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Functional Characterisation of Gonadotropin-Releasing Hormone-Estrogen Conjugates

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"Alone we can do so little; together we can do so much." – Helen Keller

List of Figures and Tables

Abbreviations

ADT – Androgen Deprivation Therapy

AIPC – Androgen Independent Prostate Cancer

AR – Androgen Receptor

ArKO – Aromatase Knockout

BDT – Bone Density Testing

BMD – Bone Mineral Density

CA – Carbonic Anhydrase

CTSK – Cathepsin K

CVD – Cardiovascular Disease

CVS – Crystal Violet Staining

DAG - Diacylglycerol

DC-STAMP – Dendritic Cell-Specific Transmembrane Protein

DES – Diethylstilboestrol

D-Lys6 GnRH Pro9 ethylamide – GnRH agonist analogue with a D-lysine substitution at position six and substitution of the carboxyl terminal with ethylamide

DMEM - Dulbecco's Modified Eagle Medium

E2 - 17β-Estradiol

E2C – D-Lys6 GnRH Pro9 ethylamide -17β –Estradiol Conjugate

EDC – Estrogen Dimer Conjugate

ER – Estrogen Receptor

ERE – Estrogen Response Element

ERα – Estrogen Receptor Alpha

ERαAF-1 – Activation Function 1

ERβ – Estrogen Receptor Beta

FCS – Foetal Calf Serum

FSH – Follicle Stimulating Hormone

Gen - Genistein

GenC – D-Lys6 GnRH Pro9 ethylamide -Genistein Conjugate

GnRH - Gonadotropin-releasing hormone

GnRHag - D-Lys6 GnRH Pro9 ethylamide

GnRHR - Gonadotropin-releasing hormone receptor

GPCR – G protein coupled receptor

GPER - G protein-coupled estrogen receptor 1

GDP - Guanosine diphosphate

GTP - Guanosine Triphosphate

HPG - Hypothalamic–Pituitary–Gonadal

HRT – Hormone Replacement Therapy

IP – Inositol Phosphate

IP_3 – Inositol 1,4,5-trisphosphate

LB – Luria Bertani

LBD – Ligand Binding Domain

LH – Luteinizing Hormone

M-CSF – Macrophage Colony Stimulating Factor

MMP 9 – Matrix Metalloproteinase 9

NCCN – National Comprehensive Cancer Network

PC – Prostate Cancer

PIP2 - phosphatidylinositol 4,5-bisphosphate

PLB - Passive lysis buffer

PLC - Phospholipase C

PSA – Prostate Specific Antigen

RANKL - Receptor activator of nuclear factor kappa-B ligand

SERM – Selective Estrogen Receptor Modulator

SQM - Squamous metaplasia

TMD – Transmembrane Domain

TMPRSS2 - 5'-untranslated region of the androgen-regulated transmembrane protease, serine 2

TNF – Tumour Necrosis Factor

TRAP - Tartrate Resistant Acid Phosphatase

WT – Wild type

XTG – Extreme Gene Transfection Reagent

Abstract

Prostate cancer (PC) is the second most commonly occurring cancer in men, and the fourth most common commonly occurring cancer overall. Almost all PC begins in an androgen-dependent state with androgen deprivation therapy (ADT) an effective treatment at this stage. PC can overtime develop into an androgen independent state at which point it can no longer be treated with ADT. The focus of this research is on androgen dependent PC and ADT. The hypothalamic–pituitary–gonadal (HPG) axis, controlled by gonadotropin releasing hormone (GnRH) is responsible for regulating reproduction, puberty and the production of spermatozoa and androgens in men. GnRH analogues (which downregulate the axis) are the foremost ADT agents. GnRH analogues may also have direct anti-proliferative effects in some cancers. However, ADT has negative side effects including loss of bone mass, hot flushes and loss of libido, due to a concomitant decrease in estrogen which is synthesised from androgen. We hypothesise that a molecule which retains GnRH receptor (GnRHR) activation while replacing estrogen activity which activates the estrogen receptors (ERs) may be beneficial. Conjugates of GnRH analogue (GnRHag) with 17β-Estradiol (E2C) or genistein (GenC) were studied examining GnRH and estrogen activity *in vitro* to evaluate their potential as novel PC therapeutics.

Synthesis of the conjugates was commissioned from a commercial company. GnRHR activation was tested in HEK 293T cells by determining the generation of inositol phosphate in cells expressing GnRHR. ER activation was determined in MCF-7 cells using an E-screen assay in a cell line expressing ERs. Anti-proliferative effects were assessed in PC cell lines by crystal violet assay. Potential bone-protective capability was measured by ability to inhibit RANKL-induced osteoclast differentiation of Raw 264.7 macrophages.

E2C and GenC elicited potent stimulation of GnRHR. The conjugates also had estrogenic activity similar to the unconjugated estrogen and phytoestrogen in the E-screen assay. Their estrogenic activities were confirmed by their ability to inhibit osteoclast differentiation to the same degree as unconjugated 17β-Estradiol and genistein. No direct antiproliferative effects, by the conjugates or GnRH, on PC cells were observed, indicating that GnRHR may not be

expressed in these cell lines. The demonstration that E2C and GenC displayed GnRHR and ER activities similar to their unconjugated counterparts suggests they may be efficacious as ADT agents with reduced side effects of estrogen deprivation.

Key Words: GnRH analogues, Prostate Cancer, Androgen Deprivation Therapy, Estrogen Deficiency, RANKL, Hot Flushes, Libido, Bone Loss

1.0 Literature Review

1.1 Prostate cancer

Prostate cancer (PC) is the second most common male cancer worldwide, and it accounts for an estimated quarter of new cancer cases in men. Worldwide it is the third most common cause of cancer related deaths, [1,2] with 7.1% of cancer deaths being attributed to PC.[2] PC generally presents as prostate urinary symptoms, including but not limited to a slow flow of urine, trouble starting or stopping flow of urine, urinating more frequently, pain when urinating, blood in the urine or semen and a feeling of not being able to fully empty bladder.[3] It is important to note though that these symptoms could also be due to the natural enlargement of the prostate that occurs in men as they age. PC is usually suspected on the basis of symptoms and prostate specific antigen (PSA) levels.[4] PSA is a protein made by the prostate gland. A raised level of PSA is not indicative of PC in itself but can be useful in establishing whether further testing, such as a digital rectal exam is required.[5] Once diagnosed PC will be graded according to the Gleason grading system. This system is based on examination of biopsy samples and the microscopic appearance of the PC. Cancers with a higher Gleason score tend to be more aggressive with a worse prognosis.[6] This then contributes to the staging of the cancer (PC tends to be grouped into one of four stages) which evaluates the size of the tumour, the extent of involved lymph nodes, whether the tumour is confined to only the prostate or not, whether metastasis has occurred and also takes into account cancer grade.[6] PC that is confined to the prostate gland is termed localised PC, if it has extended beyond the prostate gland into the seminal vesicles or surrounding organs such as the bladder or rectum it is considered to be locally advanced PC, once spread to distant parts of the body such as bone it is termed metastatic PC.[7] The five- and ten-year survival rate for men with local or regional PC is 100% and 98% respectively. If diagnosis is made after the cancer has metastasised the five-year survival rate drops to around 30%. [8] The incidence of PC continues to increase, with the annual rate of increase estimated to be between 2- and 3%.[9] The worldwide increase in incidence and mortality due to this disease is a force driving the need to find treatments with higher efficacy and improved quality of life for patients.

1.2 Role of the Hypothalamic-Pituitary- Gonadal Axis in Prostate Cancer

The hypothalamic–pituitary–gonadal axis (HPG axis, Figure 1.1) controls reproduction and puberty, and in men is responsible for the regulation of spermatogenesis and the synthesis and secretion of androgens from the testes.[10] There are three main differentiating cell types within the developing testes, gamete forming cells (spermatogonia), support cells (Sertoli cells) and hormone secreting cells (Leydig or interstitial cells). Sertoli cells provide nourishment for the developing sperm cells; terminate defective sperm cells; secrete fluid that aids in the transport of sperm into the epididymis and they release the hormone inhibin that aids in regulation of sperm production. The luminal environment as controlled by these Sertoli cells is controlled by follicle stimulating hormone (FSH) and inhibin. Leydig cells are the endocrine cells in the testis that are responsible for the production of testosterone from cholesterol via a series of enzymatic pathways and steroidal intermediates under the control of luteinizing hormone (LH) secreted from the pituitary.[10] Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and is transported to the anterior pituitary where it acts on GnRH receptors (GnRHRs) to stimulate the secretion of LH and FSH, which through positive feedback stimulate the Leydig and Sertoli cells respectively. Testosterone under the stimulation by LH is hydrophobic and enters the cytoplasm of all tissues but only acts on tissues and cells such as the prostate which express the androgen receptor (AR). Testosterone can either bind directly to AR or can be converted to 5-alpha-dihydrotestosterone by 5 alpha reductase which binds with higher affinity to the AR than testosterone. The androgen-AR complex exerts an effect by acting as a transcription factor to influence gene expression, and thus cellular physiology, by entering the nucleus of the cell and interacting with specific DNA regions.[11,12] The HPG axis is regulated by negative feedback loops of androgens (testosterone) and proteins, such as inhibin, on the anterior pituitary and/or hypothalamus.[13] Activin and inhibin are two closely related proteins that have opposing biological effects. Activin enhances FSH biosynthesis and secretion, while inhibin downregulates FSH synthesis and inhibits FSH secretion. Decreased production of LH and FSH results in a decrease in production of testosterone and inhibin. [14,15]

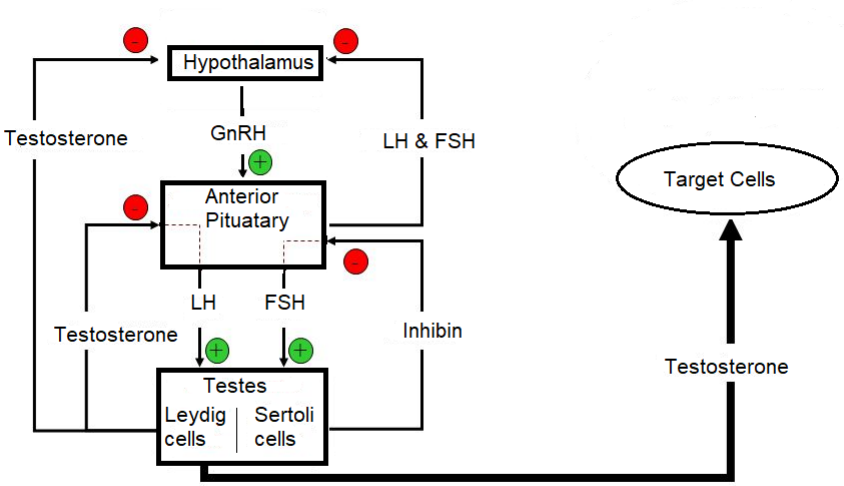


Figure 1.1 ***Major components of the hypothalamus-pituitary-gonadal axis***

Schematic representation of the major components of the hypothalamus-pituitary-gonadal axis and recognized hormone (Inhibin, testosterone, gonadotropin releasing hormone (GnRH), luteinising hormone (LH) and follicle stimulating hormone (FSH)) feedback pathways showing positive (green) and negative (red) feedback drawn by S. Leijenaar in Microsoft PowerPoint, modified from[16].

Although the etiology, risk factors and molecular mechanisms of PC are not particularly well understood, an extensive set of studies robustly shows support for the role of androgen, testosterone in particular, in the development of PC.[1,9] Almost all PC starts as an androgen-dependent disease.[17] Androgen stimulation of growth of the prostate continues throughout life, where they regulate intrinsic growth regulatory factors produced by the prostate cells.[9] Androgens can therefore promote carcinogenesis merely by promoting rounds of cell division.[18] For a portion (around 30-40%)[19] of patients initially diagnosed with androgen-dependent PC, treatment will fail, with PC reoccurring and becoming metastatic androgen independent PC (AIPC), now frequently referred to as castration resistant PC.[20] Currently no effective treatment exists to treat AIPC and it frequently progresses and metastasises leading to it being a lethal form of PC.[21]

1.3 Therapies for Prostate Cancer

A number of different PC treatment options exist depending on the stage of the cancer and the choice of treatment may be informed by PSA levels or Gleason scores .[22] More extreme measures such as orchiectomy (removal of the testes), radical prostatectomy and other less drastic measures such as watchful waiting, active surveillance and palliative intent may be used individually or in conjunction to treat PC. However, hormonal treatment through androgen deprivation therapy, (ADT) remains the primary treatment of this disease if it is in an androgen dependent state.[23] Although withdrawal of androgens through chemical or surgical means frequently leads to regression of the disease, this regression can however be temporary. Long term selection of androgen independence in ADT can cause a portion of patients to become "androgen refractory" resulting in AIPC.[9,24]

1.3.1 Androgen Deprivation Therapy

Activation of ARs by androgens is considered essential for both normal prostate development as well as PC progression.[25] Therefore, ADT or androgen suppression therapy, which removes or blocks the action of androgens is a major method used to treat PC.[26,27] ADT has increased in usage as the first line treatment against metastatic PC and has been considered the best treatment for advanced PC for more than fifty years.[9]

Diethylstilboestrol (DES), a synthetic estrogen, was the first hormonal ADT therapy used against cancer and while it has become less popular since other forms of hormone therapy have been discovered, low dose DES is still used today as a first- and second-line PC treatment. DES is an estrogenic compound, which suppresses secretion of LH (and thus androgens) through its negative feedback action on the hypothalamus and pituitary (as estrogen does in Figure 1.1). In addition, DES has some direct cytotoxic effects on the prostate, inhibiting proliferation and inducing apoptosis.[28] Administration of high levels of other sex steroids, such as progesterone, achieves similar suppression of LH release through feedback inhibition of the HPG axis.[9,28]

More recently, GnRH analogues have become the foremost therapeutic option for ADT. [29] Both GnRH agonists and antagonists have been utilised for this purpose. Agonists bind to GnRHRs and produce an initial intense stimulation leading to a marked increase in androgens, LH and FSH.[30] However, this surge or flare is transient and is followed by tachyphylaxis (a rapid decrease in response) resulting from pituitary desensitization.[31] Consequently, this leads to decreases in steroid hormone secretion. GnRH antagonists have the benefit of lacking the initial surge in LH production as well as the negative side effects associated with this flare.[32] However, antagonists need to be used at much higher doses in order to out-compete the endogenous GnRH, which presents challenges for administration of treatment. Furthermore, the first developed GnRH antagonists had histamine-releasing properties, due to a non-immunological mechanism. This causes excessive mediator release from the mast cells inducing an anaphylactoid reaction, a lack of sustained efficacy and/or solubility limitations. This has hampered the development of clinically useful drugs.[29,33] Modification of GnRH antagonist analogues by substitution of a basic residue in position 6 combined with a basic Arg in position 8 has eliminated this effect and results in no significant effect on basal histamine release (in the 3 to 300 μg ml^{-1} concentration range) (for more details regarding GnRH agonist and antagonist analogues see section 1.4).[33,34] None-the-less, GnRH agonists remain the preferred current first line treatment.[35,36] This may change should development occur of a novel delivery system for peptide antagonists or nonpeptide orally-active antagonists, or if long term survival rates are shown to be increased with the use of GnRH antagonists over agonists. [37]

In addition to their effects on the HPG axis, it has been found that patients with tumours that express GnRHRs that are treated with GnRH agonists are more likely to have a favourable outcome which is thought to be due to concurrent direct action of GnRH agonists (antitumor activity) on tumour cells expressing the GnRHRs.[38,39] The exact functioning of GnRH and its receptor at extra pituitary sites (such as malignant tumours), has not been robustly demonstrated. However, in a number of these sites it appears that they can inhibit cell proliferation and/or cause programmed cell death (apoptosis) *in vivo*. [40] However, the physiological relevance and impact of these observations remains to be fully elucidated.

1.3.1.1 Side Effects of ADT

The complex system of endocrine influences as the PC is starved of androgens has mental and physical consequences for those undergoing ADT treatment. The impact can be both immediate and long term.[41] Compared to the general population, men with PC have higher non-cancer mortality, some of this attributed to treatment.[42] It is well known that GnRH agonists used in ADT are associated with increased fat mass, and cardiovascular disease (CVD).[43] Other significant adverse side effects of ADT include bone loss, hot flushes, loss of libido, gynecomastia, serum lipid changes and memory loss need to be addressed more thoroughly.[44] Many of these side effects are due to reduced estrogen levels as a consequence of reduced androgens from which they are elaborated in men (see section 1.5).[45,46]

Early stage PC generally has a favourable prognosis with ADT treatments being highly effective in controlling metastatic PC, it therefore becomes important to assess and decide on treatment options as the impact from treatment on overall health and quality of life may be greater than that of PC itself as patient life expectancies increase. This is particularly true for men with local (cancer is retained in prostate capsule) or regional/local extension (cancer has moved out of the prostate capsule into seminal vesicles or other surrounding tissue) PC.[47] These men tend to have a more favourable prognosis and may be living with the associated side effects of treatment for many years post treatment. The use of PSA biomarker means earlier diagnosis and earlier detection of recurrent disease.[48] Therefore, the systemic side effects of androgen deprivation and quality of life have become more significant. Proactive intervention to reduce the physical and psychological consequences related to ADT is necessary. Optimizing the use of ADT therefore requires greater information and further study. Research is ongoing into possible approaches that will reduce the adverse side effects resulting from ADT.

ADT can be administered either continuously or intermittently. Two large phase III trials compared intermittent and continuous ADT using GnRH analogues. The National Cancer Institute of Canada PR-7 trial showed that the overall survival in men treated with intermittent ADT was not inferior to that of men treated with continuous ADT.[49] However,

results from The Southwest Oncology Group 9346 trial were inconclusive regarding inferiority of one method over the other.[50] Both of these studies did find that administration intermittently reduced a number of the side effects associated with ADT.[51] Therefore, the method and timing of the administration of ADT may go some-way to negating some of the associated negative side effects.[51] Tsai *et al*. found that men undergoing continuous ADT were at a higher risk for serious cardiovascular events, particularly heart failure, as well as being at a higher risk for fracture.[52] However, they found that overall survival is not significantly impacted by whether ADT is given continuously or intermittently. Intermittent ADT may also improve sexual and physical functioning when compared to continuous ADT.[41] However, further prospective studies are necessary to confirm the reduced side effects of with intermittent over continuous ADT. It should also be noted that other studies have found that intermittent ADT resulted in lower median survival. However, within these studies there were subgroups of patients, including those with symptomatic high burden disease and high initial PSA levels, in which intermittent therapy is not suitable due to the severity of the PC. [53]

1.3.1.1.1 Osteoporosis

Osteoporosis (OP) is defined as a systemic skeletal disease with low bone mass and micro-architectural deterioration of bone tissue being typical. This results in a consequent increase in bone fragility and susceptibility to fracture.[54,55] Cortical bone refers to the hard-outer layer of bones and is comparatively more dense than trabecular bone. Trabecular bone is the spongy internal tissue, it is an open cell porous network within the skeletal bones.[56] In ADT-mediated bone loss, both trabecular and cortical bone are affected, resulting in a decrease in bone mineral density (BMD).[57–60] However, the attenuation of BMD immediately after the menopause (due to decreased estrogen levels) is most pronounced in the sites of the skeleton composed of trabecular bone. Bone loss due to ADT (there is a concurrent loss of estrogen along with decreased testosterone from which it is elaborated in men) appears to be more pronounced at sites of trabecular bone.[59,61] Patients undergoing ADT have significant and long lasting decreases in BMD with effects increasing with increased time of treatment.[62] Within the first year of receiving ADT absolute BMD loss is around five percent.[57] The relationship between ADT and the incidence of osteoporosis is temporal with incidence at 49.2% at four years, 65.7% at eight years and 80.6% at ten years.[54] BMD is used as a

substitution for fracture risk, which itself is associated with an increased risk of mortality.[63] Cancer treatment-induced bone loss, including that from ADT, tends to be more severe and occurs more rapidly than does bone loss due to menopause or natural aging.[64] Men with PC undergoing ADT have 5- to 10-fold increased loss of bone density at multiple skeletal sites in comparison with healthy controls or men with PC not being treated with ADT.[65] As bone loss is greatest in the first year after beginning ADT, early preventive therapy would thus be key in addressing this side effect.[54,66] Even though organisations such as The National Comprehensive Cancer Network (NCCN) recommend that "in patients who will be undergoing therapy that lowers sex steroids, the NCCN Guidelines for Breast and Prostate Cancers recommend evaluation with baseline and periodic follow-up dual energy X-ray absorptiometry scans to evaluate bone health and risk of fracture" bone density testing (BDT) remains below ideal levels.[67] Kirk *et al*. found that BDT was only done in a minority of patients undergoing ADT (15% at the start of the three-year study and 20% at the end).[67] BDT was associated with a considerable increase in diagnosis and treatment of osteoporosis, with diagnosis of osteoporosis and fracture being increased substantially (ten and three-fold, respectively) after BDT, indicating that osteoporosis is likely under-diagnosed and under-treated among PC survivors initiating ADT. Experiencing a fracture significantly correlates with shorter survival of men with PC and in one study, the median overall survival was found to be 39 months longer in men that remained fracture free.[68]

There have been some studies exploring the use of co-therapies to try to reduce the BMD effects of ADT treatment. The activity of osteoclasts (bone-resorping cells) can be decreased, and thus induce a reduction in BMD, with the use of bisphosphonate. Bisphosphonates are synthetic compounds that inhibit the recruitment and activity of osteoclasts and induce osteoclast apoptosis reducing bone reabsorption.[68] However, this does not necessarily translate to an improved fracture risk, nor improved survival rates for those undergoing ADT.[69] Morrisey *et al*. found that in patients with castration-resistant PC, ADT induced severe bone loss, even in patients that were concurrently given bisphosphonate treatment.[70] Although other studies have shown improved BMD in PC patients undergoing ADT if bisphosphonates are given before and during therapy, there are associated negative side effects with employing this approach. Lang *et al*. found that the group in their study that gained the most benefit in terms of BMD from bisphosphonate treatment also experienced

the highest level of adverse events.[71] For example, bisphosphonates contain nitrogen and can induce the acute phase reaction resulting in influenza-like symptoms including fever, chills, fatigue, myalgia and arthralgia. These are most often experienced if the treatment is given intravenously but can occur if given orally. [72] Epigastric pain can also occur if treatment is administered orally. Furthermore pre- and post- treatment assessment is advised as bisphosphonate use can result in hypocalcaemia and osteonecrosis of the jaw.[73] Some patients are also poor candidates for bisphosphonate therapy, either due to co-morbidities including renal insufficiency, or due to ongoing fractures despite therapy.[74] Use of bisphosphonate may therefore only be appropriate depending on the patient profile including the presence of other risk factors and the ability of the patient to tolerate the treatment.

Denosumab is a monoclonal antibody that targets a stimulator of osteoclast formation/differentiation, receptor activator of nuclear factor kappa-B ligand (RANKL), which functions by impeding the bone resorbing function and survival of osteoclasts. Use of Denosumab has been shown to increase BMD and decrease fracture rates. When given in high doses for short periods there is increased time to skeletal related events as well as bone metastasis survival.[73] It has also been shown to decrease bone turnover markers such as tartrate resistant acid phosphatase (TRAP) and procollagen-1N-telopeptide.[75] In men receiving ADT for nonmetastatic PC Denosumab has been shown promising results associated with increased bone mineral density and a decrease in the incidence of new vertebral fractures among.[76,77] However, as with bisphosphonate, it carries the associated risk of hypocalcaemia and osteonecrosis of the jaw.[41]

Hormone replacement therapy (HRT) with estrogen is well documented as a treatment for post-menopausal BMD loss due to decreased levels of estrogen.[78] Indeed, a combination of estrogen replacement with bisphosphonate administration has been shown to be particularly effective at reducing trabecular bone loss.[79] Fewer clinical studies have been conducted addressing the effects of estrogen replacement in men. However, one study that compared testosterone (from which estrogen is elaborated) with bisphosphonate treatment, or a combination of both therapies, in men with low BMD found that there was no significant difference in improvement in BMD between groups treated with singular therapies or a combination of the two. All groups showed significant improvement in BMD to similar

degrees.[80] Support for the idea that this improvement in BMD is due to levels of estrogen was found in a study in which androgen supplementation was given to eugonadal men with osteoporosis for six months. The improvements in BMD in this study were positively correlated with a change in serum estradiol but not with a change in serum testosterone.[81] Further support for the role estrogen has on the male skeletal system was gleaned from studies examining the impact of aromatase deficient men. Aromatase is responsible for the conversion of testosterone to estrogen. In men lacking aromatase, BMD is lower[82] but testosterone levels can be normal, making it a useful to look at the impact of estrogen replacement. Herrmann *et al*. used this rare incidence of estrogen deficiency to demonstrate, by replacement of this lost estrogen, the importance of estrogen for bone mineralization and maturation in men.[83]

Osteoprotective therapies have become an essential component in the management of advanced PC. However, it is apparent that further research needs to be undertaken to determine the most effective way of treating BMD loss in patients undergoing ADT.

1.3.1.1.2 Cardiovascular disease

A number of retrospective and observational studies have shown an association between ADT and CVD or sudden cardiac death.[41,43] Weight gain associated with ADT (which accumulates on the hips and abdomen) can also further increase the risk of CVD. ADT causes a decrease in lean body mass with a concomitant increase in total body fat (subcutaneous rather than visceral fat) of up to ~10%.[73,74] ADT also increases, serum cholesterol and triglyceride levels, reduces insulin sensitivity and can result in dyslipidaemia (abnormal amount of lipids), all of which are risk factors for increased CVD.[84] A review of a Danish population of men with PC found a 31% increased risk of myocardial infarct and a 16% increase in the risk of cerebrovascular disease in men receiving ADT compared with men with PC not receiving ADT.[85] However, in post hoc analysis of randomised controlled trials, no statistically significant increase in CVD relating to ADT use was identified by Rhee *et al*. 2015. These discrepancies may in part be explained by the use of ADT being skewed towards those with high-risk PC as ADT has not demonstrated a survival advantage in patients with low-risk PC. Additionally, those enrolled in clinical trials may be of better health than the general population.

Notwithstanding this inconsistency, CVD is the most common cause of death among men diagnosed with PC. Thus, it is important that other risk factors for CVD should be taken into account when considering ADT, [41] and as available data suggests that complications occur within 3-6 months of starting ADT, close observation and intervention are particularly important during this period.[86]

1.3.1.1.3 Memory Loss

There is increasing evidence that demonstrates long term ADT has the potential to impact cognition (particularly memory) and a link between ADT and dementia exists.[87] Nead *et al*. found that the absolute increased risk of developing dementia among those receiving ADT when compared with those not receiving ADT was 4.4%. Additionally, it was found that the patients that had received ADT for longer than 12 months were at the greatest risk for developing dementia and that the use of ADT was found to increase the risk of dementia irrespective of age. [87] Beer *et al*. found that men undergoing ADT for PC experienced verbal memory loss, specific to processes mediated by the medial temporal lobe. Immediate and verbal memory were also found to be significantly worse in addition to slower processing speed, more fatigue and confusion as well as less vigour.[88] However no significant difference between healthy men, men with PC not on ADT and men with PC on ADT was found when undertaking working memory tasks.[88]

1.3.1.1.4 Hot Flushes

Up to 80% of patients undergoing ADT experience hot flushes to varying degrees, which ranges from mild irritation to debilitating. Up to 27% of men experiencing hot flushes report this to be the most incapacitating side effect related to their treatment.[89] Irani et al. (2010) found that out of the third of men that experienced hot flushes due to ADT, 50% of this group found this side effect had a significant adverse effect on their quality of life.[90] Thus, it remains one of the main reasons for non-compliance with treatment.[91]

Hot flushes are experienced as a rise in temperature in the face and trunk. In addition, cutaneous vasodilation occurs, usually accompanied by profuse sweating.[91] The decline in LH and FSH resulting from ADT leads to the release of hypothalamic catecholamines, particularly norepinephrine. The thermoregulation centre in the upper hypothalamus is flooded by these catecholamines causing irregular and inadequate regulation of peripheral vasodilation.[90,92] Research has also suggested that arcuate KNDy (hypothalamic kisspeptin, neurokinin B and dynorphin) neurons relay estrogen signals to preoptic structures regulating heat-dissipation effectors, lending support to the hypothesis that KNDy neurons contribute to the generation of flushes. Hot flushes therefore also appear to be mediated by elevated NKB and kisspeptin resulting from loss of regulation of estrogen negative feedback.[93,94] Therefore while estrogen replacement has been long been used to treat hot flushes[95,96], treatment with a neurokinin 3 receptor antagonist offers an alternative to effectively relieve hot flush symptoms, without the need for estrogen exposure.[97]

1.3.1.1.5 Sexual Dysfunction

Estradiol in men is necessary for regulating libido, erectile function, and spermatogenesis. Localization of ERα, ERβ, and aromatase in adult testis points to estrogen action as a likely component for Leydig, Sertoli, and germ cell development and function. Additionally, it points to a role for estrogen in the development and function of the efferent ductules and epididymis.[98] ERs, as well as aromatase, the enzyme that converts testosterone to estrogen, are plentiful in the brain, penis, and testis, all organs which play a role in sexual function.[99] In the brain, estradiol synthesis is increased in areas related to sexual arousal. In the penis, ERs are found throughout the corpus cavernosum with increased concentration around neurovascular bundles. Low testosterone and elevated estrogen increase the incidence of erectile dysfunction independently of one another.[99] Estrogen modulation impacts all levels of spermatogenesis. Estradiol regulation of testicular cells is both inhibitory as well as stimulatory.[99]

ADT frequently results in some form of sexual dysfunction.[100] This can have both physical and mental impacts on patients. A GnRH analogue study looking specifically at penial length found

that 93% of patients had a loss in length of more than one centimetre, which can negatively impact sexual activity as well as urination. [101] In men underdoing ADT, prevalence of loss of libido ranges from 58% to 91.4% and prevalence of erectile dysfunction from 73.3% to 95%.[102] Potosky *et al*. found that 80% of men with PC receiving ADT were impotent after 12 months, compared with 30% of men with PC not receiving treatment.[103] This can lead to a decrease in sexual activity with some patients not engaging in any sexual activity.[103] Furthermore, this can lead to erosion of romantic relationships.[104] Potential means of addressing these side effects include counselling, erectile dysfunction medication and even penile prothesis.[102]

Accompanying these effects is the psychological impact on patients, often experienced as feelings of loss of masculinity. Zaider *et al*. found that a third of men felt that a dimension of their sexuality was lost after undergoing treatment for PC. Psychological effects of treatment are prominent with an impact on quality of life. They remain under studied and more in-depth research is required in order to best facilitate mental as well as physical well-being following treatment for PC.[105] For example, in the Prostate Cancer Outcomes Study of the SEER program, the proportion of men that reported no sexual interest increased from 31% to 58% after GnRH agonist treatment. The proportion of men who achieved no erections increased from 38% to 74%. 80% of men in the study were unable to engage in sexual activity during ADT.[103]

The balance between testosterone and estrogen is critical when addressing erectile dysfunction.[106,107] Therefore, proactive intervention to reduce the physical and psychological consequences related to ADT or ADT with estrogen replacement is necessary.

1.4 GnRH and GnRH Analogues

GnRH was first isolated as a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.NH2) from the mammalian hypothalamus.[37] The amino-terminus (pGlu-His-Trp-Ser) and carboxy-terminus (Pro-Gly.NH2) as well as the length of the GnRH peptide (10 amino acids) have been conserved over millions of years of evolution, indicating their significance for receptor binding and activation.[37] The peptide structure (Figure 1.2) folds around a central Glycine at position

six (which is also highly conserved), allowing both the amino-and carboxy-termini to interact with its cognate receptor, the GnRHR. Interestingly, it has been found that replacement of the glycine at position six with D-amino acids increases binding affinity, presumably due to additional constraint of this horseshoe structure.[37,108] This feature is incorporated in all GnRH analogues.[109] Binding affinity is further enhanced if the substituted D-amino acid contains a large aromatic side chain, such as D-tryptophan (100 fold increase in binding affinity).[37] Additional advantages of such substitutions in this position are increased resistance to proteolytic cleavage and enhanced half-life due to binding of the GnRH to plasma proteins, such as albumin, thus decreasing the rate metabolic clearance.[110] For these reasons GnRH analogues commonly utilised for ADT therapies (e.g. triptorelin, histrelin and leuprorelin) all contain substitutions at this position. Substitution of the carboxyl terminal Gly-NH2 with alkyl moieties (for example, ethylamide) conveys further resistance to proteolysis without compromising binding affinity.[110] Agonist drugs have an affinity for and stimulate physiologic activity at cellular receptors usually stimulated by endogenous ligands. GnRH agonists are comparable in structure and function to natural GnRH, however can be many times more potent than the natural hormone.[9] Antagonist drugs nullify the action of endogenous mediators by binding to a cellular receptor without provoking a response. All antagonists have substitutions with bulky hydrophobic D-amino acids in positions 1, 2 and 3 (and some in 4).[110]

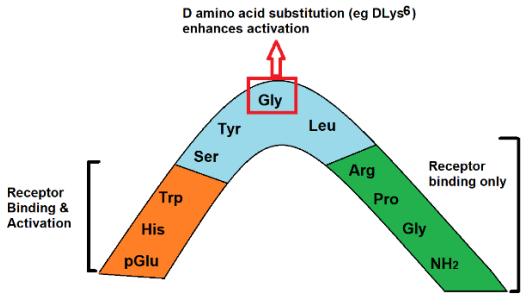


Figure 1.2 ***Mammalian GnRH in the folded conformation***

Schematic representation of mammalian GnRH in the folded conformation in which it is bound to the pituitary GnRH receptor modified from [110] by S. Leijenaar in Microsoft PowerPoint. Substitution of the glycine in position six with a D-amino acid stabilizes the folded conformation. Both terminals, Amino (red) and carboxyl (green), are implicated in receptor binding, while the amino terminal alone is implicated in receptor activation.

1.5 Role of Estrogens and Phytoestrogens in Men

It is frequently underappreciated that, in addition to androgens, a small amount of estrogen is required in men. Indeed, it is important to note that it is the ratio of estrogens to androgens is key in maintaining tissue homeostasis and prostate health.[111] Testosterone is the major precursor of estrogens in men and its conversion is due to the activity of the enzyme aromatase found in many tissues including fat tissue as well as the prostate. Aromatase is localized in the endoplasmic reticulum. It is regulated by tissue-specific promoters that are themselves controlled by hormones, cytokines, and other factors. Aromatase catalyses the final steps of biosynthesis of estrogens from androgens (in particular, it converts androstenedione to estrone and testosterone to estradiol). This involves three successive hydroxylations of the 19-methyl group of androgens, followed by concurrent removal of the methyl group as formate and aromatization of the A-ring.[112,113]

Estrogens, are a class of steroid hormones responsible for the regulation of growth, development, and physiology of the human reproductive system. Furthermore, estrogens play a role in the neuroendocrine, skeletal, adipogenesis, and cardiovascular systems. The biological functions of estrogen are mediated by binding to estrogen receptors (ERs): ER alpha (ERα), ER beta (ERβ) as well as the most recently discovered G protein-coupled estrogen receptor 1 (GPER).[114] ERα and ERβ display 59% amino acid sequence identity in their respective ligand binding domains (LBD), representing a noteworthy difference between the two. Yet, the differences within the ligand binding cavities only occur at two amino acid positions: Leu384 and Met421 in ERα are replaced by Met336 and Ile373, respectively, in ERβ. These are conservative changes between hydrophobic residues and are reciprocal, with each ER subtype having one methionine residue and then either a leucine or isoleucine residue. Most crystal structures show ERβ as having a smaller and narrower binding pocket than that seen in ERα, likely due to the sequence diversity outside of the ligand binding pocket rather than being due to the small differences in the two pocket residue positions.[115]

These proteins belong to the nuclear hormone receptor class of transcription factors that regulate gene transcription. As it takes time for genes to be transcribed into RNA and translated into protein, the effects of estrogens binding to these classical ERs is delayed.[116] Unlike the classical ERs, GPER is partly responsible for the rapid non-genomic actions of estradiol.[117] ERα and ERβ modulate the transcription of target genes by binding to estrogen response elements (EREs) in the DNA sequence. [118] Binding of ERs to EREs promotes bending and looping of the DNA, thus allowing interaction with the transcriptional machinery and co-regulator proteins.[119]

Estrogen signalling pathways are selectively stimulated or inhibited depending on a balance between the activities of these two-key classical ERs in target organs. The two ER subtypes generally have different biological functions, but dependant on cell type there can be overlap. ERα is highly expressed in the uterus, prostate stroma, ovarian theca cells, Leydig cells in testes, epididymis, breast, and liver. [120] ERβ is highly expressed in prostate epithelium, testes, ovarian granulosa cells, bone marrow, and brain. [121] ERα and ERβ have distinctive downstream transcriptional activities, resulting in their tissue-specific biological actions. [122] These differences are discussed in greater detail in section 4.3.

Estrogens can either be natural, including endogenous human estrogens and nonhuman estrogens (such as those produced from an equine source) or they can be synthetic either with or without a steroid skeleton.[123] Additionally, a group of compounds, derived from plants, known as phytoestrogens are able to mimic natural estrogens by binding and activating ERs.[124] Genistein, a soya derivative, is one such phytoestrogen.[125]

Phytoestrogens in the human diet, including genistein, are mostly found in soy products (soy-isoflavones). These compounds 'mimic' endogenous estrogens and therefore can influence several biological functions including production, metabolism and biological activity of the sex hormones.[124] In addition, it has been suggested that there is the possibility of phytoestrogens having preventative effects against a number of different forms of cancer, including PC.[126] Indeed, a correlation exists in areas in which foods containing estrogenic compounds are more regularly consumed and lower PC rates.[126]

Genistein is similar in structure to estrogen and mimics the action of estrogens on target organs.[127] A number of natural phytoestrogens, preferentially bind to ERβ with high affinity, genistein binds to ERβ with about 25 fold higher affinity than to ERα (unlike endogenous estrogens such as 17β-Estradiol, which binds to both receptor subtypes with similar affinities).[128] This difference may account for the differences in physiological activities of endogenous estrogens and phytoestrogens, which will be discussed in more detail the following sections.

1.5.1 Estrogens and Bone

Bone is a dynamic tissue. It is continuously being altered and remodelled by osteoclasts and osteoblasts, with bone resorption by osteoclasts followed by bone formation by osteoblasts. This process is a fine balance, with precursors to osteogenesis produced by the osteoblasts inducing differentiation and fusion of macrophages into resorbing osteoclasts. Should an imbalance in osteoclast/osteoblast activity occur, due to illness, or the treatment thereof, over-activity of osteoclasts may result in uncoupling of bone remodelling thus favouring bone loss over bone formation. [129] This imbalance can therefore lead to loss of trabecular (spongy) bone mass and ultimately osteoporosis. [130] Osteoporosis is a debilitating disease with a high medical and socioeconomic impact. The lifetime fracture risk of a patient with osteoporosis can be as high as 40%. Osteoporotic fractures of the hip and spine carry a 12-month excess mortality of up to 20%, due to the fact that they necessitate hospitalisation and subsequently have greater risk of other complications, including pneumonia or thromboembolic disease due to chronic immobilisation.[131]

Intercellular communication between the osteoblasts and osteoclasts is critical in preserving the structure of the bone tissue. RANKL and macrophage colony stimulating factor (M-CSF), are both produced by osteoblasts. RANKL stimulates osteoclastogenesis and prevents osteoclast apoptosis, and M-CSF is responsible for the proliferation, differentiation and survival of osteoclast precursors.[132,133] Stimulation by RANKL triggers signalling that leads to activation and expression of particular transcription factors and markers crucial for osteoclast formation, including c-Fos, NFATc1, TRAP, cathepsin K (CTSK), matrix metalloproteinase 9 (MMP-9) and dendritic cell-specific transmembrane protein (DC-STAMP).[133] Once

differentiated, osteoclasts can attach to bone, where the enzyme carbonic anhydrase (CA) acidifies the resorption micro-environment to dissolve the mineral phase of bone. Lysosomal enzymes, CTSK and MMP-9, are then released to degrade the organic matrix. The degradation products are then endocytosed by the osteoclasts before being released into the extracellular fluid.[133,134]

Estrogens and phytoestrogens have bone protective properties, such as in preventing post-menopausal osteoporosis.[135,136] However, there is some controversy regarding estrogen's role in bone physiology.[137,138] There is evidence that enhanced formation of functional osteoclasts appears to be negatively regulated by estrogen due to suppression of expression of osteoclastogenic cytokines (e.g. M-CSF) and pro-inflammatory cytokines (e.g. IL-1, IL-6, and tumour necrosis factor (TNF)). [130] Both estrogen and phytoestrogens (including genistein) have also been shown to inhibit RANKL-induced osteoclast formation (macrophage differentiation) and both cause down regulation of MMPs including MMP-9.[139–142] Studies have also found that estrogen and phytoestrogens have direct effects on both osteoclast precursors and fully differentiated osteoclasts. [143,144] In fully differentiated osteoclasts this is achieved through reducing osteoclast bone resorbing activity and reducing osteoclast lifespan, by promoting osteoclast apoptosis.[143,144] The study by Fanti *et al*. found that genistein reduced both trabecular and compact bone loss after ovariectomy.[136] Other studies have found that dietary intake of genistein is capable of bone protection.[145] The bone protection offered by genistein is twofold eliciting effects in stimulating bone synthesis via osteoblasts and preventing bone loss via osteoclasts. It stimulates osteoblastic differentiation and mineralisation and promotes protein synthesis in osteoblasts in vitro.[146,147] Genistein also inhibits osteoclastogenesis and induces apoptosis of mature osteoclasts.[148,149]

Inactivation of ERα in mice and men has demonstrated the importance of estradiol signalling via this receptor for skeletal health in males. ERα mediates estrogenic effects by translocating to the nucleus and affecting gene transcription but also by extra-nuclear actions. Activation function 1 (ERαAF-1), a domain of ERα has tissue specific roles. Farman *et al*. (2017) examined the significance of extra-nuclear estrogen effects mediated by ERαAF-1 for the skeleton in males [150]. Wild-type (WT) and ERαAF-1- inactivated (ERαAF-1^0) mice were orchiectomised and treated with equimolar doses of 17β-Estradiol (E2) or an estrogen dendrimer conjugate (EDC). EDC is unable to enter the nucleus and therefore only initiates extra-nuclear ER actions.

Here it was found that E2 increased cortical and trabecular bone volume per total volume in WT mice, EDC treatment in these mice resulted in increased cortical thickness but had no effect on trabecular bone. [150] In ERαAF-1^0 E2 treatment increased cortical bone thickness but had no impact on trabecular bone. Additionally, the effect on cortical bone by EDC was abolished in these mice [150]. Therefore, in males extra-nuclear estrogen signalling influences cortical bone mass and this is dependent on a functional ERαAF-1 [150]. ERα has been found to be strongly expressed in cortical bone whereas ERβ has been found to be strongly expressed in trabecular bone.[151] The ERβ expressed in osteoblasts may mediate the reported beneficial impact of soy isoflavones on bone metabolism.[152] As described above, genistein is capable of offering bone protection through a variety of mechanisms. It remains unclear whether this occurs predominantly through activation of ERβ. Conflicting results in the role of ERβ in cortical and trabecular loss and protection means that additional research is required to fully understand the contribution of the different ER isoforms. Furthermore, the role of the novel GPER would need to be considered as genistein is able to elicit effects by activation of this receptor[153] and this may go some way to explaining the confounding data regarding the role of the two classical ERs in bone physiology.

1.5.2 Estrogens and the cardiovascular system

Premenopausal women experience a greater degree of cardiac protection compared with age matched men or post-menopausal women. It is generally thought that estrogen is responsible for this higher level of cardiac defence.[154,155] HRT (typically consisting of an estrogen and progesterone) has previously been associated with increased risk of CVD. Participants in these earlier studies had frequently been post-menopausal for some time before initiating HRT. This along with additional health complications has made interpretation of the results of difficult.[156] Studies have found that women that started HRT within six years of the onset of menopause had improvement in some aspects of CVD, when compared with women that had started HRT more than ten years after the initiation of menopause.[157] Estrogen increases vasodilatation and inhibits the response of blood vessels to injury and the development of atherosclerosis.[158] However, rates of atherosclerosis progression did not differ between women not on HRT and those that started HRT early. [88,159] Together, these findings indicate

that HRT/estrogen replacement may not in fact be a risk factor for CVD but rather may be beneficial. Studies have indicated that phytoestrogens have similar protective effects regarding cardiovascular disease.[160] Genistein supplementation improves endothelial dysfunction induced by oophorectomy in rats and reduces infarct size in an experimental model of myocardial ischaemia-reperfusion injury. Additionally, genistein in postmenopausal women increases plasma nitric oxide breakdown products, reduces endothelin-1 levels and improves endothelial dependent vasodilation in post-menopausal women. Taken together these data suggest there are elements of cardiovascular protective function that result from genistein.[161] Another study found that 54 mg genistein given daily along with calcium, vitamin D_3, and a healthy diet was linked with favourable effects relating to glycaemic control and other cardiovascular risk markers in a cohort of osteopenic, postmenopausal women.[162]

Fewer studies have examined the impact of estrogen on cardiac function in men. However, men with genetic defects of estrogen synthesis or action develop premature atherosclerosis.[163,164] In addition, in a community-based sample study, a higher serum estradiol level was found to be associated with lower risk for CVD events in older men. [165]. These findings are therefore consistent with the notion that endogenous estrogen also has vasculoprotective properties in men.

In addition to direct effects on CVD outcomes, estrogen supplementation may also have metabolic effects that relate to reduced CVD risk. Estrogen supplementation in male mice led to a reduction in weight gain and improvements in obesogenic dietary induced metabolic changes such as enhanced glucose-insulin homeostasis, [166] which are often seen as risk factors for cardiovascular events and disease. It is thought that this is achieved by estrogen improving glucose homeostasis, insulin sensitivity and lipid profile as well as improving pathway-selective insulin resistance.[167] Additional information on the physiological effects of estrogens in men and their physiological effects is required. However, experimental and clinical evidence suggests estrogens are cardio-protective in men. Pre-existing cardiovascular risks should be considered before deciding on treatment with estrogen for men undergoing ADT. Studies have indicated that phytoestrogens, have similar protective effects to estrogen regarding cardiovascular disease.[160] Activation of both classical ERs have been found to be protective in the cardiovascular system. Arias-Loza et al. found that activation of either ERα

or ERβ was protective against the detrimental effects of aldosterone salt treatment within the cardiovascular system of rats.[168] Both ER subtypes also confer protective effects in experimental models of human heart disease, including hypertension, cardiac hypertrophy, and chronic heart failure.[169]

1.5.3 Estrogens and Memory Loss

Sex steroid receptors (including ERα and ERβ) are located in brain regions that are vital for memory.[88,170] Studies have shown that postmenopausal women had improvements in immediate recall tests after the administration of estrogen compared to women that did not receive supplementation.[171] Using a rat model Luine *et al*. were able to demonstrate that estrogen rapidly enhances visual and place memory (mediated via activation of both classical ERs and it is hypothesised that GPER may also have a role to.[172] Additionally, a study utilising male zebra finches showed that estrogen depletion affects the neuronal memory for vocalizations in the caudomedial nidopallium, suggesting that estrogen plays an essential role in auditory processing and memory for communication signals.[173] Although the mechanisms behind the neurotrophic and neuroprotective properties of estrogen are not yet fully elucidated, progress has been made in this area. It has been proposed that estrogen aids in neural repair by influencing production of new neurons under neuropathological conditions, promoting an anti-inflammatory response, decreased oxidative stress, decreased apoptosis, growth factor regulation and vascular modulation.[174,175] Indeed, in men supplementation with high dose estradiol has the potential to reverse memory deficits associated with ADT, with men treated with estradiol having a significant improvement in their recall performance.[88] The phytoestrogen genistein has also been shown to have a positive impact on cognitive decline and neurodegeneration.[176,177]

1.5.4 Estrogen and Hot Flushes

Vasomotor symptoms (VMS) such as hot flushes and night sweats are frequently encountered during menopause, these symptoms are causally related to decreasing estradiol concentrations, primarily in the serum and then also in the hypothalamic temperature

regulating centre. The lack of estrogens alters neurotransmitter activity, especially in the serotonergic and noradrenergic pathways.[178] In ovariectomised rats estradiol-17β treatment improved the maintenance of core temperature during heat exposure. Estrogen withdrawal appears to increase the sensitivity of thermoregulatory neural pathways and alters the activation of heat loss mechanisms.[179] Sex hormones in the form of progesterone and estrogen have been used, often successfully, to treat hot flushes in post-menopausal women.[180,181] Far less research has been done in males undergoing ADT. However, drugs such as megestrol acetate, a progestational agent, have been shown to markedly decrease the frequency and intensity of hot flushes in men. [182] A number of other hormonal treatments and non-hormonal treatments, such as acupuncture, have been evaluated as potential means to alleviate hot flushes but, on the whole the most successful treatment seems to be the administration of estrogen. For example, a significant decrease in severity and occurrence of hot flushes was observed when men undergoing ADT were given transdermal estrogen patches.[91] Similarly, in another study, 90% of ADT patients gained some level of relief from hot flushes when given DES.[91] Genistein has also shown some promise as a treatment compound for hot flushes in postmenopausal women but to less of an extent than treatment with estrogens.[183,184] Pan *et al*. found genistein reduced tail temperatures in ovariectomized rats by around 0.8°C compared to the control whereas estrogen reduced the tail temperature by 1.4°C compared to the control.[185]

As discussed in section 1.3.1.1.4 estrogen replacement is an established method to treat hot flushes[95,96]. In men as in women treatment with a neurokinin 3 receptor antagonist offers an alternative to effectively relieve hot flush symptoms, without requiring the need for estrogen exposure.[97]

1.6 Estrogens and Phytoestrogens in Prostate Cancer

Estrogens are usually considered as inhibitors of PC growth (Indeed, as mentioned above (section 1.3.1), estrogens (e.g. DES) are a successful form of hormone therapy used against androgen dependent PC and are still used as second line hormonal therapy.[186] Androgens are necessary for the stimulation of development, growth and secretory activities within the

prostate.[187] However, a paradox exists, in that estrogens may also be implicated in the causation of this disease, although this relationship, if any, is still unclear, and it is important to note that the ratio of estrogens to androgens is probably key in maintaining tissue homeostasis and prostate health.[111]

In several studies estrogens have been associated with the development of benign prostatic hyperplasia (prostate gland enlargement) and PC.[188] For example, Risbridger *et al*. used genetically modified mouse models, hypogonadal mice deficient in gonadotropins and androgens and aromatase knockout (ArKO) mice, deficient in estrogens due to a non-functional aromatase enzyme) to evaluate the action of estrogens alone or in combination with androgens on the prostate gland. The ArKO mice developed prostatic hyperplasia during lifelong exposure to elevated androgens but no malignant changes were ever detected. However, when androgen and estrogen were given in combination prostatic dysplasia and adenocarcinoma were detected. [187] A second study by Bosland (2005) provides additional support for estrogen having a role in PC development. In this study testosterone given to noble rats at low doses led to PC developing in 35-40% of animals. When combined with estrogen the incidence of PC increased to almost 100%.[188] Estrogen therefore appears to be a necessary component to achieve a maximal carcinogenic response to androgens in rats, but its effects are uncertain in humans.

In light of these findings, estrogen effects on PC probably need to be viewed in at least two different ways: (1) systemic endocrine effects acting through negative feedback at the pituitary gland to indirectly lower androgens, and (2) local effects that directly target prostate tissue through ERs or through other genotoxic mechanisms. The progressive emergence of the ERα and the estrogen-regulated progesterone receptor during advanced PC progression and hormone-refractory patients implies that, although estrogens may supress androgen secretion, estrogens could directly cause growth of tumours. In addition to effects mediated through ER activity, there is evidence to suggest that estrogens could act directly as carcinogenic factors through a genotoxic mechanism. Estrogen and DES can be transformed to so-called catecholestrogen metabolites by P450-mediated hydroxylation. If these metabolites aren't detoxified, they may go through a process called redox-cycling.[189] This process forms reactive oxygen species that are able to damage DNA and cause lipid

peroxidation, in addition to the formation of reactive intermediates that can directly adduct to DNA leading to mutagenesis.[189] This has been demonstrated in animal models of estrogen-induced cancer and in PC using the noble rat model.[190] Prior to cancer formation in this model, at the precise site of tumour development (the periurethral ducts), there is DNA adduction, oxidative DNA damage, and lipid peroxidation in response to combined estrogen–androgen treatment.[190,191]

Some of the differential effects of estrogens on PC noted above may be a result of the activation of different ER isoforms in different scenarios. Expression and regulation of both ERα and ERβ in the prostate are profoundly altered during PC development and tumour progression.[192] ERα in the human prostate appears to act as an oncogene, which is over expressed during the malignant transformation of the prostatic epithelium, while ERβ is indicated as a potential tumour suppressor, and there is evidence of its down regulation in high-grade prostatic intraepithelial neoplasia (widely accepted as a precursor to PC), due to methylation in the promoter of the gene.[193] Ricke *et al*. found that chronic treatment with testosterone given with estradiol was unable to induce high-grade prostatic intraepithelial neoplasia or PC when ERα was knocked out (α-ERKO), indicating that functional ERα is required for the development of PC in this mouse model.[186,192] Restoration of ERβ by adenoviral delivery has been shown to inhibit the growth of PC cells and invasiveness, and cells that over express ERβ undergo apoptosis, further supporting its anti-proliferative, anti-invasive and pro-apoptotic properties.[191,194,195] A large population-based case-control study also found an association between a single nucleotide polymorphism located in the promoter region of the *ER*β gene and risk of developing PC, also suggestive of the antiproliferative effects of *ER*β.[196]Furthermore, a large portion of PCs contain gene fusions of the 5'-untranslated region of the androgen-regulated transmembrane protease, serine 2 (TMPRSS2) promoter with erythroblast transformation specific transcription factor family members such as ERG.[197] This TMPRSS2-ERG gene fusion, is associated with a potentially aggressive molecular subtype of advanced PC, and is differentially regulated by ER-dependent signalling with its expression being found to be increased by ERα agonists (estrogens) and decreased by ERβ agonists.[186]

Interestingly, phytoestrogens, such as genistein, , which preferentially interact with ERβ[198] do

not appear to display the same carcinogenic effects as estrogen. Li *et al*. found that genistein hindered PC cell growth in the bone environment (PC frequently metastasizes to the bone) as well as down-regulated the transcription and translation of genes significantly involved in the impact of tumour cell invasion and metastasis *in vitro* and *in vivo*, implying the possible therapeutic role of genistein for metastatic PC. [140] These findings are further supported by other studies showing genistein and other isoflavones inhibiting metastasis of androgen sensitive human prostate tumours in mice and PC cell lines.[199,200] Pavese *et al*. showed that genistein inhibited cell detachment, protease production, cell invasion, and human PC metastasis. This was achievable at concentrations in line with dietary intake of genistein in humans. Furthermore, phase I and phase II clinical trials were conducted by Pavese *et al*. and showed that concentrations of genistein associated with antimetastatic efficacy in preclinical models are attainable in humans, and treatment with genistein inhibited pathways that regulate metastatic transformation in human prostate tissue.[200]

1.8 GnRH-estrogen conjugates

As described above (section 1.3) ADT utilising GnRH agonists is the primary first line therapy for treatment of androgen dependent PC. However, as previously mentioned, commonly associated negative side effects of ADT therapies include of bone loss, hot flushes and loss of libido. As these side effects are mostly due to the resulting decrease in estrogen production in these patients, as it is elaborated from testosterone, it may be beneficial to develop compounds which retain ADT activity, but which also have estrogenic activity. Other limitations of ADT therapeutics include their poor oral availability and rapid metabolic clearance.[201] As a result they are generally administered as injectable slow release formulations. This approach lessens some of the limitations but restricts the ability to vary dosages and does not allow for immediate withdrawal of treatment if desired.

Conjugation of steroid-moieties (such as estrogens) to GnRH peptide antagonists to confer plasma binding capacity, to reduce metabolic clearance, and to convey active absorption from the gastro-intestinal tract may also be a means to overcome these limitations. Genistein is lipophilic and also binds to plasma binding proteins[202], it is thought therefore that conjugation of genistein to a GnRH analogue would confer similar benefits. Ratcliffe *et al.* successfully

demonstrated that conjugation of C21 of 21- hydroxylated progesterones to a substitution at position six of GnRH analogues resulted in good plasma protein binding and high GnRH receptor and progesterone receptor activity.[203] These studies confirmed that GnRH analogues can be successfully modified through conjugation to steroid moieties and that the sites chosen for conjugation avoid disruption of crucial binding sites and steric hindrance.[203]

We hypothesised that similar molecules in which estrogens or phytoestrogens such as genistein were conjugated to GnRH analogues would produce compounds with GnRH and estrogenic activation and thus might serve as effective ADT agents with reduced side effects (due to the concurrent estrogen replacement) and improved bioactivity (due to increased half-live inferred by the steroid moiety). To this end, a GnRH agonist analogue (D-Lys6 GnRH Pro9 ethylamide) was utilised to facilitate conjugation through the epsilon amino group to an estrogen hemisuccinate. The D-Lys6 substitution also conveys resistance to an endopeptidase and substitution of the carboxyl terminal Gly.NH2 with ethylamide conveys resistance to carboxypeptidase.[203,204]

Given the controversy that exists regarding the role of 17β-Estradiol in PC development and progression it was deemed sensible to investigate use of an ERβ selective estrogen. The phytoestrogen genistein was selected as it preferentially binds to ERβ with high affinity. This is significant as ERα in the human prostate appears to act as an oncogene, which is over expressed during the malignant transformation of the prostatic epithelium, while ERβ is indicated as a potential tumour suppressor.[192,193] As the conjugates offer dual treatment, it is also worthwhile to consider alternative estrogenic compounds such as genistein or estriol with decreased estrogenic activity as this may be necessary to avoid giving excessively high doses of estrogen, discussed in greater detail in section 4.10.

1.9 Summary

Despite the success of GnRH analogue ADT as an effective first line treatment of PC, it is associated with a number of undesirable side effects including but not limited to decreased bone density and/or osteoporosis, hot flushes and loss of libido. These side effects are, in part, due to a concurrent decrease in estrogen in patients as their testosterone level, the

precursor of estrogen in men, is reduced. New antiandrogens block the AR rather than lowering levels of androgens, thus estrogens remain available as they can still be elaborated from testosterone. These non-steroidal antiandrogens are used to treat PC, but frequently are done so in conjunction with a GnRH analogue which as mentioned lead to a concurrent decrease in estrogens and so do not eliminate the side effects of ADT previously discussed.[205,206]

Previous studies have demonstrated the ability to conjugate a steroid molecule to a GnRH analogue, successfully retaining the biological activity of both. To this end, this project created new compounds in which D-Lys6 GnRH Pro9 ethylamide was conjugated to 17β-Estradiol or the phytoestrogen, genistein. These molecules were then tested to ascertain their ability to bind and activate GnRHR and ERs. Their potential to reduce the debilitating side effects of ADT, in this case their ability to offer osteoprotective characteristics, were also examined. Importantly estrogen replacement has the potential to target multiple associated side effects whereas current approaches such as the use of bisphosphonates are only directed at a single side effect.

1.9 Aims and Objectives

1.9.1 Aim

This study aims to create and test molecules of GnRH analogues, conjugated to 17β-Estradiol or the phytoestrogen, genistein, for both GnRH receptor activation and estrogenic activation, as potential novel therapies for PC which minimise side effects due to diminished estrogen resulting from conventional GnRH analogue therapy for ADT. The purpose of this study is to investigate the potential for these conjugates to ameliorate the side effects associated with GnRH analogue mediated ADT for the treatment of PC, while remaining efficacious against the prostatic disease.

1.9.2 Objectives

1.9.2.1

To test GnRH activation, *in vitro,* of the conjugates by identifying whether they are able to activate the GnRH receptor and stimulate inositol phosphate accumulation in HEK293T cells expressing the GnRH receptor.

1.9.2.2

To test ER activation of the conjugates by identifying whether they are able to activate the estrogen receptor *in vitro* using an E-screen assay.

1.9.2.3

To test the ability of conjugates to induce RANKL driven differentiation of Raw 264.7 macrophages into osteoclasts as a measure of estrogen-mediated bone protective properties.

1.9.2.4

To test the effect of conjugates on cell proliferation, in three PC cell lines, using a crystal violet cell staining assay.

2.0 Materials & Methods

2.1 Study Design

This was an experimental study design. This study conjugated an estrogen and phytoestrogen (17β-Estradiol and genistein) to a GnRH analogue (D-Lys 6 GnRH agonist). Conjugates were tested to determine whether both the GnRH analogue and the steroid components retained their respective activities at the relevant receptors. In each assay the conjugated molecule was compared to its respective unconjugated counterparts as well as a vehicle control. The 17β-Estradiol [E2C] and genistein [GenC] conjugates were also compared to each other.

2.2 Design and Synthesis of GnRH analogue- estrogen conjugates

A hemisuccinate modification to the estrogens was carried out to provide the carboxyl group for conjugation (Figure 2.1). A GnRH agonist modified at position six with D-Lysine (to provide an amine group for conjugation and the above-mentioned conferred benefits) was used (Figure 2.2). This was conjugated to 17β –Estradiol (figure 2.3) or genistein (figure 2.4. The design of the conjugates was originally based on the conjugation method was adapted from that of Mattox *et al.*[207] and Rajkowski and Cittanova[208] as described in Ratcliffe *et al*[203]. Production and purification of the conjugates was commissioned from CPC Scientific (Sunnyvale, USA).

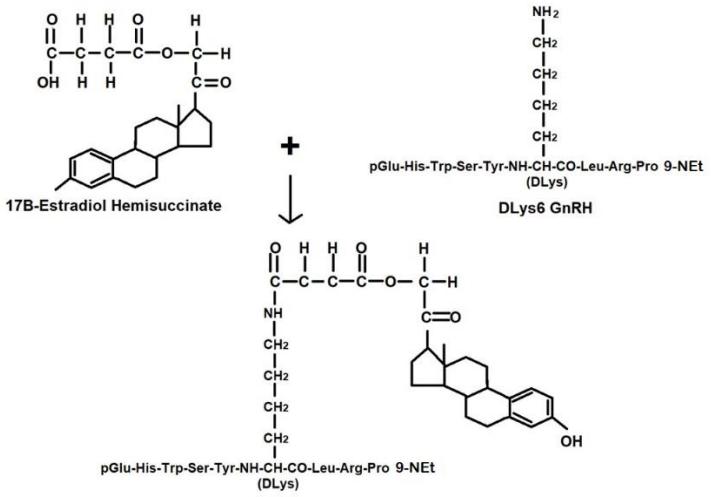
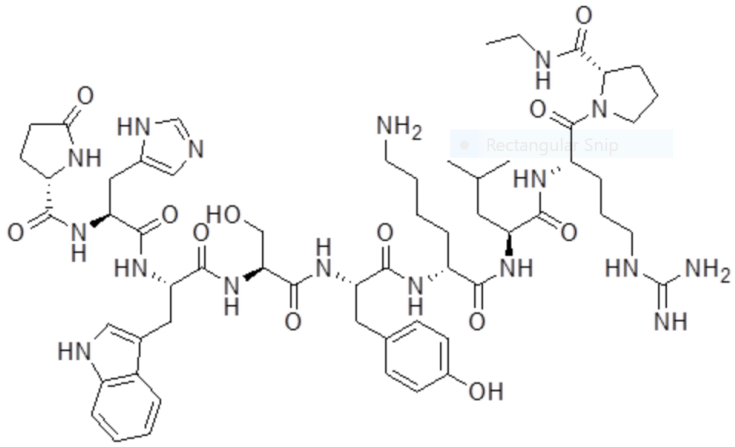


Figure 2.1 ***Representative example showing conjugation of 17β Estradiol with hemisuccinate group providing the carboxyl group for conjugation with D-Lys6 GnRH Pro9 ethylamide****, drawn by S.Leijenaar in Microsoft power point.*

A



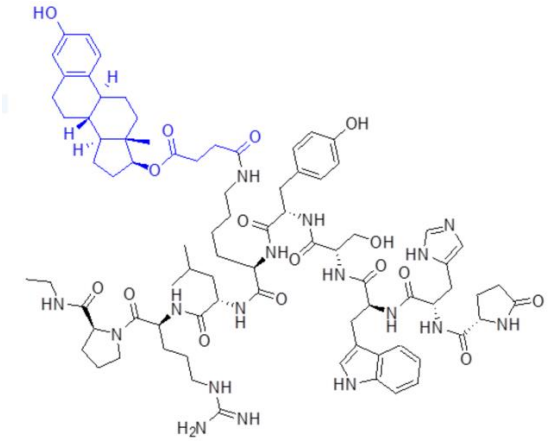
B

pGlu-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt

Figure 2.2 ***D-Lys6 GnRH Pro9 ethylamide (GnRHag) sequence and structure***

A *structure and* ***B*** *amino acid sequence of D-Lys6 GnRH Pro9 ethylamide (Chemical Formula C59H85N17O12. Exact Mass 1223.66 Molecular Weight 1224.41.) Provided by CPC Scientific.*

A



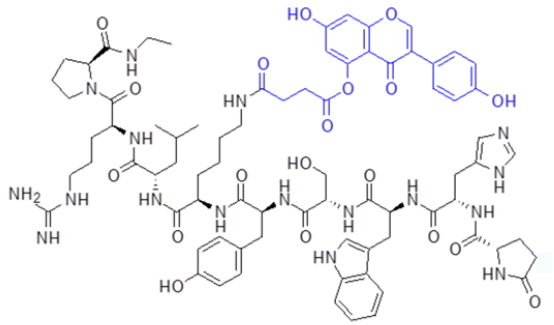
B

pGlu-His-Trp-Ser-Tyr-DLys (17β-Estradiol hemisuccinate)-Leu-Arg-Pro-NHEt

Figure 2.3 ***D-Lys6 GnRH Pro9 ethylamide -17β Estradiol Conjugate (E2C).***

A *structure and* ***B*** *amino acid sequence of D-Lys6 GnRH Pro9 ethylamide -17β-Estradiol conjugate (Chemical Formula C81H111N17O16 Exact Mass 1577.84 Molecular Weight 1578.85.) Estradiol moiety is indicated in blue, GnRHag indicated in black. Provided by CPC Scientific.*

A



B

pGlu-His-Trp-Ser-Tyr-DLys (genistein 5 hemisuccinate)-Leu-Arg-Pro-NHEt

Figure 2.4 ***An example of D-Lys6 GnRH Pro9 ethylamide -Genistein conjugate (GenC) sequence and structure via one of the hydroxyl groups***

A *structure and* ***B*** *amino acid sequence of D-Lys6 GnRH Pro9 ethylamide -Genistein (Conjugation may occur at any one of the three hydroxyl groups). Chemical Formula C78H97N17O19 Exact Mass 1575.71 Molecular Weight 1576.71). Estradiol moiety is indicated in blue, GnRHag indicated in black. Provided by CPC Scientific.*

2.3 Materials

All general laboratory reagents were purchased from Sigma-Aldrich (St Louis, USA) unless otherwise stated.

2.3.1 Ligands

Ligands (**Error! Reference source not found.**) were received and stored as lyophilized powder. Stock solutions of the ligands were made up in 100% DMSO (for Inositol Phosphate Accumulation and TRAP staining assays) or 100% HPLC-grade ethanol (for T47DKbluc, EScreen and Crystal Violet Staining assays) at a concentration of 1 mM.

Table 2.1: Ligand Details and Sources

Ligand	Manufacturer
D-Lys6 GnRH Pro9 ethylamide [GnRHag]	CPC Scientific (California, USA)
D-Lys6 GnRH Pro9 ethylamide -17β-Estradiol Conjugate [E2C]	CPC Scientific (California, USA)
D-Lys6 GnRH Pro9 ethylamide -Genistein Conjugate [GenC]	CPC Scientific (California, USA)
17β-Estradiol [E2]	Sigma-Aldrich (St Louis, USA)
Genistein [Gen]	LC-Laboratories (Massachusetts, USA)

2.3.2 Plasmids

A pcDNA 3.1 mammalian expression vector encoding human GnRHR was kindly gifted by Dr J Tello (University of St Andrews, Fife, Scotland).

The pEGFP-C1-ER alpha (ERα), pEGFP-C1-ER beta (ERβ) & 3X ERE TATA luc (ERE) plasmids were obtained from Addgene (Cambridge, Massachusetts, USA) as bacterial stabs.

2.3.3 Cell Culture Reagents

RPMI 1640 and Ham F12 media was supplied by Sigma-Aldrich, St Louis, USA. Dulbecco's Modified Eagle Medium, containing phenol red and Glutamax (DMEM) and Trypsin-EDTA solution were supplied by Life Technologies (Carlsbad, USA). Foetal calf serum (FCS) was supplied by Biochrome (Berlin, Germany). Matrigel, which was used to increase adherence during cell seeding, was supplied by BD Biosciences (Franklin Lakes, New Jersey). X-treme GENE (XTG) HP was used for transient transfections.

2.3.4 Cell lines

HEK 293T (Human Embryonic Kidney cells, stably expressing the SV40 large T antigen, a highly transfectable derivative of human embryonic kidney 293 cells), MCF7 (a breast cancer cell line) LnCAP (androgen-sensitive human prostate adenocarcinoma cells) , PC3 (high metastatic potential prostate cancer cell line) and DU 145 (hormone insensitive prostate cancer cell line) cells were available from the Centre for Neuroendocrinlogy, University of Pretoria and were originally obtained from the American Tissue and Cell Culture Collection (ATCC) (Rockville, MD). T47D (a human breast cancer cell line) cells were obtained from Dr Natalie Aneck-Hahn, Environmental Chemical Pollution and Health Research Unit, University of Pretoria. RAW 264.7 (murine macrophages) were obtained from Professor Magdalena Coetzee, Department of Physiology, University of Pretoria, originally obtained from ATCC.

2.4 Methods

2.4.1 Cell Culture

Cells were maintained in a humidified incubator at 37^{o}C with 5% CO_2 in DMEM (HEK 293T & RAW 264.7), 50% DMEM: 50% Hams F12 (MCF7, DU145, LnCaP and PC3) or RPMI 1640 media (T47D), all supplemented with 10% FCS (complete medium). To maintain a density of <90%, cells were sub-cultured twice a week. Cells were detached by incubation with 0.05% (w/v) trypsin for 1-5 minute at 37^{o}C. The trypsin was then neutralised with complete medium, where after 10% of cells were re-seeded into the flask.

2.4.2 Cell Counting

Prior to seeding of cells for each experiment, cell suspensions were counted using a haemocytometer. After cells were detached by incubation with 0.05% (w/v) trypsin for 1-5 minute at 37^{o}C in cell culture flasks, trypsin was neutralised with complete medium. 100 µl cells was removed. 900 µl complete media was added so that cells were diluted 1 in 10 to count. If the cell count was low cells were recounted without dilution to improve the accuracy of the count.

10-12 µl of cell suspension was loaded onto haemocytometer using a pipette, ensuring the entire polished surface of each chamber was covered.

The 10X objective was used to focus on the grid lines of the haemocytometer. Cells in the four corner outer squares and centre square were counted manually. Cells along the top and left edge of the counting chamber were included in the count. Cells along the bottom and right edge of the counting chamber were excluded from the count.

Cells/ml calculated using:

Number of cells X 10 (dilution factor) X 10 000 (conversion factor)
5 (number of squares)

*For low cell counts in which the sample was not diluted the number of cells was not multiplied by the dilution factor.

Prior to seeding of HEK 293T cells, the surface of the cell culture plates was prepared with Matrigel at a 1:30 dilution in serum free media, for 60 minutes at 37°C, to aid in cell adhesion. Matrigel was removed from wells prior to seeding.

2.4.3 Preparation of DNA

2.4.3.1 ERα and ERβ Preparation

A sterile inoculating loop was used to gather bacteria from the bacterial stab. Bacteria was spread onto an agar plate with the appropriate antibiotic (kanamycin for ERα and ERβ and ampicillin for ERE).

Streaking of the bacteria allowed for selection of individual colonies. Individual colonies were used to inoculate 5 ml of LB containing the appropriate antibiotic. This was placed at 37^{o}C into a shaking incubator for at least four hours.

Glycerol stocks were prepared by the addition of 500 µl glycerol (final concentration of 50% v/v) to 500 µl of stock from the overnight cultures. Glycerol stocks were stored at -80^{o}C.

2.4.3.2 Production and Isolation of plasmid DNA

A glycerol stock scrape was used to inoculate 5 ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 170 mM NaCl) containing 100 µg/ml appropriate antibiotic (amplicillin for GnRHR and ERE, kanamycin for ERα and ERβ). The resultant culture was incubated in a shaking incubator at 37^{o}C for 8 hours. 100 µl of the culture was then used to inoculate a flask containing 200 ml LB broth containing 100 µg/ml the appropriate antibiotic and the resultant culture was incubated in a shaking incubator at 37^{o}C overnight. Purification of the amplified plasmid (GnRHR, ERE, ERα and ERβ) was done with a Qiagen Plasmid Maxiprep kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

The concentration of the purified DNA was measured with a Nanodrop Spectrophotometer using the ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Absorbance of nucleotides, RNA dsDNA and ssDNA were measured at 260 nm and carbohydrates and phenols were measured at 230 nm. Nucleic acids have absorbance maxima at 260 nm. Historically, the ratio of this absorbance maximum to the absorbance at 280 nm has been used as a measure of purity in both DNA and RNA extractions. The purity of the DNA was determined based upon two ratios: 260/280 (a result of ~1.8 was accepted as a measurement of pure DNA) and 260/230 ratio (a result of ~2.0-2.2 was accepted as a measurement of pure DNA).

2.4.3.3 Transient Transfections

Following seeding, cells were grown to 60-70% confluence before transient transfection with exogenous DNA. Transfection complexes were prepared by adding appropriate plasmid DNA (10 ng/µl) with XTG HP at a 1:2 ratio (µg DNA/µl XTG) in serum-free DMEM. Following incubation for 15 minutes at room temperature, the complexes were added to the cells and incubated for 48 hours at 37°C (to allow adequate expression) prior to use in functional assays.

2.4.4 Statistical Considerations

2.4.4.1 Sample Size

The minimum sample size used for the *in vitro* assays was estimated using the standard deviation and difference in means of each parameter measured. This was based on data from current literature and previous data generated in the laboratory. Power calculations were performed to ensure that sample sizes for each parameter measured were sufficient to allow observations of statistically significant differences between the treatments. A sample size of five in each group was calculated to be appropriate to have at least 90% power to detect a difference in means of 25.000 assuming that the common standard deviation is 10.000 using a two-group t-test with a 0.050 two-sided significance level.

2.4.4.2 Data analysis

Statistical analyses were performed using a one-way ANOVA followed by Tukey's multiple comparison test for pairwise comparisons. $p<0.05$ was considered statistically significant for all data. All statistical analyses were performed using GraphPad Prism 5.0 software. (version for Windows, GraphPad Software, San Diego California USA).

3.0 GnRHR Activation by bifunctional Gonadotropin-Releasing Hormone Agonist-Estrogen Conjugates

3.1 Abstract

The annual rate of increase in the incidence of PC worldwide is estimated to be between 2 and 3%. PC is generally an androgen-dependent disease and ADT which reduces/removes androgens is currently the first line treatment against metastatic PC and has been considered the best treatment for advanced PC for more than fifty years. GnRH analogues have become the foremost therapeutic option for ADT. GnRH is a hypothalamic neuropeptide that is transported from the hypothalamus via the portal vessels to the anterior pituitary, where it activates GnRHRs on pituitary gonadotrope cells. This stimulates the release of the gonadotropins, LH and FSH, which act on the ovaries in females and testis in males to stimulate the production of gametes as well as steroid and peptide hormones. The hormone cascade is regulated through positive and negative feedback at the pituitary and hypothalamus. Both GnRH agonists and antagonists can be used for ADT, however agonists remain the preferred method.

In men androgen is the precursor for estrogen production and deprivation of estrogen by ADT has a number of associated negative side effects. Therefore, the intention of this study was to design and investigate compounds that would remain effective in the treatment of PC but have reduced associated side effects. To this end, compounds were designed in which a GnRH agonist was conjugated to two forms of estrogen (17β-Estradiol and genistein). It was first essential to determine whether the conjugated compounds (E2C and GenC) retained their activity at the GnRHR to ensure that they would still be effective in ADT. Using an inositol phosphate accumulation assay to determine activation of the GnRHR, the activities of the two conjugates were compared to D-Lys6 GnRH Pro9 ethylamide, a GnRH agonist (GnRHag). Stimulation of the GnRHR by the conjugates resulted in production of inositol phosphates, with similar or even slightly higher potency than GnRHag, although the maximal stimulation elicited was lower (72% and 85% of GnRHag max for E2C and GenC, respectively) than that

elicited by GnRHag. Both conjugates elicited similar activation of the receptor and had similar potencies, although E2C elicited a slightly lower maximal response than GenC These data indicate that the addition of the estrogen and phytoestrogen moieties did not impede the activation of GnRHR by the GnRH analogue component of the conjugates and suggest that these conjugates would remain efficacious as a treatment for PC by activation of GnRHR.

3.2 Objective

To test GnRH activation, *in vitro,* of the conjugates by identifying whether they are able to activate the GnRH receptor and stimulate inositol phosphate accumulation in HEK293T cells expressing the GnRH receptor.

3.3 Introduction

The GnRHR displays the characteristic features of the G protein-coupled receptors (GPCRs) superfamily of cell signalling proteins to which it belongs. It consists of a single amino acid chain with an extracellular amino-terminal domain and seven hydrophobic segments. The crystal structure of the GnRHR has not yet been resolved but it is believed that these segments form a bundle of membrane spanning α-helices connected by extracellular and intracellular loops, a characteristic of the GPCR family (Figure 3.1).[209–211] Activation of the GnRHR by GnRH results in Inositol 1,4,5-trisphosphate (InsP3)-dependent calcium signalling and protein kinase C activation. This is accompanied with acute regulation of gonadotropin secretion. These receptors also trigger several lipid-derived messengers and mitogen-activated protein kinases.[212]

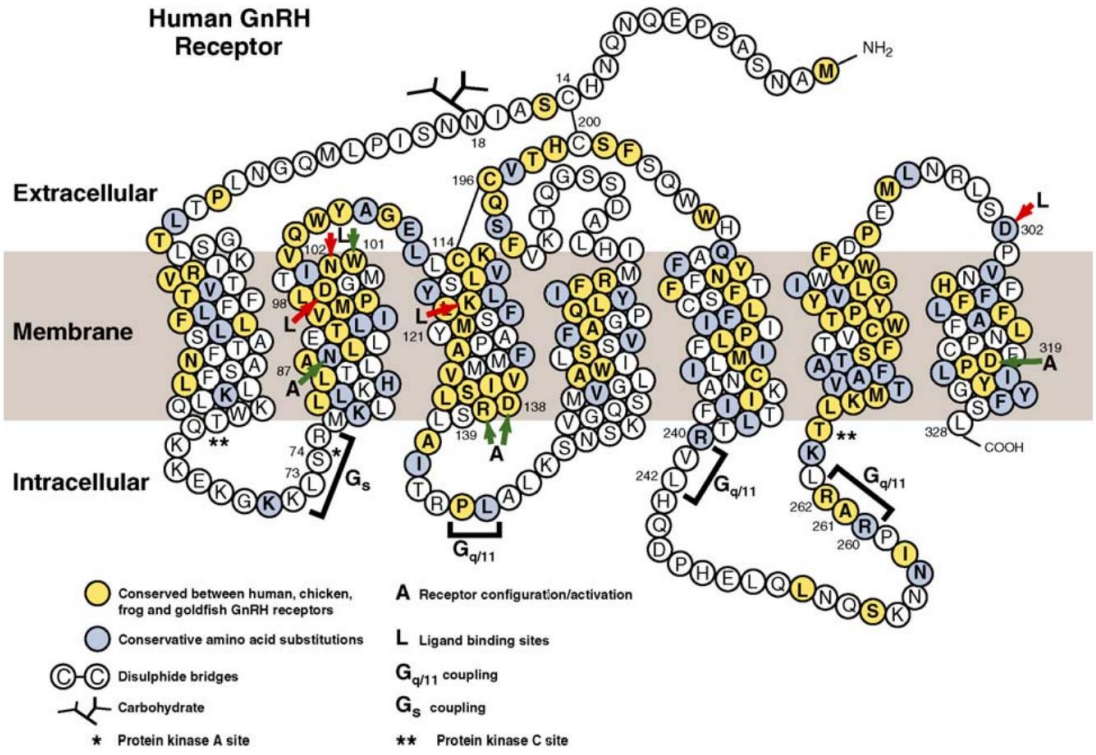


Figure 3.1 ***Two-dimensional illustration of the human GnRH receptor***

Two-dimensional illustration of the human GnRH receptor showing amino acids conserved between cloned vertebrate GnRH receptors (yellow) and conservative substitutions (blue). Assumed ligand binding sites and residues notable in receptor configuration, activation and G-protein coupling are indicated. Reproduced with permission from [108].

Peptide ligands, such as GnRH, bind principally to the extracellular domains and TMDs of GPCRs. GnRH binding to its receptor involves a number of residues namely Asp$^{2.61(98),\ 213}$, Asn$^{2.65(102,)\ 214}$, Lys$^{3.32(121),\ 215}$, and Asp$^{7.32(302,)\ 216}$, and possibly Trp$^{6.58(279),\ 217}$ and Trp$^{2.64(101),\ 218}$. These residues are conserved in all vertebrate GnRHR and are all positioned in the extracellular domains or near the extracellular boundaries of the TMDs.[219] The carboxyl domain of antagonists bind to the same amino acid residues as agonists, but but the amino terminal domain of the intact antagonist make additional contacts via the amino domain that differ from those that bind agonists.[217]

Binding of agonists to GPCRs results in marked conformational changes in the receptor protein. This enables GPCRs to promote guanosine diphosphate (GDP) release from intracellular guanine nucleotide-binding proteins (G proteins). These trimeric proteins consist of three subunits, Gα, Gβ and Gγ. The agonist-occupied GPCR interacts with guanosine diphosphate (GDP)-occupied Gα causing displacement of GDP and exchange for guanosine triphosphate (GTP).[220] Upon binding of GTP the trimeric G protein complex is disrupted, and the Gα and Gβγ subunits dissociate and can regulate the activity of various effector systems.[221] G-protein deactivation is accomplished by intrinsic GTPase activity of Gα subunits which catalyses the conversion of the bound GTP to GDP.[58,222,223]

Different GPCRs couple to different G protein subtypes which, in turn mediate different effector systems. Stimulation of the GnRHR by GnRH predominantly results in activation of Gαq and Gα11 G proteins and subsequent activation of the enzyme phospholipase C (PLC). This membrane associated enzyme catalyses the synthesis of secondary messengers inositol trisphosphate (IP3) and diacylglycerol (DAG) from the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2).[224,225] This results in IP3-dependent calcium signalling, protein kinase C activation and activation of mitogen-activated protein kinases, which together regulate gonadotropin, LH and FSH, secretion and expression (Figure 3.2).[212]

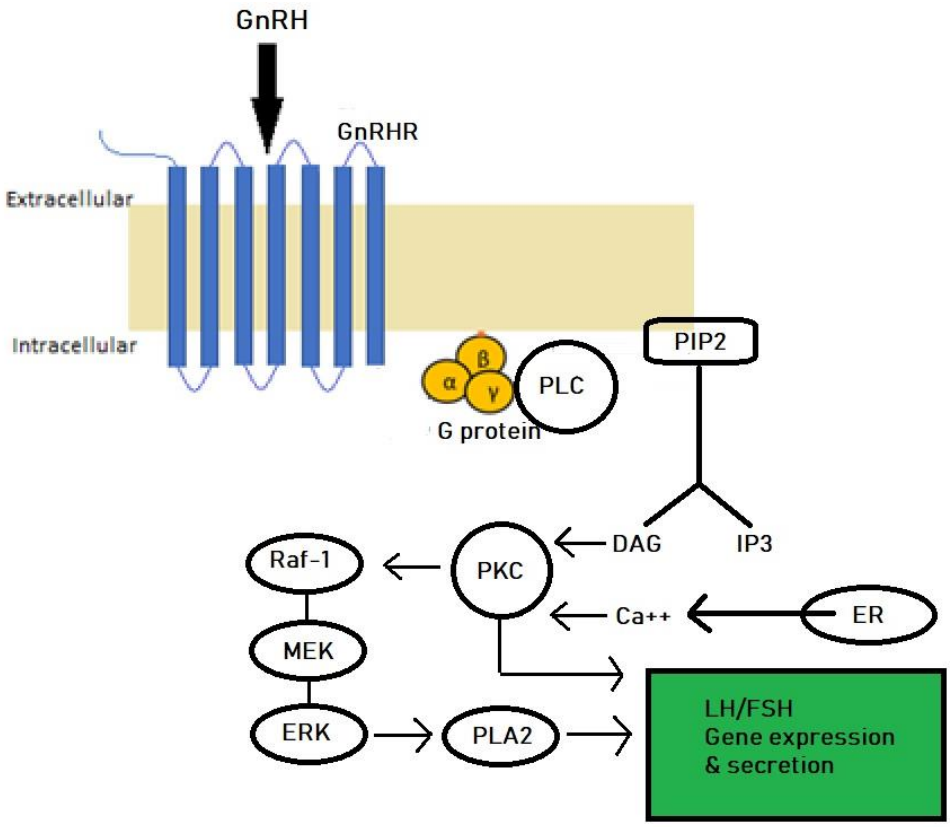


Figure 3.2 ***Ligand-induced GnRH receptor activation and G protein signal transduction***

Schematic representation of ligand-induced GnRH receptor activation and G protein signal transduction, phospholipase C (PLC) activity is induced, catalysing the generation of intracellular inositol phosphates (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 mobilizes calcium ions (Ca ++) from the endoplasmic reticulum (ER), activating protein kinase C (PKC) and mitogen-activated protein kinase (ERK), leading to luteinising hormone (LH) and follicle stimulating hormone (FSH) gene expression and secretion. Adapted in Microsoft Power Point by S Leijenaar from [226].

The modulation of reproductive hormone levels has had a considerable impact both socially (such as contraceptive use) and on medical therapeutics (such as breast and prostate cancer treatment).[110,227] GnRH is transported from the hypothalamus via the portal vessels to the anterior pituitary, here it activates GnRHR. This stimulates the release of LH and FSH which act on the ovaries in females and testis in males to stimulate the production of gametes as well as steroid and peptide hormones. The hormone cascade is regulated through positive and negative feedback at the pituitary and hypothalamus.[110] Numerous tissues are stimulated by sex steroid hormones and dysfunction related to them results in a variety of hormone-dependant diseases. All hormones acting at various sites in the cascade are potential targets for therapeutics to treat such diseases.[228]

In terms of PC treatment, GnRH agonists remain the preferred current first line treatment. However, a feature unique to GnRHR within the GPCR family is the absence of a carboxyl terminal tail (Figure 3.1). This domain in GPCRs is responsible for rapid ligand induced desensitization which leads to termination of signalling.[229] Consequently GnRHR is not rapidly desensitized. However, prolonged exposure to a GnRH agonist generates a decline in circulating levels of gonadotropin and sex steroids in humans.[110] Agonists bind to GnRHRs and result in an initial intense stimulation preceding a marked increase in androgens, LH and FSH.[30] However, this surge is temporary and is followed by tachyphylaxis resulting from pituitary desensitisation which leads to decreases in steroid hormone secretion. [31] Mechanisms of desensitisation are still unclear but suggested mechanisms include, but are not limited to, uncoupling of the GnRHR from G-proteins and desensitisation of downstream signalling such as calcium mobilization and protein kinase activation.[110]

GnRH antagonists were initially associated with histamine releasing properties, new formulations have resolved this but there are few GnRH antagonists currently on the market for treatment of PC. Additionally, one of the biggest barriers to increased use of antagonists is inconvenience as it is necessary to administer it every four weeks. Therefore, in terms of PC treatment, GnRH agonists remain the preferred current first line treatment, although this may change as new antagonists are developed.[230]

Although GnRH agonists have proven to be an effective way to treat and control PC, in conjunction with the treatment benefits, ADT is associated with a number of negative side effects. These side effects include but are not limited to loss of bone mass, hot flushes and loss of libido. Concurrent to reduced secretion of testosterone is a reduction in estrogen levels, as in men estrogen is elaborated from testosterone. This decrease in estrogen is a contributing factor to a number of the negative side effects experienced by patients undergoing ADT.[45,231]

With this in mind, we have developed conjugates of GnRH agonist analogues and estrogens to try to produce effective ADT agents with reduced side effects. First and foremost, in the creation and synthesis of a compound with dual activity was to ensure that the primary

objective – the treatment of PC would not be impeded by the conjugation of the steroid moieties, in this case estrogens, to the GnRH agonist. Therefore, the objective of this phase of the study was to test GnRHR activation by the conjugates, *in vitro*, using an inositol phosphate accumulation assay in HEK293T cells expressing the GnRH receptor. In this assay, exposure of cells to a radiolabelled precursor of PIP_2 ($[^3H]$ myo-inositol) ensures that inositol phosphates (IPs) generated thereafter (upon activation of the GnRHR and subsequent activation of Gαq G proteins and then PLC) are also radiolabelled. Ion exchange chromatography is then used to collect the radiolabelled IPs, which can be measured by liquid scintillation counting. The amount of labelled IP is measured and is therefore proportional to the signalling activation of the GnRHR (Figure 3.3).

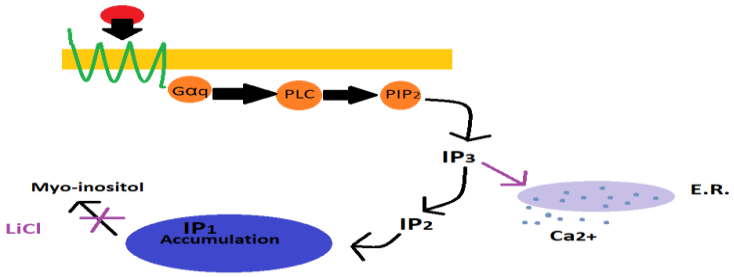


Figure 3.3 ***Gαq protein signalling in the IP accumulation assay***

Intracellular Ca2+ is triggered by inositol 1,4,5-trisphosphate (IP3). Gq coupled GPCRs activate phospholipase C (PLC) and trigger the inositol phosphate (IP) cascade. Adapted in Microsoft Power Point by S. Leijenaar from [232].

3.4 Materials and Method

3.4.1 Inositol Phosphate Accumulation Assay

HEK 293T cells were seeded into 24-well plates, at a density of $1x10^5$ cells/well, and 24hours post-seeding cells were transfected with 0.5 µg/well of plasmid encoding GnRHR.

24 hours post-transfection, media was aspirated and replaced with reduced inositol Media 199 (Gibco, Thermo Fisher Scientific, Massachusetts, United States) supplemented with 1% FCS (v/v), containing 0.5 µCi/well [^{3}H] myo-inositol (Perkin Elmer, Massachusetts, USA). Cells were incubated for 16 hours at 37^{o}C and the media was then aspirated, and cells incubated for 30 minutes with Buffer I (DMEM supplemented with 25 mM HEPES, 10 mM LiCl, and 0.1% (w/v) bovine serum albumin (BSA)) before stimulation for 60 minutes at 37^{o}C in the presence or absence of the appropriate ligand, also prepared in Buffer I. Cells were lysed by incubation for 60 minutes with 10 mM formic acid at 4°C. All isoforms of radiolabelled inositol phosphates were isolated by ion-exchange chromatography using Dowex beads (100-200 mesh, 1X8).

In short, 800 µl of cell lysates were transferred from the plates to Dowex beads (which were regenerated by incubation with 0.04 M formic acid) and were incubated for 5 minutes (vortexing the tubes gently every few minutes) before washing the beads twice in 2 ml ultra-pure water and twice in 2 ml wash buffer (60 mM ammonium formate/5 mM sodium tetraborate). Samples were then eluted from the beads in 1 ml elution buffer (1 M ammonium formate/0.1 M formic acid) and were transferred to scintillation vials. 2.5 ml optiphase HiSafe3 -scintillation fluid (Perkin Elmer, Massachusetts, USA) was added and radioactivity [counts per minute (cpm)] of the eluents was measured by liquid scintillation counting, using a Packard Tri-carb 2100TR liquid scintillation analyser.

3.4.3 Data analysis

Data were analysed by nonlinear regression and used to generate sigmoidal dose-response curves to yield values for EC_{50} and Emax. In order to minimise inter-assay variability, for each experiment values were calculated relative to the sum of all values measured. Average basal activation (measured in the presence of cells incubated in the absence of stimulating ligand) was subtracted and data were calculated as a percentage of average maximal activation measured in cells stimulated with GnRHag. Data are presented as mean ± SEM from five independent experiments.

Statistical analyses were performed using a one-way ANOVA followed by Tukey's multiple comparison test for pairwise comparisons. $p<0.05$ was considered statistically significant for all data. All statistical analyses were performed using GraphPad Prism 5.0 software. (version for Windows, GraphPad Software, San Diego California USA).

3.5 Results

To determine the ability of the two conjugates, E2C and GenC to interact with and activate the GnRHR, their ability to elicit IP accumulation in cells expressing the GnRHR was examined. IP production was stimulated by both conjugates (E2C and GenC), indicating that they were able to induce activation of the GnRHR. No stimulation occurred when unconjugated E2 or Gen were used (Figure 3.4). Although both conjugates elicited GnRHR stimulation, there were differences in the maximal responses (Emax) observed for the conjugated compounds when compared to the unconjugated GnRH agonist analogue (D-Lys6 GnRH Pro9 ethylamide; GnRHag) such that both E2C and GenC had a slightly lower Emax (72 and 85% of the response elicited by the GnRH agonist, respectively) (Figure 3.4 and Table 3.1). The maximal response level elicited by E2C was lower compared to GenC (Figure 3.4 and Table 3.1). Interestingly, the 17β-Estradiol conjugate, E2C, had a slightly (2-fold) increased potency compared to the unconjugated GnRHag and GenC conjugate (Figure 3.4 and Table 3.1).

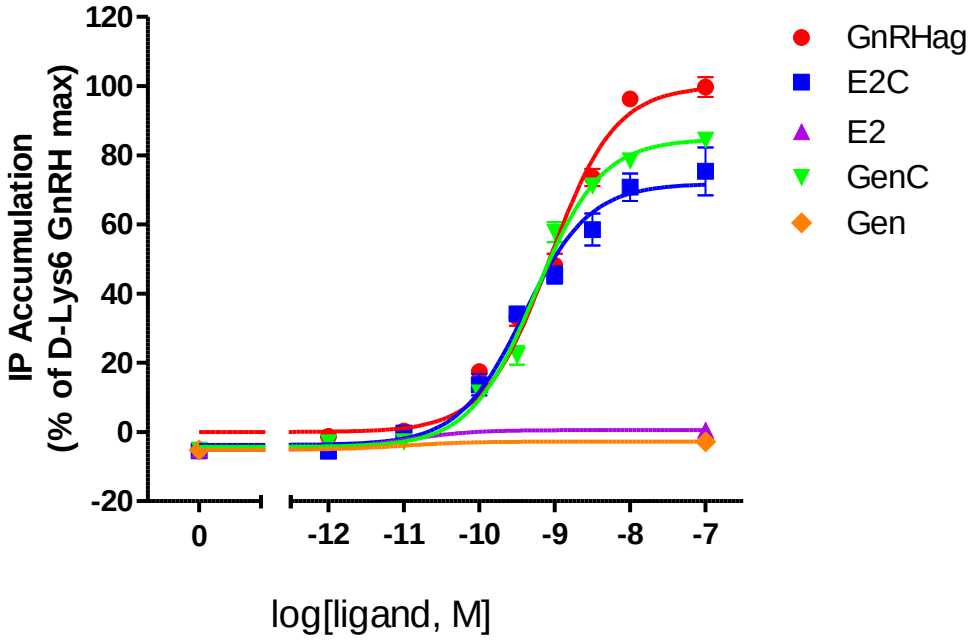


Figure 3.4 ***Stimulation of inositol phosphate (IP) accumulation by conjugated/unconjugated compounds***

HEK 293T cells transiently transfected with GnRHR were stimulated with vehicle (0) or a range of concentrations (1pm – 100nM) of D-Lys6 GnRH Pro9 ethylamide (GnRHag; red),17β Estradiol (E2,purple), 17β Estradiol- D-Lys6 GnRH Pro9 ethylamide -Conjugate (E2C, blue), Genistein (Gen, orange) and Genistein- D-Lys6 GnRH Pro9 ethylamide-Conjugate (GenC, green) and IP accumulation measured. Data are mean ± SEM from five independent experiments and are expressed as maximal % GnRHag response after subtraction of basal IP accumulation (measured in the absence of stimulating ligand).

Table 3.1: Signalling parameters (maximal responses and potencies) calculated for conjugated/unconjugated compounds (GnRHag, E2, E2C, Gen and GenC)

	Emax +/- SEM (% of GnRHag max)	**pEC_{50} +/- SEM (EC_{50}(nM))**
D-Lys6 GnRH Pro9 ethylamide (GnRHag)	100+/-3	9.07+/-0.05 (0.85)
17β-Estradiol conjugate (E2C)	72+/-5*	9.42+/-0.07** (0.38)
Genistein conjugate (GenC)	85+/-1*** #	9.25+/-0.04 (0.56)
17β-Estradiol (E2)	No stimulation	No stimulation
Genistein (Gen)	No stimulation	No stimulation

*Data are mean ± SEM of five independent experiments. *, **, *** p<0.05, 0.005, 0.001, respectively for comparison to GnRHag and # p<0.05 for comparison of 17β-Estradiol conjugate and genistein conjugate (one-way ANOVA followed by Tukey's post-test).*

3.8 Discussion

Although they were unable to elicit the same maximal response as the unconjugated GnRHag, the fact that both conjugates (GenC and E2C) were able to elicit robust IP responses, with potencies similar to (GenC) or even slightly higher (E2C) than GnRHag. This indicates that these conjugates are able to interact with and activate the GnRHR and would therefore remain efficacious as ADT therapeutics despite having the large estrogenic side chain attached. Both conjugates also displayed a very slightly increased potency in comparison to GnRHag, although this effect was only significant for the E2C conjugate. These data suggest that conjugation of GnRHag to the estrogens may enhance potency of activation but decrease the level of stimulation elicited upon receptor activation. This has been demonstrated in a number of other studies. Koch *et al*. confirmed that GnRH analogues in which Dextrarotatory amino acids were substituted for the Gly^6 displayed enhanced biological potency and resistance to enzymatic degradation.[233] Conjugation of an antiangiogenic compound (sunitinib) to a D-Lys^6-GnRH demonstrated superior efficacy due to conjugation.[234] It is therefore possible that these compounds function as partial agonists, binding and activating the receptor but with slightly reduced efficacy as they are unable to elicit the maximal response of the receptor system, in comparison to the full agonist. It is a possibility that the steroid component of the conjugates is allowing enhanced binding, due to conjugation aiding in stabilising the horseshoe structure of GnRH, but hinders interaction with the receptor required for activation to a small degree, preventing maximal activation. An interesting finding in 2018 by Mukhtasimova and Sine showed that full and partial agonists induce distinct structural changes in opening the muscle acetylcholine receptor (a GPCR) channel. According to this study the data provides information on agonist-mediated structural changes at the level of individual residue positions and provide evidence that within the protein tertiary structure juxtaposed regions may move relative to one another, implying that the α subunits flex or twist in the course of activation. The data demonstrates that different agonists prompt different structural changes during opening of the receptor channel and broaches the possibility that intermediate agonist-bound receptor states may differ from one agonist to another.[235] Although not shown in GnRHR, it does indicate one of many possible explanations for differences in efficacy of agonists and partial agonists, that the addition of an estrogenic compound may result in slight changes to the agonist-bound receptor state.

The crystal structure of GnRHR has not yet been resolved, limiting our comprehension of GnRH binding to its receptors and their consequent activation. Certainly, further research in particular binding assays would aid in the understanding of how the conjugates may affect receptor activation.

As demonstrated by Ratcliffe *et al.* the site of conjugation for a steroid hormone to a GnRH analogue is key in retaining GnRHR as well as steroid receptor binding and activation. Ratcliffe *et al.* did a comparison of conjugation via a D-amino acid in position six, an L-amino acid in position seven of GnRH antagonists, as well as the amino acid terminus in position three of a truncated GnRH antagonist, via a hydroxyl at C11 and C21 of steroids. It was found that conjugation of [D-Lys6] of GnRH antagonists with C21 of 21-hydroxyprogesterone resulted in a compound with effective plasma protein binding and high GnRH and progesterone receptor binding and activation. This indicates that this site and the long side chain attachment avoided interference of key binding sites and steric hinderance.[203] GnRH assumes a βII' conformation around Gly^6 when bound to the GnRHR. This conformation and binding affinity is enhanced in human GnRH if the Gly^6 is replaced with a D-amino acid containing a large aromatic side chain, such as D-tryptophan in the drug Triptorelin.[37,110,236] Substitutions in this position also convey the benefit of increased resistance to proteolytic cleavage. In addition, incorporating a large hydrophobic side chain leads to binding of GnRH to plasma proteins which increases the half-life in circulation and decreases metabolic clearance.[110] This led to the decision that the compounds used in our study would be produced by conjugating at the same site and indeed, conjugating in this manner had not severely impacted GnRHR activation.

4.0 Estrogen Receptor Activation by bifunctional Gonadotropin-Releasing Hormone Agonist-Estrogen Conjugates

4.1 Abstract

Early diagnosis and the use of ADT as a PC treatment has resulted in an improved prognosis and life expectancy for PC patients and thus there has been a significant rise in survivors of PC, in light of this it then becomes important to begin to address the quality of life that these survivors will experience during and after the cessation of treatment. Compared to the general population, men with PC have higher non-cancer mortality, some of this attributed to the treatment they receive. There are a number of negative side effects associated with ADT. GnRH agonists used in ADT are associated with increased fat mass, and CVD. Other significant adverse side effects of ADT include bone loss, hot flushes, loss of libido, gynecomastia, serum lipid changes and memory loss. The majority of these side effects are due to reduced estrogen levels as a consequence of reduced androgens from which they are elaborated in men.

Therefore, it is worthwhile to consider an approach that would remain efficacious against PC while simultaneously addressing a number of the side effects experienced from the use of ADT. Currently, many of these side effects are treated after the fact, making treatment more difficult and less effective. Additionally, each side effect requires a different treatment approach, such as the use of bisphosphonate to reduce loss of bone density and lifestyle modifications for cardiovascular associated side effects. As multiple side effects result from the concomitant decrease in estrogen, an approach in which estrogen is supplemented would concurrently address multiple side effects associated with ADT. To this end, this study examined the possibility of a dual treatment approach using a compound which was able to activate the GnRHR as well the ERs was pursued in this study, making use of conjugated compounds – a GnRH agonist conjugated to either 17β-Estradiol or genistein (a phytoestrogen). Following confirmation that the conjugated compounds retained GnRH receptor activity, the aim of this phase of the study was to ensure that they also had estrogenic activity.

An E screen assay which measures estrogen-induced proliferation of a breast cancer cell line was utilised and the data obtained demonstrated that all compounds containing estradiol or genistein induced significantly increased cell number compared to vehicle treated cells. The induced increase in proliferation was less when cells were treated with compounds containing genistein in comparison with compounds containing estradiol, but both conjugates induced similar effects to their respective unconjugated counterparts. In each case, the effects were negated by co-incubation with Tamoxifen, a selective estrogen receptor modulator (SERM), demonstrating that the increase in cell number was ER mediated. These results are a positive indication that the conjugates retain their estrogenic activity despite attachment to the GnRH agonist.

4.2 Objective

To test ER activation of the conjugates by identifying whether they are able to activate the estrogen receptor *in vitro* using an E-screen assay.

4.3 Introduction

The traditional ERs (ERα and ERβ) are members of a large family of nuclear hormone receptors that act as transcription factors.[237,238] These ERs share structural features that are accountable for the overlap in similar functional features. At a number of structural regions distinct amino acid compositions allow for subtype-specific properties in transmitting estrogen signalling.[238] The binding of steroids to these receptors results in dissociation of the receptor from an inactive complex allowing dimerization, high affinity DNA binding and transcriptional activation.[116] The regions, including the hormone binding domain, involved in a number of these functions are conserved, both at a structural and functional level within members of the nuclear receptor family. Activation of these receptors is associated with the cell cycle[239] and in particular the ERs are implicated in the regulation of cell proliferation.[116,239] ERs modulate the transcription of target genes by binding to estrogen response elements (EREs) in the DNA sequence. [118] Binding of ERs to EREs encourages bending and looping of the DNA, thus allowing interaction with the transcriptional machinery and co-regulator proteins.[119]

An additional estrogen receptor was discovered in 2005, G protein coupled estrogen receptor (GPER). It is thought that GPER may partly be responsible for rapid non-genomic actions associated with estradiol.[240] GPER is expressed in both normal prostate tissue as well as in PC. There is duality in the reporting of the role of GPER in in PC. Studies have argued that GPER has a causative role in prostate carcinogenesis[241], other studies have endorsed the protective nature of GPER in PC development.[242] Significant advances have been made in terms of characterisation of GPER activity in PC. However, a lack of information remains and as such a great deal more investigation needs to be undertaken to resolve the multiple actions of GPER in PC and what potential therapeutic options this receptor may offer.[243]

Estrogen signalling pathways rely on stimulation and inhibition dependant on the balance between the traditional receptor subtypes, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), in target organs (the role of GPER in this balance has yet to be fully determined).[116] The two traditional subtypes generally have different biological functions, but dependant on cell type there can be overlap. [244]

ERα is highly expressed in the uterus, prostate stroma, ovarian theca cells, Leydig cells in testes, epididymis, breast, and liver. [120] ERβ is highly expressed in prostate epithelium, testes, ovarian granulosa cells, bone marrow, and brain. [121] ERα and ERβ have distinctive downstream transcriptional activities, resulting in their tissue-specific biological actions. [122]
In addition to androgens, estrogens also have key roles in the normal growth and development of the prostate. Estrogen is important for normal tissue homeostasis in the prostate, either excessive or reduced levels of estrogen can lead to increased gland growth and disease.[245]

Testicular and serum testosterone levels both fall as men age. Estradiol however remains unchanged or may even increase as men age. It is thought that increased aromatization of adrenal androgens within peripheral adipose tissue, which also tends to increase in the older male is what contributes to these levels. As a result, during the aging process there is a decrease in the ratio of testosterone to estradiol and it is postulated that this may contribute to the onset of prostate disease, including PC.[245] Estrogens have therefore been adversely implicated in the development of PC.

Adverse actions of estrogens on the prostate gland can be separated into three categories – aberrant proliferation, inflammation and cancer (Figure 4.1). Inflammation of the prostate due to estrogens is distinct to that which is achieved by androgens, it lacks order and is rather aberrant. Inflammation resulting from estrogen administration has been noted in rodents as well as during estrogen therapy administered to transsexual males. This evidence is significant but it should be noted that this data has risen from situations in which estrogen was given at supraphysiological levels. The development of inflammation in response to estrogen is independent of androgens and is the direct response of the prostate to estrogens. HPG mice models have been used to demonstrate that mice that are completely deficient in pituitary gonadotrophins and, subsequently, of sex steroids (thus there are no confounding effects due to androgen withdrawal), have inflammatory pathology in direct response to estrogen exposure.[246] The recruitment of inflammatory material and cytokines in the prostate present an increased risk for the development of premalignant and malignant legions.[247,248] Irrespective of which model is used all the current data provide a link between estrogen and

inflammation, thus also providing a potential mechanism through which estrogen is implemented in the development of malignancy.[245] Binding of ERs to EREs advocates bending and looping of the DNA, thus allowing interaction with the transcriptional machinery and co-regulator proteins.[119]

As previously mentioned, the ratio between androgens and estrogens is key in the development of malignancy. During progression to prostatic malignancy increased local estrogen levels can result from induced aromatase expression and modified regulation. This may in turn lead to an imbalance between androgens and estrogens necessary for the maintenance of tissue homeostasis thus promoting the development and/or progression of PC.[245] The link between these three adverse effects (proliferation, malignancy and inflammation) is that the mechanism behind them involves the actions of the ER subtype – ERα (Figure 4.1).

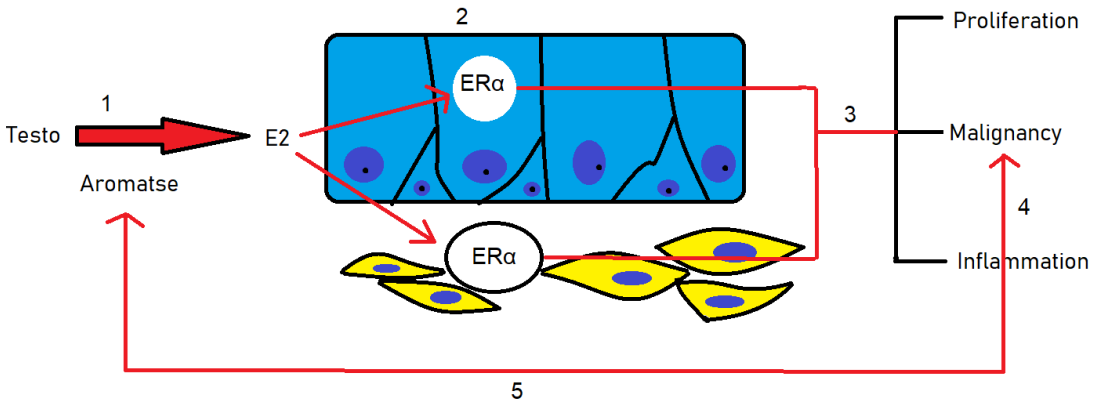


Figure 4.1 ***ERα facilitates the negative actions of estrogen in the prostate****.*

1) Locally within the prostate androgens, testosterone (testo), are metabolized to estrogens via the aromatase enzyme 2) Estrogens could signal via ERα, located and expressed within both the epithelium and stroma 3) ERα consequently mediates a number of adverse effects upon the prostate, particularly aberrant proliferation, inflammation and malignancy 4) The induction of inflammation by ERα could also foster the development of malignancy 5) and may additionally stimulate aromatase expression, which is altered in malignancy and could be driven by inflammatory cytokines stimulating a cycle of activity. Adapted by S Leijenaar in Microsoft PowerPoint from [245]*.*

In contrast to these adverse effects of estrogen in males, there is ample evidence that demonstrates that estrogen has a number of beneficial effects in men in a number of different tissues and organs. The beneficial role of estrogen in men is highlighted in men deficient in aromatase, leading to a decrease in circulating estrogens and a concurrent elevation of testosterone and gonadotrophins. These men display delayed skeletal maturation and epiphyseal closure; osteoporosis with bone pain, hyperinsulinemia, impaired lipid metabolism, and infertility.[249,250] Evidence is lacking regarding the status of the prostate gland in aromatase deficient men. However, a number of studies exist that validate the positive effects of estrogen on the prostate. Men in Asian countries are reported to have lower levels of systemic estrogens compared to their Western counterparts.[251] Additionally, reports state a much higher intake of phytoestrogens in the diet of these Asian men.[251,252] Serum estrogen levels are not measurably altered by the consumption of such compounds but there is a direct link which exists between their consumption and a reduced incidence of PC. As a result, a number of studies have been conducted in order to elucidate the mechanism by which estrogens, and in particular, phytoestrogens, can exert positive effects on the prostate (Figure 4.2).

It is postulated that the contrasting effects of estrogens in the prostate may be due to the activity of different ER subtypes. ERβ is found far in excess of ERα within the prostate.[128] The use of knock out mice lacking ERβ demonstrated that lacking this receptor resulted in a three times greater proliferative activity when compared with normal mice.[253] This link is further supported by studies utilising aromatase deficient (ArKO) mice. Here in the absence of estrogen no activation of ERβ or ERα occurs, within eight weeks of birth these mice developed prostatic hypertrophy and hyperplasia.[254] If an ERβ agonist is administered to ArKO mice there is selective suppression of prostate hypertrophy and ablation of prostatic hyperplasia. In contrast if an ERα agonist is administered suppression of hypertrophy is possible but ablation of prostatic hyperplasia cannot be achieved.[255] There is a decrease in the levels of apoptosis in β-ERKO and ArKO mice when compared with wild type tissues. Additionally, it has also been demonstrated that beta estrogen receptor knockout (β-ERKO) mice show significant increases in B cell lymphoma 2 (bcl-2) expression. This is a regulator protein, key to regulating cell death. This then implicating ERβ in the induction, or at least regulation of apoptosis.[256]

In keeping with the dichotomy between ERα and ERβ, while there is data to indicate activation of ERα is able to induce inflammation there is also research which supports the notion that activation of ERβ can lead to decreased inflammation. In animal models an ERβ-specific agonist has demonstrated beneficial effects on inflammation caused by a number of diseases including bladder cystitis, bowel disease, and microglia. The mechanism behind this has not been elucidated, however it has been speculated that it is partially due to inhibition of NF-κB transcriptional activity.[257] This is further supported by studies using β-ERKO mice. These mice have been found to develop abundant and massive inflammation, characterized by T-Cell infiltration. The wildtype littermate counterparts in this study did not develop inflammation.[258] Studies using luteinizing hormone receptor knockout mouse (LuRKO) revealed that the activation of ERβ with a specific agonist was resulted in the prevention of the development of prostatic hyperplasia and inflammation.[259] This is significant as it has been suggested that inflammation has a part to play in the etiology of malignancy and as such ERβ could be key in fulfilling a protective role in the prostate.[245]

The evidence as discussed above regarding the antiproliferative and anti-inflammatory properties relating to ERβ lends credence to the idea that this receptor may also be beneficial in the suppression of PC. Data has shown that ERβ expression is lost in PC cells.[260–262] Further more studies have also demonstrated hypermethylation of the ERβ gene in PC.[263] As previously mentioned the use of isoflavones or their derivatives such as genistein, which preferentially bind to ERβ, appear to reduce the PC burden, providing further support for the protective nature of ERβ in PC.[264,265] This demonstrates why the use of an alternative estrogenic compound such as genistein may be beneficial when conjugating to a GnRH analogue for the treatment of PC.

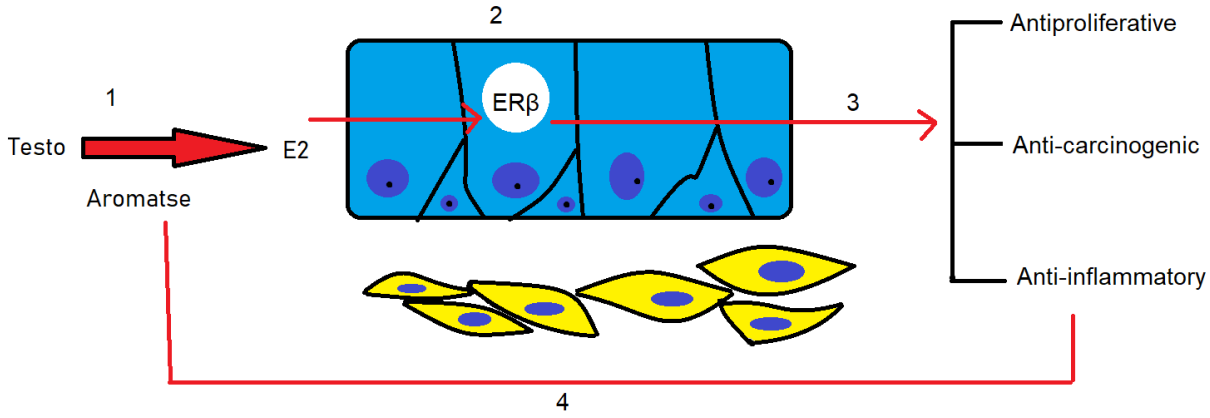


Figure 4.2 ***ERβ appears to be responsible for the beneficial actions of estrogen in the prostate.***

1) *Locally within the prostate androgens are metabolized to via the aromatase enzyme 2) Estrogens may signal via ERβ, located and expressed within the glandular epithelium 3) ERβ consequently exerts beneficial (and protective) effects upon the prostate, which could be antiproliferative, anti-inflammatory, and anti-carcinogenic 4) the anti-inflammatory actions of ERβ could possibly also result in decreased aromatase expression, and, therefore, reduced local estrogens, however this is presently not proven. Adapted by S Leijenaar in Microsoft PowerPoint from* [245].

When addressing the side effects of ADT, it is important to note that in addition to the receptor subtype's role in PC, these subtypes can influence the progression of osteoporosis as well. ERα is expressed at low levels in bone. In αERKO mice, the size and length of bone are significantly diminished and there is a reduction in mineral density compare to normal mice.[266] ERα is predominantly expressed in cortical bone, whereas ERβ shows higher levels of expression in trabecular bone, which is more active and subjected to higher levels of bone turnover and remodeling.[151] Again there are inconsistent results regarding the exact role of each receptor subtype, the differential distribution of the subtypes may be indicative of site specific variations within the skeleton relating to estrogen responsiveness.[151]

4.4 Materials and Method

4.4.1 E-Screen Estrogen Assay

MCF7 cells were seeded at a density of 2.5 x 10^4 cells/ml in a clear 96 well plate. 24 h post-seeding cells were exposed to phenol red free DMEM media containing the treatment compounds at a range of doses (0.1 pM to 1 µM), vehicle only (phenol red free DMEM) or 1 µ M test compound co-incubated with 1 µM tamoxifen for 72 h after which crystal violet staining was used to determine cell density. Media was aspirated and replaced with 100 µl/well of 1% (w/v) glutaraldehyde and plates were incubated at room temperature for 15 minutes to fix the cells. This was removed and 100 µl/well of 0.1% (w/v) crystal violet stain was added and wells incubated at room temperature for a further 30 minutes. The crystal violet stain was then removed, and plates rinsed by submerging in water. Rinsed plates were left to dry overnight. The following day 200 µl of 0.2% (w/v) Triton X-100 was added to each well and left to incubate at room temperature for 30 minutes. 100 ul from each well was transferred to a clean 96 well plate. Absorbance was measured at 595 nm on a BioRad iMark microplate reader (Biorad, Hercules, California, United States of America).

4.4.2. Data Analysis

Data were analysed by nonlinear regression and used to generate sigmoidal dose-response curves to yield values for IC_{50} and Emax. In order to minimise inter-assay variability, for each experiment values were calculated relative to control values measured in wells that were vehicle treated. Data are presented as mean ± SEM from four independent experiments. All analyses were done using the GraphPad Prism 5.0 software (version for Windows, GraphPad Software, San Diego California USA).

Statistical analyses were performed using a one-way ANOVA followed by Dunnett's multiple comparison test for pairwise comparisons. $p<0.05$ was considered statistically significant for all data. All statistical analyses were performed using GraphPad Prism 5.0 software. (version for Windows, GraphPad Software, San Diego California USA).

4.5 Results

To measure ER activity of the conjugated compounds, it was necessary to find a suitable assay. A T47DKbluc assay was initially tested, but was unable to generate consistent results and after numerous failed attempts to optimise assay it was decided that in order to assess the ER activation of our treatment compounds it would be necessary to utilise a different assay.

An ERE-luciferase assay was then designed and attempted. This assay involved transfection of cells with ERs and a ERE-luciferase reporter gene. However, despite trying many methods to try to eliminate estrogen contamination in the assay (making use of stripped serum and phenol-red-free media) a high background signal was obtained in ER transfected cells which meant that it was very difficult to measure an additional signal in response to activation by the test compounds.

To determine the ability of the conjugates, E2C and GenC, to activate the ER, their ability to increase cell number of MCF7 cells which express ERα and ERβ was measured. The E-Screen[267] generated data demonstrates all compounds containing estradiol or genistein, including E2C and GenC, were able to induce activation of the ER. The increase in cell number (indicative of increased proliferation) from estrogen treatment (E2, E2C, Gen and GenC) was significant when compared to vehicle treated wells, with percentage absorbance over vehicle ranging from 165.8 – 178.6% (Figure 4.3 and Table 2). The conjugates were able to activate the ER to the same degree as their unconjugated counterparts, with no significant difference between the E_{max} and pEC_{50} of E2 compared to E2C and no significant difference between the E_{max} and pEC_{50} of Gen compared to GenC. There was a significant difference in the EC_{50s} between treatments containing 17β-estradiol and those containing genistein (Figure 4.3, Table 2). The lack of increased cell number when treatments were co-incubated with Tamoxifen, a SERM, demonstrates that the increased cell number that occurred in the absence of tamoxifen was ER mediated. The induced increase in cell number was less when cells were treated with compounds containing genistein in comparison with compounds containing estradiol (Figure 4.3, Table 2).

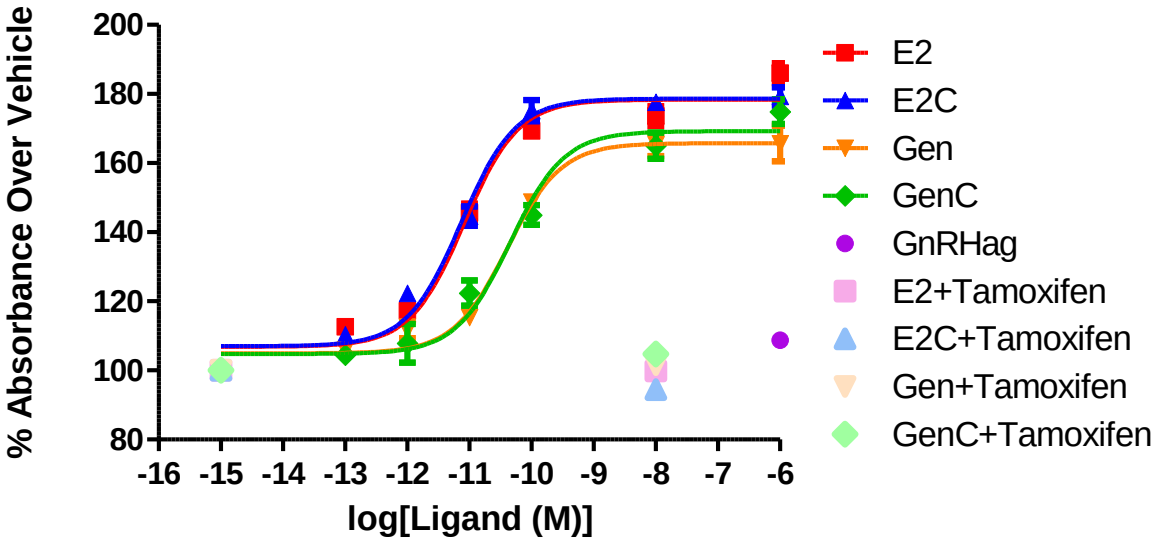


Figure 4.3 ***Stimulation of increased cell number by conjugated/unconjugated compounds on MCF7 cells***

MCF 7 cells expressing ERα and ERβ were stimulated with vehicle (0) or a range of concentrations (1 µM – 0.01pM) of D-Lys6 GnRH Pro9 ethylamide (GnRHag; purple), 17β Estradiol (E2, red), 17β Estradiol- D-Lys6 GnRH Pro9 ethylamide -Conjugate (E2C, blue), Genistein (Gen, orange) and Genistein-D-Lys6 GnRH Pro9 ethylamide-Conjugate (GenC, green). Increase in cell number was measured with crystal violet staining. Absorbance was measured at 595 nm. Data are mean ± SEM from four independent experiments and are expressed as % absorbance over vehicle.

Table 4.1: Effect of conjugated and unconjugated compounds on MCF 7 cell number

	Emax +/- SEM (% Absorbance Over Vehicle)	**pEC_{50} +/- SEM (EC_{50}(pM))**
D-Lys6 GnRH Pro9 ethylamide (GnRHag)	No stimulation	No stimulation
17β-Estradiol (E2)	178.3+/-1.632	11.08+/-0.07* (8.3)
17β-Estradiol Conjugate (E2C)	178.6+/-1.565	11.13+/-0.07* (7.4)
Genistein (Gen)	165.8+/-2.054	10.39+/-0.10* (40.8)
Genistein conjugate (GenC)	169.3+/-2.536	10.34+/-0.11* (45.3)

*Data are expressed as mean ± SEM of four independent experiments for Emax and pEC_{50} for each ligand. * $p<0.05$ E2 vs Gen, E2 vs GenC, E2C vs Gen and E2C vs GenC. No significant difference of EC50s of E2 vs E2C and Gen vs GenC. A one-way ANOVA followed by Tukey's post-test for comparison between EC50s of ligands.*

4.6 Discussion

Unfortunately, it was difficult to find an assay that directly measured ER activation that was suitable for measuring the estrogenic activity of the test compounds. Many of the issues related to the high sensitivity of the T47D-Kbluc and ER reporter gene assays, as well as estrogenic contamination which severely impacted the signal to noise ratio of the assay. This is likely because of the presence of multiple ERE response elements in the ERE - luciferase reporter and T47D-Kbluc assays which results in high sensitivity and amplification of any signal generated by contamination with estrogenic compounds in the assay reagents. Therefore, another means of measuring the estrogenic activity of the test compounds was required. A study by Leusch *et al*. (2010) compared five *in vitro* bioassays used to measure estrogenic activity. A comparison of ER-CALUX, MELN, T47D-Kbluc, yeast estrogen screen and the E-Screen assays found that all five displayed similar trends and there was good agreement with analytical chemistry results. Data from the E-Screen was found to be robust and predictable, correlating well with predictions from chemical analysis.[268] Therefore, an E-Screen was deemed an appropriate alternative to the T47DKbluc and the ERE-luciferase assays. Here the increased cell number of MCF 7 cells due to ER activity is used as a proxy to demonstrate estrogenic activity.[267]

The Escreen data shows that these conjugates (E2C and GenC) retain estrogenic activity and therefore the addition of GnRHag did not impede the activation of the ERs. Loss of response after coincubation with an anti-estrogen, tamoxifen, confirmed that the effects seen were ER mediated.

In the development of PC drugs, the design of a dual treatment drug that maintains ADT benefits while also inducing stimulation of ERβ could not only be beneficial in terms of reducing the negative side effects of ADT but may also contribute to the reduction and treatment of the disease itself. The results achieved in this study are encouraging in that genistein and the genistein conjugate were both able to activate the ER to similar levels when compared to estradiol and the estradiol conjugate.

That being said there is conflicting data on the effect of genistein on proliferation in cancer cell lines. In contrast to the data that demonstrates the preventative role of genistein on breast cancers, some studies of breast cancer cells and/or humans have indicated a growth-promoting effect by phytoestrogens including genistein.[269] In ovariectomized athymic mice implanted with ER+ MCF-7 cells, genistein has also been shown to promote tumour growth in a dose-dependent manner.[270] Genistein was shown to stimulate MCF-7 breast cancer cell growth by inducing acid ceramidase (*ASAH1*) gene expression.[271] Although Genistein binds preferentially to ERβ, it can bind to both classical ERs. It has been suggested that the presence of ERβ is associated with the positive effects of genistein whereas a high concentration of genistein in cells expressing ERα is associated with the negative impacts of phytoestrogen.[272] c-Fos proto-oncogene expression as an early molecular sensor of estrogen action in MCF7 cells as well as SKBR3 breast cancer cells (which are ER negative) was used to demonstrate the ability of 17β-estradiol and genistein to regulate the expression of growth-related genes such as c-fos even in the absence of ER. The c-fos response was repressed when the SKBR3 cells were transfected with an antisense oligonucleotide against GPER. This implicating GPER in estrogenic effects that cannot be explained by the classic model of hormone action involving the binding of the classical ERs.[273]

Future studies should take into account the level of expression of the different ER subtypes as well as the expression of the novel GPER within the cell line being used. It is possible that a cell line that expresses higher levels of ERα would proliferate in response to genistein stimulation, whereas if the cell line expressed higher levels of ERβ this might not be the case. Indeed, to gain more in-depth understanding into the role of ERα/ERβ ratio, a global gene expression profile was done on MCF-7 and T47D breast cancer cells. These cell lines were exposed to soymilk extracts. At high ERα levels, soy isoflavones demonstrated the same expression changes as those induced by estrogen, stimulating the upregulation of multiple factors involved in the cell cycle, DNA replication, chromosome segregation and inhibition of apoptosis. Upon reconstitution of the expression of ERβ by an inducible promoter, a reduction of cell division growth-promoting factors was observed as well as a stimulation of cell proliferation arrest factors.[274]

Additionally, while it is clear that the effects of genistein are in part mediated by ERβ, other molecular mechanisms have been shown to have a role. For example the inhibition of prostate cancer cell growth exerted by genistein has been coupled to a reduction of telomerase activity that is crucial for cellular proliferation capacity and immortality.[275] Inhibition of nuclear factor κB (NF-κB) activity by genistein has also been reported in prostate cancer cells.[276] These observations are indicative of the complexity of actions involved in the potential effects genistein has on both cellular proliferation and tumour growth.

It is evident that a number of factors are influential in determining the final effects of genistein, and that these effects might be quite different dependant on receptor profile and could be contradictory based on concentration of genistein. For instance, at low doses (from 10 nM to 1 μM), genistein displayed mitogenic effects on breast cancer cell growth, whereas at higher concentrations (>10 μM), it showed antiproliferative effects.[277,278] A number of these effects are clarified by interactions with the different ER subtypes bearing in mind that the ratios and the expressions of ERα and ERβ are different in various tissues depending on the period of life. It does appear that there is a level of consensus regarding the specific role of genistein in PC being beneficial, with prostate tissue strongly expressing ERβ. Thus, estrogen, genistein and their conjugates are candidates for novel treatment of PC and the associated side effects from ADT. Further research employing selective antagonists to establish the roles of the various receptors would be necessary to determine which of the conjugates may be the most beneficial as a duel treatment for PC. In vivo studies would be required to advance the understanding of the diverse pathways activated by phytoestrogens such as genistein.[198]

The plethora of effects induced by genistein stimulation can make interpretation of data complicated. However, novel cancer treatment favours a multi-target approach in order to reduce compensatory mechanisms which can lead to drug resistance. As genistein modulates multiple signalling pathways, inducing a number of effects it represents a promising agent for the management and treatment of cancers.[279]

5.0 Negative regulation of RANKL-induced osteoclastic differentiation in RAW264. 7 cells by bifunctional Gonadotropin-Releasing Hormone Agonist-Estrogen Conjugates

5.1 Abstract

Due to the concurrent decrease in estrogen as androgens are reduced, patients undergoing ADT have significant and long-lasting decreases in BMD with effects increasing with increased time of treatment. Within the first year of receiving ADT absolute BMD loss is around five percent, and men with PC undergoing ADT have 5- to 10-fold increased loss of bone density at multiple skeletal sites in comparison with healthy controls or men with PC not being treated with ADT. Levels of bone density testing in PC patients undergoing ADT are less than desirable, resulting in under or late diagnosis of osteopenia and osteoporosis There are treatment options available to try to reduce this effect, one of which is administration of bisphosphonate. However, men undergoing ADT who are treated with bisphosphonate can still experience severe loss of trabecular bone mass and this treatment can be associated with other adverse events. Therefore, alternative forms of treatment for bone loss associated with ADT are desirable. The beneficial effects of estrogen in bone physiology are well established, numerous studies of estrogen supplementation in menopausal women have yielded positive results in terms of improved bone density. This is achieved via a number of estrogen mediated mechanisms including a reduction in osteoclast differentiation. Support for the idea that improvement in BMD due to levels of estrogen in men was found in a study in which androgen supplementation was given to eugonadal men with osteoporosis for six months. The improvements in BMD in this study were positively correlated with a change in serum estradiol but not with a change in serum testosterone.

Therefore, the development of a dual treatment utilising GnRH analogue-estrogen conjugates which would effectively treated PC, while providing simultaneous estrogen supplementation was investigated. Initial results indicated the conjugated compounds retained GnRHR as well as ER activation and the aim of this phase of the study was to examine the ability of these compounds to exhibit bone-protective properties. Using a TRAP staining assay to measure

osteoclast (bone resorbing cell) differentiation, it was established that conjugates containing estrogen or genistein were able to reduce differentiation of RAW 264.7 macrophages into osteoclasts as effectively as their unconjugated counterparts. There was no significant difference between the effects of the two conjugates, with both E2C and GenC reducing differentiation into osteoclasts to a similar degree. These results offer good support for the notion that these conjugates would offer a form of protection against loss of bone density arising from ADT.

5.2 Objective

To test the effect of conjugates (E2C and GenC) and their unconjugated counterparts (GnRHag, E2 and Gen) on the ability to induce RANKL driven differentiation of Raw 264.7 macrophages into osteoclasts as a measure of potential bone protective properties.

5.3 Introduction

It is frequently underappreciated that, in addition to androgens, a small amount of estrogen is required in men, particularly to support bone physiology. A number of the side effects experienced during ADT are due to reduced estrogen levels as a consequence of reduced androgens from which they are elaborated in men.[231] The use of PSA biomarker means earlier diagnosis of PC and earlier detection of recurrent disease in patients that have already undergone treatment. Therefore, as survival rates have improved the systemic side effects of androgen deprivation and the resulting quality of life experienced by survivors have become more significant.[56] As previously discussed, (See section 1.3.1.1.1) a growing concern is the impact ADT has on the bone health of those undergoing treatment who experience loss of trabecular (spongy) bone mass and increased risk of osteoporosis and fractures. These fractures can represent life threatening events and currently there are over 70 million people worldwide that are at risk.[280] This increased risk is due to interconnected adverse side effects. The relationship between ADT and the incidence of osteoporosis is temporal with incidence at 49.2% at four years, 65.7% at eight years and 80.6% at ten years of treatment.[54] Cancer treatment induced bone loss, including that from ADT, tends to be more severe and occurs more rapidly than does bone loss due to menopause or natural aging.[64] Not only do these patients experience decreased bone mineral density (BMD) with a loss in structure and strength of bone but this is compounded by other side effects. These include loss in lean tissue mass and muscle cross-sectional area, increased fat mass and intermuscular adipose tissue and a concomitant increased risk of falls.[84,281,282] Additionally, PC is rarely (<1%) diagnosed before the age of 40. Bone health then becomes of much greater concern due to the numerous age associated changes that occur in bone strength, putting patients undergoing ADT at even higher concern for bone related side effects.[283,284] Mortality rate is doubled in ADT patients that have sustained a fracture after commencing treatment. Loss of trabecular bone mass and increased fracture risk are therefore key side effects requiring much greater attention and research.[63]

Bone is a dynamic tissue. It is continuously being altered and remodelled by osteoclasts and osteoblasts. Bone resorption by osteoclasts is followed by bone formation by osteoblasts.[285] Illnesses itself, or the treatment thereof (such as ADT), can result in an imbalance in these

processes. This imbalance can lead to loss of trabecular bone mass and ultimately osteoporosis.[286] Precursors to osteogenesis produced by the osteoblasts induce differentiation and fusion of macrophages into resorbing osteoclasts.[287] Should an imbalance occur, over activity of osteoclasts may result in uncoupling of bone remodelling, thus favouring bone loss over bone formation.[130] Intercellular communication between the osteoblasts and osteoclasts is critical in preserving the structure of the bone tissue. Receptor activator of nuclear factor kappa-B ligand (RANKL) and M-CSF, are both produced by osteoblasts. RANKL stimulates osteoclastogenesis and prevents osteoclast apoptosis. M-CSF is responsible for the proliferation, differentiation and survival of osteoclast precursors.[132,133] Stimulation by RANKL triggers signalling that leads to activation and expression of particular transcription factors and markers crucial for osteoclast formation, including c-Fos, NFATc1, TRAP, CTSK, MMP-9and DC-STAMP.[133] When osteoclasts attach to bone, carbonic anhydrase (CA) acidifies the resorption micro-environment to dissolve the mineral phase of bone. The lysosomal enzymes, CTSK and MMP-9, are released to degrade the organic matrix. The degradation products are then endocytosed by the osteoclasts and released into the extracellular fluid.[134,288]

It is accepted that estrogens have bone protective properties, such as preventing post-menopausal osteoporosis by maintaining an appropriate ratio between osteoblasts and osteoclasts.[289] There is evidence that the enhanced formation of functional osteoclasts appears to be negatively regulated by estrogen due to the suppression of osteoclastogenic cytokines (e.g. M-CSF) and pro-inflammatory cytokines (e.g. IL-1, IL-6, and TNF). [130] Both estrogen and phytoestrogens, including genistein (a soy derived isoflavone), have also been shown to inhibit RANKL induced osteoclast formation and it is thought that both cause down regulation of MMPs including MMP-9.[139–141] Additionally, studies have found that estrogen has direct effects on both osteoclast precursors and fully differentiated osteoclasts.[143,144] In fully differentiated osteoclasts this is achieved through osteoclast bone resorbing activity and osteoclast lifespan.[289] Recent studies suggest a role for Fas ligand (FasL) in estrogen-induced osteoclast apoptosis. Krum *et al*. (2008) describe a paracrine mechanism whereby estrogen impacts osteoclast survival via the up regulation of FasL in osteoblasts directing apoptosis of pre-osteoclasts.[120] Moreover estrogen has been shown to be anti-apoptotic in osteoblasts, resulting in an overall balance that supports bone building.[290] Support for the idea that

improvement in BMD due to levels of estrogen in men was found in a study in which androgen supplementation was given to eugonadal men with osteoporosis for six months. The improvements in BMD in this study were positively correlated with a change in serum estradiol but not with a change in serum testosterone.[81]

Our previous findings demonstrate that estrogen-GnRH analogue conjugates retain both GnRHR and ER activities. The aim of this phase of the study was to examine whether GnRH-estrogen and GnRH-genistein conjugates are able, in the same way, as estrogens or phytoestrogens, to inhibit osteoclast formation. These data would indicate the potential of these compounds to protect bone in PC patients.

5.4 Materials and Method

5.4.1. Osteoclast Differentiation Assay

A TRAP staining assay was performed in order to determine the impact of the test compounds on the ability of the RAW 264.7 cells to differentiate into osteoclasts, in response to incubation with RANKL treatment. TRAP, an enzyme that is expressed by osteoclast precursors during fusion and activation was assessed by staining.[285] The staining for TRAP is a technique frequently used to visualize osteoclasts. The basis for staining of TRAP includes the use of napthol AS phosphates in combination with fast garnet GBC salts for the detection of acid phosphatase.[291]

RAW 264.7 cells were seeded at 5000 cells/well in a 96 well cell culture plate. Cells were incubated at 37°C with 5% CO_2 for 5 days in the presence or absence of 15 $ng.ml^{-1}$ RANKL, and in the presence or absence of the test compounds (1 pM – 1 µ M) to allow for osteoclast differentiation, with a full medium change on the third day. Cells were fixed by removing the assay medium and replacing with 50 µl of a 3.7% (w/v) formaldehyde solution for ten minutes. TRAP analysis was performed using a leukocyte acid phosphatase kit (Sigma-Aldrich, St Louis, USA) as per the manufacturer's instructions. Cells were counter stained with haematoxylin by the removal of the kit solution (2 parts CBC base mix (1 part fast garnet to 1 part sodium nitrate), 1 part Napthol AS-BI, 4 parts acetate, 2 parts tartrate) and the addition of 100 µl/well of haemotoxylin. Plates were incubated at room temperature for five minutes and then rinsed by gentle submersion in a reservoir of water. Plates were left to air dry over night before being photographed and osteoclasts manually counted, osteoclasts were identified as large multinucleated cells stained pink. To improve accuracy of counting wells were divided into quadrants before counting took place. All wells were counted and all osteoclasts within each well were counted.

5.4.1.2 Data Analysis

TRAP data were analysed by nonlinear regression and used to generate sigmoidal dose-response curves to yield values for IC_{50} and Emax. All wells were counted and all osteoclasts present in each well were counted. In order to minimise inter-assay variability, for each experiment, values were calculated relative to control values measured in wells co-incubated with RANKL and vehicle (positive control). TRAP staining results for compound treated wells are expressed as a percentage of number osteoclasts in wells that were co-incubated with RANKL and vehicle treated. Data are presented as mean ± SEM from three independent experiments. All analyses were done using the GraphPad Prism 5.0 software (version for Windows, GraphPad Software, San Diego California USA).

Statistical analyses were performed using a one-way ANOVA followed by Dunnett's multiple comparison test for pairwise comparisons. $p<0.05$ was considered statistically significant for all data. All statistical analyses were performed using GraphPad Prism 5.0 software. (version for Windows, GraphPad Software, San Diego California USA).

5.5 Results

In order to assess the ability of the test compounds to inhibit osteoclastogenesis, cells incubated with RANKL to induce osteoclast differentiation were treated with the test compounds and osteoclast numbers assessed by counting of TRAP-positive multinucleated cells present after five days of treatment. Cells treated with either of the conjugates (E2C and GenC) showed a significant and dose-dependent reduction in osteoclast numbers (*Figure 5.1)* and in each case the conjugates resulted in similar levels and potencies of inhibition as their unconjugated counterparts, this held true when comparing estradiol compounds (E2 and E2C) with genistein compounds (Gen and GenC). GnRHag treatment resulted in no significant decrease in the number of osteoclasts (*Figure 5.1 and Table 5.1)*.

TRAP assay data demonstrates that cells treated with the test compounds containing estradiol or genistein (1 pM – 1 μ M) showed a significant decline in differentiation of RAW 264.7 cells into osteoclasts in a dose dependent manner (*Figure 5.1 and 5.2, Table 5.1)*. GnRHag treatment resulted in no significant difference in the number of cells differentiating into osteoclasts when compared to vehicle treated cells and this data has been shown as a straight line on *Figure 5.1*. There was however no significant difference between the conjugates to their unconjugated estradiol or genistein counterparts, nor between estradiol compounds when compared with genistein compounds (*Figure 5.1, Table 5.1)*.

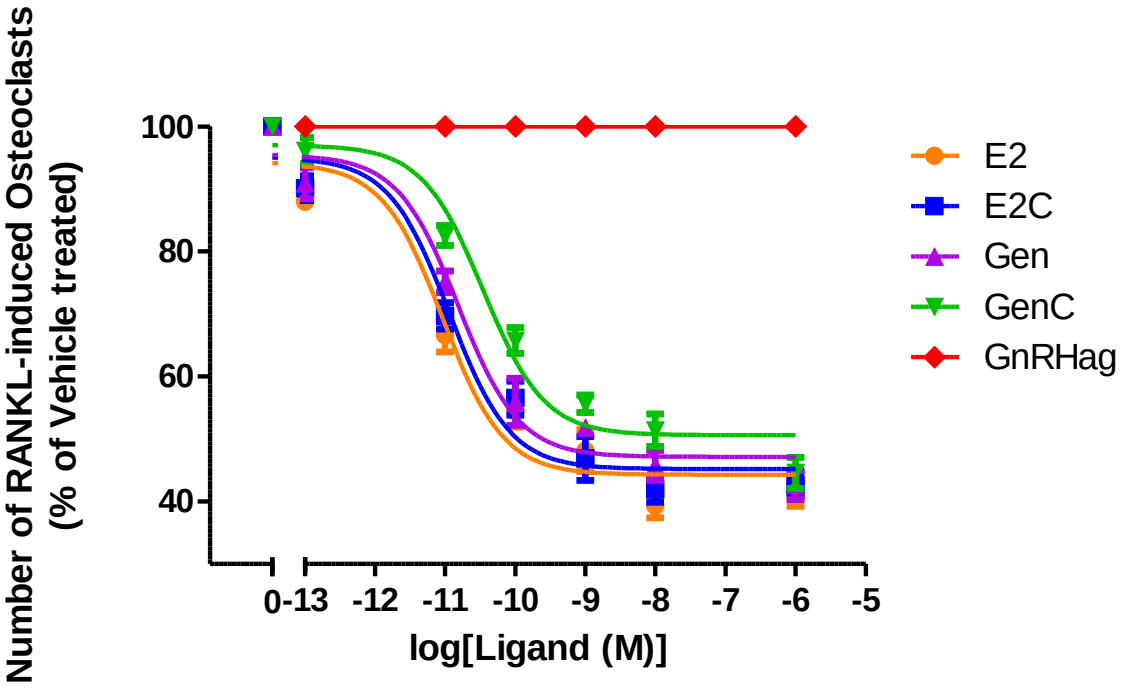


Figure 5.1 ***Number of RANKL induced osteoclasts as a percentage of osteoclasts induced when RAW 264.7 macrophages were vehicle treated***

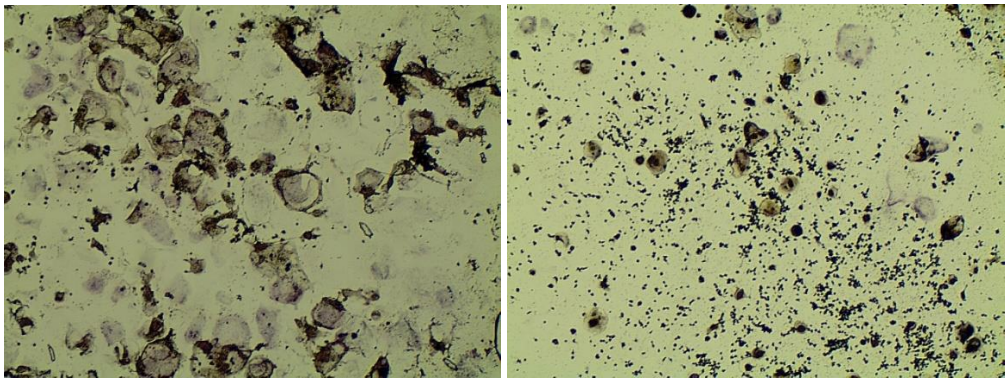
RAW 264.7 cells were incubated with vehicle (not shown as no differentiation into osteoclasts occurred), vehicle + RANKL (15 ng.ml^{-1}) or with a range of concentrations of test compounds (1 pM – 1 µ M) + RANKL (15 ng.ml^{-1}). D-Lys6 GnRH Pro9 ethylamide (GnRHag; red), 17β Estradiol (E2, orange), 17β Estradiol- D-Lys6 GnRH Pro9 ethylamide -Conjugate (E2C, blue), Genistein (Gen, purple) and Genistein- D-Lys6 GnRH Pro9 ethylamide-Conjugate (GenC, green) Data are mean ± SEM from three experiments and are expressed as percentage of osteoclasts when RAW 264.7 cells were treated with vehicle + RANKL. Data are mean ± SEM from three experiments and are presented as a % of the number of osteoclasts observed in RANKL-incubated cells treated with vehicle.

Table 5.1: IC_{50} (pM) and maximal response (percentage decrease in RANKL-induced osteoclast number) upon treatment with test compounds

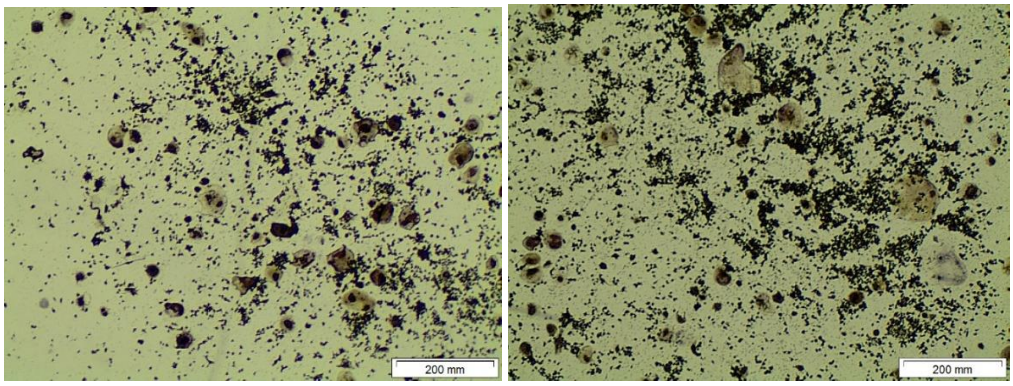
	pIC_{50} **((IC_{50}; (pM))**	**Maximal response (% RANKL-induced osteoclasts compared to vehicle treated; mean± SEM)**
Vehicle	N/A	100
17β-Estradiol	11.03± 0.10 (9.3)	44 ± 1.5*
17β-Estradiol -Conjugate	10.94 ± 0.99 (15.2)	45 ± 1.4*
Genistein	10.81 ± 0.99 (15.4)	47 ± 1.4*
Genistein-Conjugate	10.46 ± 0.97 (35)	51 ± 3*
GnRHAg	N/A	100

*Data are mean ± SEM of three independent experiments. Compounds containing 17β-Estradiol or genistein, incubated with RANKL showed a significantly reduced percentage of differentiation into osteoclasts in comparison to RAW 264.7 cells incubated with RANKL alone *, $p<0.05$. There was no significant difference between pIC50 of any of the test compounds (one-way ANOVA followed by Tukey's post-test).*

A) RANKL + Vehicle *B) RANKL + Estradiol (1 µM)*



C) RANKL + Estradiol Conjugate (1 µM) *D) RANKL + Genistein (1 µM)*



E) RANKL + Genistein Conjugate (1 µM) *F) No RANKL + Vehicle*

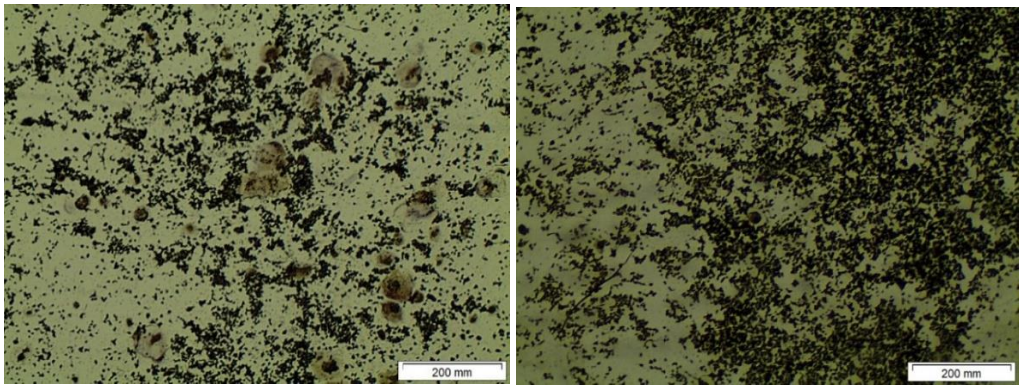


Figure 5.2 ***Representative photographs of RAW264.7 cells treated with RANKL (15 ng.ml^{-1}) + Vehicle, RANKL (15 ng.ml^{-1}) + test compounds (1 µM) and Vehicle.***

RAW64.7 macrophages were incubated in the presence or absence of *RANKL* (15 ng.ml-1) *and* test compounds (1 µM). Cells were grown for five days before staining for TRAP activity and counting of TRAP-positive (pink stained) multinucleated cells (osteoclasts). Images are representative images from three separate experiments. Wells treated with RANKL and vehicle (A) clearly show a significant increase in number of osteoclasts when compared with wells that have been exposed to the test compounds containing estradiol (B &C) or genistein (D &E). Wells that were not exposed to RANKL resulted in no differentiation of the RAW 264.7 macrophages into osteoclasts (F).

5.6 Discussion

Our study supports previous studies demonstrating the bone protective properties of estrogens and phytoestrogens. Our results indicate that both 17β-estradiol and genistein are capable of reducing differentiation of raw 264.7 macrophages into osteoclasts. The conjugation of a GnRHag did not impede either compounds ability to do so. GnRHag alone had no impact. The reduced differentiation into osteoclasts is indicative of bone protection, as the balance between osteoclasts which absorbs bone and osteoblasts that produce new bone to favour bone production.

Studies have shown that administration of estrogen can prevent ovariectomy induced trabecular bone mineral density loss in wildtype and ERβ knockout mice, and can ameliorate bone loss in ERα knock out mice to a moderate degree. This demonstrates that ERβ can at least partially compensate for loss of ERα.[292] It is thought that perhaps ERα and ERβ have redundant functions in trabecular bone, but opposing functions in femur length and response to mechanical strain.[293] GPER is expressed in osteocytes, osteoclasts and osteoblasts[294] and is also detected in chondrocytes[295], differentiation of which is regulated by GPER, additionally raw 264.7 murine macrophage cells have been shown to express the novel ER GPER[296]. Furthermore, genistein has also been shown to activate GPER[297] and has been shown to stimulate MCF-7 cell growth via induction of acid ceramidase, which occurs through a GPER-dependent mechanism.[271] The interplay and compensatory mechanisms between the classical ERs and GPER could explain the similar overall reduction in osteoclast differentiation achieved by both the estradiol and genistein treatments.

As previously mentioned, a dual system of ERs in the human prostate, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), are profoundly altered (in terms of regulation and expression) during PC development and tumour progression. ERα in the human prostate appears to act as an oncogene, whereas ERβ is indicated as a potential tumour suppressor.[191,194] Estradiol preferentially binds and activates ERα, whereas genistein, a phytoestrogen, preferentially binds and activates ERβ.[128,139]Given the debate surrounding the role of estrogen in PC development (see section 1.6) it is therefore significant that genistein is able to reduce differentiation of RAW264.7 macrophages (which express both ERα and

ERβ)[298] into osteoclasts to a similar degree when compared with estradiol, making it a valuable alternative when addressing bone loss due to ADT.

Despite the positive results put forward in this study with regards to genistein as an alternative estrogenic source for bone protection there are a number of other factors that need to be considered. Previous research on the effect of genistein as a means of bone protection in both rats and humans has been inconclusive, with results either showing no effect, little effect or positive effects.[299] One possible explanation for this is that isoflavones, such as genistein, seem to be far more effective when estrogen is deficient. A cross sectional analysis demonstrated that high dietary isoflavones were associated with higher bone mineral density (BMD) in the spine and hip of postmenopausal women, this was not found in premenopausal women.[300] This is further supported by a study which found that postmenopausal women on hormone replacement therapy (HRT) did not experience the same level of benefit from soy protein. In this study soy protein had the greatest impact on serum IGF-I (an increase of 97%) in the women not on HRT. The decrease in urinary deoxypyridinoline resulting from administration of soy protein were only observed in women not on HRT. These results suggest that soy protein could positively influence bone and calcium homeostasis in postmenopausal women, particularly those not on HRT.[301]

In animal models suppression of ovariectomy-induced femoral bone loss in young rats was comparable between soy protein isolate, purified genistein and 17β-Estradiol.[136,302,303] However, this was not the case in adult animal models of ovariectomized mice and monkeys, which showed no benefit to BMD or bone resorption markers.[304,305] Therefore it becomes important to look at models that have used skeletally mature animals that are not still undergoing bone modelling. There are a few clinical studies which have assessed the effects of isoflavones and bone health in older postmenopausal women. Results from these studies have yielded varied results with some showing no effect on BMD, these studies tended to be based on supplementation with isoflavone-enriched protein.[306] In a study using purified genistein, not as a component of isoflavone protein, it was found to have a positive effect on bone.[138] Furthermore, this study found that 54 mg/day of genistein was equally as effective as hormone therapy (1 mg of 17β-Estradiol [E2] combined with 0.5 mg of norethisterone

Acetate) in preventing bone loss. This study demonstrated the importance of giving genistein as a single bioactive isoflavone instead of as a component of soy protein. Furthermore if administered as a soy-protein, elements such as daidzein (an isoflavone extract from soy, which is an inactive analogue of the genistein) can act as an antagonistic component.[299] An unusual rat model based on an ovariectomized lactating rat showed a biphasic response to genistein with an intermediate dose being the most effective at preventing bone loss. It was hypothesised by the authors that an estrogenic agonistic effect was experienced at lower doses, and at higher doses an antagonistic effect.[302] Given that in PC estrogen levels are reduced due to ADT and that it has been hypothesised that genistein is more effective when estrogen is low, this may be a further benefit of using a genistein conjugate in this situation.

A number of different mechanisms by which genistein improves bone health have been proposed. These include positive estrogen-like effects via a mechanism that involving activation of ERβ[307], a strong inhibitory effect on protein tyrosine kinase[308] as well as acting as an osteoclast inhibitor.[308] The effects of low genistein concentration appear to be ER-mediated, whereas the impact of high concentrations (10 μM) of genistein seem to result in is tyrosine kinase inhibition.[299] The inhibition of osteoclast-like cell formation could also occur via an adenosine monophosphate signalling pathway.[309]

Our results not only agree with previous research which demonstrates the bone protective nature of estrogenic compounds, in this case 17β-Estradiol and genistein, but importantly for men receiving GnRH analogues for PC shows that these benefits are retained when estradiol or genistein are conjugated to GnRHag. The additional direct antiproliferative effects of genistein via ERβ on PC cells suggest that the GnRH analogue-genistein conjugates may be efficacious *in vivo* for achieving ADT, estrogen replacement and possibly direct antiproliferative effects.

However, this study only addressed one mechanism, inhibition of RANKL driven osteoclast differentiation, by which estrogen and phytoestrogens exert bone protection. A number of other mechanisms by which estrogenic compounds induce bone protection have been described. Including down regulation of pro-inflammatory cytokines IL-1, IL-6, TNF-α, granulocyte macrophage colony-stimulating factor, M-CSF, and prostaglandin-E2 by

estrogen.[310] TGF-β, an inhibitor of bone resorption which decreases activity and increases apoptosis of osteoclasts is upregulated by estrogen.[311] Osteoprotegerin (OPG) is a soluble decoy receptor, produced by osteoblast lineage cells, that neutralizes RANKL, OPG increases in the presence of estrogen.[312] Future studies should look at the conjugated compounds and the impact they may have on these various mechanisms to clarify in greater detail if the conjugation of a GnRHag has any effect on how estrogen and genistein influence this complex system and if so to what degree by each compound. Given the complexity of interactions involved in these systems *in vivo* studies would be necessary to investigate the overall effect of the compounds.

6.0 No Stimulation or Inhibition of Cell Survival by Conjugates in PC3, LNCaP or DU145 Prostate Cancer Cell Lines

6.1 Abstract

Estrogens are usually considered as inhibitors of PC growth (as mentioned in section 1.3.1), via an indirect mechanism of inducing androgen deprivation through negative feedback to the hypothalamic-pituitary-gonadal (HPG) axis. Indeed, estrogens (e.g. DES) are a successful form of hormone therapy used against androgen dependent PC and are still used as second line hormonal therapy.[186] Estrogens and phytoestrogens such as genistein appear to have some direct cytotoxic effects on prostate cancer cells, inhibiting proliferation and inducing apoptosis.[28,313–315] However, a paradox exists, in that estrogens may also be implicated in the causation of this disease, although this relationship, if any, is still unclear, and it is important to note that the ratio of estrogens to androgens is probably key in maintaining tissue homeostasis and prostate health.[111] This exemplified by the previously mentioned study from Bosland *et al*. which demonstrated an increase from 40% of noble rats developing prostate cancer when only given testosterone to 100% of the rats developing prostate cancer when given testosterone in combination with estrogen.[191] Furthermore, there has been consistent data produced in rodent studies which shows increased susceptibility to prostate cancer when animals were exposed to DES or pharmacologic levels of estradiol-17β *in utero* or neonatally.[316,317] Prostate progenitor cells have been found to have robust levels of the classical ERs as well as GPER, indicating their potential as direct estrogen targets.[318] There appears to be far less evidence showing genistein as a potential PC stimulant. However, a study by Mahmoud *et al*. found that in LNCaP cells that express a mutant AR, seen when hormone refractory PC arises, genistein induced a biphasic effect where physiological doses (0.5-5 µmol/L) stimulated cell growth and increased AR expression and transcriptional activity, and higher doses induced inhibitory effects.[319] Therefore, the potential of ADT combined with estrogen or phytoestrogen needs to be carefully examined to determine under which conditions it may be an appropriate treatment.

GnRH agonists, as opposed to DES, are now more commonly utilised for androgen deprivation therapy. These compounds act through overstimulation and resultant desensitisation of GnRHR activity in the pituitary which ablates HPG axis activity and therefore androgen production. In addition to GnRH agonist effects on the HPG axis, it has been found that patients with tumours that express GnRHRs and are treated with GnRH agonists (as opposed to antagonists which also block androgen production through blocking GnRH activity at the GnRHR) are more likely to have a favourable outcome. It is thought that this is due to concurrent direct action of GnRH agonists (antitumor activity) on tumour cells expressing the GnRHRs.[38,39] The exact functioning of GnRH and its receptor at extrapituitary sites (such as malignant tumours), is not entirely understood. However, in a number of these sites it appears that they can inhibit cell proliferation and/or cause programmed cell death (apoptosis). [40] The physiological relevance and impact of these observations remains to be fully elucidated.

In light of the fact that both estrogen and GnRH agonists have an impact that extends beyond their influence on the HPG axis it was necessary to investigate the effect that the conjugates may have on PC cell survival.

Three PC cell lines were selected, PC3s, LnCAP and DU145 and the effects of treatment with the compounds on cell number was assessed.

Three PC cell lines were selected, PC3s, LnCAP and DU145 and the effects of treatment with the compounds on cell number was assessed. None of the compounds significantly impacted cell number in any of the three PC cell lines. However, in HEK293T cells expressing GHRHR, incubation with the conjugates or GnRHAg reduced cell number in a dose dependant manner. The conjugates, estradiol or genistein were previously shown to increase cell number of ER-positive MCF 7 breast cancer cells, demonstrated in section 4.5.

6.2 Objective

To test the effect of conjugates on cell number, in three PC cell lines, using a crystal violet stain.

6.3 Introduction

There is evidence for both GnRH analogues and estrogens having direct effects on PC growth and viability. As mentioned in Section 1.3.1, over and above their effect on the HPG axis, GnRH analogues appear to have direct anti-tumour activity, as demonstrated by patients with GnRHR positive tumours responding more favourably to GnRH agonist treatment.[38,320] As mentioned above (section 6.1) the role of estrogen in PC in terms of its growth and viability has not been fully elaborated and there is conflicting data.[191] Although estrogenic compounds are generally considered as an inhibitor of PC it is important to evaluate this in the context of conjugation.

GnRH analogues are able to influence the *in vitro* proliferation in human cell cultures. GnRH can elicit cancer cell growth inhibition via activation of the GnRH receptor (GnRHR).[321] Cell inhibition usually occurs if the number of receptors exceed a threshold level.[321,322] The intricate interactions driving this phenomenon are only partially understood.[323] Several studies have demonstrated that tumour cell growth inhibition by a GnRH analogue could be part of a mechanism that is independent of pituitary gonadotropin release, as *in vitro* effects on proliferation have been well documented.[323,324] The mechanism of action of GnRH analogues on tumour cells does not appear to be homogenous as different cancer cells exhibit different response patterns.[325,326] This is significant as a number of prostate tumours express GnRH binding sites.[327] LNCaP cells are derived from a lymph node metastasis of prostatic carcinoma. They keep characteristics of primary human prostate carcinoma in that they produce acid phosphatase and are androgen-dependent. PC3 cells were originally derived from bone metastasis of PC. They do not respond to androgens and other growth factors.[328] DU145 cells were derived from a central nervous system metastasis, of primary prostate adenocarcinoma origin. DU145 are not hormone-sensitive and do not express PSA.[329]

Estrogens play a key role in regulation of proliferation of target tissues. The biological activities of estrogens are mediated through nuclear estrogen receptors (ERs). Estrogens interact with estrogen response elements (ERE) in the promotors of target genes, recruiting co-activators in order to mediate transcriptional regulation.[330] Within the steroid family of receptors ERs as well as androgen receptors (ARs) are unusual in their ability to stimulate cell

proliferation, highlighting their role in prostate and breast cancer.[331] A possible explanation for the protection against development of colon cancer by estrogen is the antiproliferative effect of ERβ. This has been shown in women on hormone replacement therapy where the malignant cells in the colon lose expression of ERβ.[332–334] The ventral prostate and the uterus are tissues where ERβ also exerts an antiproliferative effect. In the absence of ERβ (in ERβ -/- mice), there is hyperplasia of the prostate epithelium[335] and hypersensitivity of the uterus to the growth effects of 17β-Estradiol.[336]

As more information on the role of estrogen in proliferation becomes available it may be possible to tailor hormone therapy. Therapy could suppress ERα, so that 17β-Estradiol therefore acts through ERβ.[330] Appropriate studies of selective ERα and ERβ ligands are central to deciphering the roles of ERα and ERβ in different organs and to clarify how ligands, acting through one or the other of the ERs, can prevent or treat various age- or sex-specific diseases.[337] Drugs containing estrogenic compounds could influence the proliferation of ER positive cancers.

As there is evidence for GnRH analogues, estrogens and genistein having direct effects on PC growth and viability[28,313–315] in this phase of the study, the effects of the conjugates on prostate cancer cell number was assessed.

6.4 Materials and Method

6.4.1. Crystal Violet Staining (CVS) for Cell Number

Prior to cell seeding of HEK 293T cells, the surface of the cell culture plates was prepared with Matrigel at a 1:30 dilution in serum free media, for 60 minutes at 37°C, to aid in cell adhesion. Matrigel was removed from wells prior to seeding.

Cells were seeded into a 96-well plate, at a density of $2x10^4$ cells/well for HEK293T, MCF7, PC3 and DU145 cell lines and a density of $5x10^4$ for the LNCaP cell line.

24 hours post seeding media was aspirated. This was replaced with steroid free media containing the different treatment compounds (0.1 µM). A pilot study in order to determine length of exposure time was conducted for all three PC cell lines. Martin and Clynes found CVS to have good sensitivity but also a short linear range compared to other cell growth/survival assay methods.[338] It is therefore critical that cell numbers are within the linear range of the assay at the time of end-point determination; with the end point occurring before control wells have reached confluency.[338] Cells were grown for three days prior to conducting a crystal violet assay.

Media was removed from the plates. 100 µl/well of 1% (w/v) glutaraldehyde (Sigma-Aldrich, St Louis, USA) was added and incubated at room temperature for 15 minutes. 1% glutaraldehyde was discarded, 100 µl/well 0.1% (w/v) crystal violet stain (Sigma-Aldrich, St Louis, USA) was added and incubated at room temperature for 30 minutes. Crystal violet stain was discarded, plates were rinsed by submerging in water. Rinsed plates were left to dry overnight. The following day 200 µl/well 0.2% (w/v) Triton X-100 (Sigma-Aldrich, St Louis, USA) was added and left to incubate at room temperature for 30 minutes. 100 µl from each well was transferred to a clean 96 well plate. Absorbance was measured at 595 nm on a BioRad iMark microplate reader (Biorad, Hercules, California, United States of America).

6.4.2.2 Data analysis

In order to minimise inter-assay variability, for each experiment values were calculated relative to control values measured in wells that were vehicle treated. Data are presented as a percentage of cell number in vehicle treated cells. Data are presented as mean ± SEM from three independent experiments. All analyses were done using the GraphPad Prism 5.0 software (version for Windows, GraphPad Software, San Diego California USA).

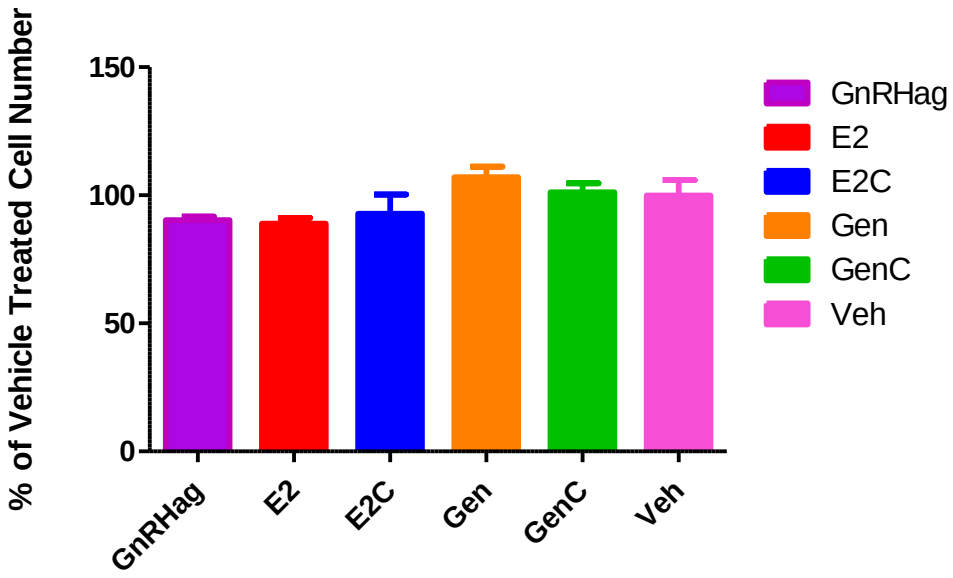
Statistical analyses were performed using a one-way ANOVA followed by Tukey's multiple comparison test for pairwise comparisons. $p<0.05$ was considered statistically significant for all data. All statistical analyses were performed using GraphPad Prism 5.0 software. (version for Windows, GraphPad Software, San Diego California USA).

6.5 Results

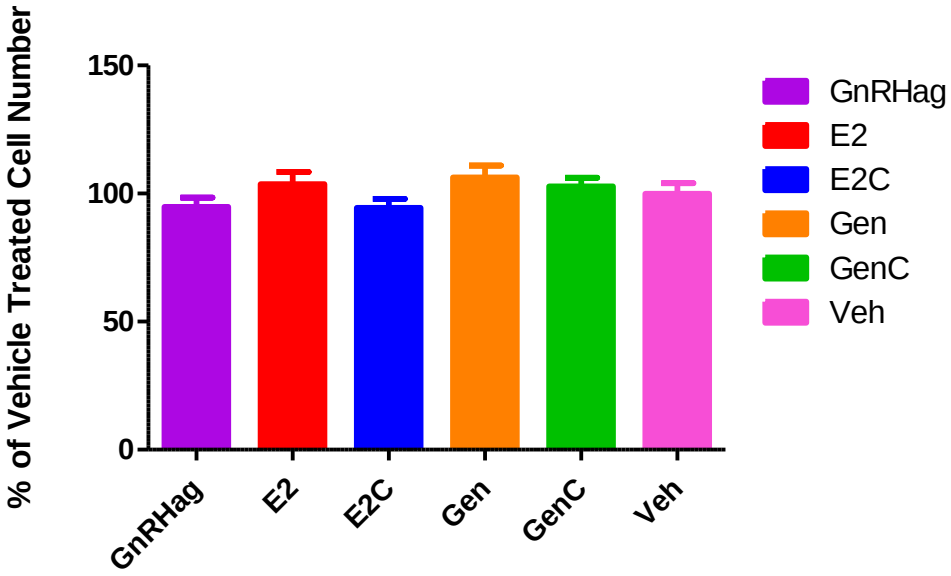
In order to assess the effects of the test compounds on cell number, it was necessary to select an appropriate cell growth/survival assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is one of the most frequently used assays to assess the efficacy and interactions of anticancer agents. However, compounds that alter cell metabolism as well as altering reaction conditions have the potential to significantly influence the results obtained from this assay. Recently Sliwa *et al*. (2016) studied whether the choice of an assay had an impact on determining the type of interaction and to identify the source of discrepancies that occurred.[339] In this study the accuracy and reliability of the MTT assay, an indirect assessment of cell viability, was compared to that obtained using crystal violet staining (CVS), a direct method measuring the DNA mass of living cells. CVS was found to be more reliable than MTT, supporting studies such as those by Maioli *et al*. (2012) which found rottlerin may enhance the production of formazan crystals, leading to false negative results in cell viability assays.[340] Factors that influence the reduction process on which the MTT assay is based include the current phase of growth, the cell cycle phase, and reaction conditions such as pH and D-glucose concentration.[341,342] CVS is not impeded by the limitations associated with assays based on enzymatic reactions and the quantity of dye absorbed is contingent on the total amount of DNA in the culture and allows for the estimation of the number of viable cells in the culture.[339] CVS was therefore deemed the most appropriate for use in our study.

Three PC cell lines (DU145, LnCaP & PC3) were grown in the presence or absence of the test compounds for three days (0.1 µM, the concentration which gave Emax for GnRHR activation, Figure 3.4). None of the compounds significantly impacted on cell number in any of the three PC cell lines (Figure 6.1 A-C). While in control cells (HEK293T cells expressing GnRHR) both the conjugates and GnRHag significantly reduce cell number (Figure 6.2, Table 6.1). As shown in Figure 4.3, both conjugates, estradiol and genistein significantly increases cell number of ER-positive MCF 7 breast cancer cells in a dose dependent manner in the Escreen assay.

(A)


% of Vehicle Treated Cell Number
150
100
50
0
GnRHag
E2
E2C
Gen
GenC
Veh
GnRHag
E2
E2C
Gen
GenC
Veh

(B)


% of Vehicle Treated Cell Number
150
100
50
0
GnRHag
E2
E2C
Gen
GenC
Veh
GnRHag
E2
E2C
Gen
GenC
Veh

(C)

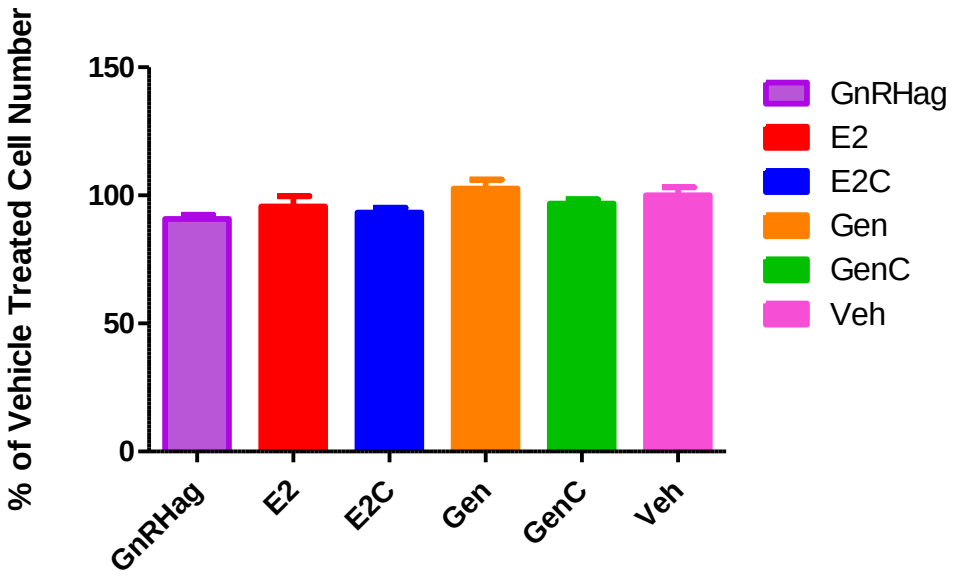


Figure 6.1 ***A-C Effect of test compounds (0.1 µM) on DU 145 (A), LNCaP (B) and PC3 (C) cell number***

A) DU 145 cells B) LNCaP cells and C) PC3 cells stimulated with Vehicle (pink) or 0.1µM of treatment compounds, D-Lys6 GnRH Pro9 ethylamide (GnRHag; purple),17β Estradiol (E2, red), 17β Estradiol- D-Lys6 GnRH Pro9 ethylamide -Conjugate (E2C, blue), Genistein (Gen, orange) and Genistein- D-Lys6 GnRH Pro9 ethylamide-Conjugate (GenC, green). Cell number was determined using a crystal violet stain. Absorbance was measured at 595 nm. Data are mean ± SEM from three independent experiments. A one-way ANOVA with a Tukey's multiple comparison test showed no significant difference (P<0.05) between all treatments in A) DU 145 cells B) LNCaP cells and C) PC3 cells.

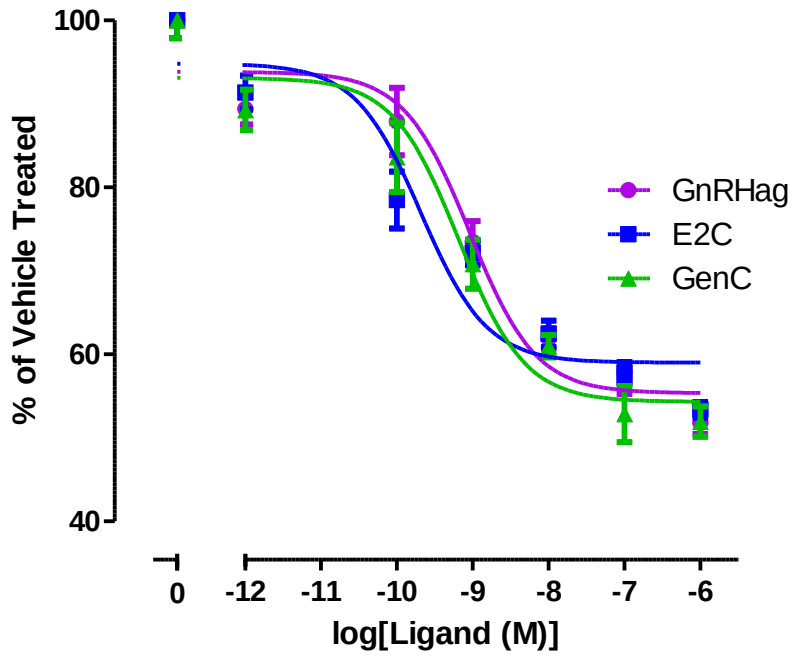


Figure 6.2 ***Effect of test compounds (1 µM – 1pM) on HEK 293T cell number****,*

HEK 293T cells transiently transfected with GnRHR stimulated with vehicle (0), or a range of concentrations (1 µM -1 pM) of test compounds , D-Lys6 GnRH Pro9 ethylamide (GnRHag; purple), 17β-Estradiol- D-Lys6 GnRH Pro9 ethylamide -Conjugate (E2C, blue) and Genistein- D-Lys6 GnRH Pro9 ethylamide-Conjugate (GenC, green), no inhibition with 17β-Estradiol (E2) or genistein (Gen) occurred and are not shown. Cell number was determined using a crystal violet stain. Absorbance was measured at 595 nm. Data are mean ± SEM from three independent experiments. A one-way ANOVA with a Tukey's multiple comparison test showed no significant difference (P<0.05) between treatments.

Table 6.1 IC_{50} (nM) and maximal response (percentage inhibition compared to vehicle treated) upon treatment with test compounds

	pIC_{50} (mean± SEM) ((IC_{50}; (nM))	**Maximal response (% compared to vehicle treated; mean± SEM)**
Vehicle	N/A	100
GnRHAg	9.04±0.13 (0.91)	55.35±1.58*
17β-Estradiol -Conjugate	9.68±0.14 (0.21)	59.01 ±1.41*
Genistein-Conjugate	9.19±0.15 (0.65)	54.32±1.85*

Compounds containing GnRHag significantly reduced cell number when compared vehicle only treated cells (, $p<0.05$). There was no significant difference in IC_{50} between treatment groups, $p<0.05$. There was no significant difference between pIC50 of any of the test compounds. Data are mean ± SEM of three independent experiments, one-way ANOVA followed by Tukey's post-test.*

6.6 Discussion

Control cells, expressing GnRHR responded to both conjugates as well as GnRHag, with cell number decreasing after exposure. Unlike the control cells in this section, which showed inhibition or the MCF-& cells (Figure 4.3) which showed proliferation, none of the three PC cell lines had altered cell number (in either direction) upon exposure to the treatment compounds.

Many PC cell lines alter with respect to their receptor expression profiles once looked at outside of *in vivo* circumstances. For example, the majority of available human PC cell lines do not express AR. This differs dramatically with human prostate carcinomas *in vivo* which express AR, even at advanced stages. Primary cultures and prostate carcinomas maintained in athymic nude mice are frequently AR-positive. Rapidly becoming androgen-independent in culture, suggesting substantial alterations in AR gene expression and/or the selection of cells under culture conditions.[327] Morgan *et al*. also found that GnRHR expression occurring in transformed cell lines was influenced by culture conditions and cell passage number and that endogenous GnRHR binding was not detectable in many metastatic PC cell lines.[343] However, expression of this receptor in native prostate carcinomas is well reported.[327,344]

Ravenna *et al*. demonstrated that Triptorelin, a D-Lys6 GnRH agonist, did not impact on proliferation of PC3 cells.[323] Furthermore, in LNCaP cell cultures, only at doses of 10^{-4} M did Triptorelin yield a significant antiproliferative effect. In cell cultures grown with FBS supplemented media there was a small but significant proliferative effect at 10^{-7} M.[323] In this study cell culture conditions as well as binding sites with different affinity may be linked with differing biological responses to the drug.[323] There is contradictory evidence, with some studies describing antiproliferative effects in cells expressing GnRHR whilst others show an absence of response to GnRH analogues *in vitro*.[327,345] It has been hypothesised that if GnRHR is not lost during cell culture then cell passage or cell culture conditions can result in fluctuating and varied levels of the receptor.[346] Cell cycle arrest and apoptosis have been established as outcomes in cells targeted by GnRH analogues.[347–349] It is not yet clear whether different cell types share a common mechanism by which GnRH-mediated inhibition occurs.[322] As the level of cell surface receptor has been shown to be a key influence on the

extent of GnRH-mediated growth inhibition[348], it could be surmised that the cell culture conditions and the passaging of the three PC cell lines has led to a loss of receptor expression in our study. Therefore, although none of the treatments impacted on proliferation of the three PC cell lines, it will be necessary to establish whether this is the case *in vivo*. This is particularly important as it has been shown that treatment with GnRH analogues has a more favourable outcome if the tumour is GnRHR or AR positive.[38]

ERα and ERβ, have structural differences (as outlined in section 4.3) and this could account for different cellular and tissue distribution and activities, having different effects at a cellular and systemic level. ERα is necessary for reproductive development and functioning. ERβ despite being present and active in granulosa cells, contributing to the provision of full fertility in mice, appears to be of greater significance in non-reproductive organs and tissues.[337] Furthermore, ERβ counteracts the activity of ERα in a number of systems. Strom *et al*. demonstrated that induced expression of ERβ in the breast cancer cell line T47D reduces 17β-Estradiol-stimulated proliferation when expression of ERβ mRNA equals that of ERα.[333]

Although the findings presented here suggest that the conjugated compounds do not have any effect (positive or negative) on growth of PC cell lines, it is clear that *in vitro* cell lines do not mimic the *in vivo* situation. *In vivo* studies will be necessary to determine the actual impact (if any) of the conjugates on proliferation of tumours that are GnRHR and/or ER positive.

7.0 General Discussion and Conclusions

GnRH has a key role in reproductive physiology, as such, there exist a vast number of clinical applications for both agonist and antagonist analogues. However, GnRH analogues tend to exhibit poor oral activity and rapid metabolic clearance. As a result, they are generally administered as either slow release injectables or daily injections.[350] Administration in this way overcomes a number of their short comings but as a consequence the ability to vary the dose is limited and immediate withdrawal of treatment is not possible.[203] Conjugating moieties to GnRH peptide analogues becomes a viable alternative to other approaches such as the pharmaceutical development of nonpeptide orally active GnRH antagonists which has proven to be diffficult.[351] This may allow for the preservation of the beneficial properties of GnRH peptide analogues, whilst addressing some of their limitations (See section 1.8). In the present study this was achieved by producing conjugates of estradiol or genistein that are bifunctional, targeting both the GnRHR and ER.

The results of this study have shown that the conjugates (E2C and GenC) retain their respective functionalities at both the GnRHR and ER but will require *in vivo* study to further assess them. Future studies could include mass spectrometry analyses to determine whether the conjugates are cleaved before activation of the respective receptors. Therefore, they have the potential to be good ADT agents which may mitigate some of the side effects associated with current therapies.

One of the other shortcomings of GnRH peptide analogues as ADT therapeutics is their rapid metabolic clearance. Another potential benefit of the GnRHAg-estrogen conjugates described herein might be an increased half-life. Indeed, Ratcliffe *et al.* have previously found that conjugating a GnRH antagonist to 21-hydroxyprogesterone resulted in a rapid decrease in plasma testosterone levels in male rabbits, the length of suppression was increased three-fold when compared with the unconjugated parent antagonist.[203] It is anticipated that the conjugates utilised in this study will display similar properties in this regard. The increase in duration of action of our conjugate will need to be confirmed with *in vivo* studies.

This study utilised a GnRH agonist within the conjugates. A disadvantage associated with GnRH agonists is the flare of the condition due to the initial stimulation of the GnRHR.[320,352] This usually lasts one to two weeks before desensitization is initiated. In contrast GnRH antagonists have the advantage of not resulting in this initial flare, as there is immediate inhibition of gonadotropin.[353,354] On the other hand it is necessary to administer antagonists at much higher doses than agonists in order for it to outcompete the endogenous GnRH, the level of which increases due to the decline in sex steroid levels and the associated negative feedback on GnRH secretion.[110] This wouldn't usually pose a problem for single therapy treatment.[1] However, in the case of the estrogen conjugates, this would make dosing challenging as it would be necessary to give high doses of estrogen to generate a therapeutic level of antagonistic activity. In addition, the initial negative effects of using GnRH agonists can be overcome by simultaneous administration of steroid antagonists or inhibitors of steroid biosynthesis such as flutamide.[355,356] Labrie *et al*. determined that a combination therapy of a GnRH agonist with flutamide resulted in improvement in the morbidity and mortality from PC.[357] For these reasons conjugates of GnRH agonists, as described here, rather than antagonists would probably be preferential.

That being said, as there is increased interest and use of GnRH antagonists as a means of addressing PC, it is important to note that while not used in this study the possibility of using a low activity form of estrogen would potentially allow for the use of an antagonist in the production of a conjugate.[358] Within naturally occurring and endogenous forms of estrogen, estradiol has the highest activity.[358] Estrone has lower activity but can be converted to estradiol in the body, making it a poor candidate to fulfil this role. Estriol is the weakest of the naturally occurring estrogens, it cannot be converted to estradiol or estrone[358], it is therefore feasible that this form of estrogen could be used to form a conjugate with a GnRH antagonist to avoid excessive dosing of estrogen during treatment.

Furthermore, as discussed in section 4.3, there may be value in using a compound which preferentially binds and activates ERβ over ERα. Zhu *et al*. found that a number of D-ring metabolites, such as 16α-hydroxyestradiol (estriol), 16β-hydroxyestradiol-17α, and 16-ketoestrone, had distinct preferential binding affinity for human ERβ over ERα, with differences of up to 18-fold.[359] Again, highlighting that estriol has notable potential for use as

part of a conjugated treatment. Furthermore estriol has been used to effectively treat a number of the symptoms resulting from decreased estrogen levels due to menopause including hot flushes.[360] Conversely, evidence on the ability of estriol to address osteoporosis remains contradictory, with some studies yielding positive results[361–364] and others finding it an ineffective treatment.[365–367] This would therefore need further investigation before it could be said with any authority that estriol would be suitable for the use in a conjugate seeking to address the side effects associated with ADT, including osteoporosis.

Another additional benefit of these conjugated compounds is the possibility of a dual effect in the down regulation of androgens both through the GnRH agonist activity and also via estrogen-mediated downregulation of the HPG axis through negative feedback.[368] Indeed, it is accepted that estradiol inhibits gonadotropin release in men by an action at the hypothalamus and pituitary, however there is a lack of consensus regarding the role and contribution of ER and AR signalling in men.[369,370] In men receiving estrogens, it has been found that estrogens preferentially inhibit LH secretion[371,372], FSH secretion, [373,374]or LH and FSH secretion to a comparable degree[375]. It is possible that these differences can be explained by the various modes, doses, or time courses of estrogen administration. Research on the relative contribution to the inhibition of gonadotropins secretion exerted by the amount of estrogens produced within the hypothalamus and/or the pituitary or by the amount of circulating estrogens remains ongoing.[376] The precise role of each sex steroid in the regulation of gonadotropin negative feedback has as yet to be determined. Studies have found that the inhibitory effect on gonadotropin secretion appears to be mediated predominantly by estradiol from endogenous conversion of testosterone rather than direct androgen action, at least in the pituitary gland.[377] Additionally, other studies suggested that *in situ* aromatization of testosterone is necessary both at the hypothalamic and pituitary levels to ensure a complete feedback mechanism of gonadotropins.[378,379] Despite the lack of a comprehensive understanding relating to the contribution of estrogen to the negative feedback of the HPG axis in men, in the case of combination with a GnRH agonist to treat PC this would not pose a significant problem as the primary objective is to reduce testosterone to castration levels.

In conclusion, use of GnRH analogues has become the preferred therapeutic treatment for PC, with these drugs rather than orchiotomy being the favoured method to achieve androgen

deprivation.[26] These drugs result in a marked decrease in PSA and an increase in the survival rates of patients with PC but are associated with an number of negative side effects.[9,22] With earlier diagnoses and increased life expectancy of PC patients, it becomes important to address the quality of life experienced. Existing guidelines for managing ADT induced side effects include monitoring known risk factors, pharmacotherapy and lifestyle interventions. These differ by country, organisation or expert opinion.[380] There is a gap in the evidence of how well these guidelines are adhered to and any improvements they may offer in mitigating the known side effects.[381] For example, guidelines advocate a fracture assessment prior to beginning ADT as well as after undergoing treatment using a DXA BMD scan. Despite these recommendations a study which included a cohort of 28 960 men found that only 10.2% were referred for a DXA scan prior to starting ADT or during the first 12 months after treatment was initiated.[382] This study provides evidence that conjugation of estrogen or estrogenic compounds to a GnRH analogue show considerable promise as a means to ameliorate a number of these side effects simultaneously, without compromising the primary objective in treating PC. Conjugated compounds that address the targeted disease and the associated side effects from treatment are proactive rather than reactive. In this scenario known side effects are pre-emptively treated, preventing harmful side effects rather than treating them once they are already apparent. A dual approach, in which GnRHR and ER are activated, delivered as a single treatment, also has the potential to improve duration of action and mode of therapeutic delivery of the GnRH agonist. Moreover, it could ensure appropriate compliance of a treatment by addressing the side effects associated with ADT which could cause patients to cease treatment. Additionally, men at risk for osteoporosis and fractures not identified due to lack of assessment will not be overlooked and will receive treatment aimed at addressing bone related side effects from the onset.

The results of this study provide a proof-of-principle for taking these compounds into *in vivo* models in which their properties can be assessed further and from which they could for the basis of an improved treatment option for PC.

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The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 28 August 2018.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee

27/10/2016

Approval Certificate
New Application

Ethics Reference No.: 375/2016

Title: Functional Characterisation of Gonadotropin Releasing Hormone-Estrogen Conjugates

Dear Mrs StaceyLee Leijenaar

The **New Application** as supported by documents specified in your cover letter dated 27/10/2016 for your research received on the 27/10/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 27/10/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 4 years
- Please remember to use your protocol number (**375/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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