Design and characterisation of novel GnRH analogues conjugated to hapten carriers

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Declaration

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for any other degree.

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Abstract

GnRH analogues are extremely useful pharmacological agents, both in the investigation of the hypothalamic-pituitary axis and in the manipulation of gonadotropins for the treatment of reproductive cancers, polycystic ovarian syndrome, endometriosis and precocious puberty, to name but a few. Most GnRH analogues are based on the substitution of the decapeptide sequence of GnRH with unnatural amino acids to modify receptor binding affinity, receptor activation and to decrease proteolysis. Nevertheless these peptides tend to be rapidly cleared from the circulation and in most cases have to be administered by injection. The aim of this study was to modify GnRH agonists and antagonists to enhance half-life by conjugation to a steroid, thereby conferring plasma protein binding affinity and also to enhance oral absorption by further conjugation a carrier molecule to achieve oral absorption.

[DLys^6]GnRH analogue-progesterone conjugates were designed and synthesized and the products were analysed by HPLC and mass spectrometry. The most successful method of conjugation was with N,N'-dicyclohexylcarbodiimide (DCC) in the presence of hydroxybenzotriazole (HOBt).

The pharmacological properties of five GnRH antagonist-21-hydroxyprogesterone 21-hemisuccinate conjugates were analysed (conjugates A, B, C, D, and E). The five conjugates were shown to bind to mammalian GnRH receptors in whole cell binding assays. The IC_{50} values of conjugates A, B, C and E were not significantly different (108 ± 22nM, 105 ± 27nM, 134 ± 26nM and 104 ± 7nM respectively), but the IC_{50} of conjugate D was significantly lower at 8390 ± 936nM ($p < 0.001$, STT). The conjugates were analysed for the ability to inhibit mammalian GnRH-stimulated inositol phosphate production as a measure of GnRH receptor antagonism. Conjugates A and B had the lowest IC_{50} values at 97 ± 40nM and 76 ± 17nM ($p > 0.05$, STT), conjugates C and E were significantly less able to inhibit IP

production ($p < 0.01$, STT), with IC_{50} values of 5580 ± 127nM and 16,000 ± 5190nM, whereas conjugate D did not show inhibition of IP production. None of the conjugates showed any evidence of partial agonism. Plasma protein binding was measured in pregnant guinea pig plasma containing high levels of the progesterone-specific plasma protein PBG. All five conjugates were able to inhibit [^{3}H]progesterone binding to plasma proteins, but with higher ED_{50} values than progesterone itself ($p < 0.05$, STT). All antagonist-progesterone conjugates were found to activate the progesterone receptor, as measured by synthesis of chloramphenicol acetyltransferase (CAT) by a CAT reporter gene linked to the progesterone receptor in stably transfected T47D cells. The conjugates induced a similar level of activation to progesterone.

Conjugate A inhibited gonadal steroid production in the marmoset. In the female marmoset, 1.0mg and 0.5mg conjugate A prematurely terminated the luteal phase when administered subcutaneously on day 8 post-ovulation. In the male marmoset, 0.5mg conjugate A inhibited testosterone concentrations for at least 72h after subcutaneous injection, whereas with the same dose of the unmodified peptide, testosterone concentrations were not significantly different from baseline levels within 24h of injection ($p > 0.05$, STT). The duration of action of conjugate A was also compared to the unmodified peptide in an ovarectomized adult macaque.

There are a number of ways to improve the oral absorption of peptides. Vitamin B_{12} is absorbed through the GIT by complexing with intrinsic factor, a large molecular weight protein and the entire vitamin B_{12}-IF complex is internalised by the intrinsic factor-cobalamin receptor. Therefore the design and synthesis of GnRH antagonist-steroid-vitamin B_{12} molecules was attempted and discussed.

In conclusion, novel GnRH analogue-progesterone conjugates have been designed, synthesized and shown to be fully bifunctional *in vitro* with respect to the GnRH receptor, plasma protein binding and progesterone receptor activation. A GnRH antagonist-progesterone conjugate was active

in vivo in a marmoset model, inhibiting gonadal steroid production for significantly longer than the unconjugated peptide. These novel molecules demonstrate that the pharmacokinetics of a peptide drug can be significantly enhanced by conjugation to steroid molecules to improve half-life and in addition, the concept that further modification may be used to increase oral absorption.

Abbreviations

AcD-Nal	acyl D-napthylalanine
AcΔPro	acyl delta-proline
AGP	alpha-1-acid glycoprotein
AMU	atomic mass units
Arg	arginine
Bo	total binding in the presence of excess ligand
BSA	bovine serum albumin
°C	degrees Celsius
CAT	chloramphenicol acetyltransferase
CBG	cortisol binding globulin
DCC	N,N'dicyclohexylcarbodiimide
D-Cpa	D-chlorophenylalanine
DHT	dihydrotestosterone
D-Fpa	D-flurophenylalanine
DMEM	Dulbecco's Modified Eagles Medium
DMF	N-dimethylformamide
D-Pal	D-pydridylalanine
DTT	dithiothreitol
EC	extracellular
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EHC	enterohepatic circulation
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
FSH	follicle stimulating hormone
GIT	gastrointestinal tract
Gly	glycine
GnRH	gonadotropin releasing hormone
GnRHR	gonadotropin releasing hormone receptor
GPCR	G-protein coupled receptor
HAS	human serum albumin
hCG	human chorionic gonadotropin
Hc	haptocorrin
HFP	1,1,1,3,3,3-hexafluoro-2-propanol
His	histidine
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
IBCF	isobutyl chloroformate
IC	intracellular

IF	intrinsic factor
IFCR	intrinsic factor-cobalamin receptor
IP	inositol phosphates
IP_3	inositol 1,4,5-triphosphate
Leu	leucine
LH	luteinizing hormone
mGnRH	mammalian GnRH
M.W.	molecular weight
NSB	non-specific binding
PBG	progesterone binding globulin
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PEI	polyethyleneimine
pGlu	pyro-glutamic acid
PKC	protein kinase C
Pro	proline
RIA	radioimmunoassay
rpm	revolutions per minute
Ser	serine
SHBG	sex-hormone binding globulin
SHBG-R	sex-hormone binding globulin receptor
SPDP	N-succinimidyl 3-(2-pyridyldithio)propionate
STT	student's T-test
TCI	transcobalamin I
TCII	transcobalamin II
TFA	trifluroacetic acid
TLC	thin layer chromatography
TM	transmembrane
Trp	tryptophan
Tyr	tyrosine
UV	ultra-violet
VB_{12}	vitamin B_{12}

All peptide sequences are shown in the amino-terminal to carboxy-terminal orientation.

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1 Literature review

1.1 GENERAL INTRODUCTION

Current estimates are forecasting a world population of 9 billion by the year 2050. The need to find new, easy to use and reliable methods of contraception for the entire world is of utmost importance. At present the most practiced methods of contraception are the combined oral contraceptive pill, the male condom and sterilization. Although the condom and barrier methods will remain important in the prevention of sexually transmitted diseases, particularly in those countries where HIV/AIDS is endemic, there remains a market for a new improved method without undesirable side effects.

Comparably few methods of contraception are currently in use, with most drug-based methods based on supraphysiological sex steroids for both men and women. Gonadotropin releasing hormone (GnRH) analogues work at the level of the pituitary to affect the release of gonadotropins, thereby controlling reproductive processes. When combined with steroid hormone replacement, GnRH analogues may provide an alternative to current therapies.

GnRH analogues have many potential clinical applications that are only beginning to be realized. GnRH can be used in a pulsatile manner to replace the endogenous pulses in polycystic ovarian syndrome (PCOS) and other causes of amenhorrea, in cryptorchidism and delayed puberty as well as in the treatment of infertility [1]. GnRH agonists (through desensitisation) and antagonists (through competition with endogenous GnRH) can be used as therapeutics in the treatment of many reproductive cancers (prostate, mammary, endometrial or ovarian) [2], in addition to treating endometriosis,

precocious puberty, PCOS, hirsutism, acne, porphyria and in the treatment of infertility [3-8]}.

The pharmaceutical drug discovery process is based on the identification of molecules possessing high target specificity, combined with desirable pharmacokinetic properties such as good water solubility, high oral bioavailability and long circulatory half-life. In this thesis an entirely different approach is explored, in which peptide analogues with high target selectivity at the GnRH receptor were modified, first to enhance half-life and to protect from rapid renal clearance and second to increase oral absorption. In this way some of the obstacles to protein and peptide pharmaceuticals such as GnRH analogues, have been overcome in a novel way.

Biologically functional molecules can be coupled to GnRH analogues without loss of GnRH receptor binding. GnRH agonists and antagonists incorporating chlorambucil and melphalan (nitrogen mustard derivatives of 4-phenylbutyric acid and L-phenylalanine) via an N-acyl moiety in position 6 of the decapeptide were designed with mixed success [9]. Only two of the modified peptides possessed high agonistic or antagonistic properties at the GnRH receptor in addition to cytotoxic effects on mammary cancer cells *in vitro* (rat and human) [9].

Modified GnRH analogues have been coupled to a range of different chemotherapeutic antineoplastic radicals through amino acid six of the decapeptides. Although GnRH agonists conjugated to a hydrophobic cytotoxic group had up to 50 times higher GnRH receptor binding affinity than GnRH, those incorporating a second cytotoxic group had reduced binding affinities [10]. Similar modification of GnRH antagonists resulted in decreased binding affinities, with the exception of the 2-(hydroxymethyl) anthraquinone-GnRH antagonist. Nevertheless all conjugates inhibited [^{3}H]thymidine incorporation *in vitro* in some reproductive cancer cell lines [10].

A highly cytotoxic derivative of the antineoplastic doxorubicin was coupled to GnRH agonists and antagonists, preserving both cytotoxicity and GnRH receptor binding affinity of the GnRH conjugates [11]. The design of the molecules was similar to that explored in this thesis, with a D-lysine in position 6 linked from the ε-amino group, via a five-atom glutaric acid spacer, to the doxorubicin derivative 14-hydroxyl group.

Rahimipour and colleagues designed a molecule incorporating an inactive molecule known to bind to albumin to extend the short half-life of GnRH agonists [12]. An inactive form of emodic acid, (a naturally occurring polyhydroxylated anthraquinone used in the preparation of laxatives) was conjugated to [DLys^6]GnRH. These conjugates retained GnRH receptor binding and had significantly extended duration of action *in vivo*, despite the large reduction in total binding to human serum albumin in comparison to emodic acid. Therefore GnRH analogues can tolerate the conjugation of bulky moieties through a D-Lys^6 without abolishing GnRH receptor binding.

This review concentrates on the aspects of the GnRH system that are directly relevant to this work as a comprehensive review of all aspects of GnRH biology are beyond the scope of this project.

1.2 GONADOTROPIN RELEASING HORMONE

1.2.1 General aspects of GnRH

GnRH is a decapeptide hormone important in the control of reproductive processes. It is released from the neurosecretory cells of the medial basal hypothalamus in a pulsatile manner [13] and is transported through the hypophyseal-portal system to the anterior pituitary. Here it acts on specific membrane-bound receptors on gonadotrope cells to stimulate the biosynthesis and release of luteinizing hormone (LH) and follicle stimulating

hormone (FSH). LH and FSH interact directly with the gonads to induce ovulation in the female and spermatogenesis in the male.

GnRH is produced by cleavage of the precursor molecule prepro-GnRH (molecular weight 10kDa) [14] to result in a 10 amino acid peptide. Multiple forms of GnRH may be present in any one species, with the majority of vertebrates possessing at least two or three different forms [15]. In humans mammalian GnRH (mGnRH), with the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is the main form of GnRH present. However the most universal form within vertebrates is chicken GnRH II (or cGnRH II) in which histidine replaces tyrosine, tryptophan replaces leucine and tyrosine replaces arginine. Chicken GnRH II was first isolated from chicken brain and has been conserved for over 400 million years [16]. Multiple forms of GnRH are proposed to have arisen from gene duplication [17]. The relationship between these structural variants has recently been confirmed by gene analysis [18] and is illustrated in figure 1.1.

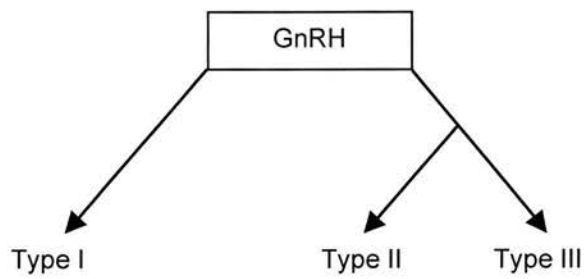


Figure 1.1: Evolution of GnRH subtypes.

In vertebrates GnRH performs a diverse range of functions in a number of different tissues. Although the primary role of GnRH is the control of gonadotropin release, GnRH also affects the central and peripheral nervous systems in a neuromodulatory role [19] and has putative roles in peripheral tissues such as the gonads, placenta, breast and prostate [20].

1.2.2 GnRH structure

The structure of GnRH is relatively well conserved throughout evolution. The maintenance of peptide length, amino-terminal and carboxy-terminal sequences indicates that these features are essential for receptor binding and activation. Structure-activity analysis of large numbers of ligands has shown that the ligand conformation requirements of the mammalian pituitary GnRH receptor are highly stringent [13]. Furthermore, these studies have identified that the amino and carboxy-terminals of the ligand must be in close apposition during receptor binding [13, 21]. Figure 1.2 illustrates the proposed folded arrangement of the GnRH peptide backbone achieved by a β-II type turn involving residues 5-8 [22, 23].

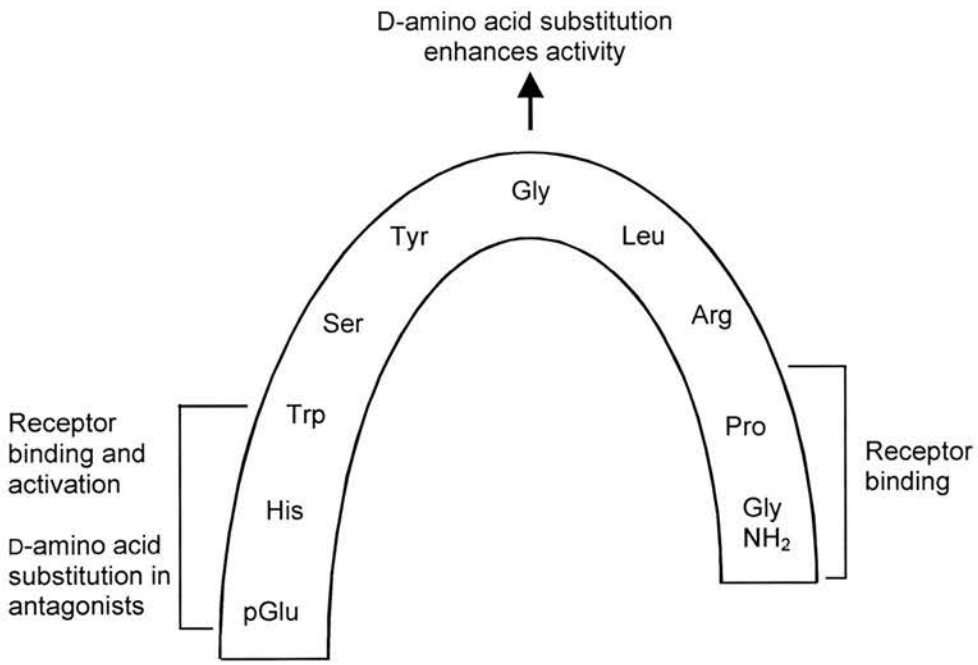


Figure 1.2: Schematic representation of the primary amino acid sequence of GnRH in the folded conformation in which it is bound to the GnRH pituitary receptor (reproduced from [19]).

GnRH has a relatively short circulatory half life, due to its small size (renal clearance) susceptibility to proteolytic cleavage and a lack of plasma protein

binding [24, 25]. Rapid dilution in the general circulation is the major contributor to the short duration of action of GnRH.

1.2.3 GnRH physiology

Pulsatile GnRH release is important in stimulating the required patterns of gonadotropin secretion [26]. Generation of GnRH pulses is a result of integration of both neural and endocrine inputs. Photic input is important in connecting GnRH pulses to light and dark cycles, in addition to input from the autonomic and limbic areas of the brain [14]. In the female the sex steroids oestradiol and progesterone are also involved in the control of GnRH pulsatility, with the feedback being primarily negative. However immediately prior to ovulation, oestradiol exerts a positive feedback effect on the release of GnRH to initiate the LH surge, when combined with an increase in progesterone secretion 12 hours later [27]. The result is GnRH pulses every 60min during the follicular phase of the menstrual cycle, slowing to every 90min during the luteal phase.

In the pituitary gonadotrope cells, the liberation of GnRH stimulates an initial release of LH and FSH, with circulatory half-lives of 30-60 minutes. This also stimulates the movement of gonadotropin-containing secretory granules to the vicinity of the release site, resulting in an increased response to further stimulation of GnRH receptors [14]. In the male, GnRH-stimulated FSH release controls spermatogenesis and LH indirectly affects androgen production in the testis [28].

1.3 GnRH RECEPTORS

1.3.1 General aspects of GnRH receptors

The GnRH receptor is a member of the rhodopsin-like G-protein coupled receptor (GPCR) family. It is located on gonadotropes, comprising approximately 5-10% of pituitary cells [29]. It is a serpentine receptor of 328 amino acids in the human, organised into seven α-helical transmembrane domains (TMs) and a series of connecting intra-cellular (IC) and extra-cellular (EC) loops [30]. The ligand (GnRH) binds to the external portion of the receptor, which includes both the extracellullar loops and the outermost regions of the TM domains. Binding of GnRH alters the receptor conformation, transmitting the signal to the internal domains of the receptor (IC domain), activating signal transduction pathways in the gonadotrope [19].

More than one type of the GnRH receptor is present in most investigated species, although there appears to be only one fully functional GnRH receptor in humans (type I) (Prof R. Millar, personal communication). These can be categorized into two separate families. Type I GnRH receptors have a highest affinity for mammalian GnRH in mammals and type II GnRH receptors have greatest affinity for cGnRH II [13, 31]. The mammalian type I GnRH receptors lack the carboxy terminal tail present in all other GPCRs, whereas the type II receptor subfamily retains this region.

A number of key features are conserved between the type I and type II GnRH receptors. These include amino acids important in both ligand binding and activation of downstream signaling molecules, in addition to the disulphide bridges between EC loop 1 and EC loop 2 [31, 32].

1.3.2 GnRH Receptor Signalling

The mammalian GnRH receptor is predominantly coupled to G_q/G_{11}, a heterotrimeric G-protein that activates phospholipase C β1 (PLC β1), generating a number of second messenger molecules, primarily diacylglycerol (DAG) and inositol triphosphate (IP_3) [31]. The ultimate result of these signalling cascades is an increase in IP_3, elevation of intracellular calcium from both internal and external stores and activation of protein kinase C (PKC). These signalling events result in the biosynthesis and secretion of stored vesicular LH or FSH [33] into the systemic circulation.

Most GPCRs desensitise and internalise as a result of sustained exposure to agonist. Agonist binding stimulates specific G-protein receptor kinases (GRKs), second messenger-regulated kinases (e.g. PKC) or casein kinases to phosphorylate the receptor [31, 34, 35]. This phosphorylation stabilises or facilitates the association of the receptor with β-arrestin, preventing receptor activation by inhibition of G-protein binding within minutes [36, 37]. β-arrestin contains recognition sites for clathrin and AP-2, which when bound to the β-arrestin-receptor complex, targets the receptor for internalisation via clathrin-coated vesicles [31].

1.4 GnRH ANALOGUES

1.4.1 Synthesis of GnRH analogues

Peptide analogues of GnRH are based on substitution of the native GnRH decapeptide structure. GnRH agonists are generally produced by modifying the amino acids in positions 6 and 10 (Figure 1.3), whereas alteration of positions 1, 2, 3, 6, 8 and 10 results in antagonists [38]. Combination of the substituents conferring greatest potency has resulted in significantly more effective and useful GnRH analogues [39].

1.4.1.1 Incorporation of D amino acids

Incorporation of dextro (D) isoforms of amino acids, particularly in position six, increases agonistic potency of GnRH analogues [40], with a 40-fold maximum increase with hydrophobic amino acid substitutions [39]. The most effective natural amino acid substitution is observed with a D-Trp in this position [41, 42].

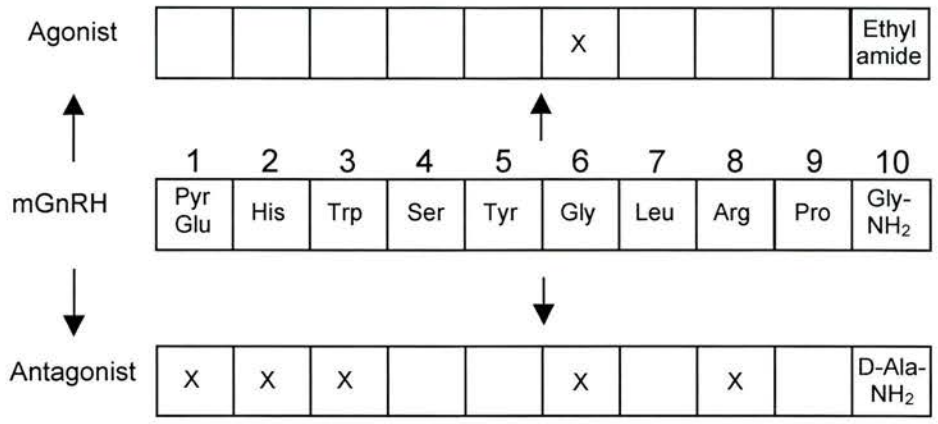


Figure 1.3: Amino acid sequence of GnRH (adapted from [38]). Alterations to the endogenous GnRH peptide sequence that create agonists and antagonists are denoted 'X'.

1.4.1.2 Modification of C-terminal

Replacing the glycine in position 10 of mammalian GnRH with ethylamide [43] results in a significantly more potent agonist of GnRH when combined with other changes to the peptide sequence. The asparagine 102 residue of the GnRH receptor is believed to be involved in the docking of the glycinamide C-terminus, probably through hydrogen bonding. GnRH analogues with a C-terminal ethylamide appear to be less dependent on this asparagine residue [44] and ethylamide features in many GnRH analogues. An aza-Gly substitution at position 10 also increases potency of GnRH agonists [45]. Total chain length appears to be important in receptor binding affinity and the ethylamide and aza-Gly substitutions are proposed to maintain this aspect [21].

In contrast, an ethylamide substitution reduces the potency of GnRH antagonists [46]. It appears that the enhanced potency of GnRH agonists is due to secondary structure stabilization in the active conformer, through dipole-dipole interaction of the ethylamide with the histidine imidazole ring [21].

1.4.1.3 Modification of N-terminal

Substitutions at positions 1, 2, 3, 4 and 5 reduce the potency of GnRH agonists [21], with the exception of a two-fold increase with 1-L-Nal incorporated in position 3 [47].

The first reported GnRH antagonist was des-His^2-GnRH [48]. Comparisons with [Gly^2]GnRH, [Phe^2]GnRH and [Trp^2]GnRH revealed that des-His^2-GnRH acts as a weak antagonist because functionality at position 2 is removed, without disrupting the features important for recognition at the GnRH receptor [21]. The first GnRH antagonist with significant anovulatory potential *in vivo* was formed by combining a weakly active antagonist ([D-Phe^2]GnRH [49]) with [D-Ala^6]GnRH to create [D-Phe^2 D-Ala^6]GnRH [46, 50]. Incorporation of halogenated phenylalanine residues (first investigated in [4-F-D-Phe^2, D-Ala^6)GnRH [51]) was a major breakthrough in the quest to further increase the potency of GnRH antagonists [21].

1.4.2 Functioning of GnRH agonists

Sustained agonist stimulation induces slow desensitisation of the GnRH receptor (described in section 1.3.2), resulting in decreased gonadotropin and gonadal sex steroid secretion [6]. However the slow desensitisation rate results in an initial stimulation of gonadotropin release before the receptors are internalised [52]. This can be detrimental in clinical situations, causing an initial flare of symptoms before long-term desensitisation occurs. Nevertheless GnRH agonists are administered in gonadal replacement

therapy or as long-term continuous treatment to reduce FSH and LH secretion.

Potent, long acting GnRH agonists are active in the microgram range, compared with the milligrams of GnRH antagonist required for clinical effects [8]. Agonist-stimulated desensitization is enhanced by the positive feedback effect of endogenous GnRH, but endogenous GnRH competes with GnRH antagonists for receptor binding, resulting in the need for higher doses with GnRH antagonists.

1.4.3 Functioning of GnRH antagonists

GnRH antagonists compete with endogenous GnRH for GnRH receptors present on pituitary gonadotrope cells. An instant block of LH and slower block of FSH secretion occurs, dependent on continued, near-complete receptor occupation [53, 54]. The equally rapid reversal of inhibition is also of benefit in comparison to GnRH agonists and may be advantageous to contraceptive use of antagonists, where gonadotropin inhibition is only required for a short period.

The absence of the initial stimulatory phase is a notable benefit of GnRH antagonists over agonists. Avoidance of this supraphysiological secretion of LH and FSH before desensitization to GnRH has major advantages in clinical practice. The immediate and persistent block of gonadotrope and extra-pituitary GnRH receptors provides the basis for a theoretical use for GnRH antagonists as anti-fertility agents [55].

1.4.4 Clinical aspects of GnRH agonists and antagonists

In clinical practice, GnRH agonists are used to suppress gonadotropin secretion through GnRH desensitization [53]. However the more rapid

inhibition of gonadotropin secretion induced by GnRH antagonists is often preferable. Current indications include follicular stimulation in vitro fertilisation (IVF) treatment cycles and in the treatment of endometriosis, uterine fibroids, PCOS, precocious puberty and cancer of the prostate, breast and ovary [53].

An alternative approach to developing GnRH antagonists with improved pharmacokinetic parameters is to design nonpeptide antagonists, thereby avoiding some of the problems associated with the peptide nature of many current analogues. The first nonpeptide GnRH antagonist was disclosed in a patent in 1987 [56]. This fused tetracyclic benzodiazepine blocked ovulation in rats at a dose of 0.5mg/kg [57]. Tryptamine-derived GnRH antagonists have been described recently by Ashton *et al* [58] with inhibitory binding affinities at the human GnRH receptor of around 1nM. The same group has also published studies on 2-arylindoles [59] and quinolones that act as GnRH antagonists [60-62]. However it is important to note that unlike peptide GnRH analogues, nonpeptide GnRH antagonists are extremely species-specific and drug development may be hindered because inexpensive rat or mouse *in vivo* studies are not possible [57]. Abbott, Merck and Alanex Corp. are the major companies currently involved in the development of nonpeptide GnRH antagonists [57] and it is likely that the successful molecules will revolutionise the role of GnRH analogues in the clinic.

A range of peptide GnRH agonists is currently available for clinical use, for example Leuprolide (Abbott), Zoladex (AstraZeneca) and Buserelin (Hoechst). These agonists vary in mode of administration (depots, subcutaneous injection or nasal spray) and dose frequency.

Clinical use of peptide GnRH antagonists was initially thwarted by adverse reactions at injection sites, however there are now several potent antagonists available with minimal histamine-releasing properties [53, 55, 63]. A number of antagonists have recently been licensed, such as Cetrorelix (Asta Medica) and Ganirelix (Organon) [64].

1.4.5 Side effects of GnRH analogues

Long term oestrogen deprivation resulting from prolonged GnRH analogue administration can result in significant side effects in females. Initial clinical side effects include hot flushes, vaginal dryness, decreased libido and headache [65]. Long-term clinical side effects are considerable. Hypo-oestrogenic bone loss can occur, although recovery is likely after cessation of treatment [66].

Prolonged uterotrophic effects of oestrogen in the absence of systemic progesterone can induce reversible endometrial hyperplasia during prolonged periods of treatment with GnRH agonists [67]. The same study failed to find a similar adverse effect with GnRH antagonists over 90 days treatment, although both agonist and antagonist were equally effective in suppressing ovulation in rhesus monkeys [67].

1.4.6 Difficulties with GnRH analogues

The hydrophobicity of some GnRH analogues, particularly GnRH antagonists, can be problematic in drug delivery. Gel formation at injection sites [55] during subcutaneous or intramuscular injection is a particular problem with such analogues. This can be advantageous in long-term administration by prolonging diffusion into the general circulation. However this is not a major problem with other routes of administration such as oral delivery.

Short-term GnRH antagonist administration can induce luteolysis, terminating the luteal phase. The effect is dependent on the stage of the cycle, in the human [68-71] rhesus macaque [72, 73] and stumptailed macaque [74] and may culminate in premature menses [68]. This would be undesirable in a medium to long-term contraceptive. However combination of GnRH

antagonists with low concentration sex-steroid replacement therapy may result in a more patient-acceptable contraceptive for repeated cycles.

One of the major drawbacks of long-term GnRH agonist or antagonist administration is the suppression of gonadotropin-dependent oestrogen production. The extent of this hypo-oestrogenic state is dependent on both the dose and duration of treatment [65], but co-administration of appropriate amounts of oestrogen with GnRH analogues may avoid this effect. It is for this reason that GnRH antagonists have been proposed for short term use (3 to 4 months duration) as post-partum contraceptives [75]. In this situation the peptide nature of the antagonist has the advantage of being inactive when orally consumed by nursing infants in breast milk [76].

The dose-dependence of GnRH antagonists [55] could be of clinical benefit with some routes of administration, allowing dose and duration of treatment optimisation for each condition [53] and perhaps also on an individual patient basis. This would be of particular benefit in the female, permitting a balance of pituitary gonadotropic function suppression with gonadal oestrogen production, minimising the risk of hypo-oestrogenic side effects [53].

Newer, more potent GnRH antagonists are widely employed in assisted reproduction, avoiding the initial increase in gonadotropin release and with more rapid effects than occur with GnRH agonists [54, 55]. The use of GnRH antagonists has led to a decrease in the number of treatment cycles terminated because of ovarian hyperstimulation syndrome and loss of oocytes due to spontaneous LH surges. An increase in patient satisfaction without compromising oocyte retrieval, fertilisation rates and embryo quality [77] has also been observed with GnRH antagonists. However decreases in pregnancy and implantation rates have recently been identified [78]. GnRH receptors are also present in the periphery. They have been identified in the reproductive tract, oocyte and embryo [20, 33, 79-81]. It is entirely possible that pharmacological doses of GnRH antagonists may

significantly affect the synthesis of growth factors [82-85] necessary for successful pregnancy through interaction with extrapituitary GnRH receptors. This effect may be of benefit in using GnRH antagonists as contraceptive agents, through decreasing the likelihood of implantation of a viable embryo.

1.4.7 Effect of short-term GnRH antagonist treatment

GnRH antagonists have also been used to investigate many aspects of reproductive function. Most studies to date have used a relatively short duration of treatment, either as single doses or repeated administration over several days to examine physiological effects and potential contraceptive use. Only a few studies have examined longer-term effects for applications where a continued dose of antagonist is required, such as in the treatment of reproductive cancers and as potential contraceptives.

Administration of GnRH antagonists at any stage of the menstrual cycle or to postmenopausal women has similar effects at the level of the pituitary. The inhibitory effect on LH release is more pronounced than on FSH, reflecting the longer half-life of FSH and its dependence on synthesis, rather than secretion. In contrast, the ovarian response to GnRH antagonists is more variable, depending on the phase of the cycle and the maturation of the developing follicle or corpus luteum [70].

1.4.7.1 Follicular phase GnRH antagonist treatment

Early evidence in macaques suggested that intermittent administration of low-potency antagonists during the late follicular stage could prevent the midcycle LH surge and thus ovulation [65]. In contrast, more recent work has suggested that the dominant follicle is relatively independent of LH support at later stages of development [54, 68].

GnRH antagonist treatment for 3 days during the early follicular phase did not significantly prolong the duration of the follicular phase, cautiously suggesting that early ovarian follicles may be relatively independent of LH and FSH support [69]. However few studies have attempted to confirm this finding. Administration of Detirelix (a GnRH antagonist) during the midfollicular phase also prevented the LH surge, inducing menses. This was followed by a lengthened time to the luteofollicular transition of the subsequent cycle [70]. A second study employing the GnRH antagonist Nal-Glu also found an increase in follicular phase length after a treatment period of 3 days. This was attributed to the demise of the dominant follicle and reinitiation of follicular growth and selection in the contralateral ovary [68]. In normal women, antagonist treatment during the periovulatory period (defined as a circulating oestradiol level exceeding 550pmoll^{-1}) delays the LH surge, the duration of which depends on the dose and frequency of the antagonist [86]. Similar results were observed with the injection of Detirelix for 3 consecutive days to a small group of normal cycling women during this period of the follicular phase [70].

1.4.7.2 Luteal phase GnRH antagonist treatment

The tolerance of the corpus luteum to GnRH antagonist treatment varies during the luteal phase. Suppression of gonadotropin levels in primates during the early luteal phase for up to 72 hours can decrease the level of circulating progesterone, but does not impair the functioning of the corpus luteum sufficiently to induce luteolysis [73, 74].

There is a variable response to GnRH antagonist treatment during the midluteal phase in both humans and primates [54, 70, 72-74]. This could be partially due to differences in the relative potencies of the antagonists used in the studies. In women, midluteal phase GnRH antagonist administration decreases both oestradiol and progesterone produced by the corpus luteum. If the dose is sufficient, luteolysis occurs, resulting in premature menses, usually within 48h of the last dose of a 3-day treatment schedule [68, 69, 71].

The corpus luteum can recover from short (single dose) and lower doses of antagonist and continues to function normally after drug treatment.

The effect of GnRH antagonists on the corpus luteum may be overcome by human chorionic gonadotropin (hCG) at physiological levels characteristic of early pregnancy [69, 72]. McLachlan *et al*[87] demonstrated that hCG, but not human menopausal gonadotropin could prevent GnRH antagonist-induced luteolysis of the human corpus luteum. This may prevent the potential use of GnRH antagonists as post-coital contraceptives.

1.4.8 GnRH antagonists as potential contraceptives

It is possible that GnRH antagonists could be used for female contraception, if the problems with long-term oestrogen and progesterone deprivation can be overcome. There are two main ways in which this could be achieved, either by using GnRH antagonists for short periods (days), or by using prolonged treatment over several months with concurrent oestrogen or progesterone replacement therapy. In the case of short-term GnRH antagonist treatment, the variable LH dependence of both the follicle and corpus luteum may be problematic.

The ability of both GnRH agonists and antagonists to reliably inhibit ovulation is widely accepted, however maintenance of this effect is dependent on continued occupation of pituitary GnRH receptors. Once the production of LH and FSH is no longer inhibited, folliculogenesis is reinitiated, resulting in ovulation within days after the cessation of antagonist treatment [69, 88-90].

Prolonged exposure to GnRH antagonists could also be used as a possible method of preventing pregnancy, in combination with oestrogen and progesterone replacement. The effects of long-term GnRH agonist treatment have been investigated in primates [67, 91] and humans [92, 93]. Most

subjects remained anovulatory throughout the treatment period, although endometrial abnormalities arising from oestrogen deprivation were observed. It is likely that GnRH antagonists could also be used in this way as contraceptives if suitable long acting formulations can be produced with little side effects.

The GnRH agonist Nafarelin successfully inhibited ovulation in 48 normal women over a period of six months, causing oligomenorrhea in the majority of participants [93]. The side effects of intranasal administration of the peptide were notable in several women (primarily hot flushes) leading to discontinuation of the trial for these women. Despite this issue, the GnRH agonist provided a rapidly reversible and reliable method of contraception.

GnRH antagonists also have potential as male hormonal contraceptives. Suppression of FSH and LH secretion in the male inhibits the steroidogenic and spermatogenic activity of the testis [28]. Administration of supraphysiological doses of androgens or progestagens could be used to capitalise on the negative feedback effect of testosterone on the hypothalamic-pituitary axis (figure 1.4). Alternatively, blocking the GnRH receptor directly with a GnRH antagonist would have the same effect on the pituitary. The most successful regimes are based on the 'block and replace' theory in which a GnRH antagonist is administered in conjunction with physiological doses of testosterone.

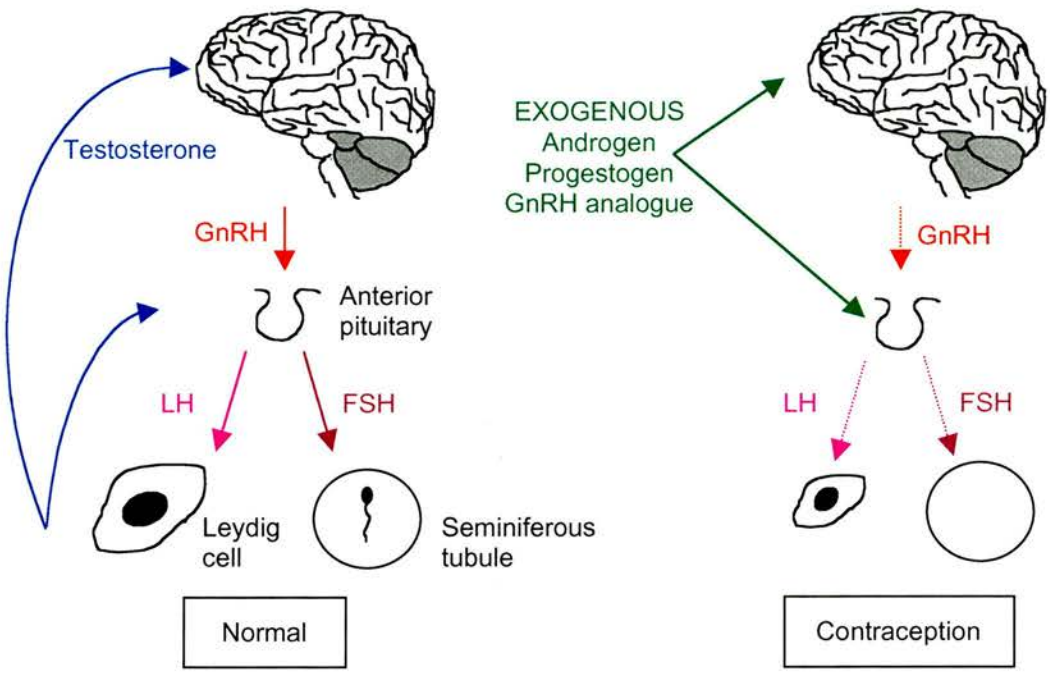


Figure 1.4: Relationship of hypothalamic-pituitary axis with gonads in a normal male and in the presence of hormonal contraception (adapted from [28]).

1.5 PLASMA BINDING PROTEINS

Binding of both drugs and endogenous molecules to plasma transport proteins is important to the half-life of these molecules in the circulation. Drugs and other molecules that bind to plasma proteins have a longer half-life than similar molecules that lack this characteristic. Therefore conjugating a plasma protein binding molecule to a non-plasma protein binding-molecule is a potential method of enhancing half-life and resistance to metabolic degradation, in this case, increasing the half-life of peptide GnRH analogues.

1.5.1 General aspects

Plasma protein binding affects the pharmacokinetics of many molecules through a combination of specific and non-specific interactions. Non-specific plasma proteins consist of serum albumin, alpha-1-acid glycoprotein and lipoprotein. Sex steroid-specific plasma proteins include cortisol binding globulin (CBG), sex-hormone binding globulin (SHBG) and progesterone binding globulin (PBG). This review will concentrate on the plasma proteins important in sex steroid physiology, CBG, SHBG and PBG.

1.5.2 Serum albumin

Most steroids, many other endogenous compounds and xenobiotics bind to albumin, the most abundant plasma protein. Current theories suggest there are at least six distinct binding sites on human serum albumin (HSA), binding small organic compounds (sites I and II), long-chain fatty acids (sites III and IV) and metals (sites V and VI) separately with normal ligand/albumin concentrations [94].

HSA has a molecular weight of 69kDa and is present in a high concentration of 550μM, almost a thousand times more than the specific steroid binding proteins [95], resulting in a high capacity. Although albumin binds to many steroids, the association constants for these interactions are generally low.

Progesterone binds to HSA with an association constant of $0.36\mu M^{-1}$ at 4°C [96], compared with an association constant for CBG of $1300\mu M^{-1}$ [97]. The affinity constant of cortisol for albumin is considerably lower at $5000\mu M^{-1}$ [98] but oestradiol binds with a similar affinity to progesterone ($0.1\mu M^{-1}$) [99]. Despite the low affinity of the interaction, HSA is a major steroid transport protein, contributing to the high proportion of steroids that are protein bound and the importance of albumin to the transport of the sex steroid hormones is illustrated in table 1.1.

Hormone	% Globulin-bound	% albumin bound	% free dialyzable
Cortisol	75	15	10
Progesterone	10 (CBG)	90	2
Aldosterone	10	50	40
Dihydrotestosterone	80	20	2
Testosterone	60 (SHBG)	40	2
Oestradiol	40	60	2

Table 1.1: Hormone distribution to plasma proteins in the human (from[100]).

1.5.3 Alpha-1-acid glycoprotein

Alpha-1-acid glycoprotein (AGP), also known as orosomucoid, is a protein with some unusual features including a very high carbohydrate content (45%) and a very low p*I* (2.8-3.8). It was discovered simultaneously in 1950 by Schmid [101] and Winzler and colleagues [102] and despite the subsequent publication of many articles, the exact function of this protein remains unclear. Physiologically the concentration of AGP is relatively stable (around 1g/l in humans), however during acute-phase reactions the concentration rises by several times [103].

AGP mostly binds basic and neutral endogenous molecules, for example vanilloids [104], IgG3, heparin, serotonin [101], platelet activating factor [105], melatonin [106] and histamine [107]. AGP also binds some endogenous steroids such as cortisol and synthetic steroids for example RU486 [108].

Some xenobiotics such as tamoxifen [101] and propanolol [109] also bind to AGP. Although it is generally assumed that acidic drugs are bound to serum albumin, in pathophysiological states binding to AGP may increase with a rise in the concentration of the protein. The binding capacity of AGP is related to the conformational change, ligand polarity and temperature [103]. Binding to AGP can affect the effective (free) concentration of many drugs.

1.5.4 Specific steroid-binding plasma proteins

Specific plasma proteins bind to steroids through numerous non-covalent interactions [110] to affect the transport of steroid hormones. There are two plasma proteins present in low concentrations that bind steroids with high affinity in humans, CBG and SHBG. A steroid may bind to more than one plasma protein [110], for example progesterone is bound to both CBG and albumin under physiological conditions (table 1.1).

An additional binding protein known as PBG exists in guinea pigs and other hystricomorph rodents [110, 111]. In these species, the evolution of PBG to specifically bind progesterone is coincident with a loss of CBG affinity for progesterone [112], whereas human CBG has affinities in the same range for both progesterone and cortisol [113]. It is interesting to note that most species of New World monkey have very low plasma levels of CBG [114]. The glucocorticoid system in these species is dramatically different to other primates, with greatly elevated plasma cortisol concentrations of up to 1-4μM compared to 10-300nM in Old World primates and humans. In addition, the CBG of New World monkeys appears to have a reduced affinity for cortisol, resulting in an apparent resistance to glucocorticoids [114].

The role of these macromolecules in steroid physiology is still controversial despite several decades of work. Reduction of steroidal hepatic metabolism is a proposed function of plasma proteins since androstenedione does not bind to plasma proteins and is cleared more rapidly from plasma than testosterone or dihydrotestosterone [110, 115]. Steroid binding proteins may also 'buffer' changes in hormone concentration [110] and provide an immediate source of steroid [110]. The free hormone hypothesis is the basis on which steroids are currently presumed to operate. This states that the biological activity of a given hormone is affected by its unbound (free) rather than protein-bound concentration in the plasma [116]. This is difficult to prove, but it is generally accepted to hold true for the majority of steroid hormones, at least on a tissue-specific basis [116].

In addition to steroid transport roles, recent work suggests that the binding proteins themselves have plasma membrane receptors, however the role of these receptors is yet to be elucidated. SHBG has been shown to interact with a membrane-bound receptor (SHBG-R) with high affinity when bound to certain steroids [117]. The SHBG-R appears to be a G-protein linked receptor that activates adenylyl cyclase via cAMP, but not all steroids that bind to SHBG activate the SHBG-R [117].

CBG receptors have been identified on hepatic cell membranes [118]. The receptor for the pregnancy-associated variant of CBG (pCBG) is present in the plasma membranes of decidual endometrium and placental syncytiotrophoblast [119]. Steroids that bind to CBG, such as cortisol and corticosterone affected the binding of CBG to its receptor, whereas steroids that do not interact with CBG, for example dexamethasone, had no effect on CBG-receptor binding [97].

CBG, SHBG and PBG are glycoproteins with molecular weights of the same order of magnitude. Each one has a single binding site for a steroid molecule [95]. Two main types of non-covalent forces govern the interaction between steroid and plasma protein. The first is hydrophobic (nonpolar) bonds, for instance van der Waals forces or other low-energy interactions between the lipophilic groups of both molecules. Hydrogen bonding also plays a part in the binding process. The overall affinity of binding between the steroid and plasma protein is the sum of many weak bonds, enabling rapid association and dissociation under physiological conditions [110].

The interactions between steroids and plasma proteins are temperature dependent (table 1.2), due to the contribution of hydrophilic and hydrophobic bonds in the binding process. If the majority of bonds are of hydrophilic nature, then the K_a at 4°C is greater than at 37°C. The converse is true for predominantly hydrophobic bonds between the binding site and the steroid [95]. The number and nature of these interactions determines the optimum steroid substituents for high affinity binding. Analysis of the association and dissociation rate constants shows that the temperature

dependency of CBG and PBG binding is due to an altered dissociation rate constant [95].

Protein	Species	Mol. Wt.	Serum conc. (mM)	Steroid	Ka (μM) 4°C	Ka (μM) 37°C	Ref.
CBG	Human	52,000	0.7	Cortisol	1400	40	[97]
				Progesterone	1300	30	
SHBG	Human	36,000	0.3	DHT	3100	990	[120, 121]
				Oestradiol	300	220	
PBG	Guinea pig	88,000	13	Progesterone	2200	350	[122]

Table 1.2: Comparison of steroid-binding serum proteins. Adapted from [95] DHT denotes dihydrotestosterone.

1.5.4.1 Sex Hormone Binding Globulin

SHBG, (also known as testosterone-oestradiol binding globulin, TeBG) is a homodimer with a molecular mass of around 100kDa [110, 123], consisting of two essentially identical subunits of between 45-52kDa [123, 124]. It primarily binds the most active androgen dihydrotestosterone (DHT) and testosterone (T) with affinity constants of less than $1 \times 10^{9} M^{-1}$ [115]. However it also binds to oestradiol (the affinity constant for oestradiol is approximately $5 \times 10^{8} M^{-1}$) [123] and weaker androgens such as androstenedione. The other endogenous oestrogens oestrone and oestriol do not bind to SHBG [115].

The concentration of SHBG in human plasma is sex-dependent, at $2 mgl^{-1}$ in men and $4 mgl^{-1}$ in non-pregnant women [124]. Sex differences in circulating androgens also affects the steroids bound to this particular plasma protein. In men, SHBG primarily binds to testosterone, however the lower levels of testosterone in females allows the other 17β-hydroxyandrogens to play a more significant role in this sex [115]. The balance between bound and free levels of androgens and oestrogens contributes to the production of SHBG

[115]. Age is also a factor in the synthesis of SHBG, with the peak concentration occurring in childhood and decreasing during puberty [115].

Circulating concentrations of SHBG are affected by administration of exogenous steroids, such as during oral contraception. SHBG levels rapidly increase by around 200% after several days of treatment with the combined oral contraceptive pill [125], declining to 100% during the pill-free interval. Maximum concentrations of SHBG (200-250nmol/l) are observed after three treatment cycles [125-130]. Oestrogen-stimulated increases in SHBG concentrations can be suppressed by androgens. Combined oral contraceptives containing testosterone-derived progestagens in combination with oestrogens (e.g. levonorgestrel and ethinylestradiol) do not increase SHBG levels, whereas combinations with less androgenic progestogens (e.g. desogestrel) increase SHBG capacity [131].

Structural investigations using substituted steroids have demonstrated that in order to interact with SHBG, a steroid must contain a 17β-hydroxyl group. Several other features, such as the addition of a hydroxyl or keto group at C11 also have major negative affects on binding affinity [110, 132]. Modification of carbon 2, 6, 9 and 11 in the steroid nucleus also reduces binding affinity [132].

1.5.4.2 Cortisol Binding Globulin

CBG (also known as transcortin) is a ubiquitous plasma protein synthesized in the liver [95], present in all vertebrates investigated [95, 110, 115, 133]. It has a molecular weight of around 52kDa, a relatively high proportion of which is due to carbohydrate, with low sequence homology to other steroid-binding proteins [134]. CBG has an essential role in modulating corticosteroid activity by binding to steroids, ensuring only a small proportion is 'free'. It also has the characteristic strong temperature dependence, contrasting with non-specific steroid binding by albumin [110].

In addition to corticosteroid binding, CBG also interacts with progesterone, a more significant function during pregnancy [134]. However this feature varies according to the presence or absence of a specific plasma protein with the primary role of binding to progesterone (PBG). For instance in humans, cortisol and progesterone are bound by CBG with similar affinity, whereas in the guinea pig, CBG has a far greater affinity for cortisol than for progesterone, reflecting the significant role of PBG in this species [97]. The plasma concentration of CBG also varies considerably during pregnancy, with the increase of CBG activity to some extent compensating for the elevated cortisol [135]. In the case of human CBG, the affinity of binding to cortisol is 79nM and to progesterone is 70nM (at 4°C) [113]. In contrast to the marked sex differences in SHBG levels, there is no distinction between the concentration of CBG in the male or female, nor is there a variation throughout the menstrual cycle [134]. Plasma concentrations of CBG are affected by physiological levels of oestrogens, accounting for the 2 to 3-fold increase in CBG during the last trimester of pregnancy [134].

Like SHBG, CBG levels are influenced by oral contraceptives, but the effect is due only to oestrogens [125-130]. CBG concentrations are increased from 40mg/l to 90mg/l [128, 129], but low dose ethinylestradiol treatment (20μg/day compared with a minimum of 30μg/day in most combined oral contraceptives) reduces this increase [126, 136].

The requirements for optimal binding to CBG have also been deduced by assessing the effect of single substitutions in steroids. These studies have shown that in human CBG, the 20-oxo and 10β-methyl groups are essential for binding, the 3-oxo and 4-ene are also important. Although the 11β, 17α-, and 21-hydroxy groups are relatively unimportant, hydroxyl groups impair binding at positions 11α, 6α, 6β, 12α, 14α, 16α and 19 [97]. Guinea pig CBG selectivity is similar, but an 11β-hydroxy is required and the necessity for a 10β-methyl is replaced by a need for the same substituent in position 19 [112]. The essential features of steroids for binding to the specific plasma

proteins were taken into account in the design of sites of conjugation to GnRH analogues, in order to retain binding to the globulins.

1.5.4.3 Progesterone Binding Globulin

PBG is a steroid binding protein found in unusually high concentration in the serum of pregnant hystricomorph rodents, such as the guinea pig. The existence of this specific plasma binding protein for progesterone appears to affect the ability of the CBG to interact with progesterone. PBG is a more stable protein than CBG, being more resistant to heat and acidic pH [137]. It has a molecular mass of 88kDa and contains a single steroid binding site [138]. The affinity of binding to progesterone is temperature dependent, in common with CBG and SHBG. The K_a for progesterone at 37°C is 35nM and at 4°C is 2nM [138].

PBG binding is proposed to predominantly consist of hydrophobic interactions with the steroid. An exception is the C-3 keto group of the A ring [95]. The high affinity of binding to progesterone suggests that at least in the case of guinea pig PBG, the binding site is adapted to bind progesterone most strongly, since almost any substitution results in reduction of binding affinity [139].

1.6 ENHANCING ORAL ABSORPTION OF PEPTIDES

Peptides are increasingly important in the pharmaceutical industry. Growing numbers of potential therapeutics are small peptides, but problems of poor oral availability have hampered the development of specific drugs for clinical use. The physicochemical constraints of size and solubility in the aqueous phase of the gut lumen affect the absorption of pharmaceuticals by this route. Several strategies have been employed to overcome this problem with a range of success.

There are also metabolic barriers to be overcome to achieve intestinal peptide absorption. The first major metabolic barrier is the stomach, containing proteolytic pepsins and acidic secretions that can hydrolyse the orally administered peptide. The second barrier is in the small intestine, where the presence of pancreatic enzymes such as trypsin, chymotrypsin, elastase and carboxypeptidases A and B [140] and the epithelial mucosa itself contribute to breakdown and lower absorption.

This review concentrates on the reported methods of enhancing oral absorption and although other methods such as buccal delivery are acknowledged, these are not discussed here except where relevant to GnRH analogues.

1.6.1 The bile acid transporter as an uptake system

Absorption of fat and fat-soluble vitamins is vital to the diet. Bile acids are secreted by the liver into bile, excreted into the ileum by the gall bladder, reabsorbed in the terminal portion of the ileum, before being taken up by hepatocytes and re-entering the cycle [141]. The process is known as the enterohepatic circulation (EHC) of bile acids (figure 1.5).

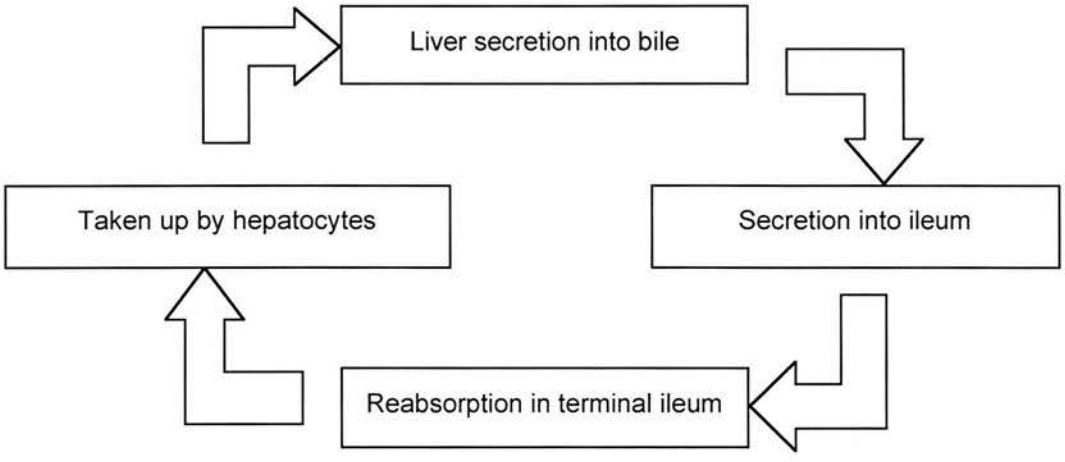
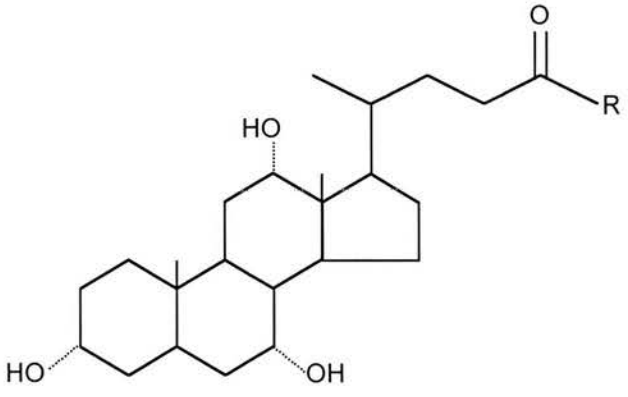


Figure 1.5: Enterohepatic circulation of bile acids.

EHC of bile acids is a highly efficient mechanism and combined with the high capacity, gives rise to the possibility of using bile acids as carrier molecules for small peptides and other poorly absorbed molecules. There is a total bile acid pool of 3-5g in humans, only 2-5% of which is not reabsorbed in the terminal ileum and is excreted in faeces [141]. In 1987 Ho addressed the possibility of using bile acids as carrier molecules "Many potential therapeutic applications are foreseen: improvement of the oral absorption of an intrinsically, biologically active, but poorly absorbed hydrophilic drug" [142].

Passive and active mechanisms are involved in the uptake of bile salts from the intestine. Ionic and non-ionic passive diffusion occurs throughout the small intestine, whereas the Na^+-gradient and basolateral Na^+/K^+-ATPase mediated uptake is localised to the ileum [140]. Physiologically, bile acids are found in conjugated states, conjugated to glycine or taurine, aided by coenzyme A to form N-acyl conjugates [141]. Conjugation in this way has several benefits, namely prevention of precipitation under acid conditions, minimization of passive transport in the jejunum and increased active transport in the terminal ileum [141].



Where R corresponds to OH in cholic acid, $NHCH_2CH_2SO_3H$ in taurocholic acid or $NHCH_2COOH$ in glycocholic acid.

Figure 1.6: General structure of the trihydroxy bile acids

Swaan and co-workers [141] attempted to use coupling to cholic acid via the 24 position (C=O) as a method of enhancing the transepithelial transport of model peptides ranging from 2-6 amino acids in length. The ability of conjugates to inhibit transepithelial transport across polycarbonate membranes coated with a human intestinal cell line (CaCo-2) was investigated by competition with [^{3}H]taurocholic acid. The affinity of modified cholic acid for the bile acid carrier was significantly reduced by conjugation to peptides rather than to a single amino acid. However after the initial decrease in affinity, additional increases in chain length did not induce further reduction. Metabolism of these conjugates was limited to 3% over 2 hours of *in vitro* studies.

The nature of the bile acid uptake system is that secreted bile acids are reabsorbed in the terminal ileum, transported to hepatocytes and re-secreted into bile. However this means that a bile acid-coupled molecule such as a peptide would also follow this route, ideal for liver-specific drug targeting [143], but not for absorption into the general circulation. Indeed this effect was observed when a fluorescent oxaprolylpeptide was coupled to a synthetic bile acid and perfused into the ileum of anesthetized rats [144].

1.6.2 Vitamin B_{12} as an uptake system

Vitamin B_{12} absorption across the GIT is significantly different from most other nutrients, although it shares some similarities with iron uptake.

Vitamin B_{12}, also known as cobalamin (VB_{12} or Cbl) is a water-soluble corrinoid molecule with a tetrapyrrolic ring-like structure. In the center of the tetrapyrrolic nucleus is a cobalt ion that can be attached to either methyl, deoxyadenosyl, hydroxy or cyano groups (figure 1.7). VB_{12} is found in all animal tissues, but not in plants, therefore all VB_{12} must originate from bacterial, fungal and algal sources [145].

Pepsin present in the stomach releases VB_{12} from consumed food. The free VB_{12} complexes with a salivary transport protein known as haptocorrin (Hc). Under the acidic conditions of the stomach it has the highest affinity for vitamin B_{12} in comparison to other transport proteins [146]. Once the [Hc-VB_{12}] complex reaches the higher pH of the duodenum it is separated by the action of trypsin and chymotrypsin, inactivating Hc by proteolysis [147, 148]. Intrinsic factor (IF) the second specific transport protein for VB_{12}, is released from parietal and enteroendocrine cells of the stomach [149]. IF interacts with vitamin B_{12} in the duodenum where it forms the second protein-VB_{12} complex [IF-VB_{12}]. Once the [IF- VB_{12}] complex reaches the ileum the entire complex binds to the intrinsic factor-cobalamin receptor (IFCR) present on the epithelium of the villus cells [150]. The binding of [IF-VB_{12}] to the IFCR requires both calcium ions and a neutral pH and results in an irreversible conformation change in the receptor. The receptor-[IF-VB_{12}] complex is then internalized by receptor mediated endocytosis [151].

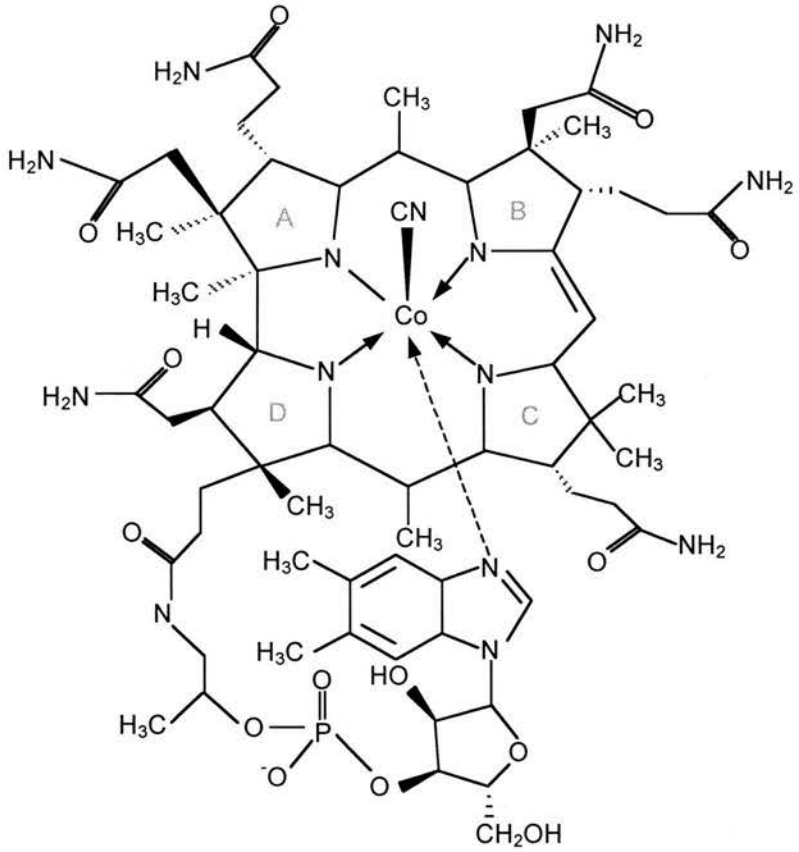


Figure 1.7: The structure of vitamin B_{12}, the A, B, C and D rings are indicated.

The mechanism by which VB_{12} dissociates from the IFCR receptor and IF itself is not yet fully understood. Cellular VB_{12} is known to bind to a third binding protein transcobalamin II (TCII) that transports the vitamin to cell surfaces. In the general circulation most VB_{12} is found associated with transcobalamin I, but interaction with TCII is thought to be necessary for tissue uptake of the vitamin. Cellular uptake of VB_{12} is mediated through surface TCII receptors and receptor-mediated endocytosis. Lysosomal degradation releases VB_{12} for metabolism to methyl-cobalamin in the cytosol or deoxyadenosyl-cobalamin in the mitochondria [151].

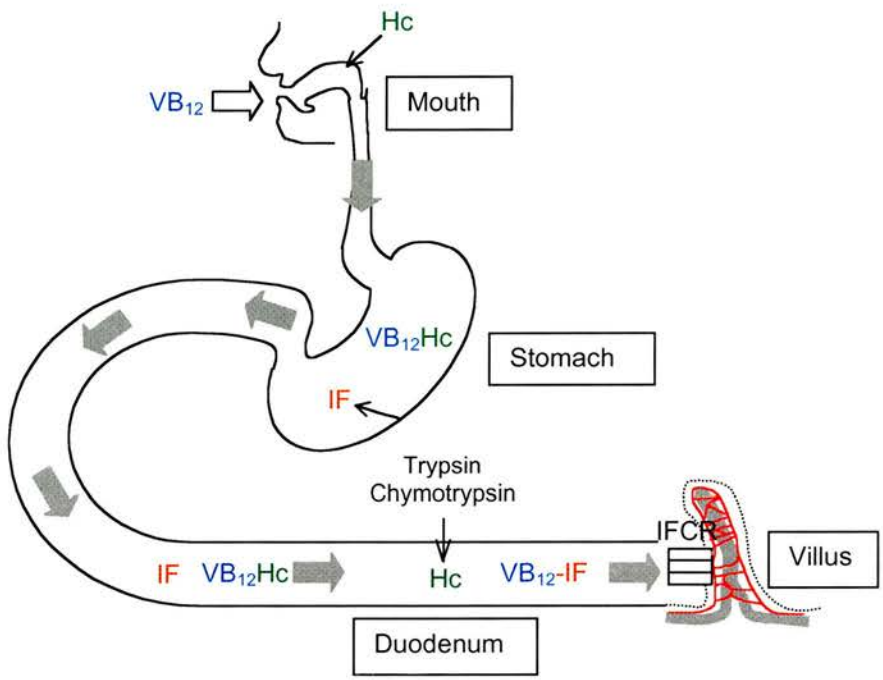


Figure 1.8: Summary of Vitamin B_{12} absorption. VB_{12} is consumed in the diet and mixes with haptocorrin (Hc) produced from the salivary glands. Hc complexes with VB_{12} in the stomach, but dissociates in the duodenum where Hc is degraded by trypsin and chymotrypsin. VB_{12} then complexes with intrinsic factor (IF) and binds to the intrinsic factor cobalamin receptor (IFCR) on the basolateral surface of the villus cells. The entire VB_{12}-IF-IFCR complex is internalized, the IF is degraded and the VB_{12} is transported out of the apical surface complexed with transcobalamin II (TCII).

The unusual uptake mechanism of VB_{12} complexed with IF and the IFCR makes this a promising candidate for a carrier molecule to use in peptide oral

absorption. Intrinsic factor is reported to have molecular weight of 45-47kDa with a carbohydrate content of 15% [152], although a conflicting report suggests that the native protein may have a molecular weight of up to 59kDa [153]. An additional factor is that VB_{12} itself, (molecular weight of 1357), is relatively inexpensive and poorly immunogenic, further adding to the suitability of VB_{12} as a carrier molecule [154, 155].

1.6.2.1 Direct conjugation to vitamin B_{12}

VB_{12} conjugates of granulocyte colony stimulating factor and erythropoetin had increased intestinal transport in comparison with the unconjugated molecules over a 24h period of continuous intraduodenal administration to rats [156]. The uptake of consensus interferon was also increased when conjugated to VB_{12} over the peptide alone [156].

Modified VB_{12} conjugates of a number of drugs and peptide/protein pharmaceuticals retained a high affinity for IF and the bioactivity of the pharmaceutical agent was not significantly reduced [154, 155, 157-159].

The GnRH antagonist Antide and derivatives have been investigated for the possibility of enhanced oral uptake by conjugation to vitamin B_{12} (VB_{12}) [158]. Antide-VB_{12} conjugates were tested by *in vitro* pituitary cell assay for inhibition of mammalian GnRH-stimulated LH release, in addition to competitive binding assay for binding to IF. *In vivo* bioactivity was assayed in a castrate male rat model, measuring serum LH after subcutaneous administration of conjugates. Therefore although the potential for oral uptake and maintenance of bioactivity was investigated, the actual *in vivo* oral bioavailability was not addressed in this paper. The group did investigate the structural requirements for bioactive conjugates. It was shown that in order to maintain both *in vitro* and *in vivo* activity, a thiol-cleavable spacer was necessary between the VB_{12} derivative and Antide [158].

Direct conjugation of metabolically stable short, but pharmacologically inactive peptides to VB_{12} via a hexyl spacer was attempted in order to prove

conclusively that absorption of radiolabelled conjugate was not in fact measuring absorption of fragments resulting from proteolytic degradation of the peptide [157]. The absorption of the model peptide-VB_{12} conjugate was investigated at all stages of the uptake process and up to 45% of the stable peptide-VB_{12} complex was taken up *in vivo*.

1.6.2.2 Other methods using vitamin B_{12}

Russell-Jones and co-workers have also published data on other ways in which VB_{12}-IF can be used as a carrier system for the oral absorption of model peptides [160]. In one method the peptide was not covalently attached to the VB_{12} molecule, but was incorporated into biodegradable nanospheres coated with VB_{12}. The peptide was thus protected from degradation and did not require chemical modification, which may hamper the bioactivity of the peptide once absorbed from the GIT.

In common with the active absorption of many other nutrients, the capacity of the uptake system is a limiting factor in the maximum possible dose. This is especially important when attempting to enhance the oral absorption of GnRH antagonists where the proportion of receptors occupied by the antagonist is vital to the desired effect. Nanoparticle incorporation of peptides could potentially increase absorption by up to 10^6-fold. Studies on optimum particle size revealed that 50nm, 100nm and 200nm particles were all transported with similar efficiency [154]. Analysis of the ideal surface VB_{12} density demonstrated that a reduction in surface VB_{12} decreased the percentage of particles transported.

1.6.3 Other methods of enhancing uptake

1.6.3.1 The peptide transporter

A specific transport mechanism exists in the duodenum for the uptake of short di- and tri-peptides [161] termed the intestinal peptide transporter or Dipeptide Transporter and is known to be involved in the intestinal uptake of some antibiotics in addition to nutrient uptake [162], however this method is obviously not suitable for large conjugated decapeptides such as GnRH analogues.

1.6.3.2 Absorption enhancers

Agents affecting the barrier function of the GI layer have been used to enhance the uptake of small peptides with a limited degree of success. In the hostile environment of the GIT, most peptides are rapidly degraded by hydrolysis and therefore any absorption enhancer must allow rapid uptake of the peptide before the peptide is inactivated [163]. Any such molecule must be non-toxic and have rapidly reversible effects in order that the integrity of the GIT is not disrupted, allowing the absorption of toxic molecules excluded under normal circumstances.

Chitosan (a non-toxic natural polysaccharide) was used to increase the absorption of hydrophilic macromolecular drugs. The paracellular permeability of peptide drugs across the mucosal epithelia was enhanced in the presence of protonated chitosan (at less than pH 6.5). Trimethyl chitosan chloride has been used in this way to reversibly widen the paracellular route for neutral and cationic peptide analogs and the permeation and absorption of heparin has been increased by mono-carboxymethylated chitosan [164-168]. However this method is not suitable for all peptides and there are concerns about using these molecules to increase GIT permeability for prolonged periods.

1.6.4 Enhancing absorption of GnRH analogues

The high binding affinity, wide range of uses, small size and variety of GnRH analogues available makes them ideal candidates for testing potential new routes of administration. The GnRH agonist Nafarelin has been tested in a controlled release injectable preparation based on microencapsulation in poly(lacticcoglycolic) acid and was found to have more profound effects in primates in comparison with the simple peptide in an injectable solution [169]. A similar technique has been used on Leuprolide (GnRH agonist) and resulted in a prostatic carcinoma treatment licensed for use [170-172].

Stable, bioavailable, readily manufacturable and patient acceptable methods of administering peptides have also been explored with GnRH analogues. Nasal delivery has the advantage of being non-invasive, with a highly vascular surface area of approximately 200cm^2, however in many cases it is necessary to co-administer absorption enhancers to achieve sufficient uptake by this route. The method by which the bile acid sodium glycocholate enhances nafarelin uptake is poorly understood, nevertheless it increased nasal uptake of the GnRH agonist from 3% to around 15% [173]. However this route has some unique problems during common upper respiratory infections, significantly hampering absorption by this route. In addition transdermal delivery of Leuprolide has been experimentally demonstrated in human cadaver skin [174].

GnRH analogues have also been tested for potential transdermal absorption by conjugation of [DLys^6]GnRH to aliphatic fatty acids of varying chain length. Analogues with twelve or less carbons had similar *in vivo* potencies to the unmodified analogue as measured by induction of LH release in proestrous rats [175]. Nicoli and colleagues attempted to achieve transdermal absorption of Triptorelin, a GnRH analogue currently used as implantable microparticles for the treatment of sex hormone-dependent tumors and benign gynecological disorders [176]. Transdermal transport through rabbit

ear skin was only identified in combination with lauric acid [177], known to modify the structure of the stratum corneum barrier [178].

1.7 CONCLUSIONS

Increasing half-life and oral absorption of peptides is subject to ongoing research. The aim of this study was to address these problems in a novel way through the conjugation of other biologically functional molecules to GnRH analogues to modify the properties of these analogues without altering sequence (except where necessary for conjugation purposes). This draws on knowledge of the three main areas outlined in this chapter, namely GnRH structure-activity relationships, sex-steroid plasma proteins and methods to enhance oral absorption of peptides, resulting in a single molecule combining all these properties.

1.8 AIMS

This thesis aims to address whether GnRH analogues can be modified to increase half-life and enhance oral absorption through conjugation to form novel bifunctional molecules. These bioconjugates will be designed, synthesised and analysed *in vitro* and *in vivo* to determine the effect of this process on the functioning of these novel molecules.

2 General Methods

This chapter details the general methods used throughout this thesis. Where methods were adapted for specific experiments, the alterations are included in the relevant chapter. All chemicals were obtained from Sigma-Aldrich Company Limited, with the exception of radiochemicals, which were purchased from Amersham Pharmacia Biotech UK Limited and unless otherwise stated. The addresses of suppliers are included in appendix I and solutions are described in appendix II.

2.1 CELL CULTURE

2.1.1 General aspects of cell culture

COS-7 cells were maintained in complete media until use (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, glutamine, penicillin and streptomycin). Research assistants Mr. R Sellar and Miss N Miller carried out routine maintenance of cell lines, including regular passage and plating of cells.

A HEK293 stable cell line expressing the rat type I GnRHR developed by Dr. L Anderson [179] and designated SCL60, was used for the inositol phosphate production assays. This cell line was maintained in complete media with G418 at 500µg/ml throughout culture. Where required, plates were coated with Poly-L-Lysine to enhance adherence to plastic ware during assay.

2.1.2 Preparation of human type I GnRH receptor cDNA for transient transfection

Plasmid DNA stocks of human GnRHR held by the laboratory were used to produce DNA for transfections. Bacteria were grown up overnight in LB Broth with 100µg/ml ampicilin for 20-24h at 37°C. The DNA was purified using Wizard® Plus Maxipreps (Promega). Bacterial cells were spun down at 5,000rpm for 10 min at room temperature and the cell pellet was resuspended in 15ml Cell Resuspension Solution. Cell Lysis Solution was added to this and thoroughly mixed for 15 min, 15ml Neutralization solution was added followed by further mixing by inversion. The cell suspension was spun once more at 4°C for 20mins at 6000rpm and filtered into a clean centrifuge bottle. 25ml isopropanol was added to this and mixed before further centrifugation at 4°C for 15 min at 6000rpm. DNA was resuspended in 2ml TE buffer and 10ml DNA Purification Resin was added. This solution was purified through a Maxicolumn and thoroughly washed with Column Wash Solution and 80% ethanol. 1.5ml TE buffer pre-warmed to 70°C was added after centrifugation of the Maxiprep column and the resulting DNA solution was assayed for purity and concentration by measuring the absorbance at 260 and 280nm.

2.1.3 Transient transfection of COS-7 cells

COS-7 cells were transfected with the human GnRH receptor (hGnRH-R) for receptor binding assay using Superfect transfection agent (Qiagen) in Optimem media (Invitrogen Life Techologies) for 4h. Briefly, 300µl optimem media was mixed with 10µg DNA per 100mm dish along with 30µl Superfect transfection reagent. The lipid was allowed to complex with the DNA for 10min at room temperature before 3.5ml complete media (see cell culture) was added. Culture media was removed and replaced with transfection media for 6h and after washing with PBS the transfection media was

exchanged for complete media. Transfected cells were assayed after a further 48h in complete media.

2.2 ANALYSIS OF GnRH RECEPTOR BINDING AND ACTIVATION

The GnRH-steroid conjugates were tested for receptor binding and effects on inositol phosphate production in the SCL60 cell line stably expressing the rat GnRHR and transiently transfected COS-7 cells expressing the human GnRHR.

2.2.1 Iodination of GnRH analogues for receptor binding assays

The GnRH analogue [His^{5}DTyr^6]GnRH was iodinated with ^{125}I for use in competition receptor binding assays (both whole cell and membrane) by Dr P Taylor or by Mr. R Sellar assisted by myself [180]. 5µl of 1mM peptide was reacted with 1000µCi ^{125}I sodium iodide in the presence of Iodogen (Pierce) for 90 seconds and washed through a G25 Sephadex column with 0.01M glacial acetic acid, 0.1% BSA. Fractions from the peak were collected and tested for specific binding in a competition whole cell binding assay before being pooled and used. A representative elution profile is illustrated in figure 2.1.

2.2.2 Competition whole cell binding assays

COS-7 or SCL60 cells were plated in 12 well plates and maintained in a 37°C incubator for 24h before assay. Cells were washed twice with PBS before

addition of 500µl HEPES modified DMEM with 0.1% BSA containing the competing ligand under test and a constant amount of radiolabelled ligand (^{125}I[His^{5}DTyr^6]GnRH). The plates were equilibrated on ice for 4h and the incubation solution was removed. The plates were washed twice in PBS and solubilized by addition of 500µl 0.1M NaOH and shaking for 20min. Samples were counted on a 1261 Multigamma (LKB Wallac) gamma-counter.

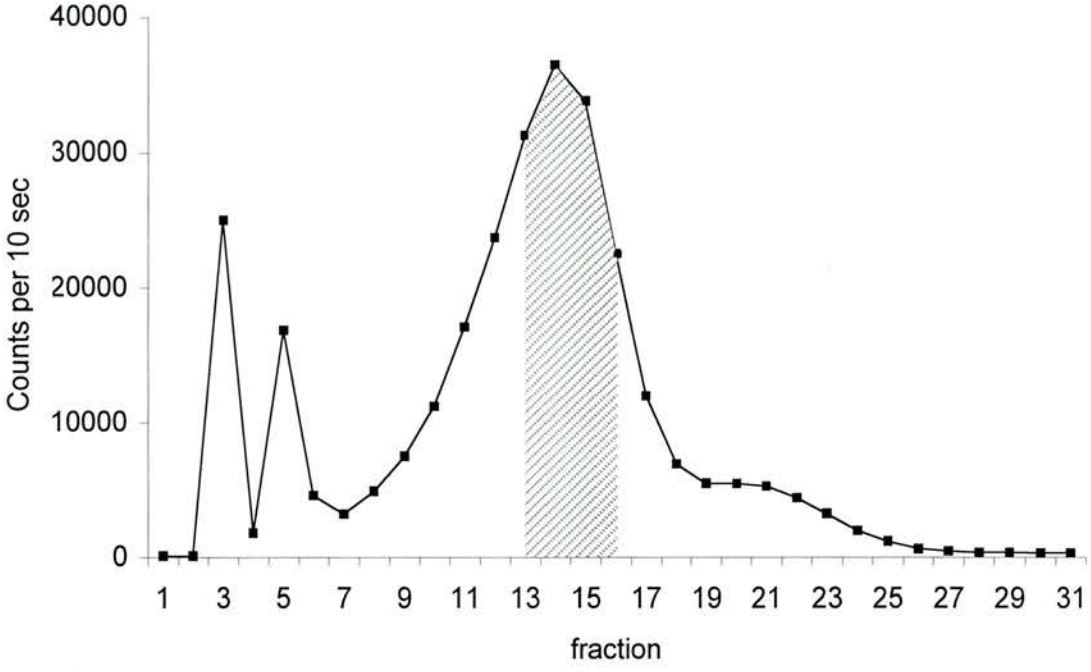


Figure 2.1: Elution profile after iodination of [His^{5}DTyr^6]GnRH on a Sephadex G25 column using 0.01M acetic acid/0.1%BSA as elution buffer. Fractions from the shaded area were checked for specific binding and pooled.

2.2.3 Competition membrane binding assays

Transfected COS-7 cells were washed in PBS, removed from plates and centrifuged at 1500rpm for 5 min to pellet the cells. The PBS was aspirated and the cells were resuspended in homogenization buffer, vortexed and left on ice for 10 min. The cell suspension was transferred to a 7ml homogeniser (Jencons (Scientific) Ltd.) and plunged 15 times with a loose plunger and 15 times with a tight plunger. The homogenized cells were centrifuged at 4°C

for 10 min at 10,000 x g and the supernatant was removed with a vacuum pump. The remaining membrane pellet was resuspended in assay buffer by repeatedly passing the membrane pellet though a 23-gauge needle. Pre-cooled 12mm glass tubes were filled with 200µl assay buffer, 50µl 1% BSA, 100µl cell membranes, 100µM ^{125}I[His^5,D-Tyr^6]GnRH in assay buffer (approximately 100,000 CPM per tube) and 50µl cold ligand (or assay buffer in Bo tubes) in increasing concentrations. The tubes were incubated for 4h at 4°C and filtered under vacuum through Whatman GF/C glass fiber filters (Whatman International Ltd.) presoaked in 1% PEI. Filters were then counted immediately on a gamma counter.

2.2.4 Measurement of total inositol phosphate production by the GnRH receptor

SCL60 cells were plated into 12 well plates and incubated at 37°C, 5% CO_2 for 24h, then incubated in special DMEM containing 1% dialysed FCS (with glutamine and penicillin/streptomycin) and 1µl/well of myo-[2-^{3}H]inositol for a further 48h. After aspiration of media and washing with incubation buffer, a further 500µl buffer containing 10mM LiCl and the antagonist or conjugate under test was added to the plates and incubated at 37°C for 30mins. Mammalian GnRH (for assay of antagonism only) was added to each well to a final concentration of 0.1µM and the plates were incubated under the same conditions for a further 1h. The reaction was terminated with 500µl 10mM formic acid, incubated at 4°C for 30min. Formic acid solutions were transferred to 12mm plastic tubes containing 500µl 50% AG-1x resin slurry (Bio-Rad Laboratories Ltd.). Inositol phosphates were eluted by addition, vortexing and removal of distilled water (1ml) and sodium tetraborate, sodium formate (1ml, 5mM, 60mM) solution. After addition of formic acid, ammonium formate (1ml, 0.1M, 1M) and vortexing, 800µl of the supernatant was counted with scintillation fluid.

2.3 ANALYSIS OF SPECIFIC PLASMA PROTEIN INTERACTION

GnRH-steroid conjugates were tested for competition with progesterone for binding to high molecular weight plasma proteins.

2.3.1 Competition plasma protein binding assay

Plasma protein binding was determined by the competitive binding of steroid conjugates, in the presence of [1,2,6,7-^{3}H]progesterone, to pregnant guinea pig plasma according to the method of Hammond and Lähteenmäki [181]. 20µl plasma was diluted with 2ml dextran-coated charcoal solution and incubated at room temperature to remove the endogenous steroids. After 30min the suspension was centrifuged at 3000 x g for 10min, the supernatant was removed and the pellet was discarded. 100µl of diluted plasma was aliquoted into centrifuge tubes, followed by 1pmol[1,2,6,7-^{3}H]progesterone/100µl PBS. 100µl PBS (total binding) or 200pmol/100µl unlabelled progesterone (specific binding) was added to diluted plasma in duplicate. The conjugates were dissolved in 100µl PBS and added to centrifuge tubes containing 100µl diluted plasma and 1pmol[1,2,6,7-^{3}H]progesterone. The tubes were vortexed and incubated at room temperature for 1h, then for an additional 15min on ice. 750µl dextran-coated charcoal suspension was added to the plasma solution and incubated for 10min on ice, followed by centrifugation (3000 x g) at 4°C for 5min. 700µl of the supernatant fluid was counted with scintillation fluid on a 1450 Microbeta Wallac-Trilux liquid scintillation counter.

2.3.2 Purification of progesterone binding globulin from pregnant guinea pig plasma

PBG present in the pregnant guinea pig plasma was semi-purified for further analysis of plasma protein binding. This was achieved by ion-exchange chromatography, utilizing the unusually low pI of 2.3 of PBG and a strong cation exchange column according to the method of Westphal *et al* [138, 182].

Pregnant guinea pig plasma anticoagulated with lithium-heparin was supplied by Charles River Laboratories and stored at –20°C until use. The plasma was dialyzed in 20ml aliquots for 16h in 2L 20mM sodium acetate, 0.02% azide at pH 7.2 at 4°C. Dialyzed plasma was titrated to pH 4.5 ± 0.02 with 50% acetic acid and the precipitate removed by centrifugation at 2000rpm for 10min. The plasma supernatant was then applied to a sulfopropyl-Sephadex (SP-Sephadex) column equilibriated with 20mM sodium acetate, 0.02% azide at pH 4.5. Fractions were collected every 2min for 2h and tested for specific [^{3}H]progesterone binding and total protein content. Most serum proteins are adsorbed by the SP-sephadex or inactivated by the low pH except PBG, which elutes in the void volume. [^{3}H]progesterone binding was measured in 20µl aliquots of fractions collected from the column, which were incubated with 50µl [^{3}H]progesterone for 1h and stripped with 750µl dextran-coated charcoal suspension. Protein measurement at 580nm revealed more than one protein was eluted from the column as at least 3 protein peaks were observed, however a large proportion of the proteins present in the plasma were removed. Fractions 22 to 36 were pooled for use in assays.

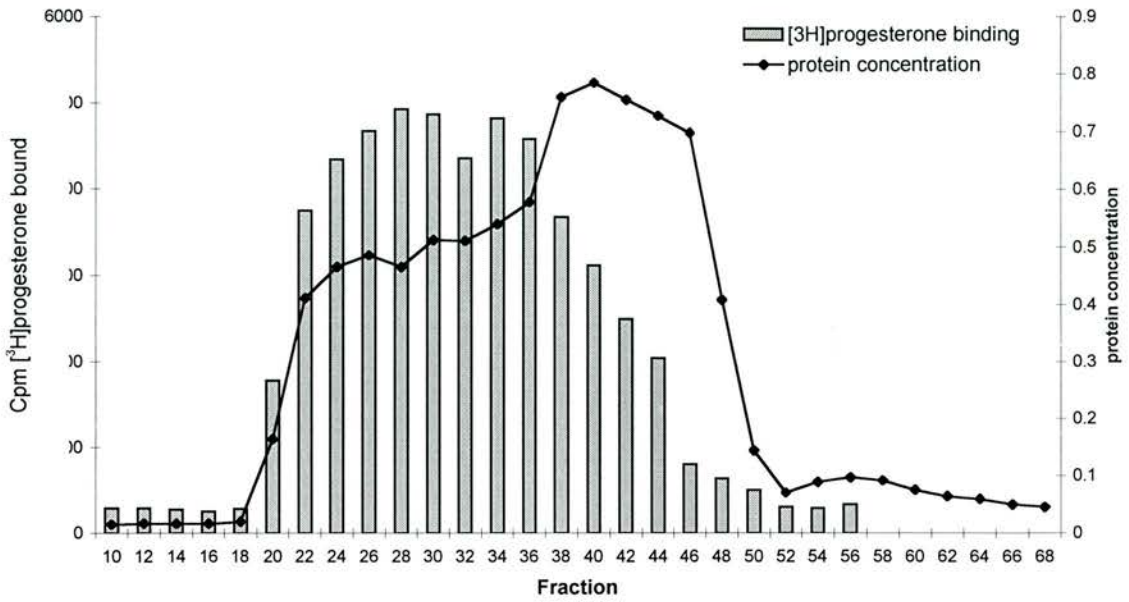


Figure 2.2: [^{3}H]progesterone binding and total protein content in fractions collected from a SP-Sephadex column with a running buffer of 20mM sodium acetate, 0.02% azide at pH 4.5.

2.4 ANALYSIS OF PROGESTAGENIC ACTIVITY

The conjugation of GnRH antagonists to progesterone to enhance half-life and confer plasma protein binding affinity also introduced the possibility that the conjugated progesterone may retain progesterone receptor binding affinity.

2.4.1 T47D cell line

Progestagenic activity was tested on the epithelial breast cancer cell line T47D, stably expressing the progesterone receptor linked to a chloramphenicol acetyltransferase (CAT) reporter gene. This cell line was kindly supplied by Prof. Rodney Kelly (HRSU), but was originally produced by Dr. M Beato (Institut fur Molekularbiologie und Tumorforschung IMT,

Philipps-Universitat Marburg, Germany). CAT is a bacterial drug-resistance gene absent from eukaryotic cells and functions to inactivate chloramphenicol by acetylation at the two hydroxyl groups (Promega Technical Bulletin 084). An enzyme assay system was available (Promega) in which the n-butyryl moiety from Coenzyme A is transferred to [^{3}H]chloramphenicol and this was used to test the activation of the progesterone receptor by the synthesized conjugates.

2.4.2 Treatment of T47D cells

T47D cells were maintained in RPMI 1640 medium supplemented with 2mM glutamine, 10% FCS, penicillin, streptomycin and insulin transferrin sodium selenite supplement. Cells were plated in 100mm dishes at a density of 2 million cells per dish, 24h in advance of treatment with progesterone or progesterone-GnRH antagonist conjugates. 100µl of steroid or conjugate solution was added to 10ml of media and cells were left for a further 24h before CAT enzyme assay.

2.4.3 CAT enzyme assay of treated T47D cells

A cell extract was prepared, washing the cells first in PBS (magnesium and calcium free) and applying 900µl Reporter Lysis Buffer to the cells. After 15 min incubation at room temperature, the cells were scraped from the plate and transferred to a microcentrifuge tube on ice. Each tube was vortexed for 10-15 seconds, then heated at 60°C for 10 min to inactivate endogenous deacetylase activity. The cell lysates were then spun at top speed in a microcentrifuge for 2 min and the supernatant was transferred to a fresh tube.

Reaction mixtures were prepared in microcentrifuge tubes containing cell extract, [^{3}H]chloramphenicol and n-butyryl CoA, made up to a total volume

of 125µl with water. Positive and negative controls were prepared containing a known amount of chloramphenicol acetyltransferase or no cell extract respectively. The reaction mixtures were incubated at 37°C for 3h and then spun briefly in a microcentrifuge. The reaction was terminated by addition of 300µl mixed xylenes and the partition of [^{3}H]chloramphenicol was measured by liquid scintillation assay.

2.4.4 T47D cell lysate liquid scintillation analysis

In order to measure the presence of n-butyryl-[^{3}H]chloramphenicol in the xylene layer, the xylene/reaction mixture was vortexed thoroughly and spun at top speed for 3 min. 200µl of the xylene phase was removed and 100µl 0.25M Tris-HCl (pH 8.0) was added, before repeating the vortex and spin procedure. 150µl of the xylene layer was transferred to a scintillation vial, scintillation cocktail was added and the cpm was measured over 1 minute. The counts measured in the negative control were subtracted from all other values.

2.5 ANIMALS

All primates used in this study were housed at the MRC Human Reproductive Sciences Unit Primate Centre under the care of primate centre staff. All procedures were in agreement with the Animals (Scientific Procedures) Act 1986 under the license of Dr H. Fraser.

2.5.1 Marmosets

Adult marmoset monkeys (*Callithrix jacchus*) were housed in cages measuring 0.6 x 1.1 x 1.15m (W, D, H) in rooms artificially lit between 07.00

and 19.00hrs. Female marmosets were housed together with a younger sister or prepubertal female and had regular ovulatory cycles. Male marmosets were housed in family groups.

Marmosets were held in a restraining device [183] to allow for blood samples (300µl) to be collected by femoral venepuncture without anaesthesia. The sealed 1ml heparinized syringes were centrifuged for 20 min at 1000 x g to extract plasma, which were then stored at –20°C until use. In the case of the female marmosets, blood samples were collected three times per week during test and control cycles. More frequent sampling was carried out immediately following the injection. Blood samples were collected from the male marmosets in the same way on three occasions during the week prior to treatment.

Female marmosets have an approximately 28 ± 2 day ovarian cycle. This is split into a 8 day follicular phase and 20 day luteal phase. Ovulation was identified by a progesterone concentration greater than 30nmol/L.

2.5.1.1 Steroid assays

Progesterone enzyme-linked immunosorbent assay (ELISA) and testosterone radioimmunoassays on marmoset blood samples were carried out by the in-house assay service.

2.5.1.2 Marmoset progesterone ELISA plate assay

Briefly, plates were coated with coating antibody for a minimum of 4h and then washed three times with ELISA washing solution. 2.5µl of marmoset plasma in phosphate/citric assay buffer was added to the plate alongside standards, Bo and NSB for quantification (for standards, stripped marmoset plasma was used). 50µl of primary antibody was then added to each well (except NSB) and incubated at room temperature for a minimum of 3h. The incubation solution was removed and the plates were washed five times with

ELISA washing solution. 100µl streptavidin-horseradish peroxidase was added to each well for 1h, followed by 200µl substrate solution, then the colour was allowed to develop over a period of 15 to 30 minutes. Once developed, the reaction was stopped with 50µl stopping solution and the plate was read at 490nm on a Victor Plate Reader.

2.5.1.3 Marmoset testosterone radioimmunoassay

50µl plasma under test was added to glass test tubes and vortexed for 10 minutes with 1ml hexane:diethyl ether (4:1). The aqueous layer of each sample was snap frozen and the supernatant transferred to a fresh glass tube where the sample was dried down under nitrogen. Each extract was reconstituted in 250µl RIA buffer. 100µl sample was combined with an equal volume of tracer and antibody and made up to 800µl with RIA assay buffer. This mixture was incubated overnight at 4°C. To this 100µl second antibody (anti-testosterone-3-CMO-BSA) and 100µl normal sheep serum was added and incubated again overnight at 4°C. 1ml 0.9% saline, 0.2% Triton X was added to all tubes except total counts and the tubes were centrifuged at 3000rpm for 30min. The supernatant was counted on a gamma counter.

2.5.2 Macaque

An adult female stumptailed macaque (*Macaca arctoides*) was ovarectomized by a veterinary surgeon and allowed to recover from the operation for three weeks before treatment. Baseline LH and FSH levels were determined by collecting blood samples three times per week for two weeks prior to injection.

2.5.2.1 Macaque LH radioimmunoassay

Macaque LH RIA assays based on the Recombinant Cynomolgus Monkey LH Immunoreactants for RIA supplied by NIH (National Institute of Health) were carried out by the in-house assay service. Briefly, total counts (TC),

non-specific binding (NSB), total binding (Bo), standards, quality controls and samples were set up in plastic tubes in duplicate. Primary antibody (100µl) was added to all tubes except TC and NSB. The tubes were vortexed and incubated overnight at 4°C. ^{125}I-LH tracer was added to each tube before a second overnight incubation at 4°C. Normal rabbit serum (100µl of 1:400 dilution in assay buffer), followed by donkey anti-rabbit serum (100µl of 1:32 dilution in assay buffer) was added to each tube (except TC) and the tubes were incubated overnight at 4°C. Wash solution (1ml) was added to each tube except TC. The tubes were centrifuged at 3000rpm for 30min at 4°C. The supernatant was decanted and the tubes were inverted on absorbent towelling to drain. All tubes were counted on a 1261 Multigamma (LKB Wallac) gamma-counter.

3 Design and synthesis of GnRH-steroid conjugates

3.1 INTRODUCTION

GnRH analogues are extremely useful pharmacological agents, with high receptor binding affinity at the GnRH receptor. However most analogues are peptides and this has limited the use of these agents in the clinic. Peptide GnRH agonists and antagonists are most commonly given by injection to avoid degradation by gastrointestinal protease enzymes, renal clearance and first-pass metabolism. Efforts are underway to address this problem by novel methods of administration such as transdermal delivery [177, 184, 185] or slow-release depot preparations [170-172], but oral absorption of peptide GnRH analogues remains an elusive goal.

It is possible that the pharmacokinetics of peptide GnRH analogues could be improved by conferring plasma protein binding properties onto peptides lacking this feature. The GnRH antagonist Antide can inhibit gonadotropin production for up to four weeks in the macaque, probably due to association with large molecular weight plasma proteins and formation of a depot gel [186]. This duration of action is considerably longer than analogues that do not bind to plasma proteins [187-189]. Therefore a method by which a GnRH antagonist could acquire this feature without requiring sequence modification (affecting GnRH receptor interaction) would be a distinct advantage.

Coupling any molecule that binds to plasma proteins onto a GnRH analogue is possible, however a molecule such as a steroid would also have some additional benefits, including reduced toxicity concerns and possible low-level steroid replacement, an advantage during long-term gonadotropin suppression. Rahimipour *et al* [12] used a similar concept, although a toxic molecule with albumin binding affinity was modified to remove the toxic

and antiproliferative effects, retaining plasma protein interaction. This molecule was coupled to a GnRH agonist, but the toxicological implications of such a molecule could be significant.

Increasing the circulatory half-life of a GnRH analogue through plasma protein binding would reduce administration frequency and the overall cost of therapy, an obvious advantage with peptides. It would also make achieving a steady concentration far simpler than with rapidly cleared peptides.

3.2 DESIGN OF GnRH ANALOGUE-STEROID CONJUGATES

To investigate the properties of novel GnRH analogue bioconjugates, entirely new molecules were synthesized. Although other bioconjugates have been produced in the past, the approach and design of the current GnRH analogue conjugates is novel.

The design of the GnRH analogue-steroid conjugates was based on consideration of a number of factors, including GnRHR binding affinity, the requirements for steroid binding to plasma proteins and the overall molecular structure. These factors were combined to produce molecules that would be expected to retain biological activity.

3.2.1 Selection of GnRH analogue: GnRH receptor binding affinity

Native GnRH is proposed to interact with the GnRH receptor in a folded conformation. Both the N- and C-terminals are required for binding, but the N terminus is crucial for receptor activation [13, 22, 23]. Previous studies on

the conjugation of GnRH analogues to cytotoxics [9-11] and to other molecules such as tetramethylrhodamine [52] and emodic acid [12] have used position six of the decapeptide and retained GnRH receptor binding.

In order to incorporate a suitable reactive group and sufficient space between the steroid and peptide, a D-lysine was used in position 6 of the initial peptide sequences. The free ε-amine group of lysine is a convenient functional group for conjugation chemistry, resulting in a relatively stable amide group when coupled to carboxylic acids. Other amino acids could potentially be used in this position, but the four-carbon side chain assisted in minimizing steric hindrance on both the steroid and the peptide. The D isoform of lysine was selected because D amino acids enhance the biologically active conformation of GnRH and the side chain is orientated away from the receptor. Incorporating a D amino acid also enhances metabolic stability by preventing the action of endopeptidases that cleave GnRH between the fifth and sixth amino acids [53, 188].

3.2.2 Selection of steroid: Steroid-plasma protein interaction

The binding affinities of a large number of steroids for specific binding proteins have been extensively studied and reported in the literature. The steroid derivative for peptide conjugation was chosen on the basis of this published data. The choice of progesterone derivative was limited by commercial availability of steroids with a free hydroxyl group for reaction with a linker. The derivative selected for the initial conjugations was 11α hydroxyprogesterone.

In the later studies 21-hydroxyprogesterone 21-hemisuccinate (also known as deoxycorticosterone 21-hemisuccinate or 21-hydroxy-4-pregnene-3, 20-dione 21-hemisuccinate) was used. The important aspects of steroids for binding to human CBG are the 20-oxo, 10β-methyl and the C4 double bond, with cortisol having the highest affinity constant (at pH 7.4, 4°C) for this specific

binding protein. Deoxycorticosterone has a similar affinity constant to cortisol (6.8μM versus 7.1μM respectively) [97] and possesses all three important features, but lacks the 11β and 17-hydroxy groups of cortisol. Although the affinity constant of deoxycorticosterone 21-hemisuccinate for human CBG could not be determined from the literature, the affinity constant of deoxycorticosterone for guinea pig PBG was only 2-fold less in comparison to progesterone [139] and the hemisuccinate group only decreased the affinity of deoxycorticosterone for PBG by a further 4-fold [139].

3.2.3 Overall structure of molecule

The overall molecular structure was designed to incorporate sufficient space between the steroid molecule and the GnRH antagonist, minimizing steric hindrance. This was achieved with a combination of the four-carbon chain of D-lysine and the hemisuccinate 'linker' between the peptide and steroid.
The following molecules were designed and produced in this thesis (the design of the antagonist conjugates is featured in section 3.4):

1. [DLys^6(11α hydroxyprogesterone 11-hemisuccinate)]GnRH
2. [DLys^6(21-hydroxyprogesterone 21-hemisuccinate)]GnRH
3. [DLys^6(β-oestradiol 17-hemisuccinate)]GnRH
4. [AcDNal^1, DCpa^2, DPal^3, Arg^5, DLys^6(21-hydroxyprogesterone 21-hemisuccinate), DAla-NH_2^{10}]GnRH (antagonist conjugate A)
5. [AcΔPro^1, DFpa^2, DTrp^3, DLys^6(21-hydroxyprogesterone 21-hemisuccinate), Gly-NH_2^{10}]GnRH (antagonist conjugate B)
6. [AcDNal^1, DCpa^2, DPal^3, Arg^5, DLys^6, Lys^7(21-hydroxyprogesterone 21-hemisuccinate), Leu^8, Arg^9, D-Ala-NH_2^{10}]GnRH (antagonist conjugate C)
7. (21-hydroxyprogesterone 21-hemisuccinate)DPal, Ser, Arg, DLys, Leu, Arg, Pro, DAla-NH_2 (antagonist conjugate D)
8. [AcDNal^1, DCpa^2, DPal^3, Arg^5, DLys^6, Lys^7(21-hydroxyprogesterone 21-hemisuccinate), DAla-NH_2^{10}]GnRH (antagonist conjugate E)

3.3 SYNTHESIS OF GnRH ANALOGUE-STEROID CONJUGATES

Conjugates were successfully prepared by two out of three methods attempted with different coupling reagents. The coupling reagents used were a mixed anhydride (isobutyl chloroformate, IBCF), a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDAC) and an organic carbodiimide (N,N'dicyclohexylcarbodiimide, DCC). No conjugation was observed using EDAC, probably due to difficulties arising from the hydrophobicity of the steroid. The two successful methods, mixed anhydride and organic carbodiimide, are detailed in this section.

3.3.1 Mixed anhydride conjugation to progesterone

The progesterone derivative 11α-hydroxyprogesterone was first converted to 11-succinylprogesterone to introduce a carboxyl group as described in figure 3.1. 11α-hydroxyprogesterone and succinic anhydride were each dissolved in equal volumes of N-dimethylformamide (DMF) to 1.5M and 5M respectively. The progesterone solution was added into the succinic anhydride solution to ensure a constant excess of the anhydride and the pH was maintained alkaline by addition of 200µl tributylamine. After 2h on ice and 12h at room temperature, an equal volume of water was added to precipitate the 11α-hemisuccinate-progesterone product, with the excess succinic anhydride remaining in solution. After 48h the crystalline product was removed and tested by thin-layer chromatography on a 10 x 20cm glass TLC plate coated with silica containing fluorescent indicator. The solvent system was 30% ethanol, 70% ethyl acetate. A product more polar (migrating less) than the starting material was identified absorbing in the UV range and analysis of the UV absorption spectra between 200 and 400nm on a Kontron Uvikon 860 UV photometer revealed the same absorption spectra as the starting material.

The derivative retained the characteristic progesterone absorption at 240nm (figure 3.2A and 3.2B).

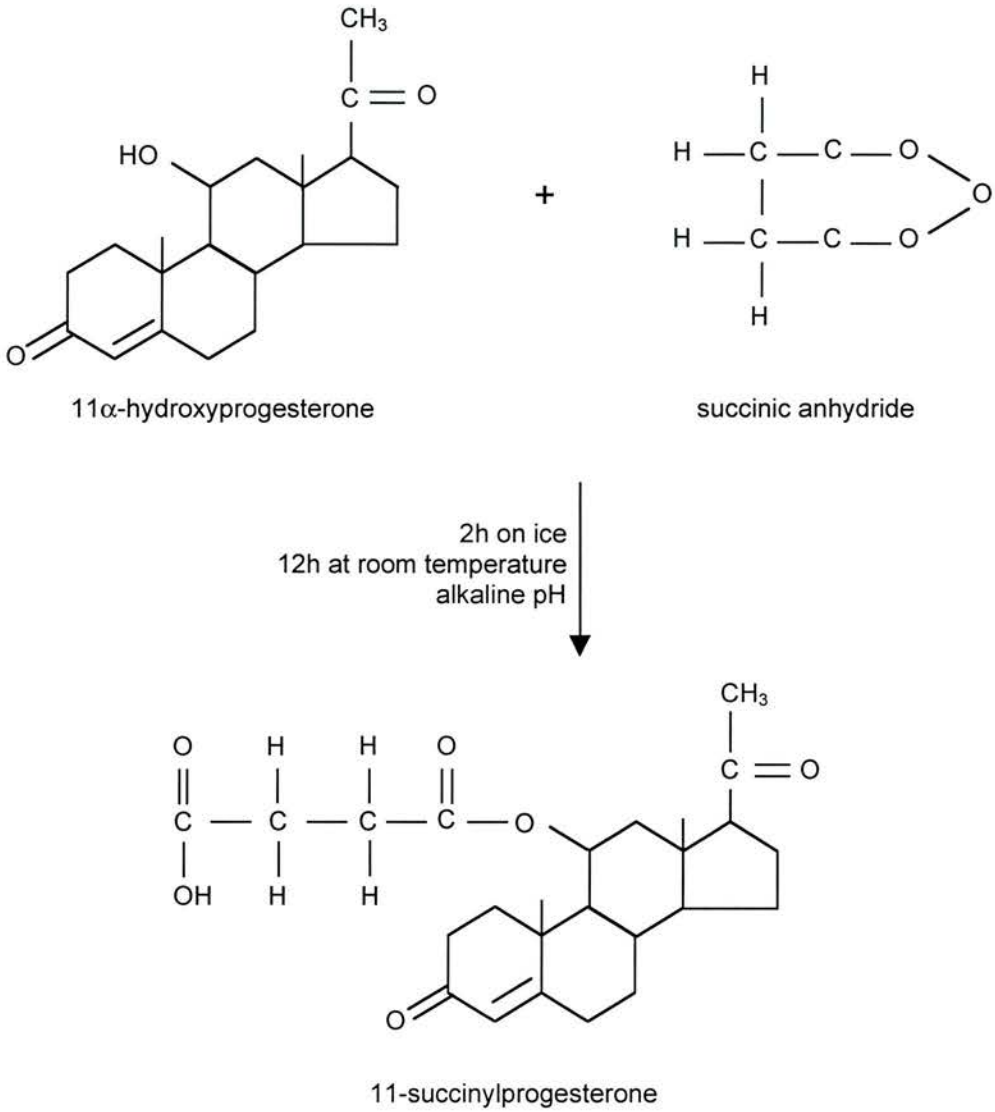


Figure 3.1: Conversion of 11α-hydroxyprogesterone to 11-succinyl-progesterone by succinic anhydride.

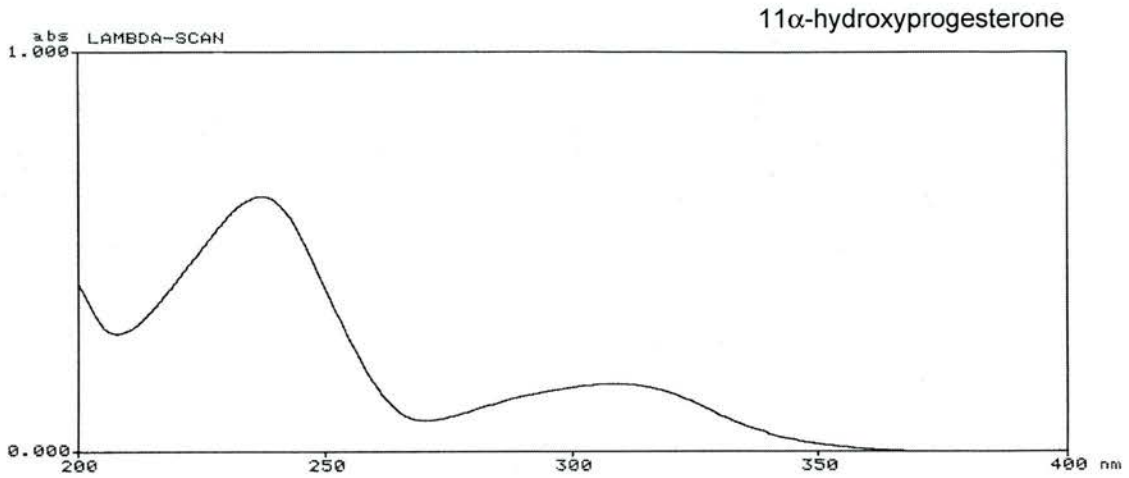


Fig 3.2A: UV absorption of 11α-hydroxyprogesterone before modification, measured at 1nm intervals between 200 and 400nm. The maximum absorption occurred at 240nm as expected for progesterone due to the C3 keto/C4 double bond.

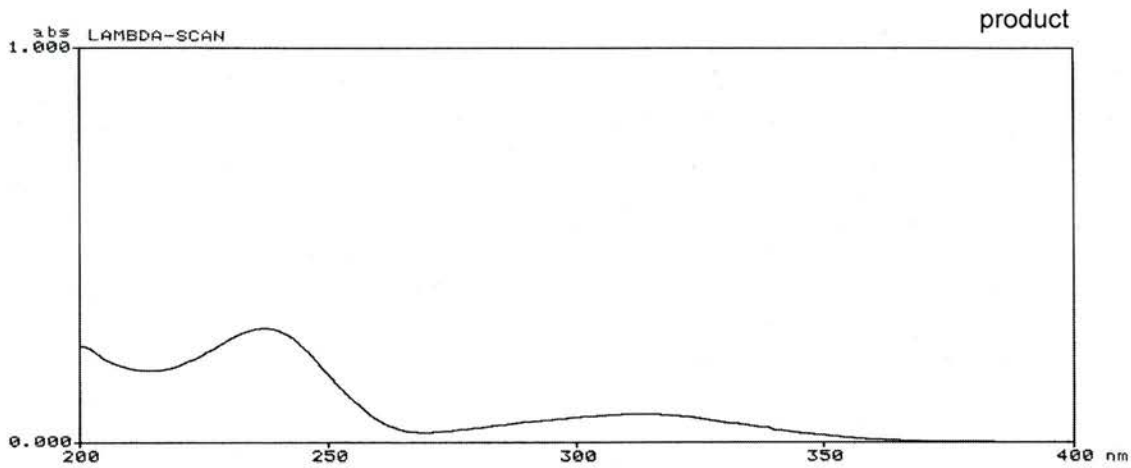


Fig 3.2B: UV absorption of crystalline product from reaction of succinic anhydride and 11α-hydroxyprogesterone, measured at 1nm intervals between 200 and 400nm. The absorption pattern remained the same after modification to add a hemisuccinate group to the 11α-hydroxyl group of the progesterone derivative.

The carboxyl group of 11-succinylprogesterone was activated with isobutyl chloroformate and reacted with [DLys^6]GnRH. The steroid and peptide were both dissolved in 50% phosphate buffer (pH 7.0) 50% DMF. The reaction mixture was left on ice for 30min and transferred to room temperature for a further 2h.

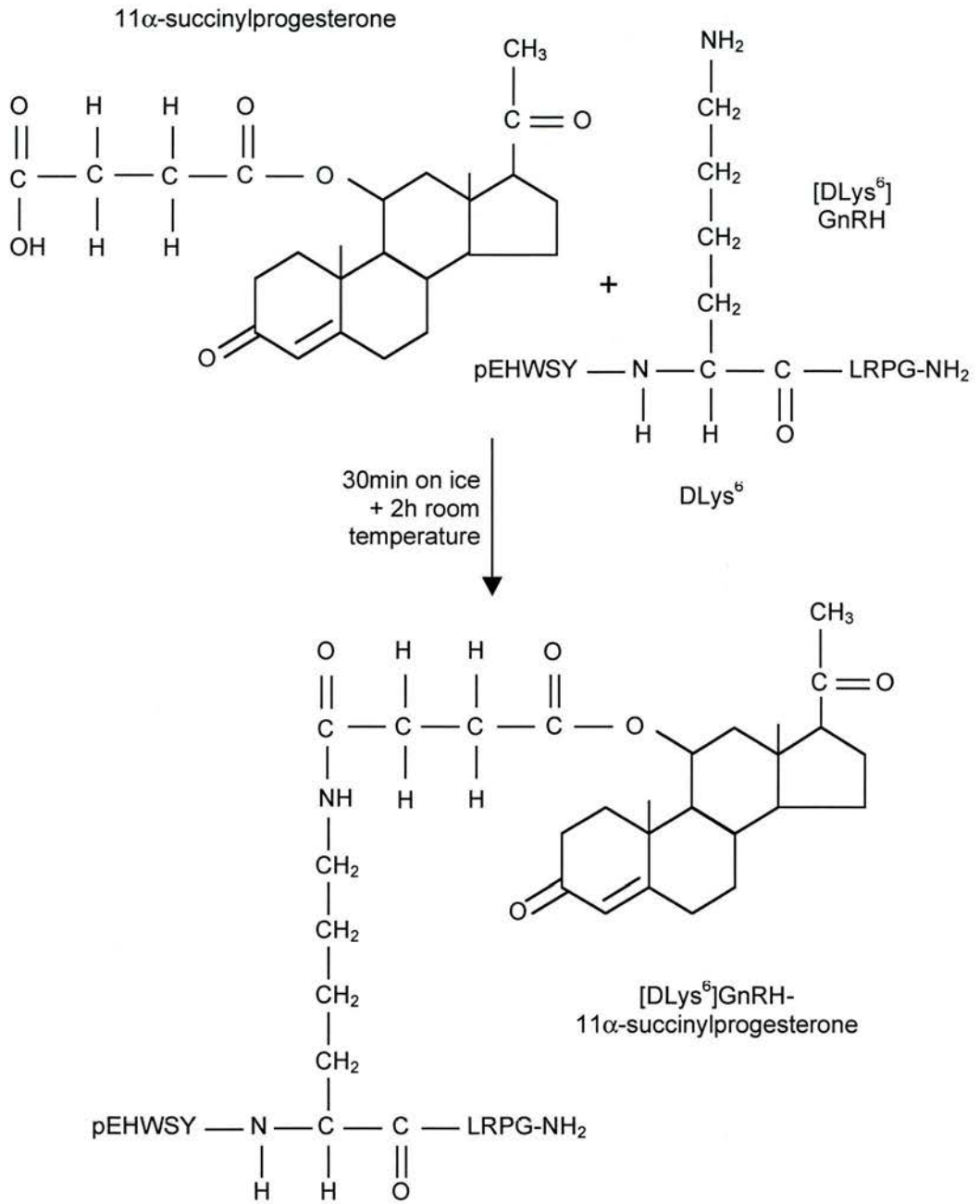


Figure 3.3: Reaction of 11-succinylprogesterone and [DLys^6]GnRH. The other amino acids are represented by the universally accepted single-letter amino acid code as follows: pE; pyro-glutamic acid, H; histidine, W; tryptophan, S; serine, Y; tyrosine, L; leucine, R; arginine, P; proline and G-NH_2; glycine-amide.

Initial purification was carried out on a Sep-Pak C18 syringe column (Millipore UK Ltd.) primed with 2ml ethanol. The reaction mixture was first washed through the column in 20ml water, then the excess steroid was eluted with 2ml ethyl acetate and the peptide-steroid conjugate was recovered from the column with 2ml 1,1,1,3,3,3-hexafluro-2-propanol (HFP). The HFP-eluted products were dried down under nitrogen and re-dissolved in acetonitrile/water (30:70). This was then analyzed on a LKB HPLC with a twin-pump gradient system and a fixed-wavelength UV absorption detector. The column was a Novapak C18 column (Millipore UK Ltd.) containing 4μm beads, measuring 3.9 x 150mm. A two buffer system was used, buffer A being water (with 0.1% trifluroacetic acid, TFA) and buffer B being acetonitrile (with 0.1% TFA). The column was developed with a linear gradient of 30-100% buffer B over 40min at a flow rate of 1ml/min. Fractions were collected every 30s for the duration of the run. Two peaks were observed close together between fractions 30 and 39, equivalent to retention times of 15 to 19.5 min (data not shown). These fractions were lyophilized and dissolved in acetonitrile/water (50:50) for mass spectroscopy analysis.

Mass spectrometry was carried out on a Tofspec 2E matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Micromass UK Ltd.) with a matrix of α-cyano-4-hydroxycinnamic acid mixed in equal volumes with the sample (dissolved in acetonitrile/water 50:50) and co-crystallized on a stainless steel plate. Data from several different areas of the spot were combined to produce a single scan with enhanced signal-to-noise and improved mass accuracy. The background was subtracted to lessen the effect of chemical noise and finally the spectrum was smoothed by the Savitzky Golay method to reduce high frequency noise.

Products with the same molecular mass as that calculated for the [DLys^6]GnRH-11α-succinylprogesterone conjugate (1665 AMU) were identified in 7 of the 10 HPLC fractions (fractions 33 to 39 corresponding to retention times of 16 to 19 minutes at 30 second intervals). The results from mass spectrometry analysis of fraction 35 are shown in figure 3.4. In addition to the expected products present mass, there were several other products

present, lowering the yield of the desired peptide-steroid. In particular, the molecular masses of 1352 and 1452 were equivalent to that of [DLys^6]GnRH and an additional 100 or 200 AMU. This could possibly be due to formation of additional hemisuccinate groups on the peptide, not connected to a steroid, increasing the mass by 100 AMU. Likewise, the 1766 peak is equivalent to the [DLys^6]GnRH-11α-succinylprogesterone conjugate with an additional hemisuccinate group, increasing the mass from 1666 to 1766 AMU. The presence of these and other minor products reduced the yield of [DLys^6]GnRH-11α-succinylprogesterone.

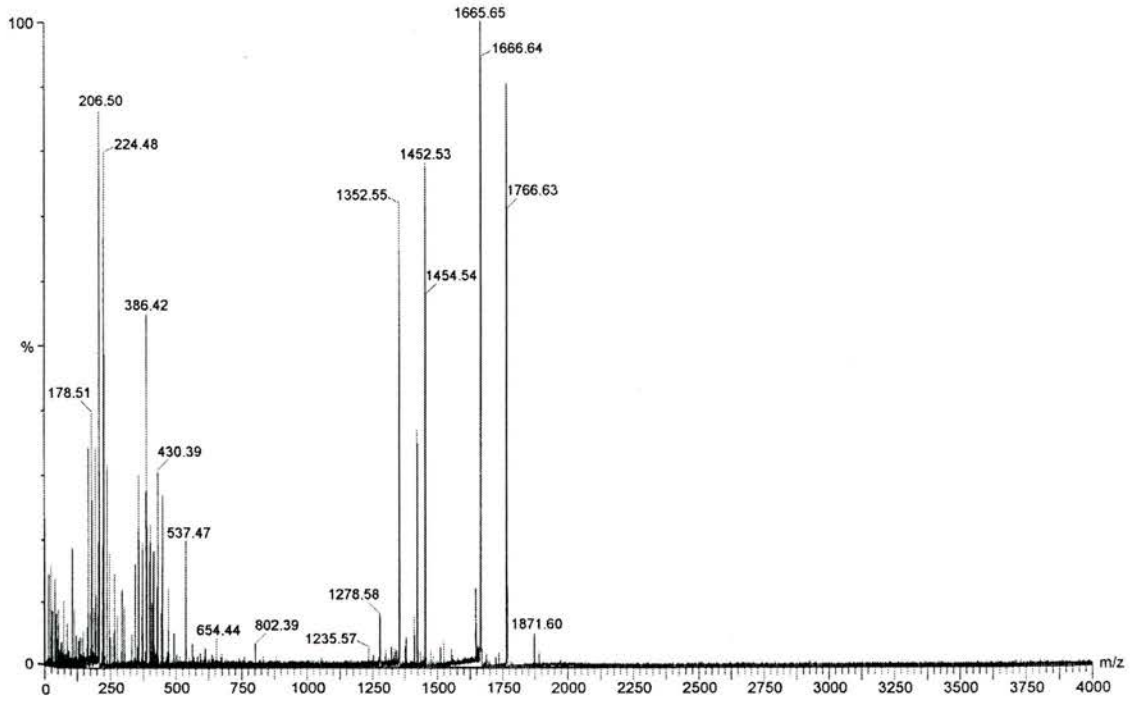


Figure 3.4: Mass spectroscopy analysis of fraction 35 of purified [DLys^6]GnRH-11 succinylprogesterone conjugate. The expected molecular weight was 1666 A.M.U. and the observed molecular weight was 1665.6 and 1666.6.

The methods used to identify the conjugates in this chapter could only identify on the basis of molecular mass (mass spectrometry) and absorption spectra (HPLC). This cannot be taken as conclusive proof of conjugate structure as this would require further nuclear magnetic resonance analysis (NMR), but the data suggest that the desired D-Lysine ε-amine conjugate would most likely be a component of this mixture.

3.3.2 Mixed anhydride conjugation to estradiol

The same method was attempted to conjugate 11α-estradiol hemisuccinate to [DLys^6]GnRH. The expected product with a molecular weight of 1622 AMU was observed in the HFP fraction from the Sep-Pak column, but other peaks were also present at 1353, 1454 and 1553 AMU, a similar pattern to that observed with conjugation to 11α-hydroxyprogesterone 11-hemisuccinate (data not shown). The peaks at 1725 and 1824 AMU present in the reaction mixture correspond to the desired product with an additional 100 or 200 AMU as observed previously.

3.3.3 Additional work converting 11α-hydroxyprogesterone to 11-hydroxyprogesterone 11-hemisuccinate

There was some difficulty in producing the hemisuccinate derivative of 11α hydroxyprogesterone in subsequent attempts. At the second attempt the hemisuccinate derivative failed to crystallize when water was added to the reaction mixture of succinic anhydride and 11α-hydroxyprogesterone. It was hypothesized that this may have been due to insufficient purity of the 11-hydroxyprogesterone 11-hemisuccinate for the crystallization process. This was despite scratching the glass and adding crystals from the previous successful reaction to facilitate crystallization.

The succinic anhydride-progesterone mixture from the second attempt was purified on an ion exchange column of Sephadex LH-20 (Amersham Pharmacia) with a mobile phase of 50:50 ethanol: ethyl acetate. This column separates molecules on the basis of hydrophobicity. Eleven-α hydroxyprogesterone is less hydrophobic than the 11-hemisuccinate progesterone derivative, therefore 11α-hydroxyprogesterone elutes first. The column eluent was connected to a UV detector set to 260nm. This wavelength was selected because although the maximum absorption of progesterone is at 240nm, at this wavelength there would also be significant absorption of the solvent system. A single composite peak resulted from this

separation, indicating that the solvent system used was too polar. An alternative solvent system was then employed, comprising 50% ethyl acetate and 50% chloroform. This resulted in two peaks, the initial peak corresponding to 11α-hydroxyprogesterone and the second provisionally identified as 11-hemisuccinate progesterone. The fractions collected from the second peak were tested by mass spectrometry. However a molecular weight corresponding to 11-hemisuccinate progesterone could not be identified by this method.

It was possible that the succinic anhydride was reacting with the C3 keto group, interfering with the UV absorption. The peaks observed could be minor peaks whilst the major progesterone products were not detected. An attempt was made to protect the carbonyl group prior to reaction with succinic anhydride by converted the ketone to a ketal (figure 3.5). The 11α-hydroxyprogesterone was dissolved in 4 molar equivalents of triethylorthroformate. Two molar equivalents of ethylene glycol were added to this mixture, followed by 0.1 molar equivalents of *p*-toluenesulfonic acid. The mixture was left overnight to crystallize. The ketal product was identified by mass spectrometry with a molecular weight of 374.5 AMU (data not shown).

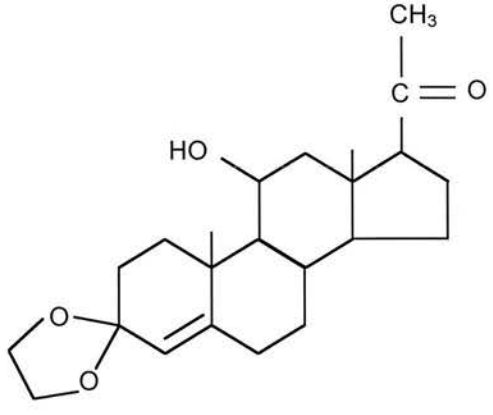


Figure 3.5: Predicted ketal progesterone product.

Time constraints prevented further analysis of the reaction between the ketal progesterone product with [DLys^6]GnRH. A hemisuccinate derivative of progesterone at C21 (deoxycorticosterone 21-hemisuccinate) was readily

available from commercial suppliers at low cost and therefore further attempts to produce a [DLys^6]GnRH-progesterone conjugate used this derivative, in place of an in house-synthesized C11 derivative.

The [DLys^6]GnRH-deoxycorticosterone 21-hemisuccinate conjugate was produced by the mixed anhydride method used with 11-hemisuccinate progesterone. After syringe column separation with 2ml ethyl acetate and 2ml HFP the conjugate was detected in the reaction mixture by mass spectrometry (data not shown). However HPLC analysis revealed a large proportion of unreacted [DLys^6]GnRH remained in the mixture. The yield (not calculated) was therefore low and could not be improved despite modifying the reaction time, incubation temperature and duration of activation stage. Thus this method was not pursued further.

3.3.4 Attempts to conjugate with EDAC

Online literature (PE Biosystems) suggested the use of 1.5 molar equivalents of steroid, in the minimum volume of 10% DMF: 90% phosphate buffer at pH 8 to 8.5 to conjugate the steroid (21-hydroxyprogesterone 21-hemisuccinate) to a peptide ([DLys^6]GnRH) using EDAC. [DLys^6]GnRH was first dissolved in phosphate buffer and the pH was raised to between 8 and 8.5. The steroid solution was added into the [DLys^6]GnRH solution and the EDAC solution was added into the peptide-steroid mixture and left for up to 24h. No evidence of conjugation was observed with this method.

The reaction was repeated in the same proportions but the steroid carboxyl group was activated with EDAC at pH 5 for 10min before addition to the peptide. This did not result in a [DLys^6]GnRH-steroid conjugate and this method was not pursued further.

3.3.5 Organic carbodiimide conjugation

The DCC coupling method was the most successful for the conjugation of [DLys^6]GnRH to a steroid. There were fewer side-products with this reaction in comparison to conjugating with the mixed anhydride. The method was devised by adaptation of two protocols previously published by Mattox *et al*[190] and Rajkowski and Cittanova[191] and basic chemical techniques.

Twenty-fold molar excess (17mM) of 21-hydroxyprogesterone 21-hemisuccinate or β oestradiol 17-hemisuccinate was dissolved in anhydrous DMF with equimolar 1-hydroxybenzotriazole (HOBt) and DCC. The mixture was mixed and left at room temperature for 1h. [DLys^6]GnRH was dissolved in 0.1M phosphate buffer (pH 7.0) to 0.8mM and to this an equal volume of N,N-dimethylformamide (DMF) was added. 50µl aliquots of the steroid solution were transferred into the [DLys^6]GnRH solution, mixing between each one. After all the steroid solution had been added, the pH was increased to at least pH 8 with tributylamine and the mixture was left at 4°C for 20h. The overall reactions are summarized in figure 3.6 (progesterone) and figure 3.6 (oestradiol). The expected [DLys^6]GnRH-progesterone (1665 AMU) and [DLys^6]GnRH-oestradiol conjugates (1607 AMU) were positively identified by mass spectrometry.

Reacted [DLys^6]GnRH was initially tested for the presence of peptide-steroid conjugate by mass spectrometry. Reactions positive for the conjugate(s) were purified by HPLC and the components of each fraction were analyzed by mass spectrometry for identification purposes.

Initial purification was carried out with a Sep-Pak C18 cartridge. The excess steroid was removed with 2ml ethyl acetate, followed by 2ml 70% HFP/30% DMF to elute the peptide-steroid conjugate. The HFP/DMF fraction was dried down under nitrogen and redissolved in 1ml 90% water/10% acetonitrile for HPLC analysis. Analytical RP-HPLC was carried out on a Novapak C18 column (4µm beads, 3.9 x 150mm) connected to a Beckman

Coulter System Gold® LC125 pump and LC168 diode array detector. The column was eluted using a two-buffer system of water with 0.1% TFA (buffer A) and acetonitrile with 0.1% TFA (buffer B). The column was developed with a linear gradient of 10% to 100% acetonitrile over 30min at a flow rate of 1ml/min. [D Lys^6]GnRH-21-hydroxyprogesterone 21-hemisuccinate conjugates were analyzed at 220nm and 240nm to measure the peak absorption of the peptide and progesterone molecules respectively. Similarly, [DLys^6]GnRH-β-oestradiol 17-hemisuccinate conjugates were analyzed at 220nm and 225nm respectively reflecting the different absorption spectra of the two steroids.

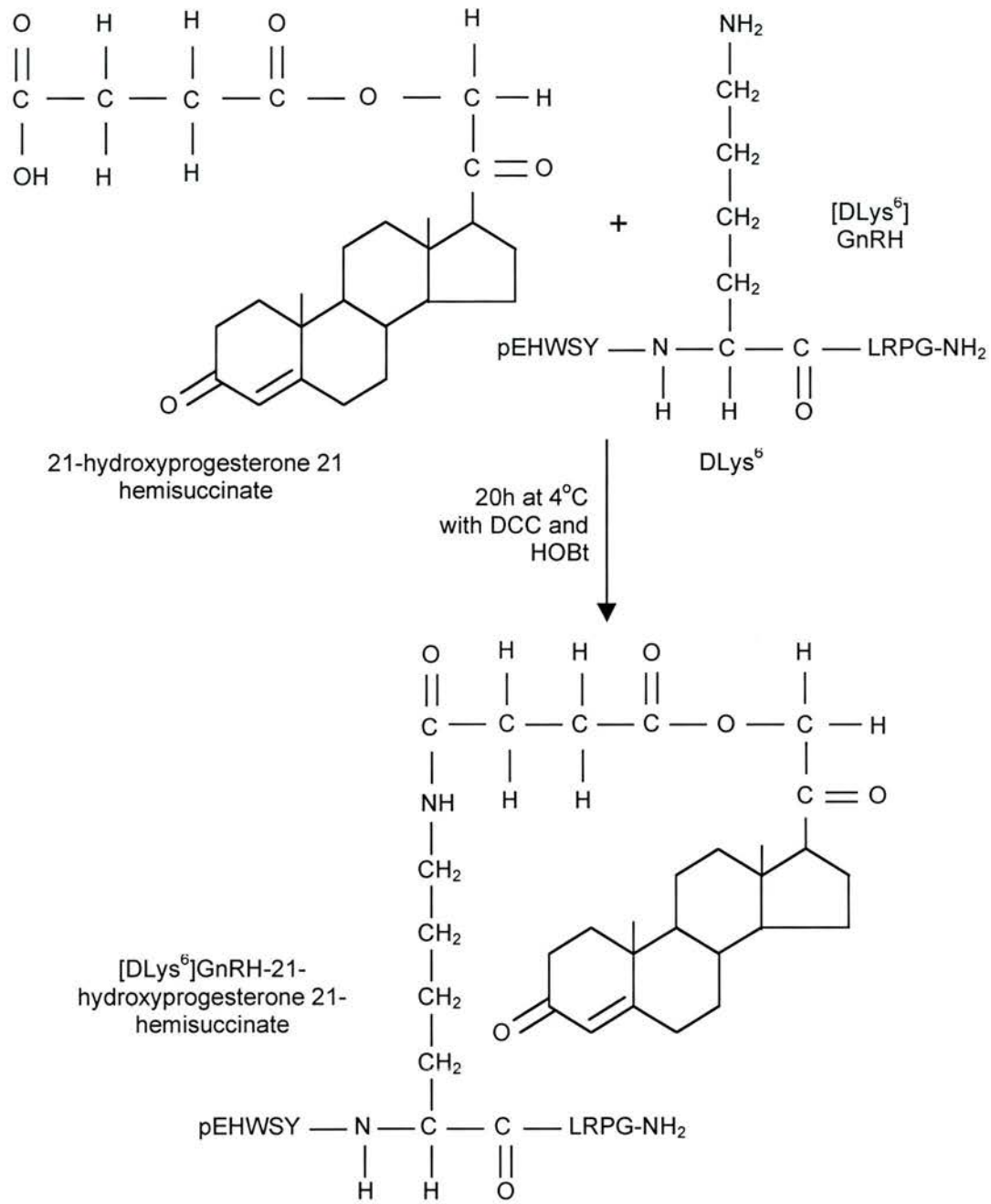


Figure 3.6: Reaction of [DLys^6]GnRH and 21-hydroxyprogesterone 21 hemisuccinate by the DCC conjugation method. The amino acids of the peptide are represented by single letter abbreviations as before.

Three molecules were identified by molecular mass (figure 3.7). The peak at 1253 AMU corresponded to unmodified [DLys^6]GnRH. The exact identity of a peak at 1275 was not clear, however this additional peak at 1275 also appeared in analysis of unreacted [DLys^6]GnRH and therefore must have been a component of this compound. A hydrated form of the peptide would not account for this peak, the mass difference was 22 AMU and a water molecule would only add 18 AMU to the molecular mass, therefore the identity remained unknown.

The peak at 1665 AMU was identified as the desired conjugate of [DLys^6]GnRH-21 hydroxyprogesterone 21-hemisuccinate. The 2075 AMU peak was consistent with a conjugate of one [DLys^6]GnRH molecule coupled to two steroid molecules, most probably due to conjugation through a less reactive amino acid side group. In some experiments a fourth minor peak was observed at 2485, possibly due to conjugation of a third steroid molecule onto the peptide. The free hydroxyl groups present in the side chains of serine and tyrosine (amino acids four and five of the decapeptide) could potentially react with the steroid hemisuccinate, although these hydroxyl groups would be less reactive than the lysine amine group. This could account for the additional peaks corresponding to two and three steroid molecules coupled to [DLys^6]GnRH. The presence of peptide-steroid conjugates with more than one steroid implies that the serine and tyrosine of the decapeptide are reactive under these conditions. Therefore the peak at 1665 could consist of [D Lys^6]GnRH-21-hydroxyprogesterone 21-hemisuccinate connected through the D-lysine amine, serine hydroxyl or tyrosine hydroxyl, all resulting in conjugates with the same molecular mass, appearing as a single composite peak.

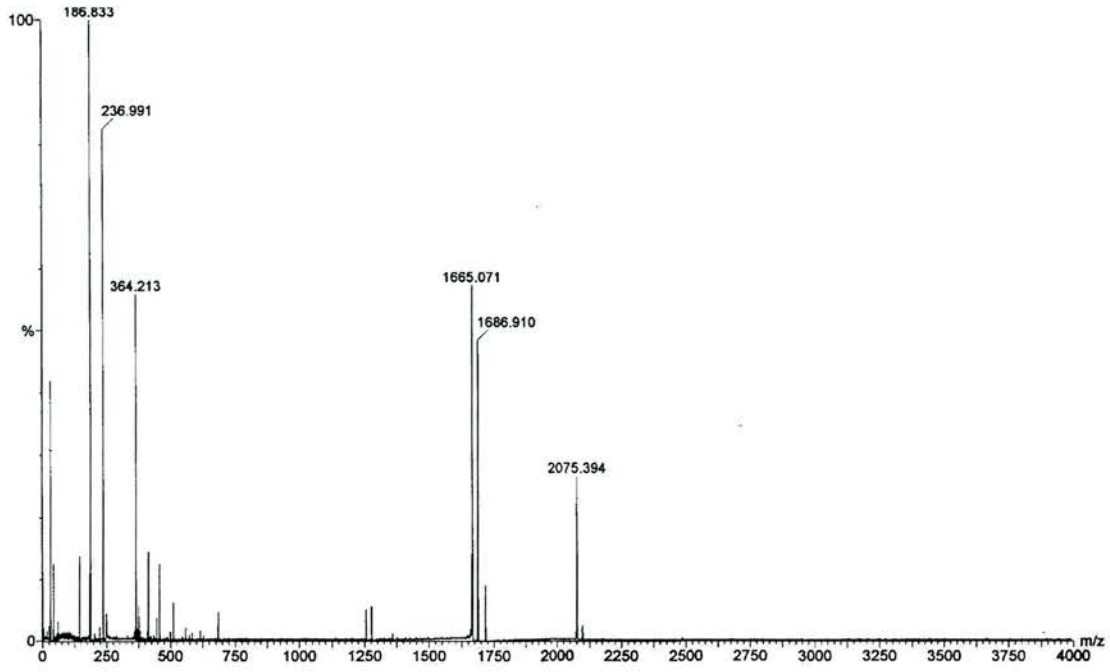


Figure 3.7: Mass spectrometry analysis of the mixture of [DLys^6]GnRH reacted with 21 hydroxyprogesterone 21 hemisuccinate coupled by DCC, before purification by HPLC.

HPLC analysis was carried out on the reaction mixture to assess the relative proportions of these molecules (figure 3.8).

HPLC analysis confirmed the presence of more than one molecule in the mixture, verifying the mass spectrometry results. Six peaks were identified by HPLC, three eluting earlier and three eluting later the unmodified steroid. It is likely that these peaks represented the major components of the mixture identified by mass spectrometry. The [DLys^6]GnRH-progesterone conjugates eluted in fractions 30 and 31 (15.3 and 15.9 minutes), with intermediate polarity between the peptide and the steroid. Both these fractions had a molecular weight corresponding to a single steroid molecule coupled to the peptide. However as indicated earlier, isomers could not be distinguished since all would result in conjugates of the same molecular weight. In order to conclusively identify the conjugate produced by this method, NMR analysis would have to be undertaken to produce a purified sample of the D-lysine ε-amine-21-hydroxyprogesterone 21-hemisuccinate conjugate. The compound

eluting later than 21-hydroxyprogesterone 21-hemisuccinate at 19.1min was identified as being [DLys^6]GnRH coupled to two steroid molecules with a molecular weight of 2075 AMU.

The area of each peak was calculated by System Gold® HPLC software and expressed as a percentage of the total area. This allowed estimation of the yield of the products (table 3.1). However the large injection artifact artificially reduced the total percentage area. Omitting this from the calculation resulted in the total areas listed in table 3.1. The greatest proportion of product eluted at 15.9min, with 46% of the total area. This product had the same molecular weight as that calculated for [DLys^6]GnRH-21 hydroxyprogesterone 21-hemisuccinate. The peaks at 15.3min and 19.1min (30 and 17% of the total area) were found to have masses equivalent to [DLys^6]GnRH-21 hydroxyprogesterone 21-hemisuccinate and [DLys^6]GnRH coupled to two 21 hydroxyprogesterone 21-hemisuccinate molecules respectively.

Retention time (min)	Corrected % total area	Molecular mass (AMU)	Identity
15.3	30	1666	[DLys^6]GnRH-progesterone
15.9	46	1666	[DLys^6]GnRH-progesterone
19.1	17	2075	[DLys^6]GnRH-2x progesterone
21.1	7	-	-

Table 3.1: Retention times and corresponding area expressed as a percentage of the total area of the major peaks, calculated from detection at 240nm by System Gold® HPLC software and the molecular weight of each peak. Removing the injection artifact and recalculating the percentage total area was carried out to calculate the corrected total area. Where no molecular mass is given, the mass could not be determined by mass spectrometry, but for those peaks that could be identified, the identity is given.

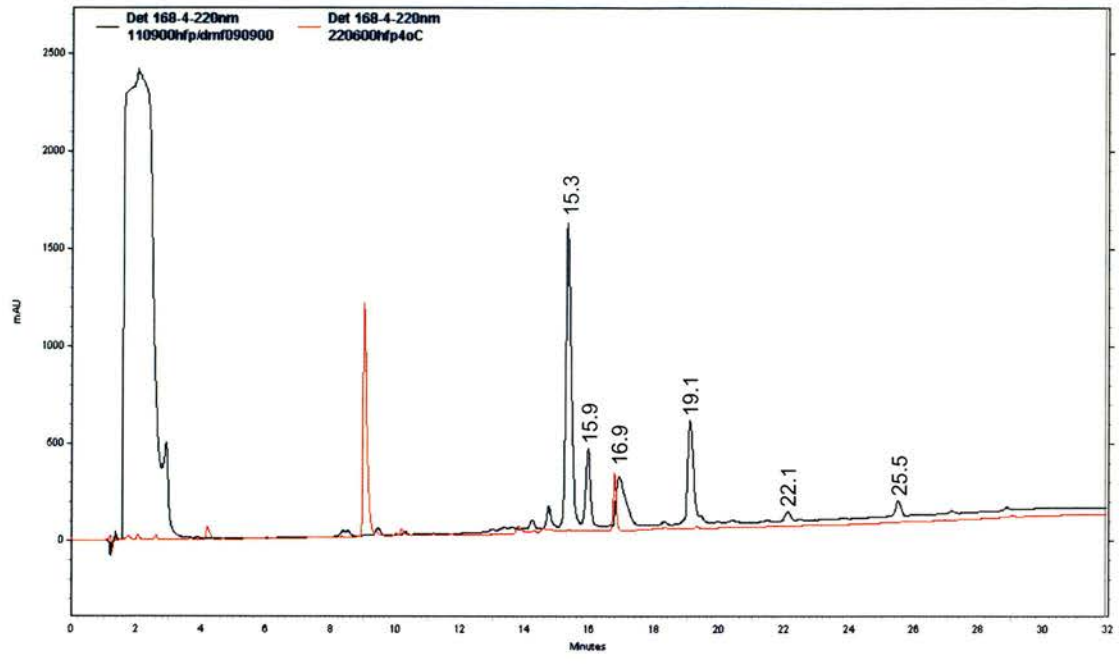


Figure 3.8: HPLC analysis of [DLys^6]GnRH reacted with 21 hydroxyprogesterone 21-hemisuccinate (black) as measured by absorption at 220nm over a 30 minute gradient of 10 to 100% acetonitrile in water at 1ml/min. The retention times of the peptide and steroid before reaction at 220nm are represented in red. Retention times are marked by each peak.

The same process was carried out to conjugate [DLys^6]GnRH to β-oestradiol 17-hemisuccinate (figure 3.9). This steroid was successfully coupled to [DLys^6]GnRH agonist analogue, with the products identified by both HPLC and mass spectrometry. The final product had a molecular weight of 1607 AMU, but a similar pattern emerged to that found with 21 hydroxyprogesterone 21 hemisuccinate, with a second conjugate with two oestradiol molecules per [DLys^6]GnRH molecule being observed (MW 1960).

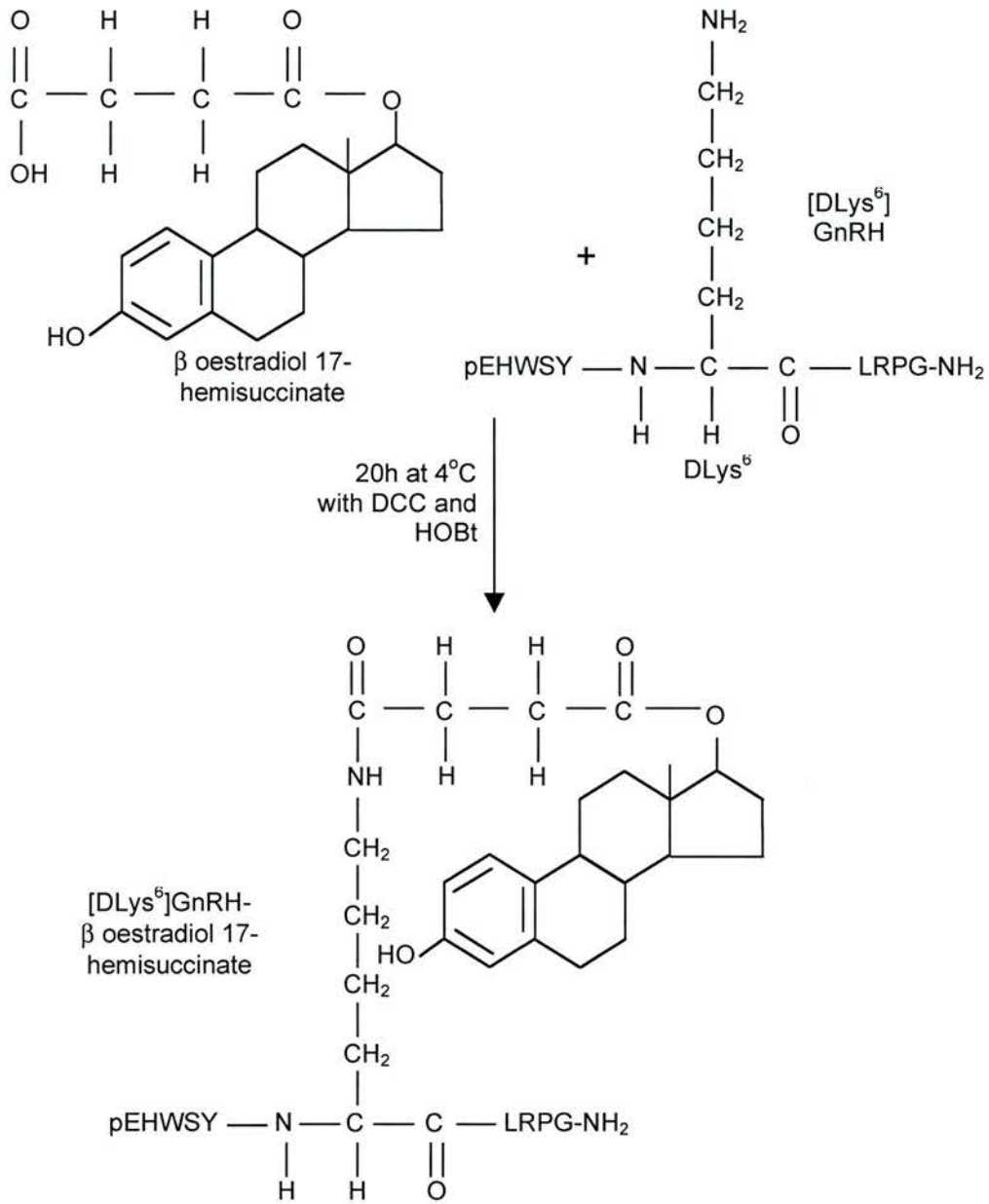


Figure 3.9: Reaction of β oestradiol 17-hemisuccinate and [DLys⁶]GnRH by the DCC conjugation method. The amino acids of the peptide are represented by single letter abbreviations as before.

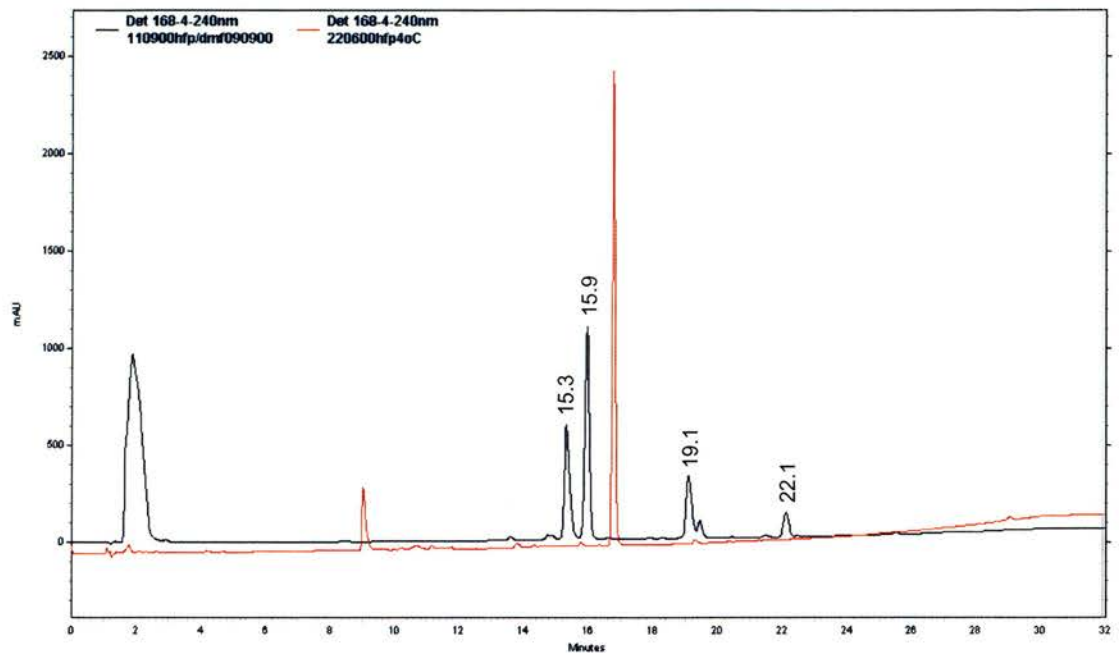


Figure 3.10: HPLC analysis of [DLys^6]GnRH reacted with 21 hydroxyprogesterone 21-hemisuccinate (black) as measured by absorption at 240nm over a 30 minute gradient of 10 to 100% acetonitrile in water at 1ml/min. The retention times of the peptide (9.3min) and steroid (17.1min) before conjugation at 240nm are represented in red.

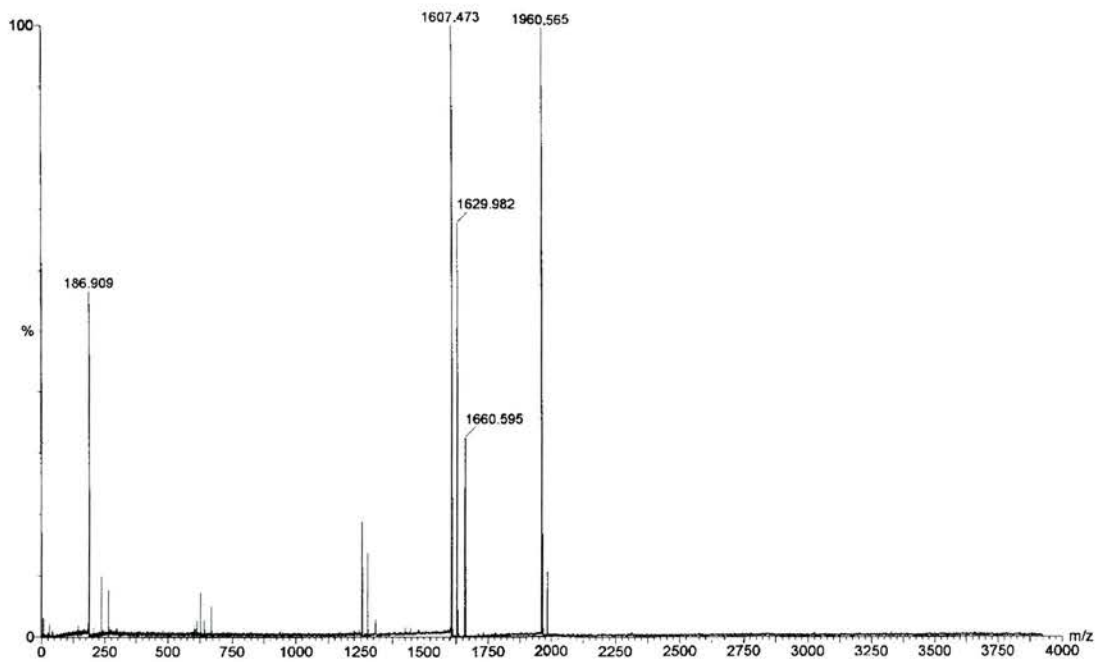


Figure 3.11: Mass spectrometry analysis of products from [DLys^6]GnRH reacted with β-oestradiol 17 hemisuccinate on an α-cyano matrix.

The total percentage area calculated by the System Gold® HPLC software was corrected to remove the injection artifact, but in addition, the considerable quantity of unreacted β-oestradiol 17-hemisuccinate that was not removed by the Sep-pak column (identified by both retention time and molecular mass) was also omitted from the calculation to obtain the percentage total area of peptide-containing components.

The excess β-oestradiol 17-hemisuccinate eluted at 16.3min, with a retention time identical to the steroid alone (red trace in figure 3.12). The major product from the reaction between [DLys^6]GnRH and β-oestradiol 17-hemisuccinate eluted at 18.7min and represented 47% of the total area. This product eluted later than the unreacted β-oestradiol 17-hemisuccinate and was found to correspond to a single [DLys^6]GnRH molecule coupled to two oestradiol molecules (mass 1960 AMU). Two peaks were observed with shorter retention times than the β-oestradiol 17-hemisuccinate, at 15.0 and 15.7min, accounting for 5 and 43% of the total area respectively. Mass spectrometry revealed that both of these peaks had identical molecular masses at 1607 AMU and therefore could be isomers resulting from conjugation to D-lysine or other reactive amino acids.

Retention time (min)	Corrected % total area	Molecular mass (AMU)	Identity
15.0	5	1607	[DLys^6]GnRH-oestradiol
15.7	43	1607	[DLys^6]GnRH-oestradiol
16.3	-	372	β oestradiol 17 hemisuccinate
18.7	47	1960	[DLys^6]GnRH-2x oestradiol
22.5	5	-	-

Table 3.2: Retention times and corresponding area of reaction between [DLys^6]GnRH and 17β oestradiol 17-hemisuccinate expressed as a percentage of the total area of the major peaks, calculated from the absorption at 225nm by System Gold® HPLC software and the molecular weight of each peak. The corrected percentage total area was calculated by omitting the injection artifact and unreacted β-oestradiol 17-hemisuccinate from the calculation. Where no molecular mass is given, the mass could not be determined by mass spectrometry, but for the remaining peaks the identity is stated.

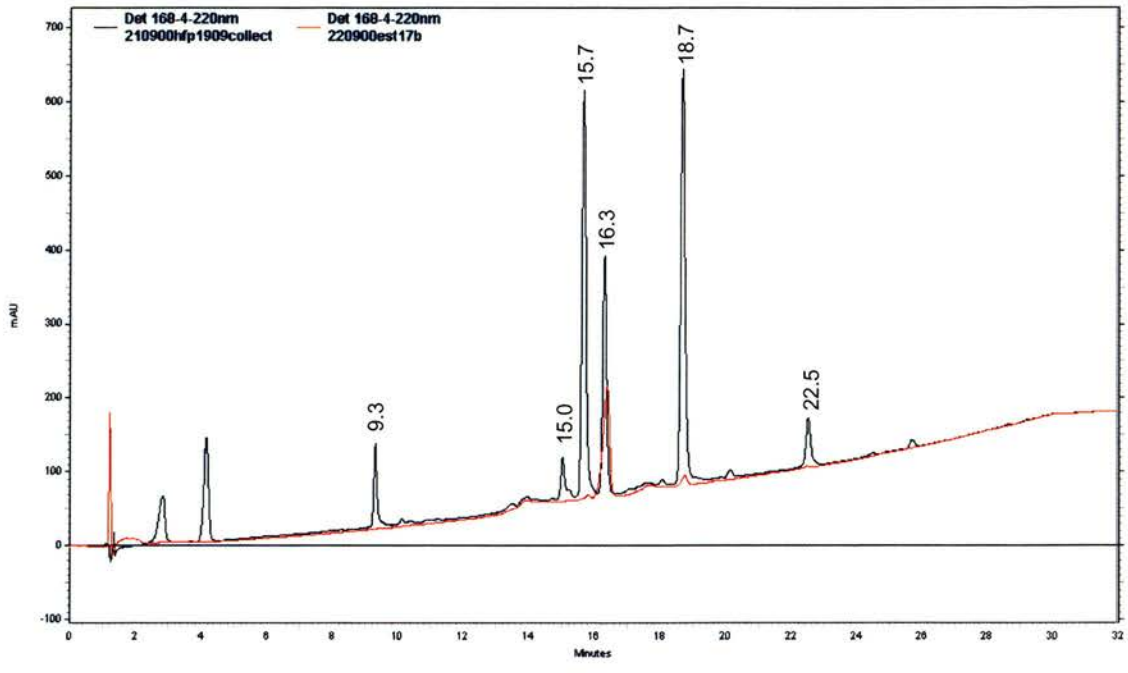


Figure 3.12: HPLC analysis of [DLys^6]GnRH reacted with 17β oestradiol 17-hemisuccinate as measured by absorption at 225nm over a 30 minute gradient of 10 to 100% acetonitrile in water. The rising baseline can be attributed to the increasing proportion of acetonitrile, which also absorbs at this wavelength. The elution profile of the unreacted steroid at 225nm is shown in red.

3.4 DESIGN OF GnRH ANTAGONIST-STEROID CONJUGATES

GnRH agonist-progesterone conjugates are interesting molecules, but GnRH antagonist conjugates are potentially more useful for rapid gonadotropin inhibition. These conjugates were also designed and produced in this thesis. As mentioned earlier (section 3.2.1), a D-lysine in position six was preferable, therefore antagonists with a single DLys^6 were sought. Dr J Rivier (Salk Institute, California) kindly donated two antagonists fulfilling these requirements. These were [AcDNal^1, DCpa^2, D Pal^3, Arg^5, D Lys^6, DAla-NH_2^{10}]GnRH (peptide A) and [AcΔPro^1, D Fpa^2, DTrp^3, D Lys^6, Gly-

NH_2^{10}]GnRH (peptide B). These sequences had a single D-lysine for conjugation, initially used for conjugation to 21-hydroxyprogesterone 21-hemisuccinate, forming antagonist conjugates A and B. However for later studies it was necessary to include a second amino acid with a reactive side chain.

One of the aims of this study was to modify peptide GnRH analogues to enhance oral absorption. Different methods have been used to achieve this, ranging from co-administration with absorption enhancers[164-168] to coupling to carrier molecules such as bile acids[141] or vitamin B_{12} [158]. GnRH antagonists are too large to be transported coupled to bile acids and the most suitable alternative method currently available is conjugation to vitamin B_{12} derivatives through a thiol- cleavable spacer [158]. Vitamin B_{12} is significantly larger than the steroid and therefore it was conjugated to the D-lysine in position six and a second lysine (L-lysine) was substituted for the leucine in position seven of antagonist conjugate A. This resulted in antagonist conjugates C and E, differing only in amino acids eight and nine of the decapeptide. Antagonist C was [AcDNal^1, DCpa^2, DPal^3, Arg^5, DLys^6, Lys^7, Leu^8, Arg^9, DAla-NH_2^{10}]-GnRH. This sequence was produced in error, with the wrong amino acids in positions eight and nine of the peptide (leucine and arginine instead of arginine and proline). Antagonist E was the correct sequence, with arginine and proline in positions eight and nine, [AcDNal^1, DCpa^2, DPal^3, Arg^5, DLys^6, Lys^7, DAla-NH_2^{10}]GnRH. Both antagonists C and E were linked to 21-hydroxyprogesterone 21-hemisuccinate though the ε-amine of the Lys^7 to form conjugates C and E.

Conjugate D was constructed from the same peptide sequence as conjugate A, with the D-lysine maintained for vitamin B_{12} conjugation, but the first two N-terminal amino acids were omitted and replaced with the 21-hydroxyprogesterone 21-hemisuccinate, conjugated via the terminal amine group of the pyridylalanine. The sequence of conjugate D was therefore 21-hydroxyprogesterone 21-hemisuccinate-D-Pal, Ser, Arg, D-Lys, Leu, Arg, Pro, D-Ala-NH_2.

The chemistry of conjugation could have been simplified by using a D-cysteine in position six in place of the D-lysine, which would easily react directly with a vitamin B_{12} derivative containing a sulphydryl group. However the side group of D-cysteine is significantly shorter than D-lysine (figure 3.13) and this would bring the peptide and steroid in close proximity to each other, increasing the likelihood of steric hindrance to both GnRH receptor and plasma protein binding. A homocysteine was another possibility, having an additional carbon in the side chain in comparison to cysteine, however this is not commercially available in the D isoform and therefore could not be used in this instance. Thus a D-lysine was used in antagonists C, D and E, with the amine group reacted with a crosslinking reagent to couple the peptide to the sulphydryl group of the vitamin B_{12} derivative.

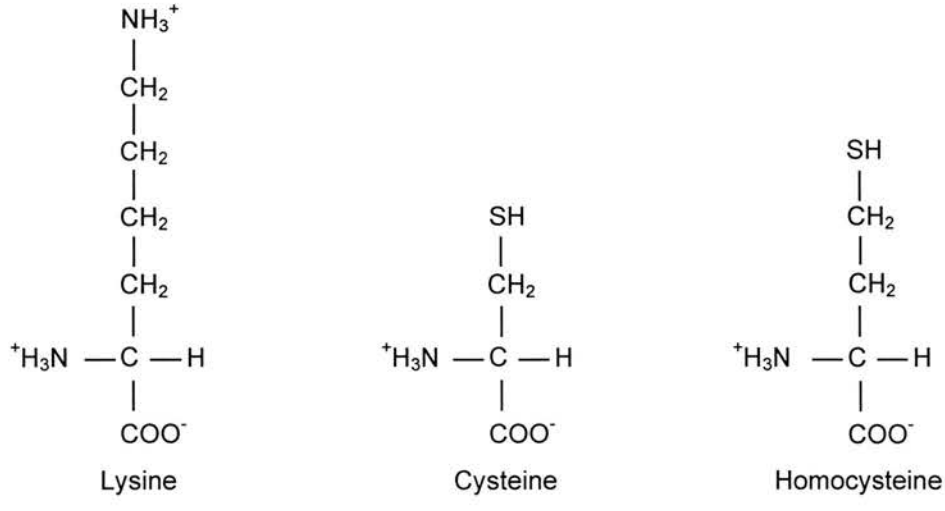


Figure 3.13: Structure of lysine, cysteine and homocysteine.

The five antagonist-progesterone conjugates are summarized in table 3.3, with the conjugates shown aligned to mGnRH for comparison.

The [DLys^6]GnRH agonist was used to test the principle of conjugation methods, but custom synthesis companies were used to synthesize GnRH antagonists for later studies. This had the advantage of conjugating the steroid during peptide synthesis whilst the peptide was still attached to the

solid resin support. The protection of other functional group side chains during this process ensured the desired conjugate could be produced at high yield and purity. The organic carbodiimide (DCC) method was used for the conjugation of all five antagonists to 21-hydroxyprogesterone 21-hemisuccinate by Bachem (UK) Ltd (conjugates A and B) and Albachem Ltd (conjugates C, D and E).

Amino Acid	1	2	3	4	5	6	7	8	9	10
mGnRH	Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Conjugate A	AcD-Nal	D-Cpa	D-Pal	Ser	Arg	D-Lys*	Leu	Arg	Pro	D-Ala-NH_2
Conjugate B	Ac-ΔPro	D-Fpa	D-Trp	Ser	Tyr	D-Lys*	Leu	Arg	Pro	Gly-NH_2
Conjugate C	AcD-Nal	D-Cpa	D-Pal	Ser	Arg	D-Lys	Lys*	Leu	Arg	D-Ala-NH_2
Conjugate D			*D-Pal	Ser	Arg	D-Lys	Leu	Arg	Pro	D-Ala-NH_2
Conjugate E	AcD-Nal	D-Cpa	D-Pal	Ser	Arg	D-Lys	Lys*	Arg	Pro	D-Ala-NH_2

Table 3.3: The amino acid sequences of the GnRH antagonists. The following abbreviations are used: Glu; glutamic acid, His; histidine, Trp; tryptophan, Ser; serine, Tyr; tyrosine, Gly; glycine, Leu; leucine, Arg; arginine, Pro; proline, AcD-Nal; acyl D-napthylalanine, D-Cpa; D-chlorophenylalanine, D-Pal; D-pyridylalanine, D-Lys; D-lysine, D-Ala; D-alanine, Ac-ΔPro; acyl delta-proline, D-Fpa; D-fluorophenylalanine, D-Trp; D-tryptophan. The site of conjugation to 21-hydroxyprogesterone 21-hemisuccinate is indicated by the asterisk.

3.5 CONCLUSIONS

Conjugates of [DLys^6]GnRH linked to progesterone were successfully prepared by two different methods, both utilizing zero length cross-linking reagents, IBCF (mixed anhydride) and DCC (organic carbodiimide). The mixed anhydride and organic carbodiimide methods were modified to adjust for the conjugation of a hydrophobic steroid to a hydrophilic peptide, on the basis of established chemistry techniques and similar methods published in

the literature. The DCC method proved to be the most successful for this application, resulting in fewer unwanted products and thus a higher yield of the peptide-steroid conjugate. Conjugates of [DLys^6]GnRH coupled to β-oestradiol 17-hemisuccinate were also produced by the DCC method. The conjugates were identified by a combination of HPLC and mass spectrometry, but additional NMR analysis would ideally have been performed to conclusively identify the structure of the conjugates.

These reactions were carried out with [DLys^6]GnRH to identify the most successful method and reaction conditions for conjugating [DLys^6]GnRH to progesterone and oestradiol derivatives. This provided a proof of concept, but for further studies two commercial companies (Bachem UK Limited and Albachem Limited) were employed to use the same methods to conjugate 21-hydroxyprogesterone 21-hemisuccinate to five GnRH antagonists. Although this thesis concentrated on the study of GnRH antagonist-progesterone conjugates, the [DLys^6]GnRH agonist-progesterone conjugates would also be interesting molecules to study and may also transform the properties of the peptide alone.

It may have been of benefit to design the conjugates to incorporate a radioisotope into the conjugate at the outset, enabling the molecule to be traced throughout analysis. This would have been advantageous to measuring both GnRH receptor binding (where competition with [His^{5}DTyr^6]GnRH was measured) and plasma protein binding. Iodination of conjugate B was attempted but was unsuccessful despite the presence of a tyrosine residue in position 5 of the decapeptide. The cause of the iodination failure was unknown. However the iodine radioisotope is of sufficient size to affect the conformation of the antagonist and could affect GnRH receptor binding. This type of radiolabelling was therefore not ideal in these circumstances. The selected isotope should be small in size and have a useful half-life beyond a few months. A possible isotope is ^{3}H, a beta emitter with a half-life of 12.4 years. Progesterone derivatives containing this radiolabel could have been used for conjugation to the peptide.

4 Properties of GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates *in vitro*

4.1 INTRODUCTION

The GnRH antagonist and steroid moieties comprising the conjugates were investigated for GnRH receptor binding, GnRH receptor antagonism, plasma protein binding and progesterone receptor activation. This enabled the effect of the conjugation process on the two constituent parts to be assessed.

Five GnRH antagonist-21-hydroxyprogesterone 21-hemisuccinate conjugates were analyzed (table 4.1).

Amino Acid	1	2	3	4	5	6	7	8	9	10
Conjugate A	AcD-Nal	D-Cpa	D-Pal	Ser	Arg	D-Lys*	Leu	Arg	Pro	D-Ala-NH_2
Conjugate B	Ac-DPro	D-Fpa	D-Trp	Ser	Tyr	D-Lys*	Leu	Arg	Pro	Gly-NH_2
Conjugate C	AcD-Nal	D-Cpa	D-Pal	Ser	Arg	D-Lys	Lys*	Leu	Arg	D-Ala-NH_2
Conjugate D			*D-Pal	Ser	Arg	D-Lys	Leu	Arg	Pro	D-Ala-NH_2
Conjugate E	AcD-Nal	D-Cpa	D-Pal	Ser	Arg	D-Lys	Lys*	Arg	Pro	D-Ala-NH_2

Table 4.1: The amino acid sequences of the antagonists tested for pharmacological properties. The site of conjugation to 21-hydroxyprogesterone 21-hemisuccinate is indicated by the asterisk. Abbreviations as before.

4.2 MEASUREMENT OF GnRH RECEPTOR BINDING AFFINITY AND RECEPTOR ACTIVATION

4.2.1 Competition whole cell binding assay

Conjugates A and B and the corresponding parent peptide sequences A and B were tested for GnRH receptor binding in COS-7 cells transiently transfected to express the human GnRH receptor. Whole cell binding was determined 48h after transfection as described in chapter 2. Total binding (Bo) was calculated as the binding of ^{125}I[His^{5}DTyr^6]GnRH in the absence of competing ligand. The binding of ^{125}I[His^{5}DTyr^6]GnRH in the presence of excess unlabelled ligand (non-specific binding, or NSB) was subtracted from the total binding to calculate the IC_{50} values.

The IC_{50} value of conjugate A was 7-fold lower than the parent peptide A at the human GnRH receptor ($p < 0.01$, student's t-test) at 108 ± 22nM (Standard error, $n = 4$) and 16 ± 4nM ($n = 4$) respectively (figure 4.1). In contrast, the IC_{50} of conjugate B, 105 ± 27nM ($n = 5$), did not differ significantly ($p > 0.05$, STT) from the parent peptide B at 47± 11nM ($n = 4$) (figure 4.2).

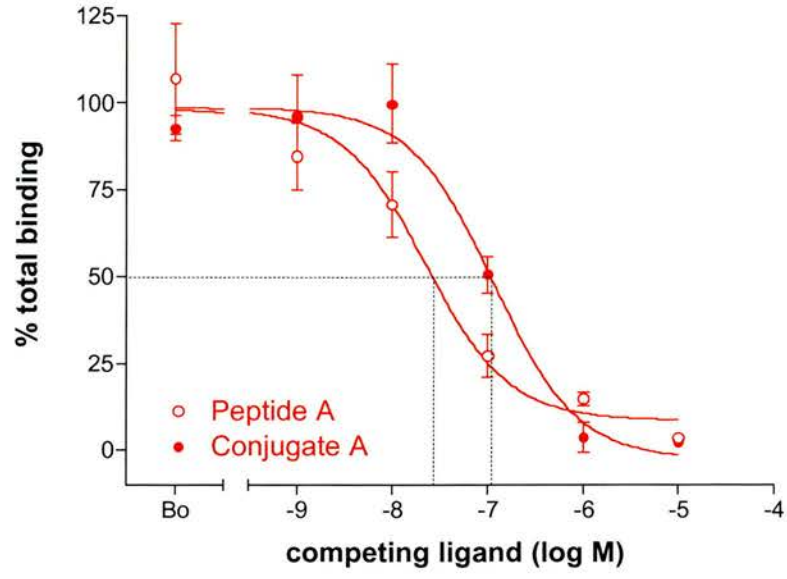


Figure 4.1: Representative result of the effect of parent peptide A (○) and conjugate A (●) on the displacement of ^{125}I[His^{5}DTyr^6]GnRH agonist bound to intact COS-7 cells transiently transfected with the human GnRH receptor. The dotted lines identify the IC_{50} values.

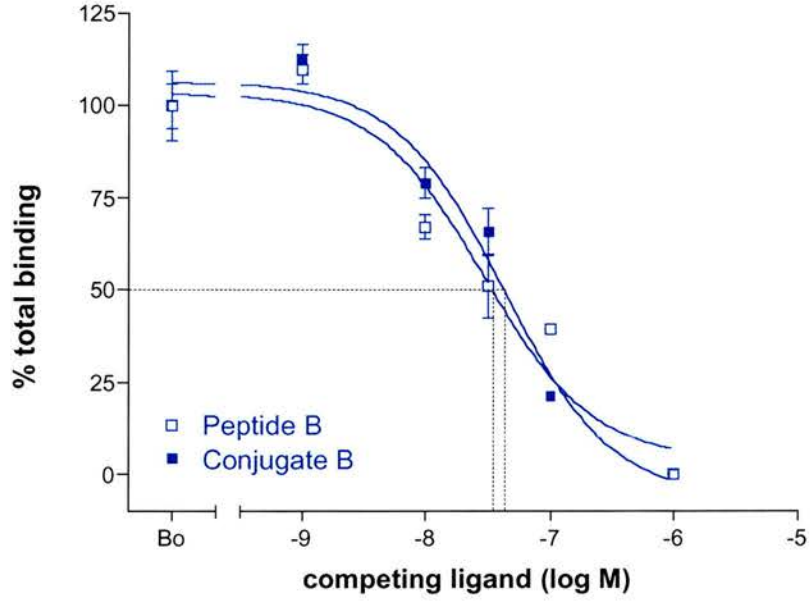


Figure 4.2: Representative results of the effect of parent peptide B (□) and conjugate B (■) on the displacement of ^{125}I[His^{5}DTyr^6]GnRH agonist bound to intact COS-7 cells transiently transfected with the human GnRH receptor. The dotted lines identify the IC_{50} values.

Conjugates C, D and E were tested for whole cell binding in SCL60 cells (HEK293 cells stably expressing the rat type I GnRH receptor, described in chapter 2). This stable cell line was used instead of transiently transfected COS-7 cells to minimize variation resulting from the transfection procedure. The IC_{50} values of conjugate C (134 ± 26nM, n = 3) and conjugate E (104 ± 7nM, n = 3) did not differ significantly ($p > 0.05$, STT), despite the sequence differences at positions eight and nine of the peptide (figure 4.3). Conjugation of progesterone in place of the first two amino acids (napthylalanine and chlorophenylalanine, see table 4.1) resulted in a significant increase ($p < 0.001$, STT) in the IC_{50} value of conjugate D (8390 ± 936nM, n = 3), compared with conjugates C and E (figure 4.3).

The GnRH receptor binding affinities of all five conjugates are shown in table 4.2.

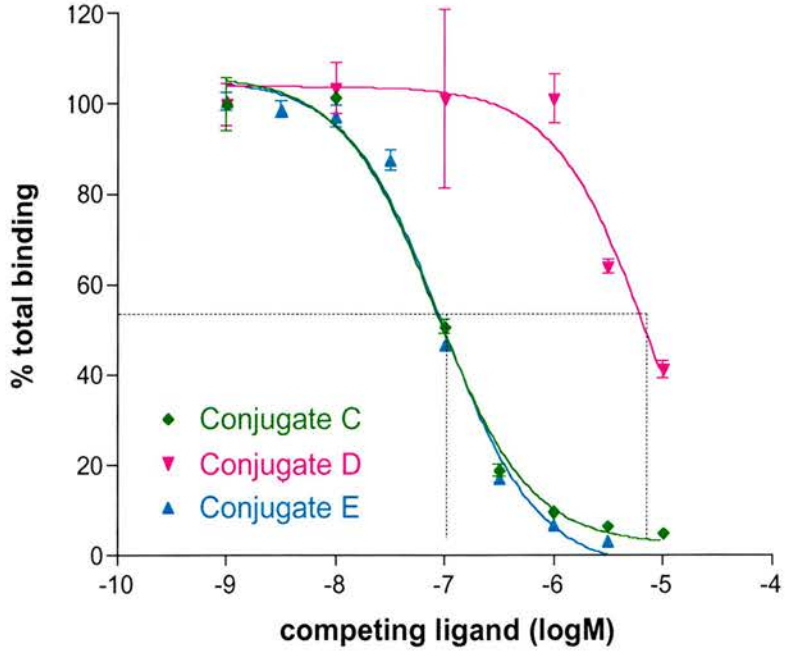


Figure 4.3: Representative result of the effect of conjugate C (◆), conjugate D (▼) and conjugate E (▲) on the displacement of ^{125}I[His^{5}DTyr^6]GnRH bound to intact SCL60 cells transiently transfected with the human GnRH receptor. The dotted lines identify the IC_{50} values.

Conjugate	Whole cell binding IC_{50}
A	108 ± 22nM[a]
B	105 ± 27nM[a]
C	134 ± 26nM[b]
D	8390 ± 936nM[b]
E	104 ± 7nM[b]

Table 4.2: Whole cell binding of conjugates A, B, C, D and E competing with [His^{5}DTyr^6]GnRH. [a] at the human GnRH receptor in COS-7 cells, [b] at the rat type I GnRH receptor in HEK293 cells.

4.2.2 Inhibition of GnRH-stimulated inositol phosphate production

The ability of the conjugates to inhibit mammalian GnRH-stimulated inositol phosphate (IP) production was measured in SCL60 cells. This confirmed that conjugates A, B, C and E remained antagonists at the GnRH receptor for this intracellular signaling pathway (figure 4.4). The IC_{50} values of conjugate A and conjugate B were 97 ± 40nM (n = 6) and 76 ± 17nM (n = 7) respectively.

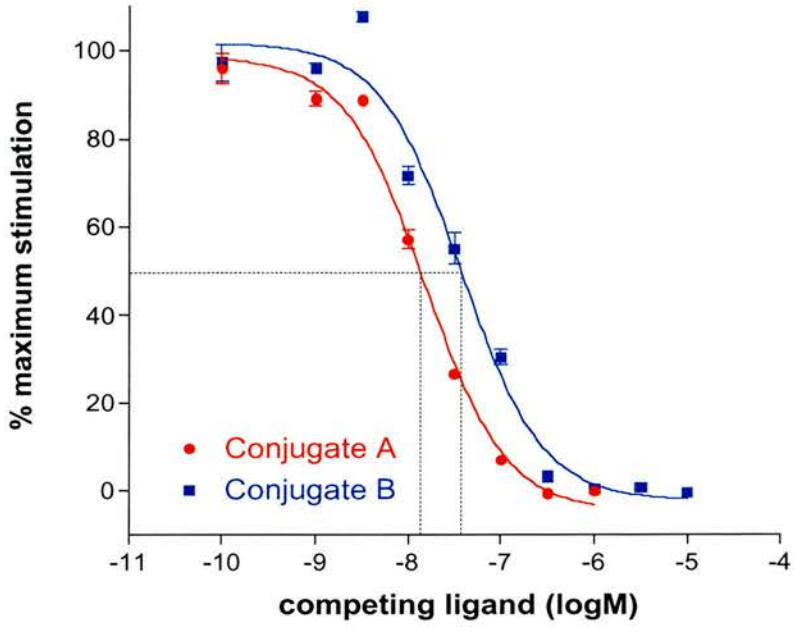


Figure 4.4: Representative result of the effect of conjugate A (●) and conjugate B (■) on 0.1μM mammalian GnRH-stimulated IP production in SCL60 cells.

Conjugates C, D and E were also tested for inhibition of mammalian GnRH-stimulated IP production and stimulation of IP alone (i.e. agonism). Conjugates C and E, differing only in amino acids eight and nine of the decapeptide sequence, had no significant difference in inhibition of IP production ($p > 0.05$, STT) with IC_{50} values of 5580 ± 127nM (n = 4) and 16,000 ± 5190nM (n = 4) respectively (figure 4.5). Conjugate D showed no evidence of inhibiting IP production, despite a limited ability to compete with ^{125}I[His^{5}DTyr^6]GnRH for binding to the GnRH receptor (figures 4.3 and 4.5). The IC_{50} values of all conjugates are summarized in table 4.3.

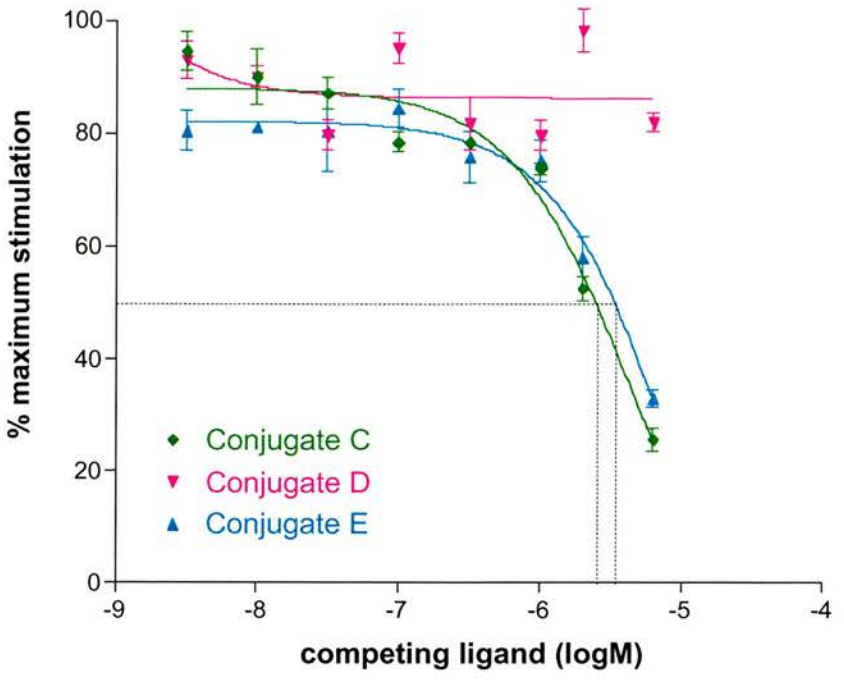


Figure 4.5: Representative result of the effect of conjugate C (◆), conjugate D (▼), and conjugate E (▲) on 0.1μM mammalian GnRH-stimulated IP production in SCL60 cells.

Inhibition of 0.1μM mammalian GnRH-stimulated IP production by the five conjugates is summarized in table 4.3.

Conjugate	IC_{50} of inositol phosphate production
A	97 ± 40nM
B	76 ± 17nM
C	5580 ± 127nM
D	No inhibition
E	16,000 ± 5190nM

Table 4.3: IC_{50} values of inhibition of 0.1μM mammalian GnRH-stimulated IP production.

None of the antagonist-progesterone conjugates were found to stimulate IP production alone (figure 4.6) in SCL60 cells.

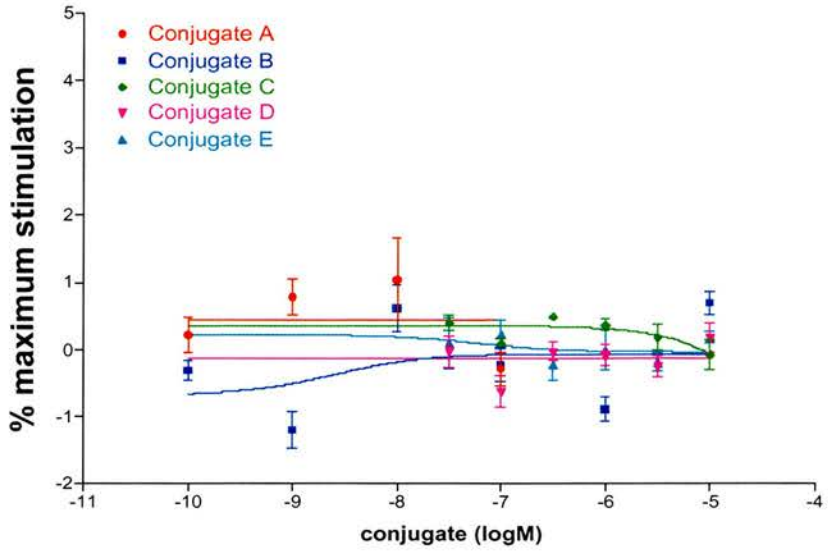


Figure 4.6: Representative result of the effect of conjugate A (●), conjugate B (■), conjugate C (◆), conjugate D (▼), and conjugate E (▲) on IP production in SCL60 cells.

The IP assay demonstrated that the conjugates acted as antagonists at the GnRH receptor on this particular intracellular signaling pathway. However it is known that many receptors can activate different pathways leading to different downstream signaling events. Human and murine GnRH receptors are known to activate a single class of G proteins (Gq/11) [192]. However there are a number of second messengers involved downstream of G-proteins, including IP_3, diacylglycerol, Ca^{2+}, protein kinase C, arachidonic acid and leukotriene C4 [193, 194]. The cross-talk of these molecules culminates in gonadotropin release and gene expression through complex signaling networks [193].

Although the information gained from this assay gives some evidence of GnRH receptor antagonism, it would have been better to assay of another second messenger molecule to confirm the IP assay result. Alternatively, a simpler method to conclusively demonstrate antagonism would have been to measure the final effect of GnRH receptor activation, i.e. production of LH by a gonadotrope cell line or a primary culture of dispersed pituitary cells, measuring LH production by RIA.

4.3 PLASMA PROTEIN BINDING

To investigate whether GnRH antagonist-progesterone conjugates bind to plasma proteins, whole serum and plasma were tested for competitive binding of [^{3}H]progesterone. The most concentrated source of human CBG is found during the second trimester of pregnancy or during treatment with estrogens, however there is limited availability of these types of serum for experimentation. First trimester serum samples were readily available from the NHS laboratories, collected for pregnancy tests with known hCG levels and these were tested as a substitute. The most concentrated source of a progesterone-binding plasma protein (PBG) is found in hystricomorph rodents such as the pregnant guinea pig.

Total specific plasma protein binding of [^{3}H]progesterone was compared in pregnant guinea pig plasma and human serum with low (5,000-20,000IU/ml) or high (20,000-90,000IU/ml) hCG levels. This revealed pregnant human serum had a low specific binding of [^{3}H]progesterone, whereas a high specific binding was observed with pregnant guinea pig plasma (figure 4.7).

High molecular weight plasma proteins were shown to bind [^{3}H]progesterone by removal of unbound [^{3}H]progesterone with dextran-coated charcoal suspension. Unlabelled progesterone competed for this binding with an IC_{50} value of 96 ± 18nM (n = 4), by conjugate A with an IC_{50} value of 1020 ± 284nM (n = 6) and by conjugate B with an IC_{50} value of 534 ± 103nM (n = 4) as illustrated in figure 4.8. The IC_{50} values of both conjugates A and B were significantly greater than progesterone ($p < 0.05$ and $p < 0.01$ respectively, STT). Parent peptides A and B were also tested for interaction with specific plasma proteins. No inhibition of [^{3}H]progesterone binding to plasma proteins was seen with either peptide A or peptide B (figure 4.9).

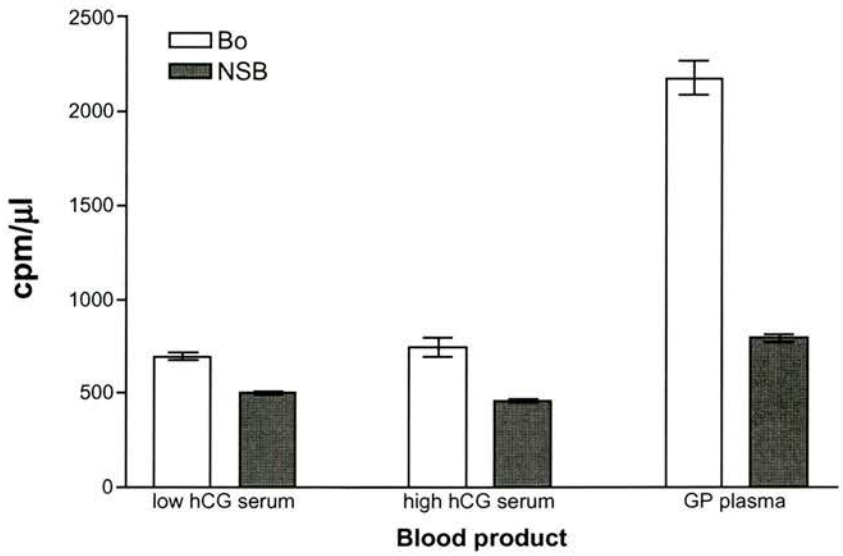


Figure 4.7: Total binding of [1,2,6,7-^{3}H]progesterone to human pregnant serum with hCG 5,000-20,000IU/ml (low hCG) and 20,000-90,000IU/ml (high hGC) and pregnant guinea pig plasma (GP plasma), as measured by separation of bound and free [^{3}H]progesterone with dextran-coated charcoal suspension. The white bars represent the total binding in the absence of a competing ligand (Bo) and the gray bars denote the binding in the presence of excess (10µM) progesterone (NSB).

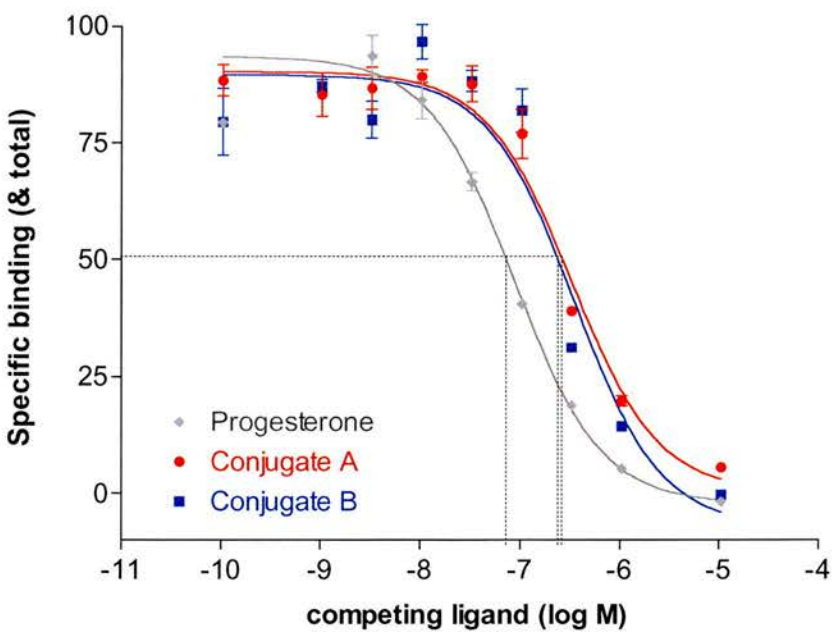


Figure 4.8: Representative result of the effect of progesterone (◆), conjugate A (●) and conjugate B (■) on the binding of [1,2,6,7-^{3}H]progesterone to pregnant guinea pig plasma, as measured by separation with dextran-coated charcoal suspension.

Inhibition of specific [^{3}H]progesterone plasma protein binding was also determined for conjugates C, D and E. All three conjugates bound well to the plasma proteins, but had higher IC_{50} values in comparison to progesterone ($p < 0.01$ for conjugate C, $p < 0.001$ for conjugate D and $p < 0.001$ for conjugate E, STT). Conjugate D inhibited [^{3}H]progesterone binding with an IC_{50} value of 264 ± 25nM (n = 3) in comparison to significantly higher IC_{50} values of 1020 ± 230nM (n = 4) and 779 ± 11nM (n = 3) for conjugates C and E respectively (figure 4.10).

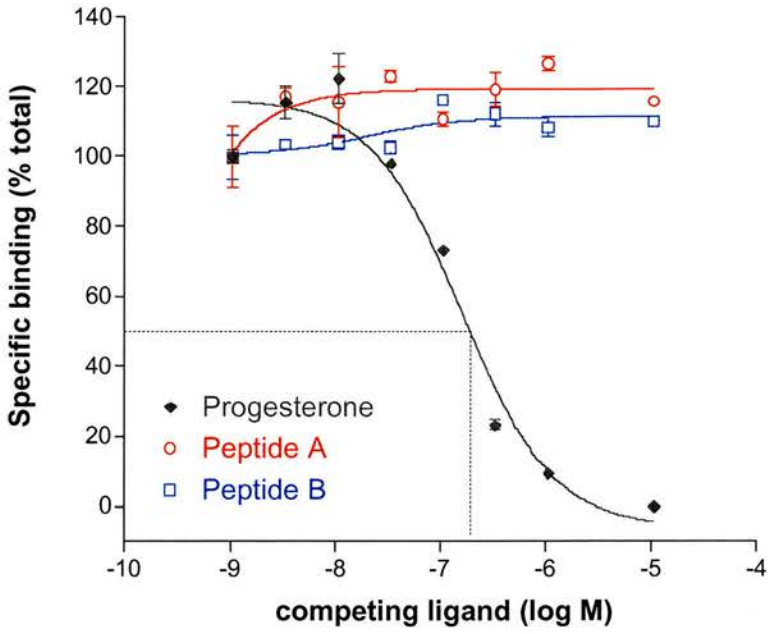


Figure 4.9: Representative result of the effect of progesterone (◆), parent peptide A (○) and parent peptide B (□) on the binding of [1,2,6,7-^{3}H]progesterone to pregnant guinea pig plasma, as measured by separation with dextran-coated charcoal suspension.

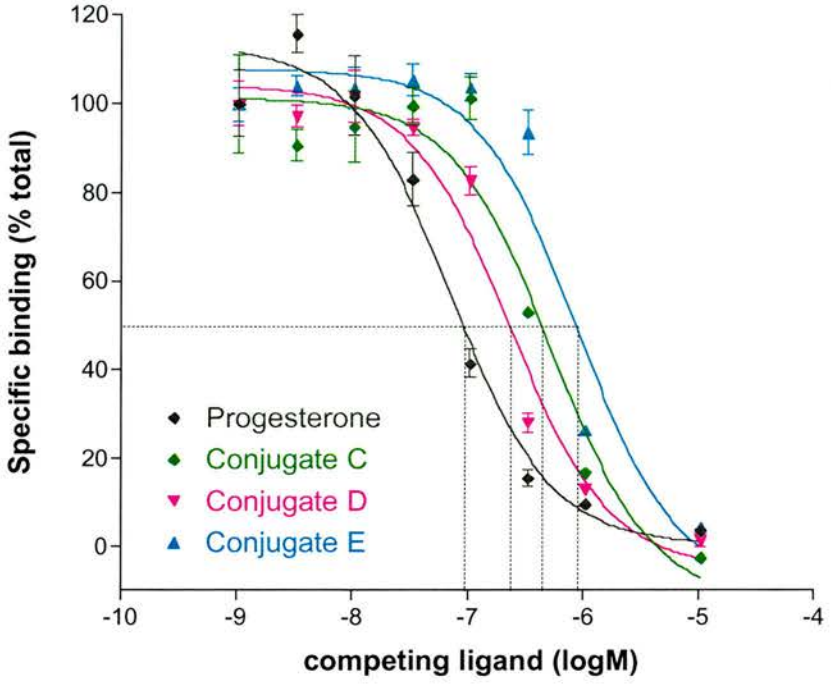


Figure 4.10: Representative result of the effect of progesterone (◆), conjugate C (◆), conjugate D (▼) and conjugate E (▲) on the binding of [1,2,6,7-^{3}H]progesterone to pregnant guinea pig plasma, as measured by separation with dextran-coated charcoal suspension.

Conjugate	IC_{50} of competition for plasma protein binding sites
A	1020 ± 284nM
B	534 ± 103nM
C	1020 ± 230nM
D	264 ± 25nM
E	779 ± 11nM

Table 4.4: IC_{50} of competition with [^{3}H]progesterone for plasma protein binding sites in pregnant guinea pig plasma of conjugates A, B, C, D and E.

The dextran-coated charcoal method is suitable for separating bound and free steroids when the binding is of high affinity, such as the interaction between steroids and the specific binding proteins CBG and PBG [181]. It is however less suitable for separating molecules bound with low affinity to plasma proteins, such as albumin. Since both specific plasma proteins and

albumin interact with sex steroids under physiological conditions, a method that can detect both types of binding would be more relevant to this investigation.

This may have been the reason why the measured specific binding in pregnant human serum was much lower than expected. A better method would have been to use equilibrium dialysis to determine the proportions of bound and free steroids in both human and guinea pig plasma. In this technique free (unbound) ligand is dialyzed through a membrane until its concentration across the membrane is at equilibrium. The free ligand concentration, concentration of binding component (i.e. plasma protein), the starting concentration of ligand and final concentration of free ligand can then be measured to determine the extent of plasma protein binding as a proportion of ligand present.

4.3.1 Specificity of progesterone plasma protein binding

The inability of cortisol to inhibit specific [^{3}H]progesterone binding clearly demonstrated the specificity of the progesterone-PBG interaction (figure 4.11). The small decrease in [^{3}H]progesterone binding to the plasma proteins at concentrations of cortisol above 1µM can most probably be attributed to interactions with non-specific plasma proteins such as albumin. This effect was not observed when [^{3}H]progesterone binding was tested in purified pregnant guinea pig plasma (data not shown).

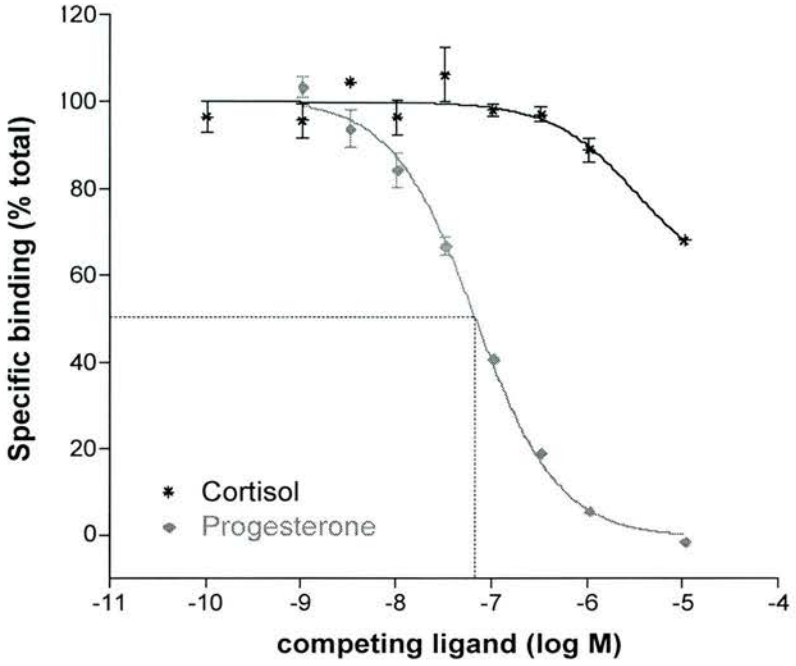


Figure 4.11: Effect of increasing concentrations of cortisol on the binding of [1,2,6,7-^{3}H]progesterone to pregnant guinea pig plasma, as measured by separation with dextran-coated charcoal suspension.

Although these results confirmed that specific plasma protein binding was retained, the relevance of this information is limited by the use of PBG as the binding protein. These results do not measure the interactions of the conjugates with human (or primate) binding proteins. As stated earlier, the use of equilibrium dialysis to measure plasma protein binding would perhaps have allowed the investigation of human or primate interactions, yielding more important information.

4.4 PROGESTERONE RECEPTOR ACTIVATION

It was important to determine whether the 21-hydroxyprogesterone 21-hemisuccinate was still able to bind to and activate the progesterone receptor when conjugated to a GnRH antagonist. Activation of the progesterone

receptor was tested in the breast cancer epithelial cell line T47D, stably expressing a CAT enzyme reporter gene linked to the progesterone receptor.

Assay of CAT enzyme activity revealed that all five GnRH antagonist-21-hydroxyprogesterone 21-hemisuccinate conjugates were able to bind to and activate the progesterone receptor in T47D cells, as measured by an increase in CAT enzyme activity (figure 4.12). The potencies of all conjugates were similar in this respect, with virtually no CAT activity at 1nM ligand and increasing CAT activity up to 1µM. The GnRH antagonist progesterone conjugates were able to activate CAT synthesis with similar potencies to that observed for progesterone itself (figure 4.12).

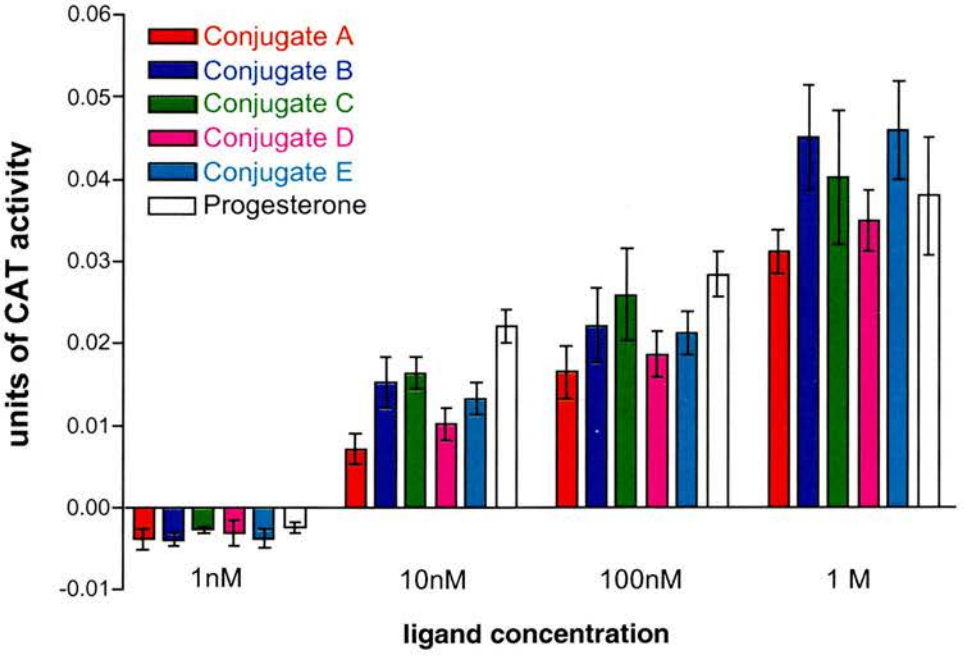


Figure 4.12: Activation of the progesterone receptor by conjugates A, B, C, D and E as measured by assay of CAT reporter gene enzyme activity in T47D cells.

A dose-response curve of progesterone receptor activation was constructed to calculate the ED_{50} values of the five conjugates in comparison to progesterone (figure 4.13). The ED_{50} value of progesterone was the lowest at

7 ± 2nM (n = 3). The five antagonists had higher ED_{50} values, identified in table 4.5.

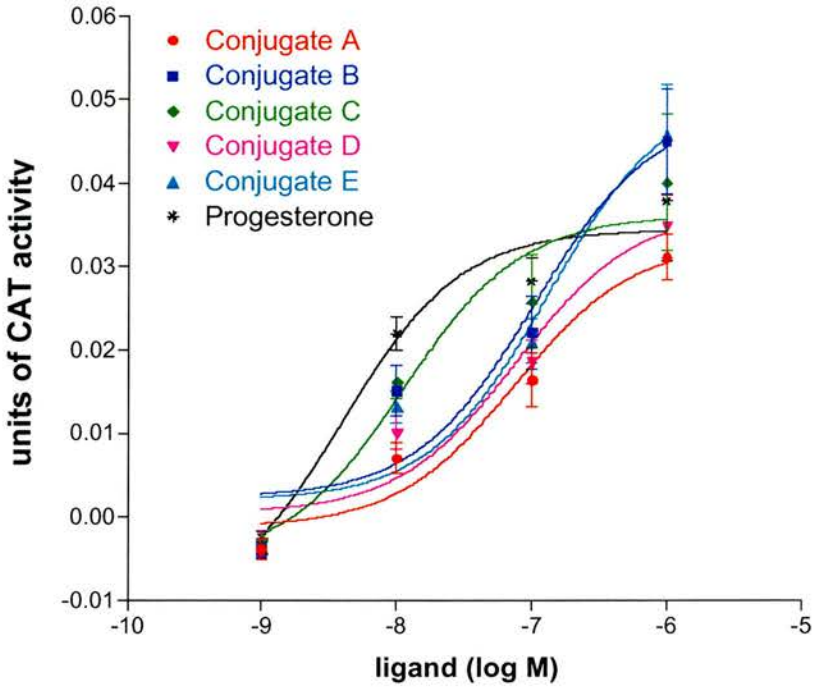


Figure 4.13: Activation of the progesterone receptor by conjugates A, B, C, D and E as measured by assay of CAT reporter gene enzyme activity in T47D cells.

Conjugate	ED_{50} of activation of progesterone receptor
A	109 ± 28nM
B	142 ± 35nM
C	66 ± 38nM
D	35 ± 17nM
E	96 ± 2nM

Table 4.5: ED_{50} of activation of the progesterone receptor by conjugates A, B, C, D and E.

The ED_{50} values of whole cell binding affinity, inhibition of mammalian GnRH-stimulated inositol phosphate production, competition with [^{3}H]progesterone for binding to plasma proteins and activation of the progesterone receptor for all five conjugates are summarized in table 4.6.

Conjugate	Whole cell binding	Inositol phosphate production	Plasma protein binding	Progesterone receptor activation
A	108 ± 22nM	97 ± 40nM	1020 ± 284nM	109 ± 28nM
B	105 ± 27nM	76 ± 17nM	534 ± 103nM	142 ± 35nM
C	134 ± 26nM	5580 ± 127nM	1020 ± 230nM	66 ± 38nM
D	8390 ± 936nM	No inhibition	264 ± 25nM	35 ± 17nM
E	104 ± 7nM	16,000 ± 5190nM	779 ± 11nM	96 ± 37nM

Table 4.6: Summary of the *in vitro* data obtained for the five GnRH antagonist-steroid conjugates.

4.5 DISCUSSION

Four out of the five GnRH antagonist-21-hydroxyprogesterone 21-hemisuccinate conjugates investigated bound to the GnRH receptor and inhibited IP production. In addition, all five conjugates bound to specific plasma proteins and activated the progesterone receptor.

All conjugates bound to the GnRH receptor

All five GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates bound to the GnRH receptor in whole cell binding assay. Conjugation of a large hydrophobic moiety to the side chain of a central lysine amino acid (DLys^6) in conjugates A and B and L-Lys^7 in conjugates C and E) did not significantly affect binding to the GnRH receptor. Conjugate D also bound the GnRH receptor, albeit with reduced affinity, when the steroid was conjugated to the N-terminal of the peptide (figure 4.14).

GnRH is believed to interact with the GnRH receptor in a folded conformation, with the side group of amino acid six orientated away from

the receptor [22, 23] (figure 4.14). Conjugation of other molecules onto this position is possible since the molecule would be also be orientated away from the receptor and thus unlikely to interfere with the receptor binding process. Position six has also been used as a conjugation site for coupling GnRH analogues to other molecules of different sizes by other groups [9, 12, 158, 175, 195] for the same reasons.

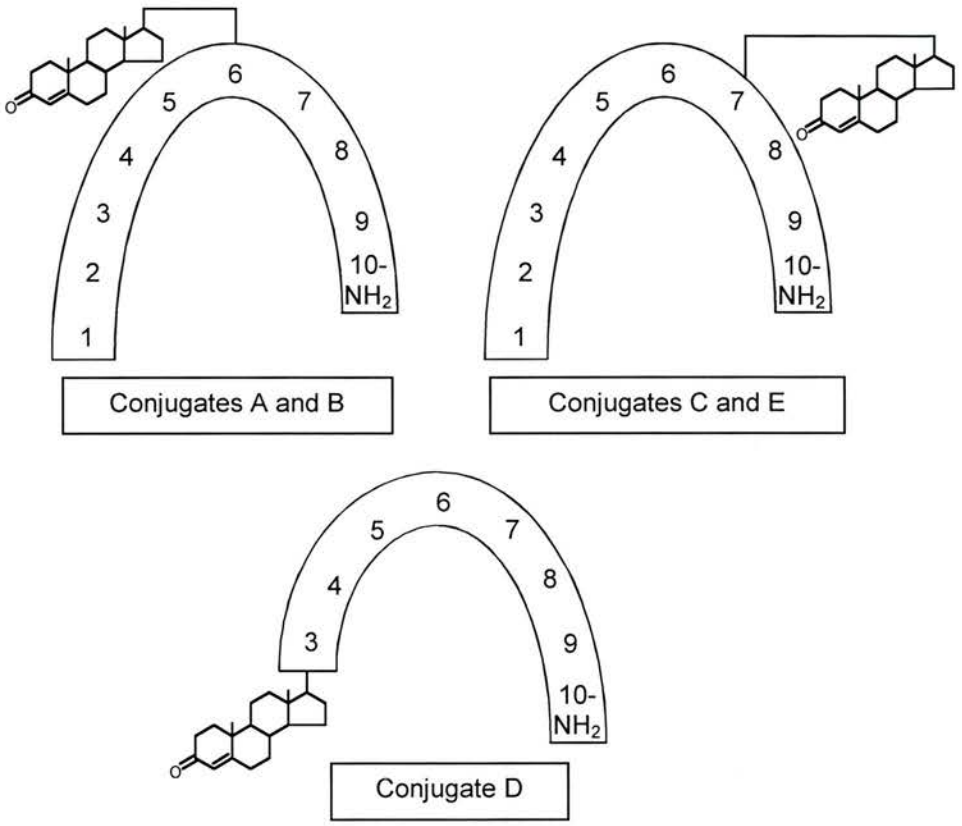


Figure 4.14: Diagramatic representation of the structure of conjugates A, B, C, D and E showing the position of steroid conjugation.

Conjugate D bound poorly to the GnRH receptor

Peptide antagonists of the GnRH receptor have been designed by replacing the N-terminal amino acids with bulky, hydrophobic amino acids [21]. The hydrophobic steroid hemisuccinate has a molecular weight of 413.5 (when coupled to the DLys^6), approximately the same size as the first two amino acids (total M.W. 416.5). To create conjugate D, the first two amino acids of

peptide A were replaced with 21-hydroxyprogesterone 21-hemisuccinate conjugated to the N-terminal amine of pyridylalanine (equivalent to amino acid three of the decapeptide). Although conjugate D was able to compete with [His^{5}DTyr^6]GnRH for binding to the GnRH receptor, significantly lower binding affinity was observed in comparison to the other four conjugates and therefore the usefulness of conjugate D is severely limited.

Entirely novel sequence of conjugate C lacking 'essential' position nine proline retained GnRH receptor binding

Although conjugate C was synthesized in error, comparison with conjugate E leads to an important finding. The conserved C-terminal proline in position nine of the decapeptide was believed to be essential for GnRH receptor binding. In conjugate C the C-terminal proline was replaced with arginine. Proline is cyclic and neutral whereas arginine is a linear basic amino acid (figure 4.15). The contrasting structures make a substitution of this kind seem incompatible with GnRH receptor binding affinity. However this study has proven that replacing the proline with an arginine had no effect on the receptor binding of this antagonist sequence. It may be that arginine can only be substituted for proline when a leucine is also present in the adjacent position eight of the decapeptide an additional change to the sequence under analysis, but this was not tested.

Conjugates A, B, C and E inhibited IP production, but conjugates C and E may be partial agonists

With the exception of conjugate D, the GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates were able to inhibit mammalian GnRH-stimulated IP production.

The IC_{50} values of conjugates A and B were close to the GnRH receptor binding affinity, with an approximate ratio of 1:1 for whole cell binding affinity compared to inhibition of IP production. However the IC_{50} values for conjugates C and E were dramatically reduced, resulting in ratios of 1:41 and 1:154 respectively. Therefore it appears that conjugates C and E are more able to compete with [His^{5}DTyr^6]GnRH for binding to the GnRH receptor than

with mammalian GnRH for inhibiting inositol phosphate production. However it is more probable that conjugates C and E act as partial agonists at low concentrations (not measured), but this effect is overcome at higher concentrations were the antagonistic effect is dominant.

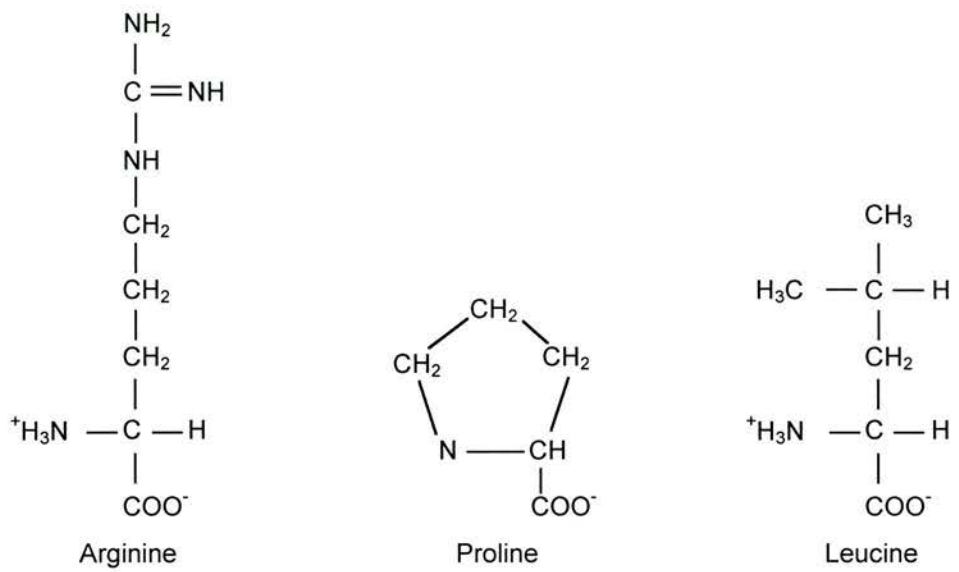


Figure 4.15: Structures of arginine, proline and leucine.

In addition, the 3.75-fold reduction in inhibition of IP production in conjugate E compared to conjugate C implies that a C-terminal sequence of Arg-Pro-DAla-NH_2 (conjugate E) is a significantly poorer antagonist of mammalian GnRH-stimulated IP production than Leu-Arg-DAla-NH_2 (conjugate C). This is in contrast to similar competition with ^{125}I[His^{5}DTyr^6]GnRH for binding to the GnRH receptor.

Conjugate D did not inhibit inositol phosphate production

Conjugate D showed no evidence of GnRH antagonism, despite a limited ability to compete with ^{125}I[His^{5}DTyr^6]GnRH for binding to the GnRH receptor. Conjugation of 21-hydroxyprogesterone 21-hemisuccinate to the N-terminal in place of amino acids one and two of the decapeptide significantly hampered GnRH receptor interaction, most probably by altering the conformation of the peptide.

All conjugates compete with [^{3}H]progesterone for plasma proteins with lower affinity than progesterone

All five conjugates investigated bound to large molecular weight plasma proteins present in pregnant guinea pig plasma. Progesterone competed with [^{3}H]progesterone for plasma protein binding with an IC_{50} value significantly lower than the five conjugates.

Steroid plasma protein binding affinity is controlled by a number of overlapping factors; hydrophobicity/hydrophilicity, spatial requirements, optimal contact and steric hindrance [139]. These factors will also affect the binding of GnRH-antagonist progesterone conjugates to plasma proteins.

The important features for steroid binding to human CBG are the 20-oxo, 10β methyl, 3-oxo and 4-ene groups [97]. Therefore a steroid suitable for conjugation had to maintain these features. In addition, hydroxyl groups at the 11α, 6α, 6β, 12α, 14α, 16α and 19 positions are not tolerated [97] and result in dramatic decreases in affinity constants. A hydroxyl group is required for reaction with succinic anhydride to form the hemisuccinate derivative of progesterone. A 21-hydroxy group on progesterone does not reduce the affinity constant for human CBG in comparison to progesterone [97] and provides a site for further conjugation unlikely to alter the steroid conformation.

Plasma protein binding affinity was assessed in pregnant guinea pig plasma in which progesterone is predominantly bound to PBG instead of CBG. The requirements for binding to PBG differ from CBG. The C3 keto group is still essential, but the 20-oxo group is less important [139]. The binding groove of PBG suggested by Blanford *et al* [139] indicates that the A ring is tightly bound via a hydrogen bond at the C3-keto and that the C13-C17-C16 edge is subject to tight hydrophobic bonding. Consideration of these factors lead to the selection of 21-hydroxyprogesterone 21-hemisuccinate as the steroid for conjugation, maintaining the features required for binding to both CBG (for *in vivo* studies) and PBG (for *in vitro* studies).

There is an eight-fold reduction in the affinity constant of 21-hydroxyprogesterone 21-hemisuccinate in comparison to progesterone for binding to PBG [139]. This is partly due to the strong polarity of the carboxylate anion of the hemisuccinate group [139]. In this study the ED_{50} values for the conjugates binding to PBG was reduced by between 3 and 10-fold in comparison to progesterone. This demonstrates that conjugation of 21-hydroxyprogesterone 21-hemisuccinate to the peptide did not have a major detrimental effect on plasma protein binding affinity compared to 21 hydroxyprogesterone 21-hemisuccinate alone. In some cases (conjugates B and D) it appeared binding was enhanced in comparison to the eight-fold reduction observed with 21 hydroxyprogesterone 21-hemisuccinate. Therefore the conjugation process must not have significantly altered the conformation of the steroid and did not impair plasma protein interaction.

This study demonstrates that a large molecule conjugated at C21 of progesterone reduces plasma protein binding affinity, but this process does not completely eliminate binding. This is an important discovery for the design of other progesterone bioconjugates and progesterone molecules with large substitutions.

Conjugate D had the lowest IC_{50} value for competition with [^{3}H]progesterone for plasma protein binding. The position of conjugation in conjugate D was considerably different to the other four conjugates. In conjugates A, B, C and E, 21-hydroxyprogesterone 21-hemisuccinate was conjugated to the side chain of a lysine in position six or seven of the decapeptide, whereas in conjugate D the amino acid was conjugated to the N-terminal amine. The four-carbon side group of lysine increased the distance between the GnRH antagonist and the steroid, but this was not sufficient to overcome the detrimental effect of the peptide on plasma protein binding. In conjugate D, conjugation of the steroid to the N-terminal situated the steroid close to the peptide, but this exerted less steric hindrance than conjugation to the apex of the peptide (figure 4.14).

All conjugates activate the progesterone receptor, but with higher ED_{50} values than progesterone

All five conjugates activated the progesterone receptor. Conjugation of a GnRH antagonist to 21-hydroxyprogesterone 21-hemisuccinate introduced a large, relatively hydrophilic (in comparison to the steroid) moiety at C21. The ligand-binding domain of the progesterone receptor has been crystallized [196], allowing the analysis of some aspects of ligand-receptor interaction.

The most important feature for binding to the progesterone receptor is the C3 keto group. A hydrogen bond is formed between the conserved receptor glutamine 725 (figure 4.16) and the C3 keto group in all steroid hormones except oestradiol, which has a C3–hydroxyl [196]. Arginine 766 and phenylalanine 778 also make van der Waals contacts with the A ring (through intervening fixed water sites) to tightly couple the ligand to the receptor (figure 4.16). The interactions at the methyl-ketone substituent (projecting from C17) are far less well understood [196, 197]. It appears from this study that vital contacts are not made around C21 since coupling at this site did not eliminate progesterone receptor binding and activation.

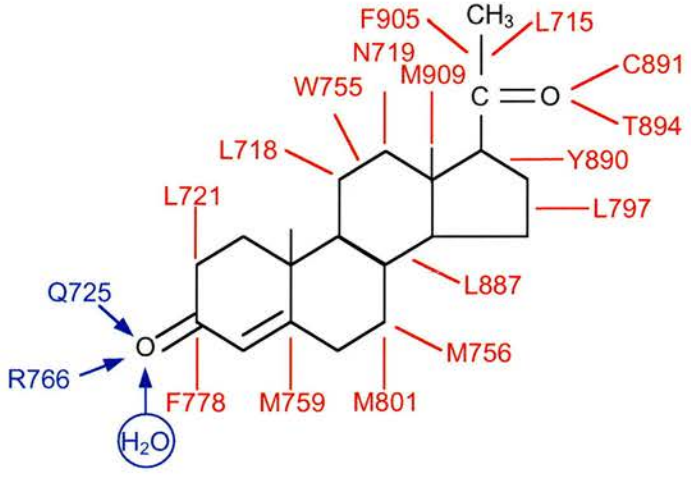


Figure 4.16: Interactions between the progesterone receptor ligand binding domain and bound progesterone. Hydrogen bonds are indicated in blue and van der Waals contacts (4 Å cutoff) in red (taken from [196]).

It is interesting to note that the IC_{50} and ED_{50} values of conjugate D were lowest for both plasma protein binding and progesterone receptor activation,

implying that the structure of this conjugate was superior to the other conjugates tested for these two interactions. However the GnRH receptor binding affinity of conjugate D was considerably reduced compared to the other four conjugates. The optimum conjugate structure may therefore be a GnRH antagonist conjugated to 21-hydroxyprogesterone 21-hemisuccinate at the N-terminal, but not the particular structure investigated in this study.

The progesterone receptor must tolerate large side groups at the D ring of 21-hydroxyprogesterone via C17. A hemisuccinate linker was used to connect the steroid to the GnRH receptor antagonist, increasing the distance between the two functional parts of the molecule. It is not possible to determine whether this was essential to retaining GnRH and progesterone receptor functionality, but these particular conjugates remained functional in *in vitro* assays.

This study has demonstrated that GnRH antagonist-steroid conjugates can be designed to retain the functionality of both parts. Some aspects of GnRH receptor-ligand interaction have been elucidated in this process. Specific plasma protein and progesterone receptor binding of these conjugates have also been confirmed. These conjugates are useful in proof of concept studies, but it is possible that other GnRH antagonist sequences based on the knowledge gained from this study would have improved properties and may be potentially more valuable.

5 Properties of a GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugate in the marmoset and macaque

5.1 INTRODUCTION

The GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates remained active at the GnRH and progesterone receptors *in vitro* (chapter 4). The activity of one of the conjugates was also tested *in vivo*. Female common marmosets were used in the initial bioactivity studies. Subsequent experiments were carried out on male marmosets and an ovariectomised adult macaque to identify differences in activity between the conjugated and non-conjugated antagonists. The duration of action of conjugate A was compared in a species without functional CBG (common marmoset) with an Old World primate (macaque) with a sex-steroid physiology more similar to humans.

One of the GnRH antagonist-21-hydroxyprogesterone 21-hemisuccinate conjugates was selected for *in vivo* analysis. At the time of selection only conjugates A and B were available for analysis. Both conjugates had similar affinities for the GnRH receptor and for plasma protein binding. Conjugate A was selected on the basis that it was lower cost to produce commercially. This conjugate was [AcD Nal^{1}, ClD Phe^{2}, D Pal^{3}, Arg^{5}, D Lys^{6}(21-hydroxyprogesterone 21-hemisuccinate), DAla^{10}-NH_2]GnRH.

5.2 BIOACTIVITY STUDIES IN THE FEMALE COMMON MARMOSET

To identify whether conjugate A was active in an *in vivo* model, 1.0, 0.5 or 0.25mg of the GnRH antagonist-steroid conjugate was administered to adult female marmosets as a subcutaneous bolus at two sites, on day 8 or 9 of the luteal phase. A 1ml saline:ethanol vehicle was used, with the proportion of ethanol ranging from 16% in the 1.0mg injection, 8% in the 0.5mg injection, to 4% in the 0.25mg injection. One 300µl blood sample was withdrawn on the day prior to GnRH antagonist injection. Further blood samples of equal size were withdrawn at 0, 4 and 8h on day of injection, daily for the following 3 days and three times per week until the next ovulation was identified by a progesterone concentration greater than 30nM. Ovulatory cycles were monitored in female marmosets by progesterone assay as described previously (section 2.5.1.2).

Conjugate A was tested in the progesterone RIA to test for cross-reactivity with the primary antibody (data not shown). The conjugate was not read as progesterone in this assay, therefore the conjugate did not interfere with the measurement of plasma progesterone in the marmoset.

5.2.1 1mg conjugate A

Administration of 1mg conjugate A to a female marmoset caused an immediate decrease in plasma progesterone concentrations and reduced the duration of the luteal phase from 24.8 ± 2.2 (n = 6) to 9 days. Progesterone concentrations did not increase until 10 days post-injection, implying complete luteal regression, followed by a 7-day follicular phase occurred. The next ovulation proceeded after a short delay.

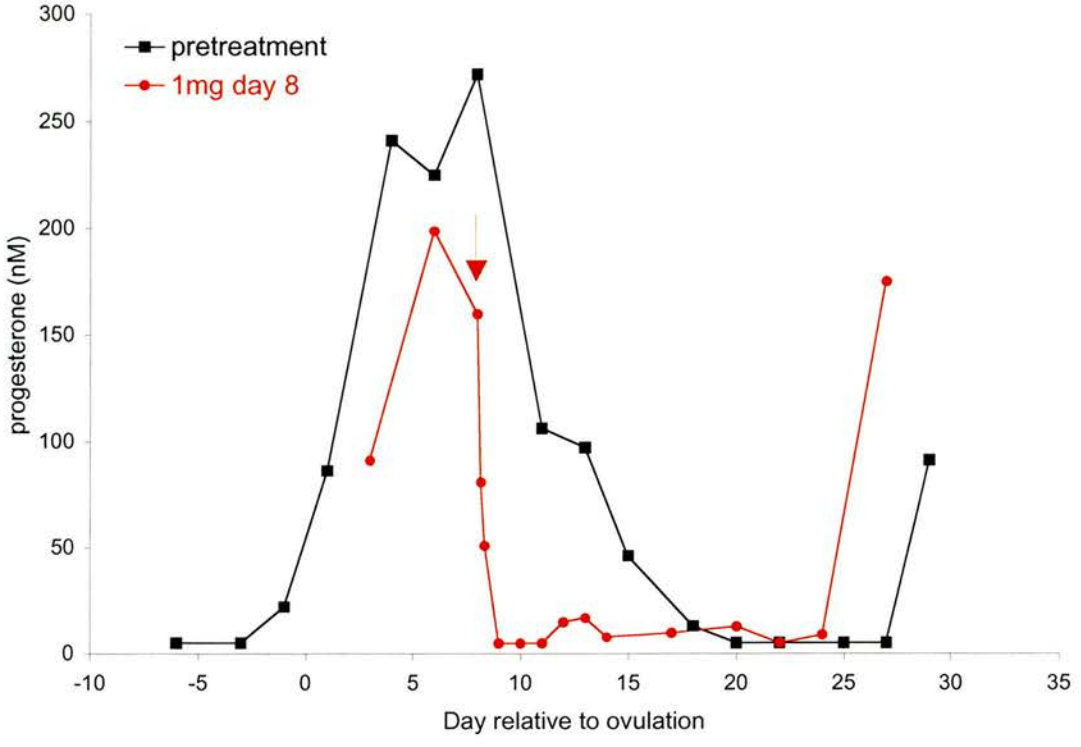


Figure 5.1: Progesterone concentrations in a female marmoset receiving 1.0mg conjugate A on day 8 of the luteal phase, normalized to the day of ovulation (day 0). The progesterone concentrations during the cycle prior to treatment are represented in black. The progesterone concentrations during the treatment cycle are shown in red and the arrow indicates the time of injection.

5.2.2 0.5mg conjugate A

0.5mg conjugate A induced a rapid decrease in plasma progesterone concentration to follicular phase levels, shortening the luteal phase from 21.0 ± 1.2 (n = 7) to 11 days. This was maintained for 10 days before progesterone concentrations indicated the next ovulation. This effect was not distinguishable from the 1mg dose of antagonist.

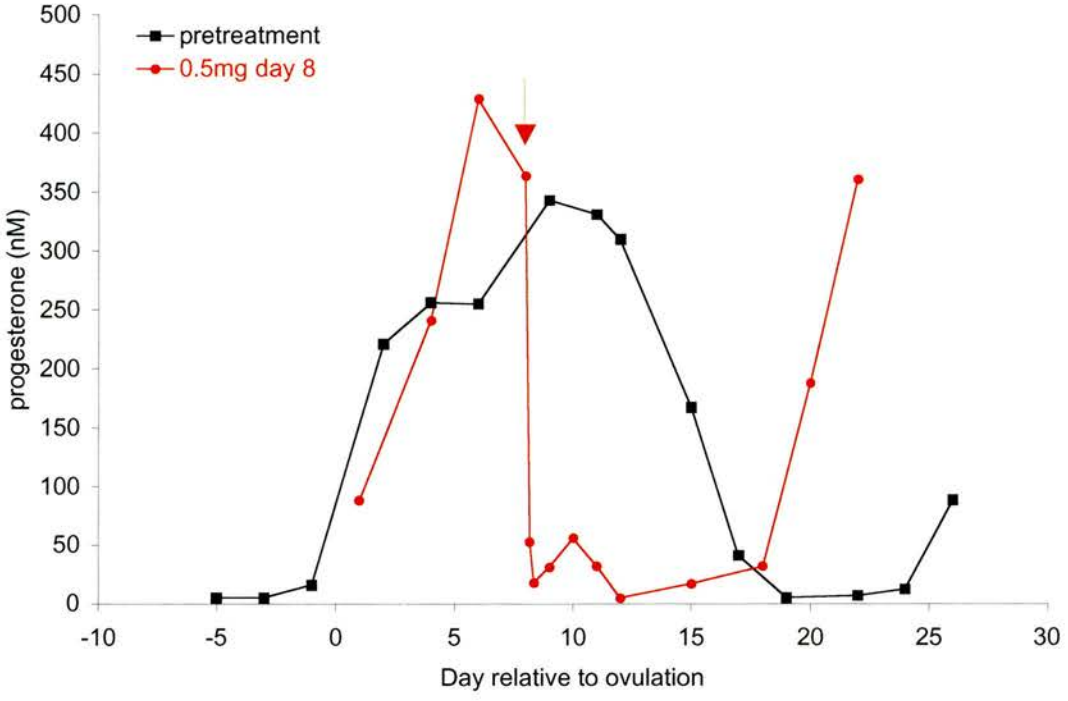


Figure 5.2: Progesterone concentrations in a female marmoset receiving 0.5mg conjugate A on day 8 of the luteal phase, normalized to the day of ovulation (day 0). The progesterone concentrations during the cycle prior to treatment are represented in black. The progesterone concentrations during the treatment cycle are shown in red and the arrow indicates the time of injection.

5.2.3 0.25mg conjugate A

The lowest dose of conjugate A also caused a rapid reduction in plasma progesterone levels, however unlike the 1mg and 0.5mg doses, the effect was transient. Within 24h of injection the progesterone concentration returned to 132nM and this was followed by the normal slow reduction in progesterone concentration as the corpus luteum regressed.

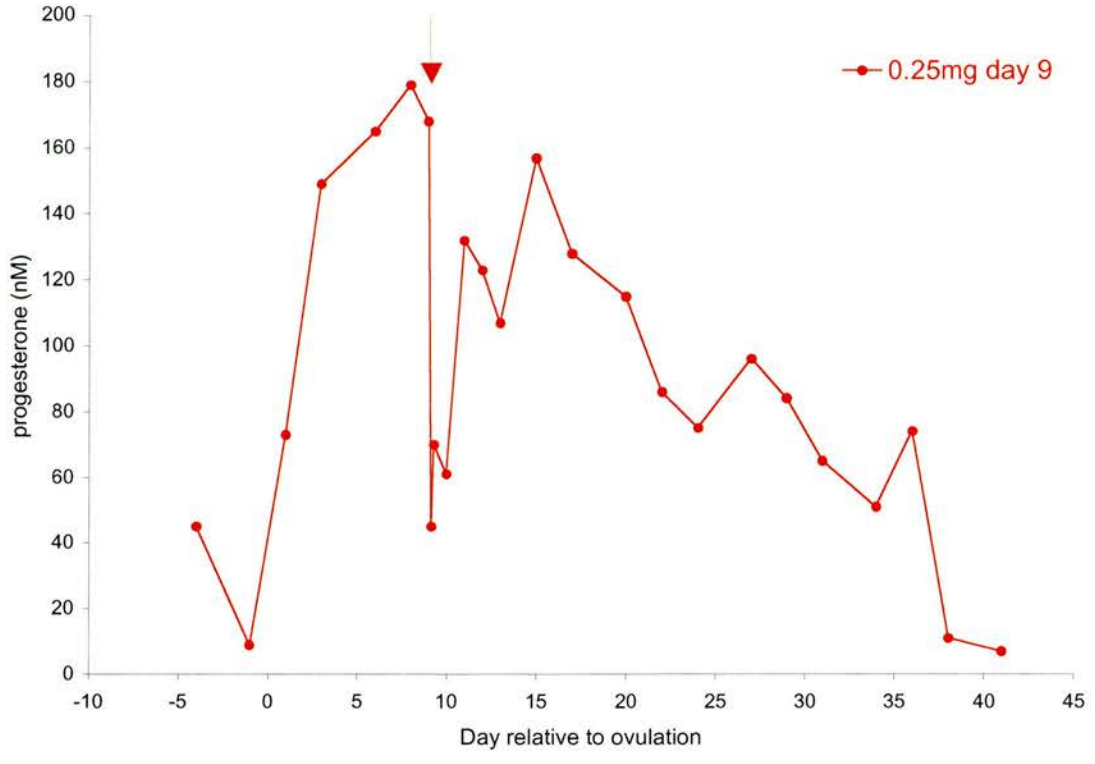


Figure 5.3: Progesterone concentrations in a female marmoset receiving 0.25mg conjugate A on day 9 of the luteal phase, normalized to the day of ovulation (day 0). The arrow indicates the time of injection.

A normal cycle has not been superimposed onto the treatment cycle in this case because this animal was not cycling in a regular pattern. However prior to treatment luteal regression (indicated by progesterone concentrations lower than 30nM) followed by ovulation was identified.

5.3 BIOACTIVITY STUDIES IN THE MALE COMMON MARMOSET

The duration of action of conjugate A was compared to parent peptide A in the common marmoset (without functional CBG). 0.5mg conjugate A was

administered as subcutaneous bolus at two sites in six adult male marmosets. The vehicle was 8% ethanol in saline in a total volume of 1ml. Testosterone concentrations were monitored as described in general methods (section 2.5.1.3). One 300µl blood sample was withdrawn on the day prior to GnRH antagonist injection, at 0h, 4h and 8h on the day of injection and on the following 3 days. Three further samples were taken during the subsequent week. The same protocol was used to analyze the effect of 0.5mg peptide A in three male marmosets (chosen from the previous group of six) after a one month rest period.

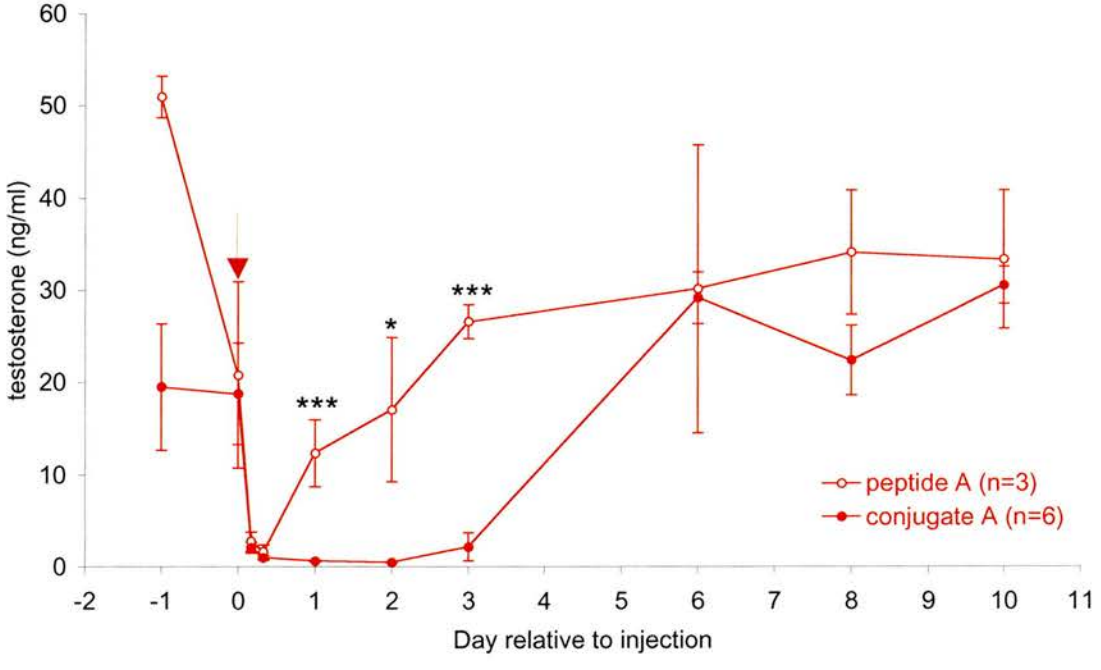


Figure 5.4: Testosterone concentrations of male marmosets injected subcutaneously with either 0.5mg peptide A (○) or 0.5mg conjugate A (●). The arrow indicates the time of injection. The testosterone concentrations at each time point after injection of peptide A and conjugate A were compared with Students t-test. A single asterisk represents a significant difference of $p < 0.05$ and a triple asterisk represents a significant difference of $p < 0.001$ (both STT).

0.5mg conjugate A ($n = 6$) rapidly decreased testosterone concentrations in the male marmoset. This was maintained until at least 72h post-injection ($p < 0.05$ versus 24h post-injection, STT) and recovered to normal levels by day 6.

The constraints of existing Home Office licensing prevented additional blood samples on days 4 and 5. Injection of 0.5mg peptide A (n = 3) also resulted in a rapid decline in plasma testosterone concentrations (figure 4.5). However the reduction in testosterone concentration with peptide A was only maintained until 8h post-injection and increased by 24h.

5.4 DURATION OF ACTION STUDIES IN THE FEMALE MACAQUE

The duration of action of conjugate A and parent peptide A were compared in an ovariectomized adult macaque to analyze the difference in duration of action in a species with functional specific binding proteins. Oophorectomy was carried out 3 weeks before commencing treatment. Conjugate A and peptide A were compared over a one-month period with a one-month rest between treatment cycles. The LH concentration was monitored for the week prior to injection and for four weeks following injection.

Conjugate A was given at a dose of 417µg/kg and the unmodified antagonist peptide A at 322µg/kg (weight of animal 17kg), equivalent to 230nmoles peptide/kg for both conjugate A and peptide A. Both conjugate A and peptide A were dissolved in a 1ml 50:50 propylene glycol:water vehicle and were administered subcutaneously at a single site.

The overall trend observed after injection of conjugate A was a rapid decline in LH immediately following the injection (within 4h), followed by a slow return to normal LH concentrations. The duration of gonadotropin inhibition appeared to be between five and ten days, although the day three LH concentration was greater than the same day measurement after peptide A. Peptide A also rapidly reduced the LH concentration within four hours of injection, but the LH concentration was highly variable in the week following the injection. In particular, the LH concentration on day six after injection of

peptide A seemed to be lower than baseline LH levels. The pulsatile nature of LH secretion may be a cause of the observed variation in plasma LH concentration.

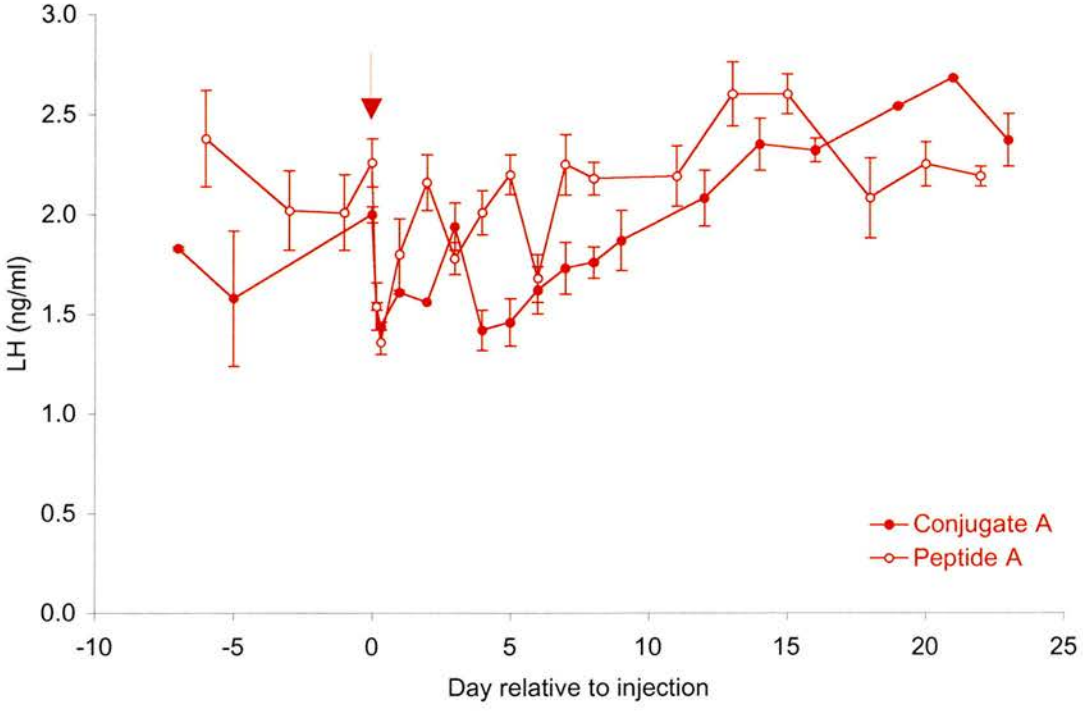


Figure 5.5: LH concentrations in an ovariectomised adult macaque receiving 417µg/kg conjugate A (●), followed by 322µg/kg peptide A (○) separated by a one-month rest period. The arrow indicates the time of injection.

The LH concentrations over short periods within the treatment cycles were pooled and compared (figure 5.6). The LH concentrations in the week preceding each injection were not significantly different ($p > 0.05$, STT), as were the LH concentrations on day 0 (0, 4 and 8h on day of injection) for both injections. On days 1 to 3 there was no difference between the LH concentrations after conjugate A or peptide A. On days 4 to 6 the LH concentration after conjugate A was significantly lower than for the same days after peptide A ($p < 0.01$, STT). The difference between days 7 to 9 (conjugate A) compared with days 7 to 11 (peptide A) was also significant ($p < 0.001$, STT).

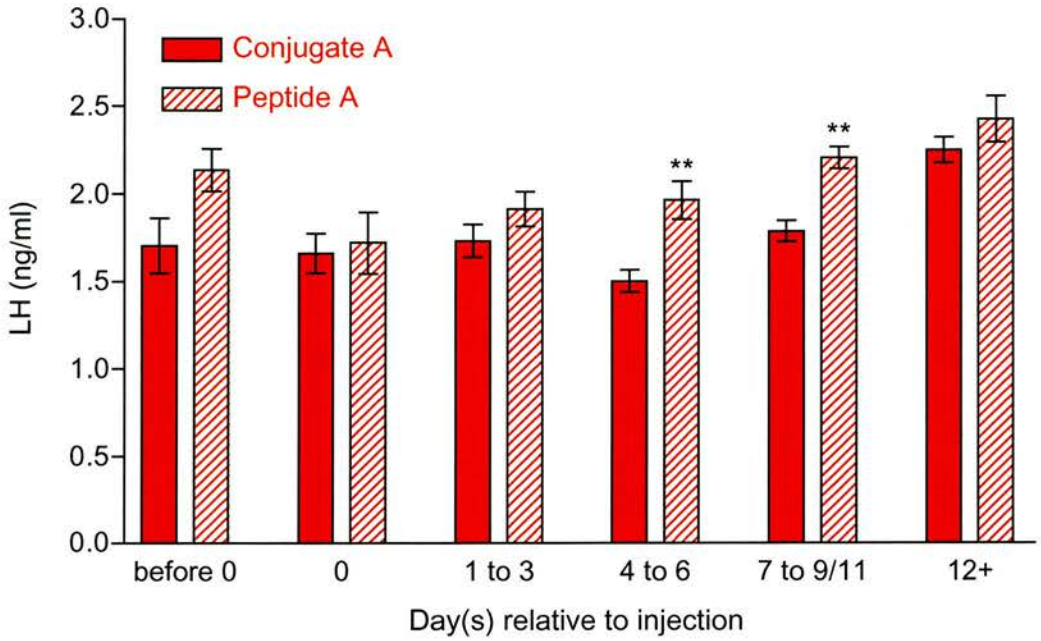


Figure 5.6: Pooled LH concentrations in an ovariectomised adult macaque receiving 417µg/kg conjugate A (solid bars), followed by 322µg/kg peptide A (hatched bars) in separate treatment cycles. The LH concentrations are pooled to compare the duration of LH inhibition after each injection. The double asterisk represents a significant difference of $p < 0.01$ (STT) and the triple asterisk marks a significant difference of $p < 0.001$ (STT).

The LH concentrations were considerably lower (approximately 2ng/ml) than would be expected in an ovariectomised animal (normally around 6ng/ml). It is therefore possible that the oophorectomy was incomplete and some ovarian tissue remained. Observation of the animal by Primate Centre staff also suggested incomplete oophorectomy.

It is difficult to draw conclusions from a single animal. Three to six ovariectomised animals should be tested to obtain a more accurate determination of any difference in the duration of action between conjugate A and peptide A.

5.5 DISCUSSION

Conjugate A terminated the luteal phase in the female common marmoset
Conjugate A retained biological activity *in vivo* in the female marmoset, reducing progesterone concentrations after a single injection. The effects were dose-dependent, with complete regression of the corpus luteum with 1mg and 0.5mg conjugate A and a transient inhibition of progesterone secretion with the lowest dose (0.25mg). The corpus luteum of the marmoset is capable of recovering from a transient withdrawal of gonadotropin support [68, 73]. This effect was observed with the lowest dose of conjugate A. Interruption of LH and FSH support for longer periods (around 3 days) in the midluteal phase can result in premature luteolysis, termination of the luteal phase and resumption of folliculogenesis after elimination of the GnRH antagonist [54]. This occurred with the 1.0 and 0.5mg doses of conjugate A, suggesting that gonadotropins were suppressed for at least three days.

These findings demonstrate that a GnRH antagonist conjugated to 21-hydroxyprogesterone 21-hemisuccinate was functional *in vivo*. Conjugation of the steroid to the peptide via DLys^6 was tolerated at the GnRH receptor in an *in vivo* model, as had been shown by binding and inhibition of IP at the GnRH receptor. Thus the four-carbon side chain of the DLys^6 and the four-carbon hemisuccinate linker was sufficient to minimize any steric hindrance the steroid may have otherwise exerted on the peptide. Previous conjugates of [DLys^6]GnRH have also used this position for conjugation [11, 195, 198], but it is important to identify that conjugation at this position is also tolerated in GnRH antagonists.

Investigation of Antide-vitamin B_{12} conjugates revealed that a thiol-cleavable spacer was necessary between the GnRH antagonist and the vitamin B_{12} derivative to retain *in vivo* activity [158]. In contrast the GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugate examined here did not

require a cleavable spacer to retain *in vivo* activity. It is likely that the GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugate was not cleaved or metabolized prior to GnRH receptor binding because the conjugate was shown to bind to mammalian GnRH receptors *in vitro*. However metabolic studies would be required to confirm this.

Conjugate A decreased testosterone concentrations in the male common marmoset for significantly longer than peptide A

There was a substantial increase in the duration of action of conjugate A (over three days) in the male marmoset in comparison to the unmodified peptide (less than 24h). This increase is significantly greater than that recently shown by Rahimipour and colleagues [12]. The duration of action of intraperitoneal [DLys^6]GnRH was enhanced by conjugation to emodic acid, resulting in significantly greater LH release after six hours in the rat. However no further measurements were taken after this time point, so duration of action could not be accurately determined.

Glucocorticoid physiology in the marmoset and other New World primates is significantly different from Old World primates and humans. In humans the major proportion of progesterone is found bound to albumin, with CBG accounting for around 10% and the remaining 2% unbound [100]. In most New World primates 40-50% of cortisol is bound to albumin, less than 5% binds to CBG and the remainder is unbound [114]. The marmoset lacks functional CBG [114, 199] and therefore the observed increase in duration of action was likely to be due to non-specific interactions with albumin.

Conjugation of progesterone to the GnRH antagonists significantly increased the hydrophobicity of the GnRH antagonists. This was evident from the poor water solubility of conjugates A and B in comparison to unmodified peptides A and B. Moreover the HPLC retention time of [DLys^6]GnRH increased from 9.3 minutes to over 15 minutes when conjugated to 21-hydroxyprogesterone 21-hemisuccinate. Increased hydrophobicity could have contributed to the increase in duration of action seen in the male marmoset. It is believed that a depot effect can occur with hydrophobic GnRH analogues, where binding of

such analogues to tissue membranes and hydrophobic carrier proteins throughout the body reduces the rate of clearance from the circulation, thereby prolonging biological half-life [39].

Effect of conjugate A in ovariectomised macaque

The limited data on the effect of conjugate A in comparison to peptide A in an ovariectomised adult macaque suggests that the conjugate may have a longer duration of action than the unmodified peptide. However the LH concentration on day three after injection of conjugate A must first be confirmed as an anomaly by further studies. The difference in duration of action between conjugate A and peptide A could be as long as 9 days. This is probably due to interactions with both specific (CBG) and non-specific (albumin) plasma proteins, protecting the conjugate from rapid clearance from the general circulation. It is expected that a similar increase would be observed in the human since Old World primate CBG physiology is more comparable to humans than New World primates [114].

Progesterone can also inhibit LH production through a negative feedback mechanism at the pituitary, mimicking the effect of a GnRH antagonist. Therefore it is possible that the inhibition of progesterone (female marmoset), testosterone (male marmoset) or LH (female macaque) is due to the effect of progesterone and not the GnRH antagonist to which it was conjugated. In order to assess the effects of progesterone and the GnRH antagonist the equivalent doses of the two molecules in unconjugated states should be investigated in the *in vivo* models. Ideally there would be four treatment cycles: GnRH antagonist; GnRH antagonist-progesterone conjugate; progesterone and vehicle.

The increase in duration of action of conjugate C, probably resulting from association with plasma proteins, has supported the concept that the conjugation of a peptide with a short circulatory half-life to a molecule that binds to plasma proteins can protect the peptide from rapid elimination. This discovery could be of great importance to both the design of other GnRH analogues where a prolonged inhibition of gonadotropin secretion is

required. More importantly, this concept could be applied to other peptides with short half-lives to extend their duration of action, reducing both cost and administration frequency. This would have notable advantages over unmodified peptides.

6 Enhancing oral absorption of GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates through conjugation to vitamin B_{12}

6.1 INTRODUCTION

Low peptide oral bioavailability currently limits clinical use of peptides to injectable administration. Development of a method to enhance oral absorption of peptides would be of great importance to the pharmaceutical industry. It is likely that the method used would be determined by peptide size, hydrophobicity and absolute amount to be transported.

The GnRH antagonist-steroid conjugates described in chapter 3 are relatively large molecules, consisting of a peptide linked to a steroid molecule via a hemisuccinate spacer, resulting in molecular weights of around 1800 AMU, depending on the amino acid sequence. Conjugation of a hydrophobic steroid onto a GnRH antagonist increased the hydrophobicity of the molecule, as shown by the increase in HPLC retention time of [DLys^6]GnRH from 9.3 to over 15 minutes when conjugated to 21-hydroxyprogesterone 21-hemisuccinate. Consequently the method selected would have to be suitable for increasing the oral absorption of a relatively large, hydrophobic peptide molecule.

Several possible methods of increasing oral absorption of peptides are detailed in chapter 1, including conjugation to bile acids [142, 144, 200, 201], co-administration with absorption enhancers [166, 202] and coupling to vitamin B_{12} [154, 155, 157-159]. The GnRH antagonist-progesterone conjugates are too large for the bile acid transporter, since only peptides consisting of four amino acids or less are transported coupled to bile acids [141]. There are concerns about the long-term use of absorption enhancers, as

the increase in permeability may allow absorption of toxic agents otherwise excluded by the GIT barrier [163].

Vitamin B_{12} is not absorbed directly, but is transported across the epithelial cell layer lining the duodenum coupled to a large molecular weight protein (intrinsic factor) [203]. The GnRH antagonist-progesterone conjugates are small in comparison to intrinsic factor (approximately 50kDa, [152, 153]) and thus could possibly be co-transported in this way [151, 204] utilizing the intrinsic factor-cobalamin receptor (IFCR).

6.2 DESIGN OF GnRH-STEROID-VITAMIN B_{12} CONJUGATES

Previous studies on coupling GnRH analogues to vitamin B_{12} derivatives [157, 158] have used a D-lysine in position six as the conjugation site. Therefore new analogues incorporating an additional conjugation site were required in order to conjugate the GnRH antagonists to both a steroid and a vitamin B_{12} derivative.

Conjugation of GnRH analogues via a D-Lys^6 to a variety of molecules and functional groups is well documented [9, 11, 12, 158, 195]. GnRH is proposed to interact with the GnRH receptor in a folded conformation, with the side chain of D-Lys^6 orientated away from the receptor [13, 22, 23], allowing conjugation at this site without compromising GnRH receptor binding affinity. Vitamin B_{12} (M.W. 1357) was assigned to the D-Lys in conjugates C, D and E because it is significantly larger than the steroid (M.W. 430.5).

Alternative conjugation sites for the steroid were investigated on the basis of current understanding of GnRH analogue structure-activity relationships. Modification of the amino and carboxy-terminal amino acids was likely to reduce binding affinity at the GnRH receptor [13, 21-23]. An L-lysine was

substituted in position seven (replacing leucine) and conjugated to the steroid in conjugates C and E. The sequences of conjugates C and E were as follows:

Conjugate C: [AcDNal[1], DCpa[2], DPal[3], Arg[5], DLys[6], Lys[7](21-hydroxyprogesterone 21-hemisuccinate), Leu[8], Arg[9], DAla-NH_2[10]]GnRH

Conjugate E: [AcDNal[1], DCpa[2], DPal[3], Arg[5], DLys[6], Lys[7](21-hydroxyprogesterone 21-hemisuccinate), DAla-NH_2[10]]GnRH

The second option investigated was to replace the first two amino acids at the N-terminal with the steroid since hydrophobic substitutions in positions one and two are a feature of GnRH antagonists [21]. This left the D-Lys[6] available for conjugation to the vitamin B_{12} derivative. The structure of conjugate D was therefore:

Conjugate D: 21-hydroxyprogesterone 21-hemisuccinate-DPal, Ser, Arg, DLys, Leu, Arg, Pro, DAla-NH_2

Low binding affinity at the GnRH receptor and an absence of antagonism of mammalian GnRH-stimulated IP production (figure 4.4) was observed with conjugate D and therefore this conjugate was not investigated further.

6.3 CHEMISTRY OF CONJUGATION OF GnRH-STEROID MOLECULES TO VITAMIN B_{12} DERIVATIVES

Conjugates C and E, only differing at amino acids eight and nine of the decapeptide, were similarly effective at GnRH receptor binding and antagonism. The IC_{50} values of plasma protein binding of conjugates C and E were not significantly different and activation of the progesterone receptor in T47D cells was identical. Therefore both conjugates were suitable for conjugation to vitamin B_{12}. Only a single conjugate was required to prove the concept of oral absorption though the vitamin B_{12} uptake system. Conjugate

C (AcDNal[1], DCpa[2], D Pal[3], Arg[5], D Lys[6], Lys[7](21-hydroxyprogesterone 21-hemisuccinate), Leu[8], Arg[9], DAla-NH_2[10]) was selected because the sequence was more unique and this would be beneficial should any commercial interest develop.

Australia Biotech holds patents on the use of the vitamin B_{12} uptake system to enhance the oral absorption of peptides. Australia Biotech created direct peptide-vitamin B_{12} conjugates with *in vitro* and in some cases *in vivo* activity [158, 159]. More recently they have published promising results with encapsulating peptides in vitamin B_{12}-coated nanospheres to achieve oral uptake [160]. Dr G. Russell-Jones (Australia Biotech) kindly supplied a DTP-aminohexyl vitamin B_{12} derivative for conjugation to GnRH antagonist-progesterone conjugates (figure 6.1). According to recent data, this spacer gives better results in direct conjugations than those used in earlier studies ([158], Russell-Jones, personal communication). In 1995 Russell-Jones and colleagues [158] demonstrated that a thiol-cleavable spacer between the vitamin B_{12} derivative and the GnRH antagonist was necessary to retain *in vivo* activity. The DTP-aminohexyl vitamin B_{12} derivative includes a disulphide bond, which could be conjugated to a D-lysine ε-amino group modified to incorporate a sulphydryl group, thus creating the spacer.

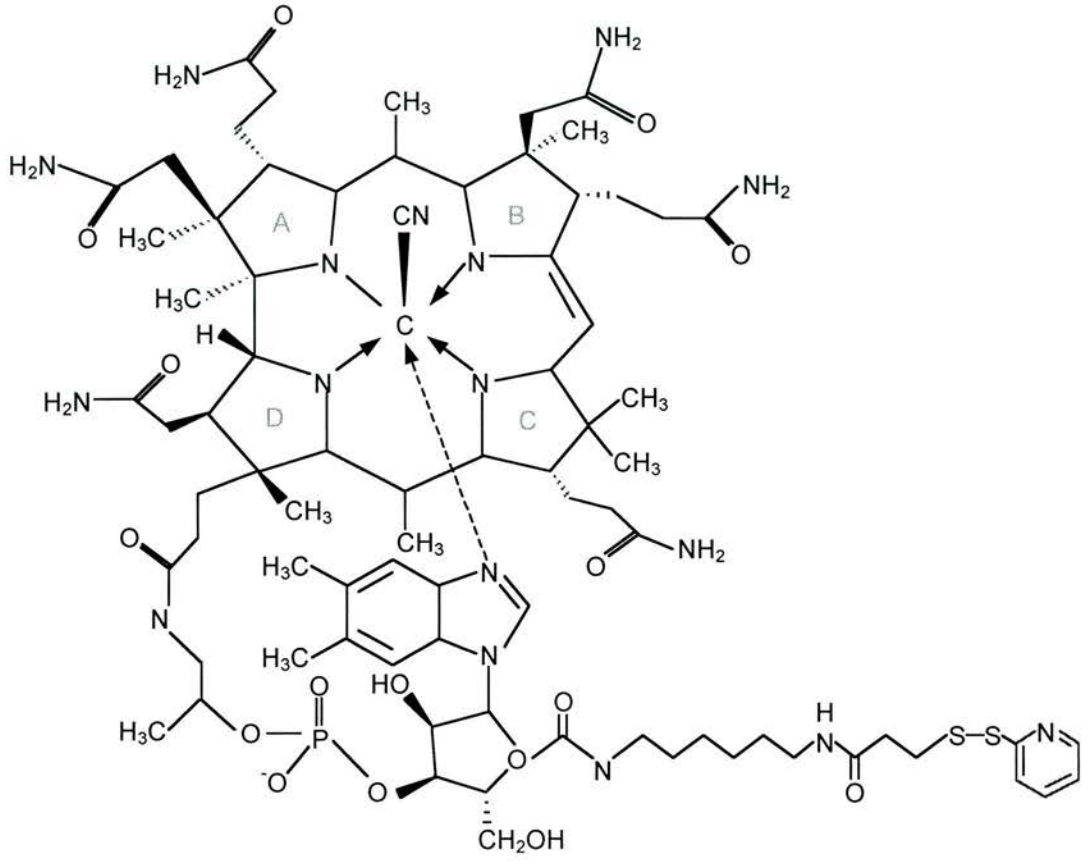


Figure 6.1: Structure of DTP aminohexyl-vitamin B_{12}. The DTP-aminohexyl group was connected via the 5′ hydroxyl of the ribose in vitamin B_{12}.

Proof of concept conjugation was attempted by reaction of [DLys^6]GnRH with a cleavable heterobifunctional cross-linking reagent (N-succinimidyl 3-(2-pyridyldithio)propionate, SPDP) and reduction with dithiotreitol (DTT). This would be expected to react with the ε-amine of a lysine as shown in figure 6.2, introducing a disulphide linked to a pyridyl group. The pyridyl group should then react with a sulphydryl-containing vitamin B_{12} derivative under mild acidic conditions, resulting in the final [DLys^6]GnRH-DTP aminohexyl-vitamin B_{12} derivative (figure 6.3).

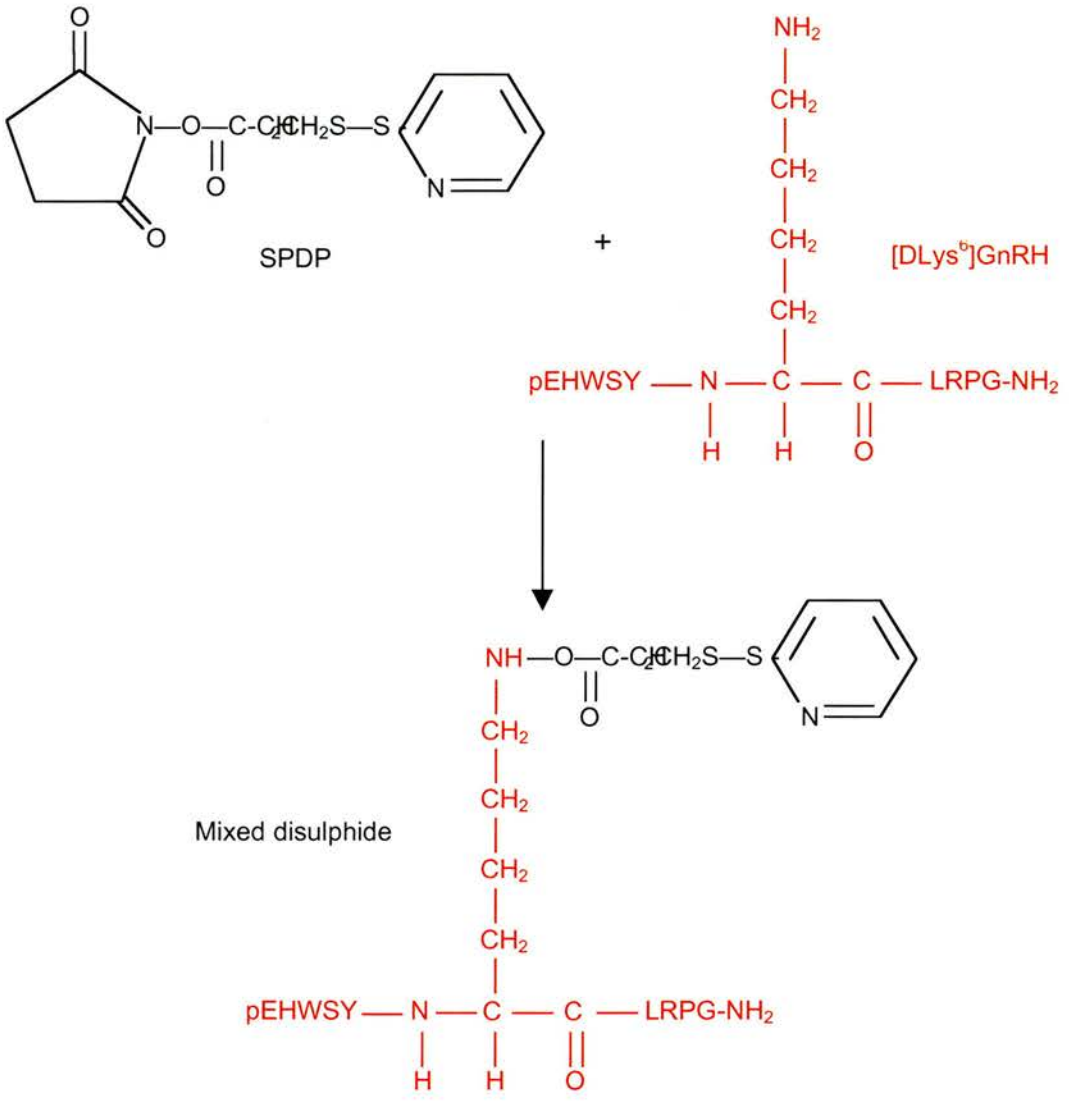


Figure 6.2: Synthesis scheme of creating a mixed anhydride intermediate by reaction of [DLys^6]GnRH with the heterobifunctional cross-linking reagent SPDP.

The initial stage of reacting SPDP with [DLys^6]GnRH was unsuccessful despite repeated attempts under different reaction conditions and mass spectrometry analysis. This was possibly due to the instability of the three-carbon spacer under these conditions.

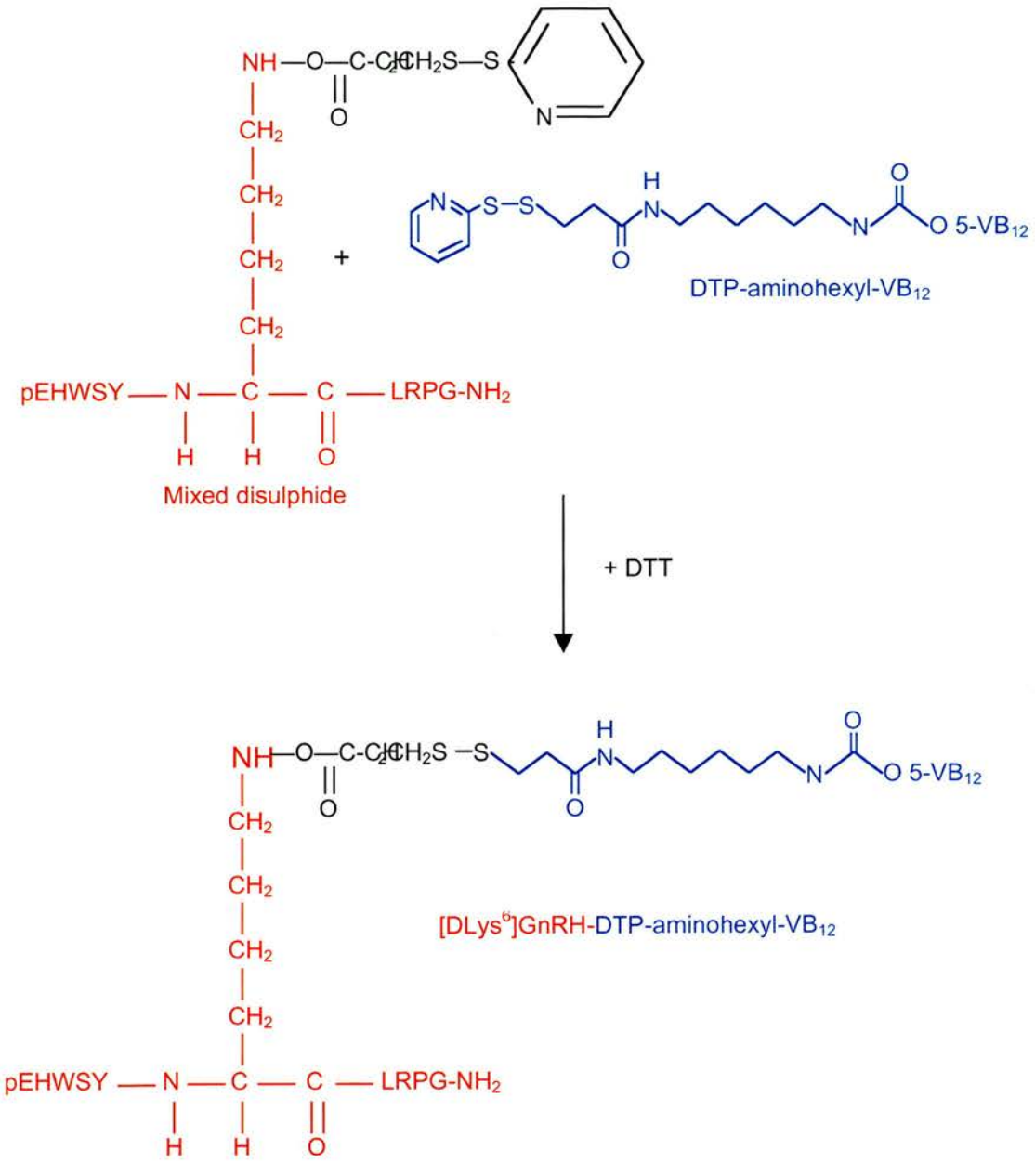


Figure 6.3: Synthesis scheme of [DLys^6]GnRH linked to DTP aminohexyl-vitamin B_{12} after reduction of the mixed anhydride with DTT.

A commercial company (Albachem Ltd.) also attempted the synthesis of the GnRH antagonist-progesterone-vitamin B_{12} conjugate. Twenty-one hydroxyprogesterone 21-hemisuccinate was conjugated to the Lys^7 by the

organic carbodiimide (DCC) method during the peptide synthesis process (section 3.2.2). The vitamin B_{12} derivative DTP aminohexyl vitamin B_{12} was coupled to the DLys^6 (after position 7 since peptides are synthesized from the C to N-terminus) via a three-carbon spacer.

The conjugate-C DTP aminohexyl-vitamin B_{12} compound could not be produced by this method. The vitamin B_{12} molecule did conjugate with the side chain of the D-Lys^6, but a second vitamin B_{12} molecule conjugated to the C3 keto of progesterone. The progesterone C3 keto group is essential to both plasma protein interaction [97, 205] and progesterone receptor activation[196] and therefore this molecule was not suitable for investigation.

6.4 PROPOSED *IN VITRO* EXPERIMENTS WITH GnRH-STEROID-VITAMIN B_{12} CONJUGATES

After successful conjugation of conjugate C to vitamin B_{12}, a series of proposed experiments would determine whether the molecule could be transported by the vitamin B_{12} uptake system.

In order to bind to the IFCR, the conjugate-VB_{12} molecule must first bind to intrinsic factor. This can be tested in a similar way to plasma protein binding (section 2.3.1). Ideally the conjugate would first be radiolabelled, but a competition binding curve with [^{57}Co]cyanocobalamin could also be constructed, measuring the proportion of intrinsic factor-bound and free label after treatment with dextran-coated charcoal suspension[158].

Transport from the basolateral to apical surfaces of villus cells can be assessed with either polarized monolayers of the CaCo-2 cell line or everted gut sacs. In the case of the CaCo-2 cell line, these colorectal carcinoma cells express the IFCR after approximately 20 days in culture on permeable cell inserts[206]. After this time, transport of IF-bound radiolabelled conjugate-

vitamin B_{12} could be measured in competition with unlabeled IF-bound cyanocobalamin (or vice versa) to assess whether transport of the conjugate occurred in a saturable manner. The movement of radiolabelled conjugate from the outside (now basolateral) to inside (apical) of an everted gut sac could be calculated to measure transport of conjugate C-vitamin B_{12}.

Once the conjugate-vitamin B_{12} molecule was prepared it would have been important to assess the stability of the conjugate in the gastrointestinal environment. Published evidence suggested that a thiol cleavable bond was required between the vitamin B_{12} molecule and the GnRH analogue[158]. It is possible that this bond may not be sufficiently stable to survive the extremes of pH and variety of enzymes present in the GIT. The amine bond between the peptide and the steroid is relatively stable, however the ester bond between the steroid and the hemisuccinate group is of more concern. Esterase enzymes could possibly act on this bond and degrade the conjugate into peptide-hemisuccinate and steroid and it is unlikely that the peptide-hemisuccinate would retain plasma protein binding activity.

These *in vitro* assays would determine whether the conjugate could be transported by the vitamin B_{12} uptake system, but *in vivo* experiments would be required to confirm these results. These would include feeding studies in a small animal model such as the rat, measuring circulating conjugate C-vitamin B_{12} metabolite levels, plasma LH and gonadal steroids after oral consumption of conjugate C-vitamin B_{12}.

6.5 DISCUSSION

Methods to enhance the oral absorption of peptides were researched in the literature, taking into account the unique structure and properties of the GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate molecules.

Synthesis of GnRH antagonist-progesterone-vitamin B_{12} derivative conjugates was identified as the most promising method.

In order to synthesize these conjugates, new antagonist sequences were designed, based on GnRH structure-activity relationships and previous work with GnRH analogue conjugates. This involved conjugating a vitamin B_{12} derivative to a DLys^6 and 21-hydroxyprogesterone 21-hemisuccinate to a Lys^7.

A number of technical difficulties arose during attempts to synthesize GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate-DTP aminohexyl vitamin B_{12} molecules. These occurred with both conjugation of the vitamin B_{12} derivative in solution after solid phase peptide synthesis of the GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate molecule and with attachment of the vitamin B_{12} molecule during the peptide synthesis process.

There are a number of ways in which the problems encountered with producing the conjugate C-DTP aminohexyl vitamin B_{12} compound could be addressed. Prior to conjugation to the Lys^7, the C3 keto group of 21-hydroxyprogesterone 21-hemisuccinate could possibly be converted to a C3 ketal to render it inactive during the vitamin B_{12} conjugation process, preventing conjugation of the DTP aminohexyl vitamin B_{12} compound at this site. This would have to be reversed after the conjugation to retain progestagenic activity.

A D-cysteine residue could be used in place of the D-lysine in position six. This would greatly simplify the conjugation process, as the sulphydryl group would already be present on the side group. However the short single-carbon side chain, compared with the four-carbon side chain of lysine would bring the vitamin B_{12} molecule close to the attached steroid. This might increase the steric hindrance exerted on the peptide. A D-homocysteine in position six would add an additional carbon to the side chain, which would potentially reduce this steric hindrance. These peptides would require full *in vitro* investigation of GnRH receptor binding, plasma protein binding and

progesterone receptor activation to ensure these peptides were fully functional. Alternatively, a vitamin B_{12} derivative could be sought that was less reactive with the progesterone C3 keto group.

Although a GnRH antagonist-progesterone-vitamin B_{12} conjugate was not successfully produced because of technical difficulties arising from conjugating to the steroid, the theory and proposed experiments suggest the approach is feasible and that the synthesis difficulties could be overcome.

7 Concluding discussion

7.1 GnRH RECEPTOR INTERACTION OF GnRH ANTAGONIST-21 HYDROXYPROGESTERONE 21-HEMISUCCINATE CONJUGATES

GnRH agonist conjugates have been synthesized by other groups [12, 175, 195, 198, 207], however few have investigated the properties of GnRH antagonist bioconjugates. This could be partly due to the inherent difficulties with working with GnRH antagonists, primarily hydrophobicity and cost, and is compounded by the significantly higher doses of antagonist required to induce biological effects.

GnRH analogue conjugates have been synthesized for various uses. Coupling to carrier molecules has been used to enhance oral uptake, for example vitamin B_{12} [158] or to fatty acids for transdermal absorption [175]. Conjugation to emodic acid allowed interaction with plasma proteins and thus extended the half-life of [DLys^6]GnRH [12] and targeted chemotherapeutic agents were created by conjugation of GnRH to cytotoxics [9, 11, 198, 207].

The GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates remained capable of binding to the GnRH receptor when directly conjugated to a steroid. The use of a spacer between the GnRH antagonist and steroid was avoided by careful design of the molecule, ensuring the steroid was conjugated in a central position, hypothesized to be orientated away from the GnRH receptor during receptor interaction. This appeared to be successful when position six of the decapeptide was conjugated to the steroid, but was less so when the seventh amino acid was used. Position six was also used with GnRH conjugates produced by other groups [9-12, 158, 195, 198]. No other positions were reported in the literature, but it is unclear whether other

positions have been attempted and deemed inactive or if position six has been used as the first choice.

7.2 STEROID ACTIVITY OF GnRH ANTAGONIST-21 HYDROXYPROGESTERONE 21-HEMISUCCINATE CONJUGATES

The functioning of the steroid moiety of GnRH antagonist-progesterone conjugates was examined with respect to plasma protein association and activation of the progesterone receptor. All conjugates examined retained both of these properties and in the case of four of the five conjugates, this was combined with GnRH receptor binding and antagonism. Therefore these conjugates were fully bifunctional.

The binding of the progestagen to both specific plasma proteins and the progesterone receptor indicates that large substitutions at C21 are tolerated in both interactions. The requirements for binding to the specific plasma proteins CBG and PBG are well documented with regard to small substitutions such as additional hydroxyl or methyl groups. Larger substitutions are less well understood, although the steroid used for conjugations (21-hydroxyprogesterone 21-hemisuccinate) has been tested for guinea pig PBG binding affinity [139]. This study has demonstrated that even larger substitutions are tolerated well by PBG.

Elements of progesterone receptor binding have also been clarified. The requirements for binding to the receptor around C21 were not well characterized by crystallization of the ligand binding domain of the progesterone receptor [196]. This study has demonstrated that significant binding to the progesterone receptor was maintained by conjugates of 21-hydroxyprogesterone 21-hemisuccinate. There was a slight reduction in the ED_{50} of receptor activation, but the effect was relatively minor. Therefore

large substitutions at this position do not significantly reduce receptor binding.

7.3 PROPERTIES OF A GnRH ANTAGONIST-21 HYDROXYPROGESTERONE CONJUGATE IN THE MARMOSET AND MACAQUE

The conjugation of a steroid molecule to the GnRH antagonists conferred the longer half-life of the steroid onto the antagonist, without the need for extensive modification to enhance resistance to metabolic degradation and rate of clearance from the circulation. It is therefore possible that this method could be used to enhance the half-life of other GnRH analogues, or possibly even completely unrelated molecules.

It is unlikely that the biological activity was due to cleavage of the amide bond between the hemisuccinate and the peptide, although the possibility that the ester bond between the steroid and the hemisuccinate could be hydrolyzed is acknowledged. It is most probable that cleavage *in vivo* did not occur since a large increase in the duration of action was seen in the marmoset studies. This result would not be expected to occur without a large change in the properties of the GnRH antagonist molecule.

The increased hydrophobicity of the GnRH antagonists when conjugated to the steroid may at least partially account for the increased duration of action since hydrophobic molecules are known to exhibit a depot effect, resulting in slower release [39], particularly after subcutaneous injection.

GnRH-BSA conjugates can be used to elicit an immune response for the purposes of medical castration [208-210]. In order to achieve this, the GnRH-BSA conjugate must be structurally similar to native GnRH, although conjugation directly to BSA can reduce the immunoreactivity towards

monoclonal and polyclonal antibodies[211]. It is possible that GnRH analogue-steroid conjugates may induce a similar response when bound to plasma proteins (either CBG or albumin). But even if GnRH antagonist conjugates provoke the formation of antibodies, the considerable structural differences between the GnRH antagonist and hypothalamic 'self' decapeptide GnRH means that cross-reactivity is unlikely with the conjugates investigated here.

It is more likely that antibodies formed to the GnRH antagonist-steroid conjugate would cause problems with repeated administration of the conjugate. GnRH immunization is designed to provoke a rapid response with a minimum number of doses. It is envisaged that GnRH antagonist-conjugates would be used repeatedly over long periods to inhibit gonadotropin production. This could result in immunoreactivity to the conjugate itself, leading to histamine responses and possibly anaphylaxis. This was not a major concern at this stage of developing GnRH antagonist-progesterone conjugates since each primate was only injected with a conjugate on a single occasion. However the immunological effects of the conjugate should be addressed in later studies.

7.4 ENHANCING ORAL ABSORPTION OF GnRH ANTAGONIST-21 HYDROXYPROGESTERONE 21-HEMISUCCINATE CONJUGATES THROUGH CONJUGATION TO VITAMIN B_{12}

Methods to enhance the oral absorption of peptides were examined in the literature with the aim of identifying the most suitable way to increase oral absorption of the GnRH antagonist-progesterone conjugates. The selected method was to use the intrinsic factor-mediated uptake of vitamin B_{12} by synthesizing GnRH antagonist-progesterone-vitamin B_{12} conjugates, but these conjugates could not be produced because of chemical synthesis difficulties.

The vitamin B_{12} uptake system remains one of the most viable methods of enhancing oral absorption of peptides, including the GnRH antagonist-steroid conjugates. Recent advances in technology have enabled peptides to be temporarily enclosed in vitamin B_{12}-coated nanospheres, which protect the peptides from the low pH of the stomach and hydrolytic enzymes of the GIT [160, 212]. Nanospheres of up to 200nM diameter are transported across CaCo-2 cells via the IFCR, with significantly greater amounts of peptide transported than with direct peptide-vitamin B_{12} conjugates [160]. The advantages of this method are that chemical modification of the peptide is not required and the greater amount of peptide transported is in the range required for GnRH antagonists [160]. Thus this method may be more suitable for the oral absorption of GnRH antagonist-steroid conjugates.

7.5 *IN VITRO* BIOTRIGGERED RELEASE OF GnRH ANTAGONIST-STEROID CONJUGATES

Conjugation of a GnRH antagonist to a steroid hormone was shown to introduce plasma protein binding affinity onto the GnRH antagonist peptides. The majority of progesterone is found bound to both specific and non-specific plasma proteins, with only around 2% free in the circulation [100]. One of the objectives of my studies was to determine if this feature could be utilized to accomplish a biologically timed inhibition of gonadotropins, a biotriggered release.

If a fixed plasma protein capacity is assumed, then an increase in progesterone concentration would saturate the plasma protein binding sites. Increases in progesterone concentration occur at midcycle immediately prior to ovulation (0.5ng/ml) and a larger increase occurs during the luteal phase (10ng/ml).

If a GnRH antagonist-progesterone conjugate was present throughout the menstrual cycle, during the follicular phase it would be predominantly

bound to plasma proteins. But during periods of high progesterone concentration, these binding sites would be predominantly occupied by progesterone, since progesterone was found to have a higher affinity for plasma proteins than the conjugates (figures 4.8 and 4.10). The presence of progesterone would therefore 'trigger' the release of the GnRH antagonist-progesterone conjugate from the plasma protein (figure 7.1A). Providing plasma protein bound conjugate is not able to interact with the GnRH receptor, the GnRH antagonist would only inhibit LH release during periods of high progesterone concentrations.

If sufficient GnRH antagonist-progesterone conjugate is released from the plasma protein during the midcycle progesterone rise, the luteal phase would be prematurely terminated, thereby preventing ovulation. Alternatively, if a larger increase in progesterone was required to release sufficient GnRH antagonist-progesterone conjugate from the plasma protein, the luteal phase increase in progesterone concentration would interrupt gonadotropin support of the corpus luteum, halting the luteal phase and inducing menses.

This principle would enable a GnRH antagonist-progesterone conjugate to be 'released' from plasma only at these times, allowing transient inhibition of gonadotropin suppression, either terminating the LH surge or interrupting gonadotropin support of the corpus luteum. This biologically timed release would have significant advantages over the current use of GnRH antagonists, allowing release of the GnRH antagonist-progesterone conjugate only during periods of high progesterone concentration. This would enable highly specific timing of gonadotropin inhibition to either inhibit ovulation or the functioning of the corpus luteum.

The effects of late-luteal GnRH antagonist treatment can be overcome by the presence of hCG [72] in the rhesus macaque, but it is unclear whether this can also occur in humans. This could have major implications to the biotriggered release of the GnRH-antagonist conjugate if an early embryo has already implanted into the endometrium, stimulating the release of hCG.

This may prevent the GnRH antagonist from inhibiting corpus luteum function, limiting the effectiveness of the GnRH antagonist-steroid conjugates as contraceptives.

This theory does not take into account the large proportion of progesterone bound to albumin [100]. This plasma protein has an immense capacity for binding and despite the low affinity of interaction, has major effects on the distribution of progesterone and a GnRH antagonist-progesterone conjugate. There may be sufficient plasma protein binding sites to bind both the progesterone and the GnRH antagonist-progesterone conjugate simultaneously and therefore biotriggered release may not occur. However the conjugate would still be of value because of the enhanced half-life in comparison to the parent GnRH antagonist peptide.

One of the fundamental assumptions of this theory was that the proportions of bound and free progesterone (or oestradiol) vary during the menstrual phase. Although the absolute concentration varies, the proportion of bound and free hormone remains unchanged [213]. Therefore the capacity for binding progesterone through both specific and non-specific plasma protein interactions exceeds maximum physiological steroid concentrations. Comparing bound and free hormone levels across the menstrual cycle with equilibrium dialysis could have provided the evidence to refute the biotriggered release theory without further analysis.

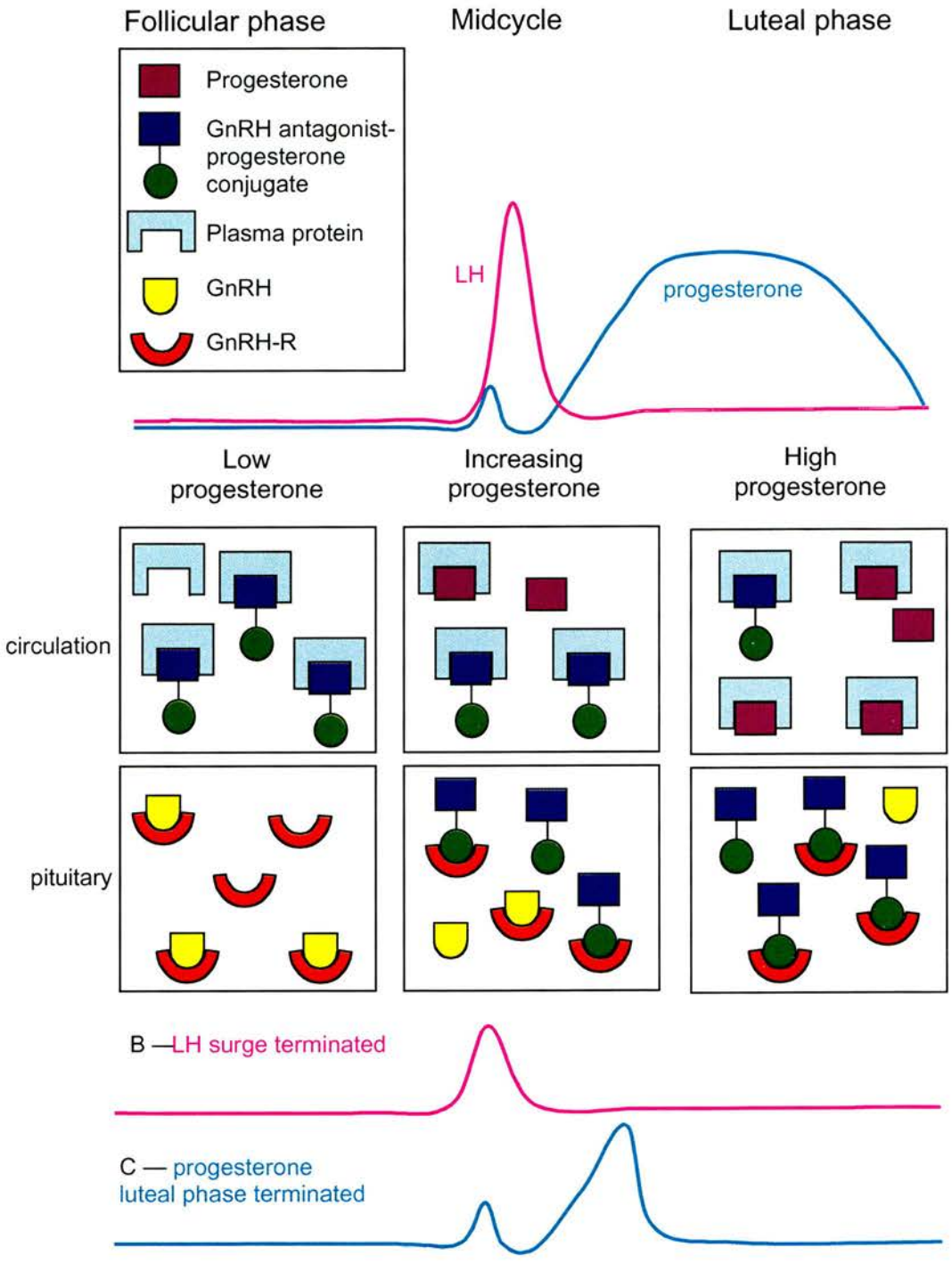


Figure 7.1A: Summary of proposed biotriggered release mechanism of GnRH antagonist-progesterone conjugates.

B - Effect on LH if the midcycle progesterone rise releases sufficient GnRH antagonist-progesterone conjugate from plasma proteins to prevent ovulation.

C -Effect on progesterone if the luteal progesterone increase releases enough GnRH antagonist-progesterone conjugate from plasma proteins to interrupt gonadotropin support of the corpus luteum, resulting in luteolysis.

This theory was tested in three types of *in vitro* assays in the presence of a combination of human serum or pregnant guinea pig plasma, conjugate, control peptide and progesterone, in incubation media.

Whole cell binding assay

Whole cell binding assays are more physiological than some other types of receptor binding assay since binding is assessed in intact cells expressing the GnRH receptor. The assay was modified to include human serum and conjugate in the incubation media, with or without progesterone. The conjugate/progesterone mixtures were incubated in this media for periods of up to 12 hours at 37°C before application to the cells and assay.

Initially changes to the IC_{50} curve were analysed. The presence of plasma or serum containing the binding proteins should have reduced the ability of the conjugate to compete with [His^{5}DTyr^6]GnRH, resulting in a right hand shift of the dose-response curve. Saturating the binding sites with progesterone before adding the conjugate should have reversed this effect (figure 7.2). However no consistent result could be identified despite altering duration and temperature of incubation and the concentration of progesterone. It was concluded that possible reasons for this failure were variation in CBG capacity in the human serum, small variations in plating density or the effect was too small to observe as a shift in the IC_{50} curve.

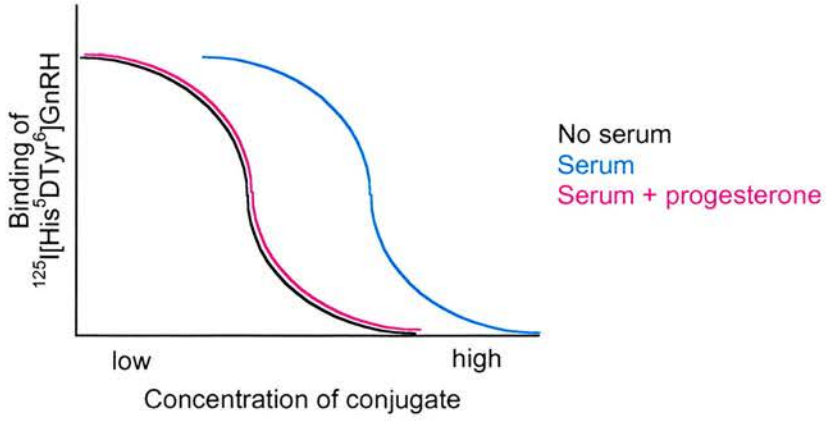


Figure 7.2: Theoretical effects of serum and serum with progesterone on competition whole cell binding assay of a GnRH antagonist-steroid conjugate.

The same assay was carried out using a single concentration of conjugate (approximating the IC_{50} value in normal whole cell binding assay) with a high number of replicates to measure small changes more accurately. However this also did not result in a significant change in [His^{5}DTyr^6]GnRH competition in the presence of serum, plasma or progesterone.

Membrane binding assays

Membrane binding assays are more suitable for modification since the binding assay is carried out in solution with homogenized membranes. The concentration of purified PBG solution included could be varied without the risk of cell detachment from a solid support as occurred in whole cell binding assays.

The effect of adding purified PBG solution, alone and in combination with progesterone to the incubation medium was measured with a single concentration of conjugate. The conjugate was present at approximately the IC_{50} value calculated under normal membrane binding assay condition. A high number of replicates were carried out to reduce error values. In the presence of PBG solution, most of the conjugate would be expected to bind to plasma proteins, reducing the ability of the conjugate to compete with [His^{5}DTyr^6]GnRH for binding to the GnRH receptor. Excess progesterone would occupy the plasma protein binding sites, with the unbound conjugate able to compete with [His^{5}DTyr^6]GnRH for GnRH receptor binding.

The expected reduction in competition of the conjugate with [His^{5}DTyr^6]GnRH in the presence of purified PBG solution was observed on two separate occasions (an example is shown in figure 7.3). The presence of progesterone was seen to reverse this effect as expected. There was no effect of PBG solution or progesterone on the competition of mammalian GnRH (control peptide) with [His^{5}DTyr^6]GnRH. This effect was not repeatable despite numerous attempts and therefore it must be concluded that the effect was not sufficiently robust.

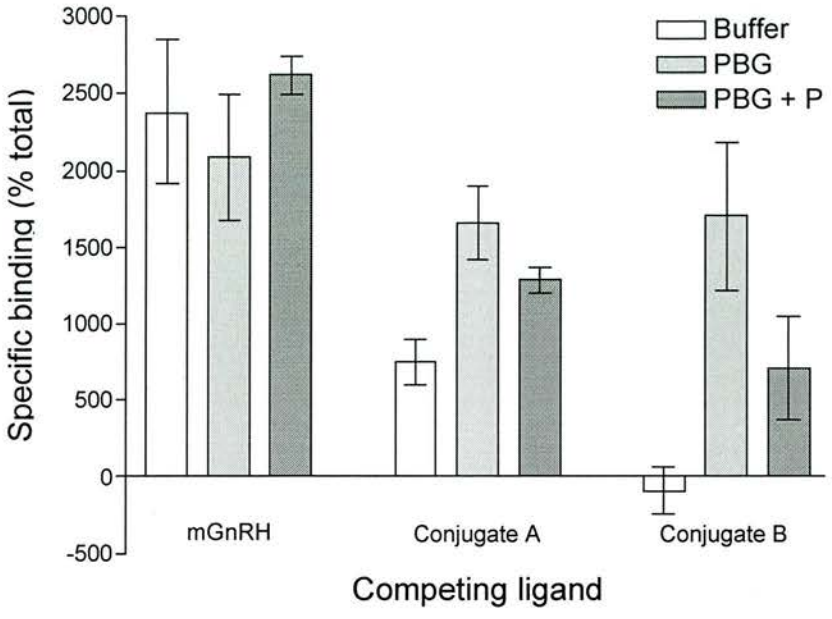


Figure 7.3: Example of biotriggered release measured with membrane binding assay. Total specific binding of ^{125}I-[His^{5}DTyr^{6}]GnRH in the presence of constant concentrations of competing ligand (mGnRH 31nM, conjugate A 3nM and conjugate B 100nM). 'Buffer' corresponds to PBG purification buffer (20mM sodium acetate, 0.02% sodium azide), 'PBG' represents PBG purification buffer containing PBG and 'PBG + P' denotes PBG purification buffer containing both PBG and progesterone.

Inositol phosphate assay

IP assays are used to measure the activation or inhibition of downstream signalling events at the GnRH receptor due to the activation of PLC. The signalling cascade amplifies receptor binding events and therefore this assay is more sensitive to detecting an effect of serum or plasma on the competition of the conjugate with mammalian GnRH for receptor binding.

There were several problems with attempting to use the IP assay to assess the effects of plasma or serum. Cell detachment from plastic ware was observed even at low concentrations of pregnant human serum. Replacing the serum with pregnant guinea pig plasma produced an interesting result. Incubation media comprising 10% pregnant guinea pig plasma produced a 100-fold right hand shift in the dose-response curve of mammalian GnRH in

comparison to the curve in normal incubation media. Pregnant guinea pig plasma must therefore contain an agent capable of blocking the effects of mammalian GnRH. An inactivating enzyme may be responsible for this effect. Thus this assay could not be used to measure biotriggered release.

Mammalian cells are routinely cultured in foetal calf serum (FCS) because it contains the additional factors that are required for cell growth and metabolism that cannot be found in any other source or produced synthetically. There are however a large number of other constituents in serum that can affect cell culture. It is not surprising that cell detachment was observed when addition of human serum into the IP assay was attempted. It would perhaps have been better to use another source of plasma binding proteins to test the biotriggered release assay, such as FCS or BSA.

These attempts to prove biotrigger release were unsuccessful, primarily because of the technical difficulties arising from the number of effects involved. Nevertheless the biotriggered release principle remains a possibility, but further investigations are required to confirm this.

7.6 FUTURE WORK

GnRH agonist-21 hydroxyprogesterone 21-hemisuccinate conjugates

[DLys^6]GnRH-21 hydroxyprogesterone 21-hemisuccinate conjugates were synthesized as detailed in chapter 3, however these molecules were designed to prove the method of conjugation and were not analysed *in vitro* or *in vivo*. It would be interesting to investigate how the properties of GnRH agonists could potentially be modified by conjugation to a steroid molecule since the requirements for agonist binding to the GnRH receptor are fairly well understood.

GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates

The GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates designed and synthesised were investigated for GnRH receptor binding, affects on IP production, plasma protein binding and activation of the progesterone receptor via a reporter gene construct. It was demonstrated that conjugating the steroid to amino acid six of the peptide retained bioactivity, however conjugation through amino acid seven markedly reduced the GnRH receptor binding and inhibition of inositol phosphate production. Therefore these conjugates would not be suitable for further investigation, but there are other sites that could be used and incorporation of linker regions may reduce the effect of the steroid conjugation on GnRH receptor activity. It is unlikely that this would be necessary to maintain steroid activity since activation of the progesterone receptor remained similar to progesterone for all five conjugates.

GnRH analogue-testosterone or oestradiol conjugates

Although the current work has concentrated on the synthesis and analysis of GnRH antagonist-21 hydroxyprogesterone 21-hemisuccinate conjugates, the ability of other endogenous steroids such as testosterone and oestradiol to increase the half-life of GnRH analogues is acknowledged. Steroids binding to SHBG would potentially have the same effect on duration of action as steroids binding to CBG (and PBG).

Conjugates of GnRH analogues linked to other molecules

The method of enhancing GnRH analogue duration of action is not limited to endogenous steroids and any other plasma protein-binding molecule would potentially have the same effect. A good candidate molecule would be mifepristone (also known as RU 486). Mifepristone is known to have a long duration of action, derived primarily from binding to alpha 1-acid glycoprotein [108]. The conjugation of this progesterone antagonist to a GnRH antagonist would result in a very interesting molecule, combining these two effects to result in a highly effective contraceptive with an unusually long duration of action.

7.7 FINAL CONCLUSIONS

GnRH analogues are important tools for reproductive medicine. Design of GnRH antagonists with reduced side effects has overcome many of the earlier problems with these peptides. In this thesis two of the remaining problems were addressed in a novel way to overcome the short half-life and low bioavailability after oral administration. The information gained from this study is of importance to future work with GnRH analogues, by proving the concept of enhancing the properties of a GnRH antagonist without sequence modification or compromising binding to the GnRH receptor. This method could also be used with other peptides to improve their properties.

The results obtained in this study provide preliminary information on the functioning of GnRH antagonist-progesterone conjugates. However some assays would be of benefit to further analyze the properties of the conjugates and the effect of the conjugation process on the constituent parts. For example antagonism at the GnRH receptor could be conclusively demonstrated by analysis of LH production (rather than just inhibition of IP production) and non-specific plasma protein binding could be measured by equilibrium dialysis. It is also important to investigate plasma protein binding of the conjugates in human serum. The information contained herein is the first step in developing GnRH antagonist-steroid conjugates and proves that the concept is possible.

This concept has advantages over similar attempts to increase the half-life of GnRH analogues by Rahimipour *et al* [12]. Conjugation of [DLys^6]GnRH to emodic acid was shown to prolong the activity of the GnRH analogue in the rat. However conjugation to emodic acid raises several issues. Emodin is an anthraquinone derivative used in some laxatives with known toxic effects. Although the conjugate was shown to be devoid of toxic effects in this study, the possibility of toxicity would be of some concern. The use of a modified endogenous molecule (a progestagen) has distinct advantages, particularly when the biological effect of the molecule is advantageous. Toxicity is

unlikely with a modified steroid and thus the conjugates described herein are superior to those described elsewhere.

Combining a GnRH antagonist with progesterone has several advantages over currently available peptide GnRH antagonists. The pharmacodynamic properties of the peptides were improved by conferring plasma protein binding onto the peptide, reducing the need for frequent doses. Combining low dose sex-steroid replacement (dependent on the dose of conjugate required) with a GnRH antagonist would provide two methods of inhibiting gonadotropin release, through negative feedback (progesterone) at the pituitary and competition with endogenous GnRH (GnRH antagonist). This will result in a highly effective contraceptive. Low-concentration progesterone replacement may also counteract some of the problems associated with long-term GnRH antagonist treatment. These conjugates could therefore have a major impact in reproductive medicine in the future.

Appendix I

Supplier addresses.

Name of supplier	Town/city	Country
Albachem Ltd.	Gladsmuir	UK
Amersham Pharmacia Biotech	Little Chalfont	UK
Bachem UK Ltd.	St. Helens	UK
Beckman Coulter UK Ltd.	High Wycombe	UK
Bio-Rad Laboratories	Hemel Hempstead	UK
Charles River Laboratories	Margate	UK
ICN Biomedicals Ltd.	Thame	UK
Invitrogen Life Technologies	Paisley	UK
Jencons (Scientific)	Leighton Buzzard	UK
Merck Ltd.	Lutterworth	UK
Micromass Ltd.	Manchester	UK
Millipore UK Ltd.	Harrow	UK
NEN Life Science Products	Hounslow	UK
National Institute of Health	Washington	USA
Perbio Scicence UK Ltd (Pierce)	Chester	UK
Promega Ltd.	Southampton	UK
Qiagen Ltd.	Crawley	UK
Sigma-Aldrich Company	Poole	UK
Whatman International	Maidstone	UK

Appendix II

Solutions used throughout thesis.

COMPETITION MEMBRANE BINDING ASSAY

Homogenization buffer

Tris	1.21g
Magnesium chloride hexahydrate	0.204g
Deionised water to 500ml	
pH adjusted to 7.2	

Assay buffer (10x)

Tris	48.46g
Magnesium chloride hexahydrate	4.07g
Deionised water to 1L	
pH adjusted to 7.4	

INOSITOL PHOSPHATE ASSAY

Incubation buffer

Sodium chloride	8.18g
HEPES	4.76g
Potassium chloride	0.298g
D-Glucose	1.44g
Magnesium chloride hexahydrate	0.203g
Calcium chloride	0.147g
BSA	1.0g
Deionised water to 1L	
pH adjusted to 7.2	

PLASMA PROTEIN BINDING ASSAY

Dextran-coated charcoal solution

Dextran T-70	0.25g
Charcoal decolorizing powder	2.5g
PBS to 500ml	

MARMOSET PROGESTERONE ELISA

Coating buffer

Sodium carbonate	4.24g
Sodium bicarbonate	4.04g

Deionised water to 1L

pH adjusted to 9.6

Washing buffer (25x)

Tris	302g
Sodium chloride	450g
Tween 20	25ml

Deionised water to 2L

pH adjusted to 7.5

Phosphate/citric assay buffer

Disodium hydrogen phosphate	17.85g
Citric acid	7.75g
Gelatin	1.0g
Thiomersalate	0.1g

Deionised water to 1L

pH adjusted to 6.0

Substrate buffer

Citric acid	10.3g
Disodium hydrogen phosphate	14.19g

Deionised water to 1L

Coating antibody

Rivanol purified DARS

Primary antibody

SAPU anti-P4 serum (from rabbits immunised with 11a-progesterone-BSA conjugate)

Label constituents

Progesterone 11α-glucuronide-biotin complex

Diluted to 1: 20,000 with assay buffer

2mg/ml 8-anilino-1 naphthalene sulphonic acid

Amdex; streptavidin-horse radish peroxidase (HRP)

Diluted to 1: 2000 with phosphate buffer pH 7.4 containing 1% casein

Substrate

O-phenylenediamine dihydrochloride	30mg tablet
Hydrogen peroxide solution	30µl
Substrate buffer	75ml

Stopping solution

Concentrated sulphuric acid	50ml
Deionised water	450ml

MARMOSET TESTOSTERONE RIA

Assay buffer

Disodium hydrogen phosphate anhydrous	8.66g
Disodium hydrogen phosphate dihydrate	6.08g
Sodium chloride	9.0g
Thiomersalate	0.1g
Gelatin	1.0g

Deionised water to 1L

Second antibody

Donkey anti goat/sheep (SAPU) 1:25 diluted

Normal sheep serum (SAPU) 1:800 diluted

MACAQUE LH RIA

Assay buffer

50ml 0.5M phosphate buffer stock solution

Sodium chloride	4.5g
BSA	5.0g
Thiomersal	0.1g

Distilled water to 500ml

Wash solution

4% PEG, 0.2% TritonX in 0.9% saline

Bibliography

1. **Filicori, M, *et al.*,** (1994). Treatment of anovulation with pulsatile gonadotropin-releasing hormone: prognostic factors and clinical results in 600 cycles. *J Clin Endocrinol Metab*, **79**(4): 1215-1220.

2. **Emons, G and Schally, AV,** (1994). The use of luteinizing hormone releasing hormone agonists and antagonists in gynaecological cancers. *Hum Reprod*, **9**(7): 1364-1379.

3. **Casper, RF,** (1991). Clinical uses of gonadotropin-releasing hormone analogues. *CMAJ*, **144**(2): 153-158.

4. **Coelingh Bennink, HJT.** 1996 Potential clinical applications of GnRH antagonists. in *Treatment with GnRH analogues: Controversies and perspectives. The proceedings of a satellite symposium of the 15th world congress on fertility and sterility.* Bologna, Italy 77-80.

5. **Conn, PM and Crowley, WF,** (1990). Gonadotropin-releasing hormone and its analogues. *N Engl J Med*, **324**(2): 93-103.

6. **Conn, PM and Crowley, WF,** (1994). Gonadotropin-releasing hormone and its analogues. *Ann Rev Med*, **45**: 391-405.

7. **Moghissi, KS,** (1992). Clinical applications of gonadotropin-releasing hormones in reproductive disorders. *Endocrinol Metab Clin North Am*, **21**(1): 125-140.

8. **Millar, RP, *et al.*,** (1998). GnRH and GnRH analogues: Structure, actions and clinical applications. *Horm Frontier Gynecol*, **5**(4): 77-83.

9. **Bajusz, S, *et al.*,** (1989). Highly potent analogues of luteinizing hormone-releasing hormone containing D-phenylalanine nitrogen mustard in position 6. *Proc Natl Acad Sci U S A*, **86**(16): 6318-6322.

10. **Janaky, T, *et al.*,** (1992). Analogues of luteinizing hormone-releasing hormone containing cytotoxic groups. *Proc Natl Acad Sci U S A*, **89**(3): 972-976.

11. **Nagy, A, *et al.*,** (1996). Cytotoxic analogs of luteinizing hormone-releasing hormone containing doxorubicin or 2-pyrrolinodoxorubicin, a derivative 500-1000 times more potent. *Proc Natl Acad Sci U S A*, **93**(14): 7269-7273.

12. **Rahimipour, S, *et al.*,** (2001). Design, synthesis, and evaluation of a long-acting, potent analogue of gonadotropin-releasing hormone. *J Med Chem*, **44**(22): 3645-3652.

13. **Sealfon, SC, *et al.*,** (1997). Molecular mechansims of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr Rev*, **18**(2): 180-205.

14. **Johnson, MH and Everitt, BJ,** The regulation of gonadal function, in *Essential Reproduction*. 1995, Blackwell Science: Oxford. p. 79-110.

15. **King, JA and Millar, RP,** (1995). Evolutionary aspects of gonadotropin-releasing hormone and its receptor. *Cell Mol Neurobiol*, **15**(1): 5-23.

16. **Sherwood, NM, *et al.*,** (1993). Origin of mammalian gonadotropin-releasing hormones. *Endocr Rev*, **14**(2): 241-254.

17. **Troskie, B, *et al.*,** (1998). Identification of three putative GnRH receptor subtypes in vertebrates. *Gen Comp Endocrinol*, **112**(3): 296-302.

18. **White, SA, *et al.*,** (1994). A second gene for gonadotropin-releasing hormone: cDNA and expression pattern in the brain. *Proc Natl Acad Sci U S A*, **91**: 1423-1427.

19. **Millar, RP,** Gonadotropin releasing hormones and their receptors, in *Reproductive Medicine: molecular, cellular and genetic fundamentals*, Fauser, BCJM, Editor. 2002, Parthenon Publishing: Lancaster. p. 199-224.

20. **Hsueh, AJW and Jones, PBC,** (1981). Extrapituitary actions of gonadotropin-releasing hormone. *Endocr Rev*, **2**(4): 437-461.

21. **Karten, MJ and Rivier, JE,** (1986). Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: Rationale and perspective. *Endocr Rev*, **7**(1): 44-66.

22. **Momany, FA,** (1976). Conformational energy analysis of the molecule, luteinizing hormone-releasing hormone. I. Native decapeptide. *J Am Chem Soc*, **98**(10): 2990-2996.

23. **Momany, FA,** (1976). Conformational energy analysis of the molecule, luteinizing hormone-releasing hormone. 2. Tetrapeptide and decapeptide analogues. *J Am Chem Soc*, **98**(10): 2996-3000.

24. **Chan, RL and Chaplin, MD,** (1985). Plasma binding of LHRH and nafarelin acetate, a highly potent LHRH agonist. *Biochem Biophys Res Commun*, **127**(2): 673-679.

25. **Tharandt, L, *et al.*,** (1979). Binding of luteinizing hormone releasing hormone to human serum proteins--influence of a chronic treatment with a more potent analogue of LH-RH. *Horm Metab Res*, **11**(6): 391-394.

26. **Karsch, FJ, *et al.*,** (1997). Gonadotropin-releasing hormone requirements for ovulation. *Biol Reprod*, **56**: 303-309.

27. **Hoff, JD, *et al.*,** (1983). Hormonal dynamics at midcycle: a reevaluation. *J Clin Endocrinol Metab*, **57**(4): 792-795.

28. **Anderson, RA,** (2000). Hormonal contraception in the male. *Brit Med Bull*, **56**(3): 717-728.

29. **Zhou, W, *et al.*,** (1995). A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites. *J Biol Chem*, **270**(32): 18853-18857.

30. **Chi, L, *et al.*,** (1993). Cloning and characterization of the human GnRH receptor. *Mol Cell Endocrinol*, **91**(1-2): R1-6.

31. **McArdle, CA, *et al.*,** (2002). Signalling, cycling and desensitisation of gonadotrophin-releasing hormone receptors. *J Endocrinol*, **173**: 1-11.

32. **Troskie, B, *et al.*,** (2000). Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotrophin-releasing hormone receptor expressed in the pituitary and midbrain of *Xenopus laevis*. *Endocrinology*, **141**: 1764-1771.

33. **Ortmann, O and Diedrich, K,** (1999). Pituitary and extrapituitary actions of gonadotropin-releasing hormone and its analogues. *Hum Reprod*, **14**(Supplement 1): 194-206.

34. **Hanyaloglu, AC, *et al.*,** (2001). Casein kinase II sites in the intracellular C-terminal domain of the thyrotropin-releasing hormone receptor and chimeric gonadotropin-releasing hormone receptors contribute to beta-arrestin-dependent internalisation. *J Bio Chem*, **276**: 18066-18074.

35. **Tobin, BE, *et al.*,** (1997). Stimulus-dependent phosphorylation of G-protein-coupled receptors by casein kinase 1 alpha. *J Bio Chem*, **272**: 20844-20849.

36. **Ferguson, SS,** (2001). Evolving concepts in G-protein coupled receptor endocytosis: the role in receptor desensitisation and signalling. *Pharmacol Rev*, **53**: 1-24.

37. **Zhang, J, *et al.*,** (1997). Molecular mechanisms of G-protein-coupled receptor signalling: role of G protein-coupled receptor kinases and arrestins in receptor desensitisation and resensitisation. *Receptor Channel*, **5**: 193-199.

38. **Thau, RB,** (1984). Luteinizing hormone-releasing hormone (LHRH) and its analogs for contraception in women: a review. *Contraception*, **29**(2): 143-162.

39. **Nestor, JJJ, *et al.*,** LHRH agonists and antagonists containing very hydrophobic amino acids, in *LHRH and its Analogs: contraceptive and therapeutic applications*, Vickery, BH, *et al.*, Editors. 1984: Lancaster. p. 23-33.

40. **Monahan, MW, *et al.*,** (1973). Synthetic analogs of the hypothalamic luteinizing hormone releasing factor with increased agonist or antagonist properties. *Biochemistry*, **12**(23): 4616-4620.

41. **Rivier, J, *et al.*,** Hypothalamic hypophysiotropic hormones, in *Peptides 1976*, Loffet, A, Editor. 1977, Editions de l'Universite de Bruxelles: Brussels. p. 427-451.

42. **Coy, DH, *et al.*,** (1976). Analogs of luteinizing hormone-releasing hormone with increased biological activity produced by D-amino acid substitutions in position 6. *J Med Chem*, **19**(3): 423-425.

43. **Fujino, J, *et al.*,** (1972). Structure-activity relationships in the C-terminal part of luteinizing hormone-releasing hormone (LH-RH). *Biochem Biophys Res Commun*, **49**: 863-.

44. **Davidson, JS, *et al.*,** (1996). Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide. *J Biol Chem*, **271**(26): 15510-15514.

45. **Dutta, AS, *et al.*,** (1978). Potent agonist and antagonist analogues of luliberin containing an azaglycine residue in position 10. *Biochem Biophys Res Commun*, **81**(2): 382-390.

46. **Yardley, JP, *et al.*,** (1975). Antagonism of luteinizing hormone release and of ovulation by an analog of the luteinizing hormone-releasing hormone. *J Med Chem*, **18**(12): 1244-1247.

47. **Yabe, Y, *et al.*,** (1976). Analogues of luteinizing hormone-releasing hormone with modification in position 3. *Chem Pharm Bull (Tokyo)*, **24**(12): 3149-3157.

48. **Vale, W, *et al.*,** (1972). Synthetic polypeptide antagonists of the hypothalamic luteinizing hormone releasing factor. *Science*, **176**(37): 933-934.

49. **Rees, RW, *et al.*,** (1974). Synthesis and biological activities of analogs of the luteinizing hormone-releasing hormone (LH-RH) modified in position 2. *J Med Chem*, **17**(9): 1016-1019.

50. **Corbin, A and Beattie, CW,** (1975). Inhibition of the pre-ovulatory proestrous gonadotropin surge, ovulation and pregnancy with a peptide analogue of luteinizing hormone releasing hormone. *Endocr Res Commun*, **2**(1): 1-23.

51. **Beattie, CW, *et al.*,** (1975). Luteinizing hormone-releasing hormone. Antiovulatory activity of analogs substituted in positions 2 and 6. *J Med Chem*, **18**(12): 1247-1250.

52. **Hazum, E, *et al.*,** (1983). Receptor-mediated internalization of LHRH antagonists by pituitary cells. *Mol Cell Endocrinol*, **30**(3): 291-301.

53. **Henzl, MR,** (1993). Gonadotropin-releasing hormone and its analogues: from laboratory to bedside. *Clin Obstet Gynecol*, **36**(3): 617-635.

54. **Hall, JE,** (1993). Gonadotropin-releasing hormone antagonists: effects on the ovarian follicle and corpus luteum. *Clin Obstet Gynecol*, **36**(3): 744-752.

55. **Reissmann, T, *et al.*,** (1995). Development and applications of luteinizing hormone-releasing hormone antagonists in the treatment of infertility: an overview. *Hum Reprod*, **10**(8): 1974-1981.

56. **Ho, CY,** *Method for the Treatment of LHRH Diseases and Conditions*. 1987: US Patent 4,678,784.

57. **Millar, RP, *et al.*,** (2000). Progress towards the development of non-peptide orally-active gonadotropin-releasing hormone (GnRH) antagonists: therapeutic implications. *Br Med Bull*, **56**(3): 761-772.

58. **Ashton, WT, *et al.*,** (2001). Orally bioavailable, indole-based nonpeptide GnRH receptor antagonists with high potency and functional activity. *Bioorg Med Chem Lett*, **11**: 2597-2602.

59. **Chu, L, *et al.*,** (2001). SAR studies of novel 5-substituted 2-arylindoles as nonpeptidyl GnRH receptor antagonists. *Bioorg Med Chem Lett*, **11**(4): 515-517.

60. **Young, JR, *et al.*,** (2000). Quinolones as gonadotropin releasing hormone (GnRH) antagonists: simultaneous optimization of the C(3)-aryl and C(6)-substituents. *Bioorg Med Chem Lett*, **10**(15): 1723-1727.

61. **Walsh, TF, *et al.*,** (2000). Potent antagonists of gonadotropin releasing hormone receptors derived from quinolone-6-carboxamides. *Bioorg Med Chem Lett*, **10**(5): 443-447.

62. **DeVita, RJ, *et al.*,** (2001). A potent, nonpeptidyl 1H-quinolone antagonist for the gonadotropin-releasing hormone receptor. *J Med Chem*, **44**(6): 917-922.

63. **Rivier, JE, *et al.*,** (1986). New effective gonadotropin releasing hormone antagonists with minimal potency for histamine release in vitro. *J Med Chem*, **29**(10): 1846-1851.

64. **Bouchard, P and Fauser, BCJM,** (2000). Gonadotropin-releasing hormone antagonist: new tools vs. old habits. *Fertil Steril,* **73**(1): 18-20.

65. **Fraser, HM,** (1988). LHRH analogues: their clinical physiology and delivery systems. *Bailliere Clin Ob Gy,* **2**(3): 639-658.

66. **Waibel, S, *et al.*,** (1988). Bone density in patients treated with GnRH agonists for myomata and endometriosis. *Gynecol Endocrinol,* **2**(Supplement 2): abstract 96.

67. **Balmaceda, JP, *et al.*,** (1984). The effects of chronic administration of LH-RH agonists and antagonists on the menstrual cycle and endometrium of the rhesus monkey. *Contraception,* **29**(1): 83-90.

68. **Hall, JE, *et al.*,** (1991). Variable tolerance of the developing follicle and corpus luteum to gonadotropin-releasing hormone antagonist-induced gonadotropin withdrawal in the human. *J Clin Endocrinol Metab,* **72**(5): 993-1000.

69. **Mais, V, *et al.*,** (1986). The dependency of folliculogenesis and corpus luteum function on pulsatile gonadotropin secretion in cycling women using a gonadotropin-releasing hormone antagonist as a probe. *J Clin Endocrinol Metab,* **62**(6): 1250-1255.

70. **Fluker, MR, *et al.*,** (1991). Variable ovarian response to gonadotropin-releasing hormone antagonist-induced gonadotropin deprivation during different phases of the menstrual cycle. *J Clin Endocrinol Metab,* **72**(4): 912-919.

71. **Dubourdieu, S, *et al.*,** (1991). Suppression of corpus luteum function by the gonadotropin-releasing hormone antagonist Nal-Glu: effect of the dose and timing of human chorionic gonadotropin administration. *Fertil Steril,* **56**(3): 440-445.

72. **Balmaceda, JP, *et al.*,** (1983). Suppression of postovulatory gonadotropin levels does not affect corpus luteum function in rhesus monkeys. *J Clin Endocrinol Metab,* **57**(4): 866-868.

73. **Hutchison, JS and Zeleznik, AJ,** (1985). The corpus luteum of the primate menstrual cycle is capable of recovering from a transient withdrawal of pituitary gonadotropin support. *Endocrinology,* **117**(3): 1043-1049.

74. **Fraser, HM, *et al.*,** (1986). Effects of an LH-releasing hormone antagonist on the secretion of LH, FSH. prolactin and ovarian steroids at different stages of the luteal phase in the stumptailed macaque (Macaca arctoides). *J Endocrinol,* **111**: 83-90.

75. **Vickery, BH,** (1985). Comparisons of the potential utility of LHRH agonists and antagonists for fertility control. *J Ster Biochem,* **23**(5B): 779-791.

76. **Dewart, PJ, *et al.*,** (1987). LRH agonist buserelin as a post-partum contraceptive: lack of biological activity of buserelin in breast milk. *Acta Endocrinol-Cop*, **114**: 185-192.

77. **Hernandez, ER,** (2000). Embryo implantation and GnRH antagonists (Embryo implantation: the Rubicon for GnRH antagonists). *Hum Reprod*, **15**(6): 1211-1216.

78. **Felberbaum, RE and Diedrich, K,** (1999). Ovarian stimulation for IVF/ICSI with gonadotropins and GnRH analogues: agonist and antagonist. *Hum Reprod*, **14**(Suppl. 1): 207-221.

79. **Dekel, N, *et al.*,** (1988). Receptors for gonadotropin releasing hormone are present in rat oocytes. *Endocrinology*, **123**(2): 1205-1207.

80. **Minaretzis, D, *et al.*,** (1995). Gonadotropin-releasing hormone receptor gene expression in human ovary and granulosa-lutein cells. *J Clin Endocrinol Metab*, **80**(2): 430-434.

81. **Murdoch, WJ,** (1995). Immunolocalization of a gonadotropin-releasing hormone receptor site in murine endometrium that mediates apoptosis. *Cell Tissue Res*, **282**(3): 527-529.

82. **Emons, G, *et al.*,** (1993). High affinity binding and direct antiproliferative effects of LHRH analogues in human ovarian cancer cell lines. *Cancer Res*, **53**(22): 5439-5446.

83. **Hershkovitz, E, *et al.*,** (1993). Luteinizing hormone-releasing hormone antagonists interfere with autocrine and paracrine growth stimulation of MCF-7 mammary cancer cells by insulin-like growth factors. *J Clin Endocrinol Metab*, **77**(4): 963-968.

84. **Kleinman, D, *et al.*,** (1993). Regulation of endometrial cancer cell growth by insulin-like growth factors and the luteinizing hormone-releasing hormone antagonist SB-75. *Regul Peptides*, **48**(1-2): 91-98.

85. **Moretti, RM, *et al.*,** (1996). Luteinizing hormone-releasing hormone agonists interfere with the stimulatory actions of epidermal growth factor in human prostatic cancer cell lines, LNCaP and DU 145. *J Clin Endocrinol Metab*, **81**(11): 3930-3937.

86. **Dubourdieu, S, *et al.*,** (1994). Effect of administration of a gonadotropin-releasing hormone (GnRH) antagonist (Nal-Glu) during the periovulatory peroid: The luteinizing hormone surge required secretion of GnRH. *J Clin Endocrinol Metab*, **78**(2): 343-347.

87. **McLachlan, RI, *et al.*,** (1989). The importance of luteinizing hormone in the control of inhibin and progesterone secretion by the human corpus luteum. *J Clin Endocrinol Metab*, **68**(6): 1078-1085.

88. **Ditkoff, EC, *et al.*,** (1991). The gonadotropin-releasing hormone antagonist (Nal-Glu) acutely blocks the luteinizing hormone surge but allows for resumption of folliculogenesis in normal women. *Am J Obstet Gynecol,* **165**(6 Pt 1): 1811-1817.

89. **Leroy, I, *et al.*,** (1994). A single injection of a gonadotropin-releasing hormone (GnRH) antagonist (Cetrorelix) postpones the luteinizing hormone (LH) surge: further evidence for the role of GnRH during the LH surge. *Fertil Steril,* **62**(3): 461-467.

90. **Sommer, L, *et al.*,** (1994). Seven-day administration of the gonadotropin-releasing hormone antagonist Cetrorelix in normal cycling women. *Eur J Endocrinol,* **131**(3): 280-285.

91. **Borghi, MR, *et al.*,** (1983). Administration of agonistic and antagonistic analogues of LH-RH induce anovulation in Macaca fasicularis. *Contraception,* **27**(6): 619-626.

92. **Fraser, HM, *et al.*,** (1990). Long-term suppression of ovarian function by a luteinizing-hormone releasing hormone agonist implant in patients with endometriosis. *Fertil Steril,* **53**(1): 61-68.

93. **Gudmundsson, JA, *et al.*,** (1986). Intranasal peptide contraception by inhibition of ovulation with the gonadotropin-releasing hormone superagonist nafarelin: six months' clinical results. *Fertil Steril,* **45**(6): 617-623.

94. **Carter, DC and Ho, JX,** (1994). Structure of serum albumin. *Adv Protein Chem,* **45**: 153-203.

95. **Westphal, U,** (1983). Steroid-protein interaction: From past to present. *J Ster Biochem,* **19**(1): 1-15.

96. **Westphal, U and Harding, GB,** (1973). Steroid-protein interactions. XXVII. Progesterone binding to polymers of human serum albumin. *Biochim Biophys Acta,* **310**(2): 518-527.

97. **Mickelson, KE, *et al.*,** (1981). Steroid-protein interactions. Human corticosteroid binding globulin: Some physicochemical properties and binding specificity. *Biochemistry,* **20**: 6211-6218.

98. **Yates, FE and Urquhart, J,** (1962). Control of plasma concentrations of adrenocortical hormones. *Physiol Rev,* **42**: 359-443.

99. **Daughaday, WH,** (1959). Steroid protein interactions. *Physiol Rev,* **39**: 885-902.

100. **Pardridge, WM,** (1981). Transport of protein-bound hormones into tissues in vivo. *Endocr Rev,* **2**(1): 103-123.

101. **Schmid, K, *et al.*,** (1950). Preparation and properties of an acid glycoprotein prepared from human plasma. *J Am Chem Soc,* **72**:2816

102. **Weimer, HE, *et al.*,** (1950). Studies on the mucoproteins of human plasma. V. Isolation and characterization of a homogenous mucoprotein. *J Bio Chem,* **185**: 561-568.

103. **Fournier, T, *et al.*,** (2000). Alpha-1-acid glycoprotein. *Biochim Biophys Acta,* **1482**(1-2): 157-171.

104. **Szallasi, A, *et al.*,** (1992). Identification of alpha-1-acid glycoprotein (orosomucoid) as a major vanilloid binding protein in serum. *J Pharmacol Exp Ther,* **262**(3): 883-888.

105. **McNamara, PJ, *et al.*,** (1986). Autacoid binding to serum proteins. Interaction of platelet activating factor (PAF) with human serum alpha-1-acid glycoprotein (AAG). *Biochem Pharmacol,* **35**(4): 621-624.

106. **Morin, D, *et al.*,** (1997). Melatonin high-affinity binding to alpha-1-acid glycoprotein in human serum. *Pharmacology,* **54**(5): 271-275.

107. **Chachaj, W, *et al.*,** (1980). Histamine binding proteins separated from human sera by the chromatographic method. *Arch Immunol Ther Exp (Warsz),* **28**(6): 947-951.

108. **Grimaldi, B, *et al.*,** (1989). In vitro study of the binding of RU 486 and RU 42 633 to human serum proteins. *Prog Clin Biol Res,* **300**: 445-448.

109. **Albani, F, *et al.*,** (1984). Stereoselective binding of propranolol enantiomers to human alpha 1-acid glycoprotein and human plasma. *Br J Clin Pharmacol,* **18**(2): 244-246.

110. **Burton, RM and Westphal, U,** (1972). Steroid hormone-binding proteins in blood plasma. *Metabolism,* **21**(3): 253-276.

111. **Heap, RB, *et al.*,** (1981). Progesterone-binding proteins in plasma of guinea-pigs and other hystricomorph rodents. *J Reprod Fertil,* **63**(2): 477-489.

112. **Mickelson, KE and Westphal, U,** (1980). Steroid-protein interactions. Influence of steroid structure and temperature on the binding of steroids to guinea pig corticosteroid-binding globulin. *Biochemistry,* **19**(3): 585-590.

113. **Stroupe, SD, *et al.*,** (1978). Kinetic and equilibrium studies on steroid interaction with human corticosteroid-binding globulin. *Biochemistry,* **17**(1): 177-182.

114. **Klosterman, LL, *et al.*,** (1986). Cortisol levels, binding, and properties of corticosteroid-binding globulin in the serum of primates. *Endocrinology,* **118**(1): 424-434.

115. **Anderson, D,** (1974). Sex-hormone-binding globulin. *Clinical Endocrinology,* **3**: 69-96.

116. **Mendel, CM,** (1989). The free hormone hypothesis: A physiologically based mathematical model. *Endocr Rev,* **10**(3): 232-274.

117. **Rosner, W, *et al.*,** (1999). Sex hormone-binding globulin mediates steroid hormone signal transduction at the plasma membrane. *J Ster Biochem Mol Bio,* **69**(1-6): 481-485.

118. **Maitra, US, *et al.*,** (1993). Corticosteroid-binding globulin receptor of the rat hepatic membrane: solubilization, partial characterization, and the effect of steroids on binding. *Endocrinology,* **133**(4): 1817-1822.

119. **Strelchyonok, OA and Avvakumov, GV,** (1991). Interaction of human CBG with cell membranes. *J Ster Biochem Mol Bio,* **40**(4-6): 795-803.

120. **Mickelson, KE, *et al.*,** (1978). Characterization of the sex steroid binding protein of human pregnancy serum. Improvements in the purification procedure. *Biochemistry,* **17**: 1409-1415.

121. **Rosner, W and Smith, RN,** (1975). Isolation and characterization of the testosterone-estradiol-binding globulin from human plasma. Use of a novel affinity column. *Biochemistry,* **14**: 4813-4820.

122. **Stroupe, SD and Westphal, U,** (1975). Steroid-protein interactions. Stopped flow fluorescence studies of the interaction between steroid hormones and progesterone binding globulin. *J Bio Chem,* **250**: 8735-8739.

123. **Fernlund, P and Gershagen, S,** (1990). 2-Iodoestradiol binds with high affinity to human sex hormone binding globulin (SHBG). *J Ster Biochem,* **36**(1/2): 75-81.

124. **Gershagen, S, *et al.*,** (1978). Subunits of human sex hormone binding globulin (interindividual variation in size). *J Bio Chem,* **262**(17): 8430-8437.

125. **Weigratz, I, *et al.*,** (1995). Effect of two oral contraceptives containing ethinylestradiol and gestogene or norgestimate upon androgen parameters and serum binding proteins. *Contraception,* **51**: 341-346.

126. **Coenen, CMH, *et al.*,** (1996). Changes in androgens during treatment with four low-dose contraceptives. *Contraception,* **53**: 171-176.

127. **Kuhl, H, *et al.*,** (1995). Prodrug versus drug effects of 150 micrograms desogestrel or 3-keto-desogestrel in combination with 30 micrograms ethinylestradiol on hormonal parameters: relevance of the peak serum level of 3-keto-desogestrel. *Horm Res,* **44**(3): 126-132.

128. **Dibbelt, L, *et al.*,** (1991). Group comparison of serum ethinyl estradiol, SHBG and CBG levels in 83 women using two low-dose combination oral contraceptives for three months. *Contraception*, **43**(1): 1-21.

129. **Humpel, M, *et al.*,** (1990). Comparison of serum ethinyl estradiol, sex-hormone-binding globulin, corticoid-binding globulin and cortisol levels in women using two low-dose combined oral contraceptives. *Horm Res*, **33**(1): 35-39.

130. **Jung-Hoffmann, C and Kuhl, H,** (1989). Interaction with the pharmacokinetics of ethinylestradiol and progestogens contained in oral contraceptives. *Contraception*, **40**(3): 299-312.

131. **Cullberg, G,** (1985). Pharmacodynamic studies on desogestrel administered alone and in combination with ethinylestradiol. *Acta Obstet Gynecol Scand Suppl*, **133**: 1-30.

132. **Cunningham, GR, *et al.*,** (1981). Steroid structural requirements for high affinity binding to human sex steroid binding protein (SBP). *Steroids*, **38**(3): 243-262.

133. **Bardin, CW, *et al.*,** (1981). Extracellular androgen binding proteins. *Ann Rev Physiol*, **43**: 189-198.

134. **Heyns, W and Coolens, JL,** (1988). Physiology of corticosteroid-binding globulin in humans. *Ann N Y Acad Sci*, **538**: 122-129.

135. **Rosenthal, HE, *et al.*,** (1969). Transcortin: A corticosteroid-binding protein of plasma. X. Cortisol and progesterone interplay and unbound levels of these steroids in pregnancy. *J Clin Endocrinol*, **29**: 352-.

136. **Basdevant, A, *et al.*,** (1993). Hemostatic and metabolic effects of lowering the ethinyl-estradiol dose from 30mcg to 20mcg in oral contraceptives containing desogestrel. *Contraception*, **48**(3): 193-204.

137. **Burton, RM, *et al.*,** (1971). Steroid-protein interactions. XXIII. Nonidentity of cortisol-binding globulin and progesterone-binding globulin in guinea pig serum. *Steroids*, **17**: 1-.

138. **Westphal, U, *et al.*,** (1977). Steroid interactions with progesterone-binding globulin. *J Ster Biochem*, **8**: 367-374.

139. **Blanford, T, *et al.*,** (1978). Steroid-protein interactions-XXXVIII. Influence of steroid structure on affinity to the progesterone-binding globulin. *J Ster Biochem*, **9**: 187-201.

140. **Fricker, G and Drewe, J,** (1996). Current concepts in intestinal peptide absorption. *J Pept Sci*, **2**(4): 195-211.

141. **Swaan, PW, *et al.*,** (1997). Enhanced transepithelial transport of peptides by conjugation to cholic acid. *Bioconjugate Chem*, **8**: 520-525.

142. **Ho, NFH,** (1987). Utilizing bile acid carrier mechanisms to enhance liver and small intestine absorption. *Ann N Y Acad Sci*: 315-329.

143. **Kramer, W, *et al.*,** (1992). Liver-specific drug targeting by coupling to bile acids. *J Bio Chem*, **267**(26): 18598-18604.

144. **Kramer, W, *et al.*,** (1994). Intestinal absorption of peptides by coupling to bile acids. *J Bio Chem*, **269**(14): 10621-10627.

145. **Battersby, AR,** (1994). How nature builds the pigments of life: the conquest of vitamin B12. *Science*, **264**(5165): 1551-1557.

146. **Sourial, NA and Waters, AH,** (1992). Effect of pH on binding of B12 to IF and R-protein. *Br J Haematol*, **81**(1): 136-138.

147. **Allen, RH, *et al.*,** (1978). Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor. In vitro evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *J Clin Invest*, **61**(1): 47-54.

148. **Marcoullis, G, *et al.*,** (1980). Cobalamin malabsorption due to nondegradation of R proteins in the human intestine. Inhibited cobalamin absorption in exocrine pancreatic dysfunction. *J Clin Invest*, **66**(3): 430-440.

149. **Howard, TA, *et al.*,** (1996). Human gastric intrinsic factor expression is not restricted to parietal cells. *J Anat*, **189 (Pt 2)**: 303-313.

150. **Kapadia, CR and Essandoh, LK,** (1988). Active absorption of vitamin B12 and conjugated bile salts by guinea pig ileum occurs in villous and not crypt cells. *Dig Dis Sci*, **33**(11): 1377-1382.

151. **Seetharam, B,** (1999). Receptor-mediated endocytosis of cobalamin (vitamin B12). *Annu Rev Nutr*, **19**: 173-195.

152. **Allen, RH and Mehlman, CS,** (1973). Isolation of gastric vitamin B 12 -binding proteins using affinity chromatography. I. Purification and properties of human intrinsic factor. *J Biol Chem*, **248**(10): 3660-3669.

153. **Gueant, JL, *et al.*,** (1985). Purification of human intrinsic factor using high-performance ion-exchange chromatography as the final step. *FEBS Lett*, **184**(1): 14-19.

154. **Russell-Jones, GJ,** (1998). Use of vitamin B12 conjugates to deliver protein drugs by the oral route. *Crit Rev Ther Drug*, **15**(6): 557-586.

155. **Russell-Jones, GJ,** Oral delivery of therapeutic proteins and peptides by the vitamin B_{12} uptake system, in *Peptide-based drug design. Controlling transport and metabolism*, Taylor, MDA, G.L., Editor. 1995, American Chemical Society. p. 181-198.

156. **Habberfield, A, *et al.*,** (1996). Vitamin B12-mediated uptake of recombinant therapeutic proteins from the gut. *Int. J Pharmacol*, **145**: 1.

157. **Alsenz, J, *et al.*,** (2000). Oral absorption of peptides through the cobalamin (vitamin B12) pathway in the rat intestine. *Pharmaceut Res*, **17**(7): 825-832.

158. **Russell-Jones, GJ, *et al.*,** (1995). Synthesis of LHRH antagonists suitable for oral administration via the Vitamin B12 uptake system. *Bioconjugate Chem*, **6**: 34-42.

159. **Russell-Jones, GJ, *et al.*,** (1995). Vitamin B12 mediated oral delivery systems for granulocyte-colony stimulating factor and erythropoietin. *Bioconjugate Chem*, **6**(4): 459-465.

160. **Russell-Jones, GJ, *et al.*,** (1999). Vitamin B12-mediated transport of nanoparticles across Caco-2 cells. *Int J Pharm*, **179**(2): 247-255.

161. **Mathews, DM and Adibi, SA,** (1976). Peptide absorption. *Gastroenterology*, **71**(1): 151-161.

162. **Navab, F and Asatoor, AM,** (1970). Studies on intestinal absorption of amino acids and a dipeptide in a case of Hartnup disease. *Gut*, **11**(5): 373-379.

163. **Fix, JA,** (1996). Strategies for delivery of peptides utilizing absorption-enhancing agents. *J Pharm Sci*, **85**(12): 1282-1285.

164. **Thanou, M, *et al.*,** (2001). Mono-N-carboxymethyl chitosan (MCC), a polyampholytic chitosan derivative, enhances the intestinal absorption of low molecular weight heparin across intestinal epithelia in vitro and in vivo. *J Pharm Sci*, **90**(1): 38-46.

165. **Thanou, M, *et al.*,** (2001). Oral drug absorption enhancement by chitosan and its derivatives. *Adv Drug Deliv Rev*, **52**(2): 117-126.

166. **Thanou, M, *et al.*,** (2001). Chitosan and its derivatives as intestinal absorption enhancers. *Adv Drug Deliv Rev*, **50 Suppl 1**: S91-101.

167. **Thanou, M, *et al.*,** (2001). Enhancement of the intestinal absorption of low molecular weight heparin (LMWH) in rats and pigs using Carbopol 934P. *Pharmaceut Res*, **18**(11): 1638-1641.

168. **Thanou, M, *et al.*,** (2001). Intestinal absorption of octreotide using trimethyl chitosan chloride: studies in pigs. *Pharmaceut Res*, **18**(6): 823-828.

169. **Sanders, LM, *et al.*,** (1986). Prolonged controlled-release of nafarelin, a luteinizing hormone-releasing hormone analogue, from biodegradable polymeric implants: influence of composition and molecular weight of polymer. *J Pharm Sci*, **75**(4): 356-360.

170. **Okada, H, *et al.*,** (1996). Persistent suppression of the pituitary-gonadal system in female rats by three-month depot injectable microspheres of leuprorelin acetate. *J Pharm Sci*, **85**(10): 1044-1048.

171. **Okada, H, *et al.*,** (1994). Sustained suppression of the pituitary-gonadal axis by leuprorelin three-month depot microspheres in rats and dogs. *Pharmaceut Res*, **11**(8): 1199-1203.

172. **Okada, H, *et al.*,** (1994). Preparation of three-month depot injectable microspheres of leuprorelin acetate using biodegradable polymers. *Pharmaceut Res*, **11**(8): 1143-1147.

173. **Sanders, LM,** (1990). Drug delivery systems and routes of administration of peptide and protein drugs. *Eur J Drug Metab Pharmacokinet*, **15**(2): 95-102.

174. **Mazer, NA,** (1989). Pharmacological and biophysical considerations in polypeptide delivery: input functions and routes of administration. *J Pharm Sci*, **78**: 885-887.

175. **Yahalom, D, *et al.*,** (1999). Synthesis and bioactivity of fatty acid-conjugated GnRH derivatives. *Life Sci*, **64**(17): 1543-1552.

176. **Sandow, J,** (1983). Clinical applications of LHRH and its analogues. *Clin Endocrinol*, **18**(571-592).

177. **Nicoli, S, *et al.*,** (2001). Physical and chemical enhancement of transdermal delivery of triptorelin. *Pharmaceut Res*, **18**(11): 1634-1637.

178. **Smith, SW and Anderson, DB,** (1995). Human skin permeability enhancement by lauric acid under equilibrium aqueous conditions. *J Pharm Sci*, **84**: 551-556.

179. **Anderson, L, *et al.*,** (1995). Rapid desensitization of GnRH-stimulated intracellular signalling events in aT3-1 and HEK-293 cells expressing the GnRH receptor. *Endocrinology*, **136**(11): 5228-5231.

180. **Flanagan, CA, *et al.*,** (1998). A high affinity gonadotropin-releasing hormone (GnRH) tracer, radioiodinated at position 6, facilitates analysis of mutant GnRH receptors. *Endocrinology*, **139**(10): 4115-4119.

181. **Hammond, GL and Lahteenmaki, PL,** (1983). A versatile method for the determination of serum cortisol binding globulin and sex hormone binding globulin binding capacities. *Clin Chim Acta*, **132**(1): 101-110.

182. **Westphal, U, *et al.*,** (1977). Progesterone binding to serum proteins. *Ann N Y Acad Sci*, **286**: 10-28.

183. **Hearn, JP, *et al.*,** (1978). Use of the common marmoset, Callithrix jacchus, in reproductive research. *Primates Med*, **10**: 40-49.

184. **Miller, LL, *et al.*,** (1990). Transdermal iontophoresis of gonadotropin releasing hormone (LHRH) and two analogues. *J Pharm Sci*, **79**: 490-493.

185. **Nicoli, S, *et al.*,** (2001). Design of triptorelin loaded nanospheres for transdermal iontophoretic administration. *Int J Pharmaceut*, **214**: 31-35.

186. **Danforth, DR, *et al.*,** (1990). Extended presence of antide (Nal-Lys GnRH antagonist) in circulation: prolonged duration of gonadotropin inhibition may derive from antide binding to serum proteins. *J Clin Endocrinol Metab*, **70**(2): 554-556.

187. **Barron, JL, *et al.*,** (1982). Metabolic clearance and plasma half-disappearance time of D-Trp6 and exogenous luteinizing hormone-releasing hormone. *J Clin Endocrinol Metab*, **54**(6): 1169-1173.

188. **Handelsman, DJ and Swerdloff, RS,** (1986). Pharmacokinetics of gonadotropin-releasing hormone and its analogs. *Endocr Rev*, **7**(1): 95-105.

189. **Rabinovici, J, *et al.*,** (1992). Endocrine effects and pharmacokinetic characteristics of a potent new gonadotropin-releasing hormone antagonist (Ganirelix) with minimal histamine-releasing properties: studies in postmenopausal women. *J Clin Endocrinol Metab*, **75**(5): 1220-1225.

190. **Mattox, VR, *et al.*,** (1979). A comparison of procedures for attaching steroidal glucosiduronic acids to bovine serum albumin. *J Ster Biochem*, **10**: 167-172.

191. **Rajkowski, KM and Cittanova, N,** (1981). The efficiency of different coupling procedures for the linkage of oestriol-16a-glucuronide and pregnanediol-3a-glucuronide to four different enzymes. *J Ster Biochem*, **14**: 861-866.

192. **Grosse, R, *et al.*,** (2000). Gonadotropin-releasing hormone receptor initiates multiple signaling pathways by exclusively coupling to G(q/11) proteins. *J Bio Chem*, **275**(13): 9193-9200.

193. **Naor, Z, *et al.*,** (1995). Signal transduction of the gonadotropin releasing hormone (GnRH) receptor: cross-talk of calcium, protein kinase C (PKC), and arachidonic acid. *Cell Mol Neurobiol*, **15**(5): 527-544.

194. **Naor, Z, *et al.*,** (1998). Mechanism of GnRH receptor signaling: combinatorial cross-talk of Ca2+ and protein kinase C. *Front Neuroendocrin*, **19**(1): 1-19.

195. **Rahimipour, S, *et al.*,** (2001). Generation of free radicals by emodic acid and its [D-Lys6]GnRH-conjugate. *Photochem Photobiol*, **74**(2): 226-236.

196. **Williams, SP and Sigler, PB,** (1998). Atomic structure of progesterone complexed with its receptor. *Nature*, **393**(6683): 392-396.

197. **Bursi, R and Groen, MB,** (2000). Application of (quantitative) structure-activity relationships to progestagens: from serendipity to structure-based design. *Eur J Med Chem*, **35**(9): 787-796.

198. **Nagy, A, *et al.*,** (2000). Stability of cytotoxic luteinizing hormone-releasing hormone conjugate (AN-152) containing doxorubicin 14-O-hemiglutarate in mouse and human serum in vitro: Implications for the design of preclinical studies. *Proc Natl Acad Sci U S A*, **97**(2): 829-834.

199. **Robinson, PA, *et al.*,** (1985). A phylogenetic study of the structural and functional characteristics of corticosteroid binding globulin in primates. *J Endocrinol*, **104**(2): 251-257.

200. **Kagedahl, M, *et al.*,** (1997). Use of the intestinal bile acid transporter for the uptake of cholic acid conjugates with HIV-1 protease inhibitory activity. *Pharmaceut Res*, **14**(2): 176-180.

201. **Swaan, PW, *et al.*,** (1996). Use of intestinal and hepatic bile acid transporters for drug delivery. *Adv Drug Deliv Rev*, **20**: 59-82.

202. **Hoogstraate, AJ, *et al.*,** (1996). In vivo buccal delivery of the peptide drug buserelin with glycodeoxycholate as an absorption enhancer in pigs. *Pharm Res*, **13**(8): 1233-1237.

203. **Russell-Jones, GJ and Alpers, DH,** (1999). Vitamin B12 transporters. *Pharmaceut Biotechnol*, **12**: 493-520.

204. **Seetharam, B, *et al.*,** (1999). Cellular import of cobalamin (Vitamin B-12). *J Nutr*, **129**(10): 1761-1764.

205. **Westphal, U,** (1983). Corticosteroid-binding globulin. A review of some recent aspects. *Mol Cell Biol*, **55**: 145-157.

206. **Meunier, V, *et al.*,** (1995). The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. *Cell Biol Toxicol*, **11**(3-4): 187-194.

207. **Nagy, A, *et al.*,** (1993). Selective coupling of methotrexate to peptide hormone carriers through a g-carboxamide linkage of its glutamic acid moiety: Benzotriazol-1-yloxtris(dimethylamino)phosphonium hexafluorophosphate activation in salt coupling. *Proc Natl Acad Sci U S A*, **90**: 6373-6376.

208. **Malmgren, L, *et al.*,** (2001). Effect of GnRH immunisation on hormonal levels, sexual behaviour, semen quality and testicular morphology in mature stallions. *Equine Vet J*, **33**(1): 75-83.

209. **Hotzel, MJ, *et al.*,** (1997). Effects of nutrition on testicular growth in mature Merino rams actively immunized against GnRH. *J Reprod Fertil*, **110**(2): 307-313.

210. **Garza, F, Jr., *et al.*,** (1988). Effects of active immunization against gonadotropin releasing hormone on gonadotropin secretion after ovariectomy and testosterone propionate administration to mares. *J Anim Sci*, **66**(2): 479-486.

211. **Singh, V,** (1991). Effect on immunoreactivity of gonadotropin releasing hormone (GnRH) in the GnRH-BSA conjugates of variable stoichiometry. *Biochem Int*, **25**(3): 509-520.

212. **McEwan, JF, *et al.*,** (1999). Synthesis and biological activity of ribose-5'-carbamate derivatives of vitamin B(12). *Bioconjug Chem*, **10**(6): 1131-1136.

213. **Minassian, SS and Wu, CH,** (1993). Free and protein-bound progesterone during normal and luteal phase defective cycles. *Int J Obs Gyn*, **43**(2): 163-168.