnRH receptors are particularly marked with GnRH antagonist analogs. For instance analogs which were pure antagonists for the mammalian GnRH receptor were found to be agonists for the avian (Millar and King, 1984; Jacobs *et al,*. 1995, Troskie *et al.*, 1997b), goldfish (Murthy *et al.*, 1993), and frog (Troskie *et al.* 1997b) GnRH receptors.

Post-translational modification of GnRH to the less active hydroxyproline analogue is proposed to have regulatory significance. It is noteworthy that the ratio of hydroxyproline GnRH to mammalian GnRH is high in foetal brain relative to that found in adult brain (Gautron *et al.,* 1992 a & b).

1.1.3 Structural features of the mammalian GnRH receptor[1]

The development of the αT3-1 mouse gonadotrope cell line (Windle *et al.,* 1990), which expresses abundant GnRH receptors coupled to a robust cytosolic calcium signalling response (Merellin *et al.,* 1992), was a major step towards the cloning of the GnRH receptor. The first step was to obtain functional expression of GnRH receptor in Xenopus oocytes by injection of αT3-1 RNA, which gave rise to a measurable electrophysiological response to GnRH (Tsutsumi *et al.,* 1992). In the oocyte, GnRH receptors were evidently functionally coupled to G-proteins, leading to PLC activation, a rise in cytosolic Ca^{2+}, and finally activation of Ca^{2+} activated chloride channels, which was measured by the patch-clamp technique. Antisense oligonucleotides, which inhibited GnRH receptor expression in this system, were used to screen an αT3-1 cell cDNA library, leading finally to identification of a full-length mGnRH receptor cDNA clone. Subsequently, the mouse GnRH receptor sequence was confirmed by Reinhart *et al.,* (1992). This was shortly followed by the cloning of the human (Kakar *et al.,* 1992; Chi *et al.,* 1993), rat (Eidne *et al.,* 1992; Kaiser *et al.,* 1992; Perrin *et al.,* 1993), sheep (Brooks *et al.,* 1993; Illing *et al.,* 1993), bovine (Kakar *et al.,* 1993) and porcine (Weesner and Matteri, 1994) GnRH receptors.

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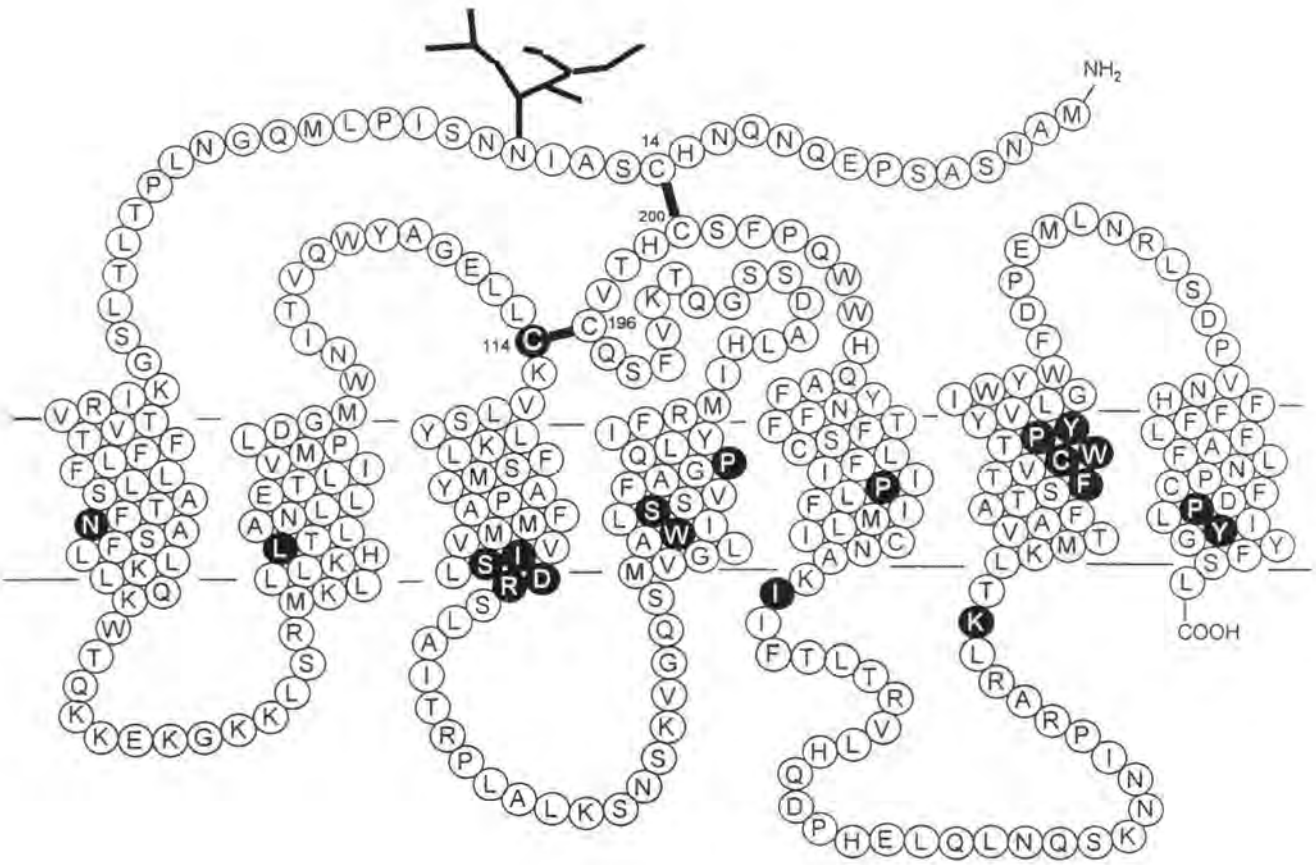
[1] Except where otherwise indicated, numbering of amino acid residues will follow that of the **human** GnRH receptor, throughout this thesis.

Figure 1.1.3 Schematic diagram of the human GnRH receptor: Highly conserved residues among the rhodopsin-like GPCRs are shown in black. The branched structure represents glycosylated site. Thick lines connecting cysteine residues indicate disulfide bridges. Putative α-helical transmembrane domains are indicated.



The putative protein structure predicted from the amino acid sequence of the cDNA indicates that it is a single polypeptide chain containing seven membrane spanning domains (TM1 to TM7) identified by hydrophobicity analysis (Figure 1.1.3). The TM domains are connected by three extracellular (EC1 to EC3) and three intracellular (IC1 to IC3) loops. The amino acid sequence of the GnRH receptor demonstrates the presence of several highly conserved TM residues, indicating that it belongs to the rhodopsin subfamily of G-protein coupled receptors (Baldwin, 1993). The TM domains are believed to be predominantly α-helical and arranged in close approximation with each other, forming a central hydrophilic core (Baldwin, 1993). However, TM1 may in fact be the only truly α-helical TM domain, as all the others contain one or more Pro

residues. The boundaries of these TM domains are unclear.

The sequence of human and sheep GnRH receptors contains two conserved potential N-linked glycosylation sites at Asn^{18} and Asn^{102} (numbering according to the human GnRH receptor), while the rodent receptors have an additional potential site at Asn^{4}. The functional relevance of the carbohydrate moieties attached at these sites will be discussed is section 1.3.4.

The GnRH receptor shares a number of amino acid residues, mostly located in the TM domains, which are highly conserved in the rhodopsin-like family of GPCRs. These include proline residues in TM2, TM4, TM5, TM6 and TM7 ($Pro^{96,173,223,282,316,320}$). Other conserved residues include Asn^{53} (TM1), Arg^{139} (IC2), Trp^{164} (TM4), Ser^{167} (TM4), Asn^{315} (TM7) and Tyr^{323} (TM7). A highly conserved Phe-X-X-Cys-Trp-X-Pro-Tyr motif also exists in TM6. Like other GPCRs, the GnRH receptor has a conserved Cys residue, Cys^{114} at the extracellular end of TM3, which has been shown to connect to a Cys in EC2 in some other GPCRs (Figure 1.1.3).

The mammalian receptors exhibit more than 85% conservation at the amino acid level with respect to each other, and are nearly identical in their transmembrane domains. However, the mammalian GnRH receptor has unique structural features that make it distinct from other GPCRs:

(a) The GnRH receptor is one of the smallest GPCRs with only 327 amino acids in the case of the rat and mouse receptors, and 328 amino acids in other mammalian species.

(b) Mammalian GnRH receptors lack an intracellular C-terminal tail, which is present in all other GPCRs. Recently, GnRH receptors from chicken, frog and goldfish (Troskie *et al.*; 1997b) and from catfish (Tensen *et al.*, 1997) have been cloned which have a more typical structure including a C-terminal tail. The absence of a C-terminal tail in the mammalian GnRH receptors is especially interesting because in other GPCRs, short-term desensitization is mediated by phosphorylation of Ser and Thr residues in

the tail by protein kinases (Hausdorf *et al.*, 1990; Palczewski and Benovic, 1991). Indeed, GnRH stimulated inositol phosphate production in GH_3 cells transiently expressing mouse GnRH receptor and in αT3-1 cells does **not** show a short-term desensitisation (Davidson *et al.*, 1994b; Forrest-Owen *et al.*, in press). This may be in keeping with the pulsatile nature of GnRH secretion and the fact that gonadotropin secretion is frequency and amplitude modulated. Recently, a critical role of the C-terminal tail of the chicken GnRH receptor in mediating agonist promoted internalisation of the receptor has been shown (Pawson *et al.*, 1997). The internalisation of the receptor as measured by following the internalisation rates of receptor bound [His^5,^{125}I-D-Tyr^6]GnRH was 11.3 % min^{-1} for the wild type chicken receptor and 0.7% min^{-1} for wild type human GnRH receptor. A truncated chicken receptor that lacks the C-terminal 38 amino acid residues showed an internalisation rate of 0.5% min^{-1}, which was comparable to that of the human receptor.

It has been suggested that the loss of the C-terminal tail during evolution of the mammalian GnRH receptor was an adaptive change (Pawson *et al.*, 1997). During the ovulatory surge, maximal LH output is required from the pituitary over a sustained period of many minutes, during which time desensitization of the GnRH receptor would be inappropriate. The absence of a tail could be a mechanism to ensure that rapid desensitization and sequestration of the GnRH receptor does not occur during the ovulatory LH surge (Pawson *et al.*, 1997)

(c) Most GPCRs, have a highly conserved "DRY" (Asp-Arg-Tyr) motif at the cytosolic end of TM3. These residues have been shown to play a critical role in coupling to G-proteins in the case of the β_2-adrenergic receptor (Wang *et al.*, 1990), rhodopsin (Franke *et al.*, 1990) and the angiotensin II receptor (Ohyama *et al.*, 1992). In the GnRH receptor, the Tyr in this motif is replaced by a Ser residue (Ser^{141}). This raised the possibility that Ser^{141} could be a phosphorylation site. However, mutation of this residue to the non-phosphorylatable alanine residue did not cause any appreciable effect on GnRH receptor-stimulated IP production (Davidson *et al.*, 1994b). In a related study, conversion of the DRS to the more usual DRY motif caused only a slight increase in agonist affinity with no detectable change in signal transduction (Arora *et*

of the plasma membrane, phosphatidylinositol-4,5-bisphosphate, generating two second messengers: inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1987; Catt *et al.*, 1991). The second stage of the signalling cascade involves the generation of different Ca^{2+} oscillatory patterns, depending on the cell types in which the receptor is expressed and modulation of these responses by various components of the signal transduction apparatus (Stojilkovic *et al.*, 1990; Stojilkovic and Catt, 1995; McArdle *et al.* 1996 a & b; Hille *et al.*, 1995). Ca^{2+} oscillations are observed in primary gonadotropes, whereas the gonadotrope-derived αT3 cell line shows a biphasic spike-and-plateau response to GnRH.

GnRH-stimulated Ca^{2+} oscillations are characterised by the following features:

- the latency (time lag) of the response is inversely related to GnRH concentration
- the frequency of oscillations is dependent on GnRH concentration
- a transient suppression of the oscillatory Ca^{2+} response is seen at high agonist concentration (Hille *et al.*, 1995; Tse *et al.*, 1992, 1994a & b).

Soon after the discovery of IP_3 as a second messenger, it was shown that addition of IP_3 caused a dose-dependent release of calcium from pituitary membrane preparations (Guillemette *et al.*, 1987). More directly, the injection of IP_3 into single cells through a patch-clamp pipette induced Ca^{2+} oscillations similar to those observed during GnRH stimulation in intact cells. This action was found to be independent of the extracelluar Ca^{2+} and was abolished in the presence of the IP_3 receptor blocker heparin (Stojilkovic and Catt, 1995). These findings established that IP_3 release is an early signalling event that occurs after stimulation of GnRH receptors, and that the first Ca^{2+} spiking is due to release of Ca^{2+} from intracellular stores. The Ca^{2+} releasing activity of IP_3 is mediated by the IP_3 "receptor" which bind IP_3 with high affinity and specificity. The IP_3 receptor is now known to be a Ca^{2+} channel located in the endoplasmic reticulum membrane (Berridge, 1993).

The GnRH-induced changes in cytosolic Ca^{2+} involve release of Ca^{2+} from intracellular stores, as well as Ca^{2+} entry via membrane Ca^{2+} channels. At least two types of Ca^{2+}

al., 1995).

(d) There is a reciprocal interchange of the highly conserved TM2 Asp and TM7 Asn residues in other GPCRs. In the GnRH receptor, the TM2 Asp is replaced by an asparagine and the TM7 Asn is replaced by an aspartate residue (Asn^{87} and Asp^{318}). Site-directed mutagenesis studies involving mutation of Asn^{87} to Asp^{87} and Asp^{318} to Asn^{318} (i.e. reversion to the usual GPCR arrangement) have shown that the two residues interact in a manner suggesting close proximity to each other (Sealfon *et al.*, 1993; Zhou *et al.*, 1994). This finding has been of particular importance for the construction of molecular models of the GnRH receptor.

1.1.4 Signalling mechanisms of gonadotropin-releasing hormone

1.1.4.1 Calcium Signalling

Stimulation of primary gonadotropes with GnRH elicits a spike of LH secretion lasting a few minutes, followed by a plateau of sustained LH release (Davidson *et al.*, 1988). Under extracellular Ca^{+2}-deficient conditions, the spike phase of secretion was decreased in magnitude, while the plateau phase secretory response was totally abolished. Furthermore, depletion of intracellular Ca^{2+} stores totally abolished the spike phase as well (Davidson *et al.*, 1988). Thus, both extracellular and intracellular sources of Ca^{2+} are involved in GnRH signalling.

Early studies suggested that the cAMP second messenger system was utilized in stimulus-secretion coupling in the gonadotrope (Borgeat *et al.*, 1972; Bonney and Cunningham, 1977). However, there is now a consensus among many workers that phopholipase-C is the primary effector system utilised by the GnRH receptor in the cascade of post-receptor events (Naor and Catt, 1981; Stojilkovic and Catt, 1995; Kiesel, 1993; Hille *et al.*, 1995). There is also general agreement that pertussis toxin-insensitive G_q/G_{11} subfamily of G-proteins mediate the control of phopholipase-C activity by GnRH in gonadotropes (Hsieh and Martin, 1992).

The binding of GnRH to its receptor results in the G-protein mediated activation of phospholipase-C, which catalyses the breakdown of a minor phospholipid component

channels are activated by GnRH and can be distinguished by their sensitivity to dihydropyridine (DHP) Ca^{2+} channel blockers (Davidson *et al.*, 1988). LH release during the early "spike" phase is insensitive to DHP channel blockers, whereas the plateau phase of LH release involves Ca^{2+} entry via both DHP-sensitive and insensitive channels (Davidson *et al.,* 1988).

In cells showing Ca^{2+} oscillations (primary gonadotropes), plasma membrane electrical potential oscillations involving apamin-sensitive K^+ channels provide a possible mechanism for the activation of DHP-sensitive Ca^{2+} channels (Hille *et al.,* 1995; Stojilkovic and Catt; 1995). In these cells, GnRH induces oscillatory electrical activity in parallel with the oscillatory Ca^{2+} fluctuations (Tse and Hille, 1992). Agonist stimulation in these cells is followed by transitory cessation of AP firing and activation of a complex pattern of changes in membrane potential (V_m), in current-clamped cells or plasma membrane current in voltage-clamped cells. The similarities in $[Ca^{2+}]$ and current / V_m oscillation in terms of their frequencies and patterns indicates that V_m changes are dependent on the rise in the concentration of Ca^{2+} in gonadotropes (Stojilkovic *et al,.* 1992; Kukuljan *et al.,* 1992, 1994). The hyperpolarization that occurs as a result of the rise in Ca^{2+} may serve to re-set the channels so that they are again responsive to depolarization (i.e. remove inactivation).

However, in αT3-1 cells GnRH stimulation is associated with a non-oscillatory rise in $[Ca^{2+}]$, with an amplitude determined by the GnRH concentration (Stojilkovic and Catt, 1992). The mechanism of activation of Ca^{2+} channels in this case remains unclear. In αT3-1 cells, depletion of intracellular Ca^{2+} stores can stimulate nifedipine-insensitive Ca^{2+} influx (McArdle *et al.,* 1996a). This process, called store-dependent-Ca^{2+} influx (SDCI), is widespread in many cell types (Putney, 1992, 1997) and may be the mechanism for activation of the DHP-insensitive Ca^{2+} channels by GnRH.

Store-dependent-Ca^{2+} influx however, can not account for GnRH activation of DHP-sensitive channels (McArdle *et al.,* 1996a). In cells showing non-oscillatory elevations in cytosolic Ca^{2+}, no clear mechanism has yet been proposed for the activation of DHP-sensitive Ca^{2+} channels by GnRH.

1.1.4.2 Protein kinase C (PKC)

GnRH stimulation of gonadotropes is followed by an increase in diacylglycerol (DAG) production, which initiates the other arm of the bifurcating signal transduction system by activating protein kinase C (Nishizuka, 1992). DAG induces the translocation of soluble PKC to the plasma membrane, where the enzyme is activated (Hirota *et al.*, 1984; McArdle and Conn, 1986). In rat primary gonadotropes and αT3-1 cells, GnRH causes a biphasic DAG release (Conricode *et al.*, 1992). There is evidence to show that the two peaks of DAG are derived from two different phospholipid sources. The initial component of DAG is derived from hydrolysis of PIP_2 by PLC, whereas in the sustained phase DAG is generated by phopholipase-D (PLD) mediated cleavage of phosphatidylcholine, which is present in greater amounts in the membrane. Interestingly, PKC itself is involved in the control of the sustained component of DAG release, by activating PLD (Conricode *et al.*, 1992; Zheng *et al.*, 1994). This constitutes a positive-feedback loop by which PKC activation is sustained.

PKC is involved in the regulation of several other processes leading to gonadotropin release, including:

- gonadotropin gene expression (Weck *et al.*, 1998; Park *et al.*, 1997)
- calcium signalling (Naor *et al.*, 1998; Tse *et al.*, 1995)
- exocytosis of secretory granules (Van der Merwe *et al.*, 1990; Davidson *et al.*, 1991; Billiard *et al.*, 1997)

Several subtypes of PKC have been identified in gonadotropes, in particular the α and β-II isoenzymes (Naor, 1990). The roles of the individual isoenzymes in the above processes are not clear, but it is known that they have different properties with respect to their mechanisms of activation and down regulation (Nishizuka., 1995).

1.1.4.3 Cyclic AMP

There is much evidence that cAMP plays a modulatory role in GnRH action. Both early (Borgeat *et al.*, 1972; Bonney and Cunningham, 1977) and more recent (Macrae *et al.*, 1990, Wun *et al.*, 1998) studies have shown that primary pituitary cell cultures

show a rise in cAMP production when stimulated by GnRH. Stimulation of cAMP production by GnRH could be explained by several "crosstalk" pathways e.g. Ca^{2+} stimulated adenylyl cyclase, or PKC-stimulated adenylyl cyclase. It has also been shown that in the gonadotrope-derived αT3-1 cell line the expression of catalytic and regulatory subunits of PKA can be modulated by activation of adenylyl cyclase by forskolin and PKC (Garrel *et al.,* 1997). In the goldfish pituitary, it was concluded that cAMP may not be utilized in GnRH action, but may constitute an independent pathway that interacts positively with GnRH-dependent gonadotropin release (Jobin *et al.,* 1996).

Cyclic AMP is also involved in gonadotropin gene transcription. Melamed *et al.,* (1996) showed that in *Talapia*, the activation of either cAMP-PKA or PKC pathways can, possibly by different mechanisms, increase mRNA levels of Gonadotropin IIβ. This may be mediated by a putative cAMP response element which has been described in the 5'- untranslated sequence at nucleotide position -1490 (Fan *et al.,* 1995).

1.1.4.4 MAP kinase

Stimulation of αT3-1 cells with GnRH agonist was shown to result in activation of MAPK-kinase which was dependent on the activation of PKC and tyrosine kinases (Reiss *et al.,* 1997). This MAPK system may be a possible target for modulation by cAMP as is the case in GT-1 cells.

1.1.4.5 Arachidonic Acid

The role of arachidonic acid (AA) in GnRH action is controversial. GnRH has been shown to stimulate the release of AA in pituitary cells, by a mechanism involving the activation of DAG lipase and phopholipase A_2 (Stojilkovic and Catt, 1995). Arachidonic acid and some of its lipo-oxygenase or epo-oxygenase products have been reported to cause gonadotropin release in cultures of pituitary cells (Naor *et al.,* 1985; Kiesel, 1991), and this action could be mimicked by the exogenous application

of phopholipase A_2. Furthermore, the action of GnRH on LH release was attenuated in the presence of several leukotriene antagonists. In addition, increased synthesis of LTC_4, LTD_4, and LTE_4 as well as 5- and 15- HETE in [^{3}H]AA-prelabelled pituitary cells have been observed upon stimulation with GnRH (Cohen *et al.*, 1992). The effects of the cyclo-oxygenase pathway products however seems to be indirect since prostaglandins do not consistently stimulate LH release, and GnRH-stimulated gonadotropin secretion is not prevented by specific cyclo-oxygenase inhibitor (Kiesel, 1993). Though all this evidence points towards an important role of AA and/or its metabolites in the GnRH signalling cascade, the mechanisms by which these factors cause release of secretory granules remains to be clarified.

It is important to note that AA, at low micromolar concentrations has non-specific detergent-like effects on cell membranes, and studies showing arachidonic acid induced hormone release must be cautiously interpreted. Kaye *et al.* (1990) showed using α-toxin-permeabilized pituitary cells, that arachidonic acid-induced LH release was ATP-independent and N-ethyl maleimide (NEM)-insensitive, indicating that it did not represent true exocytosis. It is therefore possible that several of the previous observations of AA-induced LH release were also artefactual effects.

1.1.4.6 Gonadotropin exocytosis

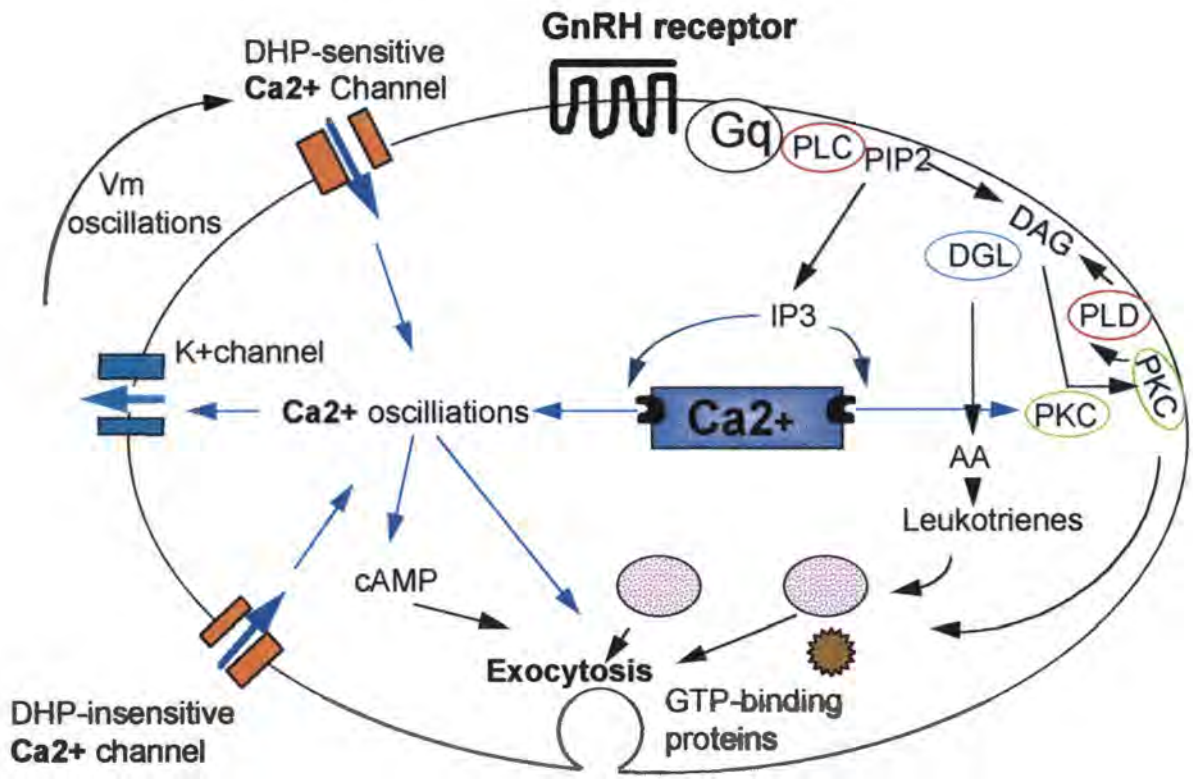
The final stage in the action of GnRH consists of the effects of the intracellular signalling molecules on the exocytotic process. An early observation was that Ca^{2+} and PKC activation are synergistic in their stimulation of LH release (Davidson *et al.*, 1987a, Van der Merwe *et al.*, 1990). Interestingly, Ba^{2+} can substitute for Ca^{2+} at the exocytotic "site" (Davidson *et al.*, 1987b; Smith *et al.*, 1989), and it is also synergistic with PKC activation (Davidson, 1989). Studies in α-toxin permeabilzed pituitary cells, where the level of Ca^{2+} can be directly controlled, have shown that Ca^{2+} and PKC stimulate regulated exocytosis of LH by separate mechanisms (Van der Merwe *et al.*, 1990). Exocytosis was stimulated by free Ca^{2+} at concentrations between 1 and 10 μM. PKC activation by phorbol ester was able to stimulate LH release in the virtually complete absence of Ca^{2+} (free $Ca^{2+} \cong 1$ nM; Van der Merwe *et al.*, 1989). In the α-toxin permeabilized cell system, cAMP also stimulated LH release, and its action was

powerfully synergistic with PKC activation (Macrae *et al.*, 1990).

These studies indicate that LH exocytosis can be triggered either by Ca^{2+} or in a Ca^{2+}-independent manner. Using the α-toxin permeabilized cell model, both pathways were shown to be ATP-dependent, and sensitive to inhibition by N-ethyl maleimide (Davidson *et al.*, 1991).

Complex effects of GTP analogs on LH exocytosis in permeabilized pituitary cells indicated that GTP-binding proteins play multiple roles in the process. The non-hydrolyzable GTP analogue GTPγS showed dual stimulatory and inhibitory effects on LH exocytosis (Van der Merwe *et al.*, 1991). Subsequent analysis of these phenomena indicated that the stimulatory effect of GTPγS was mediated by activation of heterotrimeric G-proteins ($G_{q/11}$ and G_s), while two distinct inhibitory effects of GTPγS could be distinguished, mediated by GTP-binding proteins at a site distal to second messenger generation (Davidson *et al.*, 1992). The involvement of a small GTPase of the Rab family in gonadotropin exocytosis (Hall, 1990) was supported by the finding that a peptide related in sequence to the effector domain of Rab3 caused inhibition of LH (as well as growth hormone) release (Davidson *et al.*, 1993) in streptolysin-O permeabilized cells. Figure 1.1.4 summarises the main signal transduction pathways initiated after the activation of the GnRH receptor by its ligand.

Figure 1.1.4. Schematic representation of the principal elements of the GnRH signalling cascade and exocytosis in gonadotropes: DAG = Diacylglycerol, DGL = Diacylglycerol lipase, DHP = Dihydropyridine, IP3 = Inositol-1,4,5-trisphosphate, PIP2 = Phosphatidylinositol-2,4- bisphosphate, PLC = Phosphopholipase C, PLD = Phosopholipase D, SDCI = Store-dependent Ca^{2+} influx, V_m = Membrane potential.



1.1.5 GnRH secretion

The most dense bed of GnRH-containing neurones is found in the preoptic and septal regions of the hypothalamus. Pulses of gonadotropins from the pituitary gland are the direct result of pulses of GnRH secreted from the median eminence into the hypophyseal portal blood. A one-to-one relationship between spikes of GnRH in portal blood and LH in peripheral blood has been described in sheep (Clarke and Cummins, 1982). This pulsatile GnRH secretion implies the existence of a neural pacemaker. In relation to this, recordings of activity in the mediobasal hypothalamus have shown that neural activity can be correlated with the pulsatile discharges of LH from the pituitary (Thiery and Pelletier, 1981). Interestingly, perifused primary cultures of rat hypothalamic neurones, as well as the GnRH neurone-derived GT-1 cell line, exhibit a

spontaneous episodic GnRH secretion of comparable frequency to that observed with perifused hypothalamus (Krsmanovic *et al.*, 1992).

A number of neuronal systems have been implicated in the regulation of GnRH secretion. Catecholaminergic neurones make synaptic contact with GnRH neurones in the preoptic and other areas of the brain (Nakai *et al.,* 1985). Isolation or de-afferentiation experiments involving the hypothalamus have also suggested that either an extrahypothalamic pathway or the arcuate nucleus could generate pulses, and perhaps one system may function in the absence of the other (Soper and Weick, 1980; Terasawa and Weigand, 1978).

More recently, the role of glutamate in the generation of GnRH pulses has been described. Previously it had been shown that stimulation or inhibition of the N-methyl-D-aspartate (NMDA) subtype glutamate receptor affected GnRH secretion in GT-1 cells (Bourguignon *et al.*, 1992). In addition, anatomical connections between glutamate immunoreactive neurones and GnRH neurones have been shown in the hypothalamus (Bourguignon *et al.,* 1995). Glutamate and its endogenous precursor glutamine, elicited GnRH secretion from hypothalamic explants of male rats. It was also shown that the synthesis of glutaminase increases during physiological states requiring increased gonadotropin secretion (Bourguigon *et al.,* 1995). Hence, the biosynthesis of glutamate from glutamine in the hypothalamus provides a way of recycling glutamate in the brain, a self-regulated mechanism which is known to operate in astroglial cells and glutaminergic neurones (Kvamme, 1983; Albrecht, 1989).

The pulsatility of GnRH is also affected by the opioid peptides (endorphin, met-encephalin, dynorphin), neuropeptide Y (Pau and Spies, 1997) and bradykinin (Shi *et al.*, 1998).

GnRH is also able to modulate its own secretion. In GT-1 neuronal cells, GnRH causes an initial rapid mobilisation of intracellular Ca^{2+}, which is sustained by a further entry of Ca^{2+} from extracellular compartment through voltage-sensitive calcium

channels, accompanied by the secretion of GnRH. This is followed by inhibition of GnRH secretion and then again succeeded by a delayed but prominent phase of GnRH secretion (Krsmanovic *et al.*, 1993). GnRH can also suppresses the K^+ inward rectifier channel in GT-1 cells (Bosma, 1993), an action that can lead to depolarisation of cells and a long-lasting increase in their excitability. This bi-directional effect of GnRH on Ca^{2+} current and secretory response modulates the basal pulsatile pattern, resulting in a decrease in the frequency and an increase in the amplitude of GnRH spikes, with an overall increase in the release of GnRH.

The operation of similar autoregulatory mechanism has been suggested to occur in hypothalamic GnRH neurones (Stojilkovic and Catt, 1995). The effect of intracerebroventricular GnRH infusions on the GnRH pulse generator activity of the hypothalamus was investigated in rhesus monkey. However, no changes in frequency of MUA (multi-unit electrical activity), which are electrical correlates of GnRH pulses were observed, despite significant alterations in LH secretion. These results suggested that, in the rhesus monkey, GnRH might not be involved in the operation of the GnRH pulse generator (Ordog *et al.*, 1997).

1.2 Gonadotropin-releasing hormone analogs

1.2.1 Clinical application of GnRH analogs

The pulsatility of discharge of GnRH is important to its physiological activity. A pulsatile pattern of administration of GnRH can be used to stimulate the reproductive axis. This allows treatment of conditions such as hypogonadotropic hypogonadism and infertility (Millar *et al.*, 1987). In contrast, continuous administration of GnRH or its agonist analogs results in a profound desensitization of gonadotropes (Belchetz *et al.*, 1978; Sandow, 1982, Hsueh *et al.*, 1983). This approach is currently widely used clinically to suppress gonadotropin secretion. The more direct approach of using GnRH antagonists as inhibitors of reproductive function has lagged behind because of the relatively high concentrations of such peptides required and correspondingly greater expense, compared with agonists. The GnRH analogs currently in use clinically are all peptide and require parenteral (intramuscular or subcutaneous) administration.

There are many clinical conditions, which require the suppression of the pituitary-gonadal axis by GnRH agonists or antagonists. These include inhibition of ovulation, pre-menstrual syndrome, menorrhagia, endometriosis, fibromyomata, polycystic ovary syndrome, ovulation induction, precocious puberty and prostatic, premenopausal breast, ovarian and endometrial cancers. The uses of GnRH antagonists to protect spermatogonia from effects of radiation and chemotheraputic drugs, in male contraceptive regimens involving the administration of GnRH antagonist with androgens and in oocyte donation programmes (Sauer *et al.*, 1997) are also being explored. In addition, these peptides are increasingly being used as neurophysiological probes in the studies of the hypothalamic-pitutary-gonadal axis. Extensive discussions on this subject can be found in Jaffe *et al.,* 1993; Mortola, 1993; Parmar *et al.*, 1993; Barbieri, 1992; Moghissi, 1992; Filicori, 1994; Filicori and Flamigini, 1988; Emons and Schally, 1994; and Millar *et al.*, 1987; 1998).

1.2.2 Development of GnRH antagonists

The development of GnRH antagonists was initiated by the recognition that this group of peptides has potential use as contraceptives, and the expectation that they would be relatively free of the side effects of the oestrogen-progesterone contraceptives. GnRH antagonists have the advantages that they act promptly, unlike GnRH agonists which may take several days to 4 weeks to desensitize gonadotropes (Filicori *et al.*, 1988). In addition they do not produce an initial stimulation of gonadotropin release as do agonists. However, the development of GnRH antagonists has been slow compared with agonists. All of the GnRH antagonists so far developed are peptide analogs of GnRH, though there is considerable current activity aimed at developing non-peptide GnRH antagonists.

The first competitive antagonist of GnRH to be discovered was [des-His^2]GnRH (Vale *et al.*,1972). In dispersed rat pituitary cells, this peptide inhibited GnRH-stimulated LH secretion but was not very potent, as concentrations of 1000-10000 times that of GnRH were required. [Phe^2]GnRH was more potent, but had a 2-4% of agonist activity when compared with GnRH while Trp substitution at position two resulted in a partial agonist that maintained 40% of the activity of GnRH. This suggested that

although the deletion of His^2 generated an antagonist, the presence of His^2 is not an absolute requirement for GnRH activity (Grant *et al.*, 1974). Both [des-$pGlu^1$]GnRH and des-[$pGlu^1$, His^2]GnRH showed very low, if any, GnRH agonist or antagonist activity in rat pituitary tissue cultures. Other deletion analogs of GnRH were also tried but des-[His^2] was the only analogue with pure antagonist activity in the absence of any other structural changes (Karten and Rivier, 1986).

The finding that [D-Ala^6]GnRH has a high agonist affinity led to the synthesis of [des-His^2-D-Ala^6]GnRH in an attempt to increase the affinity of the antagonist (Monahan *et al.*,1973). The high potency of [D-Ala^6]GnRH has been attributed to the conformational stabilization of a β-II type bend involving Tyr^5-Gly^6-Leu^7-Arg^8 residues (Monhan *et al.*, 1973; Grant and Vale, 1972, Momany, 1976 a & b, 1978; Kopple, 1981). As predicted, the new analogue had 3 times greater antagonist potency when compared to [des-His^2]GnRH. This was an important turning point since it demonstrated the effect of D-amino acid substitutions in enhancing antagonist potency. [Leu^2, Leu^3] GnRH is the only described full-length antagonist without a D-amino acid substitution and it is of a very low potency in *in vitro* (Humphries *et al.*, 1976).

This was followed by the development of antagonists which combined the weak antagonist property of [Phe^2]GnRH with a D-Ala^6 amino acid substitution. [D-4-F-Phe^2, D-Ala^6]GnRH was the first in a long series of antagonists with a halogenated phenylalanine residue in position 2 (Beattie *et al.*, 1975). The incorporation of multiple aromatic residues at the N-terminal end of the peptide resulted in analogs with increased antagonist potency (Coy *et al.*, 1978; De la Cruz *et al.*, 1976). A member of this group of antagonists [D-Phe^2, D-Trp^3, D-Phe^6]GnRH with three D-amino acid substitutions showed an 83% inhibition of ovulation in rats with a single dose of 1 mg peptide (Rivier and Vale, 1978). Rivier and Vale (1978) synthesized [D-$pGlu^1$-D-Phe^2, D-Trp^3, D-Trp^6]GnRH, an antagonist which had complete antiovulatory activity at low doses (250 μg/rat) and exhibited an *in vitro* potency in molar ratio of 3:1 to GnRH.
The next step in the synthesis of an improved GnRH antagonist came from modifications that increased the hydrophobicity of the peptide. This led to antagonists

with hydrophobic and progressively bulky aromatic D-amino acids at positions 2, 3 and 6. One such analogue [Ac-Δ^3-Pro^1, D-4-Cl-Phe^2, D-Trp^{3,6}]GnRH showed complete inhibition of ovulation in rats at doses of 7.5 μg (Rivier *et al.*, 1981). Further improvement came from incorporation of β-(2-naphthyl)-D-Ala (2-D-Nal) into positions 3 and 6. An example is [Ac-Δ^3-Pro^1, D-4-F-Phe^2, 2-D-Nal^{3,6}]GnRH. This family of antagonists were able to inhibit ovulation completely at doses as low as 2.5 μg/rat .

Introduction of D-AlaNH_2 at position 10 resulted in a further increase in the potency of antagonists (Erchegye *et al.*, 1981). Studies with other substitutions including Ser^{10} and Leu^{10} suggested that perhaps side chain branching at positions 10 hinders receptor binding.

The next goal was to try to improve the solubility of antagonists as the presence of Ac-D-4-Cl-Phe^1 and D-Ala^{10} resulted in very hydrophobic analogs. A very potent analogue [Ac-D-4-Cl-Phe^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-Ala^{10}]GnRH, was developed by the inclusion of Arg at position six (Coy *et al.*, 1982). This led to the development of [Ac-2-D-Nal^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-Ala^{10}]GnRH which was 3 times more potent than the previous compound.

In an attempt to combine basicity, aromaticity and hydrophilicity into a single amino acid, Folkers *et al.* (1983) introduced the heterocyclic amino acids, β-(3-pyridyl)-alanine (3-Pal) and β-(3-quinolyl)-alanine. This led to antagonists such as [Ac-2-D-Nal^1, 4-Cl-D-Phe^2, 3-Pal^3, D-Arg^6, D-Ala^{10}]GnRH which could inhibit ovulation in rats at doses as small as 500 ng.

Folkers *et al.* (1983) suggested that hydrophobic substitutions at position 7 would improve potency. A number of analogs with position 7 substitutions were tried, with the conclusion that hydrophobic substitutions improve potency up to a certain point, but beyond that increasing hydrophobicity has little effect on antagonist potency. Transposition of position 5 and 6 residues has also been tried with variable effects (Roeske *et al.*, 1985).

Alterations of chain length and backbone modification have been tried in order to improve antagonist potency. Most analogs that had longer chain lengths were found to be less potent than the parent compound (Wasiak *et al.*, 1979). A number of psuedo-dipeptide backbone modifications of antagonists have also been made, e.g. replacing the peptide bond with a methylene sulphide (-CH_2S-) bond (Spatola *et al.*, 1981). Replacement at position 6-7 was least active and those at 1-2 or 9-10 showed equal potency with parent substances *in vitro* but no activity *in vivo*. This was attributed to impaired absorption, which may have resulted from decreased solubility. A CH_2HSO (methylene sulfoxide) replacement, while being more polar than -CH_2S- did not improve the situation. Of all the suphur-based amide bond replacements the methyl-substituted chiral bond amide replacement, -$CHCH_3S$-, was the most successful in terms of having high antagonist potency with *in vitro* potencies equivalent to the parent peptide bond analogs. However these analogs were considerably less potent *in vivo* than expected (Spatola *et al.*, 1981).

Because GnRH is thought to adopt a folded conformation with a β-II type bend at positions 6-7 (Monhan *et al.*, 1973; Grant and Vale, 1972; Momany, 1976a & b, 1978; Kopple, 1981), analogs which were constrained in a similar bent conformation might be expected to show high potency. Two strategies for constraining peptides in this way have been applied to GnRH analogs; cyclic peptides and γ-lactam bridges. Of several cyclic GnRH antagonists which have been synthesized, the most potent was [Ac-2D-Nal^1, D-4-Cl-Phe^2, 3-D-Pal^3, Dpr^4, Arg^5, 3-D-Pal^6, Asp^{10}]GnRH (Rivier *et al.*, 1985). The use of γ-lactam conformational constraints has led to the development of potent agonists and antagonists e.g. [Ac-2D-Nal^1, α-Me-D-4-Cl-Phe^2, D-Trp^3, 2-(3'-amino-2'-oxo-1'-pyrrolidino-4-methyl-pentanoic acid^{6,7}, D-Ala^{10}]GnRH (Freidinger *et al.*, 1980).

The use of GnRH antagonist peptides in humans had been complicated by the development of numerous side effects mostly associated with the release of histamine (Nekola *et al.*, 1984; Hahn *et al.*, 1985, Hocart *et al.*, 1988; Morgan *et al.*, 1986; Schmidt *et al.*, 1982). The most potent analogs in triggering histamine release had a structural combination of a basic D-amino acid side chain at position 6 (in close

proximity to Arg^8) and a cluster of hydrophobic aromatic amino acids at the N-terminus.

Recently a novel group of GnRH antagonists have been synthesized and tested. (Rivier *et al.*, 1992, 1995; Campen *et al.*, 1995). These have been based on structural modification of [Ac-2-D-Nal^1, D-4-Cl-Phe^2, 3-D-Pal^3, Nic-Lys^5, D-Nic-Lys^6, Ipr-Lys^8, D-$AlaNH_2$]GnRH (antide). In most instances the $Lys(Ipr)^8$ group was retained while attempts were made to increase the overall hydrophilic character of the antagonist. The Azaline group of compounds are the results of exploration of modifications at position 5 and 6. They are essentially aminotriazolyl (atz) derivatives consisting of antagonists with N-triazolylornithine, -lysine or p-aminophenylalanine residues at positions 5 or 6. The most studied are Azaline A [Ac-D-Nal^1, D-4-Cl-Phe^2, D-Pal^3, Lys^5(atz), D-Lys^6(atz), Ipr-Lys^8, D-Ala^{10}]GnRH and Azaline B [Ac-D-Nal^1, D-4-Cl-Phe^2, D-Pal^3, Aph^5(atz), Ipr-Lys^8, D-Ala^{10}]GnRH (Rivier *et al.*, 1992). These peptides have longer half-life in rats after either subcutaneous or intravenous administration compared to any of the previously tested antagonists. The longer half-life after subcutaneous injection was attributed to slow release from the injection site. Azaline B found to be a more potent antagonist than "antide" which is among the most potent GnRH antagonists. In addition, the histamine release effect of Azaline B as measured by the cutaneous anaphylactoid assay (local wheal response) in rats was shown to be less than that of antide. A similar observation was also made in the guinea pig cardiopulmonary anaphylactoid assay after intravenous administration indicating its higher selectivity (Campen *et al.*, 1995).

The azaline group of antagonists tend to form gels due to formation of beta-sheet structures hence are not readily amenable to formulation for long-term therapy. In an attempt to increase hydrophilicity while maintaining potency and selectivity, a number of substitution analogs were constructed. These include betidamino acids ("beta" "amide") where N'-monoacylated or N'-monoacylated and N-mono or N,N'dialkylated amino glycine derivatives are used to mimic side chains of amino acids or introduce new functionalities. (Jiang *et al.*, 1997). In most cases these resulted in slightly decreased duration of action with increased hydrophilicity (Rivier *et al.*, 1995).

1.2.3 Interactions of GnRH agonists with the GnRH receptor

The identification of the binding sites of GnRH analogs on the GnRH receptor and the description of the three dimensional structure of the ligand receptor complex will enhance the design and development of both peptide and non-peptide analogs. Ideally such studies are made by X-ray crystallography. However, the fact that the GnRH receptor is an integral membrane protein and that it is expressed at low abundance by cells makes this approach particularly difficult. Nevertheless the molecular interaction of the ligand receptor complex can be studied using other methods, such as site-directed mutagenesis (sections 1.2.3 to 1.2.5) and photoaffinitiy labelling (section 1.3)

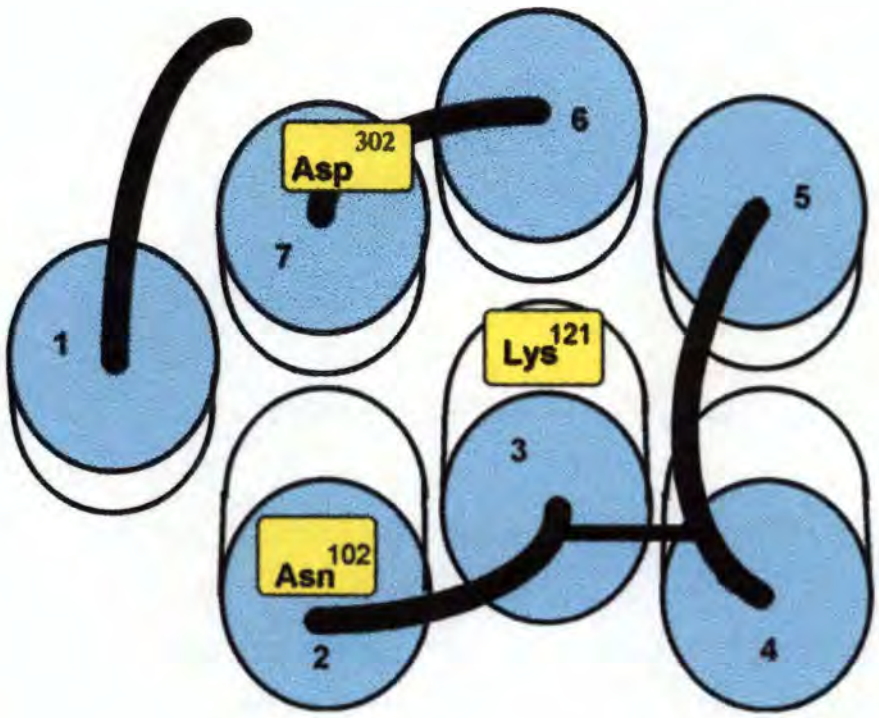
Mammalian GnRH, which has Arg at position 8 (Figure 1.1.2), binds to the mouse GnRH receptor with 100-fold higher affinity than [Gln^8]GnRH (Millar and King, 1983; Millar *et al.*, 1989). Based on this observation, it was hypothesised that Arg^8 of the peptide might interact electrostatically with an acidic residue on the receptor. Flanagan *et al.*, (1994) studied a series of mouse GnRH receptors constructs mutated at acidic residues, and found that the Glu^{301}Gln mutant receptor had lower affinity for analogs possessing an Arg residue in position 8 and maintained its affinity for ligands with a neutral amino acid at this position. This suggested that the Arg^8 residue of mGnRH interacts with Glu^{301} of the mouse receptor (equivalent to Asp^{302} of the human receptor) which is located in the third extracellular loop (Figure 1.1.3). Interestingly the GnRH agonist [des-Gly^{10}, D-Ala^6, Pro^9-ethylamide]GnRH showed a lesser dependence on the presence of an acidic residue at position 301 of the GnRH receptor, than GnRH it self (Flanagan *et al.*, 1994), suggesting that the conformational change produced by the D-Ala^6 residue in some way lessens the importance of this electrostatic interaction.

Lys^{121}, located in TM3 of the GnRH receptor (Figure 1.1.3), is conserved in all GnRH receptors for which sequences are known. This residue corresponds to Asp^{113} of the β-adrenergic receptor (and other adrenergic receptors) which has been shown to be required for a high affinity catecholamine binding (Strader *et al.*, 1988). By introducing a range of mutations at Lys^{121}, it was found that a charge-strengthened hydrogen bond

donor side-chain is required at this locus for high affinity binding of GnRH as well as the GnRH agonist [des-Gly^{10}, D-Ala^{6}, Pro^{9}-ethylamide]GnRH (Zhou *et al.,* 1995). However, Lys^{121} was not critical for binding of the antagonist [Ac-2-D-Nal^{1}, D-α-Me-Cpa, D-Trp^{3}, N-ε-Ipr-Lys^{5}, D-Tyr^{6}, D-Ala^{10}]. As described in the preceding sections, GnRH analogue development has indicated that His^{2} and Trp^{3} are the residues which mediate agonism (i.e. receptor activation), and therefore one of these has been proposed as the residue in GnRH most likely to be involved in the interaction with Lys^{121}. In both cases, a hydrogen bond may be formed by the interaction of Lys^{121} with the electron dense aromatic rings of Trp^{3} or the polar imino group of His^{2} (Zhou *et al.,* 1995).

Early work had shown that the C-terminal glycinamide residue of GnRH was important in receptor binding, as removal of this residue led to a marked loss of potency in GnRH analogs (Karten and Rivier, 1986). Recently, evidence has been obtained indicating that Asn^{102}, located at the top of TM2, may interact with the C-terminal glycinamide. Mutation of Asn^{102}, a residue conserved among mammalian as well as non-mammalian GnRH receptors, to Ala resulted in a much greater loss of affinity for GnRH analogs with C-terminal glycinamide residue (like GnRH) when compared with analogs having ethylamide C-terminus. This finding suggests that Asn^{102} may be involved in the docking of the glycinamide C-terminus of GnRH (Davidson *et al.,* 1996a). The molecular determinants of GnRH binding to its receptor suggested by these site-directed mutagenesis studies are summarised in Figure 1.1.5.

Figure 1.2.1 Schematic representation of the human GnRH receptor, viewed from the extracellular face: The numbered cylinders represent transmembrane helices, while the curved solid lines represent the extracellular interconnecting loops and N-terminal domain (truncated). The short solid line represents the C^{114}-C^{196} disulphide bridge. Site-directed mutagenesis studies have suggested that the residues Asn^{102}, Lys^{121} and Asp^{302}, shown in boxes, are involved in the binding of GnRH.



1.2.4 Interaction of GnRH antagonists with the GnRH receptor

From the kinetic point of view, the mechanism of action of peptide GnRH antagonists is that of classical competitive inhibition. According to this model, peptide antagonists inhibit agonist-mediated GnRH receptor stimulation by preventing access of the agonist to the ligand-binding pocket. Such inhibition can be overcome by a high concentration of agonist.

Possible antagonist binding mechanisms are less constrained than that of agonists, since in the case of antagonists, only binding to the receptor and blocking agonist access is required without stimulating the receptor. This means that the antagonist

and agonist binding sites may be overlapping but they may also have some contact sites, which are different. Because there is no requirement for antagonists to activate the receptor, it is conceivable that antagonists may have a larger selection of specific binding or contact sites on the receptor when compared to that of agonists.

In contrast, non-peptide antagonists may bind to totally different sites, which are non-overlapping with the peptide ligand-binding site. For example, in the neurokinin-1 receptor, the binding sites of non-peptide antagonists were completely different from the peptide-binding site (Fong *et al.,* 1992, 1993; Schwartz, 1994).

A number of studies have characterised the interaction of antagonist peptides with the GnRH receptor. Generally, antagonists have lower rates of dissociation than agonists. Loumaye *et al.* (1984), compared the binding properties of a GnRH agonist, [D-Ala^6, des-Gly^{10}, Pro^9-ethylamide]GnRH and antagonist [N-Ac-D-4-Cl-Phe^{1,2}, D-Trp^3, D-Lys^6, D-Ala^{10}]GnRH. The antagonist, which had a K_a of 8.4 x10^{-9} M^{-1}, displayed half-times for dissociation of 245 min at 4°C and 67 min at 22°C. At 37°C the dissociation half-time was between 30-60 min for the antagonist while it was 3-6 min for the agonist. In addition the dissociation of the antagonist was found to be incomplete and there was a residual fraction of tightly bound antagonist which was proportional to the duration of incubation. This 'irreversible' binding was ascribed to the extremely hydrophobic nature of the peptide (Loumaye *et al.* 1984).

Agonists and antagonists may also show a difference with regard to the effect of ions on receptor binding. For example, inclusion of 140 mM NaCl in the binding medium increased the binding of mGnRH more than the antagonist [N-Ac-D-4-Cl-Phe^{1,2}, D-Trp^3, D-Lys^6, D-AlaNH$_2$^{10}]GnRH. On the other hand, increasing concentration of KCl and LiCl (40 to 120 mM) decreased agonist binding while antagonist binding was not affected (Loumaye *et al.,* 1984).

A comparison of the receptor binding characteristics of two peptides, an agonist [D-Ala^6, N-α-Me-Leu^7, Pro^9-ethylamide]GnRH, and an antagonist [Ac-Δ^3-Pro^1-D-4-F-Phe^2, D-Trp^3, Lys^6]GnRH was made in rat anterior pituitary membrane homogenates (Perrin

et al., 1982). The two peptides had comparable affinity constants (K_a = 3 x 10^{-9} M^{-1}) at equilibrium, with similar association (1.3 x 10^7 min^{-1} M^{-1}) and dissociation (0.015 - 0.018 min^{-1} M^{-1}) constants. However, the number of high affinity sites for the antagonist was slightly higher than for the agonist. In addition, the antagonist also bound to a much larger number of low affinity sites, so that the number of total binding sites for the antagonist was 2.3 fold more than for the agonist. Although some of these sites could be non-specific binding sites, the analysis of the data for the antagonist still showed more than one binding site even after the exclusion of the non-specific binding from the computation.

A related observation in the same study was that, in binding displacement experiments, a small fraction of labelled antagonist was not displaced by high dose of agonist while the antagonist did displace all of the bound labelled agonist. Similar observations were also made with two other labelled antagonists, [Ac-Δ^3-Pro^{4-} -D-4-F-Phe^2, D-$Trp^{2,6}$]GnRH and [Ac-Δ^3-Pro^1, D-4-Cl-Phe^2, D-$Trp^{3,6}$, N-α-Me-Leu^7]GnRH.

These results suggest that, in addition to a site, which overlaps and is competitive with agonist, antagonists may bind to a site on the receptor that is not accessible to the agonist. The authors have suggested that both agonist and the antagonist may bind to the same high affinity sites but to different low affinity sites (Perrin *et al.*, 1982).

Other evidence of differential interaction of agonists and antagonists with the GnRH receptor includes the following observations:

1. N-methylation of Tyr^5 of GnRH antagonists maintains or enhances receptor binding affinity while N-methylation of the same residue in agonists causes a 2-to 10-fold decrease in binding affinity, indicating a difference in contact sites between the two analogs (Haviv *et al.*, 1993).
2. As discussed above (section 1.2.3), Lys^{121} of the GnRH receptor was shown to be important for high affinity binding of an agonist while it was not involved in antagonist binding (Zhou *et al.*, 1995).
3. As will be discussed later (section 1.3.4), differential binding orientations of

agonist and antagonist have been demonstrated using photoreactive analogs (Janovick *et al.*, 1993).

1.2.5 Non-peptide GnRH antagonists

The clinical use of peptide GnRH analogs is hampered by the fact that they must be administered parenterally (intramuscular or subcutaneous) or intranasaly. The development of a non-peptide GnRH analogue that can be taken orally is thus an attractive prospect for clinical application, and a number of pharmaceutical companies are actively pursuing this goal. Some experimental non-peptide compounds have been discovered that can interact with the GnRH receptor, but no non-peptide GnRH agonists or antagonists have yet been made available to the public domain.

Recently, a new non-peptide GnRH receptor antagonist isopropyl 3-(N-benzyl-N-methlaminomethl)-7-(2,6-difluorobenzyl)-4,7-dihydro-2(4-isobutyrylaminophenyl)-4-oxothienol[2,3-b]pyridine-5-carboxylate hydrochloride (T-98475) was shown to be active *in vivo* (Cho *et al.*, 1998). In a competition binding assay where [^{125}I]leuprorelin was used as a tracer, the compound T-98475 exhibited an IC_{50} of 0.2 nm (50 higher affinity than mammalian GnRH). Its binding to other GPCRs was found to be insignificant indicating its specificity to the GnRH receptor. In addition, oral administration to castrated cynomologus monkeys (60mg/kg) exhibited more than 70% inhibition of plasma LH levels 8 hours after administration and the effect lasted for more than 10 hours. The fact that the compound had 20 fold lower affinity to the monkey receptor than to the human makes it likely that a much lower dose will be needed for humans. The mode of interaction of T-98475 with the GnRH receptor is not known although a possible role of a positively charged amino group in an interaction with the EC3 Asp^{302} residue of the GnRH receptor has been suggested (Cho *et al.*, 1998)

1.3 Photoaffinity Labelling.

1.3.1 General considerations

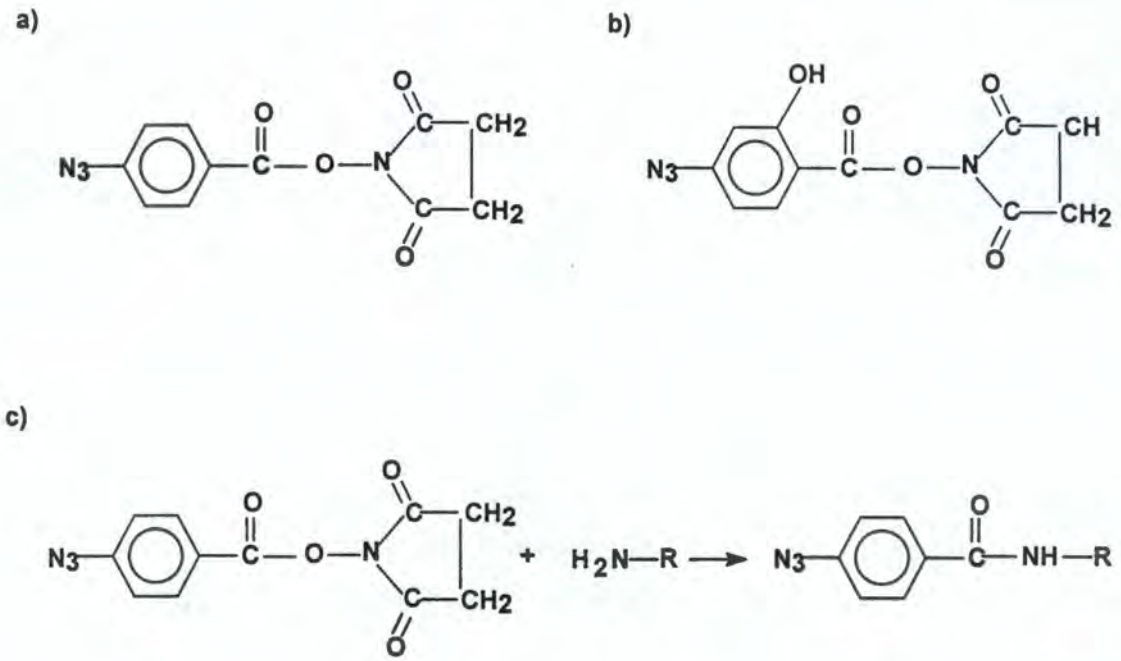
The technique of photoaffinity labelling, first described by Singh *et al.* (1962), provides an important experimental tool for the elucidation of molecular interactions involved in

biological processes. The principle involves the use of a chemically inert but photoreactive ligand which, when activated by light, produces a highly reactive species that binds to the biological receptor irreversibly at the site of interaction. In many instances, radiolabelled photoreactive ligands have allowed the characterization of target proteins on SDS gels. In some cases these experiments led to the identification of the amino acid residues labelled by the photosensitive probes. For example, in the renal V_2 vasopressin receptor, the tritiated ligand was found to be cosslinked to Thr^{102} and Arg^{106} which are conserved residues found in the first extracellular loop (Kojro *et al.*, 1993). The identification of the amino acids at the crosslinking sites provides precise data on the interaction of ligands with their receptors.

The photo activation of photoreactive reagents constitutes a complex set of reactions, the details of which are still unclear in most cases. Some photoactivated reagents rearrange to yield additional reactive species, resulting in multiple different modifications of a biological target. Most photoreactive reagents are based on nitrene or carbene chemistry and the photolabile precursors are azides, diazirines and diazo compounds. Carbenes and nitrenes have essentially the same chemistry, since they are isoelectronic species. Their singlet species are powerful elecrophiles, and interactions with double bonds or with hetero atoms (S, O, N etc.) possessing non-bonding electron pairs are extremely rapid, while in addition they are able to attack aliphatic C-H bonds (Brunner, 1993; Kotzyba-Hibert *et al.*, 1995).

The most widely used photoreactive crosslinking agents are the aryl azides (Figure 1.3.1), which yield nitrene intermediates that are highly reactive and are capable of insertion into all protein amino acid side chains. The price paid for such high reactivity is the fact that these intermediates can also interact with undesired components of membrane e.g. lipids and sugars.

Figure 1.3.1 Chemical structures of photoreactive heterobifunctional aryl azides used in the present study: a) 4-Azidobenzoic acid N-hydroxysuccinimide ester; b) 4-azidosalicylic acid N-hydroxysuccinimide ester; c) reaction of azidobenzoyl hydroxysuccinimide with an amino group.



1.3.2 Incorporation of photoreactive groups into peptides by modification of reactive residues

There are numerous bifunctional crosslinking reagents currently available. A homo-bifunctional crosslinking agent contains two groups of the same kind, while the heterobifunctional reagents contain two different active groups. One group may be photoreactive, while the other is a chemically active group with reactivity towards defined functional groups. This arrangement allows the control of the activity of the two functional groups in a differential manner. For instance, the reagent can first be coupled to a hormone or an enzyme in the dark and then crosslinked to the target receptor molecule by photolysis.

By the choice of the reactive groups and manipulation of the reaction conditions, photoreactive groups can be attached specifically to specific sites in a peptide or protein. An example is shown in Figure 1.3.1c, which shows the reaction scheme for

the coupling of an arylazido group to an amino group using a N-hydroxysuccinimide (NHS) ester as reactive group, as performed in the present study. Other reagents are available for coupling to sulfhydryl (pyridyl disulfides), guanidino (Glyoxals) or indole carboxyl groups (Carbodiimides). Reagents specific for particular functional groups can be useful in evaluation of the location and role of each type of group and for mapping the binding sites within a macromolecule. For peptides lacking suitable residues for attachment of a photoreactive group, analogs with the appropriate substitution must be synthesized. In the case of proteins, a susceptible amino acid residue can be introduced by site-directed mutagenesis. This approach, using the introduction of Cys residues, has been used successfully in the labelling of the E-subunit of the F_1 part of *E.coli* $F_1 F_0$ ATP synthase (Aggeler *et al.*, 1992).

If a cleavable photoreactive reagent is used, the reagent may be selectively removed and the subsequent purification of the native target protein may be facilitated. The most commonly used cleavable bonds introduced in photoreactive reagents are disulphide bonds (Hazum, 1983; Kotzyba-Hibert, 1995). Identification of the crosslinked product is most often achieved by labelling the derivatized photoreactive peptide, or in some cases the photoreactive reagent, with radioactive tracers such as ^{3}[H] or ^{125}[I] (Hazum, 1983).

Aryl azide-derivatized peptides exhibit strong UV absorption (250-350 nm), often with characteristic shoulders on the long wavelength of the peak (400-500). These bands usually disappear or are considerably diminished upon photolysis (Hazum, 1983). The generated arylnitrenes have a half-life of the order of 10^{-2} to 10^{-4} seconds, hence crosslinking reactions are terminated essentially immediately after irradiation. The wavelength, intensity and time of exposure required for crosslinking depend on the particular photoreactive reagent, and are also influenced by the screening effect of the biological preparation being used (Resier *et al.*, 1968). It is usually possible to obtain irradiation conditions, which achieve adequate crosslinking without excessive damage to proteins.

In some cases, individual labelled amino acids have been identified by isolation of

proteolytically derived fragments of the labelled product, followed by sequencing by Edman degradation (Kojro *et al.*, 1993). Problems may arise with this when there is a low molar ratio of labelling or heterogeneity of labelled products.

When photoreactive reagents are coupled to a peptide by way of their interaction with the side chain of an amino acid, the photoreactive group will be at an appreciable distance form the peptide back bone, depending on the chain length of the bifunctional reagent. This may result in crosslinking to a site, which is at some distance form the actual binding site of the ligand. For this reason, when coupling photoreactive groups to peptides, it is advantageous to use photoreactive reagents with short chain lengths.

1.3.3 Incorporation of photoreactive groups during peptide synthesis

Photoreactive groups can also be introduced into synthetic peptides at the time of synthesis. Two such compounds are 4'-trifluromethyl-diazirinyl-o-phenylalanine and 4'-benzoyl-phenylalanine (Bpa) (Brunner, 1993; Dorman and Prestwich, 1994). An example of this method was the use of Bpa in the place of Arg^{27} of atrial natriuretic peptide (ANP), to localize the ligand binding site on the ANP receptor (McNicoll *et al.*, 1996)

Two approaches have been described in which site-directed biosynthetic incorporation of photoreactive amino acids into proteins achieved:

a) **Modification of a natural aminoacyl tRNA:** The high specificity of the aminoacyl tRNA synthase enzyme is a problem when one tries to incorporate an unnatural photoreactive amino acid into a protein sequence. To circumvent, this one can chemically modify an amino acid which is already attached to aminoacyl tRNA. For instance, N-hydroxysuccinimide ester of azidobenzoic acid can be coupled to the ε-amino group of lysyl-tRNA. Thus when this substrate is available the modified amino acid will be incorporated in the protein at sites where lysine would have been incorporated. This has an obvious disadvantage if there are multiple lysine residues in the protein.

b) **Artificial synthesis of a special aminoacyl-tRNA:** This involves the chemical

aminoacylation of the dinucleotide pCpA with an amino acid, and then the T_4 RNA ligase-mediated coupling of this aminoacyl-pCpA to "abbreviated" tRNA which is missing the pCpA dinucleotide at the amino acid acceptor (3') end. This system of protein synthesis utilising chemically "mischarged" tRNA is restricted to *in vitro* systems such as extracts form *E. coli* (Heckler *et al.* 1984; Brunner, 1993).

1.3.4 Previous photoaffinity labelling studies of the GnRH receptor

Photoaffinity labelling has previously been used in the studies of the GnRH receptor. The early studies were done before the receptor had been cloned (in 1992) and no structural information on the receptor was then available. Using a radiolabelled photoreactive GnRH analogue, [^{125}I-Tyr^5-N-Azidobenzoyl-D-Lys^6]GnRH, Hazum (1981a) identified a specifically labelled 60 kDa GnRH receptor candidate protein in rat pituitary cells, in a pioneering study. The labelling was inhibited by unlabelled GnRH analogue Buserelin, while the non-related peptide TRH did not displace it, indicating the specificity of the labelling. In addition, the incorporation of radioactivity in this 60 kDa band was shown to vary across the estrus cycle. Female rats in diestrus and proestrus cycle showed increased labelling compared to that found in metestrus and estrus, reflecting the expected change in GnRH receptor expression. In this study, another labelled band of 48 kDa band was also identified in some preparations (Hazum, 1981, Hazum and Keinan, 1982a).

This work was followed by the demonstration that crosslinking of photoreactive agonist [N-Azidobenzoyl-D-Lys^6]GnRH to GnRH receptors of dispersed pituitary cells resulted in persistent secretion of luteinizing hormone even after extensive washing of the preparation (Hazum, 1983). The efficiency of photoaffinity labelling measured by following the dissociation of photoreactive agonist form the receptor at 37°C was 7%. The low efficiency of photoaffinity labelling in this case was similar to previously described work (Balyel and Knowles, 1977). Though the labelling efficiency was low, the level of LH secretion due to crosslinked ligand was equivalent to a saturating concentration of agonist, demonstrating the presence of spare receptors in the cell preparation.

Interestingly, [N-Azidobenzoyl-D-Lys^6]GnRH had a higher binding affinity than both GnRH and [D-Lys^6]GnRH, and was three to four times more potent than GnRH in terms of LH release from pituitary cell cultures (Hazum and Keinan, 1983). The authors believed the increased potency of the photoreactive analogue was due to both increased affinity for the receptor as well as increased resistance to peptidases. This work demonstrated for the first time that photoaffinity labelling could anchor the ligand to the GnRH receptor in a functionally relevant manner.

In the search for a more efficient photoreactive analogue, another high affinity analogue [2-nitro-azidophenyl sulfenylchloride-D-Lys^6, Pro^9-ethylamide]GnRH was shown to cause prolonged LH secretion from cultured rat pituitary cells (Nikolics *et al.*, 1984). In this instance also, the derivatized peptide was more potent than the parent peptide and than the previously used [N-Azidobenzoyl-D-Lys^6]GnRH, possibly due to the ethylamide substitution. The meta-nitro substitution in the aryl azide may also have facilitated the photolysis of the azide and increased the reactivity of the nitrene formed from the azide.

Using the photoreactive agonist [N-Azidobenzoyl-D-Lys^6, Pro^9-ethylamide]GnRH, Iwashita and Catt (1985) observed numerous labelled bands in pituitary membrane preparations from different species. The major bands were relatively broad bands of 59 kDa (rat), 62 kDa (rabbit), and 60 kDa (mouse), while minor components of between 40 and 43 kDa were also labelled. In bovine and ovine pituitary glands a single band of 42 and 39 kDa, respectively, was observed. Bands with a slight difference in electrophoretic mobility were seen in preparations from rat testis and ovary (Iwashita and Catt, 1985); however these authors did not observe an additional 60 kDa band which was detected in ovary by Hazum and Nimrod (1982). These differences could be due to experimental differences, or could reflect a difference in post-translational modification of the GnRH receptor between the cell types. The efficiency of photolabelling was estimated by acid dissociation and it was found that 50 % of the bound label was crosslinked, a much higher efficiency than reported by Hazum (1983).

A number of studies have also been carried out using photoreactive GnRH antagonists. Photoaffinty labelling of pituitary GnRH receptor with the radioiodinated photoreactive GnRH antagonist [D-pGlu^1, D-Phe^2, D-Trp^3, N-Azidobenzoyl-D-Lys^6]GnRH, resulted in the identification of a single specific band with an apparent molecular weight of 60 kDa (Hazum and Keinan, 1982b). The size of the band was the same as that labelled with photoreactive agonist, suggesting as expected that the photoreactive agonists and antagonists bind to the same receptor. The photoreactive antagonist retained its antagonist activity and bound to the receptor with an apparent higher affinity than GnRH a fact, which was suggested to be due to a greater resistance of the photoreactive derivative to peptidase activity than the native GnRH.

Janovick *et al.* (1993), reported a study comparing the labelling of GnRH receptors from rat pituitary membranes with a photoreactive agonist [^{125}I-Tyr^5-N-Azidobenzoyl-D-Lys^6, Pro^9-ethylamide]GnRH and a photoreactive antagonist [N-Ac-D-2-Nal^1, D-4-Cl-Phe^2, D-3-Pal^3, ^{125}I-N-Me-Tyr^5, N-Azidobenzoyl-D-Lys^6, Ipr-Lys^8, D-AlaNH$_2$^{10}]GnRH. When crosslinked receptors were digested with trypsin and the resulting fragmentsanalysed by SDS-PAGE, different fragments were obtained with the two analogs, suggesting that the orientation of the two analogs on the receptor protein was different.

The effect of attaching the photoreactive group at varying positions in the GnRH molecule was studied by Nikolics *et al.* (1984). In that study, a [2-nitro-4 (5)-azidophenylsulfenyl] group was introduced at positions 1, 3, 6, or 8 of the peptide and LH releasing activity of the peptides were measured. The Trp^3 modified peptides showed a complete loss of agonist activity while the peptide labelled at position 6 retained potent agonist activity. [2-nitro-4-azidohenylsulfenyl-Orn^8]GnRH, modified at position 8, retained only 7% of GnRH potency. Interestingly the [2, nitro-5-azidophenylsulfenyl-Orn^8]GnRH which has equipotent activity with [2, nitro-4-azidophenylsulfenyl-Orn^8] GnRH did not produce prolonged activation of LH release after photoaffinity labelling, implying the requirement for specific orientation of the photoreactive group in order to successfully crosslink with the receptor.

Photoaffinity labelling has also been used in the study of glycosylation of the GnRH receptor (Davidson *et al.*, 1995). In this study, Asn residues located in three N-glycosylation consensus sequences in the mouse GnRH receptor were mutated to Gln in order to identify the sites of glycosylation. COS-1 cells expressing these mutated receptors were labelled with [N-Azidobenzoyl-D-Lys^{6}]GnRH. SDS-PAGE analysis showed that the Gln^{4} and Gln^{18} mutant receptors migrated with smaller apparent molecular weight than the wild type receptor, demonstrating that Asn^{4} and Asn^{18} were in fact utilized for glycosylation. Interestingly, Asn^{102} was not glycosylated, demonstrating that the consensus sequence (N-X-S/T) is necessary but is not sufficient for glycosylation to occur. The non-glycosylated mutants had normal binding affinity, but decreased expression, indicating that glycosylation may have a role in GnRH receptor stability, folding or membrane insertion, rather than in ligand binding. This was supported by a reciprocal study where the introduction of a second glycosylation site in the human receptor, mimicking the mouse glycosylation sites, resulted in the increased expression of the human receptor (Davidson *et al.*, 1996a).

Photoreactive GnRH analogs have also been used in the study of ligand distribution on the surface of the gonadotrope and ligand internalization (Hazum *et al.*, 1982; 1983; Hazum, 1983; Hazum and Keinan, 1984). Electronmicroscopic autoradiography of GnRH receptors on rat pituitary cells that had been crosslinked with a photoreactive agonist showed that 25% of the bound hormone was internalized at 37°C in 15 minutes and after 45 min only 50% remained on the surface. The rest was localised to lysosome-like structures, secretory granules and the golgi complex (Hazum *et al.*, 1982).

Recently, using a series of mutant GnRH receptors we have been able to localize the crosslinking site of the GnRH photoreactive agonist [N-Azidobenzoyl-D-Lys^{6}] GnRH to Cys^{14} in the N-terminal portion of the GnRH receptor. In addition, by analyzing proteolytic receptor fragments under reducing and non-reducing conditions we were able to determine the presence of a disulfide bridge between Cys^{114} and Cys^{196} (Davidson *et al.*, 1997).

1.4 Aim of the thesis

The aim of the present thesis was to try to define the structural determinants on the GnRH receptor that are involved in the recognition of GnRH antagonists. Two approaches were used: a) studying the binding characteristics of selected antagonists to wild type and chimeric GnRH receptors, followed by site directed mutagenesis of selected residues in order to identify residues that are critical for ligand binding and b) developing novel photoreactive GnRH analogs and trying to define crosslinking site by peptide mapping;

CHAPTER TWO

MATERIALS AND METHODS

In this chapter, general materials and methods used through out most of the work are described. Specialised methods that particularly pertain to work described in chapters three and four are elaborated in the respective chapters.

2.1 GnRH analogs

2.1.1 GnRH antagonists and antagonists: GnRH receptor antagonists used in this study are listed below (Table 2.1.1). All antagonist peptides except antide were synthesized by Dr. Roger Roeske (Department of Biochemistry and Molecular Biology, Indiana University, School of Medicine, Indiana, USA). Antide was synthesized by Dr. Karl Folkers (Institute for Biochemical studies, University of Texas, Austin, USA) and FE 101177 (Figure 2.1.1) was a gift from Ferring Research Limited (Southampton, UK).

The GnRH agonists, mammalian GnRH, [His^5, D-Tyr^6]GnRH, [D-Trp^6, Pro^9-ethylamide]GnRH and D-Ala^6, N-Me-Leu^7, Pro^9-ethylamide]GnRH were all synthesized by R.C.deL. Milton (MRC, Molecular Reproductive Endocrinology Research Unit, University of Cape Town, Cape Town, South Africa).

Figure 2.1.1 The chemical structure of the non-peptide GnRH antagonist FE 101177: (published with permission from Ferring Research Limited).

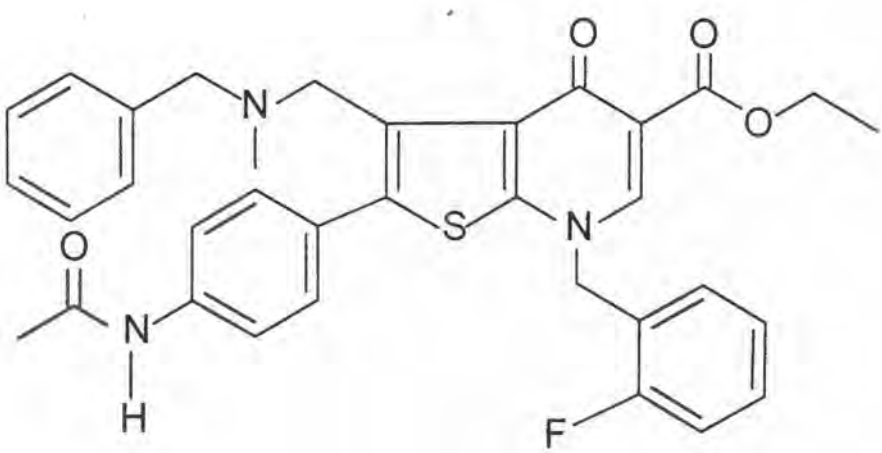


Table 2.1.1 List of GnRH agonists and antagonists used: Amino acid sequences are expressed in relation to that of mammalian GnRH. Where available, code names used in the MRC, Molecular Reproductive Endocrinology Research Unit (University of Cape Town) or by producers of the compound are indicated.

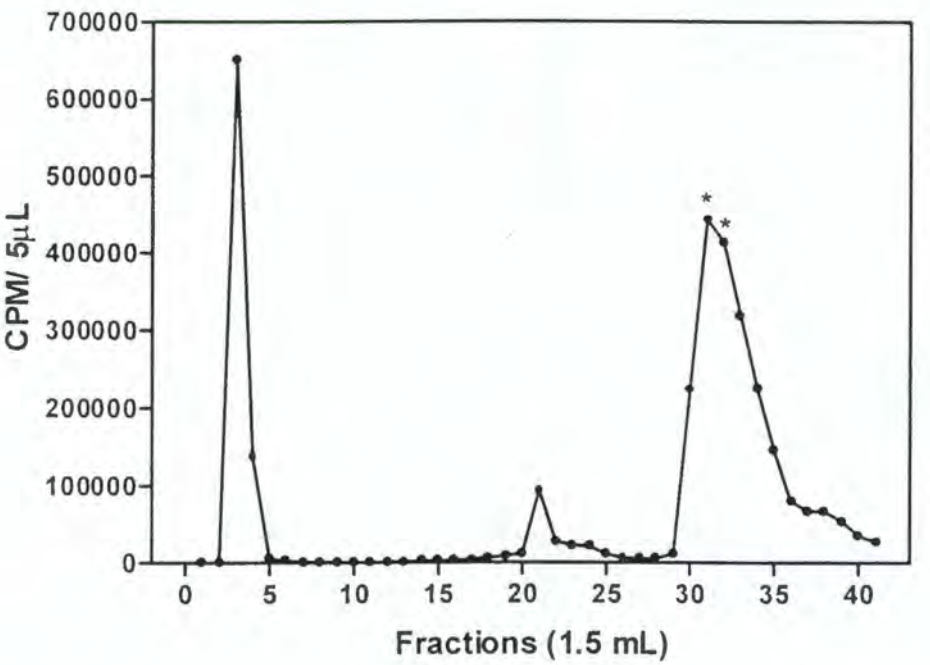
Name	*Sequence*
Antagonists	
Antagonist 137/32	[Ac-3-Pro^{1}, D-4-F-Phe^{2}, 2-D-Nal^{3}, D-Ipr-Lys^{6}, Ipr-Lys^{8}]GnRH
Antagonist 13	[2-Ac-D-Nal^{1}, D-Me-4-Cl-Phe, 3-D-Pal^{3}, D-Arg^{6}, D-AlaNH$_2$^{10}]GnRH
Antide	[2-Ac-D-Nal^{1}, D-4-Cl-Phe^{2}, 3-D-Pal^{3}, Nic-Lys^{5}, D-Nic-Lys^{6}, Ipr-Lys^{8}, D-AlaNH$_2$^{10}]GnRH
Antagonist 197/52	[Ac-D-Nal^{1}, D-4-Cl-Phe^{2}, D-Pal^{3}, D-Lys(3-pAc)^{6}, Ipr-Lys^{8}, D-AlaNH$_2$^{10}]GnRH
Antagonist 133/63	[2-Ac-D-Nal^{1}, D-4-Cl-Phe^{2}, 3-D-Pal^{3}, 1-Me-Pal^{5}, D-Trp^{6}, D-AlaNH$_2$^{10}]GnRH
FE 101177	See Figure 2.1.1
Agonists	
Mammalian GnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$
-	[His^{5}, D-Tyr^{6}]GnRH
-	[D-Trp^{6}-Pro^{9}-ethylamide]GnRH
-	[D-Ala^{6}, N-Me-Leu^{7}, Pro^{9}-ethylamide]GnRH

2.1.2 Iodination of peptides

The peptides were iodinated with Na^{125}I (Amersham) using chloramine T (Millar *et al.*, 1984). Five μg of peptide was dissolved in 40 μL of 0.5 M phosphate buffer (section 2.5) or in 50 μL of 0.1 M phosphate buffer containing 20% methanol. To this, 1mCi (10μL) of Na^{125}I was added, followed by the addition of 85 nmoles of chloramine T in 10μL 0.5 M phosphate buffer. After 10 seconds, 84 nmoles of sodium metabisulphite in 20μL of 0.5 M phosphate buffer was added, the solution was immediately injected into a C-18 reverse-phase HPLC column, and samples were eluted with 0-80% linear gradient of acetonitrile in 0.01M ammonium acetate (pH 4.6). 1.5 mL fractions were

collected and those with the highest radioactivity count were stored at -70°C. Radioiodinated peptides were generally used within 3 weeks of iodination. Figure 2.14 shows the HPLC elution profile of [His^5, ^{125}I-D-Tyr^6]GnRH which was used as a radiolabelled tracer in subsequent receptor binding studies.

Figure 2.1.2 HPLC purification of [His^5, ^{125}I-D-Tyr^6]GnRH: [His^5, ^{125}I-D-Tyr^6]GnRH radioiodinated as described in section 2.1.2 was purified using a C-18 reverse phase HPLC column and eluting with a linear gradient (0-80%) of acetonitrile/0.01M ammonium acetate (pH4.6) solution. The first peak represents free ^{125}I, and fractions marked (*) were used for receptor binding studies.



2.2 Cell culture and transfection

2.2.1 Cell culture: COS-1 and αT3-1 cells (American Type culture collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal calf serum (Highveld, Johannesburg, South Africa) in a 10% CO_2 atmosphere. Poly-D-Lysine coating was performed by adding 250 µl of sterile aqueous solution of 120 µg/L poly D-Lysine (Sigma) per well in 12-well plates, or 1 mL per 10 cm dish and incubating at room temperature for 10 minutes. The solution was then removed and the plates/dishes were washed twice with 5 volumes of distilled water and allowed to dry under sterile conditions.

2.2.2 Transfection of cells: Cells were seeded one day before transfection either into 12 well plates 2 x 10^5 cells per well or 10 cm dishes (3 x 10^6 cells per dish). Cells were transfected by a modification of the DEAE-dextran method (Keown *et al.*, 1990). The cells were washed with Hepes buffered DMEM (H-DMEM) pH 7.4, and incubated with 0.5 mL/well or 4 mL/dish of serum-free DMEM containing 2.5 μg plasmid DNA/well or 15 μg plasmid DNA/dish and 0.3 mg/mL DEAE-dextran for 4 hours at 37°C under 10% CO_2. This was replaced with DMEM containing 2% fetal calf serum and 150 μM chloroquine and incubation was continued for 1 hour at 37°C. After washing once, cells were incubated for 2 minutes in 0.5 mL of H-DMEM with 10% DMSO. Finally the cells were washed with H-DMEM twice and cultured in DMEM with 10% fetal calf serum with penicillin and streptomycin for 48 hours before inositol phosphate assays, photoaffinity labelling or receptor binding experiments.

2.3 Site-directed mutagenesis of the GnRH receptor

2.3.1 Site directed mutagenesis: The construction of GnRH receptor cDNAs with mutations at acidic residues (Flanagan *et al.*, 1994) has been previously described. h F225L receptor was constructed by David Myburgh (MRC, Molecular Reproductive Endocrinology Research Unit). A modified human GnRH receptor cDNA (Davidson *et al.*, 1996) cloned into the *Eco*RV site of the phagemid pcDNA1/Amp (Invitrogen) was used for the N19E mutation. This human GnRH receptor cDNA has an additional glycosylation site that enhances its expression in COS-1 cells and the cDNA is shortened by removal of 1.3 kilobase of the 3' untranslated sequence from the original cloned cDNA (Chi *et al.*, 1993). Oligonucleotide-directed mutagenesis was performed using the method of Kunkel *et al.*. (1987). After passage through *E. Coli* dut$^-$ung$^-$F' strain CJ236, uridine containing single-stranded DNA template was prepared using VCS M13 helper phage (Stratagene). A mutant oligonucleotide 5'-CAGCCATCAACGAGAGTATACCACTGAT G-3' was designed to encode the amino acid substitution N19E, as well as a silent *Acc*1 restriction site. The oligonucleotide was phosphorylated by T4 polynucleotide kinase (Promega), hybridised with the template, and second strand synthesis was completed using T7 DNA polymerase (Biorad) and T4 ligase (Biorad). Products of the reaction were used to transform competent cells of XL-1 blue or DH10B *E. coli* strains. Plasmid DNA was prepared

from ampicillin-resistant colonies using Qiagen columns and was digested with *Acc*I. One of three clones showing the additional restriction site was sequenced to confirm the N19E mutation. Table 2.3.1 shows mutant receptors constructed using similar methods using wild type human GnRH receptor and the primers used in their synthesis.

Table 2.3.1 List of mutant human GnRH receptors constructed by the method of Kunkel: The mutated amino acids and primers used for the introduction of the mutations are indicated. Restriction enzymes used in detecting mutant receptors are given in parenthesis.

Mutant GnRH receptor	*Primer*
hN19E	5'-CAGCCATCAACGAGAGTATACCACTGATG-3' (*Acc*I)
hL78V	5'-CTCTCAAGAATGAAGGTACTCTTAAAACATCTGACC-3' (*Rsa*I)
hR179G	5'-GACCACAGTTGTACATCTTCGGGATGATTC-3' (*Bsr*GI)
h(V160I,I165L,V169I)	5'GGACAGTCCATGATTGGCCTGGCCTGGCTCCTCAGTAGTATCTTTGCAGGACCAC-3' (*Bsr*GI)
h(A50T,T51I,A54T)	5'-CTTCCTTTTTCTGCTGTCGACGATCTTTAATACTCTTTCTTGTTG-3' (*Sal*I)

2.3.2 Restriction enzyme digestion: Purified plasmid preparations containing the cloned DNA of interest or PCR products were incubated with the appropriate restriction enzymes at 2 units /μg of DNA for one hour at a temperature recommended for the optimal activity of the enzyme. In cases where restriction enzyme digestion was carried out to expose sites for subsequent ligation, a second aliquot of enzyme was added after the second hour and digestion was allowed to proceed overnight. Samples containing 0.5 μg of DNA digests were analysed by 1 % agarose Gel electrophoresis. Gel was pre-stained with 0.5 μg/mL of ethidium bromide and stained

DNA fragments were visualised under UV light.

2.3.3 Transformation of competent cells: 200 ul of competent XL-1 Blue or DH10B *E. coli* strains were mixed with GnRH receptor cloned into pcDNA1/Amp vector at the concentration of 20 ng of plasmid/10μL of competent cell culture suspension. The mixture was put on ice for 30 minutes followed by 1 minute at 37°C. After a second cooling on ice for 5 minutes, 800 μL of 2YT medium was added and then incubated at 37° for 1 hour. 100 μL of this was inoculated on Luria-Bertani/agar plate containing ampicillin. Single colonies were picked after overnight incubation and used to initiate subsequent cultures.

2.3.4 Plasmid Preparation: A single colony of bacteria containing the plasmid of interest was used to inoculate 10 mL 2YT medium supplemented with ampicillin (0.1mg/mL). The bacteria were grown 8-10 hours, in a rotary shaker at 200 rpm, at 37° C after which the culture was added to 500 mL 2YT medium and grown for a further 16 hours. Bacterial cells were harvested by centrifuging at 6000 x g in Beckman J-6B centrifuge for 20 minutes. The plasmids were purified using Nucleobond AX Plasmid purification kits (Macherey-Nagel) according to the manufacturer's instructions. The purified DNA was finally dissolved in TE buffer (section 2.5) and quantitated by measuting absorbance at 260 nm. Protein content of the preparation was also estimated by measuring absorbance at 280 nm. The ratio of the absorbance at 260 nm to that at 280 nm was greater than 1.8 in all preparations.

2.3.5 DNA sequencing: DNA was sequenced manually by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase 2.0 DNA (United States Biochemical Corporation) or SequiTherm EXCEL™ II (Epicentre Technologies) according to manufacturer's instructions or by an automatic sequencer (model 373A, Applied Biosystems).

2.4 Receptor binding and inositol phosphate assays

2.4.1 Radioligand binding

2.4.1.1 Membrane preparations: αT3-1 cells were homogenised in binding buffer and centrifuged at 15000 x g for 30 minutes at 4°C. The pellet was resuspended in binding buffer with 0.1% w/v BSA and incubated at (1.5 x 10^5 cell equivalents/tube) with 10^5 c.p.m. (specific activity of 1500 c.p.m./ fmol) [His^5, ^{125}I-D-Tyr^6]GnRH (Flanagan *et al.*, 1998) (Figure 2.1.2) and varying concentrations of unlabelled PAnt-1 for 5 hours on ice in the dark. The incubation was terminated by addition of 3 mL of ice cold 0.01% v/v polyethylenimine (PEI) and immediate filtration through glass fibre filter (GF/C, Whatman) pre-soaked in 1% PEI solution. The filters were washed twice with 0.01% PEI solution and retained radioactivity was quantitated in a γ- counter (Riastar, Packard). Nonspecific binding was determined in the presence of 1 μM unlabelled [D-Trp^6, Pro^9-ethylamide]GnRH.

2.4.1.2 Intact cell preparations

a) To measure the loss of binding sites following crosslinking with photoreactive GnRH analogs, αT3-1 cells in 100 mm dishes were incubated with or without 100 nM of photoreactive ligand at 22° C for 30 minutes in the dark. Excess medium was removed and cells were UV irradiated as described above and washed three times with 10 mL buffer C (section 2.5) at 37° C. Cells were scraped off, resuspended in buffer C at concentration of 3.25 x 10^5 /tube, and incubated with 10^5 c.p.m. (specific activity of 1500 c.p.m./fmol) of [^{125}I-Tyr^5, D-Ala^6, N-Me-Leu^7, Pro^9-ethylamide]GnRH for 1hour at 4°C, followed by filtration as described above.

b) COS-1 cells transfected with GnRH receptor cDNA or αT3-1 cells, plated out in 12 well plates were incubated in buffer C with [His^5, ^{125}I-D-Tyr^6]GnRH (10^5 c.p.m/well with specific activity of 1500 c.p.m./ fmol) (Figure 2.1.4) for 5 hours at 4°C in the presence of different concentrations of competing unlabelled ligand. At the end of the incubation the dishes were washed 3 times with 1 mL/well of buffer D. The cells were then solubilized with 0.5 mL of 0.1M NaOH and radioactivity was counted. Where unlabelled photoreactive ligand was used to inactivate receptors, cells were pre-incubated with 100 - 200 nM of the photoreactive ligand and UV irradiated for 30

seconds followed by three washes of 5 minutes each with 1 mL of buffer B before additions of radioligand.

2.4.2 Inositol phosphate production: αT3-1 cells were labelled for 20 hours with 2 μCi/mL myo-[2-^{3}H] inositol (Amersham) in 0.5 mL/well Medium 199 (Gibco) containing 2% fetal calf serum. After washing twice with buffer A (section 2.5) cells were incubated with GnRH and PAnt-1 as indicated for 10 minutes in the presence of 10 mM LiCl. Where indicated, cells were pre-incubated with 100-200nM photoreactive ligand for 30 minutes at 37°C and then UV irradiated using a Spectroline TR-312 A UV light box at a distance of 4 cm for 60 seconds, followed by removal of excess ligand by washing three times with buffer A (section 2.5). The assay in COS-1 cells transfected with a GnRH cDNA was carried out in exactly the same manner as above but the stimulation with GnRH was for one hour. Incubations were stopped with 500 μL of perchloric acid stopping solution (PCA) and 50μL of 2 mM phytic acid solution. Total inositol phosphate was extracted by letting the plates stand for 10 minutes at room temprature. The solution was then removed from the cells and added into tubes containing 760 μL of 0.5 M KCL. After 30 minutes at 4°C the solution was chromatographed on Dowex-50W columns as described (Davidson *et al.*, 1990), and radioactivity counted. Breifly 1 mL of sample was applied to the column and washed with 10 mL of water followed by 5 mL of 5 mM Myo-inositol /0.1 M Formic acid solution and eluted with 3 mL of 1M ammonium formate/0.1 M Formic acid solution. The eluent was mixed with 14 mL of scintillation fluid (Zinsser Analytic) and radioactivity was counted.

2.5 Buffers and solutions

a) Buffer A :

140mM NaCl, 4 mM KCl, 20 mM hepes, 0.1 % bovine serum albumin, 8.3 mM glucose, 1mM $CaCl_2$,, 1mM $MgCl_2$, 6 mg/L phenol red, pH 7.4.

b) Buffer B:

140mM NaCl, 4 mM KCL, 20 mM hepes, 8.3 mM glucose, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$, pH 7.4.

c) Buffer C:

140mM NaCl, 4 mM KCl, 20 mM hepes, 8.3 mM glucose, 0.5mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% bovine serum albumin, pH 7.4.

d) Buffer D:

140mM NaCl, 4 mM KCl, 20 mM hepes, 8.3 mM glucose, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5% bovine serum albumin, pH 7.4.

e) Binding buffer:

10mM hepes, 1mM EDTA, pH 7.4.

f) Phosphate buffer:

0.5 M NaH_2PO_4, pH 7.6.

g) TE buffer:

10 mM Tris/HCl, 1mM EDTA, pH 8.

h) 2x SDS sample loading buffer:

1M Tris/HCl, 4% SDS w/v, 20% glycerol v/v, 10%v/v 2-mercaptoethanol.

i) SDS/PAGE fixer solution:

40 % methanol (v/v), 10 % TCA (w/v) in water.

j) Coomassie blue dye solution:

0.1 % coomassie brilliant blue, 40% methanol (v/v), 10% acetic acid (v/v) in water.

k) Destaining solution:

30% ethanol (v/v), 10% acetic acid (v/v), in water.

l) PCA stopping solution:

4.5 mM EDTA, 4.8 mM DTPA (Diethylenetraminepentaacetic acid), 3.6% (v/v) perchloric acid (v/v) in water.

2.6 Data Reduction: Peptide concentrations required to stimulate half-maximal IP production (EC_{50}) and to half-maximally inhibit binding of radioligand (IC_{50}) were estimated by non-linear regression using Graphpad prism (Graphpad software incorporated). For the determination of EC_{50} the following equation was used:

$Y= a + (b-a)/ [1+10^{\wedge} Log (EC_{50} - X)]$ where Y= Response, X= log concentration of agonist, a = minimum value and b= maximum value. For the determination of IC_{50}, the equation $Y= a + (b - a)/ [1 + 10^{\wedge}(X- Log IC_{50})]$ where Y= Bound c.p.m, X = log concentration of competing ligand, a= minimum value and b= maximum value, was

used. Binding constant (K_i) were calculated from inositol phosphate stimulation data by the equation EC_{50}'/EC_{50} - 1= [Antagonist]/K_i where EC_{50} and EC_{50}' are EC_{50} of agonist with and without the presence of a fixed concentration of antagonist respectively (Arunkakshana and Schild, 1947), and K_i = K_i of the antagonist (Leslie, 1987). K_i values were also obtained independently from binding assay data using the Cheng-Prusoff correction (Cheng and Prusoff, 1973). Statistical tests were performed using *student's t*-test.

CHAPTER THREE

DIFFERENTIAL ACTIVITIES OF PEPTIDE AND NON-PEPTIDE ANTAGONISTS IN MAMMALIAN GnRH RECEPTORS

3.1 Introduction

Previous work on GnRH analogs has revealed that GnRH antagonists may behave in different manners towards GnRH receptors in different species. The peptide antagonist 135/18 (Ac-D-Nal(2)-D-4-Cl-Phe-D-Pal-Ser-Ile-D-Ipr-Lys-Leu-Ipr-Lys-Pro-D-AlaNH_2), for example, was shown to be a full agonist in the avian GnRH receptor while it behaved as a full antagonist in the mammalian GnRH receptor (Jacobs *et al.*, 1995). The study of such gross differences in the behaviour of a ligand towards receptors that are structurally related may be used to analyse the structural determinants on the receptor that are responsible for such phenomenon. These comparative studies may also provide insight into the mechanism of receptor activation.

In this study, an attempt is made to identify an antagonist exhibiting a large difference in potency towards three related GnRH receptors and then use this difference for the mapping of the regions on the receptor that are responsible for this differential potency. The mapping was carried out by comparing the potency of antagonists in mutant and chimeric receptors with that of wild type receptors. Two groups of antagonists; peptides (section 3.3.1) and a non-peptide (section 3.3.2) were used in this study.

3.2 Materials and Methods

General materials and methods used have been described in chapter two. In this section, only those pertaining to this chapter will be described.

3.2.1 Construction of receptor chimeras: Receptor chimeras containing the N-terminal part of sheep GnRH receptor and C- terminal part from human GnRH

receptor were constructed by a PCR-based method. Antisense primers that correspond to sheep sequences, except for changes, which introduced restriction enzyme cutting sites found in the human receptor, were made (Table 3.1.1). Sense primers containing *Eco*R1 restriction enzyme cutting sites and the 5' bases of the sheep GnRH receptor DNA sequence were also synthesized. All primers were synthesized in the Department of Biochemistry, University of Cape Town. The PCR mixture contained, 25 pmol of each of the two primers, 200 ng of template DNA, 340 μM deoxynucleotide triphosphate mix and 2 units of Deepvent DNA polymerase in 30 μL of ThermoPol reaction buffer (Biolabs, New England). The denaturing temperature was 94°C, annealing temperature was 55° C and extension temperature was 74°C.

The PCR products were precipitated using 2.5 v/v ethanol and 0.1 v/v 3M sodium acetate (pH 5.2). After washing with 75% ethanol and resuspending in water, they were then digested with the appropriate restriction enzymes to expose their cohesive ends for subsequent ligation. Corresponding digestions were performed using wild type human GnRH receptor or human receptor with multiple engineered restriction enzyme cutting sites (Thomas Ott, MRC, Molecular Reproductive Endocrinology Research Unit, University of Cape Town, unpublished) cloned in pcDNA1/Amp. Whenever needed, a parallel digestion of pcDNA1/Amp was performed in order to expose the *Xho*1 and *Eco*R1 restriction enzyme cutting sites for cloning purposes. The resulting fragments were analyzed by agarose gel (1%) electrophoresis and the desired bands were extracted from the gel using USBioclean MP kit (United States Biochemical Corporation) according to the manufacturer's instruction. DNA fragments, which were less than 100 kilobase pairs were extracted and purified, from the agarose gel as follows. Desired bands were cut out of the gel and passed through 26-gauge needle into a tube. To this, one volume of phenol (saturated with TE) was added and after mixing thoroughly, was frozen in liquid nitrogen for 1 minute. This was followed by phenol/chloroform extraction. The final extract was precipitated with 2.5 % v/v of 100 % ethanol and 0.1 v/v of 3M sodium acetate (pH 5.2) at -70°C for 30 minutes. The precipitate was collected by centrifuging at 12000 x g for 20 minutes and washed with 75% ethanol and resuspended in sterile water.

Subsequently the DNA fragments were ligated into the vector in one or two steps using T4 DNA ligase (United States Biochemical Corporation) by incubating for 14 hours at 16° C. The ligation mixture contained a 1:1 molar ratio of receptor fragments and a 3:1 molar ratio of receptor fragment to vector ratio. The resulting product was used to transform competent bacteria (section 2.3.3). Clones of interest were identified by restriction enzyme digestion and PCR using gene specific primer (sstrt-1, section 3.2.2) and the DNA sequence of the altered regions were verified by sequencing. Table 3.2.1 lists the receptor chimeras constructed and the primers used in their construction.

Table 3.2.1 Receptor chimeras and the primers used in their construction

In the receptor chimera designation, the letters S and H refer to sheep and human GnRH receptors respectively, and the combination of the letters indicate the type of chimera. The subscript refers to the number of N-terminal amino acid residues contributed by the receptor denoted by the preceding letter. E.g. $S_{32}H$ (N-terminal 32 residues from sheep, the rest human).

The primers are named as follows: the first letter indicate the receptor type e.g. s = sheep, this is followed by the name of the restriction enzyme whose cuttings site has been introduced. The number following the hyphen indicates the position of the corresponding site in the receptor cDNA sequence and the terminal "s" and "as" stand for sense and antisense respectively.

Receptor chimera	***Primers used***
$S_{32}H$	s*Eco*RI-1s: 5'-TAGAGAATTCCCAGAAACACGAGT-3'
	s*Tth*111I-130as:5'-AATGACAAGGTCAGGGTGGGGAGGCT-3'
$S_{97}H$	s*Eco*RI-1s: 5'-TAGAGAATTCCCAGAAACACGAGT-3'
	s*PflM*I-324as: 5'-GAGACCATCCAGTGGCATAACAATCAG-3'
$S_{115}H$	s*Eco*RI-1s: 5'-TAGAGAATTCCCAGAAACACGAGT-3'
	s*Sca*I-384as: 5'-GAGAAGTACTTTGCAAAGGAGCTC-3'
$S_{171}H$	S*EcoR*I-1s: 5'-TAGAGAATTCCCAGAAACACGAGT-3'
	s*BsrG*I-562as: 5'-GAGATGTACAACTGTGGTCCAG-3'

3.2.2 Screening of bacterial colonies by PCR: Colonies of transformed bacteria

were picked with a sterile toothpick and suspended in 20 μL of water. Five microliter of this suspension was used in a PCR reaction using sense primer T7(5'-TAATACGACTCACTATAGG-3') or a sheep cDNA specific primer sstrt-1 (5'-CCAGAAACACGAGTC-3') and an antisense sp6 primers (Table 3.2.1) (Department of Biochemistry, University of Cape Town) to determine the presence of full-length GnRH receptor cDNA insert in the plasmid. The PCR mixture contained 0.2 mM deoxynucleotide triphosphate mix, 25 pmol of each of the two primers, 1.5 mM $MgCl_2$, 2.5 units Taq DNA polymerase (Taq polymerase purified by Dr. Arieh Katz, MRC, Molecular Reproductive Endocrinology Research Unit, University of Cape Town) in 20 μL of Taq DNA polymerase buffer (Promega, USA). 30 cycles of reaction at denaturing temperature of 94°C, annealing temperature of 50° C and extension temperature of 74°C were carried out.

3.2.3 Determination of potencies of selected peptide antagonists towards the mouse, sheep and human GnRH receptors

The potency and the relative potencies of the peptide antagonists were determined by measuring the ability of the antagonist to inhibit GnRH-stimulated inositol phosphate production by cells expressing GnRH receptors. In order to get information on the binding affinity as well as the functional characteristics of the different peptides, GnRH-stimulated inositol phosphate production was measured in the presence and absence of a fixed concentration of an antagonist (partial Schild plot) (Arunkakshana and Schild, 1947). The affinities of the antagonists were subsequently calculated from the inositol phosphate production data (section 2.5) and compared between receptors.

3.3 Results

3.3.1 Activities of five GnRH receptor peptide antagonists

As shown in Figure 3.3.1a-e, co-incubation of GnRH with any one of the GnRH antagonists resulted in an inhibition of GnRH stimulated inositol phosphate production. This inhibition could be overcome by higher concentrations of GnRH resulting in a shift of the EC_{50} for the GnRH dose response curves to the right with comparable maximum production of inositol phosphate. This indicates that the inhibition is competitive in nature. The degree of the shift of the EC_{50} to the right for each receptor

is proportional to the potency and concentration of the antagonist. The Ki (Binding constant of antagonist) for each of the antagonists for the mouse, sheep and human wild type GnRH receptors were calculated from the IP response data using the equation;

$$EC_{50}'/EC_{50} - 1 = [Antagonist]/K_i$$

where EC_{50} and EC_{50}' are EC_{50} of agonist in the presence and absence of a fixed concentration of antagonist respectively (Arunkakshana and Schild, 1947). Table 3.3.1 shows Ki values and relative potency of for each of the antagonists. The antagonists are identified on the graphs.

3.3.2 Comparison of the potencies of GnRH receptor antagonist peptides

All of the peptide antagonists tested showed a higher potency towards mouse and sheep receptors when compared to that for the human receptor. The greatest difference in potency was observed with antagonist 13, which showed 54 and 68 fold higher potency in inhibiting IP production with the mouse and sheep receptors respectively when compared to the human receptor. All the antagonists except antide had comparable potencies towards the sheep and mouse receptors. These screening assay results identified at least one peptide antagonists (antagonist 13) which exhibits a significant difference in potency for inhibition of IP production by two closely related mammalian GnRH receptors, the human and mouse/sheep GnRH receptors. A subsequent screening of a non-peptide antagonist FE 101177 using receptor binding assay revealed a much higher degree of difference in binding affinity between the three mammalian receptors (Section 3.3). Because of this large difference all subsequent studies were performed using FE 101177 and no further exploration of the structural determinants of peptide GnRH antagonist activity was undertaken.

Figure 3.3.1 (a-e) Partial Schild plot analysis of selected peptide antagonists: COS-1 cells were transfected with wild type mouse, sheep and human GnRH receptors. GnRH stimulated inositol phosphate production in the presence and absence of a fixed concentration of a peptide antagonist was measured. The identity of the antagonist and the concentration used is shown as inset. Circles (O,●) represent mouse, triangles (∇, ▼) human and squares (□, ■) sheep GnRH receptors. Open symbols are dose response curves in the absence of antagonist and filled symbols show responses in the presence of a fixed concentration of an antagonist. "Basal" refers to inositol phosphate produced in the absence of a ligand. The peptide sequences of the different antagonists are given in Table 2.1.1. Data points represent mean ± range of duplicate values and one out of 2-4 independent experiments performed in duplicate. Values are expressed as (%) of maximum IP production in the absence of antagonist for each receptor.

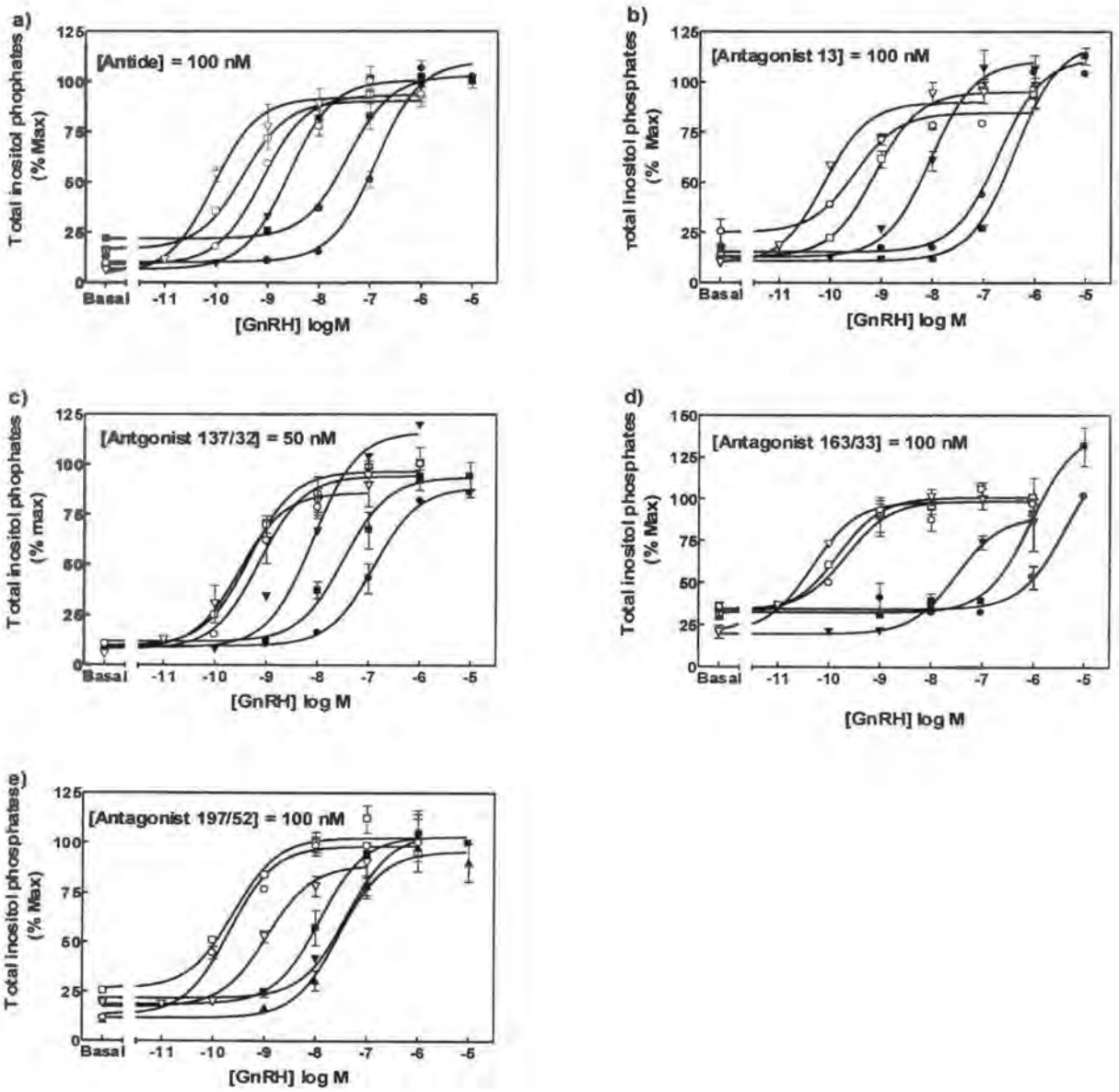


Table 3.3.1 Calculated Ki and relative potencies of five peptide antagonists for the mouse, human and sheep GnRH receptors: EC_{50} derived from dose response curves of GnRH stimulated inositol phosphate production in the presence and absence of a fixed concentration of antagonists were used to calculated the Ki values of the antagonists for the mouse, sheep and human GnRH receptors. Values are given as mean ± SEM or mean ± range, and are derived from two to four independent experiments each performed in duplicate. The number of experiments are indicated in parenthesis. Relative potency is derived by dividing the mean Ki value of the antagonists for human by that for mouse or sheep receptors. mGnRHR and sGnRHR represent mouse and sheep GnRH receptors respectively.

Antagonist	*Human GnRHR(nM)*	*Mouse GnRHR (nM)*	*Sheep GnRHR (nM)*	*Relative potency*	
				mGnRHR	*sGnRHR*
137/32	2.16±0.49 (4)	0.19±0.12 (2)	1.36±0.26 (3)	2.37	1.55
13	2±0.85 (4)	0.037±0.06(3)	0.029±0.006 (4)	54	68
Antide	0.85±0.1 (2)	0.062±0.012(2)	0.31±0.12 (3)	13.7	2.74
197/52	0.17±0.05 (3)	0.049±0.015(3)	0.07±0.02 (3)	3.4	2.4
163/33	0.21±0.03 (4)	0.036±0.005(4)	0.037±0.08 (4)	5.8	5.6

3.3.3 FE 101177 a non-peptide GnRH receptor antagonist

3.3.3.1 Differential affinities of FE 101177 to mouse, sheep and human GnRH receptors

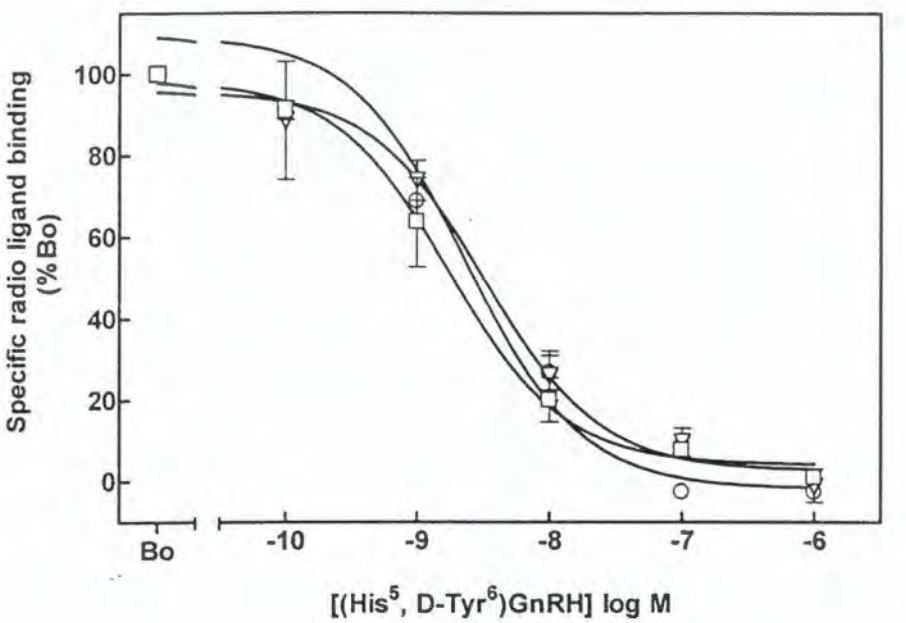
In earlier screening tests of various chemicals from a chemical library for their ability to inhibit GnRH binding to its receptor, FE 101177 (Figure 2.1.1), a non-peptide, was shown to inhibit GnRH agonist binding at a nanomolar concentration range (MRC, Molecular Reproductive Endocrinology Research Unit, University of Cape Town, unpublished data). Since the structure of the compound is markedly different from the other peptide antagonists it was postulated that FE 101177 might exhibit a different receptor binding behaviour. In order to study the interactions of FE 101177 to GnRH receptors in detail, receptor binding studies were performed using COS-1 cells transiently expressing the mouse, sheep or human GnRH receptors (Figures 3.3.1a and b).

Agonist binding to the human GnRH receptor was found to be more sensitive to inhibition by FE 101177 than either the mouse or sheep receptor and FE 101177 was least potent in inhibiting agonist binding to the sheep receptor. In the case of the sGnRH receptor, an appreciable degree of inhibition was only seen at concentrations of FE 101177 above 1 μM (Figure 3.2.1 a and b).

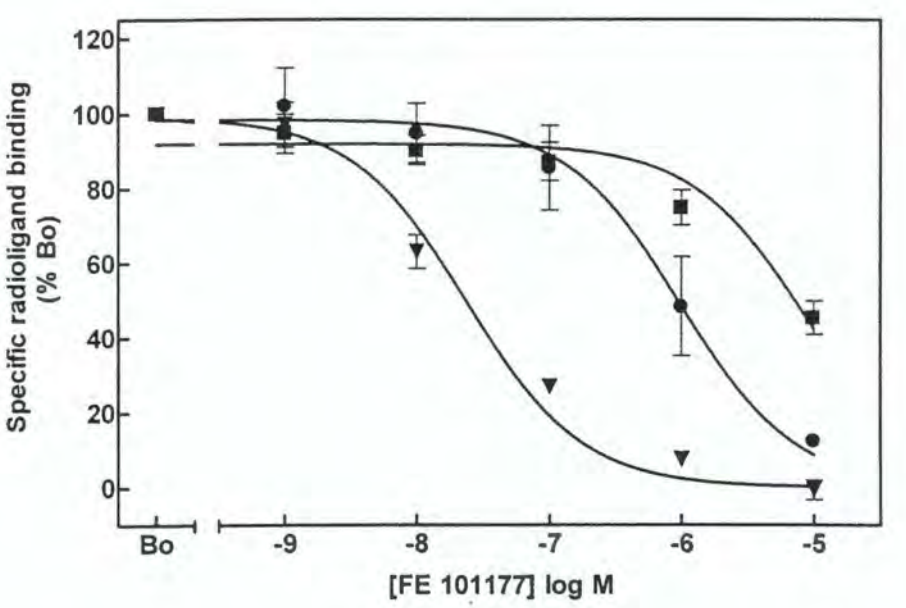
The Ki calculated from the binding data were 24.7 ± 4.1, 1965 ± 108 and 7559 ± 177 for the human, mouse and sheep receptors respectively. The data shows that there is a >300 fold difference ($P < 0.05$) in affinity between the human and sheep receptors. The difference in affinity towards the mouse and human receptors was about 80 folds ($P < 0.05$). The Kd for the [His^5,D-Ala^6]GnRH was found to be comparable for all the three receptors (Figure 3.3.1a and Table 3.3.2).

Figure 3.3.1 (a) and (b) [His^5-D-Tyr^6]GnRH and FE 101177 binding to GnRH receptors: COS-1 cells transiently expressing the mouse (O,●) sheep (□,■), or human (▽,▼) GnRH receptors were incubated with [His^5, ^{125}I-D-Tyr^6]GnRH in the presence or absence of increasing concentrations of the agonist [His^5, D-Tyr^6]GnRH (Figure 3.2.1a, open symbols) or the antagonist FE 101177 (Figure 3.2.1b, closed symbols). Binding assay was performed as described in section 2.5.1.2b. The Data is derived from 3-5 independent experiments performed in duplicate and expressed as % of maximum specific binding (Bo). "Bo" represents specific binding of radioligand in the absence of a competing ligand. Non-specific binding was determined by measuring radioligand binding in untransfected COS-1 cells.

a)



b)

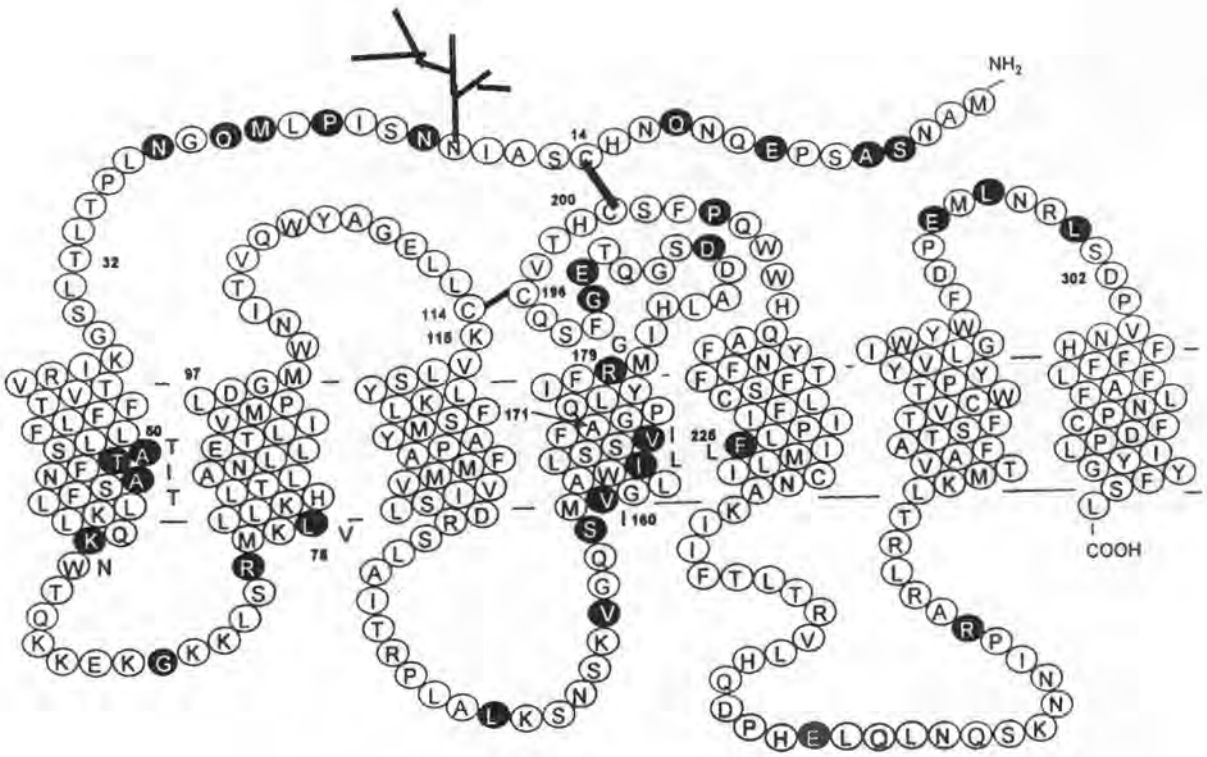


3.3.3.2 Comparison of the amino acid sequences of human and sheep wild type GnRH receptors

The sheep and human GnRH receptors showed a large difference in their susceptibility for the inhibition of agonist binding by FE 101177. Therefore subsequent studies were directed towards the understanding of the molecular basis for this difference.

The sheep and human GnRH receptors are closely related receptors exhibiting a 90% amino acid identitiy. The proximal part of the N-terminus contains the largest number of residues that are not conserved between the two receptors while EC1, TM6 and TM7 domains are completely conserved (Figure 3.3.2). Most of the amino acid differences involve conservative substitutions. In the TM regions for instance, only one (at position 225) out of seven unconserved residues contains a non-conservative substitution. Since the structures of the two receptors are very similar, any apparent difference in their interaction with ligands may be attributed to one or more of the few unique residues found in their primary structure.

Figure 3.3.2 Amino acid sequence of the human GnRH receptor:
Residues that are not conserved in the sheep GnRH receptor are shown in black. Corresponding residues in the TM domains of the sheep GnRH receptor are also indicated by their single letter designation. The thick branched lines indicate glycosylation sites and disulfide linkages are shown with connecting lines. Residues mentioned in the text are numbered.



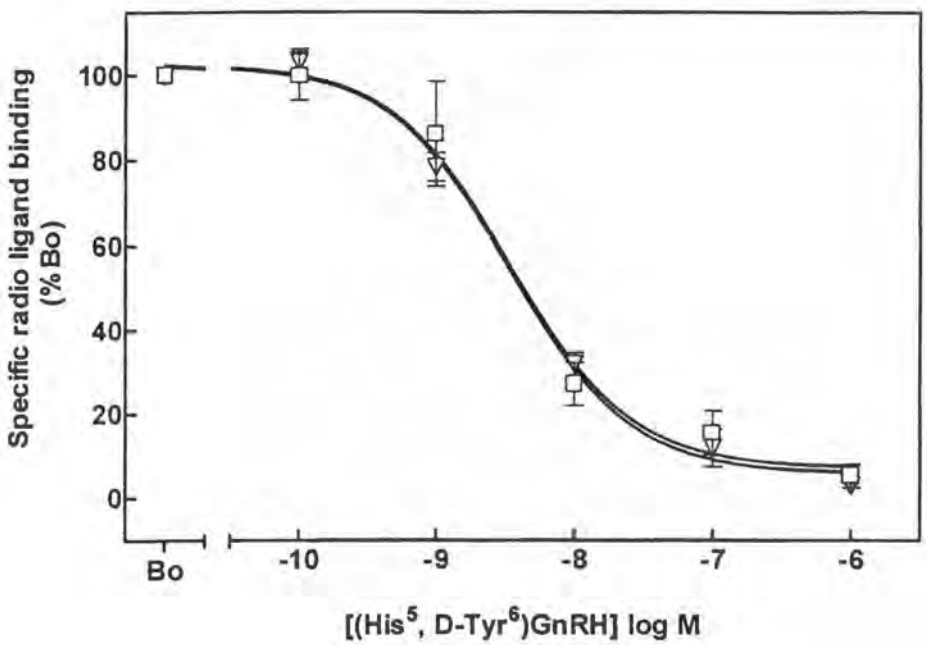
3.3.3.3 Binding characterisitcs of FE 101177 to the $S_{171}H$ chimeric receptor

A chimeric receptor composed of sheep and human cDNA derived amino acid residues was constructed as described in section 3.2.1 in such a way that the first N-terminal half of the receptor is derived from the sheep and the remainder from the human cDNA. This receptor designated $S_{171}H$ had the N-terminal 171 amino acids (up to the upper third of TM4) from the sheep receptor cDNA (Figures 3.3.2 and 3.2.4). This receptor chimera introduces the non-conserved residues found in the N-terminus, TMI, TM2, IC1, IC2 and TM4 of the sheep receptor into the human GnRH receptor. The binding affinities of FE 101177 and [His^5, D-Tyr^6]GnRH to $S_{171}H$ GnRH receptor were dermined (Figure 3.3.3 a and b). The Ki of FE 101177 was found to be

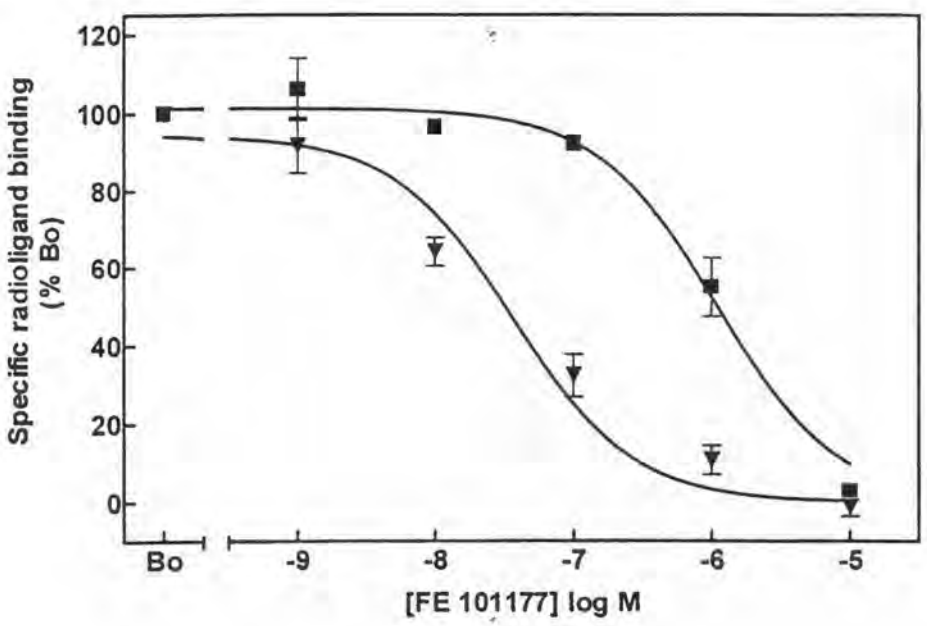
1050 ± 157 (mean ± SEM) which was about >40 folds lower than that for the wild type human receptor (Figure 3.2.3 b). The Kds for the agonist binding for human 3.58 ± 0.65 and for $S_{171}H$ 4.05 ± 0.82 (mean ± SEM) receptors were very similar. The results show that the introduction of the new domains to the human receptor caused a change in the affinity of the antagonist selectively without affecting the affinity for the agonist.

Figure 3.3.3 The binding of [His^5-D-Tyr^6]GnRH and FE 101177 to the S_{171}H GnRH chimeric receptor: COS-1 cells transiently expressing human wild type (▽, ▼) or S_{171}H (□,■), GnRH receptors were incubated with [His^5,^{125}I-D-Tyr^6]GnRH in the presence or absence of increasing concentrations of the unlabelled agonist [His^5, D-Tyr^6]GnRH (Figure 3.3.3a) or the antagonist FE 101177 (Figure 3.3.3b). After washing off the unbound radioligand, radioactivity on the cell surface was measured. The data is derived from 4 independent experiments performed in duplicate and normalised as % of maximum specific binding (Bo). "Bo" represents specific binding of radioligand in the absence of a competing ligand. Non-specific binding was determined by measuring radioligand binding in untransfected COS-1 cells.

a)



b)



3.3.3.4 The affinity of FE 101177 for and $S_{32}H$, $S_{97}H$, $S_{115}H$ and chimeric GnRH receptors

According to the results presented in section 3.3.3.3, of the total difference in affinity for FE 101177 between the human and the sheep receptors (300 folds) a large part of it (>40 folds) seems to be accounted for by residues on the N-terminus side of residue 171 of the GnRH receptor. In order to narrow down the region responsible for the loss of affinity of the $S_{171}H$ receptor to FE 101177, a series of chimeric receptors were made. These receptors contained progressively less of sheep receptors cDNA derived N-terminal amino acids. These newly constructed receptors were designated $S_{32}H$, $S_{97}H$ and $S_{115}H$. Figure 3.3.4 shows schematic diagrams of the receptor chimeras constructed for this study. The binding affinities of FE 101177 and [His^5, D-Tyr^6]GnRH to these receptors was determined and results were compared with that of the wild type human GnRH receptor (Figure 3.3.5).

Figure 3.3.4 Schematic diagram of chimeric GnRH receptors constructed: The thick lines indicate amino acids derived form the Sheep GnRH receptor cDNA while the thin lines indicate amino acids derived from the human GnRH receptor cDNA. The designated name of each receptor is indicated at the left upper corner of each diagram.

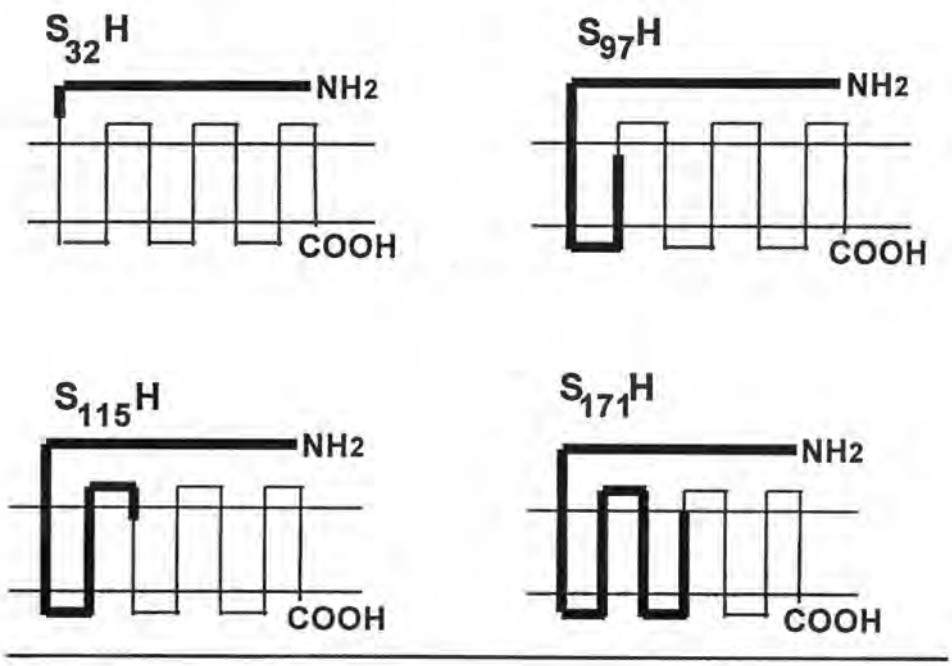
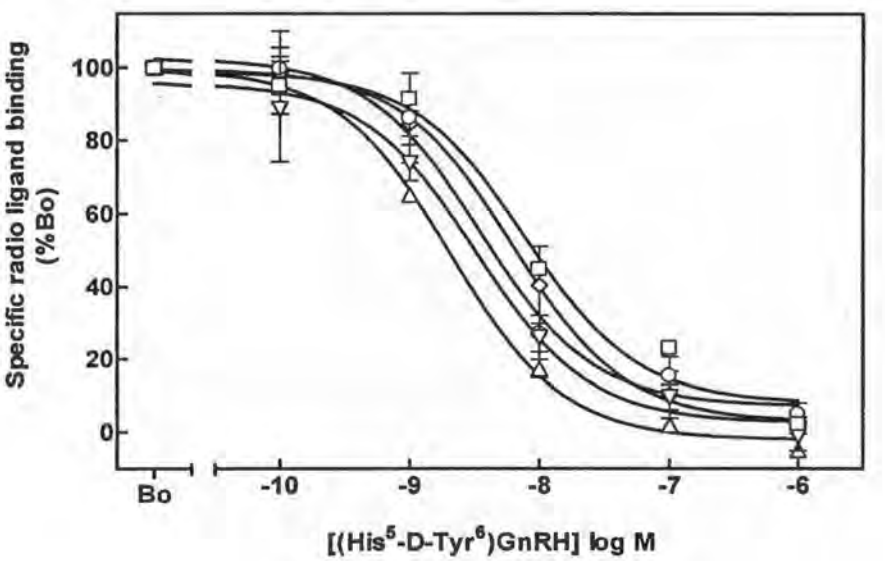
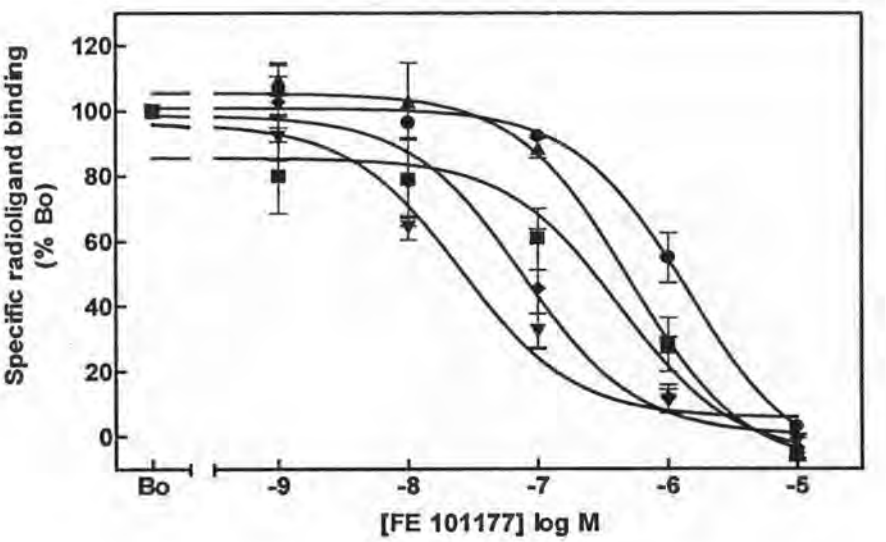


Figure 3.3.5 The binding of [His^5-D-Tyr^6]GnRH and FE 101177 to the human wild type, $S_{32}H$, $S_{97}H$, $S_{115}H$ and $S_{171}H$, chimeric GnRH receptors: COS-1 cells transiently expressing the sheep wild type human wild type (▽,▼) $S_{32}H$ (◊,◆), $S_{97}H$ (□,■), $S_{115}H$ (△,▲) or $S_{171}H$ (○, ●)chimeric GnRH receptors were incubated with [His^5-^{125}I-D-Tyr^6]GnRH in the presence or absence of increasing concentrations of the agonist [His^5,D-Tyr^6]GnRH (Figure 3.2.5a) or the antagonist FE 101177 (Figure 3.2.5b). Unbound ligand was washed off and remaining radioactivity counted. The Data is derived from 2-5 independent experiments performed in duplicate and expressed as % of maximum specific binding (Bo). Number of experiments are indicated on Table 3.3.2 "Bo" represents specific binding of radioligand in the absence of a competing ligand. Non-specific binding was determined by measuring radioligand binding in untransfected COS-1 cells.

a)



b)



The $S_{32}H$ receptor, which contains the sheep, sequences in the N-terminus extracellular regions exhibited a similar agonist and antagonist binding affinity to that of the wild type receptor (Figure 3.3.5, Table 3.3.2). Extending the sheep cDNA contribution to the top of TM2 as in the $S_{97}H$ receptor caused 18 fold decrease in affinity to FE 101177. A comparable degree of loss in affinity (20 folds) was also observed in the $S_{115}H$ receptor. In both $S_{97}H$ and $S_{115}H$ receptors the affinity to the agonist [His^5,D-Tyr^6]GnRH was found to be comparable to that for the wild type receptor (Table 3.3.2). A further increase in sheep domains to the top of TM4 further decreased the affinity of the receptor to FE 101177 as evidenced by the 40 fold difference observed in the $S_{171}H$ receptor compared to the wild type human receptor.

3.3.3.5 The effect of selected sheep amino acid residue substitutions in the human GnRH receptor on the affinity of FE 101177 to the receptor

The structure of FE 101177 contains a large hydrophobic component, which most likely may interact with other hydrophobic elements of the GnRH receptor. The transmembrane domains of the GnRH receptor contain many of the hydrophobic residues that can possibly interact with the antagonist. Comparison of the sheep and human GnRH receptors shows that there are a number of residues in TM1, TM2, TM4 and TM5 that are not conserved between the two receptors while TM3, TM6 and TM7 are completely conserved. In order to study the relative effects of these residues single or multiple amino acid substitutions that correspond to the sheep sequence were introduced into the human receptor and the binding affinities of these mutant receptors were determined. These mutant receptors studied were h(A50T, T51I,A54T), hL78V, h(V160I, I165L, V169I), hR179G and hF225L. Figure 3.3.6 show the competitive receptor binding curves of [His^5, D-Tyr^6]GnRH and FE 101177 to each of the these receptors.

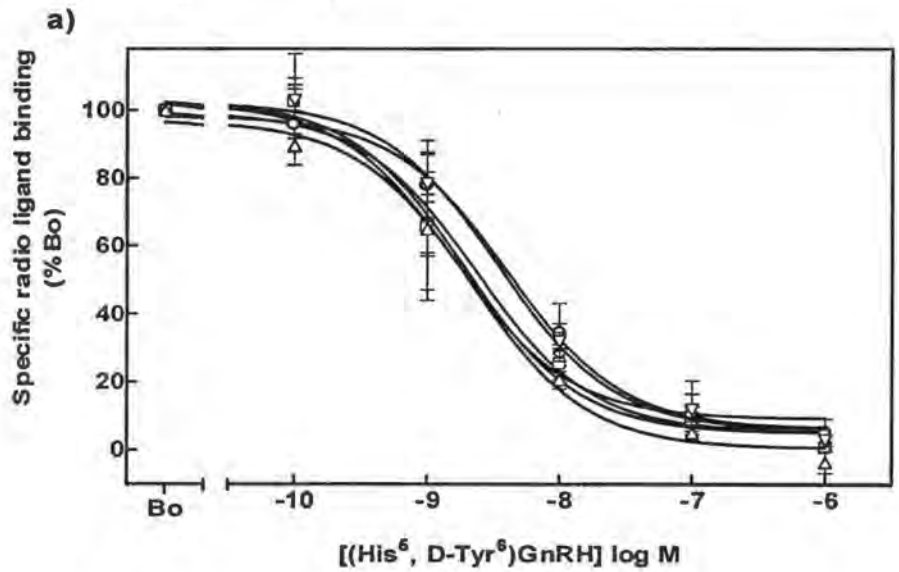
The results indicate that the Kd for agonist binding in all cases were similar to the wild type human receptor (Table 3.3.2). This absence of change in affinity towards the agonist in the presence of these mutations suggests that the structure of the receptor that is required for the recognition of the agonist [His^5, D-Tyr^6]GnRH has not been disturbed by the introduction of these mutations.

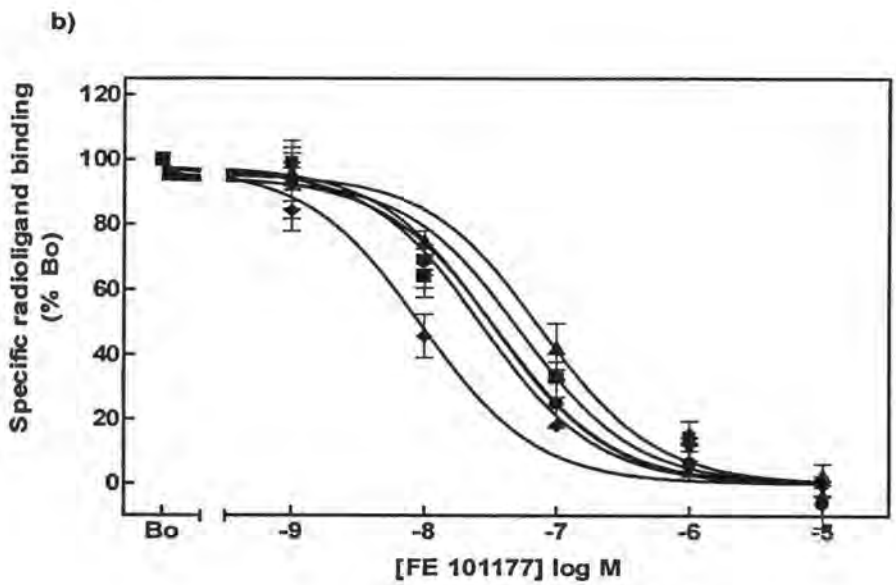
The hL78V receptor showed a small loss in affinity for FE 101177 while in the h(A50T,T51I,A54T) a slight enhancement of the affinity was observed (Figure 3.3.6, Table 3.3.2). The h(V160I,I165L,V169I) mutant receptor containing multiple mutations in TM4 also not show any significant change in affinity to both [His^5, D-Ala^6]GnRH and FE 101177. The results suggest that these residues may not be crucial in determining the differential affinity of FE 101177 to sheep and human receptors (Figure 3.2.6).

Substitution of the charged arginine 179 with the corresponding sheep residue glycine which is smaller and uncharged, did cause a 4 fold change in the affinity of FE 101177 ($P<0.05$) (Figure 3.3.6b, Table 3.3.2). However, the substitution of the TM 5 unconserved residue phenylalanine to the non-aromatic leucine did not change the affinity of the receptor to [His^5, D-Ala^6]GnRH nor FE 101177 implying that the residue may not account for the observed difference in affinity of FE 101177 to the human and sheep receptors (Figure 3.2.6).

Figure 3.3.6 The binding of FE 101177 to hR179G, hF225L, h(V160I, I165L,V169I) and h(A50T,T51I, A54T) mutant GnRH receptors.

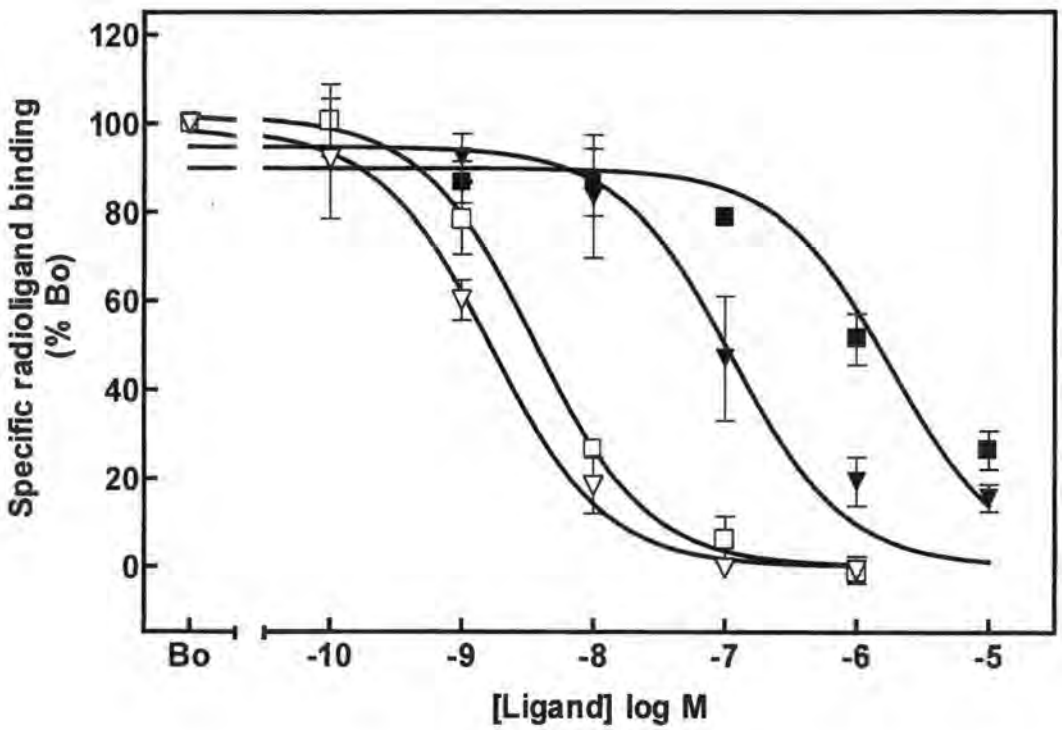
COS-1 cells transiently expressing the human wild type (▽,▼) and hR179G(△, ▲), hF225L (○, ●), h(V160I,I165L,V169I) (□, ■), h(A50T,T51I,A54T)(◇, ◆)), L78V (+, *) mutant GnRH receptors were incubated with [His^5, ^{125}I, D-Tyr^6]GnRH in the presence or absence of increasing concentrations of the agonist [His^5,D-Tyr^6]GnRH (Figure 3.2.6a) or the antagonist FE 101177 (Figure 3.2.6b). Unbound ligand was washed off and remaining radioactivity counted. The Data is derived from 3-4 independent experiments performed in duplicate and normalised as % of maximum specific binding (Bo). "Bo" represents specific binding of radioligand in the absence of a competing ligand. Non-specific binding was determined by measuring radioligand binding in untransfected COS-1 cells.





Recently Cho *et al.*, (1998) using computer generated models has proposed that the basic amino moiety on 3-methyl group (methylaminomethyl) of a non-peptide GnRH antagonist (T-98478) may interact with the negatively charged Asp^{302} in a manner analogues to Arg^{8} of GnRH and Glu^{301} of the mouse GnRH receptor (Flanagan *et al.*, 1994). A related basic amino group is present at the same position in FE 101177. The possible role of the negatively charged Asp^{302} in EC3 in the binding of Fe 101177 was investigated using the iso-steric mutant hD302N. The binding affinities of wild type human GnRH receptor and that of hD302N mutant receptor compared. Since the expression level of hD302N is much lower than that of the wild type receptor, binding assays were performed on membrane preparations (section 2.4.1.1) in order to improve the signal. As shown in figure 3.3.7 a slight difference in the affinity to the agonist was observed between the wild type human and hD302N mutant GnRH receptors. However the affinity of FE 101177 toward hD302N receptor was found to be 12 fold lower than for the wild type human receptor.

Figure 3.3.7 The binding affinity of 101177 to hD302N mutant GnRH receptor: COS-1 cells transfected with wild type or hD302N mutant GnRH receptor were homogenised and membrane preparations were incubated with [His^5, ^{125}I-D-Tyr^6]GnRH in the presence of various concentrations of unlabelled [His^5, ^{125}I-D-Tyr^6]GnRH or FE 101177. Incubation was stopped after 16 hours and excess label was removed and the remaining radioactivity was measured. Data represent mean ± SEM derived from 3 independent experiment performed in triplicate. Values are expressed as % Bo where Bo is specific radioligand binding in the absence of competing ligand. The non-specific binding was determined by measuring radioactivity in untransfected COS-1 cells.



The binding and relative affinities of the different wild type mutant and chimeric receptors used in this study to FE 101177 and [His^5, D-Tyr^6]GnRH are summarised in the table below (Table 3.3.2).

Table 3.3.2 The binding affinities FE 101177 to human, sheep, mouse and mutant GnRH receptors: Ki are derived from the receptor binding data using the Cheng-Prusoff correction (Cheng and Prusoff, 1973). Kd is derived from homologues binding data. Relative affinity is derived by dividing mean Ki or Kd values for the human receptor by the corresponding values for the test receptor (mouse and sheep wild type, mutant and chimeric receptors). Data is given as mean ± SEM of independent experiments performed in duplicate or triplicate and number of experiments is indicated in parenthesis.

GnRH receptor	*Kd for [His^5, Tyr]GnRH*	*Relative affinity (Agonist)*	*Ki for FE 101177*	*Relative affinity (Antagonist)*
Human wild type	3.58±0.65 (3)	1	24.7±4.1 (5)	1
Sheep wild type	2.2±0.63 (3)	1.6	7559±1776 (5)	0.003
Mouse wild type	2.5±1.16 (2)	1.4	1966±108 (4)	0.013
$S_{32}H$	7.35±2.8 (2)	0.48	19.25±1.2 (3)	1.28
$S_{97}H$	7.8 (1)	0.45	440.5±111 (3)	0.056
$S_{115}H$	2.47±0.6 (2)	1.44	489±35 (2)	0.05
$S_{171}H$	4.05±0.82 (3)	0.88	1050±157 (3)	0.023
hA50T,T51I,A54T	5.5±0.9 (3)	0.65	10.6 ± 3.1 (2)	2.3
hL78V	2.77±1.3 (3)	1.29	54.2±14 (3)	0.45
hV160I,I165L,V169I	2.08±1.1 (2)	1.2	31.15±5.7 (3)	0.79
hR179G	2.85±0.76 (3)	1.2	101±8.6 (3)	4.08
hF225L	3.76±1.9 (4)	0.95	31.6±8.48 (3)	0.78
HWT*	1.62±0.21 (3)	1	161±64 (3)	1
hD302N*	3.5±0.5 (3)	0.46	2110±400 (3)	0.07

* Receptor binding assay was performed on membrane preparations (section 2.4.1.1)

3.4 Discussion

All of the peptide antagonists tested show an increased potency towards the mouse receptor when compared to the human receptor (Table 3.3.1). This probably extends to many other peptide antagonists. A possible reason for this is that most of the antagonists currently in use were synthesised and selected for their activity in rodents before the human GnRH receptor was cloned in 1993. Much of the screening of these compounds were made *in vitro* or *in vivo* using rat models which select for those compounds showing the highest potency towards the rat receptor. The results presented emphasise the need to verify such finding using human receptor models. The cloning of the human receptor and the generation of cell lines that stably express the human GnRH receptor solve this problem by allowing drugs to be tested directly on the target receptor. Future screening of active analogs using the human GnRH receptor is likely to identify compounds appropriate for clinical application. The relatively small difference in potency seen for some of the antagonists (antagonist 163/33, antagonist 197/52, and antagonist 13) between mouse and sheep receptors suggest that similar domains of the receptor are utilised in the binding or recognition of these antagonists. The large difference in potency of antagonist 13 between sheep and human receptors may allow it to be used in future studies to map binding sites.

The non-peptide antagonist FE 101177 shows greater than 300 fold lower affinity for the sheep GnRH receptor when compared to that for the human receptor. This difference is remarkable since the primary structures of the two receptors show a 90% identity. More drastic differences in the responses to GnRH antagonists have been described between other less related GnRH receptors. For instance, the antagonist 135/18 which is a potent inhibitor of GnRH-stimulated inositol phosphate production of the mammalian GnRH receptor was found to act as a full agonist for the chicken, Xenopus and goldfish GnRH receptors (Jacobs *et al.,* 1994, Troskie, B., MRC, Molecular reproductive Endocrinology Research Unit, University of Cape Town, unpublished). Responses to natural GnRH subtypes also vary between different wild type receptors. The study of these differences in the context of the structural and functional differences between receptors and ligands will help in the identification of crucial domains in the receptor as well as in the understanding of the mechanistic

events that take place up on receptor activation and deactivation.

Fe 101177 is a small highly hydrophobic compound with apparently little structural similarity with GnRH peptide analogues. Hence, the interaction of the compound with the GnRH receptor is bound to be different from that of peptide ligands. The size and the hydrophobicity of the compound make it likely that it may interact with transmembrane hydrophobic residues. To understand the interaction, a series of mutant and chimeric receptors were constructed and the affinities of FE 101177 for these receptors were studied.

The substitution of the N-terminal transmembrane domain of the human receptor with that of the corresponding sheep receptor domain as in the $S_{32}H$ receptor chimera did not cause a change in the binding affinity of the receptor for FE 101177 or that of the [His^5, D-Ala^6]GnRH (Table 3.3.2). The N-terminus of the two receptors contains the most differences in amino acid sequences between the two receptors. This result suggests that the marked difference in the primary structure of the N-terminus between the sheep and human receptors does not account for the difference in affinity towards FE 101177.

When the first 171 amino acids of the human GnRH receptor were exchanged for that of the sheep as in the $S_{171}H$ receptor chimera, a marked decrease in the affinity to FE 101177 by 40 folds was noted. The affinity of $S_{171}H$ for the agonist did not change which shows that the introduction of the sheep residues did not cause a major structural disruption. This selective effect on the antagonist binding suggests that different regions in the receptors may be used for the binding of the non-peptide antagonist and the peptide agonist. The domain of the receptor from the top of TM1 up to the top of TM4 (residue 171) contains eight amino acids in the TM regions that are not conserved between the sheep and human receptors and TM4 contains most of these residues. $S_{97}H$ and $S_{115}H$ receptor chimeras contain sheep domains up to the top of TM2 and EC1 respectively and lack the changes in TM4 of $S_{171}H$. FE 101177 had a about 20 times lower affinity to these two receptor chimeras when compared to that for human wild type receptor and 2 times higher affinity when

compared to the $S_{171}H$. The isolated mutation of the three hydrophobic residues in TM4 into corresponding sheep residues h(V160I, I165L, V169I) did not cause a change in affinity to FE 101177 as well as the agonist. These findings imply that the different domains involved in the binding of FE 101177 enhance one other's contribution in the formation of the binding domain or in effecting a conformation of the receptor that maximises the binding affinity.

In the $S_{97}H$ receptor the first 97 amino acids are derived from sheep cDNA while the remainder of the receptor is derived from the human cDNA. The 18 fold difference in affinity for FE 101177 between $S_{97}H$ and human wild type receptor could not be due to disturbance of receptor structure since the agonist affinity in both receptors is comparable. It is seems unlikely that FE 101177 interacts with residues located in the extracellular N-terminus of the receptor since the $S_{32}H$ receptor showed no change in affinity to FE 101177. Therefore this observed difference might be attributed to the non-conserved residues in the TM1,TM2 and IC1. The similar results obtained for the $S_{115}H$ receptor chimera also suggest this assumption since all of the amino acids form 97-115 are conserved between the sheep and human receptors.

To investigate this, two mutant receptors h(A50T,T51I,A54T) and hL78V, that incorporate the amino acid differences found TM1 and TM2 of the sheep receptor were constructed and tested. There was however no marked alteration of affinity to FE 101177. The finding suggests that a decrease in affinity only occurs when all of the altered sheep residues (TM1 and TM2) are present at the same time.

In addition to introducing TM sheep residue differences into the human receptor $S_{97}H$, $S_{115}H$ and $S_{171}H$ chimeric receptors also introduce the non-conserved IC1 and IC2 residues. Though these residues are unable to be involved directly in the binding of FE 101177 the possibility of these residues altering receptor folding and conformation and causing an effect on FE 101177 binding sites can not be ruled out.

In the TM domains the two most structurally drastic substitutions in the sheep receptor are the substitution of Arg^{179} by Gly and Phe^{225} by Leu where a charge and a large

side chain are lost in the first instance and an aromatic ring is lost in the second instance. The hR179G receptor showed a 4 fold loss in affinity for the Fe 101177 while having similar affinity for the [His^5, D-Tyr^6]GnRH (Table 3.3.2). The absence of a negatively charged group on the FE 101177 suggests that this loss of affinity in the hR179G receptor may be due to a non-charge interaction or an effect of the residue on the folding of the receptor.

Phe^{225} in the human GnRH receptor is substituted by leucine in the sheep receptor. Since FE 101177 contains multiple cyclic structures (Figure 2.1.1), the aromatic residues in the TM domains may play a role in the interaction of FE 101177 with the receptor. It is interesting to note that Phe^{225} in the TM5 is the only TM aromatic residue that is not conserved in the sheep receptor. The hF225L mutant receptor showed binding affinities to FE 101177 and [His^5, D-Tyr^6]GnRH that are very similar to the wild type receptor suggesting that F225 is not involved in the differential binding of FE 101177.

FE 101177 is a hydrophobic molecule containing a basic amino group (Fig 2.1.1). It is conceivable that FE 101177 may use a charge interaction for anchoring itself to the receptor while allowing the bulky hydrophobic domain to go deep in to the binding pocket and be engaged in a hydrophobic interaction with residues in the TM domains of the receptor. Glu^{301} of the mouse receptor, which corresponds to the Asp302, has been shown to be important in the binding to GnRH. A recently presented computational model of the interaction of the non-peptide antagonist T-98475 to the GnRH receptor spatially puts the positively charged amino group on the 3-methyl group in the vicinity of Asp^{302} (Cho *et al.*, 1998). The hD302N mutant receptor, which has an iso-steric substitution for aspartic acid, was found to have a 12 fold less affinity to FE 101177 when compared to the wild type receptor. The affinity for the [His^5, D-Tyr^6]GnRH was only slightly lower than that of the wild type receptor (Table 3.3.2). This is in line with previous findings that the charge interaction between Glu^{301} of the mouse /Asp^{302} of the human receptors and a positively charged amino acid at position 8 of the GnRH analog is not required if the analog is structurally constrained by having a D-amino acid in position 6. However, the greater than ten fold decrease in affinity to

FE 101177 observed in the hD302N receptor implies that Asp^{301} may be involved in the interaction of FE 101177. Since both the sheep and human GnRH receptors contain the residue at the same position, the residue is clearly not responsible for to the differential affinities of FE 101177 to the sheep and human receptors.

Most of introduced point mutations, R179G, F225L and the multiple mutations h(V160I, I165L0, V169I), h(A50T,T51I,A54T) by themselves caused little or no change in the affinity of the agonist or the non-peptide antagonist. When these changes are combined together as in the receptor chimeras, a more dramatic change in affinity is observed which shows that the effects of the different mutations and alterations may not be simply additive. The data suggests that a large number of interactions are involved in bringing about the final binding behaviour of FE 101177. The N-terminal 171 residues could only account for a 40 fold difference in affinity to FE 10177 out of a total of 300 folds seen between the human and sheep receptors. This indicates that those residues present in the region from residue 172 to the C-terminus must contain additional determinants responsible for the recognition of FE 101177. The results presented also show that Asn^{302}, which is conserved in the sheep and human receptors, may be involved in the binding of FE 101177 as a common determinant.

Further work needs to be done in order to get a more complete information on the role of the different residues of the receptor in its interaction with FE 101177. FE 101177 binds to all the three mammalian receptors tested. Therefore the presence of determinants that are common to all the three receptors and which are responsible for the binding of the ligand is expected. Mutagenesis of selected conserved residues, which may be guided by previous data on receptor domains, may shed additional light. For instance recently, using receptors chimeras, the EC2 domain of the mammalian and non-mammalian receptors was found to be responsible for the recognition of 135/18 (Ac-D-Nal(2)-D-4-ClPhe-D-Pal-Ser-Ile-D-Ipr-Lys-Leu-Ipr-Lys-Pro-D-AlaNH_2) as an agonist (mammalian receptors) or an antagonist (Xenopus & chicken receptors) (Thomas Ott , MRC, Molecular Reproductive Endocrinology Research Unit, University of Cape Town, unpublished). This is particularly interesting in the light of the fact that residues in the EC2 that are not conserved between human and sheep receptors all

involve non conservative substitutions (S86P, K91E, V92G, S203P). The mouse receptor, which also has low affinity to FE 101177, has non-conservative substitutions at these sites. It would also be interesting to see if substitutions of sheep residues by human counterparts will help to gain affinity to FE 101110 by the sheep receptor. It would also be interesting to compare the binding characteristics of FE 101177 and the common peptide antagonists.

In this thesis a peptide antagonist that exhibits differential potency for two mammalian GnRH receptors has been identified. The binding characteristics of a non-peptide antagonist FE 101177 has also been studied by using mutant and chimeric GnRH receptors. The possible role of Asp^{302} in the interaction of GnRH receptor with FE 101177 has been shown. Evidence suggesting the combined roles of different residues of the GnRH receptor in providing binding sites has been presented. In addition the relative independence of the FE 101177 on agonist binding sites has been shown.

CHAPTER FOUR

INTERACTION OF PHOTOREACTIVE ANALOGS WITH THE GnRH RECEPTOR

4.1 Introduction

The determination of the three dimensional structure of a receptor or a ligand/receptor complex is of paramount importance when trying to understand a ligand/receptor interaction. Because of the low expression level of the GnRH receptor and the fact that the receptor is a membrane protein, the generation of a crystal structure of the receptor has not yet been possible. The lack of such structural information for the GnRH receptor necessitates the adoption of alternative approaches in order to understand the ligand receptor interaction. Photoaffinity labelling of receptors using reactive ligands have been extensively used in other systems in trying to delineate ligand binding sites. In GnRH receptor, photoreactive derivatives have been used to probe ligand receptor interactions, receptor internalisation, distribution and glycosylation (reviewed in section 1.3).

In this chapter, the synthesis and characterization of photoreactive analogs of GnRH and the identification of crosslinking sites is described. The emphasis of the research has been on GnRH antagonists. During the course of this work, I have also been involved as a major participant in the synthesis, characterization and determination of the crosslink site of a photoreactive GnRH agonist, [D-Ala^6, N-Azidobenzoyl-Orn^8]GnRH (PAg-1) and the identification of a second disulfide bridge in the GnRH receptor (Davidson *et al.*, 1997).

4.2 Materials and Methods

General materials and methods used through out the thesis have been described in chapter two. In this section, only methods that pertain to the work described under chapter four are outlined.

4.2.1 Synthesis of photoreactive analogs: The following peptides were used in the synthesis of photoreactive analogs. All the parent peptides were synthesized by Dr.

Roger Roeske (Department of Biochemistry and Molecular Biology, Indiana University, School of Medicine, Indiana, USA) except antagonist 26 which was synthesized by Dr. David Coy (Tulane University School of Medicine, New Orleans, Louisiana, USA) (Table 4.2.1). [D-Ala^6, Orn^8]GnRH was designed by Professor Robert P. Millar and Dr. Colleen A. Flanagan (MRC, Molecular Reproductive Endocrinology Research Unit, University of Cape Town, Cape Town, South Africa).

Table 4.2.1 List of Peptides used in the derivation of photoreactive analogs: Amino acid sequences are expressed in relation to the primary structure of mammalian GnRH. Where available, code names used in the MRC, Molecular Reproductive Endocrinology Research Unit (University of Cape Town) or by producers of the compound are indicated.

Name	*Sequence*
Antagonists	
Antagonist 26	[Ac-D-4-Cl-Phe^1, Ac-D-4-Cl-Phe^2, D-Trp^3, D-Lys^6, D-AlaNH$_2$ ^{10}]GnRH
-	[Ac-D-Nal^1, D-Dpr^2, D-Trp^3, D-Arg^6]GnRH
-	[Ac-D-Lys^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-AlaNH$_2$ ^{10}]GnRH
Agonists	
-	[L-Dpr^3, D-Ala^6]GnRH
-	[L-Dpr^2, D-Ala^6]GnRH
-	[D-Ala^6, Orn^8]GnRH

The photoreactive GnRH analogs were synthesized by a modification of the method described by Hazum (1981a). One μmol of the peptide was reacted with 10 μmol 4-azidobenzoic acid N-hydroxysuccinimide or 4-azidosalicylic acid N-hydroxysuccinimide (Sigma) in 200 μL of methanol containing 16% DMSO (v/v) and 1.2 μmol triethylamine at 24°C for 14 hours. The peptide was precipitated with 2.5 mL diethyl ether and centrifuged at 15000 x g for 20 minutes. The resulting precipitate was washed three times with ethyl acetate, dried under nitrogen, and dissolved in 50% methanol/water. The products were subsequentlyanalysed by thin layer chromatography on a 60 F_{254} silica gel plate (Merck) using a 1:1:1:1 butanol/acetic acid/water/ethylacetate solvent

and visualised using iodine vapour in order to determine the completeness of the reaction. The relative mobilities of the parent peptides and the derivatized photoreactive peptides were compared. The absorbance spectra of peptides (30-50 μM) were determined using a Shimadzu UV-160A spectrophotometer or a Hewlett/Packard 8450A diode array spectrophotometer before and after 10-60 seconds of UV irradiation (λ = 312) from a TR 312 A Spectroline transilluminator at a distance of 4 centimetres. All procedures involving photoreactive materials were performed in the dark or in dim laboratory lighting, for the shortest possible time. Table 4.2.2 lists the newly synthesized photoreactive analoges.

Table 4.2.2 List of photoreactive analogs: Designated names of the photoreactive analogs and their sequences based on the mammalian GnRH are shown. All except PAg-1 were synthesized as part of the present thesis.

Name	***Sequence***
PAnt-1	[N-Azidobenzoyl-Ac-D-Lys^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-AlaNH$_2$^{10}]GnRH
PAnt-1a	[N-Azidobenzoyl-Ac-D-Lys^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-AlaNH$_2$^{10}]GnRH
PAnt-2	[Ac-D-4-Cl-Phe^1, Ac-D-4-Cl-Phe^2, D-Trp^3, N-Azidobenzoyl-D-Lys^6, D-AlaNH$_2$^{10}]GnRH
PAnt-3	[Ac-D-Nal^1, N-Azidobenzoyl-D-Dpr^2, D-Trp^3, D-Arg^6]GnRH
PAnt-3a	[Ac-D-Nal^1, N-Salicyl-D-Dpr^2, D-Trp^3, D-Arg^6]GnRH
PAg-1	[N-Azidobenzoyl-D-Lys^6]GnRH
PAg-2	[D-Ala^6, N-Azidobenzoyl-Orn^8]GnRH
PAg-2a	[D-Ala^6, N-Salicyl-Orn^8]GnRH
PAg-3	[N-Azidobenzoyl-L-Dpr^2, D-Ala^6]GnRH
PAg-4	[N-Azidobenzoyl-L-Dpr^3, D-Ala^6]GnRH
PAg-4a	[N-Salicyl- L-Dpr^3, D-Ala^6]GnRH

4.2.2 Photoaffinity labelling and localisation of crosslinking site

4.2.2.1 Photoaffinity labelling: Intact COS-1 cells transfected 48 hours previously or αT3-1 cells in dishes were incubated in 1.5 mL/mL buffer B (section 2.5) containing 2 x 10^6 c.p.m [^{125}I]PAnt-1 in the presence or absence of 1 μM unlabelled [D-Trp^6, Pro^9-

ethylamide]GnRH at 24° C for 30 minutes, after which the buffer was removed and the dishes were immediately UV irradiated for 90 seconds using a TR 312 A Spectroline transilluminator from a distance of 4 cm. All procedures prior to UV exposure involving photoreactive peptides were performed under dim lighting. The cells were detached with a rubber scraper, homogenised in binding buffer (section 2.5) with 20 strokes in a Dounce homogeniser and centrifuged at 4° C for 10 minutes (400 x g) to remove the nuclei. The supernatant was centrifuged at 15000 x g for 30 minutes at 4° C and the membrane pellet resuspended in 0.2 mL binding buffer per original dish of cells by drawing it through a fine needle.

4.2.2.2 Localisation of crosslinking site

4.2.2.2.1 GnRH receptor cDNAs: The crosslinking sites of photoreactive GnRH analogs were investigated by studying the proteolytic products of wild type and mutant GnRH receptors. In addition to those whose synthesis is described in this work, the following wild type and mutant GnRH receptors cDNAs were used; human (Chi et al. 1993; Davidson *et al.,* 1996), sheep (Illing *et al.,* 1993), mouse (Tsutsumi *et al.,* 1992), mouse E90Q, E111Q, E8Q, E294Q mutant receptors (Flanagan *et al.,* 1994).

4.2.2.2.2 Deglycosylation and Glu-C cleavage: Membrane suspensions containing photoaffinity labelled receptors were solubilised and denatured by addition of 0.5% (final concentration) SDS and heating at 60° C for 10 minutes. The SDS concentration was then decreased to 0.2% by dilution with binding buffer (section 2.5) and a five-fold molar excess of Triton X-100 was added. Deglycosylation was performed with 5 units/mL peptide N-glycosidase-F (Boehringer-Mannheim, Cat. No. 1643037) for 18 hours at 37°C. Selected samples were further digested with 200 μg/mL endoproteinase Glu-C (V8 proteinase Boehringer-Mannheim, Cat. No. 791156) for a further 18 hours at 37° C. Samples were finally dissolved in SDS sample buffer (section 2.5) and electophoresed on 10% or 16% SDS-polyacrylamide gels (Schagger and Von Jagow, 1987). The gels were fixed (see section 2.5) for 30 minutes and then stained with Coomassie Blue for 4 hours. After destaining for 2 hours, gels were dried and autoradiographed using Kodak, X- OMAT autoradiography film exposing for 2-14 days at -70° C. Molecular masses of labelled bands were determined by graphical

interpolation from plots of Rf *vs.* log molecular weight for standard proteins. The fixing, staining and destaining steps were avoided in instances where pre-stained molecular mass markers were used.

4.3 Results

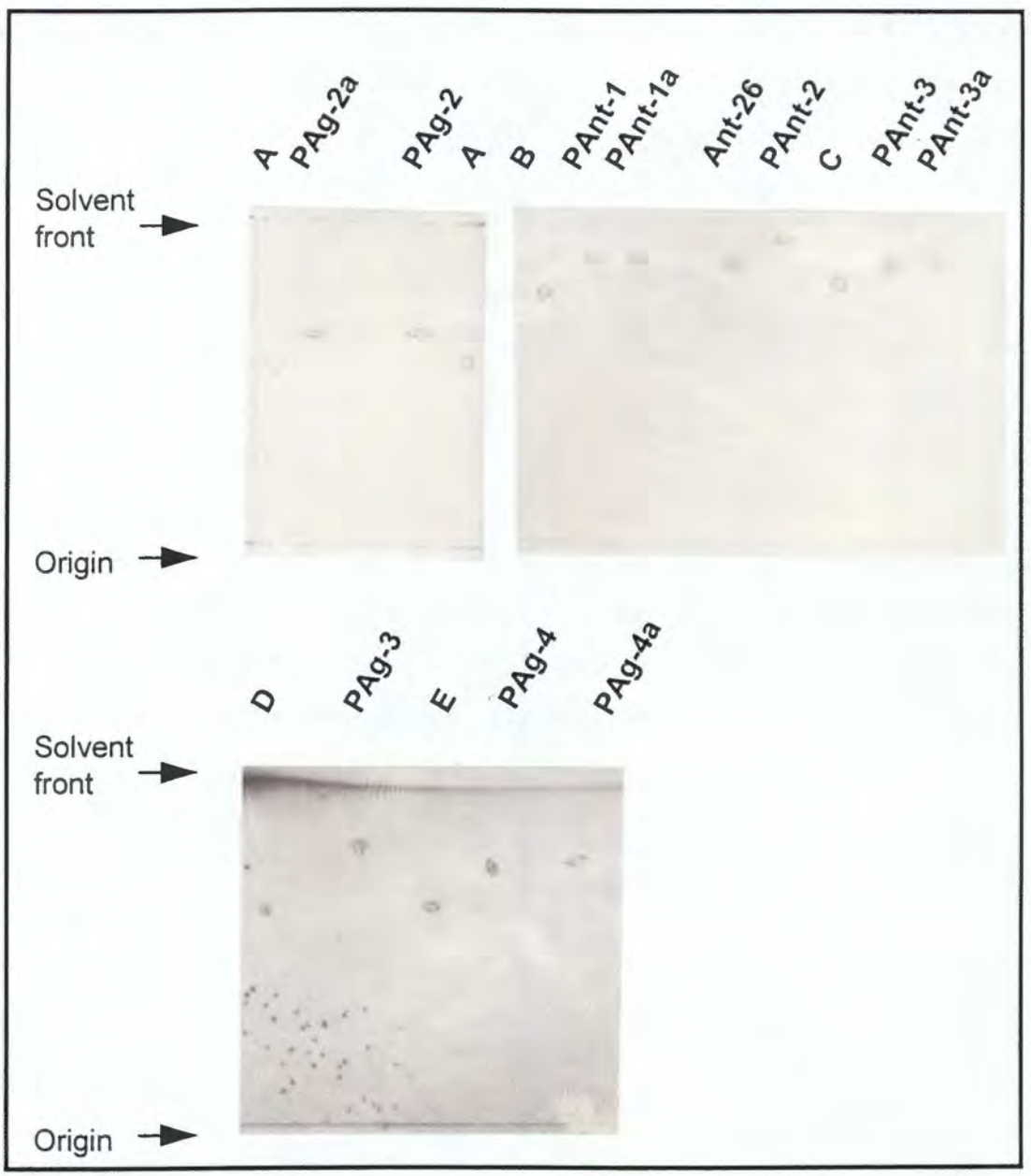
4.3.1 Synthesis and absorbance spectra of photoreactive derivatives of GnRH

Photoreactive derivatives were made from six GnRH analogs for the purpose of studying their interaction with the GnRH receptor. The synthesis of all the photoreactive derivatives was performed in an identical manner and the results are described below (section 4.3.2). Following this, characterization of each of the peptides will be described separately.

4.3.1.1 Properties of synthesized photoreactive GnRH analogs

Ten photoreactive GnRH analogs were synthesized as described in section 4.2.1. Analysis by thin layer chromatography (TLC) followed by staining with iodine vapour showed, clearly circumscribed spots representing either the parent peptide or the photoreactive derivatized species. In each case, the results indicated that the coupling reaction between the peptide and 4-azidobenzoic acid- or 4-azidosalicylic acid N-hydroxysuccinimide esters had gone to completion, and the parent peptide was not detectable by TLC of the products (Figure 4.3.1). All the photoreactive derivatives exhibited an increase in mobility on TLC when compared to the parent peptides as a result of the increased hydrophobicity conferred to them by the addition of the azidobenzoyl or azidosalicylate groups (Table 4.3.1). The new derivatives were designated as indicated in Table 4.3.1.

Figure 4.3.1 TLC analysis of parent and derivatized peptides: Three micrograms of peptide in 50% methanol/water was spotted on a TLC plate and run using a 1:1:1:1 butanol, acetic acid, ethylacetate and water solvent. The TLC plates were stained with iodine vapour. Some of the stained spots and the solvent front have been marked to aid visualisation. A = [D-Ala^6, Orn^8]GnRH; B = [Ac-D-Lys^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-AlaNH$_2$^{10}]GnRH; C = [Ac-D-Nal^1, D-Dpr^2, D-Trp^3, D-Arg^6]GnRH; D = [L-Dpr^3, D-Ala^6]GnRH and E = [L-Dpr^2, D-Ala^6]GnRH. Amino acids sequences for the derivatised peptides are given in Table 4.2.2.



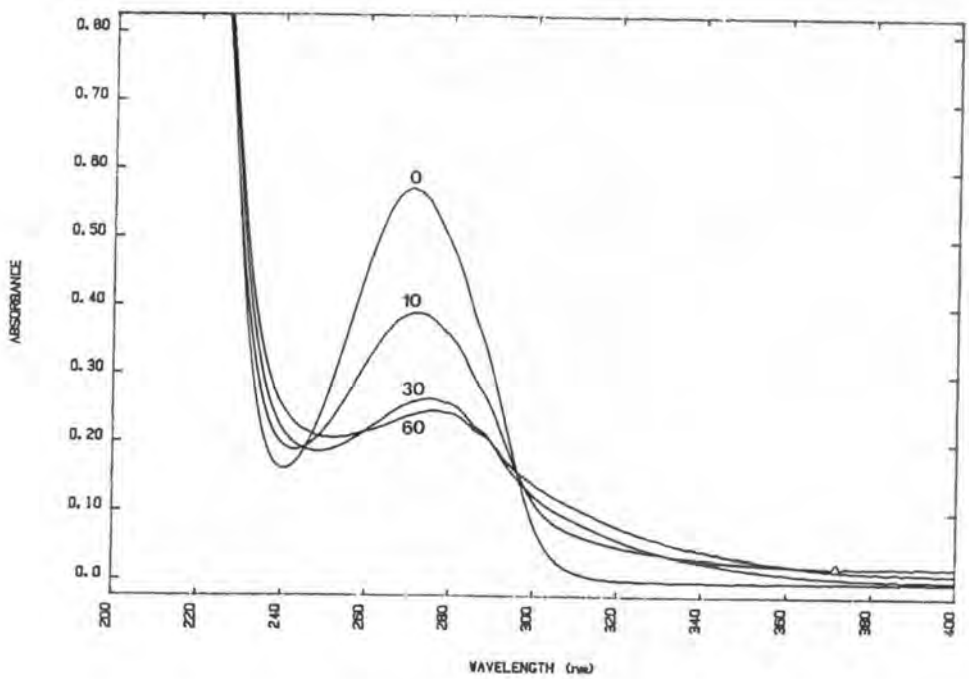
UV absorbance spectra were obtained on the derivatized peptides. The photoreactive analogs showed a small shift in the wavelength of their absorbance peaks when compared to the parent peptides (Table 4.3.1). These peaks of maximum absorbance were abolished or greatly decreased after the peptides were exposed to UV irradiation (Figures 4.3.2 - 4.3.7), confirming their photoreactivity.

Table 4.3.1 Relative mobilities on TLC (R_f) of parent peptides and their photoreactive derivatives: R_f values were measured relative to the solvent front and the wavelength of maximum absorbance of the peptides are indicated.

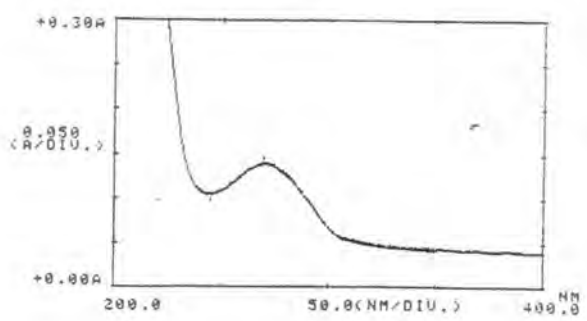
Peptide	R_f	*Maximum Absorbance (λ) nm*
[Ac-D-Lys^1, D-4-Cl-Phe^2, D-Trp^3, D-Arg^6, D-AlaNH$_2$ ^{10}]GnRH	0.75	277
PAnt-1	0.86	271
PAnt-1a	0.86	270
Antagonist 26	0.87	276
PAnt-2	0.94	275
[Ac-D-Nal^1, D-Dpr^2, D-Trp^3, D-Arg^6]GnRH	0.81	276
PAnt-3	0.85	273
PAnt-3a	0.85	274
[D-Ala^6, Orn^8]GnRH	0.55.	275
PAg-2	0.64	285
PAg-2a	0.66	280
[L-Dpr^2, D-Ala^6]GnRH	0.66	275
PAg-3	0.80	273
[L-Dpr^3, D-Ala^6]GnRH	0.65	275
PAg-4	0.77	273
PAg-4a	0.77	274

Figure 4.3.2 Absorbance spectra of PAnt-1 and PAnt-1a: The peptides at 40 μM concentration were irradiated with UV light. Absorbance was measured before and after UV irradiation. a) Absorbance spectra of PAnt-1 before (0) and after 10, 30 and 60 seconds of irradiation. b) Absorbance spectum of PAnt-1a before irradiation. c) Absorbance spectrum of PAnt-1a after irradiation for 30 seconds.

a)



b)



c)

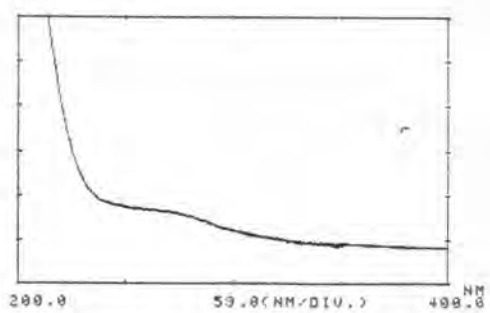


Figure 4.3.3 Absorbance spectrum of PAnt-2: Absorbance spectra of 30 μM PAnt-2 before (0) and after 10, 30 and 60 seconds of UV irradiation.

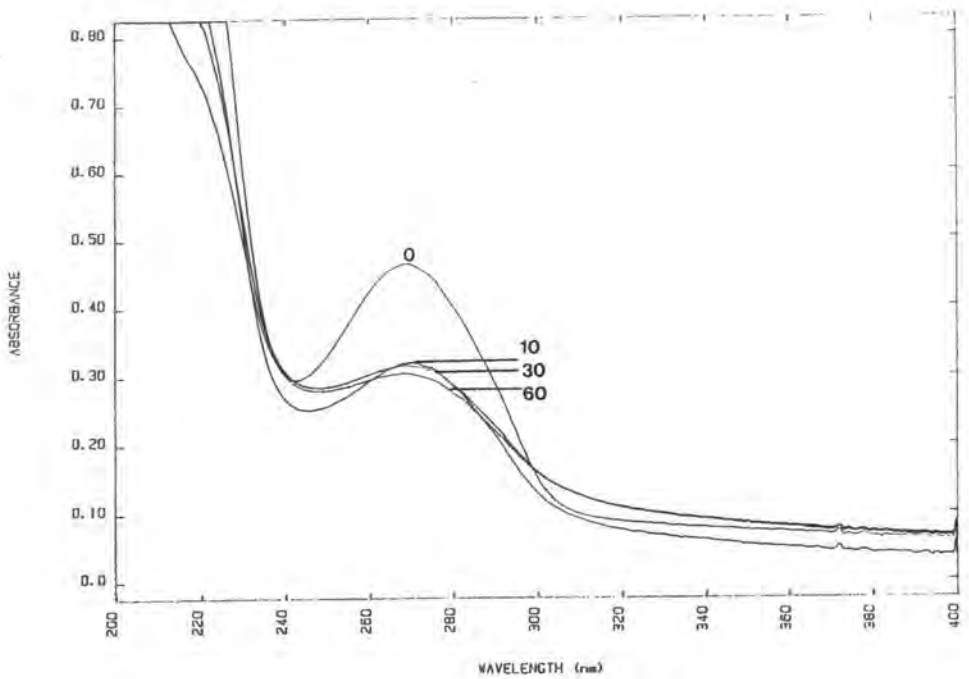
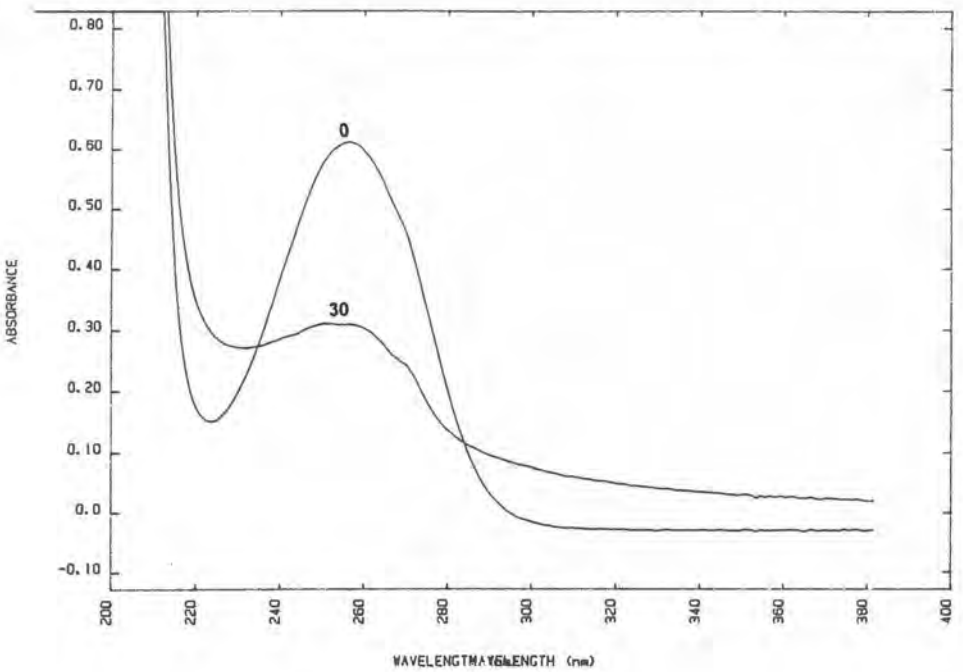


Figure 4.3.4 Absorbance spectra of PAnt-3 and PAnt-3a: Absorbance spectra of 40μM of PAnt-3 (a), and PAnt-3a (b), before (0), and after (30) seconds of, UV irradiation.

a)



b)

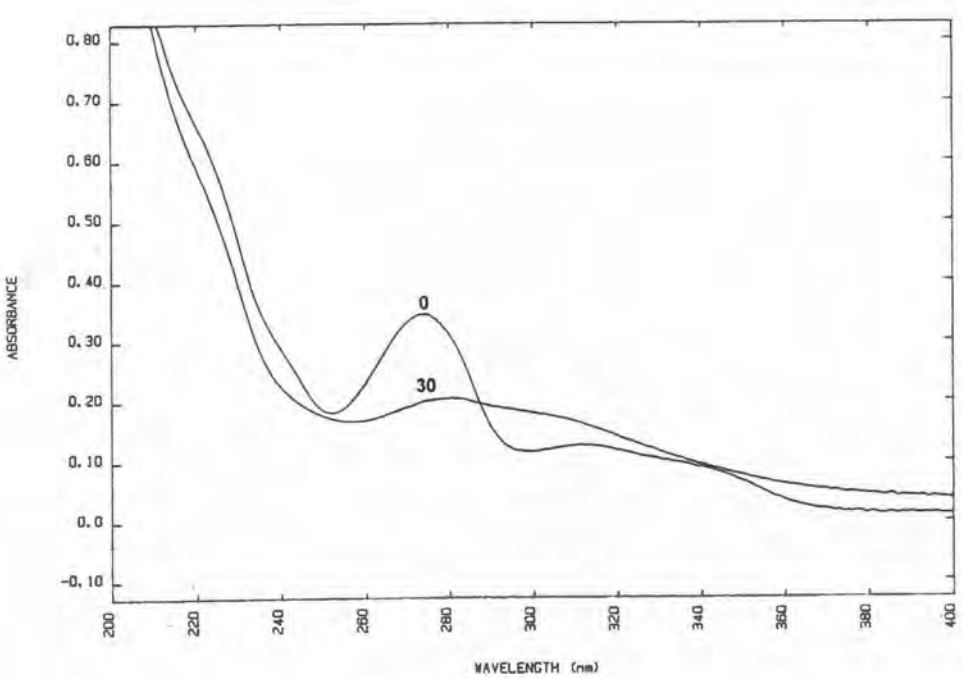


Figure 4.3.5 Absorbance spectra of PAg-2 and PAg-2a: Absorbance spectra of 40 μM PAg-2 before (a), and after (b), 30 seconds of UV irradiation; and of 40 μM PAnt-2a before (c), and after (d), 30 seconds of UV irradiation.

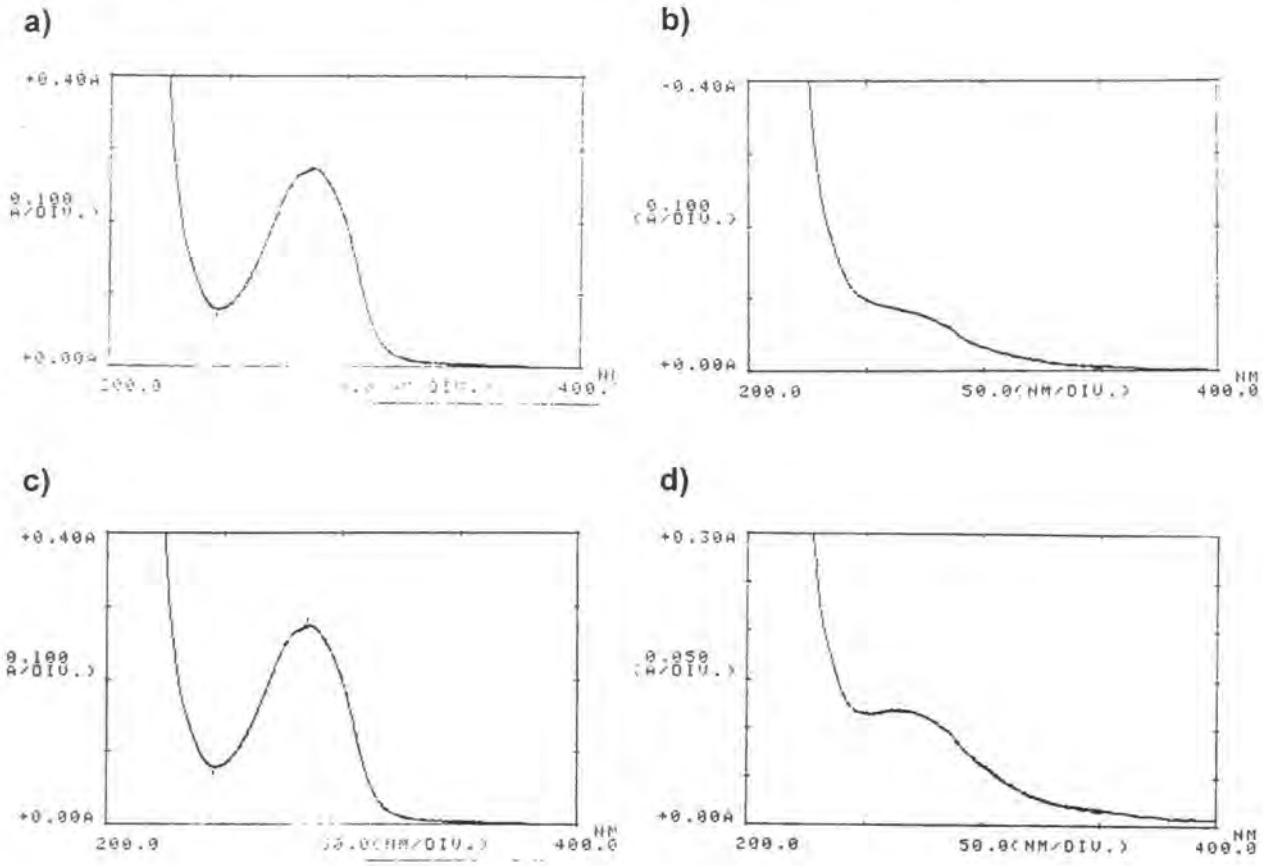


Figure 4.3.6 Absorbance spectrum of PAg-3: Absorbance spectra of 40 μM PAg-3 before (0) and after (30) thirty seconds of UV irradiation.

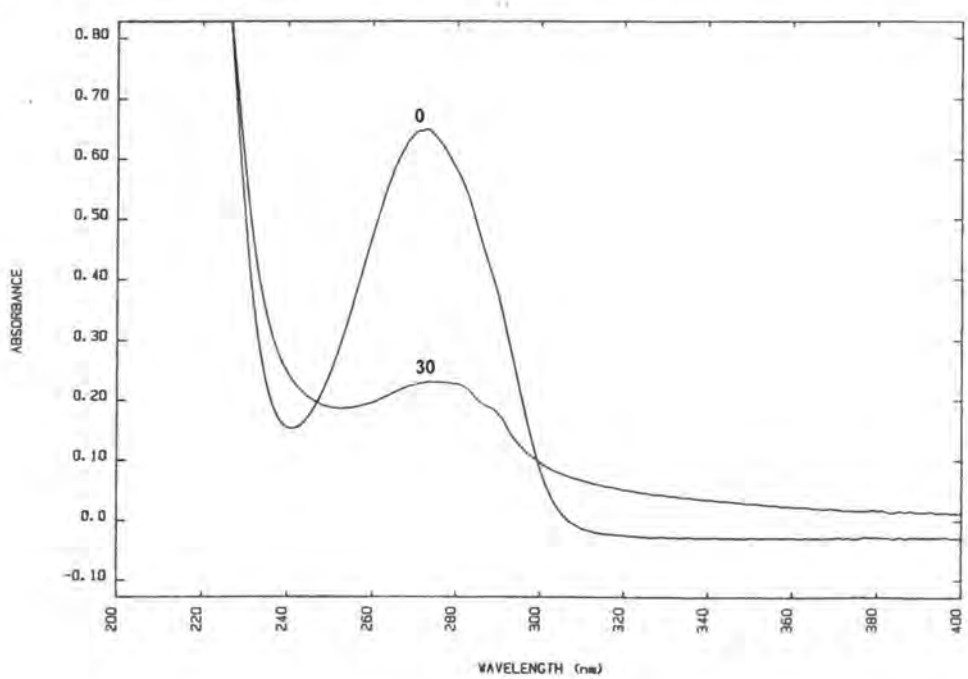
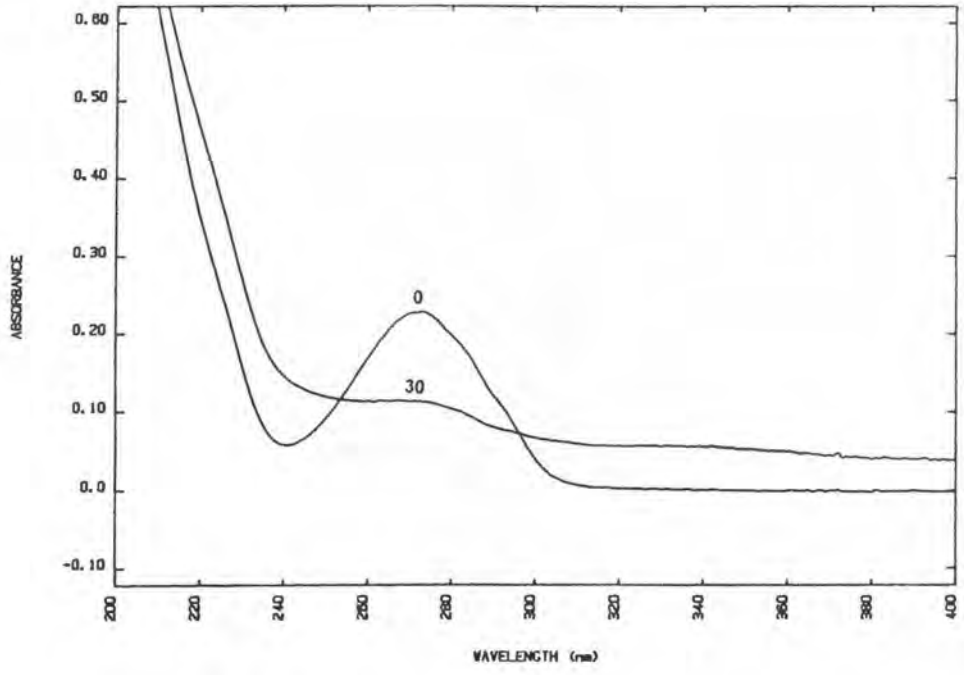
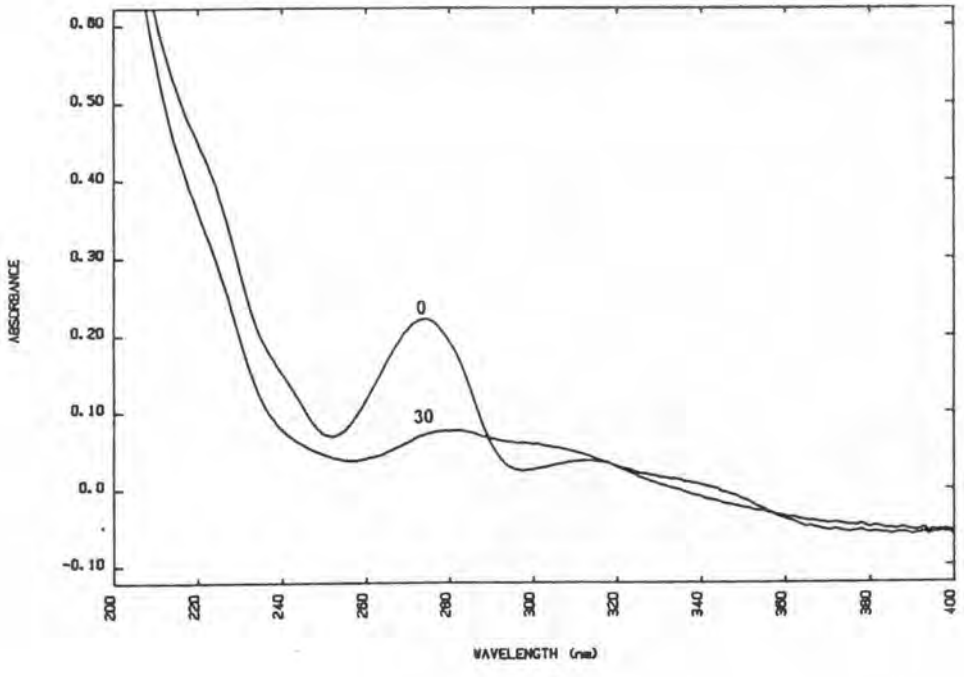


Figure 4.3.7 Absorbance spectra of PAg-4 and PAg-4a: Absorbance spectra of 40 μM PAg-4 (a) and 40 μM PAg-4a (b) before (0) and after (30) thirty seconds of UV irradiation.

a)



b)



4.3.1.2 Time - course of photolysis of photoreactive analogs

To define the length of time needed for complete photolysis of photoreactive derivatives, PAnt-1 and PAnt-2 were irradiated with UV at λ = 312 nm from a distance of 4 cm for various lengths of time. As figures 4.3.2a and 4.3.3 show, the photolysis was complete in both peptides after irradiation for 30 seconds. A similar time-course was also observed for PAg-2 and PAg-2a (results not shown).

4.3.1.3 The photolysis reaction in solution involves interaction with Trp^3

The absorbance spectrum of GnRH shows a maximum absorbance at 277 nm which is not affected by UV irradiation under conditions specified above (section 4.2.2.1) (Figure 4.3.8). A similar absorbance peak at 275 to 277 nm was also observed for all the parent peptides used for the synthesis of photoreactive analogs (Table 4.3.1). GnRH and most of these peptides contain tryptophan at position 3. In contrast, [Leu^3] GnRH, which lacks tryptophan, exhibits a much smaller absorbance peak at 275 nm (Figure 4.3.9) indicating that the peak is due mainly to the Trp^3 residue. The decrease of this peak in all Trp^3 containing photoreactive derivatives after UV irradiation suggests that the photolysis of these peptides in solution involves an intra- or inter-molecular interaction with the Trp^3 residue, with disruption of the UV-absorbing ring structure. The smaller peak at 275 nm in [Leu^3]GnRH is probably due to Tyr^5, and in PAg-4 and PAg-4a the azidobenzoyl- and asidosalicyl- groups may also contribute to it.

Figure 4.3.8 Absorbance spectrum of GnRH: Absorbance spectrum of 80 μM GnRH before (0) and after (30) thirty seconds of UV irradiation.

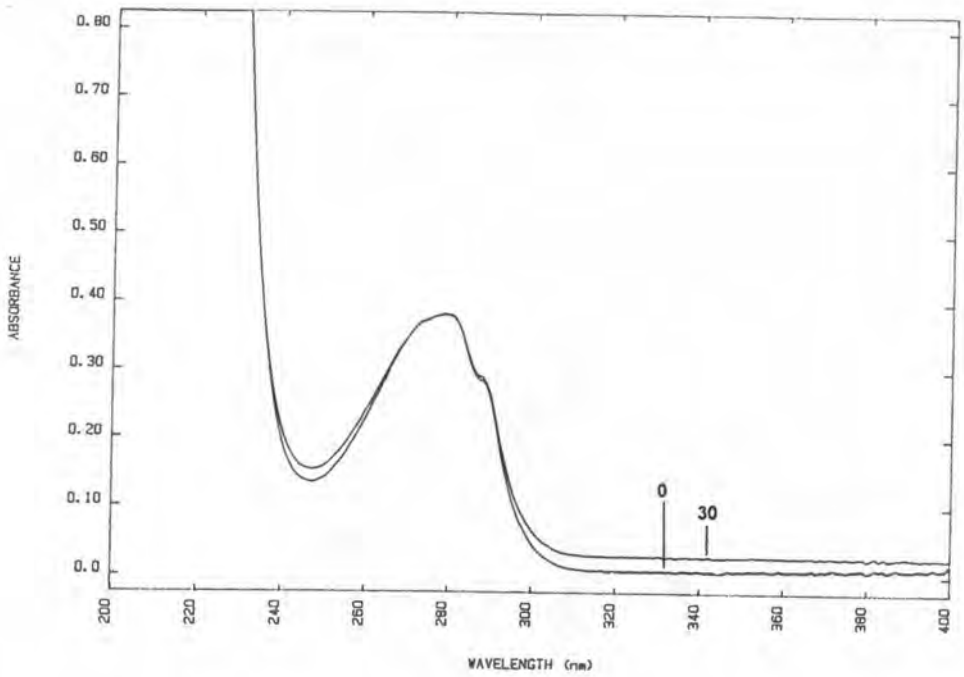
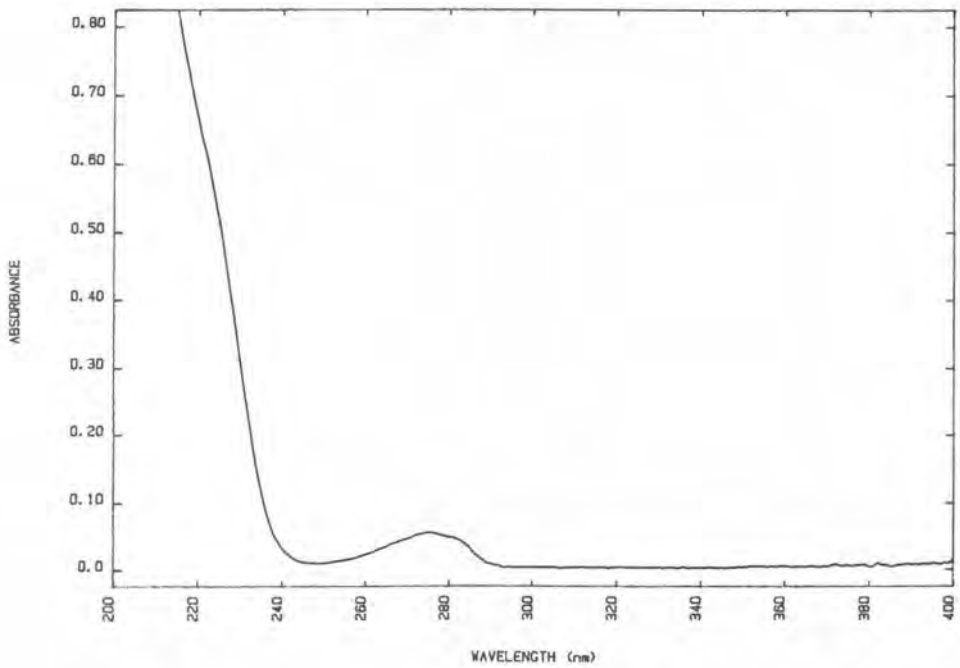


Figure 4.3.9 The absorbance spectrum of [Leu^3] GnRH: Absorbance spectrum of 80 μM [Leu^3]GnRH.



4.3.2 Evaluation of effect of UV irradiation on GnRH receptor function

Because crosslinking of the photoreactive peptides to the GnRH receptor requires UV irradiation it was necessary to evaluate the possible damaging effect of such irradiation on the cells. The effect of UV irradiation on the function of GnRH receptors was investigated by comparing GnRH-stimulated inositol phosphate production in αT3-1 cells after UV irradiation (λ=312 nm, 4 cm, for 60 seconds) with that seen in non-irradiated cells (Figure 4.3.10). At 100 nM GnRH, IP production in UV irradiated cells was 93.4 ± 1.3% ($p < 0.05$)(mean ± SEM, n = 4 independent experiments) of that of non-irradiated controls. The number and affinity of binding sites for [His^5,^{125}I-D-Tyr^6]GnRH on UV irradiated and non-irradiated αT3-1 cells was also compared. The Kd for binding of [His^5,^{125}I-D-Tyr^6]GnRH was 3.31 nM ± 0.54 nM for the UV irradiated cells which is not significantly different from the value of 4.05 nM ± 0.12 nM for the their non-irradiated controls (mean ± SEM, n = 3 independent experiments) (Figure 4.3.11). The maximum radioligand binding in the UV irradiated cells in the absence of competing ligand was 101 ± 4.5 % of the non-irradiated controls. These data show that photoaffinity labelling can be performed using UV radiation at the specified conditions without significantly damaging the GnRH receptor or its signalling mechanisms.

Figure 4.3.10 Effect of UV irradiation on inositol phosphate production: GnRH- stimulated inositol phosphate production was measured in αT3-1 cells pre-irradiated with UV for 60 seconds (+UV) and non irradiated (-UV) cells. Cells were stimulated for 10 minutes with 100 nM GnRH in the presence of Li^+. Bars represent normalized data from four independent experiments each assayed in duplicate. Values are expressed as % of inositol phosphate production in non-irradiated cells.

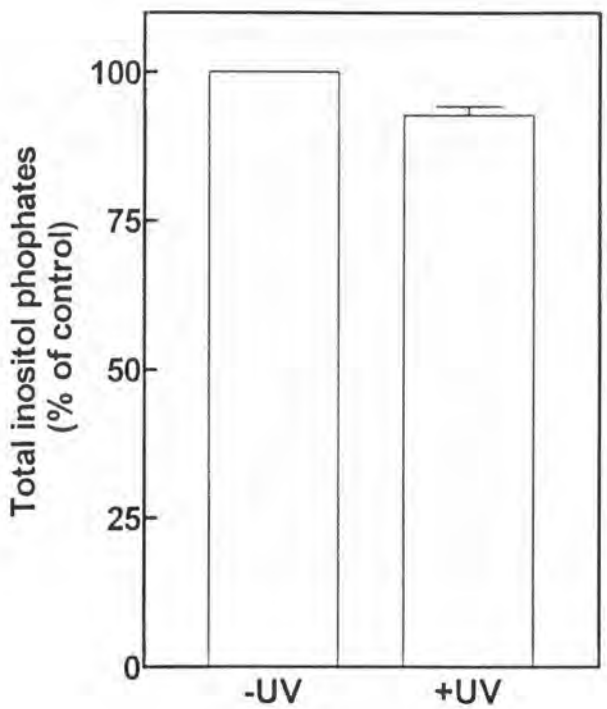
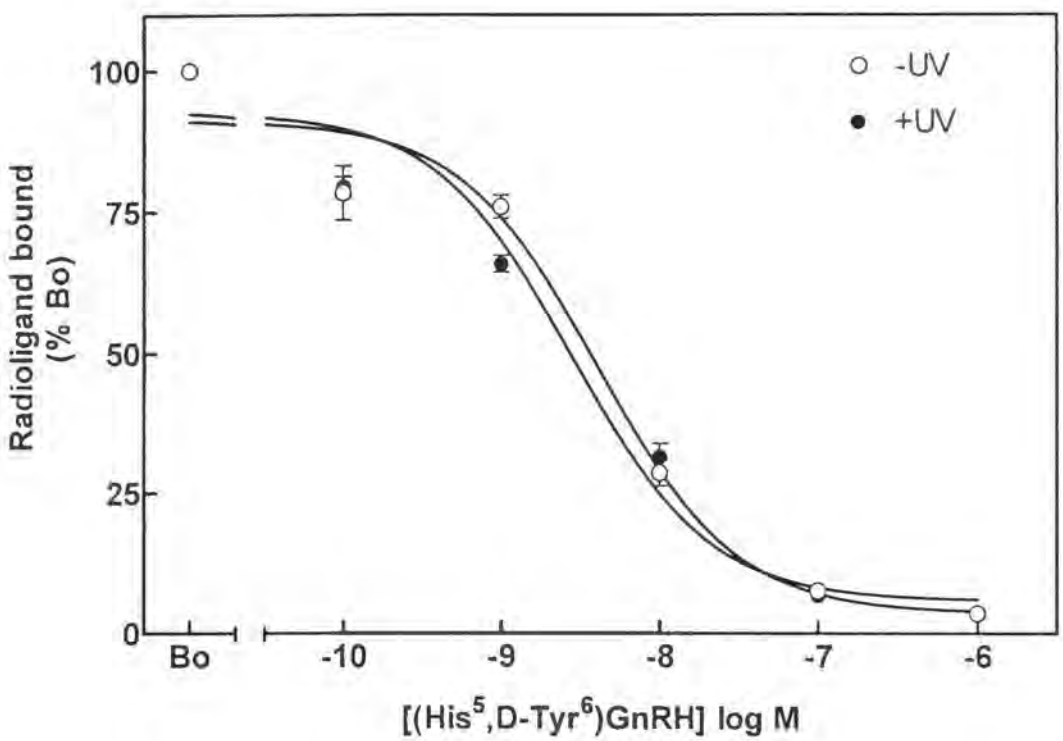


Figure 4.3.11 Receptor binding of [His^5,^{125}I-Tyr^6]GnRH to UV irradiated and non-irradiated αT3-1 cells: UV irradiated (+UV) and non irradiated (-UV) αT3-1 cells were incubated with various concentration of unlabelled [His^5,Tyr^6]GnRH in the presence of 10^5 c.p.m of [His^5, ^{125}I-Tyr^6]GnRH tracer. After 5 hours of incubation at 4°C, unbound ligand was washed off and the remaining radioactivity was measured as described in section 2.5.1.2b. Data points indicate normalized data (mean ± SEM) derived from three independent experiments each performed in duplicate. Bo represents radioligand binding in the absence of competing ligand and is taken as 100%.

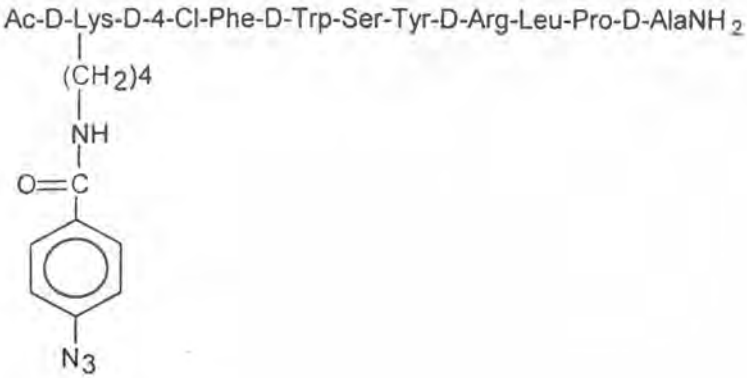


4.3.3 Characterization of PAnt-1

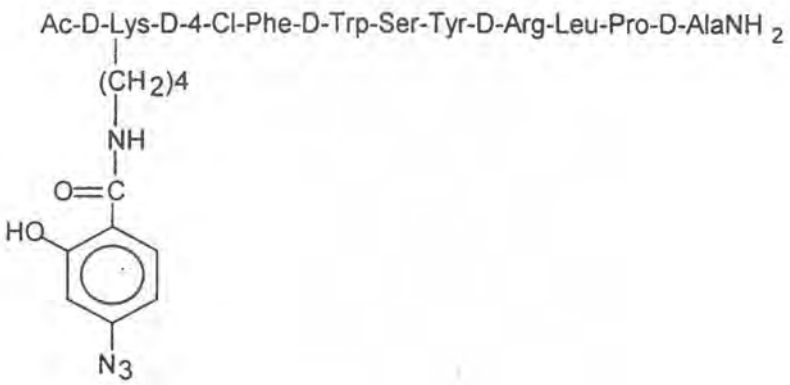
PAnt-1 and PAnt-1a have their photoreactive group on Lys^1 at the N-terminus of the peptide (Figure 4.3.12). Residues at these positions in GnRH and its analogs are involved in the high affinity binding of these peptides to the GnRH receptor (Gupta *et al.*, 1993;.Millar *et al.*, 1984). It follows that if a photoreactive group at this site crosslinks to the receptor, it is likely to attach to a residue in or near ligand binding pocket of the receptor.

Figure 4.3.12 Structures of PAnt-1 (a) and PAnt-1a (b): The side chain of Lys^1 and its attachment to the photoreactive group is shown.

a) PAnt-1



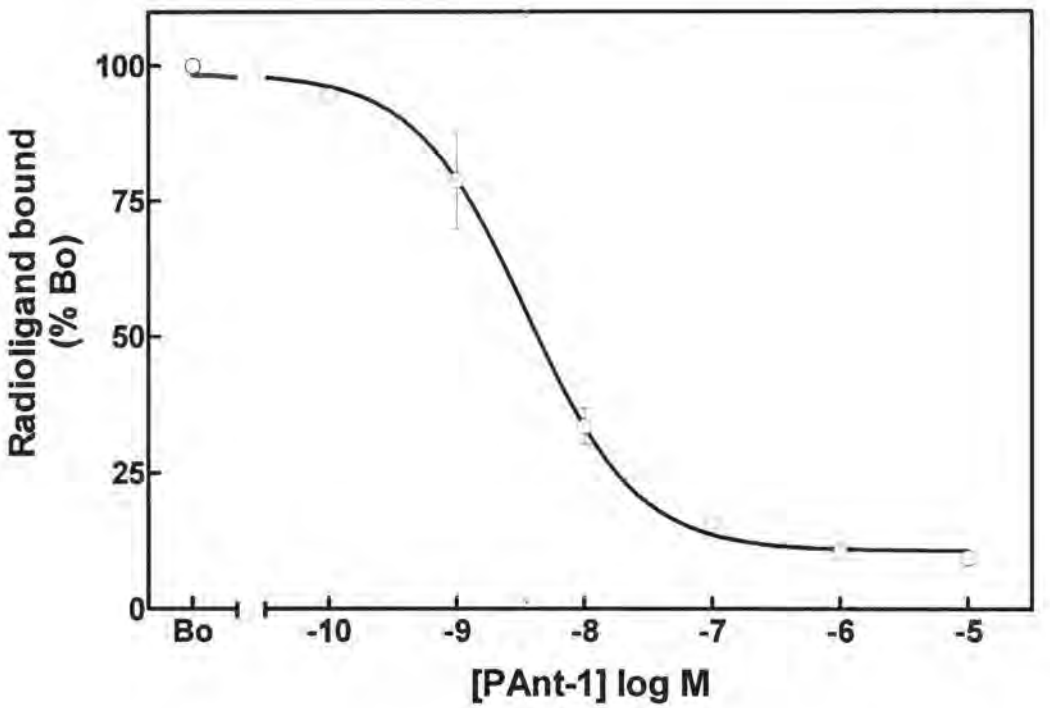
b) PAnt-1a



4.3.3.1 Pant-1 has high binding affinity for the GnRH receptor

PAnt-1 showed high receptor binding affinity, as shown by competitive binding experiments using GnRH receptors endogenously expressed in αT3-1 cells (Figure 4.3.13). Unlabelled PAnt-1 displaced the radioligand [His^5,^{125}I-D-Tyr^6]GnRH with Ki = 3.1 ± 0.8 nM (mean ± SD, n = 2 experiments). This shows that the introduction of the bulky hydrophobic azidobenzoyl group at the N-terminus of the peptide did not result in loss of affinity for the GnRH receptor.

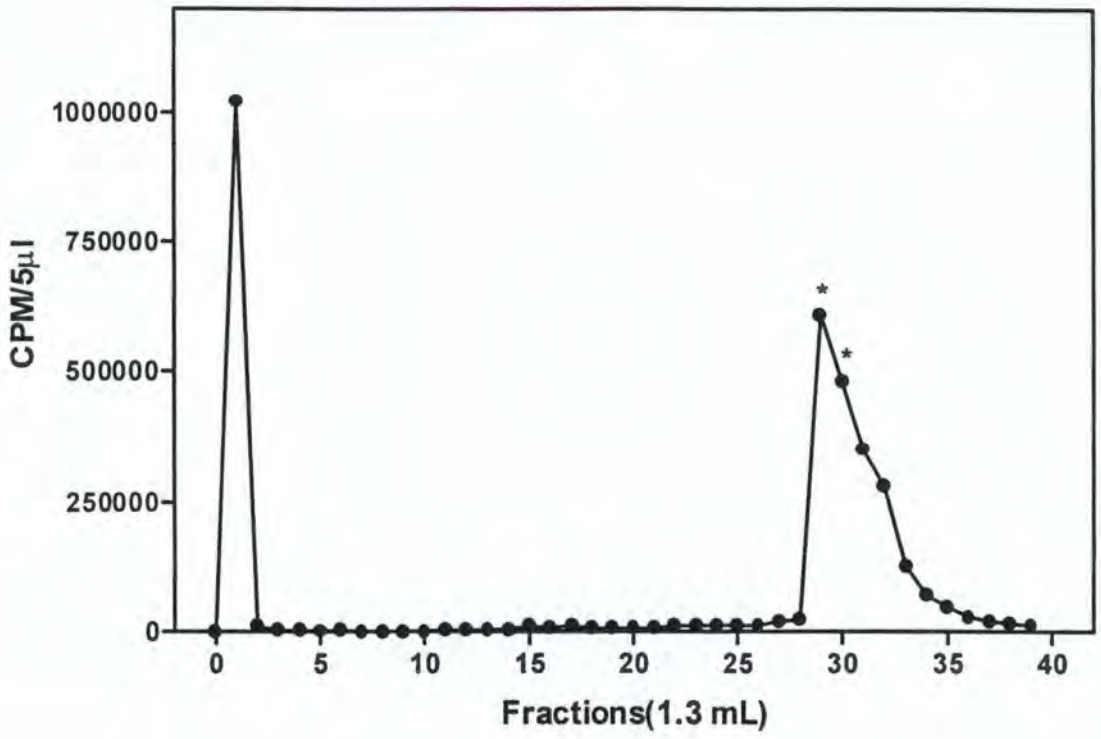
Figure 4.3.13 Competitive binding of PAnt-1: [His^5, ^{125}I-D-Tyr^6]GnRH binding to αT3-1 cell membrane preparations, displaced with increasing concentrations of unlabelled PAnt-1 as described in section 2.4.1.1. Data points are means ± SEM of 2 experiments performed in triplicate. "Bo" represents radioligand binding in the absence of a competing ligand and is taken as 100%.



4.3.3.2 PAnt-1 crosslinks to the GnRH receptor

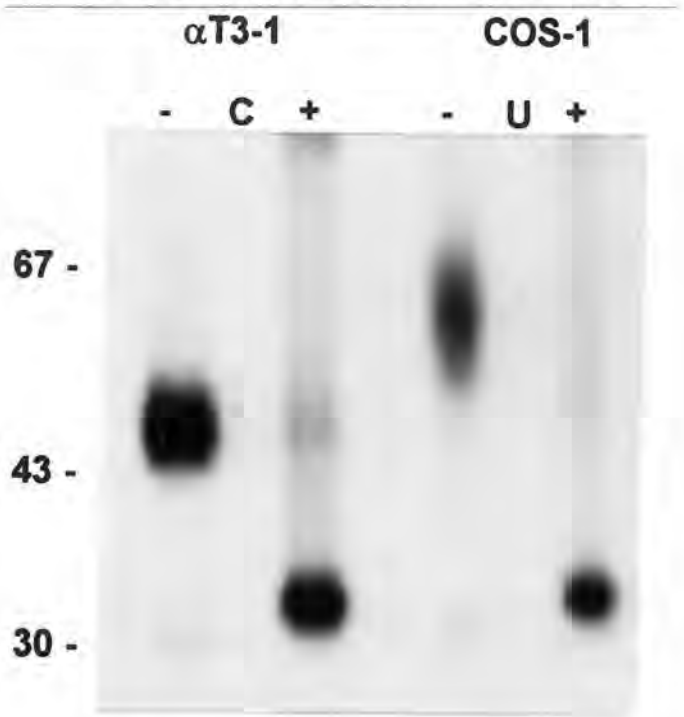
The high affinity of a photoreactive ligand does not by itself guarantee UV dependent crosslinking which is also dependent on the proximity of suitable groups on the receptor to the reactive group of the ligand. In order to determine whether PAnt-1 crosslinks to the GnRH receptor in a specific manner, PAnt-1 was radioiodinated with ^{125}I (Figure 4.3.14) and used for photoaffinity labelling of GnRH receptor expressing cells.

Figure 4.3.14 HPLC purification of [^{125}I]PAnt-1: [^{125}I]PAnt-1 was radioiodinated as described in section 2.1.2 and purified using a C-18 reverse phase HPLC column, eluting with a linear gradient (0-80%) of acetonitrile/0.01M ammonium acetate (pH 4.6). The first peak represents free ^{125}I, and fractions marked (*) were stored in the dark and used for photolabelling experiments.



UV-induced crosslinking of [^{125}I]PAnt-1 to GnRH receptors expressed in αT3-1 cells or COS-1 cells transiently transfected with mouse GnRH receptor cDNAs, followed by SDS-PAGE and autoradiography revealed specific labelling of broad bands with apparent molecular weights of approximately 45 kDa in αT3-1 cells and 63 kDa in COS-1 cells (Figure 4.3.15). These bands represented GnRH receptors, as (a) they were absent in non-transfected COS-1 cells, and (b) labelling of the 45 kDa band was displaced by excess (1 µM) unlabelled [D-Trp^6, Pro^9-ethylamide] GnRH agonist in αT3-1 cells. The size difference of the receptors observed in the two cell types was due to differential glycosylation, as deglycosylation of the labelled membranes with peptide N-glycosidase F resulted in visualisation of the GnRH receptors as sharp bands of 32 kDa in both cell types (Figure 4.3.15). Bands of identical size were seen after photoaffinity labelling with the agonist PAg-1 as reported by this laboratory (Davidson *et al.*, 1997).

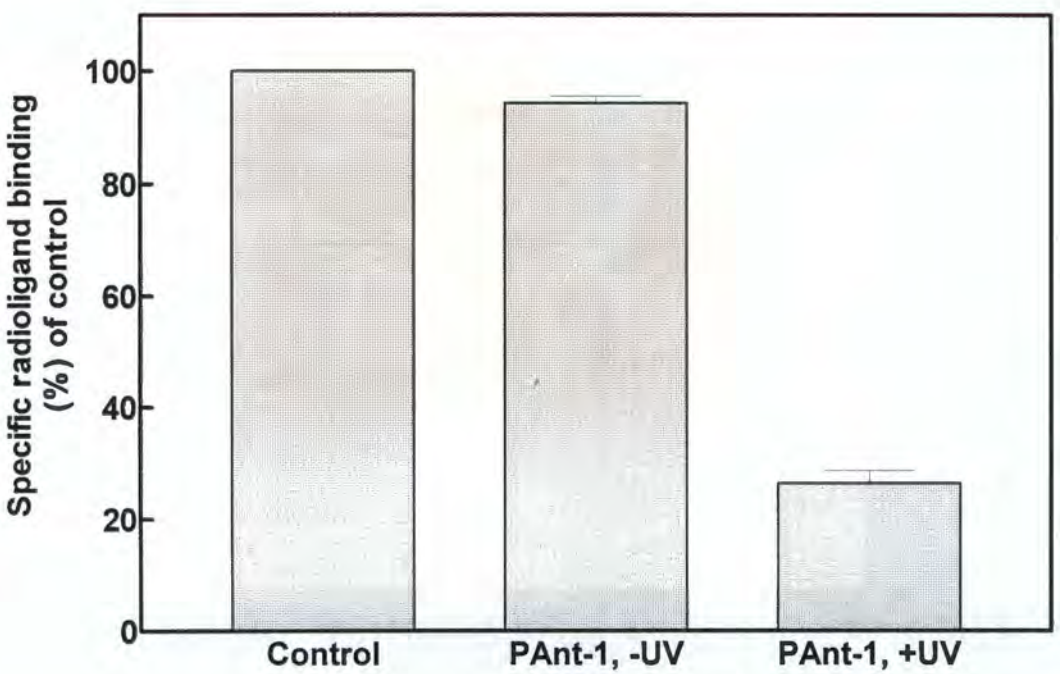
Figure 4.3.15 Photoaffinity labelling with [^{125}I]PAnt-1 and deglycosylation of GnRH receptors: [^{125}I]PAnt-1 was incubated for 30 minutes in the dark with intact αT3-1 cells, or with intact COS-1 cells transfected with mouse GnRH receptor (mGnRHR) cDNAs, then photolyzed with UV irradiation. Membranes were prepared as described in section 2.4.1. Labelled membranes before (-) and after (+) deglycosylation with N-glycosidase F (section 4.2.2.2.2) were solubilized and electrophoresed on a 10% SDS-PAGE gel, and autoradiographed. Cells labelled in the presence of 1μM unlabelled [D-Trp^6, Pro^9-ethylamide]GnRH are marked "C" and untransfected COS-1 cells "U".



4.3.3.3 PAnt-1 crosslinks to the GnRH receptor with a high efficiency in a functionally relevant manner

The efficiency of cross-linking of PAnt-1 with the GnRH receptor was estimated by quantification of the irreversible loss of binding sites for the radioligand [^{125}I-D-Tyr^5, D-Ala^6, N-Meth-Leu^7, Pro^9-ethylamide]GnRH. Intact αT3-1 cells were incubated in the presence or absence of 100 nM of unlabelled PAnt-1, then UV irradiated and extensively washed to remove noncovalently bound peptide. Crosslinking of PAnt-1 resulted in 76.3 % ± 3.2 % (mean ± SEM, n = 3) decrease in binding sites in cells which were irradiated with UV before washing, when compared to that of controls (Figure 4.4.5). Cells which were incubated with PAnt-1, but not exposed to UV light showed only a small decrease in binding sites (5.7% ± 1.7%) (mean ± SEM, n = 3), indicating that the crosslinking was UV-dependent, and demonstrating the adequacy of the washing procedure in removing non-covalently bound PAnt-1.

Figure 4.3.16 Loss of ligand binding sites on αT3-1 cells after crosslinking to PAnt-1: αT3-1 cells were pre-incubated in the presence(PAnt-1) and absence (Control) of 100 nM unlabelled PAnt-1 for 30 minutes. One group (+UV) was irradiated with UV prior to washing and all groups were washed extensively. Membrane preparations from these cells were then used to perform binding assay using [^{125}I-Tyr^5, D-Ala^6, N-Meth-Leu^7, Pro^9-ethylamide]GnRH (10^5 c.p.m). Specific binding is expressed as (%) of controls. Data represents normalised values from three independent experiments performed in triplicates. Non-specific binding was obtained by displacing radiolabelled ligand with 1 μM of unlabelled [D-Trp^6-Pro^9-ethylamide]GnRH.



The effect of PAnt-1 on GnRH receptor activation by agonist was assessed by measurement of GnRH-stimulated total inositol phosphate production in αT3-1 cells prelabelled with myo-[2-^{3}H] inositol. In the absence of UV irradiation PAnt-1 behaved as a competitive antagonist, causing an increase in the EC_{50} of GnRH for stimulation of IP production, which was overcome at high GnRH concentrations and was reversible by extensive washing before stimulation with GnRH (Figure 4.3.17). The K_i of non-photolysed PAnt-1 for the receptor was calculated based on its competitive inhibition of GnRH-stimulated IP production using the equation;

$$EC_{50}'/EC_{50} - 1 = [Antagonist]/K_i;$$

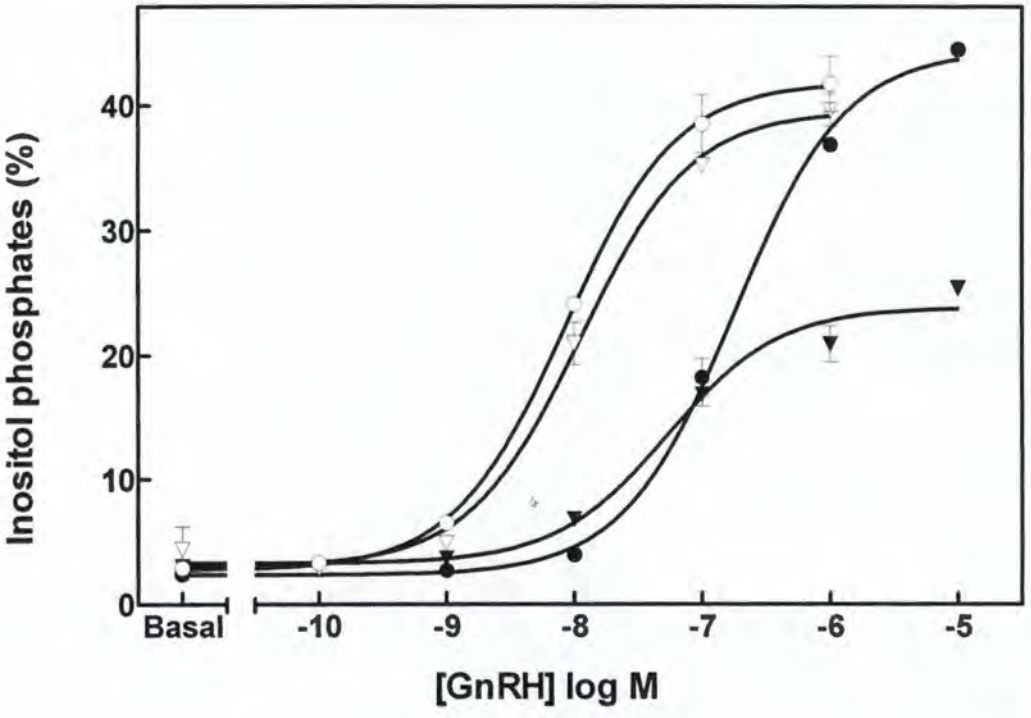
where EC_{50} and EC_{50}' are EC_{50} of agonist with and with out the presence of a fixed

concentration of antagonist respectively (Arunkakshana and Schild, 1947), and $K_i = K_i$ of the antagonist (Leslie, 1987). This yielded a value of 3.8 ± 0.4 nM (mean ± SEM, n=3), which was in good agreement with the K_i of 3.1 nM obtained from competitive binding data (Figure 4.3.13).

In contrast, PAnt-1 inhibited GnRH-stimulated inositol phosphate production in a non-competitive and irreversible manner when cells were preincubated with a saturating concentration (100 nM) of PAnt-1, followed by UV irradiation and extensive washing to remove noncovalently bound peptide (Figure 4.3.17). Crosslinking of saturating PAnt-1 caused an irreversible 57.9 ± 13.6 % decrease in maximal GnRH-simulated IP production and a 7.0 ± 0.06 fold increase in EC_{50} (mean ± SEM, n=3 independent experiments). The above sets of experiments indicate that PAnt-1 is high affinity, competitive antagonist of GnRH, with high crosslinking efficiency.

Figure 4.3.17 Inhibition of GnRH-stimulated inositol phosphate production by PAnt-1: αT3-1 cells pre-labelled with myo-[2-^{3}H]inositol were preincubated with 100 nM PAnt-1 (∇, ●, ▼) or without PAnt-1 (○), then UV irradiated (▼) or not irradiated (○, ∇, ●,). PAnt-1 was then removed by extensive washing (∇, ▼) or not washed off (●), after which cells were stimulated with GnRH in the presence of Li^+ and ^{3}H-inositol phosphate accumulation was determined. Data shown is from a single experiment representative of 3 independent experiments done in duplicate. Values are expressed as % of total inositol incorporated into cells. The following table summarises the experiment.

	Pre-incubation with PAnt-1	*UV irradiation*	*Wash*
	No	No	No
	Yes	No	Yes
	Yes	No	No
	Yes	Yes	Yes



4.3.3.4 Experiments demonstrating that PAnt-1 crosslinks to the N-terminus of the GnRH receptor between Gln^9 and Glu^{68}

The attachment site of PAnt-1 to the receptor was localized by peptide mapping of labelled fragments derived from proteolytic digestion of GnRH receptors crosslinked

with [^{125}I]PAnt-1. Natural sequence differences between human, sheep and mouse GnRH receptors, as well as a panel of mutant GnRH receptors with engineered protease cleavage sites, were used to identify the location of labelled fragments. Photoaffinity-labelled receptor preparations were deglycosylated and digested with endoproteinase Glu-C (V8 proteinase) which cleaves at the carboxyl terminus of glutamic acid residues. The resulting proteolytic fragments wereanalysed with a 16% SDS-PAGE tricine method developed for small peptides (Schagger and Von Jagow, 1987) and followed by autoradiography.

Deglycosylation and Glu-C cleavage of photoaffinity-labelled wild type mouse GnRH receptor from αT3-1 cells or transfected COS-1 cells yielded a labelled doublet of 13/14 kDa (Figure 4.3.18). The same two bands were observed in the mouse E111Q and E294Q mutants. In contrast, the mouse E8Q mutant yielded only the larger (14 kDa) fragment, and the smaller fragment was never recovered from this mutant receptor in three similar experiments. This indicates that the 13/14 kDa doublet results from partial cleavage at Glu^{8} (Figure 4.3.18 and 4.3.19), and identifies the N-terminal residue of the labelled 13 kDa fragment as Gln^{9}.

Removal of the Glu-C cleavage site at position 90 in the mouse E90Q mutant resulted in a doublet of ± 20 kDa upon Glu-C cleavage (Figure 4.3.18), indicating that the C-terminal residue of the 13 kDa labelled fragment is at Glu^{90}. These results identify the labelled 13 kDa fragment in the wild type mouse receptor as comprising residues Gln^{9}-Glu^{90}. The size of the labelled fragment (± 20 kDa) in the E90Q mutant is too large to be compatible with cleavage at E111, and suggests that the 20 kDa fragment is most likely the result of cleavage at Glu^{156}. The reason for the lack of cleavage at E111 may be the constrained conformation of this part of the polypeptide due to its proximity to the disulphide bridge at C114.

Figure 4.3.18 Endoproteinase Glu-C cleavage of photoaffinity labelled wild-type and mutant GnRH receptors: a) αT3-1 cells and COS-1 cells expressing wild type mouse (mWT), wild type human (hWT), or mutant GnRH receptors, were photoaffinity labelled with [^{125}I]PAnt-1. Membrane suspensions were solubilised, deglycosylated, digested with Glu-C and electrophoresed under reducing conditions as described in section 4.2.2.2. *Arrowheads* indicate the bands referred to in the text. A representative gel from three independent experiments is shown, b) schematic diagram showing origin of peptide fragments.

a)

b) Schematic diagram showing origin of fragments seen in (a).

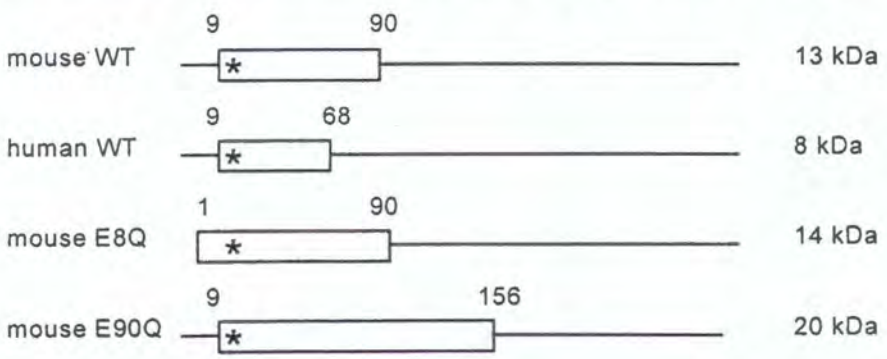
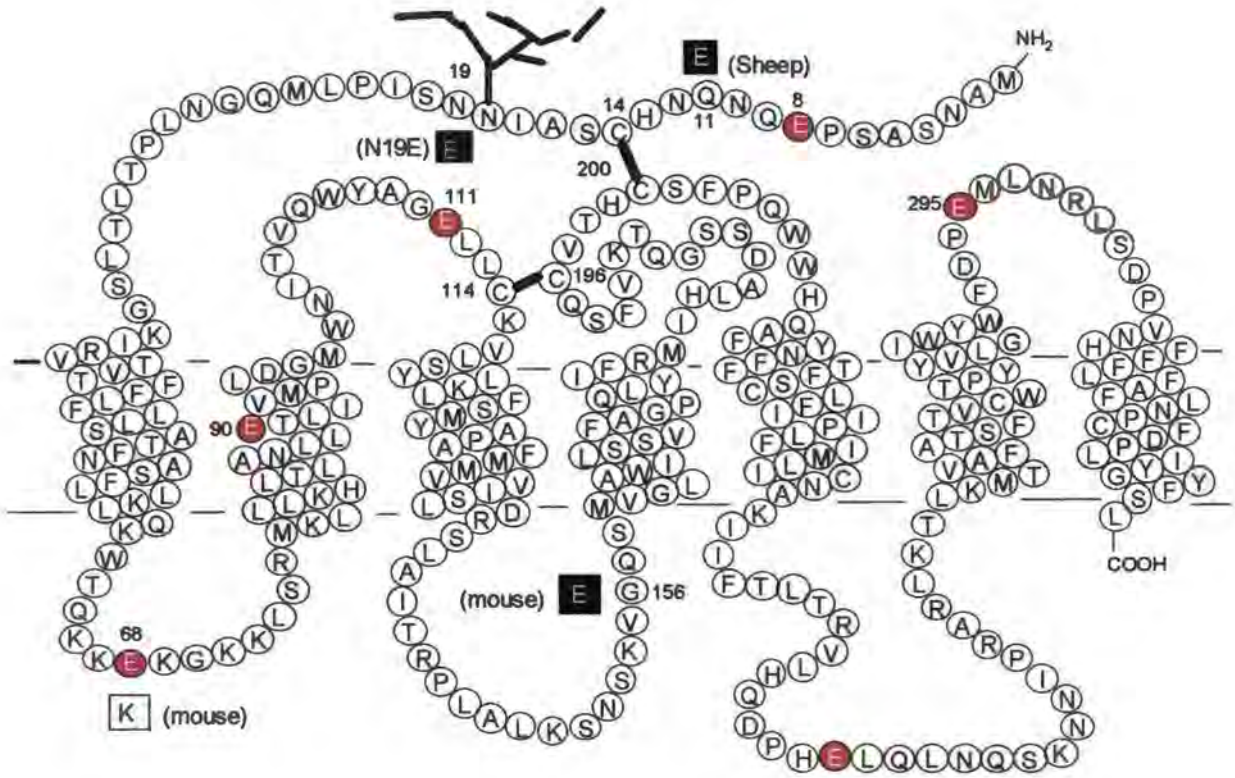


Figure 4.3.19 Amino acid sequence of human GnRH receptor: Potential Glu-C cleavage sites are coloured in red. Sequence differences in the mouse and sheep receptors, which were utilized in the study, are shown in squares. Residues referred to in the text are numbered.



The wild type human GnRH receptor differs from the mouse receptor in having a glutamic acid residue at position 68 as shown in figure 4.3.19. When PAnt-1-labelled wild type human receptor was digested with Glu-C, the resulting labelled band had a molecular size of approximately 8 kDa (Figure 4.3.18), consistent with a fragment consisting of residues Gln^{9} to Glu^{68}, which would be expected, assuming that the ligand crosslinks to the same site in both receptors.

4.3.3.5 Experiments demonstrating that PAnt-1 crosslinks between residues Asn^{12} and Asn^{18}

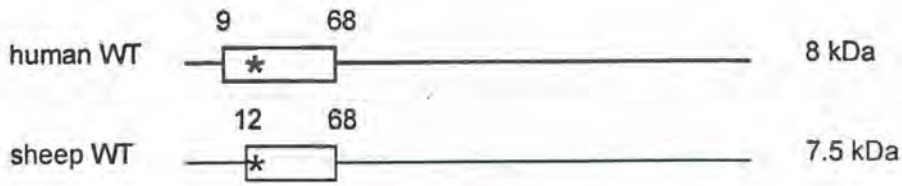
The wild type sheep GnRH receptor lacks the glutamic acid at position 8 and instead has a glutamic acid residue at position 11 (Figure 4.3.19). When the sheep receptor was digested with Glu-C, doublet labelled bands were seen at ± 7/8 kDa (Figure

4.3.20), the smaller band being slightly smaller than the Glu^{9} - Glu^{68} fragment derived from the human GnRH receptor. This indicates that the smaller band of the labelled doublet of the sheep GnRH receptor consists of residues Asn^{12} to Glu^{68}.

Figure 4.3.20 Glu-C cleavage of photoaffinity labelled wild type human and sheep GnRH receptors: (a) [^{125}I]PAnt-1 was crosslinked to COS-1 cells expressing wild type human and wild type sheep GnRH receptors. Membrane suspensions were solubilized, deglycosylated and digested with Glu-C (+) or not digested (-), then and electrophoresed under reducing conditions as described in section 2.4.2.2. *Arrowhead* indicates the 8 kDa labelled fragment referred to in the text. A representative experiment from three independently performed experiments is shown, (b) schematic diagram showing origin of peptide fragments.

a)

b) Schematic diagram showing origin of fragments seen in (a).



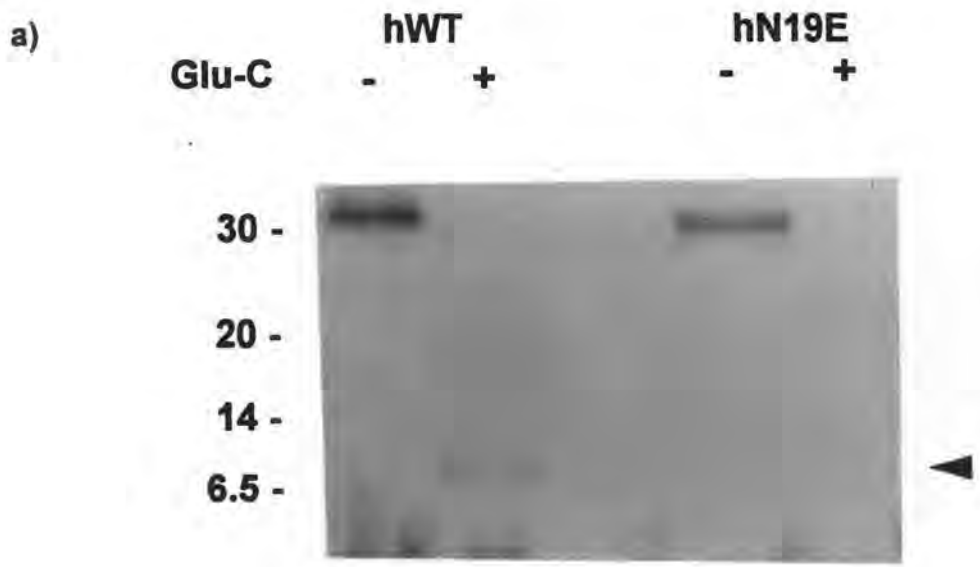
To further narrow down the attachment site of PAnt-1, a Glu-C cleavage site was introduced in the human GnRH receptor by mutation of Asn^{19} to Glu (hN19E). The ability of PAnt-1 to bind and crosslink to the hN19E mutant was tested by photoaffinity labelling of COS-1 cells expressing hN19E. When cells labelled with [^{125}I]PAnt-1 were analysed by SDS-PAGE/Autoradiography, a wide band with an average molecular mass of 63 kDa and of a comparable intensity was seen for both the wild type and hN19E mutant receptors (Figure 4.3.21). This indicates that PAnt-1 binds to the hN19E and crosslinks on UV irradiation, with similar efficiency compared to the wild type receptor.

Figure 4.3.21 SDS-PAGE analysis of photoaffinity labelled hN19E GnRH receptor: COS-1 cells expressing the modified human GnRH receptor (hWT*) which has two glycosylation sites (Davidson *et al.*, 1996) or hN19E mutant GnRH receptors were photoaffinity labelled with [^{125}I]PAnt-1 and solubilized membrane preparations were electophoresed on a 10% SDS-PAGE gel which was followed by autoradiography. In the control (C) cells expressing the wild type receptor, ^{125}I[PAnt-1] was displaced with 1 μM of GnRH.

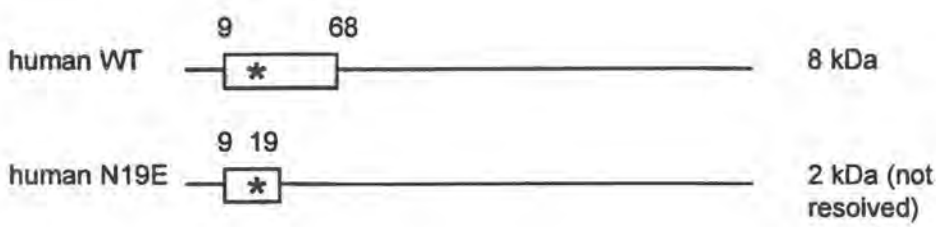
The incorporation of the N19E mutation allows the further digestion of the labelled Glu^{9} - Glu^{68} fragment which is expected to give two smaller fragments consisting of residues 9-19 and residues 20-68. Depending on where the radiolabelled PAnt-1 is attached and allowing for the size (± 1 kDa) of the bound peptide ligand, the expected

molecular weights of the two cleavage products are 2.1 and 5.6 kDa respectively. Glu-C cleavage of the hN19E mutant receptor labelled with [^{125}I]PAnt-1 resulted in a fragment too small to be resolved on the gel. A 5.6 kDa band, which would have been clearly resolved if it was labelled, was consistently not present (Figure 4.3.22), indicating that the fragment labelled was the smaller Gln^{9} - Glu^{19} peptide. Residue 19 can be excluded as the attachment site, because the attachment of a peptide to this residue (Glu19) in the hN19E mutant would abolish its recognition by Glu-C as a cleavage site, which was not the case (Figure 4.3.22). These findings taken together localize the crosslinking site of PAnt-1 on the GnRH receptor to a region consisting of eight amino acids between residues 12 and 18 (Figure 4.3.19).

Figure 4.3.22 Glu-C cleavage of photoaffinity labelled wild type human and hN19E mutant GnRH receptors: (a) [^{125}I]PAnt-1 was crosslinked to COS-1 cells expressing wild type human (hWT) and hN19E GnRH receptors. Membrane suspensions were solubilised, deglycosylated, digested with Glu-C (+) or not digested (-), and electrophoresed under reducing conditions as described in section 4.2.2.2. *Arrowhead* indicates the 8 kDa fragment referred to in the text, (b) schematic diagram showing origin of peptide fragment.



b) Schematic diagram showing origin of fragment seen in (a).

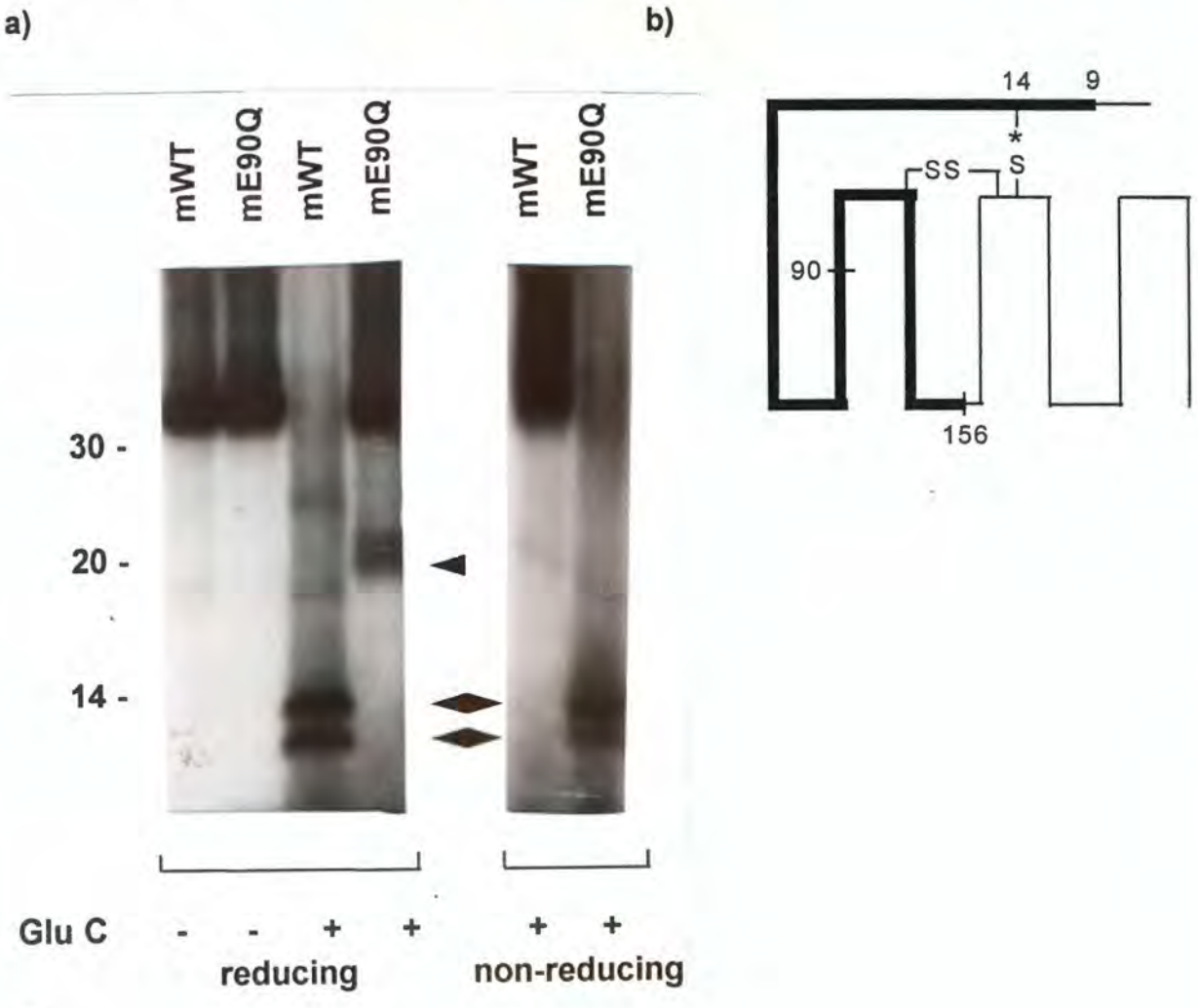


4.3.3.6 Evidence for the presence of C114-C196 disulphide bond.

Information on the presence of a disulphide bond was obtained using the same peptide mapping strategy under non-reducing conditions. PAnt-1-crosslinked mouse GnRH receptors were cleaved with Glu-C under non-reducing conditions, then electrophoresed under reducing (5% β-mercaptoethanol) and non-reducing (no β-mercaptoethanol) conditions (Figure 4.3.23). The wild type mouse GnRH receptor gave the same 13/14 kDa doublets when electrophoresed under reducing and non-reducing conditions (Figure 4.3.23). This result indicates that the C14-C200 bridge

either does not exist or was cleaved in the course of the photolysis reaction. In contrast, the mE90Q mutant yielded a ± 20 kDa band in reducing conditions which was absent in non-reducing conditions, and the smallest cleavage product from the mE90Q receptor, visualised under non-reducing conditions, was a ± 32 kDa band similar in size to the intact deglycosylated receptor protein (Figure 4.3.15). This result demonstrates directly the presence of the C114-C196 disulphide bridge (See discussion, section 4.4)

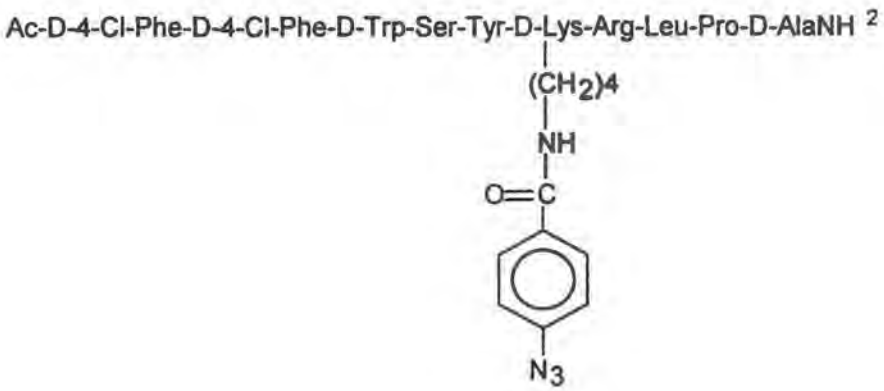
Figure 4.3.23 SDS-PAGE analysis of mouse GnRH Glu-C fragments under reducing and non-reducing conditions: (a) [^{125}I]PAnt-1 was crosslinked to COS-1 cells expressing wild type mouse and mE90Q GnRH receptors. Membrane suspensions were solubilised, deglycosylated, digested with Glu-C (+) or not digested (-), and electrophoresed under reducing or non-reducing conditions as described in section 2.4.2.2. *Arrowheads* indicate the 13/14 kDa doublet and the 20 kDa fragment referred to in the text. (b) Schematic diagram showing the 20 kDa fragment (thick line) linked to the rest of the receptor under non-reducing conditions.



4.3.4 Characterization of PAnt-2

PAnt-2 was derivatized from the high affinity antagonist, antagonist 26 (Table 4.2.1). This peptide contains a D-lysine residue at position 6 (Figure 4.3.24). The sixth residue is located at the βII type bend in the "horse-shoe" conformation in GnRH and its analogs ((Monhan *et al.*, 1973; Grant and Vale, 1972; Momany, 1976, 1978; Kopple, 1981). Thus, introduction of a bulky photoreactive group at this site was considered unlikely to interfere with the binding affinity which is primarily dependent on the N-and C-termini of the peptide (Gupta *et al.*, 1993;.Millar *et al.*, 1984).

Figure 4.3.24 Structure of PAnt-2: Side chain of Lys^6 and its attachment to the photoreactive group is shown.



4.3.4.1 PAnt-2 binds with the GnRH receptor in an "irreversible" manner.

Measurement of the efficiency of crosslinking by PAnt-2 was attempted by measuring the degree of irreversible inhibition of stimulated IP production as a result of photolabelling αT3-1 cells with PAnt-2 and washing off any un-crosslinked peptide. As shown in Figure 4.3.25, at 100 nM, PAnt-2 caused a complete inhibition of GnRH-stimulated IP production indicating PAnt-2 has high affinity and behaves as an antagonist. After incubation with PAnt-2 followed by UV irradiation about 80% inhibition was resistant to washing. However in those control cells where PAnt-2 was washed off without being exposed to UV, a comparable degree (70%) of inhibition was observed indicating inability to wash off the peptide was not due to UV-induced crosslinking. Similar results were obtained even after vigorous washes at various temperatures were tested in an attempt to increase the off rate of the peptide. This

indicates that an adequate wash could not be performed to remove PAnt-2 completely. Thus, the contribution of the UV-induced covalent attachment of PAnt-2 to the GnRH receptor leading to irreversible inactivation of the receptor could not be determined. A similar problem was also observed for the parent peptide Antagonist 26, which also showed a component of "irreversible" binding resistant to washing (results not shown).

Figure 4.3.25 Inhibition of GnRH-stimulated IP production by PAnt-2 in αT3-1 cells: 100 nM GnRH was used to stimulate four groups of αT3-1 cells. These are, cells not exposed to PAnt-2 (control), cells co-incubated with 100 nM PAnt-2 (+ PAnt-2), cells pre-incubated with 100 nM PAnt-2 and washed (PAnt-2 washed) and cells pre-incubated with PAnt-2 followed by UV irradiation and washing (UV washed) and washed afterwards. GnRH-stimulated IP production was measured. A summary of the experiment is shown below each bar. One of two experiments performed in duplicate is shown.

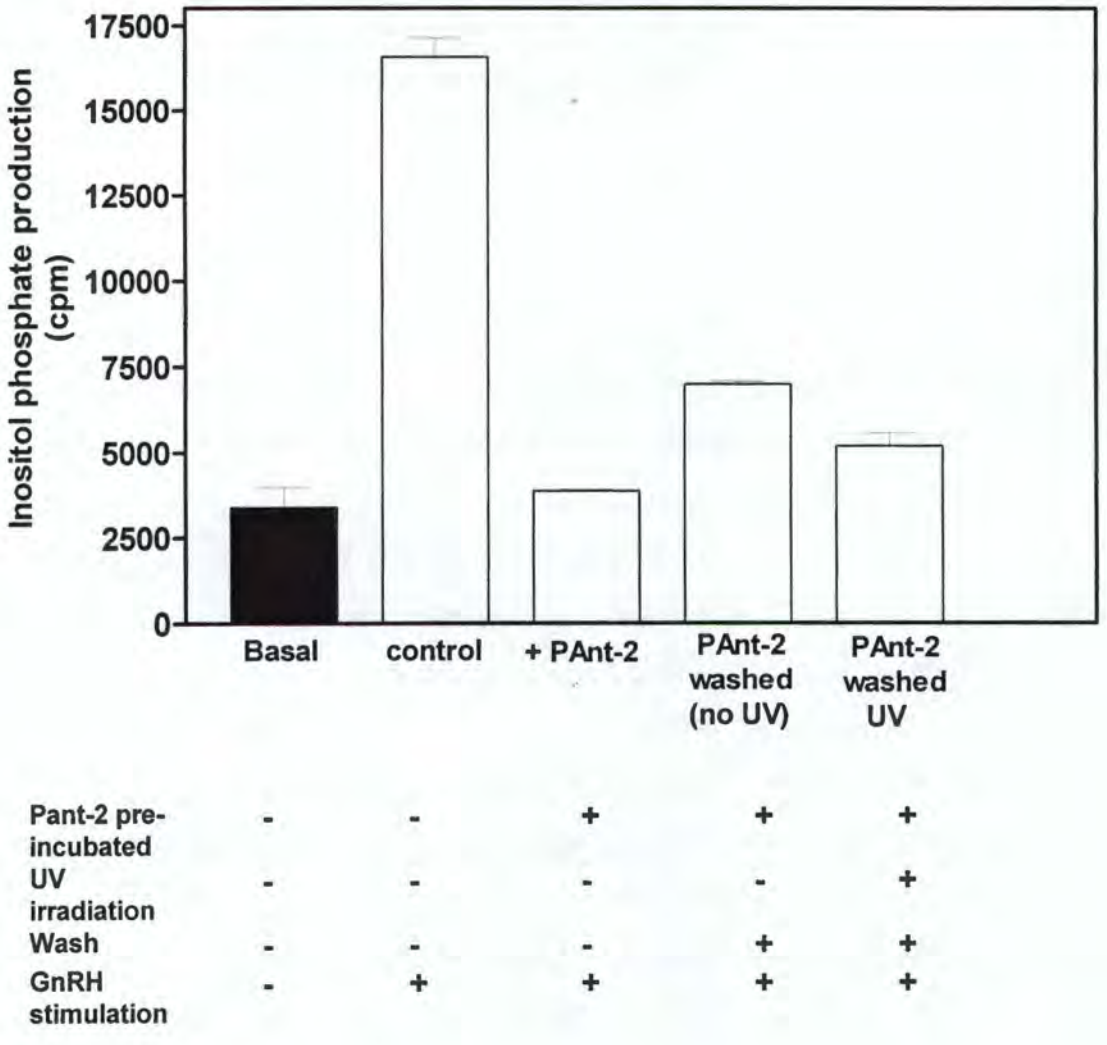
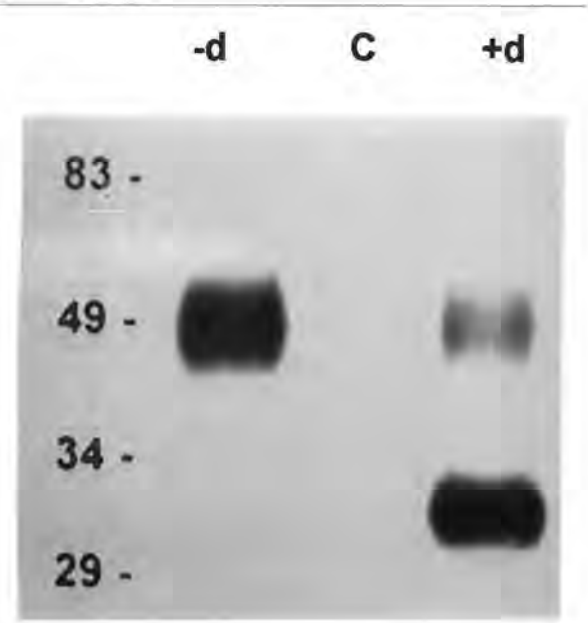


Figure 4.3.27 SDS/PAGE autoradiograph of αT3-1 cells photoaffinity labelled with ^{125}I-[PAnt-2]: αT3-1 cells were photoaffinity labelled with ^{125}I-[PAnt-2]. Cells were homogenzed and solubilized membrane fractions were electorphoresed on 10% SDS/PAGE and autoradiographed before (-d) and after (+d) deglycoslylation. Control cells (C) were co-incubated with excess unlabelled [D-Trp^6, Pro^9-ethylamide]GnRH before UV irradiation.

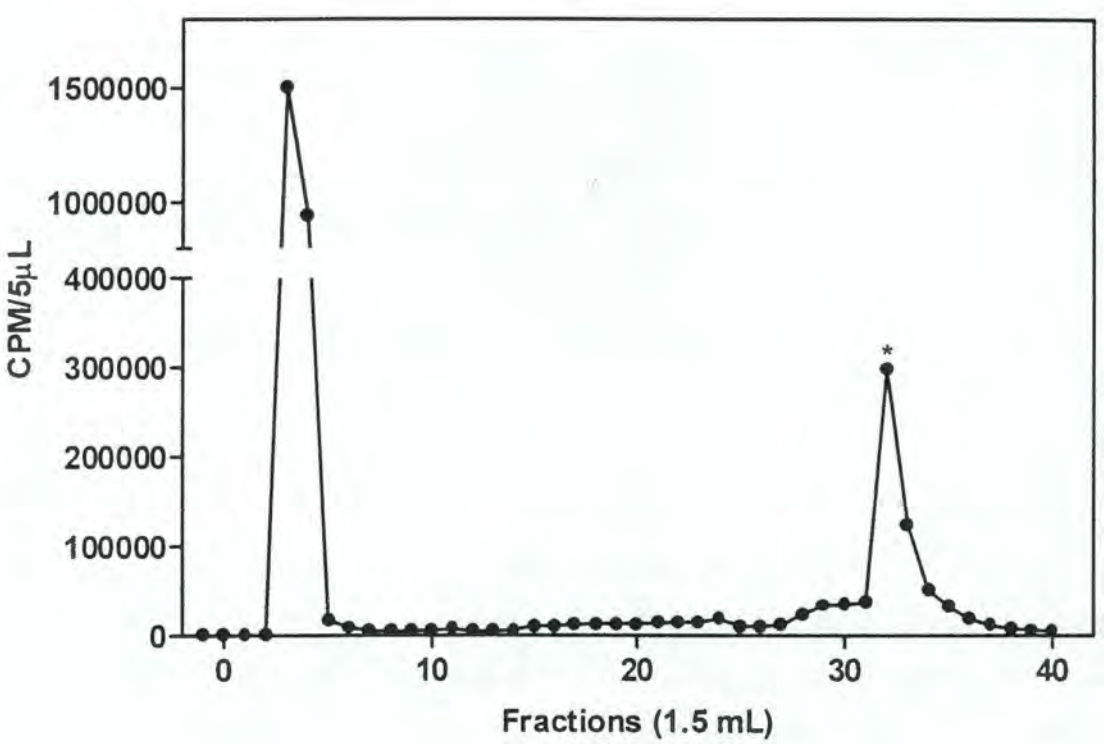


As stated in section 4.3.4.1, the tight association of PAnt-2 with the receptor even in the absence of UV irradiation makes it difficult to attribute the labelling of the 45 kDa band (Figure 4.3.27) completely to UV-dependent covalent crosslinking. In order to investigate whether the association between the receptor and PAnt-2 is covalent or not, photoaffinity labelled membrane preparations from αT3-1cells were thoroughly washed, resuspended in binding buffer and incubated for 0, 14 and 26 hours at 37°C to an attempt to promote dissociation of ligand after which SDS-PAGE/autoradiography was performed. Both the 14- and 26- hours samples of deglycosylated as well as non-deglycosylsted membrane preparations showed strong labelling of GnRH receptor (Figure 4.3.28). In addition, measurement of radioactivity (not shown) in each of the bands did not reveal any decrease in both the non-deglycosylated 14- and 26- hours samples but a small (16%) decrease was seen in the 26 hour deglycosylated samples compared to 0 hour values. This favours the conclusion that covalent labelling was achieved by UV irradiation since a non-covalent

4.3.4.2 PAnt-2 crosslinks to the GnRH receptor

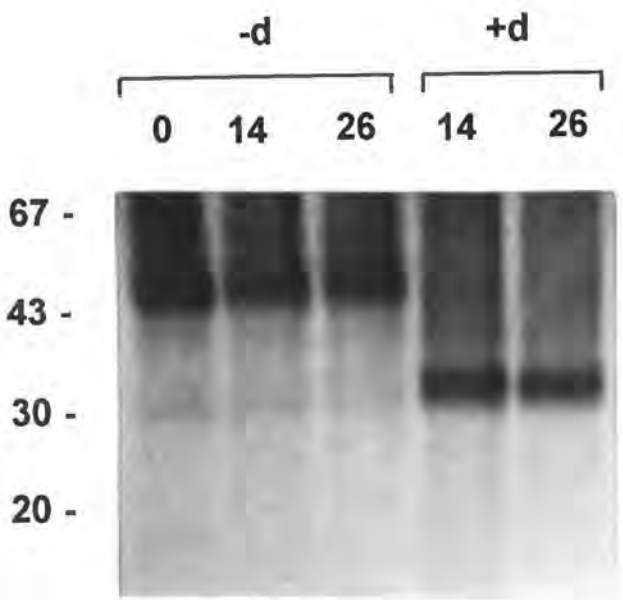
PAnt-2 was iodinated as described in section 2.1.2 (Figure 4.3.26). αT3-1 cells were preincubated with ^{125}I-[PAnt-2] in the presence or absence excess unlabelled [D-Trp^{6}, Pro^{9}-ethylamide]GnRH then irradiated with UV. Analysis of the labelled products by SDS-PAGE followed by autoradiography showed a labelled band at 45 kDa (Figure 4.3.27) which was abolished by excess unlabelled ligand. Deglycosylation of labelled membranes with N-glycosidase F resulted in a smaller and sharper band of 32 kDa corresponding to the molecular size of the non-glycosylated GnRH receptor (Figure 4.3.15). These results indicate that PAnt-2 binds and photolabels the GnRH receptor in a specific manner.

Figure 4.3.26 HPLC purification of [^{125}I]PAnt-2: [^{125}I]PAnt-2 radioiodinated as described in section 2.1.2 was purified using a C-18 reverse phase HPLC column and eluted with a linear gradient (0-80%) of acetonitrile/0.01M ammonium acetate (pH 4.6). The first peak represents free ^{125}I, and fraction marked (*) was stored in the dark and used for photoaffinity labelling.



interaction would be expected to show a decrease in the intensity of the bands incubated at 37°C over time.

Figure 4.3.28 Prolonged incubation of [^{125}I]PAnt-2/receptor complex at 37°C: Solubilized membrane preparations from αT3-1 cells photoaffinity labelled with [^{125}I]PAnt-2 were incubated at 37° C in a binding buffer (section 2.5) for 0, 14 and 26 hours followed by analysis on a 10% SDS/PAGE gel and autoradiography. Deglycosylated membrane samples are indicated by "+d" and non-deglycosylated samples by "-d".



4.3.4.3 Glu-C digestion of GnRH receptors crosslinked with PAnt-2

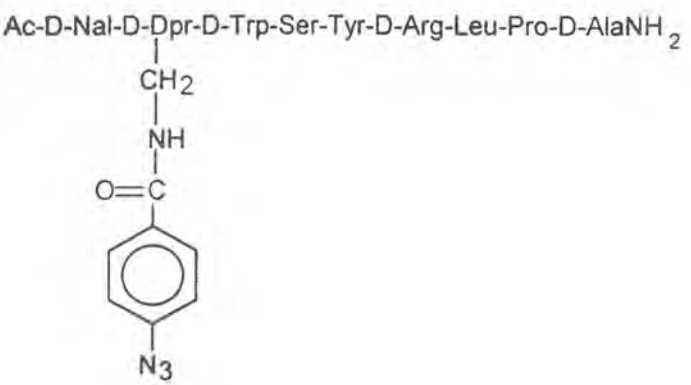
In order to determine the attachment site of PAnt-2 with the GnRH receptor, αT3-1 cells and COS-1 cells expressing wild type human, mouse, mouse E90Q, E111Q, E8Q, E294Q mutant receptors (Flanagan *et al.*, 1994) were photoaffinity labelled using [^{125}I]PAnt-2. Membrane preparations from these cells were solubilzed, deglycosylated and digested with Glu-C. The products were then analysed by SDS/PAGE. However, in six experiments, a clear and reproducible pattern of radioactive fragments could not be seen despite varying the amount of radioactive peptide, length of UV irradiation and solubilization conditions. It is possible that PAnt-2 crosslinks to several sites on the receptor, leading to a large number of fragments which were not clearly resolved from one onother.

4.3.5 Characterization of PAnt-3 and PAnt-3a

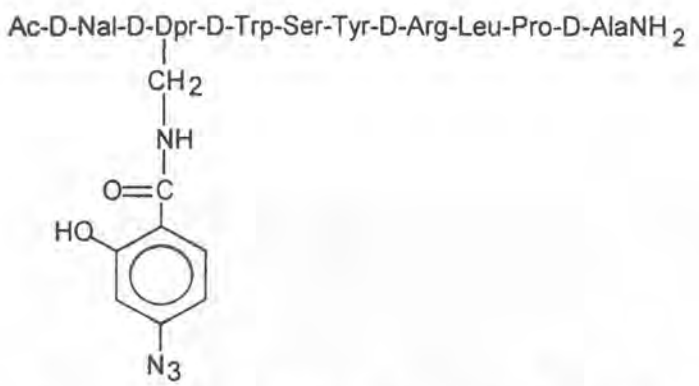
PAnt-3 and PAnt-3a have a diaminopropionic acid at position 2, which supplies the free amino group for the coupling of azidobenzoyl or azidosalisylate groups (Figure 4.3.29).

Figure 4.3.29 Structures of PAnt-3 (a) and PAnt-3a (b): Side chain of Dpr^2 and its attachment to the photoreactive group is shown.

a)



b)



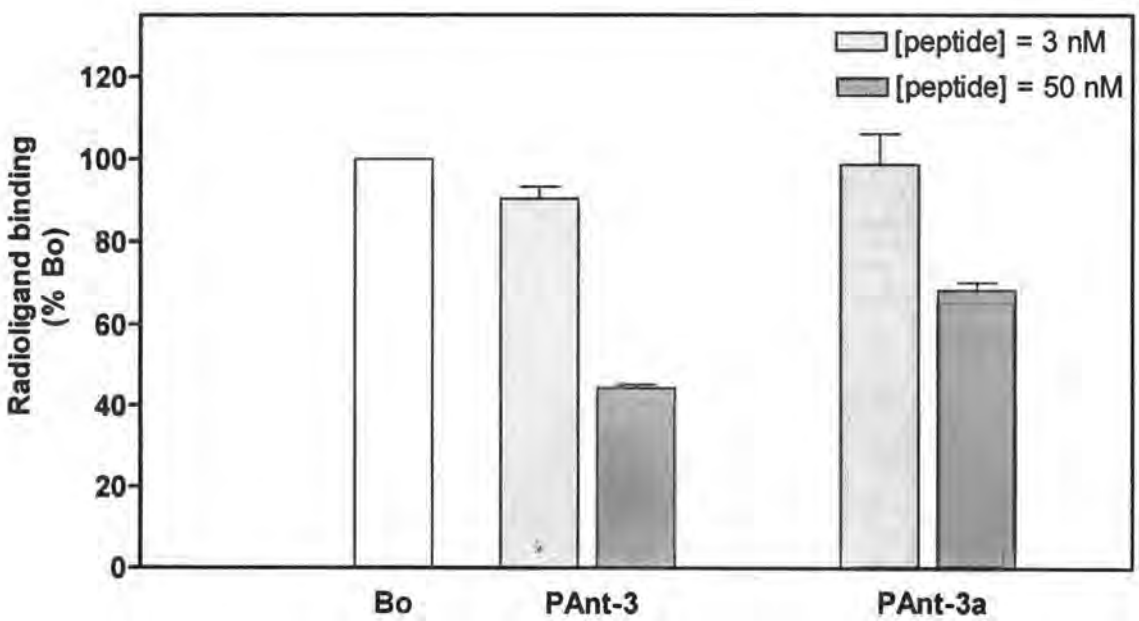
The side chain of diaminopropionic acid is shorter by three carbons compared to that of lysine, which was used in PAnt-1 and PAnt-2 for coupling to the photoreactive group. The rationale for the introduction of a shorter arm for the attachment of the photoreactive group was to enable the labelling of a residue in the receptor, which is close to the binding pocket of the ligand. Both PAnt-3 and PAnt-3a are derived from an antagonist and contain an azidobenzoyl or an azidosalicylate group respectively.

4.3.5.1 Binding of PAnt-3 and PAnt-3a to the GnRH receptor

In order to determine whether PAnt-3 and PAnt-3a had a high affinity for the GnRH

receptor, competition binding to human wild type GnRH receptor expressed in COS-1 cells was measured in the presence of 3 and 50 nM concentrations of unlabelled peptides with [His^5, ^{125}I-D-Tyr^6]GnRH as tracer. At a concentration of 50 nM, PAnt -3 and PAnt-3a caused a displacement of the tracer by (65%) and (38%) respectively (Figure 4.3.30). This indicated that both PAnt-3 and PAnt-3a have moderate affinity to the GnRH receptor.

Figure 4.3.30 Binding of PAnt-3 and PAnt-3a to GnRH receptor: The unlabelled peptides at concentrations of 3 and 50 nM were incubated with COS-1 cells expressing human wild type GnRH receptors in the presence of (10^5 c.p.m.) [His^5,^{125}I-D-Tyr^6]GnRH tracer. "Bo" represents radioligand binding in the absence of competing unlabelled ligand. Binding assay was performed as described in section 2.4.1.2. Data represents mean ± SD from two independent experiments performed in triplicate.



4.3.5.2 Photoaffinity labelling of GnRH receptors with [^{125}I] PAnt-3

Since PAnt-3 seemed to have the higher affinity of the two peptides, in order to determine if PAnt-3 could be crosslinked to the GnRH receptor, PAnt-3 was iodinated with ^{125}I (Figure 4.3.31) and the resulting [^{125}I]PAnt-3 was used in a photoafffinity

labelling experiment involving GnRH receptors expressed by αT3-1 cells.

Solubilized membrane preparations from these cells were analysed by SDS-PAGE and autoradiography (Figure 4.3.32). A very faint band could be seen for [^{125}I]PAnt-3/receptor complex on the original autoradiograph but this is not visible on the photograph shown in Figure 4.3.32. Control cells labelled with PAnt-2 showed strongly labelled band at around 45 kDa (native) and 32 kDa deglycosylated as in previous experiments. This suggests that the affinity and/or efficiency of crosslinking of PAnt-3 is too low to allow localization of the croslink site on the GnRH receptor.

Figure 4.3.31 HPLC purification of [^{125}I]PAnt-3: PAnt-3 was radioiodinated as described in section 2.1.2 and purified using a C-18 reverse phase HPLC column by eluting with a linear gradient (0-80%) of acetonitrile/0.01M ammonium acetate (pH4.6). The first peak represents free ^{125}I, and fractions marked (*) were stored in the dark and used for photoaffinity labelling.

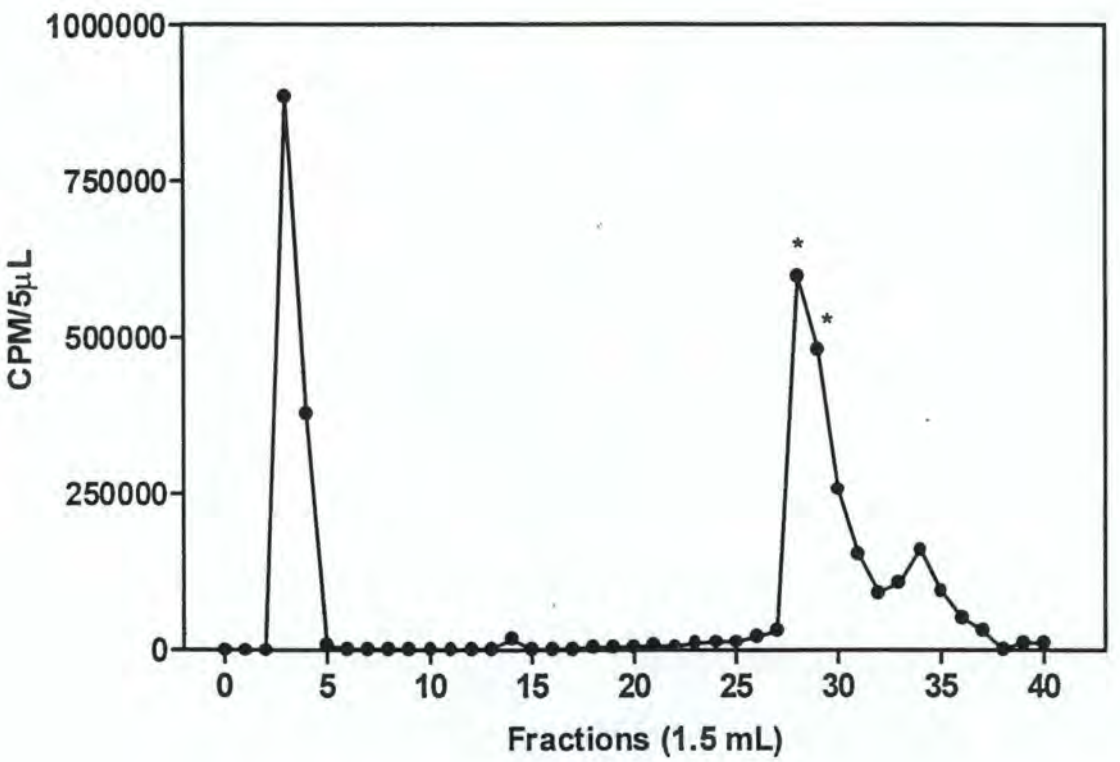
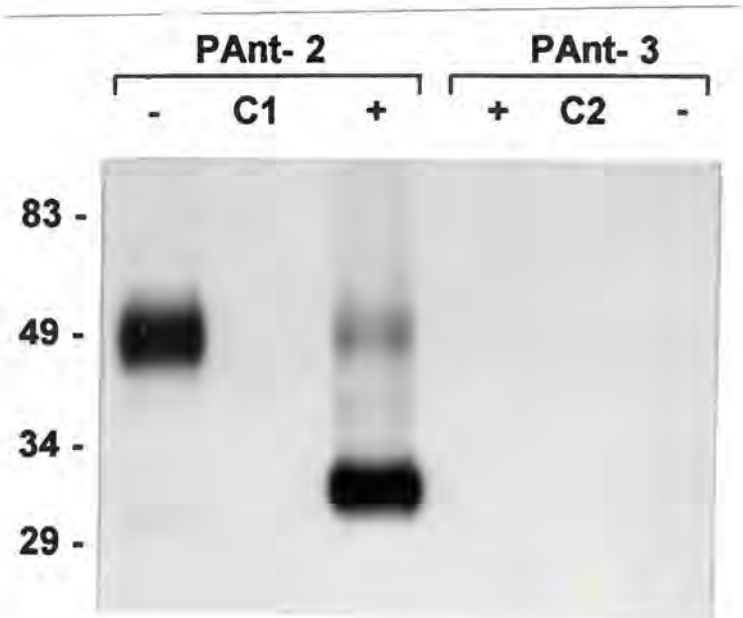


Figure 4.3.32 SDS/PAGE analysis of αT3-1 photolabelled with [^{125}I]PAnt-3: αT3-1 cells incubated with [^{125}I]PAnt-2 or [^{125}I]PAnt-3 followed by UV irradiation were solubilized and analysed on a 10% SDS/PAGE gel before "-" and after "+" deglycosylation. 1μM unlabelled [D-Trp^6, Pro^9-ethylamide]GnRH was used to displace [^{125}I]PAnt-2 "(C1)" and [^{125}I]PAnt-3 "(C2)" before UV irradiation.

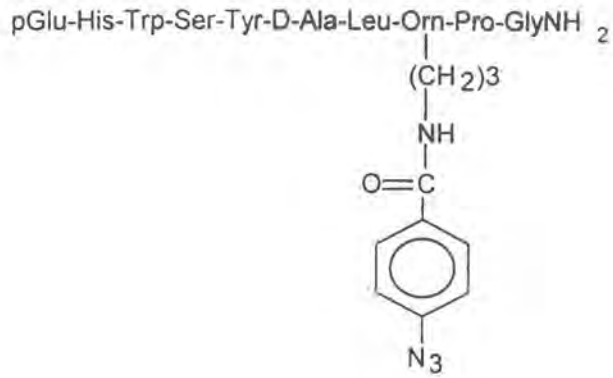


4.3.6 Characterization of PAg-2 and PAg-2a

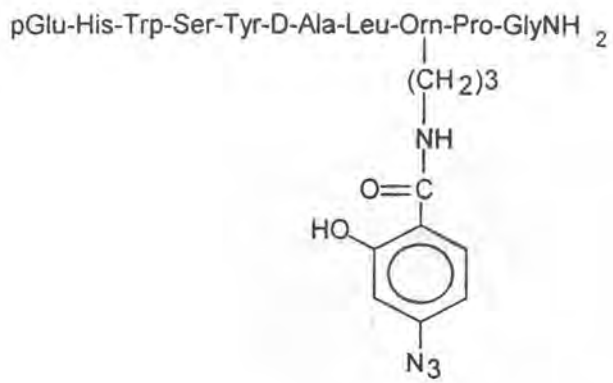
PAg-2 and PAg-2a have their photoreactive groups linked to Orn^8 at the C-terminus of GnRH agonist (Figure 4.3.33). Residues at position 8 of GnRH and its analogs are involved in the high affinity binding of these peptides to the GnRH receptor (Gupta *et al.*, 1993; Millar *et al.*, 1984). The critical role in receptor binding of Arg^8 of the mammalian GnRH has been investigated by mutagenesis of the receptor (Flanagan *et al.*, 1994). It follows that if a photoreactive group at this site crosslinks to the receptor, it is likely to attach to a residue in or near the receptor's ligand binding pocket.

Figure 4.3.33 Structures of PAg-2 (a) and PAg-2a (b): The side chain of Orn^8 and its attachment to the photoreactive group is shown.

a)



b)

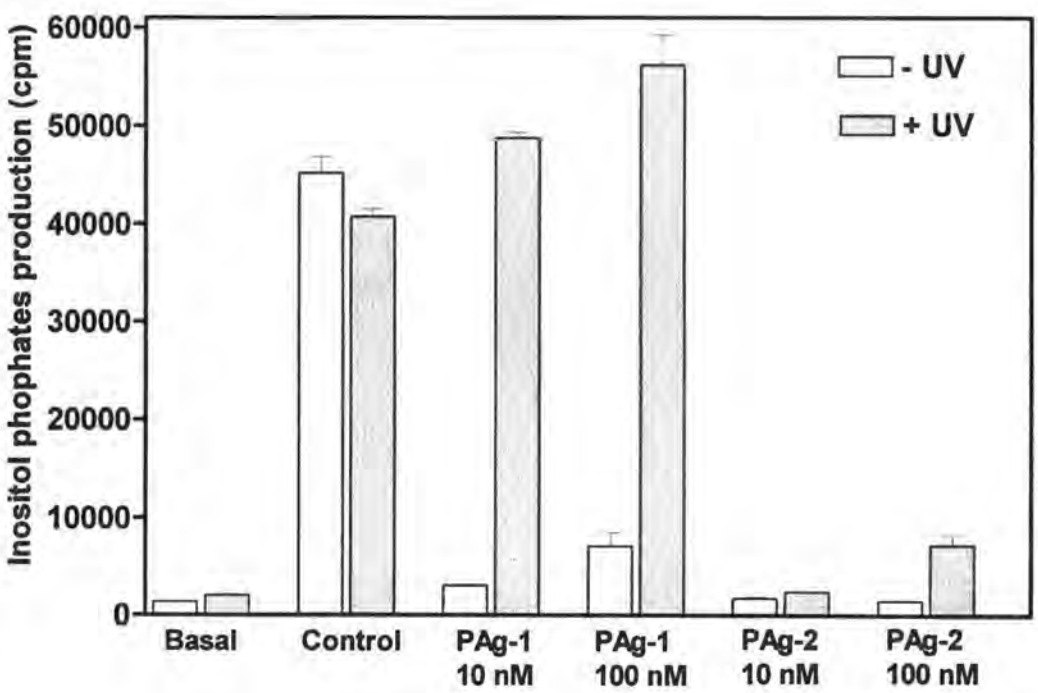


4.3.6.1 PAg-2 does not cause prolonged stimulation of inositol phosphate production in αT3-1 cells after attempted photoaffinity labelling

Previously, the photoreactive agonist PAg-1 was shown to cause irreversible activation of the GnRH receptor (Davidson *et al.*, 1997). The method used to demonstrate irreversible activation is to pre-incubate cells pre-labelled with ^{3}H-myo-inositol with the photoreactive agonist in the absence of Li^+, then to UV irradiate, wash off uncrosslinked agonist, and finally add Li^+ containing medium. Studies from this laboratory have previously shown that during stimulation with agonist in the absence of Li+, labelled inositol phosphate are efficiently recycled within the cells and the labelled phospholipid pool does not become depleted (Davidson *et al.*, 1994b).

The ability of PAg-2 to irreversibly activate the GnRH receptor was tested by evaluating its ability to cause prolonged stimulation of inositol phosphate production in αT3-1 cells after UV irradiation (Figure 4.3.34). After pre-incubation at 10 nM in the absence of Li^+ followed by UV irradiation, PAg-2 was able to stimulate IP production at only 6 % the rate produced by a similar concentration of GnRH in control cells. In contrast, irreversibly stimulated IP production by the high affinity photoreactive analogue PAg-1 after photoaffinity crosslinking labelling was even greater than the controls (119 %).

Figure 4.3.34 Stimulation of IP production by PAg-2 after UV irradiation: αT3-1 cells were preincubated with PAg-1 or PAg-2 at 10 and 100 nM in the absence of Li^+ for 30 minutes at room temperature followed by UV irradiation (+UV) or no irradiation (-UV). Subsequently the uncrosslinked ligand was washed off using buffer "A" for 3 x 10 minutes. Finally IP production was measured in the presence of Li^+ as described in section 2.4.2. Control cells were stimulated with 10 nM GnRH after washing. "Basal" indicates IP produced in non stimulated cells. Data points are given as mean ± range of duplicate.



4.3.6.2 PAg-2 has a low efficiency crosslinking to the GnRH receptor

To determine whether the lack of prolonged stimulation by PAg-2 is due to low binding affinity for the receptor or due to low crosslinking efficiency, the binding affinity of unphotolysed PAg-2 for GnRH receptors expressed by αT3-1 cells was measured. The IC_{50} for for the inhibition of radioligand binding PAg-2 was 2.37 nM ± 0.3nM (mean ± SEM, n = 4) which is similar to that for [His^5-D-Tyr^6]GnRH (2.1nM ± 0.07 nM, mean ± SEM). This indicates that the addition of a photoreactive group to the peptide at position 8 did not result in a marked loss of affinity to the GnRH receptor (Figure 4.3.35). The above result was also supported by ligand-stimulated IP response data that showed comparable response of αT3-1 cells to GnRH and PAg-2 (Results not shown).

Figure 4.3.35 Binding of PAg-2 to GnRH receptors: αT3-1 cells were incubated with various concentrations of PAg-2 or [His^5,D-Tyr^6]GnRH in the presence of 10^5 c.p.m of [His^5-^{125}I-D-Tyr^6]GnRH in the dark (5 hours at 4°C). Subsequently binding assay was performed as described in section 2.5.1.2b. Data represents normalised values from 2-4 independent experiments. "Bo" represents radioligand binding in the absence of competing unlabelled ligand and is taken as 100%.

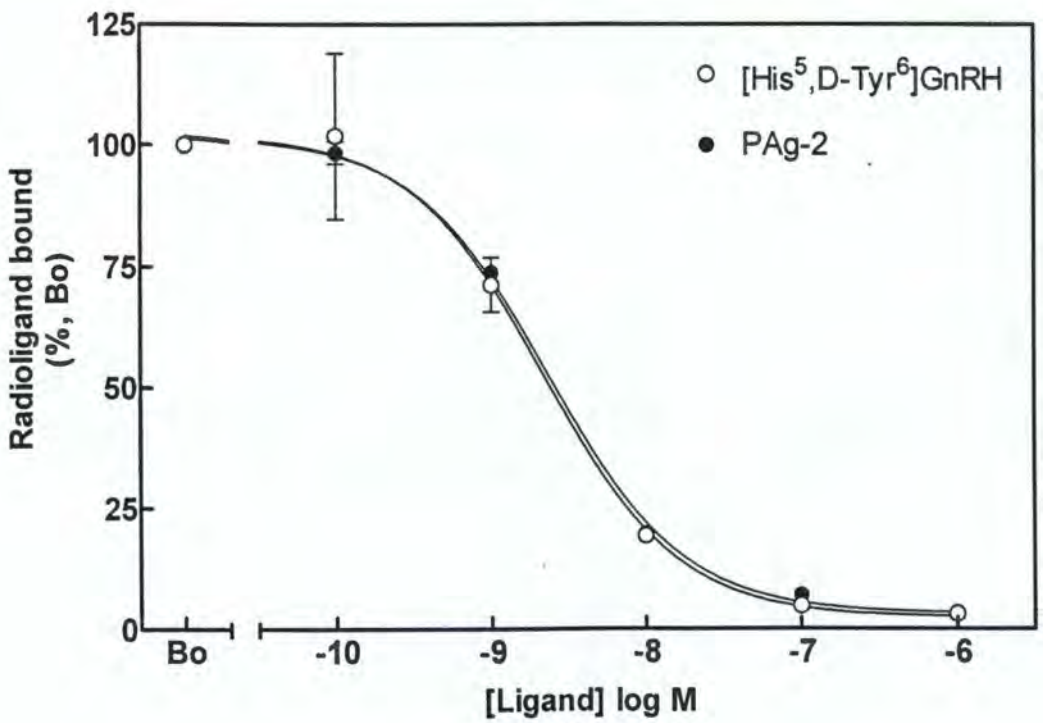
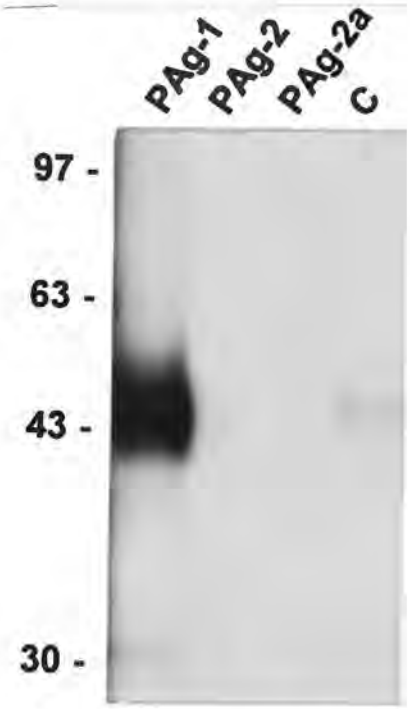


Figure 4.3.37 SDS/PAGE autoradiograph of αT3-1 cell membranes photoaffinity labelled with [^{125}I]PAg-1, and after attempted photoaffinity labelling with [^{125}I]PAg-2 or [^{125}I]PAg-2a: Membrane preparations from αT3-1 cells which were photoaffinity labelled with [^{125}I]PAg-1, [^{125}I]PAg-2 or [^{125}I]PAg-2a were solubilised and analysed on a 10% SDS/PAGE gel. In the control lane "C", [^{125}I]PAg-1 was displaced with 1 μM of unlabelled [D-Trp^{6}, Pro^{9}-ethylamide]GnRH.



4.3.7 Characterization of PAg-3, PAg-4 and PAg-4a

PAg-3 has an azidobenzoyl group at the second position while PAg-4 and PAg-4a have their photoreactive group at the third position (Figure 3.4.38). The photoreactive groups in all three peptides are coupled to diaminoproionic acid and they have sequences compatible with agonist activity.

The binding affinity of these peptides for human GnRH receptor expressed in COS-1 cells was measured using 3 and 50 nM concentrations of the unlabelled peptides to displace [His^{5}, ^{125}I-D-Tyr^{6}]GnRH tracer (Figure 4.3.39). None of the three analogs showed any displacement at 3 nM and at concentration of 50 uM the largest displacement shown was by PAg-3, which was (38%). The results indicated that PAg-4 and PAg-4a have low affinity for binding to the GnRH receptor while PAg-3 has moderate affinity. Based on these results the binding affinities of PAg-3, PAg-4, PAg-4a was concluded to be to low for the peptides to be used in photoaffinity labelling studies.

Both PAg-2 and PAg-2a were iodinated with ^{125}I (Figure 4.3.36) and attempts were made to photoaffinity label GnRH receptors on αT3-1 cells. However, when membranes from cells pre-incubated with [^{125}I]PAg-2 or [^{125}I]PAg-2a and UV irradiated, were analysed by SDS-PAGE/autoradiography, no bands corresponding to the GnRH receptor were seen (Figure 4.2.37). In the same experiments, control cells labelled with [^{125}I]PAg-1 showed a strongly labelled band at 45 kDa as in previous experiments. This band corresponded with the size of the glycosylated GnRH receptor in αT3-1 cells (Figure 4.3.15). The above results suggest that PAg-2 and PAg-2a do not crosslink with the receptor efficiently and are not suitable probes for photoaffinity labelling of the GnRH receptor.

Figure 4.3.36 HPLC purification of [^{125}I]PAg-2 and [^{125}I]PAg-2a: PAg-2 (a) and PAg-2a (b) were radioiodinated as described in section 2.1.2 and purified using a C-18 reverse phase HPLC column by eluting with a linear gradient (0-80%) of acetonitrile/0.01M ammonium acetate (pH4.6). The first peak represents free ^{125}I, and fractions marked (*) were stored in the dark and used for photoaffinity labelling.

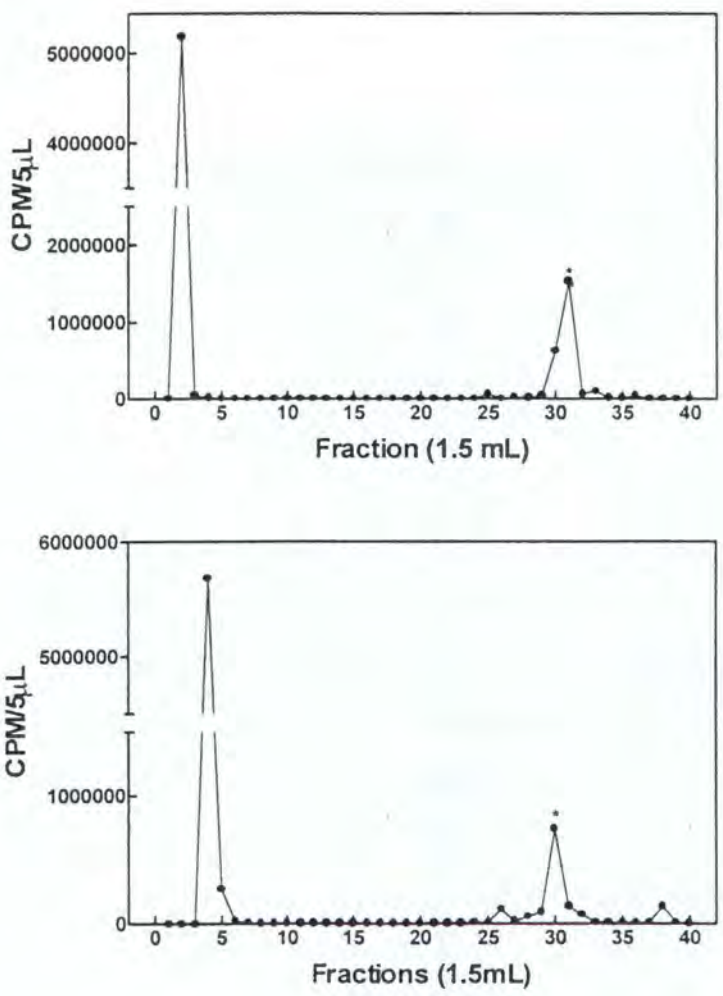
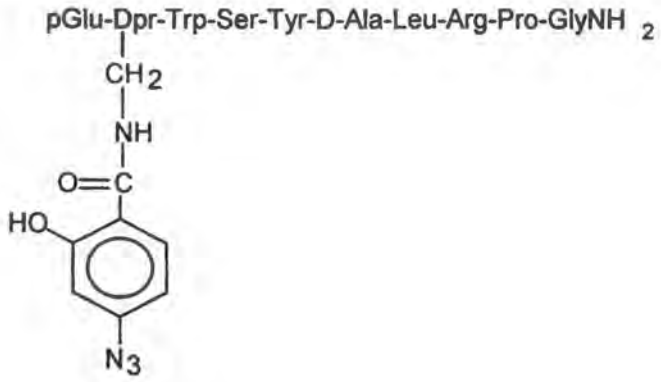
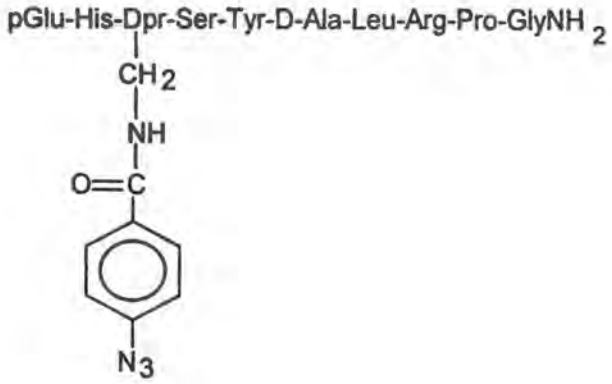


Figure 3.4.38 Structures of PAg-3, PAg-4 and PAg-4a:

a) PAg-3



b)PAg4



c)PAg4-a

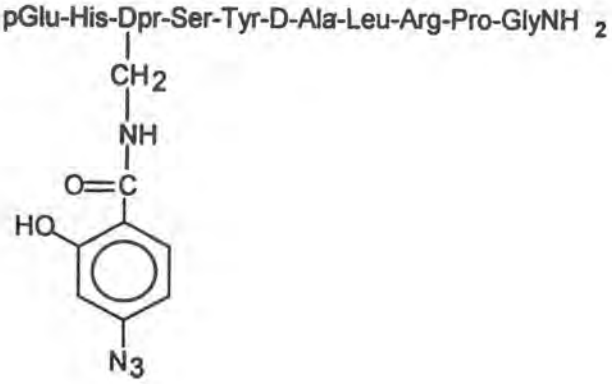
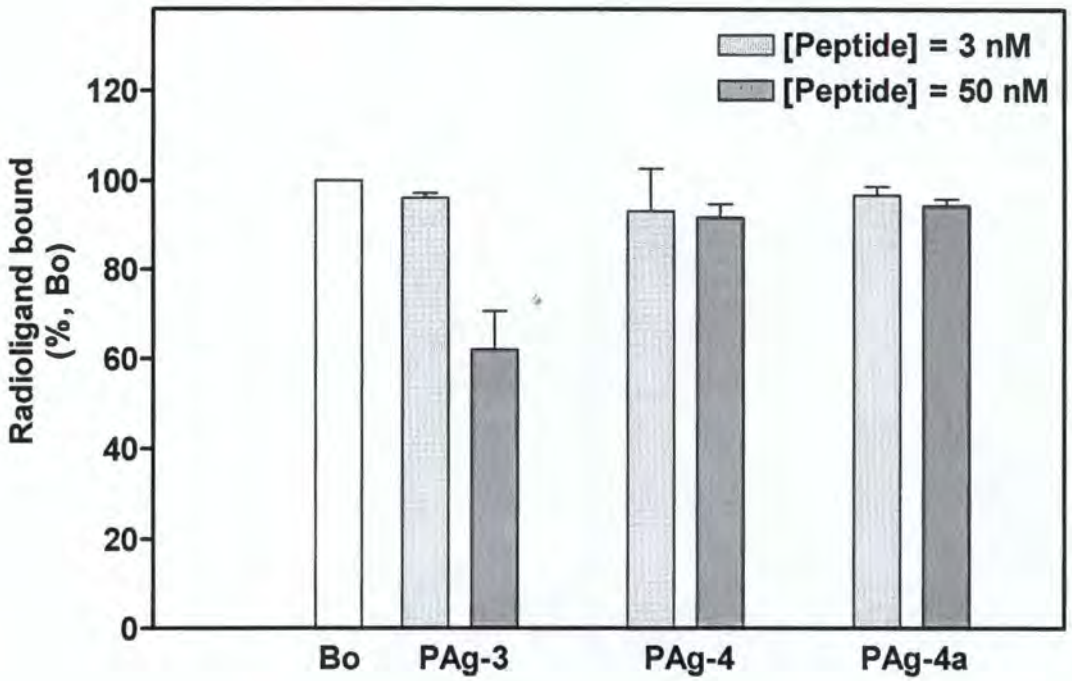


Figure 4.3.38 Binding of PAg-3, PAg-4, PAg-4a, to human GnRH receptor: The unlabelled photoreactive peptides at concentrations of 3 and 50 nM were incubated with αT3-1 cells in the presence of (10^5 c.p.m) of [His^5, ^{125}I-D-Tyr^6]GnRH tracer. Binding assay was performed as described in section 2.4.1.2. "Bo" represents radioligand binding in absence of competing unlabelled ligand. Values are expressed as % of Bo and are mean ± SD from one experiment performed in triplicates.



4.4 Discussion

The rational design of drugs requires the understanding of the structural basis of drug-receptor interaction. Photoaffinity labelling provides an important experimental tool for the elucidation of interactions involved in biological processes at the molecular level. In this section of the thesis the interactions of selected GnRH analogs with the GnRH receptor was characterized using photoaffinity labelling techniques. Peptide analogs containing either lysine or diaminopropionic acid on which 4-azidobenzoic acid or 4-azidosalicylic acid were attached were used. The choice of the reactive reagents was based on previous success of azidobenzoic acid as a photolabelling reagent (Hazum, 1983; Iwashita and Catt, 1985). The two photolabelling reagents are structurally very

similar and capable of producing a photo-generated nitrene intermediates that are highly reactive and capable of insertion into all protein amino acid side chains in the absence of any particular functional group (Kotzyba-Hibert *et al.*, 1995). 4-Azidosalicylic acid has an additional OH on the aryl azide which increases its reactivity compared with azidobenzoic acid. However, the associated increased electophilicity of the intermediate nitrene group may facilitate reaction with the solvent water and decrease efficiency of affinity labelling (Hazum, 1983).

All of the synthesised analogs showed increased hydrophobicity compared with the parent peptide as could be seen from their enhanced mobility on TLC (Figure 4.3.1, Table 4.3.1) as a result of the addition of the hydrophobic aziosalicyl or azidobenzoyl groups. This, coupled with the hydrophobic nature of most GnRH antagonist analogs produces a photoreactive peptide that is highly hydrophobic. Although increased hydrophobicity particularly on the N-terminus of GnRH analogs increases the affinity of most GnRH antagonists, this property may present a practical problem in photoaffinity labelling by increasing non-specific interactions, in particular with hydrophobic membrane components resulting non-specific labelling. In addition, because of a marked decrease in solubility in aqueous environment, iodination of the peptides in solutions becomes difficult. To increase efficiency of radioiodination and enhance subsequent purification by HPLC, the reactions described in this thesis were carried out under low salt containing buffer (0.1 M phosphate) in the presence of methanol (section 2.1.2). Of the 10 photoreactive analogues, PAnt-2 was found to have the highest hydrophobic property while PAg-2 and PAg-2a both derived from the less hydrophobic agonist analogue [D-Ala^6, Orn^8]GnRH had the least hydrophobicity (Figure 4.3.1, Table 4.3.1). The high hydrophobicity of PAnt-2 may have in part have contributed to the problems encountered when trying to localise its crosslinking site on the GnRH receptor (see below).

Photometric scan of all of the photoreactive analogs showed a characteristic absorbance peak at approximately 275 nm as has been described previously for aryl azide-containing peptides (Hazum, 1983) (Figures 4.3.2 - 4.3.7). This peak was greatly decreased or abolished after ultraviolet irradiation under conditions used.

The absorbance peak at 277 nm seen for GnRH (Figure 4.3.8) is mainly produced by Trp^3 as this peak is almost absent in [Leu^3]GnRH (Figure 4.3.9). The small peak at 280 nm in [Leu^3]GnRH is most likely due to the Tyr^5 residue. Since Trp^3 is present in most of the photoreactive analogs, it is expected to contribute similarly to the absorbance peaks observed in the photoreactive analogs at the same region. The decrease after photolysis of the 275 - 277 nm absorbance peaks in these analogs suggest that part of the photolysis reaction involves the disruption of the tryptophan ring structure due to an inter- or intra-molecular interaction. This however, does not imply that it is the only reaction that occurs during photolysis. In a situation where the peptides are constrained in a particular conformation such as occurs after binding to the GnRH receptor, this reaction may not even occur, or if it does, could be quantitatively insignificant.

In experiments where photoreactive analogs were used for localisation of crosslinking sites, a longer irradiation period of 60 to 90 seconds was used since in preliminary experiments it was found that a longer exposure resulted in a stronger labelling of bands without increasing non-specific labelling. A longer duration of irraditaion for photolysis of hormone-receptor complexes than for hormone alone has been previously described (Hazum, 1983) and this effect is presumably due to the screening of the ligands by the protein.

Photoaffinity labelling of biological preparations using UV irradiation may be complicated by the possibility of UV induced structural damage of proteins. The conditions for photoaffinity labelling used in this thesis were tested to find out if UV irradiation of cells affects receptor binding, which is a reflection of the structural integrity of the GnRH receptor, or GnRH-stimulated inositol phosphate production which is a reflection of both the integrities of the GnRH receptor as well as that of the down-stream signalling apparatus. Irradiation of cells expressing the mouse GnRH receptors did not result any change in affinity of the receptor to its natural ligand (Figure 4.3.11). The small (6.55%) decrease in total inositol phosphate production observed in UV irradiated cells indicates that down stream signalling mechanisms are damaged only slightly, if at all under the conditions used (Figure 4.3.10).

PAnt-1, which has a photoreactive azidobenzoyl group at position 1, bound with high affinity to the GnRH receptor and behaved as a competitive antagonist in the absence of UV irradiation. The binding affinity (Ki) of PAnt-1 estimated by competitive ligand binding assay (3.1 nM) (Figure 4.3.13) was in good agreement with the Ki value of 3.8 nM determined by the inhibition of GnRH-stimulated inositol phosphate production (Figure 4.3.17).

UV irradiation after the binding PAnt-1 (100 nM) to the GnRH receptor resulted in crosslinking of the peptide to the receptor with high efficiency, as indicated by irreversible inactivation of about 76% of the binding sites for a high affinity agonist [^{125}I-DTyr^5,D-Ala^6, N-Meth-Leu^7, Pro^9-ethylamide]GnRH. The small decrease (5.7 %) in radioligand binding observed in cells pre-incubated with unlabelled PAnt-1 but washed before UV irradiation is most probably caused in most part by residual PAnt-1. After correcting for this decrease, at least 70% of the decrease in radioligand binding seen in Figure 4.3.16 can be attributed to the UV induced covalent interaction of PAnt-1 with the receptor. The efficiency of the crosslinking was comparable to that of the high affinity photoreactive agonist [4-azidobenzoyl-D-Lys^6]GnRH which at 100 nm was able to decrease the specific binding of [^{125}I]Buserelin to pituitary membranes by 80% (Hazum, 1981b). These results also show that un-photolysed PAnt-1 can be washed off cellular preparation with relative ease, a feature which is unusual for peptide GnRH antagonists due to their highly hydrophobic nature. This feature makes PAnt-1 a good tool for specifically inactivating GnRH receptors in experimental situations where a decrease of surface receptors is required (e.g. in abolishing spare receptors) (Forrest-Owen *et al.*, 1998).

A related set of experiments where 100 nm PAnt-1 was used to irreversibly inactivate GnRH receptors before stimulation with GnRH showed a 57.9% decrease in the maximum IP response after crosslinking of PAnt-1 along with a 7-fold increase in the ED_{50} for GnRH. This apparent discrepancy between amount of receptors inactivated in the receptor binding studies (70%) (Figure 4.3.16) and degree of decrease in inositol phosphate production (57.9%) (Figure 4.3.17) at similar doses can be explained by the presence of spare receptors in αT3-1 cells. Thus a decrease in

number of available receptors by crosslinking with PAnt-1 such as described in Figure 4.3.17 will result in an apparent shift of the ED_{50} progressively to the right until the spare receptors are exhausted and then this will be followed by a decrease in the maximum IP production. The (▼) curve in figure 4.3.17 expresses a situation where all of the spare receptors have been made unavailable by the crosslinking of PAnt-1.

SDS-PAGE analysis of [^{125}I]PAnt-1 crosslinked GnRH receptors yielded bands with differing apparent molecular weights depending on cell and receptor types. The specificity of these bands as GnRH receptors was established by: (i) inhibition of crosslinking of [^{125}I]PAnt-1 by unlabelled GnRH analogue [D-Trp^6, Pro^9-ethylamide]GnRH (Figure 4.3.15), (ii) inhibition of the binding of GnRH analogue radioligand by unlabelled PAnt-1 with high affinity (Figure 4.3.13) and (iii) absence of crosslinking of PAnt-1 in untransfected cells that do not express the GnRH receptor (Figure 4.3.15). The difference in size and broadness of the bands are due to variations in glycosylation of the receptor in different cell types. This was shown by enzymatic deglycosylation which produced an identical band of 32 kDa in all cases, in keeping with the expected molecular mass of the deglycosylated GnRH receptor (328 amino acids) (Figure 4.3.15).

The attachment site of PAnt-1 was defined by peptide mapping using natural sequence differences between human, mouse and sheep GnRH receptors as well as a panel of mutant GnRH receptors with engineered protease cleavage sites. Three closely related receptors were used for this study since their structural similarity allows the information gathered from one receptor to be extrapolated toward the other receptor more readily. Since all the three mammalian receptors have the same natural ligand it is logical to assume that the ligand docks to the binding pocket of each receptor in very similar manner. The differences in Glu-C proteolytic cleavage sites on these receptors along with the introduced alteration of Glu-C cleavage site in mutant receptors allowed identification of the labelled peptide cleavage products. In identification of the different peptide fragments, molecular size information from SDS-PAGE data was compared with potential Glu-C derived fragments expected from the primary structure of the receptors.

Although the human GnRH receptor has the same Glu^8 residue as the mouse receptor, cleavage with Glu-C resulted in a more complete proteolysis at this site than the mouse receptor (4.3.18). This could not be due to the different glycosylation states of the mouse and human wild type receptors since the sheep GnRH receptor and the hN19E mutant receptor both of which contain two glycocylation sites also showed a more complete digestion than that of the mouse receptor. It is more likely that this difference is due the difference in their primary structure that causes a subtle structural difference that affects the susceptibility of the Glu^8 to the Glu-C endoproteinase.

The results described taken together show that PAnt-1 is crosslinked to a 7 amino acid residue segment, comprising residues 12 to 18 (Asn-His-Cys-Ser-Ala-Ile-Asn) of the N-terminal domain of the receptor, which implies that PAnt-1 is oriented in its binding site with the peptide's N-terminal end in the vicinity of these residues.

Previously we had shown the presence of a C14-C200 disulfide bridge using site-directed mutagenesis of Cys residues as well as the effects of sulfihydryl reactive reagents [Davidson *et al.*, 1997]. The present data showing a PAnt-1 labelled fragment of the same size under both reducing and non-reducing conditions indicate that this C14-C200 bridge must have been cleaved during the crosslinking reaction of PAnt-1. Therefore the results strongly suggest that C14 itself is the crosslinked residue.

Our previous study showed cross-linking of a GnRH agonist with azidobenzoyl group at position 6 of the peptide also crosslinks to the same cysteine residue [Davidson *et al.*, 1997]. The involvement of the same receptor residue in crosslinking of two different peptides with photoreactive groups in different positions indicates that the binding pockets of these two peptides differ and is consistent with the suggestion [Janovic *et al.*, 1993) that the binding configurations of GnRH agonists and antagonists are different. However, this involvement of the same cysteine residue may also reflect the particular susceptibility of disulfide bridges to cleavage during UV-

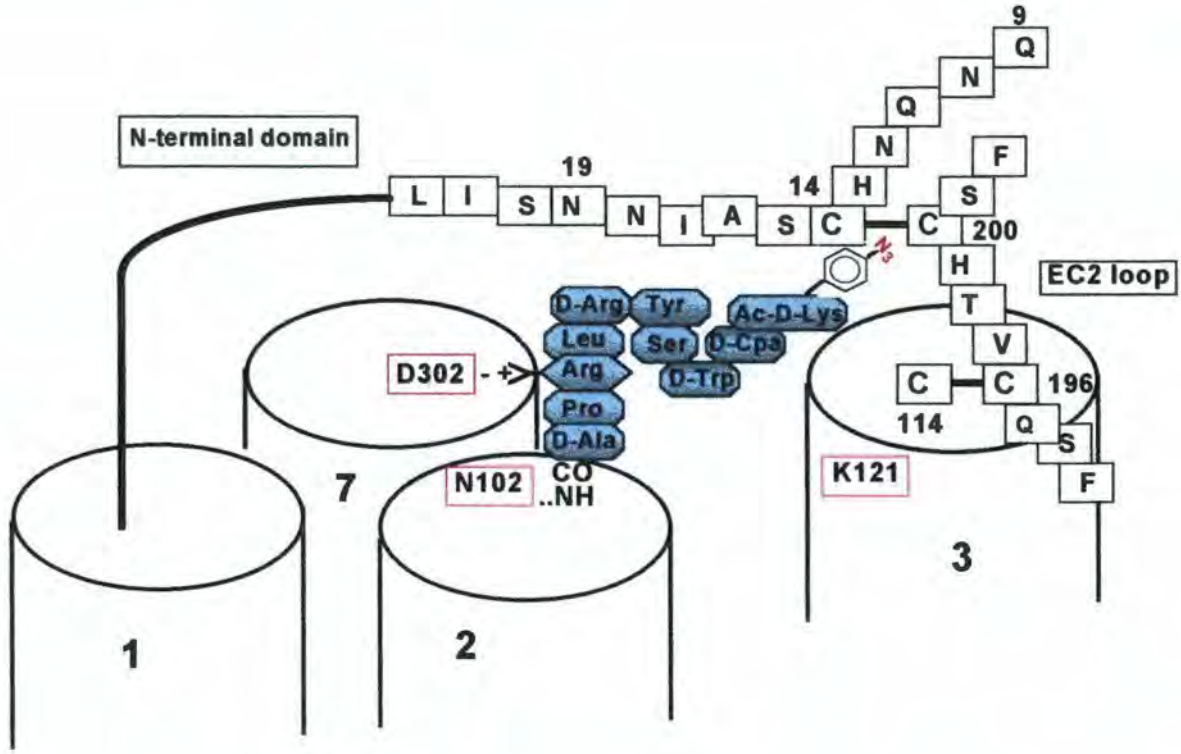
induced crosslinking of azido compound (Kotzyba-Hibert, *et al.* 1995). The crosslinking reaction mechanism occurs by hydrogen abstraction or, in the case of a disulfide bridge, by disulfide dissociation. The energy required for disulfide dissociation is considerably lower than for hydrogen abstraction, and cleavage of disulfide bridges is therefore expected to be the most favourable reaction of the nitrene free radical generated during photolysis (Kotzyba-Hibert, *et al.* 1995). Cysteine is the second most frequently labelled residue (after Tyr), and in at least one case two adjacent Cys residues involved in a disulfide bond were labelled (Dennis *et al.*, 1988).

The $pGlu^1$, His^2 and Ser^4 residues at the N-terminus, and the Pro^9, Gly^{10}-NH_2 residues of the C-terminus, are the most conserved residues of the GnRH peptide [King and Millar, 1995,1997). Most substitutions of $pGlu^1$, His^2 or Trp^3 have caused a loss of agonist activity, and multiple aromatic substitutions in the N-terminal residues are a feature of many potent GnRH antagonists. These findings have led to the concept that specific interactions of the N-terminus of GnRH agonists with the receptor are required for receptor activation (Sealfon *et al.*, 1997). There is evidence from site-directed mutagenesis that one residue which participates in this interaction may be Lys^{121} of the 3rd transmembrane helix, as mutation of Lys^{121} markedly decreases agonist but not antagonist binding (Zhou *et al.*, 1995). The corollary of these findings is that the N-terminal regions of GnRH antagonists are likely to interact with some different receptor residues from those found for agonists. The present results suggest that the N-terminus of this GnRH antagonist binds in the vicinity of the Cys^{14}-Cys^{200} disulfide bridge, as depicted in Figure 4.4.1.

Previous site-directed mutagenesis studies have suggested that Arg^8 of GnRH interacts electrostatically with Glu^{301} of the mouse receptor, equivalent to Asp^{302} in the human receptor, (Flanagan *et al.*, 1994), and that the Asn^{102} side-chain plays a role in binding of the C-terminus of the peptide, possibly by H-bonding with the C-terminal glycinamide moiety (Davidson *et al.*, 1996). The above putative contact sites are incorporated in Figure 4.4.1, which depicts Arg^8 and the C-terminal glycinamide of PAnt-1 interacting with Asp^{302} and Asn^{102} of the human GnRH receptor respectively, and the N-terminal end of the peptide positioned in a site comprising the constrained

regions of the N-terminal domain and second extracellular loop in the vicinity of the Cys^{14}-Cys^{200} disulfide bridge.

Figure 4.4.1 Schematic diagram of PAnt-1 binding site on the human GnRH receptor: Numbered cylinders represent the transmembrane helices. The solid curved line represents the portion of the N-terminal domain linking the N-terminus to the first transmembrane helix. Cys residues Cys^{14}-Cys^{200} and Cys^{114}-Cys^{196} are shown linked by disulfide bridges. The photoreactive GnRH antagonist PAnt-1 (blue) is shown docked in its binding site, defined by residues Asn^{102}, Lys^{121} and Asp^{302}, as described in the text. The azidobenzoyl group of the photoreactive peptide is shown positioned near to Cys^{14}, to which it covalently attaches after UV irradiation.



PAnt-2 contains a photoreactive group at $D\text{-}Lys^{6}$. Previously GnRH receptors have been photolabeled successfully with peptides containing reactive groups at position 6. PAnt-2 was found to associate with the GnRH receptor in a very tight manner even in the absence of UV dependent crosslinking with the receptor (Figure 4.3.25). This may in part be due to the extreme hydrophobic nature (Figure 4.3.1) of the derivatized peptide. Although the SDS-PAGE data suggests that the GnRH receptor is

photolabeled strongly by PAnt-2, subsequent endoproteinase digestion did not produce clearly labelled bands. This may be due to labelling of receptor at multiple sites.

PAnt-3 and PAnt-3a were used to explore the interactions of the residue at the second position of the GnRH analog. The second position His^2 is conserved in all naturally occurring GnRH peptides except one (Table 1.1.1) and is substituted in most antagonist GnRH analogs. The binding of an analog to the receptor requires basic and aromatic residue with hydrogen bonding capacity for agonist activity. The incorporation of a photoreactive group in position 2 was hypothesised to be compatible with antagonist properties since position 2 in antagonists is generally occupied by a bulky, hydrophobic aromatic groups. However both PAnt-3 and PAnt-3a showed low affinity toward the GnRH receptor as evidenced by the competitive binding studies (Figure 4.3.30). At a concentration of 3 nM, which is near the Ki of PAnt-1 for the mouse receptor, both PAnt-3 and PAnt-3a could hardly displace the radioligand. The addition of a spacer in the form of the side chain of diaminopropionic acid to which the azidobenzoyl of azidosalicyl groups are attached seems to have affected the binding of the peptide to the receptor. The low affinity of these peptides is the probable reason why photolabelling of the GnRH receptor could not be achieved using [^{125}I]PAnt-3 (Figure 4.3.32).

PAg-2, containing photoreactive group at position 8 was found to have high affinity. However PAg-2 had a very low crosslinking efficiently and neither PAg-2 nor PAg-2a were able to photolabel the GnRH receptor. The fact that this high affinity photoreactive ligand could not be crosslinked with the receptor shows that a close approximation and specific orientation of photoreactive groups of and target residues are required.

PAg-3 an agonist analog, which also has a photoreactive group at position 2, had a low affinity to the GnRH receptor. The low affinity of PAg-4 and PAg-4a may be explained by the facts that the peptides have L-amino acid substitutions at position three. Trp^3 is conserved in all but one of the GnRH hormones described to date.

Substitution at this position nearly always result loss of agonist activity and often loss of affinity to the receptor. Substitution with even aromatic residues gives a peptide of very low activity (Tyr 0.1% and Phe 0.5%). The aromatic interaction in a form of π - π interactions at these site is important as evidenced by the increased activity of [pentamethyl-Phe^2]GnRH which was 30-70% when compared with [Phe^2]GnRH which was 0.5% GnRH In fact even in most antagonist analogs while having multiple substitutions, a D-sterioisomer of Trp^3 is invariably preserved.

Knowledge of the location of the attachment site is less valuable in the case of ligands where the photoreactive group is attached to the peptide by a long spacer arm, since the possibility exists that the actual binding pocket may be quite distant from the covalent attachment site. Such is the case with the photoreactive GnRH analogs PAnt-1 (this thesis) and PAg-1 (Davidson *et al.*, 1997) which have the azidobenzoyl group attached to the ε-amino group of lysine residue. For this reason, the analogs PAnt-3, PAnt3a, PAnt-4 and PAnt-4a were designed incorporating a diaminopropionic acid residue, which is 3 carbons shorter than lysine. However, none of these analogs showed sufficiently high binding affinity to be useful in photoaffinity labelling, and they could not be used to map the binding site.

The aim of this section of the thesis was to obtain a mapping of the receptor binding site for GnRH antagonists, by determining the attachment sites of several photoreactive GnRH analogs with photoreactive groups located at different positions on the peptide. This aim was only partially achieved, as the attachment site of only one of the peptides, PAnt-1 was successfully localized.

CHAPTER FIVE

CONCLUDING DISCUSSION

The understanding of the physical interactions involved in ligand-receptor complex formation is important for the understanding of the biology of hormone responses as well as in the rational design of drugs for therapeutic purposes. The thousands of GnRH analogs that are available have been designed empirically in the absence of an in-depth understanding of the physical interactions of the ligand with its receptor. The increasing availability of new data on structure activity relationships of GnRH analogs and the GnRH receptor is increasingly guiding the development of potent GnRH analogs. The recent cloning of the receptor cDNA and the availability of the primary structures for the receptor and the ligand has allowed receptor mutagenesis and computational modelling of the receptor and ligand in order to gain insights into the molecular mechanisms of the actions of GnRH and its analogs. However much is still lacking in the understanding of the molecular basis of these interactions.

Many of the clinical applications of GnRH analogs depend on the inhibition of the hypothalamo-pituitary-gonadal axis. Since the binding sites of agonists and antagonists may not be the same, a separate concerted effort towards the understanding of the molecular basis of the interactions of GnRH antagonists with the receptor is warranted.

A number of residues in the GnRH receptor have been identified that are important in ligand recognition. The residues so far described are Glu^{301} of the mouse receptor (corresponding with Asp^{302} of the human GnRH receptor) (Flanagan *et al.*, 1994), Lys^{121}(Zhou *et al.*, 1995) and Asn^{102} (Davidson *et al.*, 1996). Asp^{98} was also suggested to play a role by interacting with His^2 of GnRH (Rodic *et al.*, 1996).

Glu^{301} of the mouse receptor determines the specificity of mammalian GnRH by an ionic interaction with Arg^8 (Flanagan *et al.*, 1994). An alternative or additional possibility suggested for the role of Arg^8 is that it may stabilise the active conformation of GnRH by hydrogen bonding with the side chains of His^2 and Tyr^5 of the GnRH. It

has been suggested that the more acidic nature of these residues is due to their proximity to the cationic side chain of Arg^8 (Sealfon *et al.*, 1997). The requirement for Arg^8 of GnRH analogs for binding to the mammalian receptor is however diminished if the analogues are structurally constrained by the incorporation of a D-amino acid at position 6. The importance of Arg^8 in ligand-receptor recognition is also emphasised by the fact that many of the high affinity peptide GnRH antagonists contain arginine at this position (Janecka et al., 1991). Even though a systematic investigation of the role of Arg^8 in antagonist binding is lacking, it is possible that the negatively charged residue in TM3, Glu^{301} in mouse (Asp^{302} in human) receptor is crucial both for agonist and antagonist binding. The fact that the residue is conserved in all non-mammalian receptors (chicken, goldfish, Xenopus) raises the possibility that in these receptors this residue may have a different role since their natural ligands do not contain Arg at position 8 and could not therefor be involved in ionic interaction.

Asn^{102}, located at the extracellular end of TM2, has been shown to be a critical determinant of affinity for GnRH agoinsts containing a glycinamide C-terminus (Davidson *et al.*, 1996). One possiblity is that the glycinamide C-terminus is involved in a H-bond with the side chain of Asn^{102}. Many of the peptide antagonists also contain an amide ($Ala^{10}NH_2$) at these positions and hence a similar interaction may exist.

The comparative study of agonists and antagonists with respect to their receptor binding and subsequent pharmacological effects may help shed light on the mechanism of receptor activation i.e. the transition of receptors from in-active to active state. Indeed Lys^{121} which corresponds to the highly conserved Asp in TM3 of biogenic amine receptors was identified as a crucial residue for agonist binding but not for the binding of an antagonist (Zhou *et al.*, 1995). However one must also keep in mind that GnRH antagonists are a heterogeneous group and inferences from one antagonist to another may not always be correct.

Mutation of Asp^{98} to Asn was shown to cause a decrease in the affinity of the receptor for ligands containing His at position 2 while affinity for Trp^2 containing peptides was enhanced suggesting its (Asp^{98}) role in interaction with His^2 (Rodic *et al.*, 1996). The

contribution of Asp^{98} to antagonist binding has not been investigated. However, the fact that the first GnRH antagonist to be discovered was [des-His^2]GnRH and that many antagonists including those described in this thesis lack His at position two suggests that Asp^{98} must have a different interaction with antagonists, or perhaps is not involved in antagonist recognition.

While GnRH may share some binding sites that are overlapping with agonists, they probably also have binding domains that are distinct from that of agonists. One example is Lys^{121} which has been shown convincingly not to be involved in interactions with an antagonist. The differences may be more profound with non-peptide antagonists, which are structurally less related to GnRH than their peptide counterparts. Hence it is possible that the non-peptide antagonists even use binding sites on the receptor that are totally different from the agonist binding sites. Because antagonists are only required to bind and not also to activate the receptor, there may be a larger selection of binding sites that could be utilised when compared to sites that are available for agonist binding.

Because of the difficulties associated with achieving a non-degenerative crystallisation, the knowledge of the 3-dimensional structure of the GnRH receptor and other GPCRs is limited and no member of the GPCR superfamily of receptor is known in atomic detail. The probable arrangements of the helixes have been suggested by Baldwin (1993) after comparing sequence data from more than 200 receptors. This has been supported by a low-resolution (9 Angstoms) electron density projection map of bovine rhodopsin (Schertler *et al.*, 1993). The transmembrane domains of all GPCR's are believed to be α-helical and arranged around a hydrophilic core in a manner similar to the rhodopsin map (Ballesteros and Weinstein, 1995).

A large part of the information on the ligand binding sites of the GnRH receptor is generated from receptor mutagenesis. The interpretation of data derived from mutational analysis is complicated by a number of factors. A substitution of a residue may modify the side chain that interacts with ligand or may alter the conformation of the receptor affecting the presentation of ligand binding site distant from the mutated

residue. In addition, substitution of residues may either by altering the structure of the receptor or by providing contact site may produce new compensatory sites. In situations where multiple substitutions are made the difficulties of interpretation are compounded even more. On top of these, most of the receptor binding studies on GnRH receptors utilise radioiodinated analogs. The introduction of such a bulky electron withdrawing ligand may alter the properties of the ligand. Such complication of interpretations may in the future be removed when a more through understanding of the effect of an amino acid replacement on the peptide or protein structure is achieved. Recently a number of computational modelling packages have become available (Ballesteros and Weinstein 1995). Information gained through such modelling exercises can be tested by conventional mutagenesis methods. This approach will help focus investigations on fewer domains.

In this thesis, sequence differences between wild type and mutant GnRH receptors have been utilised in order to determine the crosslinking sites of photoreactive GnRH analogs to the GnRH receptor. Ten photoreactive analogs were synthesised and tested. Of these, the crosslinking site of one antagonist (PAnt-1) was localized to a 7 residue segment of the the extracellular N-terminus of the GnRH receptor. Evidence was presented suggesting that Cys^{14} is the attachment site. This localises the N-terminus of the peptide antagonist to which the photoreactive residue is attached to the vicinity of Cys^{14} of the receptor. The characteristics of PAnt-1 determined also show that PAnt-1 can be used as a specific receptor inactivator in experimental situations where a decrease of the surface receptor number is required. Four photoreactive antagonists, PAnt-3, PAnt-3a, PAnt-4 and PAnt 4a were found to have low affinity to the receptor and crosslinking to the receptor could not be demonstrated. One of the antagonists, PAnt-2, had high affinity towards the receptor but analysis of its crosslinking site was unsucessful. The agonist analog PAg-2 while possessing a high affinity towards the receptor, had low crosslinking efficiency and crosslinking could not be demonstrated with this analog or with the similar analog PAg-2a. PAg-3, PAg-4 and PAg-4a were found to have a low affinity. Similar approaches can be used in the future to map binding sites of antagonists. Since many of the antagonists contain bulky hydrophobic and aromatic N-terminal residues it may be possible to

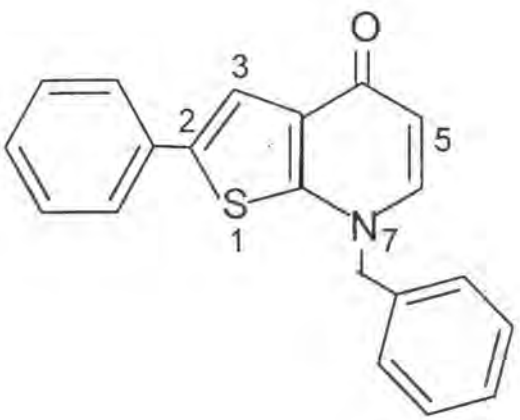
incorporate in their sequence a photoreactive residue such as 4'-trifluromethyl-diazirinyl-o-phenylalanine and 4'-benzoyl-phenylalanine. Such a peptide will have the photoreactive residue directly on the backbone of the peptide therefore, any crosslinking information derived would be very relevant.

All of the current GnRH analogs that are used clinically in order to stimulate or inhibit gonadotropin secretion are peptides and therefore suffer from two major drawbacks. Firstly, in the case of superactive agonists that are used to desensitise the pituitary and subsequently inhibit GnRH secretion, there is an undesirable initial stimulation of the reproductive system. In contrast the onset of inhibition of GnRH antagonists is immediate, although however higher (milligram) quantity perday of antagonists are required. Secondly, all of these peptide analogs have to be administered parenterally or intranasally. Currently a number of slow release depot preparations of GnRH analogs are in use eliminating the need for daily injection. While increasing patient compliance, this has the disadvantage of not being able to withdraw treatment when desired and also the inability of achieving accurate dosages. Because of these disadvantages there is a major interest in developing non-peptide GnRH analogs. Such analogs would ideally be orally administered antagonists that would be effective at low concentration and relatively free of side effects.

The information gained on GnRH binding pockets by site directed mutagenesis and computational modelling has allowed the estimation of the approximate size of molecules that might fit in to the ligand binding pocket. This information has been utilised to screen a large group of non-peptide compounds. Few of them have been found to interact with GnRH receptor in a significant manner (Millar *et al.*, 1999). Using this as a starting point, chemical modification of the compound in part guided by the knowledge of side chains of amino acids in the binding pocket can be made. Both T-98475 (Cho *et al.*, 1998) and FE 101177 contain a bicyclic heterocycle " scaffold" that mimics the main chain of the β-turn of GnRH which was latter modified to produce the final product (Figure 5.1). Similar approaches using heterocyclic compounds have been described as non-peptide antagonists for a number of G-protein coupled receptors including those for cholecystokinin (Freidinger, 1989), angiotensin (Ducia *et*

al., 1992), Substance P (Snider *et al*, 1991) Vasopressin (Yamamura *et al.*, (1991) and Endothelin (Clozel *et al.*, 1993)..

Figure 5.1: Thieno[2,3-b]pyridin-4-one scaffold structure present in FE 101177 and T-98475 non-peptide GnRH antagonists



The ability of one such compound FE 101177 to differentiate between the human GnRH receptor and the highly related sheep or mouse receptors is an indication that highly specific non-peptide antagonists can be developed. In this thesis, the effects of mutation of selected residues and swapping of receptor domains on the binding characteristics of FE 101177 has been studied. The choice of the residues for testing was mainly guided by the differential binding affinity of FE 101177 to two closely related mammalian GnRH receptors (sheep and human). The results show that the TM domains have a combinatorial effect on the binding of FE 101177. The results from the receptor chimera binding studies also show that in large part, the binding domain of FE 101177 may be distinct from that of the peptide agonist. In addition the evidence for the possible role of the charged EC3 residue Asp^{302} in the receptor binding processes has been presented. The work by Cho *et al.*, (1998) on the related compound T-98478 has already shown extremely encouraging results in terms of the therapeutic applicability of these and related drugs. The future will see an accelerated work towards the development of non-peptide antagonists and in-depth studies into the structural basis of GnRH antagonist-receptor interaction.

CHAPTER SIX

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