Gonadotropin-releasing hormone and its receptor in normal and malignant cells

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Abstract

Gonadotropin-releasing hormone (GnRH) is the hypothalamic factor that mediates reproductive competence. Intermittent GnRH secretion from the hypothalamus acts upon its receptor in the anterior pituitary to regulate the production and release of the gonadotropins, LH and FSH. LH and FSH then stimulate sex steroid hormone synthesis and gametogenesis in the gonads to ensure reproductive competence. The pituitary requires pulsatile stimulation by GnRH to synthesize and release the gonadotropins LH and FSH. Clinically, native GnRH is used in a pump delivery system to create an episodic delivery pattern to restore hormonal defects in patients with hypogonadotropic hypogonadism. Agonists of GnRH are delivered in a continuous mode to turn off reproductive function by inhibiting gonadotropin production, thus lowering sex steroid production, resulting in medical castration. They have been used in endocrine disorders such as precocious puberty, endometriosis and leiomyomata, but are also studied extensively in hormone-dependent malignancies. The detection of GnRH and its receptor in other tissues, including the breast, ovary, endometrium, placenta and prostate suggested that GnRH agonists and antagonists may also have direct actions at peripheral targets. This paper reviews the current data concerning differential control of GnRH and GnRH receptor expression and signaling in the hypothalamic–pituitary axis and extrapituitary tissues. Using these data as a backdrop, we then review the literature about the action of GnRH in cancer cells, the utility of GnRH analogs in various malignancies and then update the research in novel therapies targeted to the GnRH receptor in cancer cells to promote anti-proliferative effects and control of tumor burden.

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Introduction

Gonadotropin-releasing hormone (GnRH) is the hypothalamic factor that mediates reproductive competence (Wierman 1996, Neill 2002). Intermittent GnRH secretion from the hypothalamus acts upon its receptor in the anterior pituitary to regulate the production and release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (see Fig. 1). LH and FSH then stimulate sex steroid hormone synthesis and gametogenesis in the gonads to ensure reproductive competence. The pituitary requires pulsatile stimulation by GnRH to synthesize and release the gonadotropins LH

and FSH. Continuous stimulation of pituitary GnRH receptors (GnRHR) by exogenously administered GnRH agonists, rather than pulsatile stimulation, desensitizes and down-regulates GnRHRs (Neill 2002). The ultimate effect of this chronic stimulation of the pituitary GnRHRs is to decrease LH and FSH production, with subsequent decreases in circulating sex steroid levels (Wierman 1996, Neill 2002).

Clinically, native GnRH is used in a pump delivery system to create an episodic delivery pattern to restore hormonal defects in patients with hypogonadotropic hypogonadism (Pitteloud *et al.* 2002). On the other hand, agonists of GnRH are delivered in a continuous mode to turn off reproductive function by inhibiting gonadotropin production (Labrie *et al.* 1981), thus

Figure 1 The hypothalamic–pituitary–gonadal axis. Secretion of GnRH from the hypothalamus occurs in a pulsatile fashion and is partially responsible for controlling the number of GnRHRs in the pituitary gland and controls synthesis and secretion of FSH and LH. These gonadotropins in turn regulate function of the testes and ovaries. E, estrogen; P, progesterone; T, testosterone.

lowering sex steroid production, resulting in medical castration (Labrie 1991, Labrie *et al.* 1993, Huirne & Lambalk 2001). They have been used in endocrine disorders such as precocious puberty, endometriosis and leiomyomata, but are also studied extensively in hormone-dependent malignancies (Labrie *et al.* 1980, 1996, Huirne & Lambalk 2001). More recently, pure GnRH antagonists have been devised to directly block GnRHRs on the pituitary and inhibit LH and FSH production (Huirne & Lambalk 2001). Currently, they are indicated for the treatment of female infertility as adjunct therapy during ovarian hyperstimulation for *in vitro* fertilization and are under active investigation in various malignancies (Labrie *et al.* 1996, Huirne & Lambalk 2001).

The detection of GnRH and its receptor in other tissues including the breast, ovary, endometrium, placenta and prostate suggested that GnRH agonists and antagonists may also have direct actions at peripheral targets. This paper will review the current data concerning differential control of GnRH and GnRHR expression and signaling in the hypothalamic–pituitary axis and extrapituitary tissues. Using these data as a backdrop, we will then review the literature about GnRH action in cancer cells, the utility of GnRH analogs in various malignancies and then update the research in novel therapies targeted to the GnRHR in cancer cells to

promote anti-proliferative effects and control of tumor burden.

GnRH expression and control

GnRH expression profile

At least two isoforms of GnRH have been identified in the mammalian central nervous system (CNS), GnRH-I and GnRH-II. GnRH-I is the hypothalamic decapeptide responsible for LH and FSH secretion from the anterior pituitary originally isolated by Guillemin and Schally (Guillemin 1967). GnRH-II was initially discovered as chicken GnRH-II and displays a diffuse pattern of localization in most tissues (Densmore & Urbanski 2003, Pawson *et al.* 2003). In the CNS, GnRH-II has been hypothesized to play a role in the behavioral components of reproduction (Pawson *et al.* 2003). The genes for human GnRH-I and GnRH-II are on chromosomes 8 and 20 respectively (Wierman 1996, Limonta *et al.* 2003). Both isoforms of GnRH are decapeptides that are characterized by post-translational modifications, including the pyro-glutamic acid at the amino termini and amidated glycine at the carboxy termini. GnRH-I is conserved throughout evolution and has been identified in both vertebrates and invertebrates (Wierman 1996, Limonta *et al.* 2003). GnRH-I shares a 60% identity

between mammals and tunicates, whereas GnRH-II is even more highly conserved with 100% identity between birds and mammals (Wierman 1996, Limonta *et al.* 2003). GnRH-I is synthesized as a prohormone, human prepro-GnRH that contains 92 amino acids. A signal peptide is contained in the first 23 amino acids, followed by the GnRH decapeptide, a 3 amino acid cleavage site and a 56 amino acid product known as GnRH-associated peptide (GAP). Post-translational processing of GnRH involves cleaving by a prohormone convertase, removal of basic amino acids by a carboxypeptidase, amino-terminus modification through the action of glutamyl cyclase and carboxy-terminus modification by peptidylglycine α -amidating mono-oxygenase (Wierman 1996, Limonta *et al.* 2003). This enzymatic processing produces mature GnRH and GAP. A physiologic role for GAP has not been determined, although it has been postulated to act as a prolactin-inhibitory factor.

Control of GnRH expression

There are approximately 1500–2000 GnRH neurons in the human (in contrast to the 800 neurons in the rodent) that are diffusely arranged in a neuronal network in the basal hypothalamus after migration (Tobet *et al.* 2001). The small number and dispersed population make *in vivo* studies of the control of GnRH expression difficult. Immortalized GnRH neuronal cell culture models as well as isolated GnRH neurons demonstrate that the GnRH ‘pulse generator’ is intrinsic to the neuronal cells, and have served as model systems to define the many membrane and nuclear factors important in GnRH expression across development (Wierman *et al.* 2004). During human development, the GnRH pulse generator and subsequent gonadotropin release is active in the early neonate, but decreases by 3–4 years of age (Terasawa & Fernandez 2001, MacColl *et al.* 2002). Until puberty, the GnRH pulse generator is repressed. The exact mechanisms of repression are not known and may involve central γ -amino butyric acid neuronal activity (Terasawa & Fernandez 2001). Similarly, the factors that reactivate the GnRH pulse generator during puberty are not completely understood, but the process involves activation of glutamatergic pathways (Terasawa & Fernandez 2001).

GnRHR expression and regulation

Mammals, including humans, produce two isoforms of GnRHRs, GnRHR-I and GnRHR-II (previously called chicken GnRH-II receptor) (Clayton & Catt 1981, Grundker *et al.* 2002a, McArdle *et al.* 2002, Kang *et al.* 2003, Ruf *et al.* 2003). Both isoforms are members of the G-protein coupled receptor (GPCR) family of proteins,

couple with Gq α and function in the inositol phosphate signaling pathway (see Fig. 2). In functional studies that measured inositol phosphate production, primate GnRH-I receptors demonstrated an approximate 48-fold selectivity for GnRH-I versus GnRH-II (Gault *et al.* 2003, Pawson *et al.* 2003, Terasawa 2003). Conversely, GnRH-II receptors demonstrated a 421-fold preference for GnRH-II versus GnRH-I (Gault *et al.* 2003, Pawson *et al.* 2003, Terasawa 2003). Unlike the unique GnRH-II receptor and most other GPCRs, the GnRH-I receptor contains no large cytoplasmic C-terminal tail (Clayton & Catt 1981, Grundker *et al.* 2002a, McArdle *et al.* 2002, Kang *et al.* 2003, Ruf *et al.* 2003). The C-terminus of both types of receptors are phosphorylated in response to GnRH, leading to receptor desensitization. The GnRHR-I is on chromosome 4 and the GnRHR-II is on 1q (Pawson *et al.* 2003). Although the mRNA for GnRHR-II has been cloned from monkey as well as rodent and fish species, these investigators have suggested that it is not expressed into a functional protein in the mouse or human, making the physiologic relevance of GnRHR-II in human biology a question for further study (Pawson *et al.* 2003).

GnRHR expression is regulated in the pituitary across sexual maturation and in response to GnRH, sex steroids and gonadal peptides (Norwitz *et al.* 2002a,b, Ellsworth *et al.* 2003a,b, Sadie *et al.* 2003). Activin A augments GnRH activation of the GnRHR promoter in α T3 pituitary cells (Norwitz *et al.* 2002a,b). Differential control of LH β versus FSH β gene expression by GnRH is partially mediated by an up-regulation of GnRHR (Bedecarrats & Kaiser 2003). An increase in GnRHR number in the immortalized gonadotrope cell line L β T2 cells resulted in a disruption of the response of the FSH β but not the LH β promoter to GnRH (Bedecarrats & Kaiser 2003). Together, these cell systems suggest that, at the level of the pituitary, GnRHR-I expression is tightly regulated.

Signaling downstream of GnRHR in the pituitary

Effects on gonadotropin gene expression and secretion

GnRH activation of its receptor results in stimulation of diverse signaling pathways in the anterior pituitary (Fig. 2; see Ruf *et al.* (2003) for detailed discussion of components of the pathway). The GnRHR is coupled to Gq/11 proteins to activate phospholipase C which transmits its signal to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Kraus *et al.* 2001, McArdle *et al.* 2002, Krsmanovic *et al.* 2003, Ruf *et al.* 2003). DAG activates the intracellular protein kinase C (PKC) pathway and IP3 stimulates release of intracellular calcium. In

Figure 2 Effects of GnRH activation of its receptor in stimulating diverse signaling pathways in the anterior pituitary. (Reprinted with permission from Ruf *et al.* (2003)).

addition to the classical Gq/11, coupling of Gs is occasionally observed in a cell-specific fashion. PKC activation in response to GnRH also leads to increases in the mitogen-activated protein kinase (MAPK) pathways including ERK1,2, ERK5, p38MAPK and JNK in pituitary cells (Kraus *et al.* 2001, McArdle *et al.* 2002, Ruf *et al.* 2003). The active MAPKs move to the nucleus where they activate a variety of transcription factors, such as the Ets and/or AP1 families to modulate gene expression. These pathways then differentially regulate the synthesis and secretion of the gonadotropin subunits, α , LH β and FSH β , in the anterior pituitary, selectively modulating gonadotropin synthesis and/or release from pituitary cells.

In L β T2 immortalized pituitary gonadotrope cells, studies suggest the importance of G proteins as well as Gq/11 (shown to be critical in α T3 gonadotrope cells) to regulate gonadotropin subunit gene expression (Liu *et al.* 2002, 2003, Vasilyev *et al.* 2002, Krsmanovic *et al.* 2003). Whether signaling downstream from the GnRHR changes across pituitary development or the differences between results in the α T3 and L β T2 are related to their immortalization by SV40 T antigen, remains to be determined. Desensitization in response to chronic GnRH administration in L β T2 cells results in decreased

GnRHR and Gq/11 expression, and down-regulation of PKC, cAMP and calcium-dependent signaling (Liu *et al.* 2002, 2003). In addition, stimulation of ERK and p38MAPK, as well as c-Fos and LH β protein expression, were blocked (Liu *et al.* 2002, 2003). These studies suggested that chronic GnRH may also result in desensitization of other Gq-coupled receptors such as the epidermal growth factor (EGF) receptor (EGFR; see Fig. 2) at the level of the pituitary (Shah *et al.* 2003).

GnRHR cross-talk with other growth factor receptors

In addition to the direct effects of the GnRHR in activating intracellular signaling, recent work has suggested that cross-talk with the EGFR may occur at the level of the pituitary (Shah *et al.* 2003). In L β T2 pituitary cells, Roelle *et al.* (2003) showed that EGFR can be activated via GnRHR based upon proteolytic release of local EGF-like ligands from transmembrane precursors. In this system, matrix metalloproteinase (MMP) 2 and 9 allow shedding of the growth factors to activate EGFR. GnRH stimulation of the cells induced Src, Ras and ERK that were dependent on the action of the MMPs, whereas activation of c-Jun N-terminal kinase and p38MAPK by

GnRH was unaffected by the inhibition of EGFR or MMPs. GnRH activation of c-Jun and c-Fos was, however, blocked by interference with the MMPs or EGFR signaling. Together, these data underscore the complexities of direct and indirect effects of GnRH/GnRHR action on diverse intracellular signaling pathways.

GnRHR effects on proliferation

Recent data suggest GnRH may also modulate proliferation of gonadotropes via activation of its receptor. Miles *et al.* (2004) showed that continuous exposure to a GnRH signal or with the GnRH agonist leuprolide resulted in an anti-proliferative effect in LβT2 cells. The cells accumulated in G0/G1. The effects were receptor dependent in that they were blocked with the GnRH antagonist antide. Of interest, the agonist was maximally effective at nanomolar concentrations, yet little effect of native GnRH was observed until concentrations exceeded physiologic levels (Miles *et al.* 2004). This suggests that the mechanisms of GnRH agonist action at peripheral targets are not through normal physiologic pathways, but instead related to the potency of the agonist to bind to the receptor, the long receptor occupancy and subsequent desensitization of GnRHR, and potential cross-talk with other growth factor signaling cascades. These observations are pertinent to the possibility of direct targeting of peripheral tissues including tumors (see below).

Further support for GnRHR influence on proliferation is provided in a study by Davidson *et al.* (2004). They demonstrated that activation of the GnRHR results in both cell adhesion and cytoskeletal remodeling. GnRH (10^{-7} M) increased adhesiveness of HEK293 kidney cells overexpressing GnRHR. Cytoskeletal remodeling was dependent on focal adhesion kinase I, c-Src, ERK and Rac and independent from the classic phospholipase C signaling pathway (Davidson *et al.* 2004). These studies are compromised in that they utilized pharmacologic levels of GnRH which may act more like a GnRH agonist at the receptor in contrast to nanomolar concentrations normally effective in activating GnRHR downstream signaling.

GnRH and GnRHR in extra-hypothalamic tissues

In addition to the hypothalamus, GnRH-I has also been localized to the endometrium, placenta, breast, ovary, testis and prostate (Clayton & Catt 1981, Wierman 1996, Huirne & Lambalk 2001, Limonta *et al.* 2003). The exact function of GnRH-I in these tissues is under active investigation. The recent production of a transgenic

mouse targeting alkaline phosphatase expression with the rat GnRHR promoter (Granger *et al.* 2004) and luciferase expression with the mouse GnRHR promoter (McCue *et al.* 1997) may provide additional model systems to identify and map developmental expression of GnRHR in extrapituitary sites. Several lines of evidence suggest that the GnRHR-I is also expressed in the brain in GnRH neurons to contribute to an ultrashort loop feedback mechanism (Xu *et al.* 2004).

In the endometrium and myometrium, GnRHR has been detected by radioligand binding experiments and immunohistochemistry (Clayton & Catt 1981). In the placenta, GnRH-I and II are expressed in human placenta and are key regulators of urokinase-type plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor (PAI-1) (Chou *et al.* 2003a). In endometrial stromal cultures from first trimester decidual tissues, GnRH-I and GnRH-II increased expression of uPA mRNA and protein (Chou *et al.* 2003a). In contrast, GnRH-I increased but GnRH-II decreased PAI-1 mRNA and protein expression. A GnRH receptor antagonist inhibited the effects of GnRH-I but not GnRH-II.

GnRH-I also increased mRNA for MMP 2 and 9 in decidual stromal cultures with no effect on tissue inhibitors of metalloproteinases (TIMP)-1 (Chou *et al.* 2003b). In the placenta, GnRH-I and GnRH-II increased the production of MMPs and decreased the expression of TIMPs in trophoblasts. The effects of GnRH-I but not GnRH-II were blocked by a GnRH antagonist (Chou *et al.* 2003c). These results suggest the complex interaction of GnRH action both in the endometrium and placenta impacting on implantation. The studies also support the hypothesis that a functional GnRH-II may not be expressed in humans, explaining the divergent results with GnRH-II versus GnRH-I. Together, these data suggest that, in the endometrium and placenta, the effects of native GnRH and GnRH agonists may be similar. The down-regulation of GnRHR that occurs at the level of the pituitary in response to tonic stimulation by GnRH may not occur in these peripheral sites. Further studies are needed to clarify the control of GnRH and its receptor in these tissues.

In the ovary, *in situ* studies have shown the presence of the GnRH-I mRNA in granulosa cells of primary, secondary and tertiary follicles (Kang *et al.* 2003). Recently, investigators have shown the presence of GnRH-II in human granulosa-luteal cells (hGLCs), immortalized ovarian surface epithelial (OSE) cells and in ovarian cancer cells (Kang *et al.* 2003). Some studies suggest a physiologic role of the GnRH system in the control of atresia (Kang *et al.* 2003). GnRH can inhibit DNA synthesis, induce apoptosis and activate genes important for follicular rupture and oocyte maturation

such as plasminogen activator, prostaglandin endoperoxide synthase type 2 and progesterone receptor, and those important for matrix remodeling such as the MMPs (Kang *et al.* 2003).

GnRHR expression changes in the ovary correlate with the degree of follicular development across the estrous cycle (Kang *et al.* 2003). GnRH-I induced a biphasic effect on GnRH and GnRHR expression in hGLCs and OSE cells. Estrogen treatment resulted in an initial up- and then down-regulation of GnRH and GnRHR expression. GnRH agonist administration can also down-regulate estrogen receptors α and β in ovarian cells (Kang *et al.* 2003). Recent work has suggested that the GnRHR promoter is controlled by a unique upstream regulatory sequence in human ovarian granulosa-luteal cells which was not critical in ovarian cancer cells or pituitary cells (Cheng *et al.* 2002). Thus, there may be tissue-specific regulation of GnRH/GnRHR pathways.

In the breast, both GnRH and GnRHR have been detected by RT-PCR (Kottler *et al.* 1997). Most studies involved testing of breast cancer cell lines in the absence of normal breast samples (Kakar *et al.* 1994). GnRH agonists inhibit malignant breast epithelial cells (Kakar *et al.* 1994). In the absence of studies on normal human breast tissue or normal breast cell lines, the direct action of GnRH via its receptor in the breast in the absence of malignancy remains to be elucidated.

In the testes, GnRH mRNA is expressed in Sertoli cells while the GnRHR is expressed in the Leydig cells by RT-PCR (Bahk *et al.* 1995, Botte *et al.* 1998). In early fetal development in the rat, GnRH mRNA expression in the testis precedes that in the ovary, followed by GnRHR expression. In late fetal development, levels of GnRH and its receptor increased first in females in the ovary and then in males in the testes (Dufau *et al.* 1984, Botte *et al.* 1999). In cultured testes, GnRH increased GnRHR, and GnRH agonists blocked steroidogenesis, supporting a direct action of GnRH in the testis (Dufau *et al.* 1984, Botte *et al.* 1999). These effects of GnRH or GnRH agonists may not be physiologic. No studies are available concerning the regulation of GnRH or GnRHR in the human testis.

In the prostate, GnRHR has been detected in human samples of benign prostatic hypertrophy (BPH) (Bono *et al.* 2002). GnRHR has been detected by RT-PCR in prostate biopsies with levels lower in normal prostate than in prostate cancer specimens (Bono *et al.* 2002). No normal human prostate cell lines exist to clarify the role of GnRH signaling in normal prostate.

In summary, GnRH and GnRHR are expressed in many peripheral tissues. The functional physiologic role of the ligand and its receptor in these sites is under active investigation. Problems with the current literature include the detection of GnRH and GnRHR mRNA often with

RT-PCR techniques without functional assays of protein expression and ligand-binding assays. Studies of potential physiologic signaling via GnRHR in extrapituitary tissues are flawed by the sole use of GnRH agonists and/or antagonists with long half-lives, often at pharmacologic levels. These agents may trigger signaling via local GnRHR that is different from potential physiologic paracrine signaling from local GnRH/GnRHR activity. Cell systems serve as models for GnRHR signaling. However, many are not physiologic models. This may explain the divergent pathways detected and confuse the complexities of functional pathways *in vivo*. Finally, few cutting-edge techniques have been used to date to prove that the effects of GnRH agonists or antagonists in peripheral target tissues are via the GnRHR, such as the use of siRNA, antisense technology or tissue-specific knockouts. Further research is needed to differentiate between GnRH/GnRHR signaling in normal pituitary and extrapituitary sites and show how it differs from that observed in cancer cells.

Aberrant expression of GnRHR in cancer

Various disease and/or transformed epithelial cells are known to express the GnRHR (Friess *et al.* 1991, Kakar & Jennes 1995, Chatzaki *et al.* 1996, Kottler *et al.* 1997, Barbieri 1998, Yin *et al.* 1998, Ortmann & Diedrich 1999, Borroni *et al.* 2000, Halmos *et al.* 2000, Kang *et al.* 2000, Lee *et al.* 2000, Noci *et al.* 2000), and the GnRHRs present on hormonally responsive tumor cells appear to be identical to the high-affinity pituitary receptor. Recently, GnRHR-II, which transmits significantly stronger anti-proliferative effects than GnRHR-I, has been identified in ovarian and endometrial cancers (Kang *et al.* 2000, Bedecarrats & Kaiser 2003); the significance of this remains to be determined as the role of GnRH-II has not been elucidated.

Cancer types with cells expressing the GnRHR include breast, prostate, endometrial cells in endometriosis and endometrial cancer, ovarian, pancreatic and hepatoma (reviewed by Imai & Tamaya 2000). Analysis shows that the GnRHR-I sequences in these cancer cell types are identical to those in pituitary gonadotropes (Chatzaki *et al.* 1996, Yin *et al.* 1998), and binding studies have demonstrated the functionality of these receptors. High-affinity and low-affinity GnRH-binding sites have been found in 90% and 50% of ovarian cancer biopsies respectively (Emons *et al.* 1993, Emons & Schally 1994, Imai & Tamaya 2000). While ovarian cancer is not hormone responsive, the presence of GnRH-binding sites, as well as the intraperitoneal distribution of the tumor, makes this disease an especially attractive model for GnRH-toxin therapy.

About 80% of endometrial cancers and 50% of breast cancers express both GnRH and GnRHR as part of an autocrine system (Emons *et al.* 2003). Similarly, it has been reported that 86% of human prostate adenocarcinomas express high-affinity binding sites for GnRH and express mRNA for the receptor; higher Gleason score tumors show reduced receptor numbers, but higher affinity receptors (Halmos *et al.* 2000). Proliferation of ovarian cancer cells was shown to increase after treatment with an antiserum to GnRH, supporting the idea that GnRH produced by the tumor cells acts as a negative autocrine regulator (Emons *et al.* 2000). Native GnRH, as well as GnRH agonists and antagonists, inhibited proliferation of cancer cells in a dose- and time-dependent manner (reviewed by Grundker *et al.* 2002b).

The anti-proliferative effect of GnRH analogs is mediated, at least in part, by changes in signal transduction (recently reviewed by Emons *et al.* 2003, Limonta *et al.* 2003). When bound to its ligand in cancer cells, the GnRHR couples to G protein α_i , activating a phosphotyrosine phosphatase which dephosphorylates EGFRs (Grundker *et al.* 2001). This in turn suppresses EGF-induced activation of MAPK and c-fos, thus inhibiting proliferation (Emons *et al.* 1996, Grundker *et al.* 2000a). This mechanism of cell signaling in cancer cells is different from that observed in pituitary cells, as described in the section on 'Effects on gonadotropin gene expression and secretion'. There are also data supporting the role of GnRH-I in apoptosis in tumor cells, but this remains controversial (Grundker *et al.* 2000b, Wang *et al.* 2002).

Fewer GnRH-binding sites have been found in sex steroid-independent gynecologic tumors such as cervical carcinoma (Imai *et al.* 1994). Using RT-PCR to detect message, there was significantly lower expression of the receptor in BPH and normal prostate tissues (Straub *et al.* 2001). However, GnRH-I behaves as a negative regulator of growth in some tumors outside of the reproductive tract such as melanoma (Limonta *et al.* 2003). In these cancer cells, the biochemical and pharmacological profiles of GnRHR-I correspond to those in pituitary cells (Limonta *et al.* 2003). GnRH-I-binding sites have also been described in glioblastoma (van Groeninghen *et al.* 1998) and in leukemic T cells (Chen *et al.* 2002).

Current use of GnRH analogs in cancer

With the elucidation of the structure of GnRH-I by Guillemin and Schally (Guillemin 1967), it became possible to synthesize thousands of different analogs of the primary decapeptide. Although agonists were recognized early on (Labrie *et al.* 1980, 1981, Faure *et al.* 1982) and have been employed in clinical medicine for more than 25 years, they act as delayed inhibitors of LH and FSH secretion

following the initial agonistic activity. The mechanisms underlying this desensitization simulate continuous infusions of GnRH which down-regulate GnRHR and Gq/11 expression resulting in decreased PKC-, cAMP- and Ca-dependent signaling, possibly resulting in decreased bioactive and immunoreactive gonadotropin secretion (Labrie *et al.* 1996, Labrie 2004). The development of safe and effective GnRH antagonists required considerably longer, necessitating the substitution of three or more amino acids to achieve the desired pharmacologic profiles (van Loenen *et al.* 2002) (Table 1).

GnRH analogs in prostate cancer

Phase I trials in men with clinical stage D prostate cancer with the agonist leuprolide acetate demonstrated that doses of 1–10 mg daily effectively reduced testosterone to the castrate range (Warner *et al.* 1983). A small phase III trial was then completed in similar stage D metastatic prostate cancer patients in which the efficacy of leuprolide, administered as a single daily 1 mg injection, was compared with a single oral 3 mg dose of diethylstilbestrol (DES). Comparable numbers of patients in both groups had suppression of testosterone, dihydrotestosterone and the only tumor marker available at that time, acid phosphatase. Tumor responses were equivalent, but the DES side-effects of gynecomastia, nausea/vomiting and thromboembolism led to the Federal Drug Administration (FDA) approval of leuprolide as a safer means of achieving medical castration (Anonymous 1984). Although orchiectomy remains a safe, effective alternative, it is often rejected by patients because of the psychologic side-effects (Samdal *et al.* 1991). The excess costs (compared with surgical castration) of these psychologic decisions by individual patients are estimated to be approximately \$386/month (Chon *et al.* 2000), while the overall costs to the USA Medicare healthcare system are about \$1.2 billion dollars annually for the two available GnRH agonists, leuprolide and goserelin (Anonymous 2003).

Treatment with GnRH agonists results in transient elevations in testosterone and dihydrotestosterone. In a fraction of patients with metastatic disease, this can result in 'tumor flare' characterized by worsening of disease symptoms. These include worsening of bone pain, urethral or ureteral obstruction and, of gravest concern, spinal cord compression or even death in patients with advanced metastatic disease. These effects can be blocked (Labrie *et al.* 1984, 1987, Kuhn *et al.* 1989) with the short-term administration of an anti-androgen.

A much more controversial issue is the longer term use of either steroidal (flutamide, bicalutamide or nilutamide) or non-steroidal (megestrol or cyproterone) anti-androgens in combination with the GnRH agonists

Table 1 GnRH agonists and antagonists. Reprinted with permission from van Loenen *et al.* 2000.

Name	Amino acid sequence									
	1 pGlu	2 His	3 Trp	4 Ser	5 Tyr	6 Gly	7 Leu	8 Arg	9 Pro	10 Gly-NH ₂
Human GnRH										
GnRH-I	1	2	3	4	5	6	7	8	9	10
GnRH-II	1	2	3	4	His	6	Trp	Tyr	9	10
GnRH-III	1	2	3	4	5	6	Trp	Leu	9	10
GnRH agonist										
Nonapeptides										
Leuprorelin	1	2	3	4	5	D-Leu	7	8	9—	N-Et-NH ₂
Buserelin	1	2	3	4	5	D-SER(Bu ¹)	7	8	9—	N-Et-NH ₂
Goserelin	1	2	3	4	5	D-SER(Bu ¹)	7	8	9—	AzaGly-NH ₂
Histreltin	1	2	3	4	5	D-His(ImbzI)	7	8	9—	N-Et-NH ₂
Deslorelin	1	2	3	4	5	D-Trp	7	8	9—	N-Et-NH ₂
Decapeptides										
Nafarelin	1	2	3	4	5	(d-Nal) ₂	7	8	9	Gly-NH ₂
Triptorelin	1	2	3	4	5	D-Trp	7	8	9	Gly-NH ₂
GnRH antagonist										
Abarelix	D-Ala	D-Phe	D-Ala	4	5	D-Asp	7	Lys(iPr)	9	D-Ala
Antarelix	D-Nal	D-Phe	D-Pal	4	Phe	D-Hcit	7	Lys(iPr)	9	D-Ala
Cetrorelix	D-Nal	D-Phe	D-Pal	4	5	D-Cit	7	8	9	D-Ala
Ganirelix	D-Nal	D-Phe	D-Pal	4	5	D-hArg	7	HArg	9	D-Ala
Iturelix (Antide)	D-Nal	D-Phe	D-Pal	4	NicLys	D-NicLys	7	Lys(iPr)	9	D-Ala
Nal-Glu	D-Nal	D-Phe	D-Pal	4	D-Glu	D-Glu	7	8	9	D-Ala

in the treatment of prostate cancer. While these agents can prevent some of the physiologic effects of the androgen-induced tumor flare, they also have the theoretic advantage of blocking adrenal androgens. In addition, they would be expected to enhance tumor control in the occasional patient who experiences 'escape' from chronic GnRH agonist administration. A thorough meta-analysis of 8275 men who participated in 27 randomized trials of medical/surgical castration with or without the addition of anti-androgen therapy concluded that the addition of anti-androgens improved the 5-year survival by only 2 or 3% (Anonymous 2000). As there is some variability in the definition of 'castrate levels' of testosterone (traditionally <50 ng/dl) and the fact that men who are surgically castrated rarely have total testosterone levels exceeding 20 ng/dl, the National Comprehensive Cancer Network has recommended using the latter level as a lower threshold (Millikan & Logothetics 1997). A more recent review (Loblaw *et al.* 2004) of hormonal management for androgen-sensitive prostate cancer concludes that 'Combined androgen blockade confers a statistically significant but questionable clinical improvement in survival over orchiectomy or LHRH monotherapy' and that '... treatment with antiandrogen monotherapy appears unlikely to lead to a survival benefit in men with localized disease managed with non-definitive therapy (watchful waiting)'. As this active debate on the effects of steroidal versus non-steroidal anti-androgens is beyond the scope of this review

article, the reader is referred to Loblaw *et al.* (2004) for current guidelines and recommendations.

The first GnRH antagonist to be approved for clinical use in the USA was abarelix (Table 1). A randomized phase III multicenter study comparing its efficacy with the combination of leuprolide and bicalutamide revealed that over half of abarelix-treated patients achieved castrate (<50 ng/dl) levels of testosterone by day 4 while 79% of the combination-treated patients had still not achieved castrate levels by day 15 (Trachtenberg *et al.* 2002) (Fig. 3). Although the rate of fall in prostate specific antigen (PSA) levels among the two groups was similar, the potential avoidance of tumor flare via the use of abarelix in patients with impending cord or ureteral/urethral urinary tract obstruction or severe bone pain persisting on narcotic analgesics led to FDA approval for use in these limited circumstances. Unfortunately, the clinical trials leading to this approval also revealed a low incidence of immediate-onset allergic reactions that included hypotension and syncope. These phenomena led to a black box warning that patients who are administered abarelix need to be observed in the physician's office for 30 min following each administration and physicians must participate in a training program to ensure their competence to manage these side-effects. Nevertheless, abarelix offers an important alternative to surgical castration, the use of ketoconazole or GnRH agonist plus anti-androgen in the patient who presents

Figure 3 Median ($\pm$ S.E.M.) serum testosterone level in patients treated with abarelix depot (broken line) and those treated with leuprolide acetate and bicalutamide (solid line) on study days 1 through 169. (Reprinted with permission from Trachtenberg *et al.* (2002)).

with significant neurologic or urinary obstructive symptoms.

The duration of response and survival of patients treated with GnRH analogs or other means of androgen deprivation therapy (ADT) depends largely on their clinical status and volume of disease. Table 2 lists some of the important factors that have been found to influence responses (Oefelein & Resnick 2003). The timing of GnRH agonist ADT (i.e. when to initiate therapy and for how long to continue it) has generated considerable discussion and a variety of experimental approaches simply because, unlike surgical castration, GnRH agonist therapy is reversible.

With the advent of PSA testing, it has become possible to detect residual, advancing disease months to years before any symptoms develop. One of the few

studies on the natural history of patients who go untreated after a PSA rise is detected after successful prostatectomy revealed that radionuclide scans and other body imaging studies do not detect metastases for a median of 8 years from the time of biochemical progression (Pound *et al.* 1999). Predictors of more rapid development of clinical metastases are the initial Gleason score, the time-interval between prostatectomy and initial PSA detection and the doubling time of the PSA once detectable. In this study, the median time from radiographic metastases to death was 5 years. These data suggest that men can wait until radiographic progression is detected before initiation of ADT. Early treatment of prostate cancer is reviewed by Labrie *et al.* (2002).

Against this approach are studies showing that early use of GnRH agonist or other ADT for prostate cancer

Table 2 Clinical factors predictive of response and overall survival in patients with prostate cancer treated with androgen suppression therapy. Reprinted with permission from Oefelein & Resnick 2003.

Variable	Good prognostic factors	Reference
Nadir PSA	Undetectable	Oefelein <i>et al.</i> 2002
Time to nadir PSA	<3 months	Oefelein <i>et al.</i> 2002
Baseline PSA	Low	Sabbatini <i>et al.</i> 1999
Tumor grade	Low	Albertsen <i>et al.</i> 1995
Bone pain	No pain	de Voogt <i>et al.</i> 1989
Extent of metastases	Minimal	Sabbatini <i>et al.</i> 1999
Performance status	Low	de Voogt <i>et al.</i> 1989, Sabbatini <i>et al.</i> 1999
Alkaline phosphatase	Low	de Voogt <i>et al.</i> 1989, Sabbatini <i>et al.</i> 1999
Tumor stage	Low	de Voogt <i>et al.</i> 1989
Hemoglobin	High	Sabbatini <i>et al.</i> 1999
Body mass index	Low	Oefelein <i>et al.</i> 2002
Pretreatment testosterone	Low	Chodak <i>et al.</i> 1991

Figure 4 Eastern Cooperative Oncology Group: disease-free and overall survival data. Kaplan-Meier estimates of overall survival and disease-free survival in patients receiving immediate versus deferred hormonal therapy. (From Crawford (2003), adapted with permission from Messing *et al.* (1999)).

can improve overall survival. When combined with radiation therapy for advanced local disease, two large randomized trials showed either disease-free or overall survival advantages compared with radiation alone (Pilepich *et al.* 1995, Bolla *et al.* 1997). Similarly, in patients with nodal metastases at the time of prostatectomy, immediate medical or surgical castration improved the 5-year survival when compared with waiting until metastases (or rising PSA) were evident (Messing *et al.* 1999) (Fig. 4).

However, the GnRH agonists produce considerable side-effects including hot flashes, accelerated bone resorption, impotence, loss of libido, loss of muscle mass and, in some instances, profound psychologic effects. One approach to avoid these toxicities would be better selection of patients for early hormonal ablation. Various algorithms and neural networks have been developed to try to better predict which patients would benefit the most from early ADT (Crawford 2003). Further pharmacologic intervention can be used as well, adding additional drugs (and expense) to the patient's treatment regimen. For example, calcium and vitamin D supplementation with or without bisphosphonates can significantly reduce bone loss (Smith 2003). Hot flashes can be ameliorated in approximately half of the patients by prescribing venlafaxine (Quella *et al.* 1999), estrogens or progestins. A variety of partially effective measures can be tried to alleviate sexual dysfunction (Higano 2003).

Since the GnRH analogs are reversible, another less well-studied approach to their use is intermittent therapy. Prostate cancer cells deprived of androgen stimulation eventually become androgen independent, leading to clinical progression of metastases. Resistance to combined androgen blockade in localized disease is not yet understood; its occurrence in metastatic disease is discussed by

Labrie *et al.* (2002). The molecular events accompanying this phenomenon include mutations in or overexpression of the androgen receptor and alterations in other signaling pathways that lead to loss of the capacity for the cells to undergo apoptosis (Avila *et al.* 2001). In theory, allowing patients to recover from GnRH analog-induced ADT could delay or prevent the emergence of such cells, and prolong the duration of the hormone-sensitive state. Early experiments with this approach in animals demonstrated a 3-fold prolongation of the hormone-sensitive state (Sato *et al.* 1996). These observations led to a phase II clinical trial that found improved quality of life in men treated with intermittent ADT without obvious compromise in duration of responsiveness or survival (Goldenberg *et al.* 1999). Although the quality of life in intermittently treated patients is improved, the overall impact on survival and duration of hormone responsiveness is unknown. Two large randomized trials in the USA and Canada are being performed to answer these questions (Pether *et al.* 2003).

GnRH analogs in breast cancer

It was first recognized that surgical ovarian ablation could produce clinical responses in breast cancer in the late 19th century by Albert Schinzinger and, later, George Thomas Beatson (Love & Philips 2002). Additional endocrine ablative options including adrenalectomy, hypophysectomy and radiation ablation of the ovaries all received attention in the 20th century, but all are irreversible and invasive. Response rates to oophorectomy in premenopausal metastatic breast cancer patients vary from 30 to 75% with the highest responses in patients with estrogen receptor (ER)- and/or progesterone receptor (PR)-positive tumors (Sunderland & Osborne 1991). As with

prostate cancer, gonadal ablation with a GnRH analog was shown to be equivalent to surgical oophorectomy in premenopausal patients with receptor-positive disease although, surprisingly, there was an approximate 15-year delay in such trials, possibly due to the availability of effective anti-estrogens like tamoxifen (Taylor *et al.* 1998).

With the advent of adjuvant chemotherapy, it was widely recognized that at least some of the benefit of treatment was due to chemically induced menopause. Multiple studies did not resolve the controversy over which effect was more important, the cytotoxic effect of chemotherapy agents on the cancer cells, which could extend to the ER/PR-negative populations, or the chemotherapy-induced ovarian failure (Pritchard 2002). The value of permanent ovarian ablation by surgery or radiotherapy in premenopausal women under 50 years of age with both node-positive and node-negative disease was established by several randomized trials performed by the Early Breast Cancer Trialists' Collaborative Group (Anonymous 1996). More recently, this group reported on a randomized trial of adjuvant chemotherapy followed by goserelin versus either modality alone in premenopausal node-negative patients. The study accrued 1063 patients from 1990 to 1999 and stratified participants according to ER status. ER-negative patients fared best if they received chemotherapy with some additional improvement by the GnRH agonist treatment. In contrast, ER-positive patients had similar outcomes regardless of the treatment arm, suggesting equivalence of the therapies. A possible exception was noted for those women under 39 years of age where the combination of chemotherapy followed by goserelin resulted in superior disease-free survival (Castiglione-Gertsch *et al.* 2003). There are many nuances in considering these outcomes, such as reserving the addition of a GnRH agonist only for those patients who are not rendered menopausal by the chemotherapy and the side-effects of chemotherapy versus those of ovarian ablation. Further, newer regimens of chemotherapy which are superior to the 'classic CMF' (cytoxan, methotrexate, 5-fluorouracil) regimen used in this study complicate the interpretation of results such that some experts feel ovarian ablation should *not* be recommended routinely to premenopausal patients otherwise being treated with newer regimens (Pater & Parulekar 2003).

For premenopausal node-positive patients similar findings hold for adjuvant therapy. A large randomized study sponsored by the manufacturer of goserelin compared treatment with 'classic CMF' versus goserelin, accruing approximately 800 patients in each group between 1990 and 1996. The disease-free survival was equivalent in those patients (~60%) with ER-positive disease, while those with ER-negative tumors again fared best with chemotherapy. The quality of life was superior

in the goserelin-treated group (de Haes *et al.* 2003). This study did not include a group treated with both modalities, but did document amenorrhea rates which were approximately 65% during the first 6 months of chemotherapy. Again, the practicing clinician must weigh these findings in the light of superior chemotherapy regimens compared with the CMF regimen employed in the trial (Jonat *et al.* 2002).

Premenopausal patients with metastatic disease have often been treated with the anti-estrogen tamoxifen because of numerous studies showing equivalence with ovarian ablation (Crump *et al.* 1997). As tamoxifen is relatively inexpensive, easily administered and comparatively well tolerated, there have been few studies comparing its use with the GnRH agonists alone or in combination with tamoxifen. A meta-analysis of four small studies suggested there might be a benefit from the combination, but no large trials have been performed (Boccardo *et al.* 1999, Pritchard 2000). Most attention in this area as well as in the treatment of postmenopausal patients is now directed at the use of third generation aromatase inhibitors (Buzdar 2004).

Clinical studies with targeted toxins

The approach of coupling cytotoxic agents to GnRH has been used in model systems to test therapies for a number of tumors possessing GnRHRs (see section on 'Aberrant expression of GnRHR in cancer'). To achieve specificity in a GnRH-toxin approach, the GnRHR levels on cell types other than the targeted tumor must be considerably lower than on the tumor cells.

In animal studies, passive immunization was achieved with anti-GnRH antibodies in nude mice with human breast cancer cell xenografts (Jacobs *et al.* 1999). GnRH-*Pseudomonas* exotoxin (PE) conjugates were also shown to reduce adenocarcinoma tumor size when injected in a nude mouse xenograft model (Ben-Yehudah *et al.* 1999). In a human clinical study (Simms *et al.* 2000), GnRH decapeptide conjugated to diphtheria toxoid was injected into patients with locally advanced prostate cancer. In all patients, antibodies to GnRH and castrate levels of testosterone (which appeared to be reversible) were produced. In short, the presence of elevated levels of functional GnRHR in certain tumor types supports the notion that the GnRHR is a good candidate for targeted therapies.

Many chemotherapeutic agents used to treat cancer require interaction with DNA, transcriptional machinery or microtubules to disrupt cellular function. Therefore, potentially thousands of molecules may have to be delivered to a cell to inhibit its function completely. In the past two decades a novel approach, the use of targeted

Figure 5 Mechanism by which hormonotoxins may lead to cell death. The ligand–toxin conjugate binds to specific receptors on the surface of target cells (1) and is then internalized via receptor-mediated endocytosis (2). Once inside the cell, the conjugate (or the toxin alone) enters the cytoplasm by an unknown mechanism (3) and inhibits protein synthesis; the mechanism by which this occurs depends on the type of toxin. *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) inactivate elongation factor-2 (EF-2), whereas plant toxins (ricin toxin A (RTA); ribosome-inactivating protein (RIP)) inactivate 28S RNA. In either case, the intoxicated cell is unable to synthesize proteins which results in cell death. (Adapted with permission from Thrush *et al.* (1996)).

cytotoxins, has been developed to destroy specific tissue or cell types in the body. In the initial studies, antibodies specific for a particular cell or tissue were generated and conjugated to bacterial or plant toxins (Collier & Kaplan 1984). Following binding of the antibody–toxin conjugate to a molecular target on the cell surface, it is internalized into endosomes. In the case of many plant and bacterial toxins, acidification results in conformational changes in the toxin that permit it to exit the endosome and enter the cytoplasm. Once in the cytoplasm, most toxins inhibit protein synthesis, eventually leading to cell death, reviewed by Thrush *et al.* (1996) (Fig. 5). Many immunotoxins have been developed to target specific tumors based on unique cell-surface targets discovered through the use of monoclonal antibodies. Whereas cytotoxic small molecules can be delivered by such antibodies, the advantage of toxic proteins such as diphtheria toxin (DT), pokeweed antiviral protein (PAP) and *Pseudomonas* exotoxin (PE) lies in their enzymatic activity such that a single molecule specifically delivered to a target cell may have a much greater intracellular influence, potentially acting on thousands of substrate molecules within a cell. Thus, compared with traditional chemotherapeutic agents, only a small fraction of the number of molecules needs be delivered to cause cytotoxicity. In the past decade, targeted toxin therapy has been employed in numerous human clinical and animal studies (Brinkmann & Pastan 1994, Bast *et al.* 1996,

Nichols *et al.* 1997, Frankel *et al.* 2000, Olsen *et al.* 2001, Allen 2002, Abou-Jawde *et al.* 2003) and some agents have been approved by the FDA for use in patients (Gunther *et al.* 1993, Bast *et al.* 1996, Nichols *et al.* 1997, Uckun *et al.* 1999, Olsen *et al.* 2001, Abou-Jawde *et al.* 2003).

Studies with hormonotoxins

More recently, a similar approach has been the use of hormones rather than antibodies to target toxins to specific cells in the body (Schwartz *et al.* 1987, Singh & Curtiss 1991, 1994, Marcil *et al.* 1993). With this approach, a toxin is conjugated to a hormone that has specific receptors in a select population of target cells. The ‘homonotoxin’ then binds to those receptors, is taken into the cell by receptor-mediated endocytosis and, when delivered to the cytoplasm, results in inhibition of protein synthesis leading to cell death.

Among peptide hormones, cytotoxic analogs of somatostatin, bombesin and GnRH have been synthesized in a program headed by Andrew Schally (Letsch *et al.* 2003). These compounds have shown efficacy in ovarian, breast and renal cell carcinoma cell lines, and xenograft models. Receptors for somatostatin have been demonstrated on breast, kidney, brain and non-small cell lung cancers, and radiolabeled ^{90}Y or ^{111}In somatostatin analogs have been used for both imaging and therapy

(Virgolini *et al.* 2002). Additionally, a hormonotoxin utilizing corticotropin-releasing hormone (CRH) as the targeting agent was reported to decrease the ability of pituitary cells to respond to CRH (Schwartz *et al.* 1987, Schwartz & Vale 1988). Thus, this approach appears to have utility for numerous hormone receptors.

As discussed previously and reviewed elsewhere, elimination of gonadal function is an effective therapy for prostate and breast cancers (Miller *et al.* 1985, Vickery 1986, Nicholson & Walker 1989, Hoffken 1992, Weinbauer & Nieschlag 1992), and for other hormone-dependent abnormalities including fibroid tumors and endometriosis. GnRH analogs have successfully replaced surgery in a number of settings such as metastatic breast or prostate cancers, and their reversibility is particularly useful in settings where return of gonadal function is desirable. On the other hand, in many instances, for example, the ongoing maintenance of the castrate state after a prostate cancer patient becomes 'hormone refractory', permanent castration would be preferable. Development of a non-surgical agent that resulted in permanent suppression of gonadal activity after a single administration would thus constitute a major advance in the treatment of hormone-dependent tumors.

Potentially, hormonotoxins could be developed to attack the gonadal cells directly by attaching toxins to gonadotropins (Marcil *et al.* 1993, Singh & Curtiss 1994). If attached to LH, the toxin could potentially destroy Leydig cells in males, and theca, granulosa and luteal cells in females. Since theca and granulosa cells do not acquire LH receptors until development of a follicle is relatively advanced (i.e. follicles in early stages of development would not be affected), and since the corpus luteum is a transient endocrine organ, an LH-toxin conjugate would not lead to permanent castration in females. Likewise, in males there appears to be a stem cell population that will replace Leydig cells if they are destroyed (Keeney *et al.* 1990). Thus, an LH-toxin conjugate would only lead to a transient decrease in testicular function. If attached to FSH, again the toxin may lead to destruction of granulosa cells in follicles thus preventing follicular development. However, primordial and primary follicles do not have FSH receptors (Fortune *et al.* 1991) so as soon as the FSH-toxin conjugate is cleared from the blood stream, follicle maturation would resume. An FSH-toxin conjugate in males may result in destruction of Sertoli cells and lead to aspermia. However, the number of Sertoli cells in the adult testis is not static, at least in some species of animals (Johnson & Thompson 1983). Since Sertoli cells appear capable of dividing in adult males, it seems unlikely that the effects of an FSH-toxin in males would be permanent. Further, Leydig cells would be unaffected. Therefore, testosterone secretion would continue una-

bated and androgenic stimulation of hormone-dependent tumors would continue. Thus, an FSH-toxin conjugate also would not be useful for inducing permanent gonadal inactivity. For these reasons, 'chemical castration' using gonadotropin-toxin conjugates does not appear feasible.

To circumvent problems inherent with the use of LH and/or FSH, a hormonotoxin using GnRH as the targeting agent to achieve chemical castration would appear preferable. The GnRH-toxin would not target the gonads directly, but rather destroy gonadotropes in the anterior pituitary gland, the cells that produce the hormones (FSH and LH) responsible for stimulating gonadal activity. Gonadotropes appear to be a terminally differentiated cell line, so destruction of gonadotropes would potentially lead to a permanent loss of gonadal function. Some of the GnRH-toxin approaches used to date have not been permanently toxic to the pituitary gonadotropes. This may be due to the relatively resting G0 state of these cells and the high amounts of toxin moieties such as the doxorubicin analogs required when compared with the enzymatic toxins. On the other hand, use of PE as the toxin in conjugates has been associated with dose-limiting CNS toxicity (Pai *et al.* 1992).

There are several additional reasons why GnRH is a superior choice to the use of gonadotropins to eliminate gonadal activity: (1) GnRH is a relatively small peptide (10 amino acids) that can be prepared by chemical synthesis in large quantities for nominal cost, (2) GnRH functions similarly in both males and females so a single compound could be used in either sex, (3) numerous analogs of GnRH are available that have much higher affinity for receptors than native GnRH thus enhancing the probability that the hormone-toxin conjugate will bind to receptor, (4) the GnRH molecule can be easily modified to produce analogs that can be readily linked to toxins and (5) there is little evidence that gonadotropes actively divide once puberty has occurred; thus, if existing gonadotropes are destroyed, it does not seem likely that they will be replaced. Gonadotropins are essential for gonadal function, so this would lead to permanent gonadal inactivity in both males and females. In addition, tumor cells that aberrantly express GnRHRs could be directly affected by such a conjugate as discussed above. Therefore, it is possible that for some hormone-sensitive tumors, both direct (via GnRHRs expressed in tumors) and indirect (via reduction in sex steroids by elimination of gonadotropin secretion) effects on the tumor could be achieved.

Several investigators have attempted to develop GnRH-toxin conjugates that destroy gonadotropes. Myers & VILLEMEZ (1989) conjugated GnRH to DT toxin, but they did not report its bioactivity. Szoke *et al.* (1994) reported that a GnRH agonist conjugated to glutaryl-2-(hydroxymethyl)-anthraquinone, a cytotoxic

agent, was internalized by rat pituitary cells *in vitro*, a necessary step for causing cell death. More recently, it was reported (Kovacs *et al.* 1997) that a GnRH agonist conjugated to 2-pyrrolinodoxorubicin, a highly potent cytotoxic agent, decreased GnRH-stimulated LH secretion in rats by 63% whereas thyrotropin-releasing hormone-induced thyrotropin secretion and GHRH-induced growth hormone secretion were not significantly affected. However, their study lasted for less than 60 days so whether the reduction in gonadotrope function induced by the GnRH-toxin was permanent or temporary was not ascertained. Moreover, chemotherapeutic agents like glutaryl-2-(hydroxymethyl)anthraquinone and 2-pyrrolinodoxorubicin have two disadvantages compared with agents such as DT or similar compounds. First, even though GnRH will facilitate localization to gonadotropes, there is a significant potential for non-specific toxicity because any cell in the body could still take up the conjugate in small amounts due to the chemical nature of the chemotherapeutic agents. Secondly, many chemotherapeutic agents such as the anthracyclines must intercalate into DNA to disrupt cellular function. Potentially thousands of molecules may therefore have to be delivered to a cell to inhibit its function completely. In contrast, the enzymatic activity of some plant and bacterial toxins renders them much more efficacious once internalized. In fact, one molecule of DT has been reported to inactivate as many as five million molecules of elongation factor 6 in cells (Collier & Kaplan 1984).

If GnRH-cytotoxins actually destroy gonadotropes as they should do theoretically, then it is possible that a single dose could lead to permanent suppression of gonadal function in an individual with steroid-responsive prostate or breast cancer. Thus, these compounds would alleviate the need for repeated and continual treatment as is necessary with the GnRH agonists and antagonists in use today. Therefore, GnRH-cytotoxins have the potential to significantly decrease the number of times an individual will need to travel to a clinic to obtain treatment and decrease the lifetime cost of such treatments. In addition to these advantages, many breast and prostate cancers actually express receptors for GnRH and GnRH-cytotoxins have the potential to specifically bind to those receptors and destroy cancer cells, something that GnRH agonists or antagonists cannot do. This is discussed in greater detail in the next section of this review.

Potential use of GnRH-toxins for prostate, breast and ovarian cancers

Although GnRH analog therapy will suppress gonadotropin (and therefore ovarian/testicular steroid hormone) production and thereby slow progression of breast or

prostate cancers, many such tumors also appear to have receptors for GnRH (as discussed above). Thus, the potential exists for these cells to respond directly to hormonotoxin treatment using GnRH as the targeting ligand. Ovarian cancer cells, although not responsive to steroid hormones, express the GnRHR and their growth is inhibited by GnRH analog treatment.

Comparison of fusion and conjugate GnRH-toxins

Fusion proteins consisting of GnRH and PAP or PE have been produced and shown to inhibit growth of cultured tumor cells expressing GnRHRs (Nechushtan *et al.* 1997, Schlick *et al.* 2000); however, the activity of the fusion proteins were not directly compared with the activities of hormonotoxins produced by conjugation of a GnRH analog to the toxic protein moiety. Since recombinant fusion proteins are not chemically synthesized, they should always have one and only one GnRH associated with each PAP molecule, and it is always at the same site. For this reason, the uniformity of GnRH-PAP fusion preparations should be much better than for conjugates prepared by chemical means, thus making the generation of large amounts of GnRH-PAP for clinical studies more feasible. It is likely, however, that recombinant fusion proteins will have inferior activity to hormonotoxins produced via chemical conjugation for two reasons: (1) the ends of the GnRH molecule are known to be important for receptor binding (Karten & Rivier 1986) and are not likely to be as easily accessible to the receptor in a fusion protein and (2) the incorporation of a D-amino acid in position 6 that is known to enhance receptor binding affinity approximately 30-fold is impossible in fusion proteins.

We recently compared GnRH-PAP fusion toxin (single polypeptide chain) with GnRH-PAP chemical conjugate for binding and cytotoxic activity in Chinese hamster ovary (CHO) control cells, and CHO cells expressing a transfected GnRHR (CHO-GnRHR) (Qi *et al.* 2004). In support of the hypothesis that the conjugate would be more cytotoxic than the fusion protein, we showed that while the conjugate bound and caused specific toxicity to GnRHR-positive cells, the GnRH-PAP fusion proteins were much less active in both of these regards. We tested two different versions of fusion protein, corresponding to either full (f, containing post-translationally modified sequences) or mature (m, without these sequences). GnRH-fPAP was tested because a previous study (Schlick *et al.* 2000) demonstrated that a GnRH-PAP fusion protein containing these post-translationally modified sequences was cytotoxic to Ishikawa cells (an endometrial cell line). While the GnRH-PAP conjugate bound to GnRHR, albeit at somewhat higher

Figure 6 Clonogenic assay in (A) CHO-GnRHR or (B) CHO control cells exposed to varying concentrations of PAP, GnRH-mPAP fusion protein or GnRH-PAP conjugate. Values are means $\pm$ S.E.M. (Reprinted with permission from Qi *et al.* 2004).

concentrations compared with control GnRH alone (D-Lys⁶-GnRH), neither of the fusion proteins (GnRH-fPAP or GnRH-mPAP) were able to inhibit binding of the radioligand to GnRHR. However, both the GnRH-PAP conjugate and GnRH-mPAP fusion proteins inhibited translation to a similar extent as PAP alone in a cell-free rabbit reticulocyte translation system, demonstrating that the PAP molecule in the conjugate and fusion proteins retained toxicity. Thus, any difference in their cytotoxicity when tested in cell survival/clonogenic assays could not be attributed to disruption of PAP function.

To evaluate the ability of GnRH-PAP to inhibit growth of cells expressing GnRHR on their surface, cells were treated with increasing amounts of PAP or GnRH-PAP conjugate or fusion protein. We showed that GnRH-PAP was able to prevent the growth of cells expressing GnRHRs including several prostate and breast cancer cell lines (Qi *et al.* 2003, Yang *et al.* 2003). Results from a clonogenic assay (consisting of counting the number of colonies formed at the end of a 5-day incubation period) are shown in Fig. 6 in control CHO and CHO-GnRHR cells. As seen in the figure, PAP and

GnRH-mPAP fusion showed only non-specific toxicity, with similar results in both CHO and CHO-GnRHR cells at all concentrations ($P > 0.1$, comparing GnRH-mPAP to PAP alone at 1×10^{-8} M). Fifty percent inhibition in both cell types with these proteins was observed only at concentrations > 100 nM. In contrast, GnRH-PAP conjugate protein showed specific toxicity in CHO-GnRHR cells ($P < 0.002$ at 1×10^{-8} M compared with PAP alone), with 50% inhibition seen at approximately 2 nM; in CHO control cells, toxicity from the conjugate protein was within the same concentration range as the fusion protein and PAP ($P > 0.09$ at 1×10^{-8} M). These results showed that the conjugate protein was approximately 50-fold more cytotoxic to CHO-GnRHR cells compared with CHO control cells. The fusion protein, however, did not show any specific toxicity to CHO-GnRHR cells. This finding is consistent with the lack of fusion protein binding to GnRHR.

Although the native GnRH sequence was used in the fusion proteins while the analog GnRH (with D-amino acid in position 6, see above) was used in the conjugate protein, the difference in binding affinity (30-fold) by itself was not sufficient to explain the three to four log difference in binding between conjugate and fusion GnRH-PAP proteins, nor the approximate two log difference in cytotoxicity.

GnRH-toxin conjugates in cancer cell lines

With this information in hand, we decided to evaluate GnRH-PAP prepared by chemical conjugation both *in vivo* and *in vitro*. Two sets of studies were performed to evaluate this conjugate *in vitro*. First, ovine anterior pituitary glands were dissociated into single cells and placed in cell culture. Cultured cells served as controls or were treated with PAP alone, or with GnRH-PAP overnight. Following the overnight culture, treatments were removed by washing the cells with media and then the cells were incubated for an additional 24 h. At this time, the cells were collected, homogenized and amounts of LH (to reflect gonadotrope activity) and prolactin (to assess activity in a cell type not expressing GnRHRs) were measured. PAP alone did not alter the amounts of either LH or prolactin in the pituitary cells compared with controls. However, GnRH-PAP decreased the amount of LH in the cells by approximately 80% compared with the controls, but did not alter the amount of prolactin in the cells (Fig. 7). Secondly, we tested the chemical GnRH-PAP conjugate against selected cancer cell lines for direct toxicity. As shown in Fig. 8, both CHO cells expressing the GnRHR and α T3-1 cell lines were specifically killed by the conjugate at concentrations greater than 10 nM (Yang *et al.* 2003). We also demonstrated direct GnRH-PAP cytotoxicity in prostate cancer cell lines (Qi *et al.* 2003).

Figure 7 Effects (means $\pm$ S.E.M.) of GnRH-toxin or toxin alone on the ability of cultured anterior pituitary cells to synthesize LH (solid bars) or prolactin (shaded bars). Toxin alone did not alter LH synthesis compared with untreated cells, but GnRH-toxin substantially decreased ($P < 0.01$) synthesis of LH. In contrast, GnRH-toxin did not influence the ability of cultured pituitary cells to synthesize prolactin ($P > 0.05$).

These data demonstrate the ability of this conjugate to specifically inhibit the function of cells expressing GnRHRs without altering the function of cells lacking GnRHRs. Moreover, the pituitary cells that were affected are the cells responsible for stimulating the activity of gonads. Thus, inhibiting the function of these cells would eliminate the gonadal steroids on which many prostate and mammary tumors are dependent for their growth. As GnRH agonists alone will not result in sterilization, the ability to achieve this is an important avenue of investigation. The potential for use of this conjugate in clinical trials will clearly depend on the level of expression of the GnRHR *in vivo*, quantification of which is a current focus of our laboratories.

GnRH-toxin conjugates in animal studies

Encouraged by the results obtained in the experiments discussed above, the GnRH-PAP conjugate was next tested *in vivo*. In this study, the effect of GnRH-PAP on reproductive function was evaluated in adult, male dogs. Four dogs received GnRH-PAP hourly for 36 h (hourly) and four other dogs received GnRH-PAP as one bolus injection daily for 3 consecutive days (bolus). One dog received a single bolus (single). Three adult male dogs received GnRH without the PAP conjugate and served as controls. Twenty-five weeks after the initial treatment, all treated dogs received a second treatment with GnRH-PAP as a single bolus while control dogs received GnRH. Serum concentrations of testosterone and LH were determined by radioimmunoassay, and testis size was measured for 9 months after treatment. Stimulation tests ($5 \mu\text{g/kg}$ GnRH) were used to evaluate the ability of the pituitary gland to release LH. Serum

Figure 8 Cytotoxicity of GnRH-PAP conjugate against (A) CHO-GnRHR and (B) α T3-1 cells in the cell proliferation assay, with various concentrations of GnRH-PAP (●), D-Lys⁶-GnRH (▲) or PAP (■). Values are means $\pm$ S.E.M. (Reprinted with permission from Yang *et al.* (2003)).

testosterone concentrations were measured to evaluate testis function during the study. Concentrations of testosterone were significantly lower ($P < 0.05$) in all animals treated with GnRH-PAP than in controls after treatment (Fig. 9A). Basal LH was lower ($P < 0.05$) in all treated animals than in the control group between weeks 0 and 33 post-treatment. Likewise, treatment with GnRH-PAP reduced ($P < 0.05$) LH release after GnRH stimulation in treated animals compared with the control area under the curve (AUC) (Fig. 9B). Testis volume was lower ($P < 0.05$) in all treated versus control dogs (Fig. 9C). In conclusion, administration of the GnRH-PAP conjugate at a 25-week interval resulted in a major disruption of reproductive function in male dogs which was maintained for at least 11–12 weeks after the second GnRH-PAP injection (Sabeur *et al.* 2003).

In all animal and human studies to date from our group and others, there has been no evidence for

Figure 9 Effects (means±S.E.M.) of GnRH-PAP on reproductive parameters in male dogs. Treatment regimens are described in the text. Arrows in (A) indicate the times that treatments were administered. (A) Changes in serum concentrations of testosterone at various times after treatments. The ability of the anterior pituitary to respond to a GnRH challenge is shown in (B); data shown represent the total amount of LH released (AUC) following the challenge. Changes in testicular volume with time after treatments is depicted in (C). Since each of the treatment regimens resulted in decreased testicular volume, data from all the treatment groups were combined. (Reprinted with permission from Sabeur *et al.* 2003)).

non-specific toxicity, nor any indication that removal of GnRH inhibited the function of a variety of organs in which mRNA for GnRH receptor has been identified (i.e. gut). The fact that administration of GnRH-PAP or immunization to eliminate active GnRH from the circulation only affects reproductive tissues supports the premise that GnRHR can be safely employed as the target for hormonotoxins.

Conclusions and future prospects

Over three decades have passed since the elucidation of the GnRH molecule. Analogs are now widely employed in the treatment of prostate cancer, eliminating the need for surgical castration, and ongoing studies suggest similar benefits in selected breast cancer patients. The expression of GnRHR-I and GnRHR-II in peripheral tissues,

especially neoplastic tissues, may provide a new target for GnRH-toxin-directed therapy. This treatment could potentially achieve both permanent castration by elimination of pituitary gonadotropes as well as direct cytotoxicity to tumors. Animal studies to date support such development, revealing little toxicity to normal intact tissues, while clinical studies will depend on the development and testing of good manufacturing practices (GMP) materials. The elucidation of the differences in function of the GnRH-I and GnRH-II receptors and ligands remains an area of intense investigation.

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