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REPRODUCTIVE FUNCTION AND ANTITUMOR ACTIVITY: DIFFERENT ROLES FOR THE HYPOTHALAMIC HORMONE GnRH

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SUNTO. – Il decapeptide GnRH (Gonadotropin-Releasing Hormone, ormone di rilascio delle gonadotropine), la cui sequenza aminoacidica è stata scoperta nel 1971 dal gruppo di ricerca del Dr. A.V. Schally, è stato inizialmente identificato come l'ormone ipotalamico che svolge un ruolo chiave nel controllo delle funzioni riproduttive. Questo ormone, infatti, legandosi a recettori specifici (GnRH-R) a livello ipofisarico, stimola la sintesi e la secrezione delle due gonadotropine (LH e FSH) e, di conseguenza, la produzione di steroidi a livello gonadico. Attualmente, questi recettori rappresentano il target molecolare dei trattamenti farmacologici standard per i tumori ormono-sensibili, quale il tumore prostatico ormono-dipendente. Infatti, nei pazienti affetti da questo tipo di tumore, la somministrazione cronica di agonisti del GnRH induce la desensitizzazione dei GnRH-R ipofisarici, e di conseguenza la soppressione della produzione degli androgeni testicolari. Il ruolo fisiologico del GnRH nel sistema riproduttivo, e la sua regolazione, hanno rappresentato uno dei temi principali di ricerca del professor Martini e dei Suoi collaboratori. A partire dagli anni '80/'90 è diventato sempre più chiaro che i GnRH-R sono espressi anche in differenti tipi di tumore, sia correlati che non correlati al sistema riproduttivo; questi recettori sono coinvolti nel controllo della crescita tumorale. In particolare, i recettori del GnRH sono espressi in cellule di tumore prostatico avanzato, detto anche resistente alla castrazione (per il quale le opzioni terapeutiche sono ancora limitate) e la loro attivazione mediante analoghi del GnRH è associata ad una significativa attività antipro-

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liferativa, antimetastatica e antiangiogenica. Questi effetti biologici opposti (stimolazione delle gonadotropine a livello dell'ipofisi ed azione antiproliferativa a livello tumorale) sono correlati alle specifiche vie di trasduzione del segnale associate a questi recettori nei diversi tessuti. Sulla base di queste osservazioni, i GnRH-R espressi sulle cellule tumorali vengono attualmente considerati nuovi target per terapie innovative ('targeted' therapies). Queste si basano sull'utilizzo di molecole complesse formate da un agonista del GnRH legato ad un agente chemioterapico standard. Il razionale di questo intervento terapeutico si basa sul fatto che l'agonista del GnRH va a legarsi in modo specifico ai suoi recettori presenti sulle cellule tumorali, trascinando con sè il chemioterapico. A livello tumorale il complesso viene internalizzato e successivamente degradato nei lisosomi; in questo modo l'agente chemioterapico viene direttamente indirizzato e rilasciato dentro le cellule tumorali così da sviluppare la sua azione proapoptotica in modo specifico in queste cellule, 'risparmiando' le cellule non tumorali. In conclusione, i recettori del GnRH sono espressi non solo sull'ipofisi ma anche in diversi tipi di tessuti tumorali. I recettori del GnRH a livello tumorale vengono attualmente considerati un efficace bersaglio molecolare per lo sviluppo di nuove strategie terapeutiche.

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ABSTRACT. – The decapeptide GnRH (Gonadotropin-Releasing Hormone), whose amino acidic sequence was discovered by Dr. A.V. Schally, was initially identified as the key hypothalamic hormone involved in the control of reproductive functions. GnRH, by binding to specific receptors (GnRH-R) at the pituitary level, stimulates the synthesis and secretion of the two gonadotropins (LH, luteinizing hormone and FSH, follicle stimulating hormone) and the downstream production of steroid hormones at the gonadal level. At present, these receptors represent the molecular targets of the standard pharmacological treatments for hormone-related tumors, such as androgen-dependent prostate cancer. Actually, chronic administration of synthetic GnRH agonists induces the desensitization of pituitary receptors and, subsequently, the suppression of testicular androgen production. The physiological role of GnRH in reproductive functions, and its regulation, represented a very important line of research for professor Martini and His colleagues. In the last three decades it has become increasingly clear that GnRH-R are expressed also in a wide range of tumors, both related and unrelated to the reproductive system; in particular GnRH-R are expressed in prostate cancers after development of resistance to androgen ablation therapy (castration resistant prostate cancer, CRPC), a tumor known to be refractory to standard chemotherapy. Activation of these receptors by means of GnRH agonists is associated with a significant antiproliferative/antimetastatic/antiangiogenic activity. These different biological effects at pituitary *vs.* prostate tissues are related to specific intracellular signal transduction pathways. Based on these observations, tumor GnRH-R are presently considered an effective molecular target for novel therapies ('targeted' therapies). In particular, GnRH-based bioconjugates, in which a standard cytotoxic drug is linked to a GnRH analog, have been developed. The rationale for this 'targeted' therapy is that the GnRH analog behaves as the targeting moiety by binding to GnRH-R in tumors, thus specifically delivering (targeting) the cytotoxic drug to tumor cells. At the level of tumor cells, the bioconjugate is internalized and degraded at the lysosomal level; in this way the anticancer drug is specifically released into the tumor cells to exert its cytotoxic effects, while sparing normal cells. In conclusion, GnRH-R are expressed not only at the pituitary level but also in a wide range of tumor tissues; these receptors are at present under investigation as an effective molecular target for the development of novel therapeutic strategies.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is the hypothalamic decapeptide whose structure (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was first discovered in 1971 by Dr. A.V. Schally's group [1]. GnRH is synthesized in a small number of hypothalamic neurones and secreted, in a pulsatile way, into the hypophyseal portal system through which it reaches the gonadotrope cells in the anterior pituitary. Here, by binding to specific receptors (GnRH-R), the decapeptide stimulates the synthesis and secretion of the two gonadotropins (LH, luteinizing hormone and FSH, follicle stimulating hormone), thus regulating gonadal steroidogenesis; GnRH pulsatility is mandatory for the stimulatory activity of the decapeptide. Based on its activity, GnRH is considered the key factor in the control of the reproductive functions [2].

During the last decades, it has become increasingly clear that GnRH and its receptors are expressed also in different tumors (including prostate cancer), both related and unrelated to the endocrine system; activation of these GnRH-R significantly reduces cancer cell proliferation and metastatic behavior indicating that, in cancer cells in which it is expressed, this GnRH-based system is associated with an antitumor activity [3-7].

In addition to this classical form of GnRH, other isoforms of the decapeptide have been later discovered: GnRH-II, present in most vertebrates including humans, whose functions are still unclear; GnRH-III, isolated from sea lamprey (*Petromyzon marinus*), shown to possess a significant anticancer activity through the binding to cancer GnRH-R but being less potent than GnRH in stimulating gonadotropin synthesis/secretion [8] (*Tab. 1*).

Taken together, these data strongly support that locally expressed GnRH receptors might represent an effective target for novel therapeutic approaches in tumors, including prostate cancer.

Tab. 1. Amino acid sequences of natural GnRH isoforms.

GnRH	Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH-II	Glp-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
GnRH-III	Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2

Glp, pyroglutamic acid.

2. Pituitary GnRH receptors

GnRH-R expressed at the pituitary level belong to the family of rhodopsin-like G protein-coupled receptors (GPCR); these membrane proteins contain seven transmembrane domains with an extracellular amino terminus (35 amino acids) and a uniquely short (1-2 amino acids) carboxyl-terminal cytoplasmic tail [9].

2.1 GnRH-R signaling pathways in pituitary cells

Pituitary GnRH-R are known to be coupled to a $G_{\alpha q/11}$ protein to activate phospholipase C, which leads to the formation of 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). In turn, IP3 stimulates release of intracellular Ca^{++} while DAG activates the intracellular protein kinase C (PKC) pathway. Ca^{++} levels in gonadotropes increase as a result of this initial mobilization from IP_3-sensitive intracellular stores and, later, by an influx of extracellular Ca^{++} [10,11]. PKC activates the downstream MAPK cascades, specifically ERK1/2 and JNK, which lead to the phosphorylation (*i.e.*, activation) of specific transcription factors (*i.e.*, c-Jun, ATF2 and Elk1) ultimately responsible for the stimulation of gonadotropin synthesis and release [10,11] (*Fig. 1*).

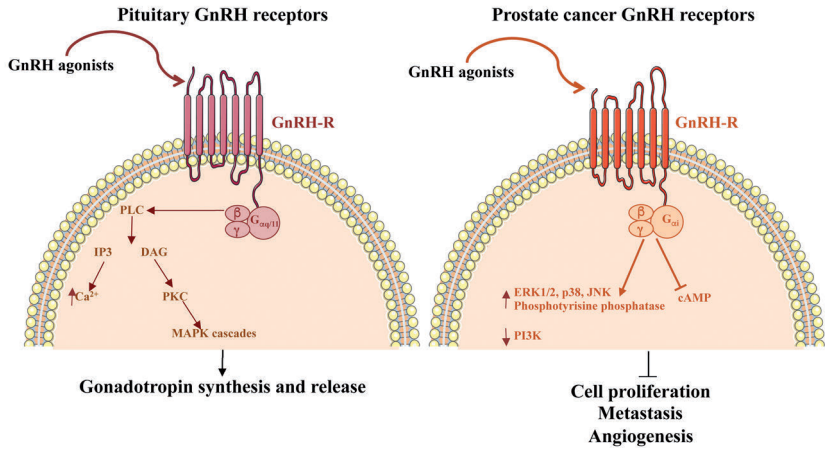

Fig. 1. Different intracellular signaling pathways of GnRH receptors in pituitary and prostate cancer cells.

2.2 Pituitary GnRH-R as molecular targets in prostate cancer

Prostate cancer is the most common cancer in men in Western countries [12]. This tumor is dependent on androgens for its growth with androgen deprivation therapy representing the most effective initial therapy [13]. As stated above, the rationale for this treatment is that, by binding to pituitary GnRH-R, these compounds desensitize these receptors thus suppressing pituitary gonadotropin and, in turn, gonadal steroid secretion [13,14].

Unfortunately, despite an initial response, most prostate cancers often progress to the condition of castration-resistant prostate cancer (CRPC) with high malignant features. Chemotherapy (docetaxel, cabazitaxel) represents the therapy of choice for CRPC patients [15]; however, development of drug resistance frequently occurs in these patients. Since the androgen receptor is still involved in the growth of CRPC, current treatments are based on novel antiandrogens (enzalutamide, darolutamide) or inhibitors of androgen synthesis (abiraterone) [16,17].

Given the key role of GnRH in the control of the reproductive functions, pituitary GnRH-R represent an effective molecular target for the treatment of different hormone-related pathologies, such as prostate cancer. Actually, it is well known that a pulsatile release of GnRH from the hypothalamus is crucial for the stimulatory effect of the peptide on gonadotropin synthesis/release; on the other hand, high doses and chronic exposure of gonadotropes to the decapeptide, or its synthetic agonists, result in suppression of the pituitary-gonadal axis through a desensitization of GnRH-R and a decrease of their number, consequently leading to a suppression of gonadal steroid secretion, the so called medical castration.

Based on the short half-life of the native decapeptide, several synthetic analogs of GnRH, both agonists and antagonists, have been developed. In particular, GnRH agonists were synthesized based on the consideration that native GnRH is rapidly degraded in blood by cleavage at the Gly^6 amino acid; thus, synthetic agonists are characterized by the presence of a D-amino acid in this position. Moreover, it is known that the first amino acids of the peptide are responsible for its biological activity; so, these amino acids are conserved in the structure of the synthetic agonists. On the other hand, the carboxy-terminal Gly^{10}-amide is usually substitute with an ethylamide residue with the aim to increase

the binding affinity of the compound to the pituitary receptor [18]. Goserelin, leuprolide, triptorelin and histrelin are the GnRH agonists mostly employed in the treatment of early-stage androgen-dependent prostate cancer (*Tab. 2*).

Administration of GnRH agonists is known to be associated with an initial undesired effect, the so called 'flare' event characterized by an increase of gonadotropin and gonadal steroid secretion; to avoid this effect, GnRH antagonists were developed. GnRH antagonists are characterized by five or more substitutions of the amino acids in position 1-3, 6 and 10. These compounds bind to GnRH-R in a competitive and reversible way, immediately suppressing gonadotropin and steroid secretion [19,20]. However, these drugs were reported to be associated with serious side effects.

3. GnRH receptors in prostate cancer

In the last decades it has become increasingly clear that GnRH-R are expressed in a wide range of cancer cells, mostly related to the endocrine system, including prostate cancer, both androgen-dependent and androgen-independent (CRPC) [3,5,7,21-25]. In particular, GnRH-R expressed in prostate cancer cells share the same DNA nucleotidic sequence and encode mRNA and protein of the same size as the pituitary receptors [26]. GnRH-R were shown to be expressed in prostate cancer cell lines, both androgen-dependent (LNCaP) and CRPC (DU145 and PC3) and in cancer specimens from patients in the early and in the late stage of the pathology [4-6,21,26,27]. Interestingly, GnRH-R expression was reported to be higher in prostate cancer than in normal prostate tissues [28].

Tab. 2. Amino acid sequences of GnRH agonists.

Compound	**Amino acid sequence**
Goserelin	Glp-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-Pro-AzaGly-NH_2
Triptorelin	Glp-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2
Leuprolide	Glp-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt
Histrelin	Glp-His-Trp-Ser-Tyr-D-His(Bzl)-Leu-Arg-Pro-NHEt

tBu, tert-butyl; Bzl, benzyl.

3.1 GnRH-R signaling pathways in prostate cancer cells

Soon after the discovery of GnRH-R in prostate cancer cells, it was demonstrated that these receptors are associated with an antitumor activity, leading to the investigation of the intracellular signaling pathways that might be associated with their activation. It was found that GnRH-R in prostate cancer cells (as well as in other cancer cells expressing this receptor) is coupled to a $G_{\alpha i}$ protein that inhibits cAMP accumulation, thus resulting in decreased cell proliferation and metastatic behavior (1). By triggering the $G_{\alpha i}$/cAMP pathway, tumor GnRH-R induce activation of different downstream signals, such as MAPK kinases (ERK1/2, JNK and p38MAPK), phosphotyrosine phosphatase and phosphatidylinositol-3-kinease (PI3K) [29] (*Fig. 1*).

3.2 Tumor GnRH-R as molecular targets in prostate cancer

GnRH agonists were widely reported to inhibit the growth of androgen-dependent prostate cancer cells in culture, suggesting that, when utilized for the treatment of this tumor, they not only suppress the pituitary-gonadal axis but they also exert a direct anticancer effect at the level of cancer cells [21,30]. Interestingly, GnRH-R activation was also shown to exert a significant antitumor effect in CRPC cells. GnRH agonists significantly reduce the growth of CRPC cells both *in vitro* and *in vivo* [31,32]. In these cells, GnRH agonists induce cell cycle arrest and apoptosis by counteracting the activity of the PI3K/ protein kinase B pathway, caspase activation and p53 expression/phosphorylation. Moreover, they also act by interfering with the mitogenic activity of growth factors, such as EGF and IGF-I [33,34].

GnRH agonists also counteract the metastatic behavior of CRPC cells, by reducing their migratory and invasive behavior, by inhibiting IGF-I activation, by affecting extracellular matrix-degrading enzymes and cell-cell adhesion molecules and by interfering with the mechanisms of actin cytoskeleton organization [35]. Finally, these compounds were shown to interfere with the process of neoangiogenesis by reducing VEGF production in cancer cells and counteracting VEGF-induced proliferation of human umbilical vein endothelial cells (HUVEC) as well as their ability to form capillary-like structures [36].

Taken together, these obervations strongly support the notion that

locally expressed GnRH-R may represent a direct and effective therapeutic target of GnRH agonists in CRPC (GnRH-R targeted therapy).

Based on the presence of GnRH-R endowed with antitumor activity in CRPC cells, during the last decades it has been proposed that these receptors might be considered as effective molecular targets of novel GnRH- based cytotoxic conjugates. Aim of this molecular targeted therapeutic approach is to increase the selectivity of chemotherapeutic agents while reducing their side effects. In these compounds, a traditional anticancer drug is chemically linked to a GnRH derivative peptide; the rationale of this 'targeted' therapy is that the GnRH analog (the targeting moiety) will specifically bind to GnRH-R expressed on cancer cells, thus directly targeting/delivering the cytotoxic drug at the tumor level. After the binding to its receptors, the GnRH analog (together with its bound drug) is internalized (by endocytosis) into the cells and degraded at the lysosomal level. Thus, the cytotoxic drug will be made free into the cells, so to exert its antitumor/proapoptotic activity (*Fig. 2*).

The first cytotoxic GnRH bioconjugates were developed in Dr. A.V. Schally's laboratory; in these hybrids, the [D-Lys^6] GnRH analog was linked to alkylating agents *(i.e.*, cisplatin) or antimetabolites (*i.e.*,

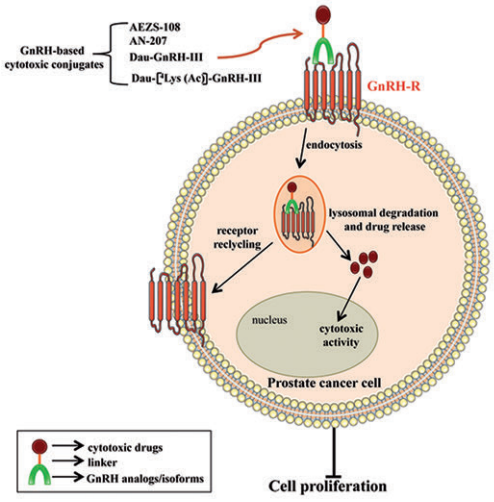

Fig. 2. Schematic representation of the receptor-mediated uptake and of the mechanism of action of cytotoxic GnRH bioconjugates.

methotrexate) [37]. Later, more efficient bioconjugates were developed in which the [D-Lys^{6}] GnRH peptide is linked to doxorubicin (Zoptarelix Doxorubicin, also known as AEZS-108 or AN-152) or to 2-pyrrolino-doxorubicin (AN-207). These cytotoxic hybrids were first shown to exert significant anticancer activities in different types of tumor cells (related to the female reproductive system), as well as in phase I and II clinical studies [38] in patients with breast, endometrial and ovarian cancers [39].

Similar results were reported in prostate cancer cells. AEZS-108 and AN-207 were shown to significantly decrease the proliferation of androgen-dependent as well as CRPC cells, being more effective than equimolar doses of doxorubicin, possibly due to the more selective delivery of the bioconjugates to tumor cells; these positive results were also obtained in preclinical studies [40].

AEZS-108 was recently investigated in CRPC men. In a phase II trial performed in men with CRPC, who had cancer progression after docetaxel-based chemotherapy, it has been observed that treatment with the bioconjugate is associated with clinical efficacy in terms of nonprogression of the disease (at 12 weeks of treatment), absence of toxicity, increased progression-free survival, response rate, and overall survivial [41].

In most recent years, novel bioconjugates were developed with the aim to decrease endocrine effects and to increase the antitumor activity. As discussed above, two additional isoforms of the GnRH peptide were discovered (GnRH-II and GnRH-III). GnRH-III (isolated from sea lamprey, *Petromyzon marinus*), in particular, was shown to possess a significant and direct anticancer activity through the binding to the classical form of cancer GnRH-R while being less potent than GnRH in stimulating gonadotropin synthesis/secretion at the pituitary level [42]. Based on these considerations, two novel promising bioconjugates were synthesized: Dau-GnRH-III, in which daunorubicin is linked to the amino acid Lys in position 8 and Dau-[^{4}Lys(Ac)]-GnRH-III in which daunorubicin is linked to the acethylated form of lysine in position 4. Both these conjugates were demonstrated to have high chemical and enzymatic stability [43]; moreover, treatment with these compounds was associated with a significant decrease of the growth of CRPC cells, both *in vitro* and *in vivo* (preclinical studies), by binding to cancer GnRH-R and subsequent internalization [44].

Taken together, these results clearly support the suitability of

GnRH-III-based cytotoxic bioconjugates as targeted chemotherapeutics for prostate cancer treatment; clinical studies are needed to confirm these experimental results.

Conclusions

1) The key role of pituitary GnRH-R for the treatment of androgen-sensitive prostate cancer is very well established. GnRH agonists, by inducing desensitization of these receptors and the subsequent suppression of the pituitary-gonadal axis, represent the therapy of choice for early stage, androgen-responsive, prostate cancer patients. *2*) GnRH-R, expressed in prostate cancer cells (especially CRPC cells), are endowed with a significant antitumor activity (antiproliferative, antimetastatic, antiangiogenic). Thus, these receptors may represent an effective molecular target for novel GnRH agonists or GnRH-based treatment strategies for this disease. *3*) GnRH-based bioconjugates, in which a GnRH-like peptide is linked to a cytotoxic drug, have been developed. These bioconjugates, by binding to cancer GnRH-R, directly deliver the chemotherapeutic drugs to prostate cancer cells, thus increasing their specificity and activity while reducing their adverse effects. The development of novel GnRH analog-based derivatives with low toxicity and high efficacy will likely improve the therapeutic options for CRPC patients.

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