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PERSPECTIVE

Challenges and Opportunities of Trapping Ligands

Mariusz W. Szkudlinski

Trophogen, Inc., Rockville, Maryland

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ABSTRACT

Because gonadotropin-releasing hormone (GnRH) analogs constitute an important class of therapeutics for various reproductive and hormone-dependent disorders, many novel compounds have been discovered and studied. Several orally active nonpeptide GnRH antagonists have recently gained increased attention. In the study published in this issue of *Molecular Pharmacology*, Kohout et al. (p. 238) used small-molecule TAK-013 (sufugolix; developed previously by Takeda Chemical Industries) as a tool to elucidate the mechanism of its insurmountable antagonism. On the basis of receptor mutagenesis combined with molecular modeling, the authors hypothesized that certain amino acid sequences uniquely present in the human GnRH receptor amino terminus and extracellular loop 2 may form a "trap door" retarding dissociation of TAK-013. Such a trapping mechanism could be both ligand- and receptor species-specific. Although analogous models were previously proposed for other G protein-coupled receptors, the study by Kohout et al. (2007) provides an important advance in the GnRH antagonists field and an illustration of the fact that preclinical studies using animal models with nonhuman receptors may have very limited value in predicting drug efficacy in human disease. There are many examples showing that high-affinity protein, peptide, or nonpeptide agonists or antagonists have also enhanced clinical efficacy. However, there are also numerous studies indicating that very high receptor binding affinity is not a guarantee of drug efficacy and that other factors, including pharmacokinetic profile, ligand-induced receptor desensitization, and "trafficking," are critical in design and development of optimal drugs.

Gonadotropin-releasing hormone (GnRH) is a polypeptide produced in specialized neurons of the hypothalamus. GnRH is released in pulses into the hypophyseal portal system, reaches the anterior pituitary, and stimulates synthesis and secretion of two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH receptor is a member of G protein-coupled receptor superfamily with seven cell membrane-spanning helices. The human GnRH receptor preferentially binds GnRH I and couples to $G_{q/11}$ protein in pituitary cells, leading to the stimulation of phospholipase C and calcium mobilization (Hazum and Conn, 1988; Millar, 2005). GnRH I is more potent in the activation of the $G_{q/11}$ protein in the gonadotrope; however, GnRH II is more potent in the stimulation of apoptosis and antiproliferative effects through activating G_i protein-mediated signaling. GnRH binding also causes up-regulation and clustering of GnRH receptors, resulting in their internalization, partial degradation, and recycling. Activation of many GPCR receptors, including GnRH receptor, is likely to be associated with breaking, by a ligand, the intrareceptor interactions that stabilize the receptor in its inactive conformations, creating new inter- and intramolecular contacts, resulting in ligand-specific set of receptor conformations (Zhang et al., 2000; Lu et al., 2005).

During the 4 decades since initial identification and sequencing of GnRH by Schally et al. (1971), several thousand peptide and nonpeptide GnRH receptor agonists and antagonists have been synthesized and tested. Long-acting GnRH agonists and antagonists are currently used in assisted reproduction and a wide spectrum of sex-hormone-dependent diseases. GnRH agonists bind to pituitary GnRH receptors and initially induce release of FSH and LH ("flare"), which is followed by enhanced internalization of GnRH receptors and subsequent decrease of FSH and LH levels. Clinical efficacy of antagonists is based on their ability to bind GnRH receptor

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ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; NBI-42902, 3-[(2*R*)-amino-2-phenylethyl]-1-(2,6-difluorobenzyl)-5-(2-fluoro-3-methoxyphenyl)-6-methylpyrimidin-2,4-dione; TSH, thyroid-stimulating hormone.

and prevent binding of endogenous GnRH by prolonged receptor occupancy (Broqua et al., 2002). In contrast to agonists, they act without induction of typical for GnRH receptor second messenger production and cellular responses.

Peptide and nonpeptide GnRH antagonists were developed in the last decade to overcome several limitations of the agonists, including "flare-up" phenomenon and limited efficacy of agonists. GnRH peptide analogs are currently widely used to treat very diverse clinical conditions, but very common application is still ovarian stimulation for in vitro fertilization (Macklon et al., 2006; Tarlatzis et al., 2006). However, many such antagonists have very poor oral activity, quite strong histamine-releasing properties, and a very high metabolic clearance rate, requiring multiple injections or depot formulation.

The first potent and orally active nonpeptide antagonist was reported in 1998 (Cho et al., 1998). Several improved GnRH antagonists, including TAK-013 (sufugolix), were later synthesized and partially characterized by researchers at the Takeda Chemical Industries (Osaka, Japan). TAK-013 entered clinical trials for endometriosis and uterine fibroids in the United States, Europe, and Japan. Researchers from Neurocrine Biosciences developed several new nonpeptide GnRH antagonists, including NBI-42902 (Tucci et al., 2005). Nonpeptide GnRH antagonists offer convenient oral administration, which is particularly important in long-term treatment of GnRH-dependent precocious puberty, endometriosis, and prostate cancer. Many new compounds are not only more potent and longer acting than older antagonists but also lack undesirable side effects related to histamine release.

In theory, action of orthosteric receptor antagonist on biological response can result in three different changes of agonist dose-response curves: (1) shifting to the right with no decrease of the maximal response (surmountable antagonism); (2) decrease of maximal response combined with a right shift in potency (insurmountable antagonism); and (3) decrease of maximal response without right shift in potency (insurmountable antagonism). Examples of the first two instances are presented in the article by Kohout et al. (2007). The authors determined amino acid residues involved in TAK-013 insurmountability at the human GnRH receptor that are absent in the Rhesus macaque GnRH receptor molecule. Comparison of GnRH receptor sequences and receptor mutagenesis combined with molecular modeling led the authors to the "trap door" hypothesis, explaining different behavior of TAK-013 at the human and Rhesus macaque receptors.

Evolutionary Adaptation of Primate GnRH Receptors

Because native GnRH I and II sequences are highly conserved in mammals, including their virtual identity between the Old World monkey Rhesus Macaque (*Macaca mulatta*) and humans, it is tempting to speculate why certain evolutionary changes in GnRH receptors occurred and why human GnRH receptors became more prone to insurmountability by antagonists such as TAK-013. Progressive elimination of basic lysine and arginine residues in the sequence of the glycoprotein hormone [thyroid-stimulating hormone (TSH), FSH, chorionic gonadotropin, and LH] common α-subunit was correlated with their decreased bioactivity (Szkudlinski et al., 1996, 2002). In the case of TSH, the attenuated bioactivity after separation of apes from Old World monkeys may be due to the greater need to conserve iodine for thyroid hormone synthesis during long periods of fasting in the nomadic life that early hominoids had to endure. Similar modifications of FSH, chorionic gonadotropin, and LH intrinsic activities in apes and humans compared with New World and Old World monkeys are probably related to slower rates of reproduction and maturation in hominoids (i.e., all apes and humans). It is noteworthy that the evolutionary rate of gonadotropin receptors is considerably slower than that of their natural ligands. In contrast to gonadotropin receptors, significant functional differences between human and Old World monkey GnRH receptors, as described by Kohout et al. (2007), may suggest adaptation of GnRH receptor to simultaneous modifications in other components of reproductive system during primate evolution. Consistent with more rigorous control of reproduction in apes and humans, significantly lower expression of human than rodent GnRH receptors was recently correlated with the presence of primate-specific lysine 191 residue, increased rate of receptor misfolding, and decreased receptor signaling (Janovick et al., 2006). Such restriction of human GnRH receptor cell membrane expression developed under strong selective pressure and quite complex pattern of cyclicity associated with primate reproduction (Janovick et al., 2007). Consequently, evolutionary changes resulted in a hu-

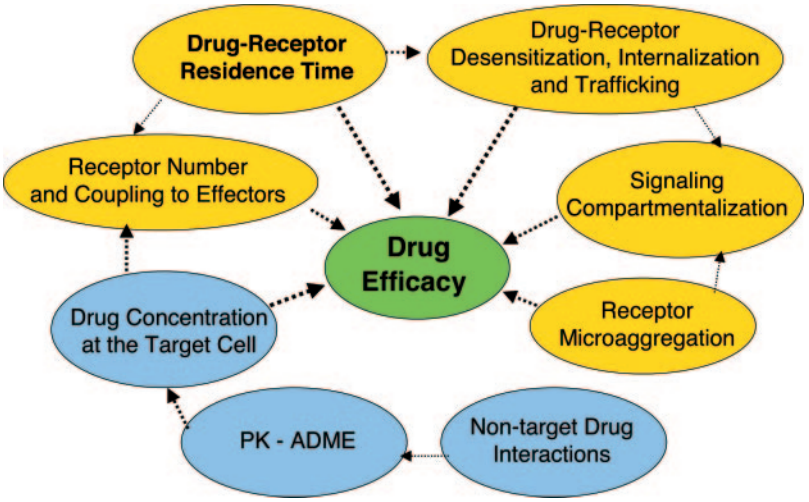

Fig. 1. Schematic diagram of factors determining drug efficacy in humans. Marked in yellow are factors assessable by in vitro studies using natural human cells and cell lines expressing recombinant human receptors. In blue are factors tested in animal studies, including drug pharmacokinetics (PK), affecting duration of action in relation to minimum effective concentration for intended response. Complex direct and indirect relationships between different factors in vitro and in vivo are shown here for illustrative purposes only and are not discussed in detail. Although previous strategies in drug discovery and development preferred answering many questions about pharmacodynamics before studies of PK, current trends include more integrated approaches, such as an early assessment of efficacy in animal models and phase 0 of clinical trials. ADME, absorption, distribution, metabolism, and excretion.

man GnRH receptor molecule that is precisely balanced between either expression at the plasma membrane or internalization and degradation in the endoplasmic reticulum. Such balance was proposed to be the reason why the human GnRH receptor, in contrast to its more highly expressed rodent counterparts, is highly susceptible to single-point mutations that result in disease (Conn et al., 2006). The challenge that lies before us is to get a deeper insight into many uniquely human or primate reproductive phenomena and integrate them with quite complex regulation of hormone and receptor function.

Receptor Binding and Efficacy

Modern drug discovery is founded on Paul Ehrlich's concept that receptor binding is critical in drug efficacy. The term efficacy, previously associated only with agonists, is now applied more broadly to all molecules causing a change in receptor function, including antagonists and inverse agonists. Many studies show that high-affinity agonists and antagonists have enhanced clinical efficacy. Studies of the kinetics of high-affinity analogs suggest that different chemical groups and residues may determine their increased association and decreased dissociation rates (Wang et al., 1997; Szkudlinski et al., 2002, 2004; Schreiber et al., 2006). A widespread view is that the most crucial factor for sustained drug efficacy in vivo is not the apparent affinity for the target receptor, but rather the residency time at the receptor that is measured by the dissociation half-life (Copeland et al., 2006). It determines the longer drug effect in vitro or in vivo and is commonly considered a key parameter predicting duration of drug efficacy. Accordingly, Kohout et al. (2007) showed a direct correlation between the degree of insurmountability and the dissociation rate of TAK-013 from the receptor. It is possible that the trapping mechanism may function not only for nonpeptide ligands but also may be responsible for delayed dissociation of many peptide and protein ligands. Such a ligand-trapping mechanism could be enhanced in certain receptor systems with receptor dimerization, coreceptor recruitment, glycosaminoglycans, and other components of cell membrane.

The question remains whether a higher degree of insurmountability results in enhanced clinical efficacy of TAK-013. In contrast to experiments performed in closed systems (e.g., static short-term in vitro studies), the clinical efficacy of a drug is influenced by many factors other than molecular determinants of ligand-receptor complex.

Other Factors Affecting Efficacy

Although receptor binding is highly critical, there are other important factors determining in vivo efficacy (Fig. 1). Because treatment with various antagonists of GnRH, including cetrorelix, leads to down-regulation of GnRH receptors, similar to the effect of GnRH agonists (Halmos and Schally, 2002), it is expected that the efficacy of GnRH antagonist is also dependent on the rate of internalization affecting total number of GnRH receptors in the cell membrane. It was shown previously that certain GnRH agonists and antagonists are internalized to a similar extent and that receptor activation may not always be necessary to initiate the rapid internalization of hormone-receptor complexes (Hazum et al., 1983; Loumaye et al., 1984). In addition, it is also critical if the antagonist-receptor complex is routed after internalization into a recycling pathway or to a degradation pathway.

Ligand-induced GPCR receptor dimerization and oligomerization has emerged recently as a factor modulating cellular signaling. Some studies indicate that GnRH agonist, but not antagonist, occupancy of the GnRH receptor promotes microaggregation of receptors (Cornea et al., 2001; Cornea and Conn, 2002). Cell specific colocalization of signaling components within lipid rafts and caveolae emerged as an additional modifier of drug responses (Ostrom and Insel, 2004). Cell surface receptor expression and receptor coupling to various effectors in the target organ is obviously critical for drug efficacy. Agonist potency and efficacy is known to decrease in systems with low receptor levels and impaired/altered effector coupling (Kenakin, 2003). If natural ligand X requires 500 receptors in a given cell to produce maximal response, whereas the high-affinity analog X requires 50, then a decrease of the receptor density to 50 per cell should theoretically not decrease maximal response to analog X, but only to the natural ligand.

In addition to the assessments based on concentration of the drug in the blood, drug concentration at the target cells depends on organ perfusion and drug distribution from blood to a given tissue. Receptor spliced variants, altered signaling mechanism, modulation of multiple targets, and unanticipated effects of ligand modification may create even more complexity in the process of predicting clinical efficacy (Sarkar et al., 2002). Based on research and development experiences with many drug candidates, it is now becoming abundantly clear that the clinical efficacy and safety in humans is never completely certain, even after completion of clinical trials.

Concluding Remarks

In pharmacology and drug design at the beginning of the 21st century, we are confronted with both challenges and opportunities related to new classes of receptor ligands. Although the most basic questions about optimal selection and design methods of peptide, nonpeptide, and protein analogs are not yet fully addressed, it is now envisioned that each binding molecule may result in a unique spectrum of receptor conformations, leading to qualitatively different receptor behavior than produced by a natural ligand. Many more studies are required to better understand the mechanisms regulating various analog-specific phenomena such as orthosteric or allosteric receptor binding, effects of various antagonists in constitutively active systems, analog-induced receptor signaling, trafficking, and degradation. Nonetheless, the data of Kohout et al. (2007) strongly suggest that using inherent receptor trapping properties may lead to design of novel insurmountable antagonists within GPCRs and other receptor superfamilies. Perhaps an era of highly specific and efficacious insurmountable receptor antagonists is just beginning.

References

Broqua P, Riviere PJ, Conn PM, Rivier JE, Aubert ML, and Junien JL (2002) Pharmacological profile of a new, potent, and long-acting gonadotropin-releasing hormone antagonist: degarelix. *J Pharmacol Exp Ther* **301**:95–102.

Cho N, Harada M, Imaeda T, Imada T, Matsumoto H, Hayase Y, Sasaki S, Furuya S, Suzuki N, Okubo S, et al. (1998) Discovery of a novel, potent, and orally active

nonpeptide antagonist of the human luteinizing hormone-releasing hormone (LHRH) receptor. *J Med Chem* **41:**4190–4195.
Conn PM, Knollman PE, Brothers SP, and Janovick JA (2006) Protein folding as posttranslational regulation: evolution of a mechanism for controlled plasma membrane expression of a G protein-coupled receptor. *Mol Endocrinol* **20:**3035–3041.
Copeland RA, Pompliano DL, and Meek TD (2006) Drug-target residence time and its implications for lead optimization. *Nat Rev Drug Discov* **5:**730–739.
Cornea A and Conn PM (2002) Measurement of changes in fluorescence resonance energy transfer between gonadotropin-releasing hormone receptors in response to agonists. *Methods* **27:**333–339.
Cornea A, Janovick JA, Maya-Nunez G, and Conn PM (2001) Gonadotropin-releasing hormone receptor microaggregation. Rate monitored by fluorescence resonance energy transfer. *J Biol Chem* **276:**2153–2158.
Halmos G and Schally AV (2002) Changes in subcellular distribution of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) after treatment with the LH-RH antagonist cetrorelix. *Proc Natl Acad Sci U S A* **99:**961–965.
Hazum E and Conn PM (1988) Molecular mechanism of gonadotropin releasing hormone (GnRH) action. I. The GnRH receptor. *Endocr Rev* **9:**379–386.
Hazum E, Meidan R, Liscovitch M, Keinan D, Lindner HR, and Koch Y (1983) Receptor-mediated internalization of LHRH antagonists by pituitary cells. *Mol Cell Endocrinol* **30:**291–301.
Janovick JA, Brothers SP, Knollman PE, and Conn PM (2007) Specializations of a G-protein-coupled receptor that appear to aid with detection of frequency-modulated signals from its ligand. *FASEB J* **21:**384–392.
Janovick JA, Knollman PE, Brothers SP, Ayala-Yanez R, Aziz AS, and Conn PM (2006) Regulation of G protein-coupled receptor trafficking by inefficient plasma membrane expression: molecular basis of an evolved strategy. *J Biol Chem* **281:** 8417–8425.
Kenakin T (2003) Predicting therapeutic value in the lead optimization phase of drug discovery. *Nat Rev Drug Discov* **2:**429–438.
Kohout TA, Xie Q, Reijmers S, Finn KJ, Guo Z, Zhu YF, and Struthers RS (2007) Trapping of a nonpeptide ligand by the extracellular domains of the gonadotropin-releasing hormone receptor results in insurmountable antagonism. *Mol Pharmacol* **72:**238–247.
Loumaye E, Wynn PC, Coy D, and Catt KJ (1984) Receptor-binding properties of gonadotropin-releasing hormone derivatives. Prolonged receptor occupancy and cell-surface localization of a potent antagonist analog. *J Biol Chem* **259:**12663–12671.
Lu ZL, Gallagher R, Sellar R, Coetsee M, and Millar RP (2005) Mutations remote from the human gonadotropin-releasing hormone (GnRH) receptor-binding sites specifically increase binding affinity for GnRH II but not GnRH I: evidence for ligand-selective, receptor-active conformations. *J Biol Chem* **280:**29796–29803.
Macklon NS, Stouffer RL, Giudice LC, and Fauser BC (2006) The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocr Rev* **27:**170–207.
Millar RP (2005) GnRHs and GnRH receptors. *Anim Reprod Sci* **88:**5–28.
Ostrom RS and Insel PA (2004) The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br J Pharmacol* **143:**235–245.
Sarkar CA, Lowenhaupt K, Horan T, Boone TC, Tidor B, and Lauffenburger DA (2002) Rational cytokine design for increased lifetime and enhanced potency using pH-activated "histidine switching." *Nat Biotechnol* **20:**908–913.
Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, and White WF (1971) Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. *Science* **173:**1036–1038.
Schreiber G, Shaul Y, and Gottschalk KE (2006) Electrostatic design of protein-protein association rates. *Methods Mol Biol* **340:**235–249.
Szkudlinski MW (2004) Recombinant human thyrotropins of the twenty-first century. *Expert Opin Pharmacother* **5:**2435–2440.
Szkudlinski MW, Fremont V, Ronin C, and Weintraub BD (2002) Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. *Physiol Rev* **82:**473–502.
Szkudlinski MW, Teh NG, Grossmann M, Tropea JE, and Weintraub BD (1996) Engineering human glycoprotein hormone superactive analogues. *Nat Biotechnol* **14:**1257–1263.
Tarlatzis BC, Fauser BC, Kolibianakis EM, Diedrich K, Rombauts L, and Devroey P (2006) GnRH antagonists in ovarian stimulation for IVF. *Hum Reprod Update* **12:**333–340.
Tucci FC, Zhu YF, Struthers RS, Guo Z, Gross TD, Rowbottom MW, Acevedo O, Gao Y, Saunders J, Xie Q, et al. (2005) 3-[(2*R*)-Amino-2-phenylethyl]-1-(2,6-difluorobenzyl)-5-(2-fluoro-3-methoxyphenyl)-6-methylpyrimidin-2,4-dione (NBI 42902) as a potent and orally active antagonist of the human gonadotropin-releasing hormone receptor. Design, synthesis, and in vitro and in vivo characterization. *J Med Chem* **48:**1169–1178.
Wang Y, Shen BJ, and Sebald W (1997) A mixed-charge pair in human interleukin 4 dominates high-affinity interaction with the receptor alpha chain. *Proc Natl Acad Sci U S A* **94:**1657–1662.
Zhang M, Tong KP, Fremont V, Chen J, Narayan P, Puett D, Weintraub BD, and Szkudlinski MW (2000) The extracellular domain suppresses constitutive activity of the transmembrane domain of the human TSH receptor: implications for hormone-receptor interaction and antagonist design. *Endocrinology* **141:**3514–3517.

Address correspondence to: Mariusz W. Szkudlinski, Trophogen, Inc., 6 Taft Court, Suite 150, Rockville, Maryland 20850. E-mail: mszkudlinski@trophogen.com