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GnRH AND STEROIDS IN CANCER

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1. ABSTRACT

Gonadotropin-releasing-hormone (GnRH) analogues are synthetic compounds derived from decapeptide neurohormones (LHRH; LH/FSH-RH). They have a key role in hormone dependent cancer, particularly breast and prostate cancer. GnRH analogues produce an efficient inhibition of gonadotropins and sex steroid hormones. Their use in cancer therapy result in a, pharmacological castration (i.e. ovariectomy and orchiectomy), providing an androgen and estrogen ablation. GnRH exert an inhibitory action on the growth of hormone-dependent human and canine mammary tumor. Mammary tumors can produce growth factor that potentially could modulate their own proliferation in an autocrine fashion (i.e. TGF-α and TGF-β ∂r with a paracrine mechanism (i.e. EGF, IGF, FGF). The expression of EGF receptors is related in mammary tissues to the action of oestrogen and progesteron and to the presence of functional receptors for oestrogen (ER) and progesteron (PR). The present review elucidate the role of GnRH receptors in cancer and their connection with steroid hormones. Besides we showed the link between GnRH and signal transductions pathways: Estrogen-receptors, GnRH-receptors, EGF-receptors signal transduction pathways. A very tight link exists between steroid hormones and GnRH analogues both on central pituitary gonadal axis and on tumor receptors peripherically. This last mechanism could be explained either locally activating GnRH receptors or locally interacting with EGF receptor-Intracellular NitricOxide system.

2. INTRODUCTION

LHRH was first isolated, sequenced and synthetized in the early 1970s (1,2,3) and showed that both natural LHRH and the synthetic decapeptide corresponding to its structure possessed major follicole-stimulating hormone (FSH)-releasing as well as LH-

releasing activity (3). It was forecast that chemical substitutions in the molecule would lead to analogues possessing antagonist or increased agonist activity, useful as anti-and profertility agents, respectively. Initial efforts were highly successful in generating highly potent agonist analogues: substitution of only one or two amino acids resulted in analogues with to 200 times the potency of the native molecule (4). However, the causes of this increase in potency, i.e. increased binding affinity and resistance to metabolism, accentuated the ability to shut down rather than simulate reproductive function.

The concept that LHRH regulates the secretion of both FSH and LH from the pituitary gland, is upheld by much experimental and clinical evidence (5,6). Indeed, it was originally suggested that the name LHRH be canged to GnRH for gonadrotopin-releasing hormone (3,6).

In the past years, more than 3000 analogs of LHRH have been synthesized (5,7). Agonistic analogs, such as Decapeptyl, Zoladex, Leuprolide and Buserelin, much more active than the LHRH itself, and available in depot preparations,have important clinical application in gynecology and oncology (5). Potent antagonist of LHRH such as Cetrolelix, Ganerelix and Abarelix, suitable for clinical use, have been likewise synthesized (8,9,10).

2.1. GnRH chemical

Lutheinizing hormone-releasing hormone (LHRH; LH/FSH-RH, GnRH) is an endogenous decapeptide neurohormone with an obligatory role in reproduction. In normal circumstances it is synthesized in hypothalamic neurones and secreted in a pulsatile fashion from neurones at the median eminence, traverses the hypothalamo-hypophysial portal system and interacts with its receptors on the gonadotrophs in a transient fashion to stimulate release (and synthesis) of the gonadotrophins (11). By binding to and by activating specific receptors on gonadotrophs, the neurohormone stimulates the synthesis and the release of the two gonadotropins LH and FSH (follicole-stimulating hormone) (12); for this reason it is also called GnRH (gonadotropin-releasing hormone). Hypothalamic neurosecretory cells release LHRH in a pulsatile wayand LHRH pulses are critical for the maintenance of gonadotropin gene expression and for the physiological pattern of secretion of LH and FSH (13). The two gonadotropins are themselves secreted in a pulsatile way in the systhemic circulation and act on the gonads to regulate gametogenesis and steroid synthesis (13).

The synthesis and secretion of both LHRH and gonadotropin are regulated by gonadal steroids which act at the hypothalamus and the pituitary level (14). The LHRH gene has been shown to carry steroid response elements (15,16). Moreover, estrogen (estrogen receptors β mainly) and androgen binding sites have been demonstrated to be expressed in a transgenically derived LHRH neuronal cell line (GT1) (17), which represents a very useful experimental tool to study the regulation of LHRH gene expression.

The gene encoding hypothalamic LHRH has been identified in several species and is organised into four exon and three introns (16). This gene encodes for a preprohormone consisting of a signal peptide followed by the sequences of LHRH and by GAP (gonadotropin-releasing hormone associated peptide), separated by a canonical cleavage site (18). In vertebrate, the sequence of the different forms of LHRH is similar but not identical: amino acids in positions 5 – 8 show a reduced degree of conservation. Recently, a gene coding for a second form of LHRH (LHRH-II) has been identified in several placental mammals, including humans. LHRH-II encode a peptide identified as [His5Trp7Tyr8]LHRH, indicating that the amino acid sequence presents three substitution in these three position. LHRH-II is mainly present in the midbrain, while peripheral tissues expressing the peptide include the prostate, bone marrow and kidney (18). The existence of the two form of LHRH may indicate specific function for the peptides and suggest the possible presence of multiple receptor subtypes.

2.2. GnRH analogues

The elucidation of the crucial role played by GnRH in the control of the pituitary-gonadal system immediately underlined the relevance of its possible clinical applications for the treatment of several reproductive-related disease. However, GnRH is rapidly degraded at pituitary level and its half-life in the circulation is about 2 – 4 min. (20); moreover, the binding affinity of native GnRH to its pituitary receptor is quite low (Kd - 10-7M) (21). Therefore, efforts have been directed at obtaining GnRH analogues with increased stability against enzymatic attack and with higher affinity for the receptor. These studies led to the synthesis of two different class of GnRH analogues:

- GnRH agonists, binding with high affinity to the GnRH - R and mimicking the action of the neurohormone on pituitary function,
- GnRH antagonist, competing with GnRH for the binding to the receptor but devoid of intrinsic activity.

2.2.1. GnRH agonist

In past years, intensive analyses of the structure-activity relationship of the GnRH molecule have been performed. These studies revealed that both the NH_2- and COOH-terminal domain plays a crucial role in receptor activation (22). Moreover, although a lack of conservation of amino acids 5 – 8 has been reported in the primary structures of GnRH peptides in different species, ARG8 seems to be important for a high-affinity binding to the mammalian receptor (22). From the enzymatic point of view, it has been widley reported that the degradation of native GnRH occurs mainly at the Gly residue in position 6 (23). Based on these experimental observations, several synthetic GnRH agonists have been developed to be clinically used in the place of GnRH itself. Available GnRH agonist are mainly characterised by the presence of a D-amino acid in position 6; some of them present a

Table 1. Amino acid sequences of GnRH agonist

Peptide	Sequence
• GnRH	pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2
• Leuprolide	pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-D-Leu[6]-Leu[7]-Arg[8]-Pro[9]-NHC_2H_5
• Buserelin	pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-D-Ser(tBu)[6]-Leu[7]-Arg[8]-Pro[9]-NHC_2H_5
• Tryptorelin	pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-D-Trp[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2
• Goserelin	pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-D-Ser(tBu)[6]-Leu[7]-Arg[8]-Pro[9]-AzGly[10]-NH_2

Table 2. Amino acid sequences of GnRH antagonists

Peptide	Sequence
• Nal-Glu- GnRH	Ac-D-Nal[1]-D-Cpa[2]-D-Pal[3]-Ser[4]-Arg[5]-D-Glu[6](AA)-Leu[7]-Arg[8]-Pro[9]-D-Ala[10]-NH_2
• Cetrorelix	Ac-D-Nal[1]-D-Cpa[2]-D-Pal[3]-Ser[4]-Tyr[5]-D-Cit[6]-Leu[7]-Arg[8]-Pro[9]-D-Ala[10]-NH_2
• Antide	Ac-D-Nal[1]-D-Cpa[2]-D-Pal[3]-Ser[4]-NicLys[5]-D-NicLys[6]-Leu[7]-ILys[8]-Pro[9]-D-Ala[10]-NH_2
• Azaline B	Ac-D-Nal[1]-D-Cpa[2]-D-Pal[3]-Ser[4]-Aph[5](Atz)-D-Aph[6](Atz)-Leu[7]-ILys[8]-Pro[9]-D-Ala[10]-NH_2
• Ganirelix	Ac -D-Nal[1]-D-Cpa[2]-D-Pal[3]-Ser[4]-Tyr[5]-D-hArg(Et2)[6]-Leu[7]-hArg(Et2)[8]-Pro[9]-D-Ala[10]-NH_2

deleted carboxy-terminal Gly10- amide with the addition of an ethylamidic residue to Pro9 (Table 1). These compounds share the same biological activity of the native decapeptide but are more resistant to enzymatic degradation and bind with high affinity (Kd - 10^{-9} M) to the pituitary receptor. This affinity can be further increased by the introduction of hydrophobic groups on the sixth amino acid (24).

At pituitary level, GnRH agonist elecit the same biological activities observed after GnRH stimulation. An low dose or a pulsatile administration of GnRH agonist leads to receptor activation and to stimulation of gonadotropin secretion. On the other hand, a prolonged application of GnRH agonist induces, after an initial release of LH and FSH (the so-called "flare-up phenomenon"), the downregulation and desensitization of the GnRH receptor, resulting in the complete suppression of pituitary-gonadal functions. This latter effect is called "chemical castration" and represent the molecular basis for several important clinical applications of these compounds (24).

2.2.2. GnRH antagonists

The search for antagonistic analogues of GnRH was initially driven by the desire to develop new family of regulatory compounds with higher specificity and lower toxicity than steroidal contraceptives. Antagonists of GnRH generally present multiple amino acid subsitutions at positions 1,2,3,6,8 and 10 (Table 2).

By competing with the native hormone, GnRH antagonists were determined to be unsuitable for clinical applications because of their undesired side effects, such as oedematogenic as anaphylactoid reactions, due to concomitant stimulation of histamine release (25). More recently, new generations of GnRH antagonists have been developed; these present a similar complex structure with several amino acid subsitutions but appear to be free of anaphylactoid side effects. However, in order to continuously counteract the activity of the endogenous hormone, these compounds must be administered at high doses and in the form of long-acting preparations. Therefore, possible limitations of the use of GnRH antagonists may reside in some of their low solubility and propensity to form gels aqueous solutions. Efforts have been made to develop antagonistics analogues of GnRH devoid of side effects and with increased solubility (26). Cetrorelix, Ganirelix and Abarelix (a short acting antagonist with low histamine-releasing activity) are now available as long acting depot formulations (26,27) and have been already tested in long-term clinical trials (28,29). More recently, more soluble GnRH antagonists have been obtained by increasing the number of hydrogen binding sites on the peptide side chains. Among these compounds, FE200486 has been reported to possess a very long duration of action, likely to be due to the unique physicochemical properties, such as solubility in aqueous milieu, low propensity to form gels and ability to diffuse slowly from the s.c.sites of administration (30). Further studies might optimise the application of these compounds for the treatment of those reproductive-related pathologies wich require a complete blockade of the pituitary-gonadal axis.

3 GnRH CLINICAL USE IN CANCER

The action of GnRH and its analogs are mediated by high-affinity receptors for GnRH found on the membranes of the pituitary gonadotrophs. An acute administration of GnRH agonists induces a marked release of LH and FSH. However, continous stimulation of the pituitary by chronic administration of GnRH agonist produce an inhibition of the hypophyseal-gonadal axis through the process of "down-regulation" of pituitary receptors for GnRH, desensitization of the pituitary gonadotrophs, and a suppression of circulating levels of LH and sex steroids (5,31,32). This down-regulation of GnRH receptors, produced by sustained administration of GnRH agonists, provides the basis for clinical applications in gynaecology and oncology of this class of compounds (32). Antagonists of GnRH exhibit no intrinsic activity, but compete with GnRH for the same receptor sites. GnRH antagonists produce a competitive blockade of GnRH receptors and cause an immediate inhibition of the release of gonadotropins and sex steroids (5,32). The principal mechanism of action of GnRH antagonist was thought to be based only on a competitive occupancy of GnRH receptors, but recently, has been demonstrated that administration of the GnRH antagonist Cetrorelix produce down-regulation of pituitary GnRH receptors and a decrease in the levels of mRNA for GnRH receptors (32,33). Other works indicates that GnRH antagonists exert their inhibitory effects on the gene expression of pituitary GnRH receptors by counteracting the stimulatory effect of endogenous GnRH

(34). Specific GnRH receptors are also found on breast, prostatic, ovarian, endometrial and even pancreatic cancers (31,32). These GnRH receptors on tumor cells can mediate direct effect of GnRH analogs (32). Thus, high-affinity binding sites for GnRH and the expression of mRNA for GnRH receptors were detected in human prostate cancer samples, human prostate cancer lines, and Dunning rat prostate cancer (35,36). The presence of GnRH receptors in various human mammary carcinoma cell lines was also reported. GnRH receptors were similarly found in human ovarian epithelial cancer specimens and human ovarian cancer lines (31,32,37,38). The presence of high-affinity membrane receptors for GnRH was also established in nearly 80% of human endometrial carcinomas and in some endometrial cancer lines (32). GnRH receptors on human cancer appear to be similar to pituitary GnRH receptors. The expression GnRH receptors gene in human breast, endometrial, ovarian tumors, and respective cancer cell lines was also demonstrated by RT-PCR (37,38,39,40). The inhibition of growth of human mammary, ovarian, endometrial and prostatic cancer cell lines by GnRH agonists and antagonists in vitro strongly support the concept of their direct effect (5). The evidence for the production of an GnRH-like peptide and/or expression of mRNA for GnRH was also demonstrated in human prostatic, mammary, endometrial and ovarian cancer lines. This suggest that local GnRH may be involved in the growth of these tumors. The existence of functional regulatory system consisting of locally produced GnRH-like peptides and specific GnRH receptors has also been postulated in prostate cancer and ovarian cancer. The authors suggested that this GnRH, produced by tumor cells, might have an inhibitory function. However, the proliferation of various cancer cells in vitro is dose-dependently suppressed by GnRH antagonists and inhibitor effects of agonists might be explained by receptor down-regulation (31). Other studies in ES-2 human ovarian cancer lines suggest that locally produced GnRH is stimulatory. Additional investigations are needed to resolve the role and the action of endogenous GnRH-like peptides produced by various tumors.

3.1. Breast cancer

Breast cancer is the most common malignancy in women. About 30% of women with breast cancer have estrogen-dependent tumors and can be treated by hormonal manipulations such Tamoxifen or oophorectomy (44). Experimental and clinical studies clearly demonstrated that agonists of GnRH can be used for treatment of estrogen-dependent breast cancer. Thus initial investigations in rat and mouse models of breast adenocarcinoma showed that chronic administration of agonist [D-Trp6] GnRH decreased tumor weight and volume (5). This suggested that agonists of GnRH should be considered for a new hormonal therapy for breast cancer in women. Various clinical trials carried out since the early 1980s, demonstrated regression of tumor mass and disappearance of metasatses in premenopausal and postmenopausal women with breast cancer treated with [D-Trp6] GnRH, Buserelin, Zoladex or Leuprolide (44,45). These studies showed that GnRH agonists are efficacious for the treatment of premenopausal women with estrogen-dependent, estrogen-receptors positive breast cancer (45). Recently, our studies, demonstred that Goserelin is efficacy on such disease producing tumor shrinkage and reducing the incidence of metastases in bitches with hormone-dependent mammary cancer (46).

3.2. Prostate cancer

The greatest therapeutic impact of GnRH analogues was in the field of prostate cancer, which is the most common noncutaneus malignant tumor in men. About 70% of human prostate cancers are testosterone-dependent and the treatment of advanced prostate cancer is based upon androgen deprivation (36). Therapy with agonist of GnRH with or without antiandrogens is currently the preferred treatment for men with advanced prostate cancer and in about 70% of cases GnRH agonist are selected for primary treatment (5,36). Administration of antiandrogens prior to and during early therapy with agonist can prevent the disease flare. Clinical trials in patients with advanced prostate cancer show that the antagonist of GnRH could be beneficial as a monotherapy for patients with prostate cancer and metastates in the brain, spine, liver and bone marrow in whom the GnRH agonist cannot be used as single drug, because of the possibility of flare-up (41,42). GnRH antagonists cause an immediate fall in the levels of gonadotropins and sex steroid and greatly reduce the time onset of therapeutic effects (36,40). In addition, treatment with GnRH antagonists can produce long-term improvement in patients with symptomatic benign prostatic hyperplasia (BPH) (42,43) and offers a therapeutic alternative to patients with BPH who are considered poor surgical risk (43).

3.3. Endometrial cancer

Endometrial cancer is a common gynaecologic malignancy in the Western world (5). Surgery or radiotherapy is successful in 75% of cases, but new methods are needed for advanced or relapsed cancer (31). Endometrial carcinoma is estrogen-dependent and thus it should respond to therapy with GnRH analogs. In addition, high affinity receptors for GnRH are present on nearly 80% of membranes of human endometrial cancer and endometrial cancer cell lines. Bioactive and immunoreactive GnRH and the expression of mRNA for GnRH were also found in these cells.

3.4. Epithelial ovarian cancer

Epithelial ovarian cancer is the fourth most frequent cause of cancer-related deaths in women (5). The treatment based on surgery or chemotherapy is not very effective and new approaches are needed. Ovarian cancer may be dependent on LH and FSH and in experimental cancer models, the suppression of the secretion of gonadotropins produced by GnRH analogs inhibits the growths of ovarian tumors (47). Studies in vivo indicate that GnRH antagonist inhibits growth of human OV-1063 and ES-2 epithelial ovarian cancer wich was xenografted into nude mice better than agonist [D-Trp6] GnRH (32). In clinical studies some patients with advanced ovarian carcinoma treated with agonists of GnRH showed stabilization of disease (5), but in a multicenter trial no beneficial effects of therapy with [D-Trp6] GnRH could be found (48).

4 GnRH ANALOGUES ACTION

The role of female hormone, especially estrogen in the development of mammary tumor in mammals including the bitchs is well established (51,52). Ovarian hormones act synergistically with pituitary hormones, especially growth hormone and prolactine to promote the development of mammary tumors (53,54). The expression of GnRH-receptors and/or their ligands, as well as other growth factors, is related in mammary tissue to the action of estrogen and progesteron and to the presence of functional receptors for estrogen (ER) and progesteron (PgR) (55,56). Consequently, several therapeutic approaches to this malignancy are aimed act achieving a blockade of ovarian hormones secretion and/or action (57,58). Gonadotropin-releasing hormone (GnRH agonist) have been shown to be effective to suppress ovarian hormones in human and in bitch, through the down-regulation of the pituitary-ovarian axis (59,60).

In addition to its function as a key hormone in the regulation of pituitary-gonadal axis, GnRH2 probably affects human extrapituitary tissues (61,62). GnRH analogues have been used in some of sex hormone-dependent cancer terapy, including breast (63,64,65), prostatic (66,67,68,69), pancreatic (70,71), endometrial (72), and ovarian (73,74) cancers. Although this effect may be mediated by an indirect mechanism based on the reduction in sex hormone secretion, there are indications that GnRH analogues suppress the growth of cancer cells in vitro (75,76,77,78). Specific binding sites for GnRH are demonstrated in certain tumors responsive to GnRH analogues (79,80,81). Moreover, GnRH analogues activate GnRH signal transduction pathways in breast cancer cells (82). Despite the fact that surgical castration showed no therapeutic effect on canine mammary tumors (83,84), our results showed that Goserelin, which acts according with a chemical castration, is efficacy on such disease producing tumor shrinkage and reducing the incidence of metastases in bitches with hormone-dependent mammary cancer (46). This suggests that GnRH analogues efficacy is not only due to the suppression of gonadal activity but also to the block of hypothalamus-pituitary axis and to a direct action on tumor cells; such hypothesis is supported by the detection of GnRH receptors in canine mammary tumors (85) as well as by the results of Vincze *et al*. (86), who have reported that the GnRH agonists significantly inhibit the growth of xenografts of the estrogen-receptors-negative of human MDA-MB 231 mammary tumor, and the effect of GnRH analogues.

On murine MXT mammary adenocarcinoma GnRH analogues which reduce significantly the concentration of binding sites (87). Moreover, recent findings showed that GnRH agonist exert both in vivo and in vitro a direct inhibitory action on the proliferation of human prostate tumor cells by interfering with the stimulatory action of EGF, reducing EGF-R and c-fos expression (88).

All these findings suggest direct inhibitory effects of GnRH analogues on the tumor growth. However, their mechanism of action remain unknown.

4.1. Pituitary action level

GnRH and /or GnRH analogues can be employed for two opposite clinical goals: to restore fertility (pulsatile administration) and to suppress the pituitary-gonadal function (chronic administration). The paradoxical antiferility effect of a chronic treatment with GnRH agonists, also called “chemical castration”, is usually reversible after the cessation of drug administration. Daily injections or monthly depot formulations of GnRH agonists have been successfully used either to interfere with the physiological LH peaks in order to improve the results of in vitro fertilisation procedures or to block an abnormal function of the pituitary-gonadal axis.

Chemical castration is also the basis for the clinical employment of GnRH agonists for the treatment of those malignancies whose progression may depend on gonadal steroids, such as breast, prostate, endometrial and ovarian cancer. GnRH agonists represent an established and well-tolerated alternative to surgical castration for some of these tumors, at least during their initial phase of hormone dependency (89). Through the down-regulation of pituitary GnRH receptors, GnRH agonists suppress the secretion of gonadal steroids, thus depriving the tumors of their mitogenic stimulus. For the treatment of these malignancies, GnRH agonists are usually combined with anti-steroidal agents to counteract the initial burst of gonadotropin secretion induced by GnRH agonists themselves.
GnRH antagonists, which have been experimented in clinical trials but are not yet available for routine clinical use, might offer the advantage of inhibiting the pituitary-gonadal axis without exhibiting the “flare up” phenomenon. (Figure 1 A and B).

4.2. Extrapituitary action level

In the last few years, evidence has been accumulated that GnRH agonists can exert a direct antiproliferative action on a number of malignancies related to the reproductive system (90,91). These compounds seem to exert their antimitogenic activity through the binding to GnRH receptors that have been consistently shown to be expressed in cancer cells. Tumor cells have been further demonstrated to express GnRH itself (or a GnRH-like peptide) which, in turn, might regulate cell proliferation in an autocrine/paracrine way by acting as a growth inhibitory factor (90). Therefore, in addition to their action at the pituitary level, GnRH agonists might affect tumor growth by modulating the activity of the GnRH system which is locally expressed in cancer tissues (Figure 2). The intracellular mechanisms mediating the direct growth-inhibiting activity of GnRH agonists have not been fully elucidated so far. A better clarification of these molecular mechanisms might improve the pharmacological treatment.

5 GnRH RECEPTORS IN CANCER CELL LINES

The expression of mRNA coding for the GnRH receptors has been reported in different hormone-related malignancies, such as prostate (92,93) breast (94,95), endometrium (96,97) and ovarian (94,98,99) cancers. The nucleotide sequence of GnRH receptor mRNA in these

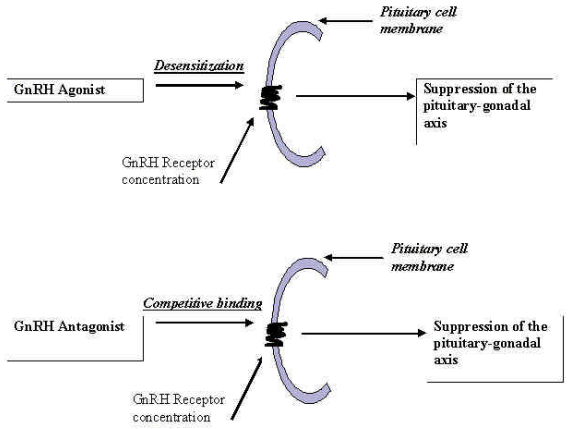

Figure 1. A GnRH agonist pituitary action levels. Receptor desensitization B GnRH antagonist pituitary action levels. Receptor competition.

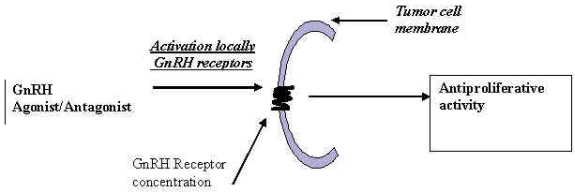

Figure 2. GnRH analogues extrapituitary action levels. Activation GnRH receptors.

tumors appears to be identical to that found in the pituitary gland. In cancer cells, the GnRH receptors is detectable also at the protein level. By Western blot analysis and by using a specific monoclonal antibody raised against the human pituitary receptor (100), Limonta *et al.* has been able to demonstrate that a protein band of approximately 64 kDa is present in membrane preparations of prostate cancer cells (101). This molecular weight is the same as that previously reported for the GnRH receptors protein at pituitary level in humans (100). Although these observations seem to support the hypothesis that the GnRH receptors in cancer tissues corresponds to that of the pituitary, at least in terms of nucleotide sequence and molecular weight; different results have been reported so far for the binding characteristics of GnRH receptors in cancer. Other authors have reported the presence of two types of GnRH binding sites, in gynaecological cancer cells, one of low affinity and the other one of high affinity (102).

In our studies, in GnRH positive canine mammary cancer cells, we observed, in binding competition experiments, that GnRH agonist was able to affect EGF binding reducing binding affinity of [^{125}I]EGF (103). These data are in agreement with the results obtained in human and murine tumor cells in which a phosphorilation of EGF receptor (86) and a significant reduction of the EGF-binding sites was demonstrated in presence of GnRH agonists (87). Finally, only one class of high affinity receptors has been identified in endometrial as well as in ovarian cancer cells (104,105). Some of these discrepancies might be due to the different experimental conditions adopted for the binding assay, or to point mutations in the receptor or in the G-protein to which it is coupled or to the different cancer etiology.

Most of the reports so far summarised have been performed in hormone-dependent cancer cells. Interestingly, the observation of the expression of GnRH receptors also in androgen-independent carcinoma of the prostate, indicate that the receptor is still present when the tumor has progressed to the more advanced phase of steroid-unresponsivenes, which is also poorly responsive to standard therapy.

6 GROWTH FACTORS AND STEROID RECEPTORS IN CELLULAR REGULATION

6.1. Growth factor in the normal and malignant gland

The natural secretory products of the mammary epithelial cell, colostrum and milk, are aboundant sources of growth factors (131). Growth factors in the normal gland probably are necessary for multiple purposes: in the newborn development, mammary growth, and mammary carcinogenesis. A large amount of literature has shown that estrogens, antiestrogens, progestins, and antiprogestins strongly regulate certain growth factors of the Epidermal Growth Factor (EGF) and Transforming Growth Factor ß (TGF-ß) families, as well as growth factors and secreted binding proteins for the IGF family (132,133) (Table 3).

EGF, apparently the most aboundant milk-derived growth factor, is an important regulator both of the proliferation and differentiation of the mouse mammary gland in vivo and of mouse mammary explant in vitro (134,135). Modulation of signal of transduction pathways of EGF and its family members, as well as other unrelated growth factors are proving to be critical during mammary development. The EGF family consist, at present, of four receptors, a half dozen growth factors, and an additional half dozen growth factors encoded only by certain viruses (135,136).

In human breast cancer lines in vitro, autocrine growth factor dependent on the TGFa-EGF receptor system as also been documented in one of ER-negative breast cancer cell line, the MDA-MB-468 cell line. It is clear in this line that a rare gene amplification of the EGF receptor sensitizes the cells to autocrine function of TGFa. This line, representative of only a small percentage of breast cancers, express more than 10^6 EGF receptors/cell. TGFa and amphireguline are also induced by estrogen in hormone-dependent MCF-7 breast cancer cells; antisense cDNA or anti TGFa antibodies partially block estrogen induced growth in vitro (137,138,139), strongly suggesting a hormone-inducible type of autocrine system. The potential clinical relevance of this type of system is under investigation.

In human breast cancer an inverse correlation was observed between ER and EGF receptor positivity (106,107,108), while a significant direct correlation was observed between the concentration of ER and EGF receptors in canine malignant tumors (109). These findings suggest that the growing mechanism of human hormone-dependent breast cancer are different from those of the canine tumors. Further studies are required to clarify this issue.

Table 3. Hormonal regulation of Growth Factor system in breast cancer

Family	Growth Factor	Receptors	Binding protein
• EGF	EGF, TGFa, amphiregulin	EGF, erB-2	Unknown
• IGF	IGF-II	IGF-I, IGF-II, insulin	BP-3, BP-4
• TGF-ß	TGF-$ß_1$, TGF-$ß_2$, TGF-$ß_3$	Unknown	Unknown
• PDGF	PDGF-1, PDGF-2	Unknown	Unknown

Paracrine tumor-host interactive functions of this family of factors may then begin to dominate its functions as the disease progresses. Strategies employing toxic, genetically engineered EGF receptors, ligand, bacterial toxin, fusion proteins termed oncotoxins or anti-EGF receptors antibodies copuled to toxin or other therapeutic drugs (immunotoxins) or the closely related c-erB2 receptor could possibly find future therapeutic utility, since a large portion of hormone-independent breast cancer express significant levels of these receptors (140,141,142,143). Recent studies have addressed the function of the heregulins acting on the EGF receptors-related c-erB-3 and c-erB-4 protein in breast cancer. The heregulins appear to act in vitro with biphasic effects: low levels are proliferative, while higher levels may inhibit epithelial proliferation. The heregulins also appear to promote differentiation and to induce casein synthesis in the developing gland and in breast cancer cells (144,145,146).

6.2. Regulation of steroid receptors

The estrogen and progesterone receptors are dimeric, gene-regulatory proteins. In mammary tissue, a single gene encodes an estrogen receptor subunit, and these subunits homodimerize and complex with additional proteins, such as heat shok proteins, to form the complete estrogen receptor (ER) assembly (110). Recent studies have complicated the picture with the identification of alternate spliced and mutated forms of the ER in breast cancer and some normal tissues such as brain and uterus (111). A single gene encodes three different isoforms of the progesterone receptor subunit in mammary tissue; there are two forms of progesterone receptor, homodimericone and heterodimericone (PR) (112,113). The multiplicity of progesterone and estrogen receptor isoforms in breast cancer may allow for significant variations in patterns of dimerization and in resultant variations in specificity of ligand recognition with respect to agonist versus antagonist and differential regulation of target genes (110,114). On top of this complexity, each receptor is able to adopt multiple conformations, depending upon the characteristic of interaction of the steroid (or nonsteroid ligand) with the receptor binding pocket. For example, the estrogen receptor can adopt at least three distinct conformations, depending on the antiestrogen bound (115).

The ER cannot be clearly classified as an oncoprotein or tumor suppressor protein. Although it clearly mediates onset and progression of the disease, unexpected results were obtanained when the ER was expressed endogenously in ER-positive breast cancer cell lines and compared with its heterotypical expression in formerly ER-negative cell lines. In striking contrast to its normal function in ER-positive cell lines, ER expressed in ER-negative cell lines suppresses cell growth, in spite of its normal action in regulating expression of certain hormonally responsive genes (116,117). Thus, the multiple differences between ER+ and ER- breast cancer seem to include incompatible growth-regulatory mechanism. We still do not understand the molecular basis for a lack of expression of PR in certain ER-positive breast cancer. A recent cell hybridization study with an antiestrogen resistant, ER-positive but PR-negative cell subline of MCF-7 has shown that PR expression last is a recessive phenotype in this system (118).

Increased expression and altered isozyme patterns of the cellular enzyme protein kinase C (PKC) family has also been implicated in the malignant progression of breast cancer (119). This enzyme family can act down-modulating ER-mRNA, activating ER function, independently inducing some estrogen-responsive genes with AP-1 sites in their promoters, and allowing more invasive cellular characteristic to be expressed (120). The PKC family contain at least nine cytoplasmic-nuclear enzymes, which possess serine-threonine specificity for phosphate addiction to other cellular proteins (119); different isotype serve different cellular functions. The activity of PKC is known to be regulated by hormones and /or growth factors during normal lactational differentiation and to contribute to casein expression regulation. PKC activity has been found to be elevated in ER-negative and drug-resistant breast cancer relative to ER-positive breast cancer. Treatment of ER-positive breast cancer with an activatory of PKC such as the phorbol ester 12-O-tetradeconyl-phorbol-13-acetic (TPA) leads to rapid down-regulation of ER, destabilization of its mRNA, and phosphorylation of the ER protein, coincident with modulation of its function (121,122,123,124). Phosphorylation of ER and PR, induced by estrogen itself, growth factor pathways (such as insulin-like growth factor-1 (IGF-1)), heregulin, cAMP, dopamine agonists, and other hormones may also constitutively activate the steroid receptors (125,126,127,128). Other current studies have suggested that receptors for other steroids (potential cancer prevention agents, retinoids and vitamin D) may modulate ER/PR function by forming heterodimers with ER or Pr or by modulating chromatin interactions of ER and PR (129,130).

7 SIGNAL TRANSDUCTION

7.1. Signal transduction and nuclear oncogenes

A unifying mechanistic link between the proliferative action of growth-promoting steroid and

Table 4. Genetic Defect in Breast cancer

A. Established Familial Breast Cancer Genes		
Gene	**Disease**	
• BRCA-1	Femal breast and ovarian cancer	
• p53	Li-Fraumeni syndrome of hereditary cancers	
• BRCA-2	Female and male breast cancer	
B. Established Breast Cancer Progression Genes		
Gene	**Class**	**Function**
• Rb-1	Suppressor Gene	Cell cycle G_1 regulator
• cyclin-D1	Oncogene	Cell cycle G_1 regulator
• myc	Oncogene	Cell cycle/cell death regulator
• erB-2	Oncogene	Growth factor receptor
• p53	Suppressor gene	Cell Cycle/cell death/DNA repair regulator

growth factor in different tissues are the nuclear protoncogenes (Table 4 A and 4B).

These transcription-regulating proteins mediate convergent pathways of growth regulatory stimuli through direct steroid action, through growth factor-induced mitogen-activated protein (MAP) kinase or phospholipase C-PKC, or through cytokine-induced JAK-STAT pathways (147,148,1150). The most important pathway for the proliferative stimuli exerted through the EGF receptor, the erB-2 receptor, and the insulin receptor families (151,152) appears to be the MAP kinase pathway. Receptors trigger this pathway through autophosphorylation and subsequent binding to PTB domains of signal transduction adaptor proteins (153,154). Following a cascade of protein phosphorylation, the products of the c-fos, c-myc, c-mib and c-jun protooncogenes are commonly observed to be induced shortly following mitogenic growth factor treatment of many types of cells, including normal and malignant breast epithelial cells. The protein products of at least three nuclear protooncogenes, c-fos, c-myc and c-jun, are also induced by both estrogen and progesterone in breast cancer (147). Progestins additionally induce a c-jun related protooncogene known as c-junB (148). Not surprisingly, tamoxifen down modulates c-yic expression during treatment-induced regression of patient tumors, (155) c-myc, c-fos and c-jun induction have also been shown to occur in human mammary epithelial cells in vitro and in the rat uterus in response to estrogen treatment in vivo (156,157,158). The protein products of c-fos, c-myc and c-jun genes form a heterodimeric complex, which interacts to form with a gene promoter consensus sequence termed AP-1. It has also been shown that the Myc protein binds retinoblastoma tumors-suppressor gene product Rb-1 or its partner to block their growth-inhibitory action (159). The Myc protein is also of particular importance in human breast cancer, since its gene is amplified in approximately 30% of the cases. As discussed below, other important signal transduction pathways are induced by oxidative stress, intracellular calcium and nitric oxide.

7.2. Signal transduction patways of Estrogen receptors

The biological activity of estrogen is mediated by specific high-affinity estrogen receptor (ER) located within target cell nuclei. In the absence of hormone, ER is associated with a host of proteins that prevent it from interacting with the cellular transcription apparatus. Upon binding estrogen, the receptor undergoes an activating conformational change, facilitating its association with target genes and permitting it to regulate gene transcription (160). In addition to the well esthablished pathway, however, it has been shown that estrogen can induce extremely rapid increase in the concentration of the intracellular second messengers, calcium and cAMP (161,162,163). The time course of these events is similar to those elicited by growth factors and peptide hormones, lending support to the hypothesis that they do not involve the classical genomic action of estrogen through its receptor. These similarities, between the nongenomic actions of steroids and growth factor-signalling pathways converge in such a manner to permit cross-talk. For instance, both estrogen and epidermal growth factor (EGF) are known to act as mitogens in promoting cellular proliferation in the breast cancer and reproductive tract (160). Furthermore, the effects of these two agents sometimes overlap: estrogen has been shown to increase the uterine levels of both EGF and its receptor (164,165,166), and EGF has been shown to mimic the effects of estrogens in the mouse reproductive tract (167). Although the molecular details of this cross-talk remain to be elucidated, it is clear that ER itself is an important point of convergence. Specifically, it has been shown that ER transcriptional activity can be activated by binding to its cognate estrogen ligand but also by a variety of other extracellular signals: EGF, TGF, insulin and dopamine (127,168,169). The activation of ER by EGF has been demonstrated to involve direct phosphorilation by mitogen-activated protein kinase or extracellular-regulated kinase (MAPK/ERK) of serine residue (170,171). A further embodiment of this cross-talk was revealed when it was demonstrated that 17ß-Estradiol (E2) causes rapid activation of MAPK in mammalian cells in an ER-dependent manner (172,173). Thus, we can consider that a feed-forward system exists where E2 activates MAPK, an event that, in turn, enhances the transcriptional activity for ER. Recently, Sica *et al.* (174) showed that Leuprorelin, a GnRH-agonist, inhibits ERK1 and 2 phosphorilation in androgen-sensitive LNCaP cells while a stimolatory activity on ERK phosphorilation in androgen-insensitive PC-3 cells has been demonstrated. Such evidences suggest a different activation of MAPK in different cellular models and that ERK don't necessary induce a cell proliferation. Although the mechanisms underlying estrogen-induced MAPK activation and its physiological significance

remains to be explained, the activation of this signalling patway may represent a potential mechanism by which estrogens regulate proliferation.

7.3. Signal transduction patways of GnRH receptors

Individual molecules of the signal transduction cascades turned on by receptor agonist binding can play important roles not only in the intracellular activation process but also in the fine feedback regulation of signalling itself. In cancer cells and tissues, GnRH and GnRH analogues exert an antiproliferative action. This observation stimulated intensive experimental research aimed at identifying the signal transduction pathways coupled to the GnRH receptor at tumor level.

GnRH binds to a G protein-coupled membrane receptor in gonadotropes (177,178,179,180) and results in activation of multiple signaling pathways (181). The initial phase of GnRH action involves G protein- mediated stimulation of phospholipase C, leading to the formation of inositol 1,4,5-triphosphate (Ins-P3) and diacylglycerol.

Although various studies suggested a possible link between the GnRH-R and PLC-mediated phosphoinositide metabolism in mammary and ovarian cancer (82,175), later reports indicated that PLC activation might not represent the crucial mechanism of GnRH receptor activation in tumors (176). In prostate, both androgen-dependent and androgen-independent cancer cells, it been demonstrated that antiproliferative action of GnRH agonists is completely abrogated by pertussis toxin. Moreover, GnRH agonists substantially antagoniste the pertussis toxin-catalysed ribosylation of a Gi protein. These data consistently indicate that, at variance with the receptor of the gonadotrophs, prostate cancer GnRH receptor might be coupled to the Gi-cAMP signal transduction patway.

Inositol 1,4,5-triphosphate induces the release of intracellular calcium, and diacylglycerol activates PKC, resulting in multiple cellular responses to GnRH. Intracellular transmission of extracellular signals is mediated in large part by several groups of sequentially activated protein kinases, which are collectively known as the MAPK cascades (182,183). In the growth factor signaling, the key elucidated MAPK cascade is the ERK (184). Recent evidence indicates that some G protein-coupled receptors can activate ERK cascade (185,186,187,188). The signals transmitted through the ERK cascade lead to activation of a set of regulatory molecules that eventually initiates cellular responses such as growth and differentiation (189,190,191). Recently, it has been shown that GnRH agonist is capable of activating ERK in the pituitary organ culture (192) and the aT3-1 gonadotroph cell line (191,192) and that ERK is involved in regulation of gene expression of the gonadotropin α-subunit (193). However, the ERK cascade is not the only link between membrane receptors and their intracellular targets, and in the past few years, several other ERK-like cascades have been identified (190). One of the most studied is JNK [also known as stress-activated protein kinase (195,196)] cascade, which is known to be activated in response to cellular stress such as apoptosis (197). ERK, JNK, and p38 (198) are members of the MAPK family. Recent data suggest that GnRH is capable of activating JNK in the αT3-1 gonadotroph cell line (199). It was reported that the signaling cascade by which GnRH acts in peripheral tumors is distinct from that in the anterior pituitary (200,201). In addition, the ERK cascade has been implicated in both cell proliferation (202,203,204) and growth arrest (205,206). In particular, ERK is reported to be involved in G1-specific cell cycle arrest of human breast cancer cells (207), NIH 3T3 murine fibroblasts (208), and human myeloblastic leukemia cells (209). Dephosphorylation of a tumor suppressor gene product, pRB, seems to be a target of the extracellular signals that induce cell cycle arrest and differentiation (210,211). In Go/Gi, pRB is underphosphorylated and complexed to the E2F transcription factor. This prevents the activation of some E2F-regulated genes required for DNA replication (212,213). Phosphorylation of pRB, during mid to late G1 by cyclin D- and cyclin E-associated kinases (214,215), is accompanied by release of E2F and activation of transcription of E2F-regulated genes, resulting in entry into S phase.

Recently Kimura *et al.* (216) showed that although GnRH agonist had no effect on the activation of the Jun N-terminal kinase (JNK), treatment of Caov-3 cells with GnRH agonist activated extracellular signal-regulated protein kinase (ERK), and its effect was more than that induced by GnRH. GnRH agonist also activated ERK kinase (mitogen-activated protein/ERK kinase) and resulted in an increase in phosphorylation of son of sevenless (Sos), and Shc. Both pertussis toxin, which inactivates Gi/Go proteins, and expression of a peptide derived from the carboxyl terminus of the beta-adrenergic receptor kinase I, which specifically blocks signaling mediated by the betagamma subunits of G proteins, blocked the GnRH agpnist-induced ERK activation. Phorbol 12-myristate 13-acetate (PMA) also induced the ERK activity, but pretreatment of the cultured cells with PMA to down-regulate protein kinase C did not abolish the activation of ERK by GnRH agonists. Elimination of extracellular Ca^{2+} by EGTA also did not abolish the activation of ERK by GnRH agonist. Inhibition of mitogen-activated protein/ERK kinase by means of PD98059 canceled the antiproliferative effect of GnRH agonist and apparently reversed the GnRH-induced dephosphorylation of the retinoblastoma protein, the hyperphosphorylation of which is a hallmark of G1-S transition in the cell cycle. All, these results provide evidence that GnRH agonist stimulation of ERK activity may be mediated by G$\beta\gamma$ protein, not by PMA-sensitive protein kinase C nor extracellular Ca^{2+} in the Caov-3 human ovarian cancer cell line, suggesting that this cascade may play an important role in the antiproliferative effect of GnRH agonist. This cascade might have a role in GnRH agonist-induced antiproliferative effect. Furthermore, post-GnRH receptor signaling cascade in ovarian cancer cells seems to be different from that in pituitary cells, suggesting that the postreceptor signaling cascade might be different, depending on the cell, although the receptor is same.

7.4. Signal transduction pathways of Growth factors

Tumor growth factor (TGFa) and Epidermal growth factor (EGF) bind to the same membrane receptor

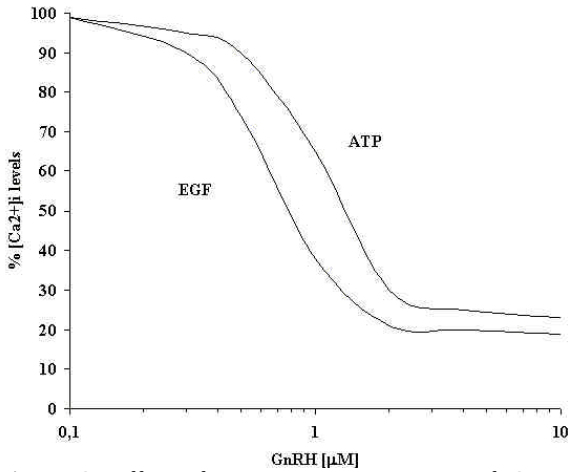

Figure 3. Effect of increasing concentration of GnRH agonist in canine mammary tumor. Cells treated with a single dose of EGF or ATP (1µM) in a 1 mM Ca2+ Medium (calcium influx).

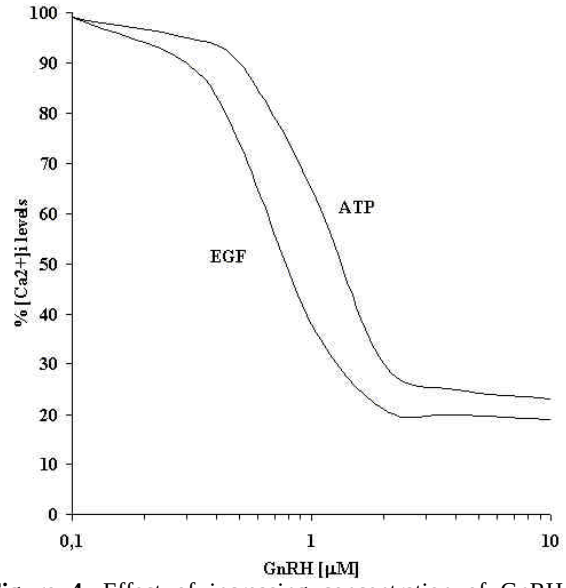

Figure 4. Effect of increasing concentration of GnRH agonist in canine mammary tumor cells treated with a single dose of EGF or ATP (1µM) in Medium without a 1 mM Ca2+ in presence of 10 µM EGTA (calcium release from intracellular stores).

(EGF-R), which is a class I receptor tyrosin kinase (244) which induce direct PIP2 hydrolysis , by a direct phosphorylation of PLCs, producing Inositol triphosphate (InsP3) and variations in $[Ca^{2+}]i$ sustained by both release from intracellular stores and influx across the plasmalemma. (218,219). The divalent cation calcium (Ca^{2+}) is used by cells as a second messangers to control many cellular process including muscle contraction, secretion, metabolism and neuronal excitability (219). Moreover cytosolic Ca^{2+} plays an important role in the regulation of several cell types (220). Importantly, it partecipates in the regulation of the cell cycle in proliferating cells and in tumor cells in particular (221). Moreover it has long been thought that Ca^{2+} and Nitric oxide (NO) work together in the control of cell homeostasis and NO could have appeared as a step in the signalling cascade initiaded by the cation. However the interaction between the two messangers does not exist as a dependence but as a true, bi-directional cross-talk. In fact currently, almost all aspects of Ca^{2+} homeostasis have been reported to involve modulation by NO (222). Our experiments (103) demonstrated that, in canine mammary tumor cells, the GnRH agonist, Goserelin, was able to reduce calcium proliferative stimuli acting both on a specific proliferative stimulus such as EGF and on an aspecific proliferative stimulus such as ATP. We found that the interaction of GnRH with ATP system in canine mammary tuomor cells showed that GnRH agonists was able to reduce both EGF and ATP induced Ca2+ rises both from released from internal stores and extracellular calcium entry (Figure 3 and 4). Interestingly, Goserelin, dit not induce a typical calcium response in canine mammary tumor cells (93). Finally, our results on GnRH activity on intracellular NO levels EGF or ATP induced (Figure 5), suggest that Nitric oxide may have a role in the chain of intracellular event elicited by activation of epidermal growth factor receptors and in the down-regulation of calcium signalling by GnRH . While the Ca2+ storage machinery is unaffected by the treatment with NO, the gaseous messengers is shown to negatively modulate PIP2 hydrolysis and the ensuing generation of IP3 (223). An important consequence is the reduction of the growth factor-induced release of Ca2+ from the intracellular stores. In fact Goserelin, used in association with proliferating stimuli such as EGF or ATP, significantly increased nitric oxide production and affecting both calcium signal as well as cell proliferation in canine mammary tumor cells (103). This observation in according with fact that NO-induced negative signal modulations were already observed in several types of neurona cell lines, strongly suggests that it may have a wider meaning (225)

8 DIRECT EFFECT OF GNRH ON TUMOR GROWTH

The observation that GnRH and its receptors are expresed in cancer cells suggest that this GnRH autocrine/paracrine system might be involved in the local control of tumor growth. This hypothesis has been confirmed by a number of reports. Antimitogenic activity of GnRH agonists has been reported for breast cancer (82,226,225), prostatic cancer (226,227), endometrial cancer (75,102,228) and ovarian cancer (26,105,229,230). These observations have been further confirmed by in vivo studies showing that GnRH agonists can significantly counteract the growth of cancer cells xenografted into nude mice (86,231). The GnRH system, which is present in cancer cells, appears then to partecipate in the local regulation of tumor growth by inhibiting cell proliferation. It has also been suggested that, when used for the treatment of hormone-dependent tumors GnRH agonist might directly reduce cancer growth by activating the locally expressed GnRH receptor, in addition to their main action at the pituitary level. Moreover GnRH analogues seem to interact on canine mammary tumor cells, not only

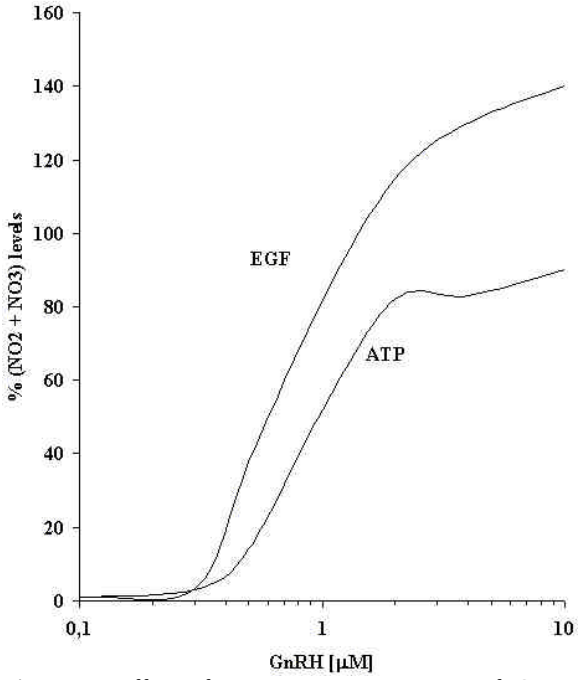

Figure 5. Effect of increasing concentration of GnRH agonist in canine mammary tumor cells treated with a single dose of EGF or ATP (5μM) on Nitric Oxide production.

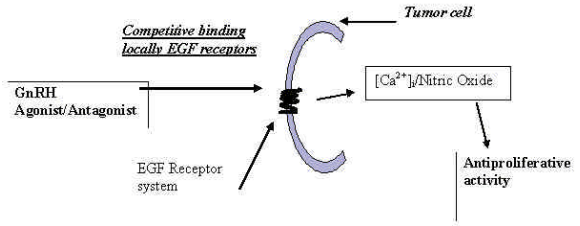

Figure 6. GnRH analogues locally action levels. Competitive binding on EGF receptors and pathways Nitric Oxide System

at pituitary level but also with Nitric Oxide system, directly to esplicate their antiproliferative activity (Figure 6).

GnRH agonists might also be considered as a possible therapy for the treatment of steroid-unresponsive tumors. However, the clinical observation so far available on the efficacy of GnRH agonist in hormone-independent cancers are poor. These observations seem to suggest a low efficacy of these compounds, at least when administrated according to the standard routes of administration. The elucidation of the concentration of GnRH agonists which might be required at the level of the tumor tissue to induce their antiproliferative action might help clarify this issue.

Unexpectedly and interestingly, Cetrorelix, belonging to the previous generation of GnRH antagonists, has been reported to have an antiproliferative activity similar to that of GnRH agonists on cancer cells, either in vitro or in vivo (105,163). Therefore, Cetrorelix seem to behave as a potent suppressor of the pituitary-gonadal axis at pituitary level and as an activator of the GnRH receptors at tumoral levels. Although the molecular mechanisms making Cetrorelix a GnRH antagonistic analogue at the pituitary level and an agonistic analogue at cancer level are still unknown. This peculiarity of this molecule and structurally related compounds, with a longer half-life, may give an advantage over GnRH agonists for the treatment of hormone-related cancers, especially as GnRH antagonists are completely devoid of the "flare-up" phenomenon. Further studies are necessary to confirm these observations. The presence of GnRH receptors in tumor tissue has recently prompted studies aimed at the development of targeted chemotherapy based on GnRH anologues. Target cytotoxic GnRH conjugates are hybrid molecules composed of a GnRH agonist and a cytotoxic part. By specifically binding to its receptor, the GnRH analogue brings the chemotherapeutic drug directly to the tumor tissue. This might increase the efficacy of standard chemotherapy while reducing its toxicity (232).

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