GnRH Receptors in Cancer: From Cell Biology to Novel Targeted Therapeutic Strategies

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The crucial role of pituitary GnRH receptors (GnRH-R) in the control of reproductive functions is well established. These receptors are the target of GnRH agonists (through receptor desensitization) and antagonists (through receptor blockade) for the treatment of steroid-dependent pathologies, including hormone-dependent tumors. It has also become increasingly clear that GnRH-R are expressed in cancer tissues, either related (i.e., prostate, breast, endometrial, and ovarian cancers) or unrelated (i.e., melanoma, glioblastoma, lung, and pancreatic cancers) to the reproductive system. In hormone-related tumors, GnRH-R appear to be expressed even when the tumor has escaped steroid dependence (such as castration-resistant prostate cancer). These receptors are coupled to a Gαi-mediated intracellular signaling pathway. Activation of tumor GnRH-R by means of GnRH agonists elicits a strong antiproliferative, antimetastatic, and antiangiogenic (more recently demonstrated) activity. Interestingly, GnRH antagonists have also been shown to elicit a direct antitumor effect; thus, these compounds behave as antagonists of GnRH-R at the pituitary level and as agonists of the same receptors expressed in tumors. According to the ligand-induced selective-signaling theory, GnRH antagonists might assume various conformations, endowed with different activities for GnRH analogs and with different intracellular signaling pathways, according to the cell context. Based on these consistent experimental observations, tumor GnRH-R are now considered a very interesting candidate for novel molecular, GnRH analog-based, targeted strategies for the treatment of tumors expressing these receptors. These agents include GnRH agonists and antagonists, GnRH analog-based cytotoxic (i.e., doxorubicin) or nutraceutical (i.e., curcumin) hybrids, and GnRH-R-targeted nanoparticles delivering anticancer compounds. (Endocrine Reviews 33: 784–811, 2012)

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I. Introduction

It was in 1971 when Dr. Schally’s group (1–3) first announced the discovery of the structure of the hypothalamic hormone GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂). Later, the pivotal role of this decapeptide in the control of reproductive functions has become increasingly clear (4). GnRH is synthesized in a small subset of neurones in the septal-preoptic-hypothalamic region (5, 6). These neurones secrete the neurohor-
mone into the hypophysial portal circulation, through which it reaches the anterior pituitary to stimulate the synthesis/release of the two gonadotropins that, in turn, regulate gonadal sex steroid production (4). In humans, the GnRH gene is located, as a single gene copy, on chromosome 8p11.2-p21 and is composed of four exons separated by three introns (7, 8). The gene encodes a preprohormone consisting of a 23-amino-acid signal peptide separated by the GnRH decapeptide by two serine residues. The decapeptide is followed by a GKR sequence (responsible for signaling amidation of the carboxy terminus and enzymatic cleavage of the decapeptide from the precursor) and by a 56-amino-acid peptide termed GnRH-associated peptide (GAP) (9, 10). After the processing of the preprohormone in neurosecretory cells, GnRH is released in a pulsatile way and reaches the anterior pituitary to exert its stimulatory effect on gonadotropin secretion (11), through the binding to its high-affinity cognate GnRH receptor (GnRH-R, a member of the seven-transmembrane, G protein-coupled receptor family) (12) (Table 1).

Currently, pituitary GnRH-R represent the molecular target of the most widely used pharmacological treatment of hormone-dependent tumors (such as prostate, breast, and ovarian cancers), based on GnRH agonists and antagonists. Chronic pharmacological administration of GnRH agonists, contrary to the natural pulsatile stimulation, induces desensitization of GnRH-R and, in turn, suppression of gonadotropin secretion, resulting in the inhibition of gonadal steroid production. This process is slow, being preceded by the so-called flare phenomenon, i.e. an initial stimulation of the pituitary gonadal axis that might be responsible for an initial acceleration in cancer activity. In contrast, GnRH antagonists competitively target the GnRH-R, eliciting an immediate inhibition of LH and FSH release (4, 13–18).

In the past two decades, it has become increasingly clear that GnRH-R are expressed in several cancer tissues, either related (prostate, breast, ovarian, and endometrial cancers) or unrelated (melanoma, glioblastoma, lung, and pancreatic cancers) to the reproductive system (Table 1); activation of these receptors by means of GnRH agonists has been consistently reported to be linked to a strong antitumor (antiproliferative, antimetastatic, antiangiogenic) activity (19–27). These opposite biological effects of GnRH-R activation (stimulation of gonadotropin synthesis/release at pituitary level vs. antitumor activity in cancer tissues) seem to be linked to specific intracellular signaling cascades that are coupled to this receptor in the different tissues: $G_{aq}/$phospholipase C pathway in gonadotropes and $G_{af}/cAMP$ pathway in cancer (28–31).

Intriguingly, in cancer cells, classical agonistic GnRH analogs have been reported to behave as GnRH agonists by exerting an antitumor effect through activation of locally expressed GnRH-R (25–27, 32–35). Taken together, these observations indicate that the pharmacology of both GnRH agonists and antagonists is different in the different cellular environments. To explain this, the concept of ligand-induced selective signaling has been proposed by Millar’s group (34, 36, 37). According to this concept, different conformations of the GnRH-R may give rise to selective binding of GnRH analogs and differential intracellular signaling pathways.

A second form of GnRH (GnRH-II) has been discovered in most vertebrates, including humans (10, 24, 38). This peptide has been suggested to act through a putative cognate receptor (type II GnRH-R), which is expressed in different tissues, including tumor cells. However, the human type II GnRH-R gene carries a frameshift and a premature stop codon, indicating that a functional full-length receptor protein does not exist in humans (Table 1). Thus, it is now believed that it is only the classical form of the GnRH-R (type I GnRH-R) that mediates the biological effects of GnRH, GnRH-II, and their synthetic analogs (10, 24, 38–40).

Another natural isoform of GnRH, GnRH-III, has been isolated from sea lamprey (Petromyzon marinus); this peptide binds to GnRH-R on cancer cells, exerting a strong antiproliferative effect, but it is much less potent than GnRH in stimulating gonadotropin release at the pituitary level (41, 42).

Based on these observations, tumor GnRH-R are now considered a very interesting candidate for novel molecular targeted strategies for the treatment of tumors that bear these receptors. These agents include GnRH agonists and antagonists, GnRH agonist-based cytotoxic (i.e. doxorubicin) or

### TABLE 1. Human GnRH receptors

<table>
<thead>
<tr>
<th>Tissue distribution</th>
<th>Type I GnRH-R</th>
<th>Type II GnRH-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary, placenta, ovary, uterus, prostate gland, breast, liver, heart, kidney, skeletal muscle, cancer cells (either related or unrelated to the reproductive system)</td>
<td>4q13</td>
<td>1q12</td>
</tr>
<tr>
<td>3 exons, 2 introns</td>
<td>3 exons, 2 introns</td>
<td>No functional full-length protein due to a frameshift in exon 1 and a premature stop codon in exon 2</td>
</tr>
<tr>
<td>328-amino-acid protein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Type I GnRH-R is the classical form of the GnRH-R.
nutraceutic (i.e. curcumin) hybrids, and GnRH-R-targeted nanoparticles delivering anticancer compounds.

In this review, we will address and discuss the molecular aspects of the antitumor activity associated with cancer GnRH-R as well as the different classes of GnRH-R-targeted agents currently under development and investigation.

II. Pituitary GnRH-R

At the pituitary level, hypophysiotropic GnRH, after being secreted in a pulsatile way from hypothalamic neurons, binds to and activates its cognate receptor to stimulate gonadotropin synthesis/release. GnRH-R was first cloned from an immortalized murine gonadotrope-derived cell line (αT3-1); subsequent cloning from pituitaries of several species, including humans, made it clear that it is a highly conserved protein (43, 44). In humans, the gene for the pituitary GnRH-R is located on chromosome 4 (4q13); it is composed of three exons and two introns and codes for a 328-amino-acid protein (12, 45, 46) (Table 1). This receptor belongs to the family of rhodopsin-like G protein-coupled receptor (GPCR) proteins containing seven transmembrane domains and an extracellular, 35-amino-acid amino terminus with two putative glycosylation sites (10, 37, 38, 47–49). The most striking cellular, 35-amino-acid amino terminus with two putative introns and codes for a 328-amino-acid protein (12, 45, 46) (Table 1). This receptor belongs to the family of rhodopsin-like G protein-coupled receptor (GPCR) proteins containing seven transmembrane domains and an extracellular, 35-amino-acid amino terminus with two putative glycosylation sites (10, 37, 38, 47–49). The most striking feature of the human GnRH-R protein is the presence of a uniquely short (1–2 amino acids) carboxyl-terminal cytoplasmic tail, a region that has been implicated in the coupling of GPCR receptors to the G proteins and to be important for receptor internalization and desensitization (46, 50).

In the anterior pituitary, the GnRH-R is coupled to a Goq11 protein to activate phospholipase C, which transmits downstream signaling to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). In turn, IP3 stimulates release of intracellular Ca2⁺ and DAG activates the intracellular protein kinase C (PKC) pathway. Ca2⁺ levels in gonadotropes increase as a result of this initial mobilization from IP3-sensitive intracellular stores followed by an influx of extracellular Ca2⁺ via nifedipine-sensitive L-type voltage-sensitive Ca2⁺ channels (10, 51–56)

Phospholipase A2 and phospholipase D are also sequentially activated by GnRH (51, 54). Phospholipase A2 provides long-chain unsaturated fatty acids, such as arachidonic acid, which has been shown to activate PKC. Moreover, arachidonic acid can be converted into downstream lipoxygenase products that have been implicated in gonadotropin synthesis and release. Phospholipase D acts on the membrane phospholipid phosphatidylcholine, converting it into phosphatidic acid, which can further metabolized into DAG, thus prolonging PKC activation (57, 58).

PKC represents the major mediator of the downstream activation of the MAPK cascades. GnRH-R-coupled intracellular signaling has been found to activate all four MAPK cascades: ERK1/2, c-Jun N-terminal kinase (JNK), p38MAPK, and big MAPK1 (also known as ERK5) (10, 51, 55, 56, 59). Both ERK1/2 and JNK have been widely shown to be activated by GnRH, whereas activation of the other two kinases is still less clearly characterized. ERK1/2 activity is stimulated through the Raf1 kinase signal, whereas the Src-CDC42/Rac1 pathway is specifically required for JNK activation. Once activated, MAPK provide the crucial link for the transmission of signals from the cell surface to the nucleus, leading to transcription factor (Elk1, c-Jun, and activating transcription factor 2) phosphorylation and ultimately triggering gonadotropin synthesis and release (10, 55, 56, 60–62). Together with PKC and MAPK, also Ca2⁺ levels are deeply involved in these processes. Specifically, ERK activation seems to be mediated by Ca2⁺ influx, whereas JNK activation is likely triggered by Ca2⁺ mobilization from intracellular stores (63, 64).

III. Pituitary GnRH-R as a Molecular Target in Endocrine-Related Cancers

The elucidation of the key functions played by gonadotrope GnRH-R in the control of the pituitary-gonadal axis underlined their crucial role as molecular targets for the treatment of reproduction-related diseases. Based on this observation, as well as on the short half-life of the native GnRH, several synthetic GnRH analogs that bind to these receptors, and display either agonistic or antagonistic activity, have been developed. As a general rule, GnRH and GnRH analogs can be used for two opposing clinical goals: 1) to restore fertility in GnRH-deficient subjects and 2) to suppress the pituitary-gonadal axis in the clinical conditions in which gonadotropin secretion and steroid activity need to be blocked.

Pulsatile release of GnRH from the hypothalamus is crucial for gonadotrope activation; in the same way, GnRH agonists need to be administered in a pulsatile way to mimic GnRH activity and maintain pituitary function. On the other hand, high doses and sustained administration of GnRH agonists, after an initial stimulation of gonadotropin and gonadal steroid release (the so-called flare phenomenon), suppress the activity of the pituitary-gonadal axis, through a desensitization of GnRH-R and a decrease in the number of GnRH-R in gonadotropic cells (4, 65–67). It is now well established that sustained activation of GPCR induces receptor desensitization and in...
ternalization. When chronically activated, the receptor represents a substrate for phosphorylation by both second messenger-dependent protein kinases and GPCR kinases that phosphorylate the receptor on its C-terminal tail, thus facilitating its interaction with arrestins (68, 69). This event prevents the receptor from activating its corresponding G protein, causing receptor desensitization and subsequent internalization; the internalized receptor is then targeted to lysosomes for degradation (i.e. reduced receptor number) (68). However, as mentioned above, it is now clear that the cytoplasmic C-terminal tail is absent in pituitary GnRH-R (46, 50), raising the question about the mechanisms underlying the desensitization of these receptors in humans. To this purpose, it is important to underline that the decrease in gonadotropin levels after sustained administration of GnRH agonists takes days or weeks to develop, whereas the process of desensitization in all the other GPCR occurs within seconds or minutes. Thus, alternative explanations have been proposed for this pituitary down-regulation, such as reduced GnRH-R expression, depletion of intracellular Ca2+ or gonadotropin stores, and changes in the activity of the different downstream effector proteins (70–72).

Pulsatile administration of GnRH or GnRH agonists is used to restore fertility in GnRH-deficient subjects or in patients with disorder of secretion of endogenous GnRH (hypogonadotrophic hypogonadism, Kallman syndrome, panhypopituitarism, etc.) (4, 73–75). On the other hand, sustained administration of GnRH agonists is widely and successfully used for the treatment of different hormone-related pathologies. For instance, GnRH agonists are employed to suppress the pathological activity of the pituitary-gonadal axis in central precocious puberty, endometriosis, adenomyosis, uterine leiomyomas, and polycystic ovarian disease. These compounds are also used for the suppression of a normal pituitary-gonadal function in situations in which circulating gonadotropins might interfere with a specific goal, such as in vitro fertilization/assisted reproductive technologies (76–80).

GnRH antagonists have a much simpler mechanism of action. They bind to pituitary GnRH-R and competitively block the binding and activation by the native peptide, thus suppressing the pituitary-gonadal axis. Therefore, these compounds avoid the initial flare phenomenon, typical of GnRH agonists, leading to an immediate therapeutic benefit (81–84). At present, GnRH antagonists are mainly used for the treatment of endometriosis, benign prostatic hyperplasia and in cases when in vitro fertilization is necessary (67, 85, 86). However, because each of these different therapeutic approaches needs a specific hormone level, it has been underlined that the doses of GnRH antagonist to be used must be carefully defined.

A. GnRH agonists

Synthetic GnRH agonists have been designed on the basis of the following criteria: 1) the half-life of native GnRH in the circulation is very short (2–5 min), 2) the degradation of GnRH occurs mainly at the Gly residue in position 6, 3) both the NH2 and the COOH termini of the decapeptide are essential for the binding to the receptor, and 4) the NH2-terminal domain also plays a crucial role in receptor activation (81, 91). Thus, superagonist derivatives of GnRH are produced by modifying the Gly6 and Gly10 amino acids in the native sequence of the peptide. Usually, Gly6 is replaced with bulky apolar side-chain amino acids [Leu, Trp, Ser(tBu), His(Blz)], whereas Pro9-Gly10-NH2 is modified as Pro-NHET or Pro-Azgly-NH2. To date, the GnRH agonists most used in clinics are leuproline, [d-Leu6, Pro9-NHET] GnRH; goserepin, [d-Ser(tBu)6, Azgly10] GnRH; nafarelin, [d-Nal(2)] GnRH;...
triptorelin, [D-Trp⁶] GnRH; and buserelin, [D-Ser(tBu)⁶, Pro⁹-NHEt] GnRH (Table 2). These compounds have been widely proven to be more potent than GnRH itself (25, 65, 66, 81, 82). GnRH agonists are predominantly administered through sc injections of slow release (1–12 months) depot preparations (92–95).

Prostate cancer is the most commonly diagnosed cancer for men and remains a leading cause of death in most Western countries (96). Most prostate cancers are dependent on the presence of androgens for growth and survival; therefore, androgen ablation therapy, aimed at blocking androgen release/activity, represents the most effective initial treatment (97). Therapy with GnRH agonists still represents the preferred pharmacological treatment for advanced, metastatic prostate cancer, leading in about 14–28 d to a complete suppression of serum testosterone levels. Antiandrogens may be administered before or during early therapy with GnRH agonists to prevent the undesired flare event but also to achieve complete androgen blockade by antagonizing the activity of androgens because they are produced not only by the testes but also by the adrenal gland and by prostate cancer cells (17, 98–100).

Breast cancer remains one of the first leading causes of death in women, and currently endocrine treatment is of major therapeutic value in patients with estrogen-receptor-positive tumors. Selective estrogen-receptor modulators such as tamoxifen, raloxifene, and toremifene and aromatase inhibitors such as anastrozole represent the drugs of choice to counteract the growth of the tumor as well as to reduce the risk of recurrence (101–103). GnRH agonists have been used either alone or in combination with tamoxifen or with aromatase inhibitors for the treatment of estrogen receptor-positive, pre- and perimenopausal, breast cancers (104–106). The combined treatments were more effective than the treatments with each drug given alone (107, 108).

GnRH agonists have been proposed also for the treatment of gynecological cancers, such as ovarian and endometrial cancer. Ovarian cancer, which usually occurs in postmenopausal women, poorly responds to chemotherapy (paclitaxel, cisplatin, carboplatin, and doxorubicin). However, it is sensitive to gonadotropins, and for this reason, GnRH agonists have been investigated as a possible alternative treatment. Several studies have been performed to study the effects of GnRH agonists in patients (mostly platinum-resistant). However, the number of patients that has achieved an objective remission or a disease stabilization was relatively low (109, 110). The possible effect of GnRH agonists as a standard therapy for gynecological cancers needs to be further investigated.

### B. GnRH antagonists

The undesired side effect of the flare phenomenon associated with acute administration of GnRH agonists emphasized the search for novel analogs with the aim to suppress gonadal steroid release without the initial stimulation of pituitary gonadotropes. Thus, pure synthetic GnRH antagonists have been developed that bind to pituitary GnRH-R with high affinity and competitively block the receptor and its activation by the native peptide (25, 35, 66, 82, 83, 86) (Table 2). These analogs cause a rapid and sustained inhibition of gonadotropin and steroid release. First- and second-generation antagonists, however, were found to be not suitable for clinical use because of solubility limitations and anaphylactic reactions caused by histamine release (65, 83, 111); thus, third- and fourth-generation GnRH antagonists have been developed.

### TABLE 2. Chemical structure of GnRH agonists and peptidic antagonists

<table>
<thead>
<tr>
<th>Structure</th>
<th>Agonists</th>
<th>Peptidic antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>GnRH</td>
<td>pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂</td>
<td></td>
</tr>
<tr>
<td>Agonists</td>
<td>Buserelin</td>
<td>pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-o-Ser(tBu)⁶-Leu⁷-Arg⁸-Pro⁹-NHC₂H₅</td>
</tr>
<tr>
<td>Goserelin</td>
<td>pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-o-Ser(tBu)⁶-Leu⁷-Arg⁸-Pro⁹-AsGly¹⁰-NH₂</td>
<td></td>
</tr>
<tr>
<td>Nafarelin</td>
<td>pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-o-Leu⁶-Leu⁷-Arg⁸-Pro⁹-NHC₂H₅</td>
<td></td>
</tr>
<tr>
<td>Triptorelin</td>
<td>pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-o-Nal (2)⁶-Leu⁷-Arg⁸-Pro⁹-NHC₂H₅</td>
<td></td>
</tr>
<tr>
<td>Peptidic antagonists</td>
<td>Abarelix</td>
<td>Ac-o-Ala¹-o-Cpa²-o-Ala³-Ser⁴-Tyr⁵-o-AsGly⁶-Leu⁷-lys⁸-Pro⁹-o-Ala¹⁰-NH₂</td>
</tr>
<tr>
<td>Acyline</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-Aph(Ac)⁵-o-Aph(AC)⁶-Leu⁷-lys⁸-Pro⁹-o-Ala¹⁰-NH₂</td>
<td></td>
</tr>
<tr>
<td>Antarelix</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-Tyr⁵-o-Hc⁶-Leu⁷-lys⁸-Pro⁹-o-Ala¹⁰-NH₂</td>
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</tr>
<tr>
<td>Antide</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-Lys(Nic)³-o-Lys(Nic)⁴-Leu⁷-lys⁸-Pro⁹-o-Ala¹⁰-NH₂</td>
<td></td>
</tr>
<tr>
<td>Azaline B</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-Aph (Atz)⁵-o-Aph(Atz)⁶-Leu⁷-lys⁸-Pro⁹-o-Ala¹⁰-NH₂</td>
<td></td>
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<tr>
<td>Cetrorelix</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-Cit⁶-Leu⁷-Arg³-Pro⁹-o-Ala¹⁰-NH₂</td>
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</tr>
<tr>
<td>Degarelix</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-Aph(5-hydroorotyl)⁵-o-Aph(carbamoyl)⁶-Leu⁷-lys⁸-Pro⁹-o-Ala¹⁰-NH₂</td>
<td></td>
</tr>
<tr>
<td>Ganirelix</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-Tyr⁵-o-IArg (Et)³-o-Leu⁷-IArg (Et)³-Pro⁹-o-Ala¹⁰-NH₂</td>
<td></td>
</tr>
<tr>
<td>Ozarelix</td>
<td>Ac-o-Nal¹-o-Cpa²-o-Pal³-Ser⁴-N-MeTyr⁵-o-hCit⁶-Nle⁷-Arg³-Pro⁹-o-Ala¹⁰-NH₂</td>
<td></td>
</tr>
</tbody>
</table>
developed subsequently. These compounds contain Ac-D-Nal-D-Cpa-D-Pal in the N-terminal part of the peptide and D-Ala in position 10; moreover, they present different amino acid derivatives in positions 5, 6, and 8 (35, 83, 112–114) (Table 2).

Some of these antagonists are already commercially available (cetrorelix, ganirelix, abarelix, and degarelix), whereas others are still in preclinical or clinical trials (antarelix, antide, azaline B, acyline, and ozarelix). As mentioned, GnRH antagonists have various clinical applications in gynecology and reproductive medicine; cetrotelix and ganirelix have been proved to be particularly efficient in the inhibition of premature LH surge in assisted reproduction technology (86, 115–118).

Oncology, more specifically prostate cancer therapy, is a field that stands to benefit greatly from the clinical application of GnRH antagonists (18, 35, 119–123). The administration of these compounds to patients results in a rapid decrease of LH, testosterone, dihydrotestosterone, and prostate-specific antigen (PSA), in the absence of the flare phenomenon. The rate of PSA fall after GnRH antagonist administration has been reported to be comparable to that obtained after treatment with GnRH agonists in combination with antiandrogens. Thus, GnRH antagonists might avoid the use of antiandrogens that have their own side effects. Moreover, it is known that the flare phenomenon can precipitate such clinical symptoms as spinal cord compression, which can often lead to paralysis. Being devoid of flare, GnRH antagonists clearly avoid these medical emergencies. However, it must be underlined that due to solubility problems, antagonists must be administered more frequently, and this represents a major inconvenience to patients.

The first GnRH antagonist to be approved by the U.S. Food and Drug Administration was abarelix, in 2004. Abarelix can be administered either sc or by depot formulation, which provides an increased bioavailability of the drug and a significantly higher inhibition of testosterone release and PSA levels. In a phase II open label study, Tomera et al. (124) reported that in prostate cancer patients who had increasing PSA levels after definitive local therapy or who had intermittent hormonal therapy for more than 6 months before starting abarelix treatment (by means of depot formulations) resulted in castration levels of testosterone in 34.5 and 82.3%, respectively, at 2 and 13 of treatment. Conversely, in the same study, castration levels could not be achieved in patients treated with the GnRH agonist leuprolide. Later studies further confirmed these first clinical observations (125–127). With respect to GnRH agonists, the advantage of abarelix treatment is represented by the absence of the flare phenomenon, a rapid reduction of testosterone and PSA levels, the avoidance of the necessity of combination therapy with antiandrogens and a safety profile generally comparable to that of leuprolide (either alone or with the antiandrogen bicalutamide) (126, 127). However, allergic reactions, accompanied by symptoms of upper respiratory tract disorders, fever, and hot flushes represented the major concern with the new compound (128, 129). Due to these side effects, abarelix was withdrawn from the U.S. market in 2005 (119); it is still commercially available in Europe.

Degarelix is another third-generation GnRH antagonist that offers to clinicians the means to reduce circulating testosterone levels, thereby antagonizing the growth of prostate cancer (130–132). Degarelix was developed by Jiang and co-workers (133) with the aim to obtain potent GnRH antagonists characterized by low histamine-release properties, by long-lasting biological activity, and by high solubility. Based on its chemical structure, and its ability to form hydrogen bonds, it was hypothesized that it might bind to pituitary GnRH-R with a particularly high stability. However, binding assays demonstrated that the affinity of this compound for GnRH-R was comparable to that of other, previously synthesized, GnRH antagonists, indicating that its binding affinity was not responsible for its duration of action (133, 134). Pharmacokinetic studies suggested that after sc administration, degarelix forms a gel depot, from which the compound is then distributed to the body. In a phase II clinical trial, degarelix was reported to cause a significant and sustained suppression of testosterone release and PSA levels, without any symptoms of allergic reactions (135). This study demonstrated that degarelix should be administered at an initial dose of 240 mg with a maintenance dose of 80 and 160 mg every 4 wk to cause maximal testosterone, LH, and PSA suppression. In a phase III trial, the efficacy of degarelix was compared with that of the GnRH agonist leuprolide in patients with histologically confirmed prostate cancer. The antagonist was found to be as effective as leuprolide in suppressing testosterone and PSA levels, with the advantage of being much faster than the agonist in expressing its activity (130–132). Degarelix was well tolerated; the most common side effects were related to androgen deprivation (i.e. hot flashes, weight increase, etc.) and mild/moderate injection-site reactions. Thus, these clinical observations strongly support the suggestion that degarelix might make an important contribution to prostate cancer treatment.

Ozarelix, a fourth-generation peptidic GnRH antagonist, is currently under investigation; however, only in vitro studies on prostate cancer cells have been performed (136).

The use of peptidic agonists and antagonists of the GnRH-R clinically exploited so far is limited by the necessity of their predominantly parenteral administration, due to their poor oral bioavailability. Recently, several
small organic molecules have been discovered offering the prospect of being orally active GnRH-R antagonistic agents (137). These compounds (furamides, thienopyrimidinediones, thienopyridinones, quinolinones, indoles, uracils, benzimidazoles, and piperazynilbenzimidazoles) were reported to block the GnRH-R in the nanomolar and micromolar range (35, 137–139). The observation that molecules with different structures bind to the same receptor suggest that they may recognize different subregions of the receptor protein (35).

One of these drugs, elagolix (sodium R-(-)4-[(2-[5-(2-fluoro-3-methoxyphenyl)-3-(2-fluoro-6-[trifluoromethyl]benzyl)-4-methyl-2,6-dioxo-2H-pyrimidin-1-yl]-1-phenylethylamino)butyrate, is currently in phase II clinical trial in women with endometriosis (140, 141) (Fig. 1). CMPD1 (AG-045572; 5-([3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthalenyl)methyl]-N-(2,4,6-trimethoxyphenyl)-2-furamide) has been investigated for the treatment of sex hormone-related diseases (Fig. 1). This compound has been shown to bind with high affinity to rat, mouse, and human pituitary GnRH-R. In experimental models, administration of this antagonist, both iv and orally, dose-dependently suppresses testosterone levels and counteracts GnRH agonist-stimulated gonadotropin and testosterone release, suggesting its possible utility for the treatment of androgen-dependent tumors (142, 143). For all these nonpeptidic GnRH antagonists, additional studies are obviously required before affirming their clinical utility.

IV. GnRH-R in Tumors

It is now well established that GnRH-R are expressed in a variety of human reproductive tissues, such as ovary, endometrium, placenta, breast, and prostate (22, 24–26, 55).

In the ovary, GnRH-R are expressed in granulosa-luteal cells, and their level of expression varies according to the stage of follicular development, suggesting that sex hormones may influence its biology and/or it may play a role in the control of ovulation and luteolysis (144, 145). In both endometrium and placenta, activation of locally expressed GnRH-R increases the activity of the urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMP) systems, supporting a crucial role of these receptors in the process of egg implantation (146–148).

Moreover, the levels of expression of these receptors have been reported to change at different weeks of gestation, indicating that their expression may be a function of pregnancy stage (149). The data available on the presence of GnRH-R in normal human breast and prostate cells are still limited. However, the presence of mRNA for these receptors has been described in prostate biopsies, with lower levels in normal prostate than in prostate cancer specimens (150).

In addition to their presence in peripheral normal tissues, it is now well established that GnRH-R are also expressed in a variety of cancers (22, 25, 55, 82, 151).

A. Expression of GnRH-R

Soon after their discovery in human tumor tissues and cell lines, GnRH-R have been characterized in terms of binding affinity for GnRH analogs, and divergent results were reported. In Limonta’s laboratory (152, 153), only one class of low-affinity binding sites could be detected in prostate cancer cells, either androgen dependent or androgen independent. Two types of receptors, one with high affinity/low capacity and another with low affinity/high capacity have been found in breast and other gynecological cancers by some authors (14, 154), whereas other described the presence of one single class of high-affinity receptors in breast, ovarian, and endometrial tumors (155–158).

These initial contrasting observations stimulated the analysis of tumor GnRH-R at the molecular level. Thus, it is now well accepted that extrapituitary GnRH-R share...
the same cDNA nucleotide sequence and encode mRNA and proteins of the same size as the pituitary receptor (31, 159, 160). In all the tumors expressing GnRH-R, also the GnRH decapeptide has been shown to be present, supporting the existence of an autocrine/paracrine GnRH/GnRH-R system that might be involved in the local regulation of tumor growth.

Interestingly, the presence of GnRH-R has been demonstrated also in melanoma (161), glioblastoma (162), lung (163), and pancreatic cancer (164, 165), indicating that this GnRH-based system might be active also in tumors that are not classically related to the reproductive system (Fig. 2).

**B. Antitumor activity (antiproliferative, antimetastatic, and antiangiogenic)**

The elucidation of the possible role of GnRH-R in tumor growth and progression has been carried out in several studies, performed on different types of tumor cells expressing the receptor. In most of these studies, GnRH-R activation was achieved by treating cancer cells with native GnRH or synthetic GnRH agonists (Table 3).

1. **Antiproliferative activity**

GnRH agonists have been shown by Limonta and her associates (152, 153, 166) to exert strong antiproliferative effects on human prostate cancer cells, either androgen dependent (LNCaP) or androgen independent (castration-resistant: DU145 and PC3) in vitro. Interestingly, the same compounds were able to counteract in vivo the growth of androgen-independent DU145 xenografts in nude mice, further supporting a direct, non-pituitary-mediated, antitumor activity (167). Similar observations were later reported in primary cell cultures from human prostate carcinoma (168).

GnRH analogs have been shown to inhibit the growth of human breast cancer cells, either estrogen dependent (MCF-7) or estrogen independent (MDA-MB-231) both in vitro and in vivo (19, 169, 170). Moreover, in breast cancer cells overexpressing high-affinity GnRH-R, the antiproliferative action of GnRH agonists on these cells was dramatically enhanced (171).

The antiproliferative activity of GnRH-R has also been investigated in other gynecological cancers, such as endometrial and ovarian cancers (20). Several studies demonstrated a growth-inhibitory activity of GnRH and its synthetic analogs in different GnRH-R-expressing ovarian cancer cell lines (27, 33, 156, 172, 173). However, contrasting results have also been reported. GnRH agonists have been shown to inhibit cell proliferation at extremely high concentrations (in the micromolar range) while exerting a significant growth stimulation at low doses (in the nanomolar range) (174–176). These discrepancies might be explained by differences in the experimental conditions adopted (cell lines, cell culture conditions, types of GnRH analogs, etc).

So far, a strong antitumor activity has been consistently reported also for GnRH-R in tumors classically unrelated to the reproductive system. In Limonta’s laboratory, it has been shown that GnRH agonists exert a strong antiproliferative effect on human melanoma (25) and glioblastoma (162) cells. Similar results have been reported for pancreatic (177) and lung (163) cancer.

In addition to reduced cell proliferation and cell cycle arrest, apoptosis has been suggested to be involved in the antitumor activity of GnRH-R; however, the data so far available on this issue are still controversial.
In prostate cancer cells overexpressing GnRH-R, GnRH analogs induce an accumulation of the cells in the G2 phase of the cell cycle without triggering apoptosis (178). In these cells, activation of GnRH-R affected the expression of apoptosis-related genes, without induction of programmed cell death (179). In nude mice bearing castration-resistant prostate cancer xenografts (DU145), GnRH agonist treatment significantly reduced tumor growth without altering the apoptotic index and the immunohistochemical staining of p53 (167). In contrast, GnRH analogs were described to induce apoptosis both in prostate cancer cells (180) and in primary cultures of human prostate cancer cells (168, 181).

In ovarian cancer cells, apoptosis was induced only when GnRH analogs were used at high doses or for a prolonged period. High doses or prolonged treatments with a GnRH agonist have been reported to induce TNF-α secretion and DNA fragmentation (172, 182). On the other hand, although the Fas/Fas ligand (FasL) system was shown to be expressed in ovarian carcinomas and ovarian cancer cell lines, and GnRH agonists were reported to increase the expression of FasL, a direct link between GnRH analogs and the Fas/FasL system has not been definitely demonstrated (183, 184).

### 2. Antimetastatic activity

Dissemination of tumor cells throughout the body (metastasis) is a complex phenomenon, involving decreased cell adhesion and increased cell motility. In the authors’ laboratory, the GnRH agonist leuprolide was reported to reduce the migration of castration-resistant prostate cancer cells by interfering with the effects of IGF-I on cell motility and by modifying actin cytoskeleton organization and αvβ3 integrin expression/cellular localization (185). In agreement with these observations, GnRH analogs were shown to decrease uPA activity in the cytosolic fractions from Dunning R3327H rat prostate tumors, suggesting the achievement of a reduced invasiveness of the tumor (186). Moreover, high doses of these compounds attenuated the invasiveness of prostate cancer cells (either androgen dependent or androgen independent) by affecting cell-cell adhesion molecules and extracellular matrix.

<table>
<thead>
<tr>
<th>GnRH analogs</th>
<th>Target cancer cells</th>
<th>Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GnRH agonists</strong></td>
<td>Prostate cancer cells and xenografts</td>
<td>Decreased proliferation</td>
<td>152, 153, 156</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell cycle arrest</td>
<td>178, 179</td>
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<td>Apoptosis</td>
<td>168, 180, 181</td>
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<td>Decreased tumor growth without apoptosis</td>
<td>167</td>
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<tr>
<td></td>
<td>Breast cancer cells and xenografts</td>
<td>Decreased migration and invasiveness</td>
<td>185–190</td>
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<td></td>
<td>Decreased proliferation and tumor growth</td>
<td>19, 169, 170, 191</td>
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<tr>
<td><strong>GnRH antagonists</strong></td>
<td>Prostate cancer cells and xenografts</td>
<td>Decreased proliferation and tumor growth</td>
<td>168, 187, 220–222</td>
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<td>Cell cycle arrest</td>
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<tr>
<td><strong>GnRH-II peptide and agonists</strong></td>
<td>Endometrial and ovarian cancer cells</td>
<td>Decreased metastasis formation</td>
<td>191</td>
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<tr>
<td><strong>GnRH-II antagonists</strong></td>
<td>Prostate, breast, endometrial and ovarian cancer cells</td>
<td>Decreased proliferation</td>
<td>29, 40, 219, 285, 286, 289</td>
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<td>Apoptosis</td>
<td>204</td>
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<td>Apoptosis</td>
<td>288</td>
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<td>Autophagy</td>
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**TABLE 3. Effects of GnRH-R activation on tumor growth and progression**

<table>
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</table>
degrading enzymes (187–189). GnRH has been shown to exert its antimitoty effects by interacting with proteins classically involved in the regulation of actin polymerization (small GTPases such as Rac1, Cdc42, and RhoA) (190). Recently, it has been shown that bone-directed invasion of human breast cancer cells in vitro is time- and dose-dependently reduced by GnRH analogs. Moreover, these drugs were reported to significantly reduce tumor growth and metastasis formation by triple-negative breast cancer xenografts in vivo (191).

In agreement with these observations, an antimetastatic activity of GnRH-R was shown also on cells derived from tumors classically unrelated to the reproductive system, such as melanoma. In particular, it has been described that the GnRH agonist goserelin significantly reduces the migratory and the invasive behaviors of melanoma cells by affecting integrin (αv) and MMP-2 expression/activity (192).

In contrast with all these findings, a proinvasive activity has been proposed for GnRH analogs [D-Ala6] GnRH in ovarian cancer cells (193). The reasons for this discrepancy are still unknown; however, it might be related to differences in the experimental conditions adopted (mainly, GnRH analogs used, doses of GnRH analogs, and duration of treatments).

3. Antiangiogenic activity

Angiogenesis, the formation of new capillaries from preexisting blood vessels, is well known to be involved in both physiological and pathological conditions. The process of angiogenesis relies on a complex series of orchestrated events with the activation of endothelial and perivascular cells (pericytes and smooth muscle cells) and the modification of the surrounding basement membrane and extracellular matrix. The formation of new vessels is initiated by proangiogenic factors, produced by both tumor and host cells, including vascular endothelial growth factor (VEGF), platelet-derived growth factor, and fibroblast growth factor. These growth factors act by binding to their cognate tyrosine kinase receptors activating intracellular signal transduction pathways that lead to increased endothelial and stromal cell survival, proliferation, invasion, and migration.

The possible involvement of extrapituitary GnRH-R in the process of angiogenesis has been first investigated in ovarian neovascularization, a physiological condition that is necessary for follicular and luteal function. It has been shown that activation of these receptors reduces neovascularization by decreasing the expression of VEGF and angiopoietin-1 and of their receptors (194).

However, angiogenesis is also well known to play a key role in pathological conditions, such as tumor growth and metastasis; the tumor vasculature provides the necessary supply of oxygen and nutrients and allows the dissemination of metastatic tumor cells to distant organs. For this reason, the acquisition of angiogenic ability by cancer cells (termed angiogenic switch), together with invasion and metastasis, is considered one of the six hallmarks of tumor progression and an important target for cancer therapy (195, 196). Thus, the identification of novel molecular pathways involved in the angiogenic process is urgently needed to increase the therapeutic strategies for highly aggressive tumors. Based on these observations, the authors' laboratory has investigated whether locally expressed GnRH-R might exert their antitumor activity not only by reducing cancer cell proliferation and metastasis but also by affecting the process of neoangiogenesis. It has been found that GnRH analogs significantly decrease the synthesis and secretion of VEGF from melanoma cells. Moreover, the ability of human umbilical vein endothelial cells (HUVEC) to move toward the conditioned media of melanoma cells was significantly reduced when these cells were pretreated with GnRH analogs. This indicates that activation of GnRH-R reduces the capacity of melanoma cells to attract endothelial cells, thus possibly interfering with the process of angiogenesis. Unexpectedly, but very interestingly, it has been found that GnRH-R are expressed also in HUVEC and that their activation reduces VEGF-induced cell proliferation and ability to form a capillary-like structure. From these data it is concluded that GnRH analogs may exert a significant antiangiogenic activity, both indirectly by reducing VEGF secretion from tumor cells and directly by interfering with the proangiogenic activity of the growth factor on endothelial cells (197) (Fig. 3).

In partial agreement with these results, GnRH agonists have been shown to reduce the expression of VEGF in human uterine myomas (198). Moreover, VEGF immunostaining was found to be decreased in prostate cancer tissues of patients undergoing complete androgen receptor blockade therapy (bicalutamide plus goserelin) (199).

Taken together, these observations indicate that locally expressed GnRH-R are endowed with a specific antitumor activity (antiproliferative/antimetastatic/antiangiogenic); this supports their role as an effective molecular target for newly developed, GnRH analog-based, therapeutic interventions in tumors (Fig. 4).

However, as pointed out, these conclusions are mainly based on data from in vitro and from in vivo (preclinical) studies. Clinical trials supporting the direct action of GnRH analogs on tumors that have escaped hormone dependence (such as castration-resistant prostate cancer) or that are hormone unrelated (such as melanoma, glioblastoma, pancreatic and lung cancer, etc.) are still lacking.
C. Molecular mechanisms involved in the antitumor activity

It is now well established that GnRH-R may be coupled to different G proteins according to the cell context. The various intracellular downstream signaling pathways are then responsible for the specific biological outcome of receptor activation in the different tissues. At the pituitary level, the $G_{\alpha_\text{q}}$ protein is the major mediator of the stimulatory effects of GnRH on gonadotropin synthesis and secretion. In cancer cells, it has been shown that GnRH analogs substantially antagonize the pertussis toxin-induced ADP ribosylation of the $G_{\alpha_i}$ protein; these compounds also counteract the forskolin-induced cAMP accumulation (20, 31, 200, 201). These findings strongly support the concept that $G_{\alpha_i}$ is the major G protein mediating the antiproliferative/antimetastatic activity of GnRH-R in cancer cells (Fig. 5).

Coupling of GnRH-R to $G_{\alpha_i}$ is then followed by the activation of different intracellular signaling cascades, such as MAPK (p38MAPK, ERK1/2, and JNK), phosphatidylinositol-3-kinase (PI3K), and phosphotyrosine phosphatase.

The stress-activated protein kinase p38MAPK mediates the apoptotic effect of GnRH analogs in benign prostate hyperplastic BPH-1 cells (202). In ovarian Caov-3 cancer cells, GnRH agonists have been reported to stimulate ERK1/2 through Shc and Sos, and this activation is crucial for inhibition of cell proliferation (203); similarly, both ERK1/2 and p38MAPK were shown to mediate the antitumor effects in another ovarian cancer cell line (OVCAR-3) (204, 205). The observation that ERK might mediate both the mitogenic effects induced by growth factors and the antiproliferative activities of GnRH analogs appears intriguing. However, it has been hypothesized that the role played by ERK in determining the cell fate (life/death) may largely depend on the conditions of kinase activation (short vs. prolonged activation) (206).

Another MAPK member, JNK, has been reported to be involved in the antitumor effects of GnRH analogs. In castration-resistant prostate cancer cells, JNK activation was found to mediate GnRH analog-induced apoptosis; this effect was shown to be mediated, at least in part, by inhibition of the upstream activator of JNK, mixed lineage kinase 3 (180, 207). In ovarian and endometrial cancer cells, the GnRH agonist triptorelin was reported to increase JNK activity, leading to c-Jun binding to DNA and, ultimately, to an antiproliferative effect (30, 208). However, in contrast with these observations, JNK was shown to mediate the GnRH-stimulated invasive behavior of ovarian cancer cells by increasing the expression of MMP-2 and MMP-9 (193). Thus, the understanding of the specific roles played by each MAPK member in the antitumor activity of GnRH analogs in different cell contexts is still rather limited, and additional investigation is required.
The PI3K/Akt pathway is another very important regulator of cell growth/survival. Increased activation of Akt has been consistently reported in prostate cancer cell lines as well as in tissues from high-Gleason-grade prostate cancer (209–211). It has been shown that, in castration-resistant DU145 prostate cancer cells, activation of GnRH-R induces apoptosis (180) and reduces the migratory and invasive behavior (185) by inhibiting this survival pathway. In uterine leiomyomas, GnRH agonists cause a significant reduction in PI3K/Akt activity by inhibiting the expression of antiapoptotic proteins, thus inducing apoptosis (212). Accordingly, in ovarian cancer cells, GnRH agonists interfere with activation of the PI3K/Akt signaling to reduce cancer cell invasive behavior (213).

The question of whether PKC might be involved in the intracellular signaling coupled to GnRH-R in cancer cells has been addressed. Actually, GnRH analogs were reported to activate MAPK via a PKC-dependent mechanism in some tumor cell lines. In ovarian cancer cells, a PKC-activating phorbol ester (12-O-tetradecanoyl phorbol-13-acetate) stimulated ERK1/2 phosphorylation, mimicking the effects of GnRH, whereas a specific inhibitor of PKC counteracted the antiproliferative activity of the peptide (214). However, the data so far available on the possible direct involvement of PKC and the mechanisms of its activation (tumor-specific G αq -coupled GnRH-R?) are still limited, and this question is at present under investigation (215).

A crucial mechanism in the antiproliferative/antimetastatic activity of GnRH-R is represented by their cross talk with receptor tyrosine kinases at the membrane level of cancer cells. In prostate cancer cells, GnRH analogs have been consistently shown to reduce the expression/activity of epidermal growth factor (EGF) and IGF-I receptors (23, 189, 216, 217), thus silencing their specific intracellualr signaling pathways (MAPK and PI3K/Akt) (185, 217). Similar observations were reported in gynecological tumors, such as ovarian, endometrial, and breast cancer cells (201, 218, 219). These effects appear to be mediated by the activation of a phosphotyrosine phosphatase (28, 151, 201, 218) (Fig. 5).

V. Tumor GnRH-R as a Molecular Target for Novel Therapeutic Strategies in Cancers

The expression of GnRH-R endowed with antitumor activity in different types of cancers, together with their limited expression in normal tissues, strongly supports the concept that they might represent a good candidate for novel targeted therapeutic strategies. To this purpose, one must be cognizant that, in hormone-dependent cancers, GnRH-R do not appear to be down-regulated after prolonged treatments with GnRH agonists. So far, several GnRH derivatives have been developed aimed at targeting these receptors in different tumor cells, both related and unrelated to the reproductive system, with the aim to deliver anticancer drugs specifically at the tumor level (Fig. 6). Their anticancer efficacy is at present under investigation.

A. GnRH analogs (agonists vs. antagonists)

1. GnRH agonists

GnRH agonists represent the first-line therapy for hormone-dependent tumors, based on their ability to suppress the pituitary-gonadal axis. As described above, these compounds have been widely used to demonstrate the antitumor activity endowed with locally expressed GnRH-R in cancer cells. GnRH agonists such as leuprolide, triptorelin, buserelin, and goserelin were consistently shown to inhibit, both in vitro and in vivo in preclinical models, the proliferation and the metastatic behavior of cell lines derived from tumors of the reproductive tract, suggesting that, when used for the treatment of hormone-dependent tumors, these compounds might exert an additional and more direct antitumor activity (19–23, 25–27, 33). Unfortunately, it is not easy to verify in quantitative terms how much the direct effects of GnRH agonists on these tumors may contribute to their efficacy, which is obviously related mainly to the suppression of the pituitary-
The gonadal axis. On the other hand, GnRH agonists also exert a strong antitumor effect on cells derived either from steroid-dependent cancers that have escaped hormone dependence (such as castration-resistant prostate cancer) or from tumors that are classically unrelated to the hormonal system (melanoma, glioblastoma, and lung and pancreatic cancer), either in vitro or in preclinical models (22, 23, 25, 104, 162, 165) (Table 3). Thus, as a general concept, GnRH agonists might be considered as an effective therapeutic strategy for the treatment of all those tumors demonstrated to express GnRH-R.

2. GnRH antagonists

The search for GnRH antagonists was initially stimulated by the need to obtain compounds that might overcome the GnRH agonists-associated flare phenomenon. They competitively bind to pituitary GnRH-R without activating the intracellular signaling cascade. At the beginning, it was thought that these agents might behave as antagonists also at the level of tumor cells expressing the GnRH-R. Surprisingly, it was found that in positive GnRH-R cancer cells, most of the GnRH antagonists exert a time- and dose-dependent antitumor effect, indicating that they might behave as agonists on these cells (Table 3).

Cetrorelix, a third-generation antagonist, has been reported to inhibit the growth of androgen-independent prostate cancer cells, both in vitro and in vivo, by interfering with the EGF system (187, 220–222). This compound was also found to reduce the migratory and invasive behavior of DU145 prostate cancer cells by decreasing the secretion and the enzymatic activity of uPA, while increasing the levels of its inhibitor plasminogen activator inhibitor-1/2 (187). In line with these observations, Castellón and co-workers (168) demonstrated that cetrorelix inhibits the growth of primary cell cultures from human prostate carcinoma, and this effect is similar to that obtained with the GnRH agonist leuprolide. In the same experimental conditions, the antagonist triggers apoptosis by activating the caspase-8-mediated extrinsic pathway (181).

Cetrorelix was shown to inhibit the proliferation of human mammary estrogen-responsive tumor cells (MCF-7) in vitro (223), as well as the growth of MCF-7 xenografts in nude mice (224). More recently, GnRH-R were found to be expressed in triple-negative breast cancer cells; activation of these receptors by the GnRH antagonist resulted in a significant decrease of cell proliferation (225). In addition, the antagonist reduced metastasis formation by triple-negative MDA-MB-435 and MDA-MB-231 breast cancer cells in nude mice (191), confirming the antimetastatic activity associated with GnRH-R activation in tumors. Finally, in ovarian EFO-21 and in endometrial HEC-1A cancer cells, cetrorelix exerted a similar antiproliferative activity as the agonist triptorelin (156, 226). Accordingly, in the OV-1063 ovarian cancer cell line, the antiproliferative activity of cetrorelix in vitro even exceeded that of the agonist triptorelin (32).

Recently, the possible direct antitumor activity of oza-relix, a fourth-generation antagonist, has also been investigated in androgen-independent DU145 and PC3 prostate cancer cells. In this study, Festuccia and co-workers (136) demonstrated that the GnRH antagonist significantly reduced tumor cell growth, both by retarding cell cycle progression (by inducing accumulation of cells in G2/M phase of the cell cycle) and, at longer exposure to the drug, by triggering the extrinsic apoptotic pathway (through TNF-related apoptosis-inducing ligand and Fas activation).
The different biological effects and pharmacological properties of GnRH agonists and antagonists in different cellular environments (i.e., pituitary gonadotropes vs. cancer cells) is still an intriguing open question. As a possible explanation, the ligand-induced selective signaling theory has been proposed by Millar’s group (34, 36, 37, 202). According to this concept, the GnRH-R might assume different conformations in a cell-context-dependent manner. GnRH agonists and antagonists can make selective contacts with the GnRH-R, thus stabilizing different receptor conformations that activate different intracellular signaling pathways and, therefore, different biological activities.

The consistent experimental and preclinical data showing direct antitumor activity of both GnRH agonists and antagonists lead to the following considerations: 1) when used for the treatment of steroid-dependent malignancies, in addition to suppression of the pituitary axis, GnRH analogs might also exert a direct antitumor activity; 2) GnRH analogs might be considered for the treatment of tumors that have escaped hormone dependence (such as castration-resistant prostate cancer); and 3) the clinical use of GnRH analogs might be considered also for tumors that are not related to the reproductive tract but do express GnRH-R. In agreement with these considerations, it must be recalled that GnRH-R are expressed in most human prostate, breast, endometrial, and ovarian (117, 227, 228) cancer tissues. In prostate cancer patients, the expression of the GnRH-R was found to correlate with disease-specific survival (188) and to persist after prolonged GnRH agonist treatment, suggesting the potential therapeutic utility to target the receptor even when the tumor has become castration resistant (227). Similarly, the level of expression of GnRH-R was shown to be related to the grade of malignancy also in ovarian epithelial cancers (228). Finally, in a recent paper, Lawrentschuk and co-workers (229) reported a retrospective (ethics approved) review of the records of patients with prostate cancer who received a GnRH agonist (goserelin or leuprolide), experienced progression, and were rechallenged with the other analog (leuprolide or goserelin). They reported that PSA levels significantly decreased after switching from one agonist to the other. This decrease appeared particularly significant in patients switching from leuprolide to goserelin; the duration of the response after switching was approximately 5 months.

In conclusion, GnRH-R may be considered as an effective molecular target for the development of novel, GnRH analog-based (both agonists and antagonists) therapeutic strategies, for tumors shown to express these receptors.

B. GnRH analog-based cytotoxic hybrids

At present, cytotoxic hybrids represent one of the most promising targeted therapeutic strategy for tumors expressing the GnRH-R (Fig. 6). The first of these compounds were developed by Schally’s group about 25 yr ago. Their development was based on the rationale that the expression of GnRH-R in certain tumors is higher than in the corresponding non-tumoral tissues. Thus, linking a GnRH analog to a traditional cytotoxic compound would allow its specific targeting to GnRH-R-expressing cancer cells. The most used GnRH analog is [D-Lys^6]GnRH, which binds GnRH-R with high affinity. In the first series of hybrids, the GnRH agonist was linked to alkylating agents such as cisplatin and mephalane or to antimetabolites such as methotrexate (230, 231). More efficient conjugates were then developed in which [D-Lys^6]GnRH is linked to the cytotoxic drug doxorubicin (AN-152) or its analog 2-pyrrolino-doxorubicin (AN-207). In these hybrids, the cytotoxic agent connected to glutaric acid by means of an ester bond is conjugated to the e-amino group of [D-Lys^6]GnRH (232). It has been shown that, after specific binding of the GnRH agonists to its receptor, AN-152 is internalized, and after cleavage, doxorubicin accumulates into the nucleus to exert its cytotoxic activity
The receptor-mediated internalization was demonstrated to be specific because it was counteracted by the GnRH agonist triptorelin (234).

The strong antitumor activity of AN-152 has been shown in different experimental tumor models, such as breast, ovarian, endometrial, prostate, pancreatic, and colon cancer cell lines (233, 235–238). Interestingly, in cancer cells of the female reproductive system, the cytotoxicity of the hybrid was found to be independent of the multidrug resistance-1 system, suggesting that the conjugate may overcome the mechanisms of chemoresistance (239, 240).

Preclinical studies were also performed to confirm the efficacy of cytotoxic GnRH derivatives in vivo. Nude mice, xenografted with androgen-sensitive (LNCaP and MDA-PCa-2b) and insensitive (C4-2) prostate cancer cells, were treated iv with AN-152. Treatment resulted in a significant reduction of tumor growth (70–80%), decreased PSA serum levels, and increased apoptosis at the tumor level (241). Similar antitumor and proapoptotic effects were also reported in mice bearing human endometrial, ovarian, and breast (MX-1 and triple-negative MDA-MB-231, HCC1806, and HCC1937) cancer cells (242–244). These preclinical data, together with the low toxicity of the conjugated compound, strongly support the clinical utility of targeted chemotherapy, based on AN-152, as a novel treatment strategy for tumors expressing the GnRH-R. Thus, AN-152 recently entered phase I/phase II clinical trials in women with gynecological cancers and in men with castration-resistant prostate cancer. In patients with endometrial, breast, or ovarian tumors, a therapeutic effect of AN-152, including remission and prolonged intervals of stable disease, was observed in six patients (of 13) in the two highest doses groups of 160 and 267 mg/m²; the cytotoxic hybrid was found to be well tolerated, with only mild hematological and nonhematological side effects even at the highest dose (245). AN-152 is also being clinically investigated in men with castration-resistant prostate cancer. Patients enrolled in this study must have shown documented progression to at least one previous treatment with a GnRH agonist and one chemotherapy taxane-based regimen. Moreover, previous prostate cancer biopsies from these patients must have demonstrated positivity for GnRH-R expression. The primary outcome of this study was the clinical benefit of the treatment defined by nonprogression of the disease as well as by no dose-limiting toxicity or other toxicity elements requiring termination of the treatment. Secondary outcome measures included time to overall disease progression, PSA response, time to PSA progression, and overall survival. A possible side effect of this GnRH-R-targeted chemotherapy may be toxicity at the pituitary level. Actually, both LH and FSH (but not cortisol or TSH) levels were found to be decreased in women receiving AN-152 (245); however, preclinical studies reported that this decrease was transient in nature, and gonadotropin secretion was soon recovered after cessation of treatment (246). For castration-resistant prostate cancer patients, a novel cytotoxic hybrid is at present under evaluation: docetaxel-deslorelin. The rationale for the development of this conjugate is that docetaxel is, at present, the first-line chemotherapy treatment for this patient population (247).

Recently, cytotoxic GnRH derivatives have been developed, in which the GnRH agonist [D-Lys⁶] GnRH has been replaced by GnRH-III (pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH₂). This decapptide isoform specifically binds to GnRH-R in cancer cells, displaying a strong antiproliferative effect, whereas it is much less potent than GnRH agonists in reducing gonadotropin secretion (41, 42, 248). In this molecule, Lys⁶ is used as a conjugation site for the attachment of the cytotoxic drugs. A hybrid compound was obtained by conjugating daunorubicin to GnRH-III via an oxime bond; because the oxime bond is relatively stable under physiological conditions, a tetrapeptide (Gly-Phe-Leu-Gly) spacer, which is cleaved by lysosomal enzymes (such as cathepsin B, known to be generally overexpressed in tumor cells), was inserted between the cytotoxic drug and the decapptide (249, 250). The significant cytostatic effects of this conjugate on cancer cells were reported in both in vitro (249, 251) and in vivo (249, 250) experiments.

C. GnRH analog-based nutraceutic hybrids

The role of diet and nutrition in cancer prevention has recently become a very popular subject (252). Phytochemicals derived from fruits and vegetables, in particular, are very important micronutrients essential for preserving the balance between antioxidant and prooxidant reactions in tissues. Based on this activity, they have been widely studied for their possible efficacy in the prevention and treatment (inhibition of progression) of cancer, a disease whose development is strictly associated with oxidative stress conditions (253). More recently, phytochemicals have been consistently reported to exert their anticancer properties also by contrasting inflammatory processes and by interfering with several intracellular signaling pathways involved in cancer cell proliferation/motility/invasion (84, 254). Targeting phytochemicals directly and specifically at the level of tumor cells might help increase their anticancer properties (Fig. 6).

Curcumin (diferuloylmethane) is a dietary pigment in turmeric (Curcuma longa, mostly known as curry powder) and is widely used as a spice and coloring agent in food. Curcumin has been shown to exert potent antiprolifera-
tive and proapoptotic activity on different cancer cells, both in vitro and in preclinical cancer models (255–257). This agent has also been shown to reduce the motility/invasive behavior of several cancer cells (such as breast, prostate, melanoma, and glioma cells) through modulation of the expression/activity of molecules involved in the mechanisms of cell-matrix and cell-cell interaction (254, 258–260). Finally, in different types of cancer cell xenografts, dietary administration of curcumin significantly decreased the incidence of metastases at distant organs (254, 261, 262).

In a recent study, Aggarwal and co-workers (263) conjugated curcumin with the GnRH synthetic agonist [d-Lys6] LHRH and evaluated the effects of the conjugate on pancreatic cancer cells in vitro and in vivo (Fig. 6). These authors reported that the [d-Lys6] LHRH-curcumin conjugate significantly reduces the proliferation of pancreatic cells, while triggering caspase-3-dependent apoptosis. In nude mice bearing pancreatic cancer cell xenografts, iv infusion of the conjugate caused a significant reduction of tumor growth (263).

These promising results might open the way to the development of novel pharmaceutical formulations, based on GnRH agonists as carriers for the specific delivery of micronutrients, with established antioxidant/anticancer activity, at the level of tumor cells expressing GnRH-R.

D. GnRH-R-targeted nanoparticles delivering anticancer compounds

The conventional parenteral systemic administration of standard chemotherapy is based on the use of high doses of cytotoxic drugs, producing severe side effects on normal tissues. To overcome this serious problem, in the last few years, different types of nanocarrier-based delivery systems have been developed with the aim to carry and deliver anticancer drugs (chemotherapeutics, oligonucleotides, small interfering RNA, DNA, and proteins) directly at the level of tumor cells. These nanocarriers differ in terms of chemical structure and, therefore, in terms of phamacokinetics, organ distribution, and ability of cell penetration. They include linear and branched polymers, dendrimers, liposomes, nanoparticles, nanospheres, etc. First-generation nanocarriers were simply based on two components: the carrier and a cytostatic drug (264–267). More recently, nanocarriers have been developed in which the drug carrier is linked to a moiety that specifically recognizes molecular targets that are expressed on the membranes of cancer cells. Based on the observation that GnRH-R are overexpressed in most tumors (117, 227, 228), but not in healthy organs, efforts have been made to use GnRH analogs as a specific targeting agent (Fig. 6). Minko and co-workers (268–270) developed three types of tumor-targeted nanocarriers (linear polymers, branched star-like dendrimers, and liposomes) containing the anticancer drug paclitaxel (a taxane compound characterized by a low aqueous solubility); a synthetic GnRH analog was then attached to the nanocarriers to be used as the targeting moiety (Fig. 6). The three nanocarriers obtained were linear Tax-polyethylene glycol (PEG)-GnRH polymers, Tax-PAMAM (polyamidoamine)-GnRH dendrimers, and Tax-DSPE-PEG (1,2-distearoylethanolamine) amine-N-aminopolyethylene glycol-GnRH liposomes. In addition, similar nanoparticles were produced in which the cytotoxic drug was substituted by an imaging agent (the near-infrared cyanine Cy5.5 dye). The efficacy of these nanoparticles in terms of cellular uptake and internalization, cytotoxic activity, specific tumor and organ distribution, and anticancer activity was investigated both in vitro and in vivo experiments. These studies (270) were carried out on human small H69 and nonsmall A549 lung cancer cells. The authors reported that GnRH-based specific targeting significantly enhance intracellular uptake, apoptosis induction, and in vivo intratumoral accumulation (accompanied by limited side effects on healthy organs) as well as a clear anticancer activity in terms of tumor growth in nude mice. Based on these encouraging results, Minko’s laboratory constructed novel nanocarriers based on a multifunctional tumor-targeted polymer-peptide-drug delivery system [polymer-peptide-drug conjugate (PPDC)]. The development of this conjugate was based on the consideration that most tumors, in their later stages, escape the cytotoxic effects of chemotherapeutic agents by developing both intrinsic and acquired resistance to these drugs. A perturbation in the balance between antiapoptotic Bcl2 protein and proapoptotic proteins, such as Bax (with an increase in the Bcl2/Bax ratio), is critically involved in the acquisition of such resistance (271). Thus, the PPDC developed consisted of the PEG polymeric carrier conjugated to an anticancer drug (camptothecin), a GnRH derivative as the tumor targeting moiety, and a suppressor of cellular antiapoptotic defense (Bcl2 homology 3 domain, BH3, peptide). Based on its multifunctional composition, this delivery system was expected not only to specifically deliver a cytotoxic drug to cancer cells and to increase its antitumor activity but also to help cancer cells overcome the development of drug resistance. In human ovarian cancer cell lines, PPDC was found to be internalized and to trigger a strong apoptotic response; moreover, in nude mice xenografted with human ovarian cancer cells, multiple treatments with the delivery system led to almost complete regression of tumors and prevented growth of malignant ascites (272).
Magnetic nanoparticles have demonstrated strong promise toward the development of novel targeted agents for cancer treatment. Especially, FePt nanoparticles, once internalized, give rise to a controlled release of Fe, which catalyzes $\text{H}_2\text{O}_2$ decomposition into reactive oxygen species within cells, causing membrane lipid oxidation and cell death. Conjugation of FePt nanoparticles with a synthetic GnRH agonist significantly increases the cytotoxicity of intracellularly released Fe in human ovarian cancer cells (273).

Finally, methotrexate-human serum albumin-conjugated nanoparticles have been constructed to deliver the cytotoxic drug to tumor cells more efficiently than the free cytotoxic drug. Covalent attachment of a GnRH agonist on the surface of these nanoparticles significantly increases both the internalization of the cytotoxic drug and its antitumor activity in GnRH-R-expressing breast cancer cells (274).

Given the reported efficacy of these GnRH-driven delivery systems, research is now focusing on the development of novel tumor-targeted nanocarrier-based formulations, with improved properties that might provide specific advantages, like an easier administration. To this purpose, a tumor targeted mesoporous silica nanoparticle (MNS)-based drug delivery system was developed for inhalation treatment of lung cancer. MNS nanocarriers were loaded with an anticancer drug (doxorubicin and cisplatin) combined with two types of small interfering RNA designed to suppress pump and nonpump drug resistance; a synthetic GnRH analog was also conjugated on the surface of MSN. These systems were effective in inhibiting the proliferation of human lung adenocarcinoma cell lines, expressing the GnRH-R. In a mouse orthotopic model of human lung cancer, local delivery of these MSN nanocarriers by inhalation led to their preferential accumulation in the mouse lungs, prevented their escape into the general circulation, and limited their accumulation in other organs (163); however, the specific involvement of lung GnRH-R in these experiments still needs to be verified.

Taken together, these observations strongly support the concept that cancer GnRH-R represent an effective molecular target for the development of innovative tumor-targeted nanoparticles delivering anticancer drugs directly and specifically at the level of the tumor.

**VI. The GnRH-II System in Tumors**

As previously mentioned, a second form of GnRH (GnRH-II) has been identified in most vertebrates, including humans. This form was first isolated from chicken brain, and its structure is uniquely conserved from fish to mammals (24, 36, 275, 276). GnRH-II is a decapeptide, encoded by a gene organized in four exons and three introns, located on chromosome 20 in humans (275, 276); it is characterized by three amino acid differences from the classical GnRH: His$^5$, Trp$^7$, Tyr$^8$. It is widely distributed in the central nervous system, where it seems to act as a neuromodulator of sexual behavior but also in the peripheral nervous system and different peripheral tissues, including tissues of the reproductive system (277–281).

The existence of GnRH-II stimulated the search for a specific receptor (called type II GnRH-R). This receptor was cloned from human cDNA and nonhuman primates (282, 283). The receptor appeared to be a classical GPCR receptor, with a classical cytoplasmic tail containing several threonine and serine residues, potential phosphorylation sites that might be important for rapid desensitization and internalization (283). However, it is now clear that a full-length functional type II GnRH-R protein is not expressed in human tissues; the gene sequence of this receptor reveals a frameshift in coding exon 1 and a premature stop codon in the sequence coding its extracellular loop (exon 2) (24, 38, 281) (Table 1).

However, type II GnRH-R and GnRH-II transcripts have been shown to be expressed in human tumors as well as in cancer cell lines derived from these tumors (21, 24, 29, 36, 38, 40, 284). Based on these observations, it was suggested that a novel GnRH-II system, distinct from the classical GnRH system, might be expressed in tumors where it could play a specific role in the control of cancer cell proliferation. Actually, GnRH-II and GnRH-II analogs (both agonists and antagonists) have been consistently reported to inhibit the proliferation or to induce apoptosis of prostate, breast, endometrial, and ovarian cancer cells (29, 40, 204, 219, 285–289) (Table 3).

However, as pointed out above, no transcripts that could be translated into a full-length, functional receptor have been found in humans (24, 38, 281). According to these observations, it was initially speculated that the type II GnRH-R gene might give rise to partial transcripts that might be translated into peptide fragments; however, the possible functions of these receptor fragments have never been demonstrated. Thus, it was speculated that GnRH-II and GnRH-II analogs might act through the activation of the classical form of the GnRH-R. Limonta and co-workers (40) reported that, in androgen-independent PC3 prostate cancer cells, silencing of the classical GnRH-R (but not of type II GnRH-R) completely counteracts the antiproliferative effects of GnRH-II as well as that of the GnRH agonist goserelin. Similar observations were reported by Kim and co-workers in ovarian cancer cells (214), whereas contrasting results have been obtained by other authors. For instance, Enomoto and Park (290) re-
ported that, in androgen-independent DU145 prostate cancer cells, the type II GnRH-R mediates the antiproliferative action of GnRH-II through a direct interaction with the classical GnRH-R. Moreover, Gründker et al. (291) showed that, in endometrial and ovarian cancer cells, GnRH-II decreases cell proliferation, even after the knockout of the classical form of GnRH-R. The reasons for these discrepancies are still unclear (214). On the basis of these observations, it is possible to conclude that the functionality of the human type II GnRH-R in tumors and its involvement in transmitting the specific signals from GnRH-II still need to be elucidated.

However, despite this still open question, it remains clear that GnRH-II analogs, both agonists and antagonists, are endowed with a strong antiproliferative effect in tumor cells. These compounds may represent an effective therapeutic approach for the treatment of cancers shown to express the GnRH-R. In addition, in these types of tumors, they may be used as a vehicle to specifically target anticancer compounds to cancer cells.

**VII. Conclusions and Future Perspectives**

GnRH-R are expressed in many types of tumors: steroid-dependent tumors, steroid-dependent tumors that have escaped hormone dependence, and tumors classically considered to be hormone unrelated, such as melanoma, pancreatic cancer, and glioblastoma.

These receptors are activated by both GnRH agonists and antagonists, indicating that the biological activity and pharmacological properties of these compounds may depend on the specific cellular environments in which GnRH-R are expressed.

Consistent data from the literature indicate that activation of these receptors triggers a strong antitumor (antiproliferative/antimetastatic/antiangiogenic) effect. These observations, together with their limited expression in normal tissues, strongly support the concept that they might represent a good candidate for novel targeted therapeutic strategies.

Consistent experimental and preclinical data show a direct antitumor activity of both GnRH agonists and antagonists, suggesting they may have clinical relevance for the treatment of tumors that have escaped hormone dependence (such as castration-resistant prostate cancer) as well as of tumors that are not related to the reproductive tract, but that do express GnRH-R.

In the last few years, cancer cell-specific pharmaceutical formulations, based on GnRH analogs as the carrier of potent anticancer drugs (GnRH analog-based cytotoxic hybrids, GnRH analog-based nutraceutic hybrids, and GnRH-R-targeted nanoparticles, which specifically deliver cytotoxic compounds to cancer cells) have been developed.

The in vitro and in vivo preclinical studies performed so far look highly encouraging, consistently reporting that these compounds provide enhanced accumulation in tumor cells and an enhanced effectiveness in delivering the anticancer drug.

The doxorubicin-\([\text{-Lys}^6]\) GnRH conjugate (AN-152) recently entered clinical trials in women with gynecological cancers and in men with castration-resistant prostate cancer; encouraging results in terms of efficacy and safety have been reported.

Based on these preliminary results, additional clinical studies are urgently needed to define the effectiveness and lack of toxicity of these novel GnRH analog-based pharmacological formulations to improve the therapeutic options for tumors bearing GnRH-R for which the available treatments are still limited.

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