

# Development of Prolactin Receptor Antagonists: Same Goal, Different Ways

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**Abstract:** Hyperprolactinemia is an endocrine pathology resulting from over-production of prolactin (PRL) by pituitary adenomas, and leading to various reproductive disorders. In addition, there is increasing evidence that PRL acts as a growth-promoter of breast and prostate tumors. Classical drugs blocking pituitary PRL production are not necessarily efficient in these pathological situations, which has encouraged the search for alternative ways of inhibiting the undesirable actions of PRL. Prolactin receptor (PRLR) antagonists, acting at the level of receptor activation rather than PRL production, are the most promising strategy. Based on the protein core of human (h)PRL or growth hormone (hGH), the other natural hPRLR ligand, a series of new variants have been engineered within the past couple of years, leading to various patent applications. Modifications of amino acid sequences involve single/multiple substitutions, truncations, or generation of fusion proteins. Three mechanisms of action have been reported for these PRLR antagonists: 1) inhibition of PRLR signaling by competition with endogenous PRL for receptor binding, 2) activation of specific PRLR signalling pathways resulting in actions opposite to those of wild-type hPRL, and 3) engineering of chimeric ligands targeting more than one receptor/cell type, in order to improve tumor-growth inhibition. Since none of these patented molecules is yet in clinical trials, their efficacy to treat PRL-dependent pathologies remains to be demonstrated in humans.

**Keywords:** Breast cancer, prostate cancer, hyperprolactinemia, growth hormone, G129R-hPRL, 1-9-G129R-hPRL, G129R-hPRL, S179D-hPRL, induced-fit

## 1. INTRODUCTION

### 1.1. Prolactin

Prolactin (PRL) is a polypeptide hormone mainly known for its role in the induction of lactation. However, more than 300 biological activities have been attributed to this hormone, which can be subdivided into 5 categories: reproduction, endocrinology and metabolism, control of water and electrolyte balance, growth and development, brain and behavior, and finally, immunoregulation and protection [1]. These multiple actions suggest that PRL has actually many other targets than the mammary gland, in which it modulates, probably more than it regulates, many physiological processes other than lactation.

PRL belongs to the PRL/GH/PL hormone family, which includes several members sharing high structural and functional similarity [2-4]. The best known are growth hormone (GH) and placental lactogen (PL). These hormones are part of the four-helix bundle hematopoietic cytokine family, that includes various polypeptides exhibiting multiple functions, such as interleukins or erythropoietin. As true for their respective ligands, the receptors for these cytokines are also closely related [5]. They belong to the Cytokine Receptor Superfamily, which involves non tyrosine-kinase, single-pass transmembrane chains [6,7]. The receptors for PRL (PRLR) and GH (GHR) are very close in sequence and structure, which correlates that they share some ligands (see below).

### 1.2. Prolactin Pathophysiology

Many experimental, clinical and epidemiological arguments strongly supporting a role for PRL in human diseases have appeared within the past couple of years, which are mentioned in this section. However, despite these data have considerably renewed the interest of the scientific community for PRL in human pathophysiology, there is still no known genetic disease linked to the PRL system (hormone or receptor mutations). The lack of a human model that clearly reflects the phenotypes resulting from the failure or, in contrast, the hyperactivation of PRL signaling largely contributes to the fact that PRL is rarely considered by clinicians to be involved in pathologies other than hyperprolactinemia.

Hyperprolactinemia is one of the most frequent endocrine diseases, especially in young women. Pathological hyperprolactinemia is defined as circulating PRL levels above normal range other than in normal physiological conditions, such as pregnancy and lactation. Excess of PRL secretion leads to various disorders of reproductive functions, including galactorrhea (spontaneous lactation), amenorrhea (lack of menstrual cycle) and sterility. It also impacts on bone mineral density (osteoporosis). Prolactinomas, which are benign pituitary tumors affecting lactotrope cells - the cell type secreting PRL -, are the most common cause of pathological hyperprolactinemia [8]. The latter can also result from various dysregulations of the neuroendocrine mechanisms governing PRL secretion, or pharmacological treatment involving dopamine antagonist, e.g. neuroleptic therapy.

Besides hyperprolactinemia, a role for PRL as a tumor growth promoter has been suggested for more than two decades. This issue has been reviewed in many recent

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articles [9-11]. Briefly, based on various *in vitro* (cell) and *in vivo* (genetically-modified animal models) studies, PRL was shown to promote the growth of mammary tumors. As the mammary gland is the main target tissue of PRL, this finding is not surprising. Recent epidemiological studies confirmed the role of PRL in breast cancer growth, since pre- as well as post-menopausal women with high-normal PRL levels were shown to be at higher risk for breast cancer compared to women with low-normal PRL levels [12]. Besides breast cancer, cell and animal studies also identified the prostate as another tissue on which PRL can exert tumor growth promoting actions, as highlighted by the fact that transgenic mice over-expressing PRL systemically or locally develop prostate hyperplasia [13,14]. There is also accumulating evidence that PRL promotes survival of human prostate cancer cells [15]. In contrast to breast cancer, however, epidemiological studies failed to confirm any relationships between circulating PRL levels and prostate cancer risk [16].

Besides these tumoral pathologies affecting the pituitary, the prostate and the breast, the involvement of PRL in autoimmune diseases has also been suggested, based on the observation that pathologies such as Systemic Lupus Erythematosus (SLE) are accentuated in post-partum, i.e. when PRL levels are elevated to maintain lactation [17]. Nevertheless, the possible correlation between circulating PRL levels and the severity of the disease remains controversial [18].

### 1.3. Therapeutical Treatment of PRL-Related Pathologies

The current therapeutical approach to inhibit excess of PRL synthesis and/or to decrease its undesirable effects involves the use of synthetic analogs of dopamine, which is the physiological negative regulator of PRL synthesis and secretion by lactotrope cells [19]. Bromocriptine was the pioneering dopamine analog, after which more potent drugs with improved characteristics (increased half-life, fewer side effects) were developed (Pergolide, Quinagolide, Cabergoline) [8].

With respect to prolactinomas, dopamine agonists normalize circulating PRL levels by decreasing its production, which among other effects, restores fertility in most of women presenting with hyperprolactinemia [8,20,21]. These drugs also lead to rapid shrinkage of the pituitary tumor, which is an important parameter for the well-being of patients, since various symptoms associated with pituitary adenomas, such as visual field disturbance or headache, are also improved [8]. However, because the target tissue is a tumor, the physiological regulations found in normal tissue may in some instance be altered, or even lost. This explains that 10 to 15% patients presenting with prolactinomas are dopamine-resistant or, at best, only partially respond to dopamine agonists without complete normalization of PRL levels [22]. This unresponsiveness most likely involves a decrease of dopamine D2 receptor expression at the membrane of lactotrope cells [22,23]. To date, the main strategies to circumvent dopamine agonist resistance include switching to another dopamine agonist or increasing the dose beyond conventional doses to see if some response is observed. Surgery or radiotherapy can also be proposed on a case-per-case basis. Since these alternatives are not always fruitful, or simply cannot be proposed to the patients, the

current treatments based on dopamine agonists remain unsatisfactory for a significant number of hyperprolactinemic patients.

With respect to breast and prostate tumors, PRL is currently not considered as a relevant target by any clinical treatment. Only a few trials involving dopamine agonists have been reported in the past, and the results obtained were disappointing. Although bromocriptine was shown to normalize PRL levels in metastatic breast cancer and prostate carcinoma patients, this was not found to provide significant benefit for the patients [24-26]. One tempting hypothesis for the failure of dopamine agonists against breast and prostate tumors relies on the potential role of locally-produced PRL in promoting tumor growth via an autocrine-paracrine mechanism of action, that is distinct from the classical endocrine route. Various cell and animal models (transgenics) have highlighted the pro-tumor role of autocrine PRL on mammary and prostate tissues [14,27,28], and there is now clear evidence that human breast and prostate express both hPRL and its receptor (for reviews, [9,10,27,29-31]). Co-expression of the ligand and its receptor by the same cells suggests that the autocrine-paracrine action could also participate to tumor growth in humans, especially if one of the two partners was over-expressed, as it has been proposed [15,32]. Since dopamine does not regulate PRL synthesis in non pituitary tissues [20], demonstration of the positive role of autocrine PRL in human tumor growth, and inhibition thereof, awaits the availability of compounds capable of blocking its synthesis and/or its effects.

### 1.4. Potential Uses of PRLR Antagonists

As briefly outlined above, there are potentially two types of pathologies in which elevated PRL levels should be of concern, and for which dopamine agonists are inefficient, or simply inappropriate. The first one involves breast and prostate tumors, in which there is urgent need to target locally-produced PRL in order to evaluate its actual promoting role on tumor growth. If such a role can be demonstrated, then PRLR antagonists could be proposed as a new therapeutical anti-hormonal approach, providing they are sufficiently efficient and tolerated. The second indication involves specific situations of hyperprolactinemia. Clearly, dopamine-resistant prolactinomas are a major target. Given the high prevalence of the disease, 10-15% of resistance accounts for a non negligible number of patients who should not remain untreated. Beyond the well known consequences of hyperprolactinemia on reproductive functions, the recent epidemiological evidence that high-normal PRL levels increase breast cancer risk [12] addresses the question whether hyperprolactinemic patients presenting only partial normalization of PRL levels under dopamine agonist treatment are also at higher risk for breast cancer. The same question should also be of concern for patients treated with drugs known to induce hyperprolactinemia, one of the most important of which involves neuroleptic medications used in the treatment of psychiatric disorders such as schizophrenia [33,34]. The mechanism of action of these drugs is to block dopamine D2 receptors, which usually result in moderate hyperprolactinemia (50-100 µg/l). For all these situations in which blockade of PRL production has failed, PRLR

antagonists could represent an alternative medical treatment, since they should be able to block the actions of PRL, rather than its production.

## 2. DEVELOPMENT OF PRLR ANTAGONISTS

### 2.1. Mechanism of Receptor Activation

The development of PRLR was achieved by merging two sets of data that have evolved in parallel since the late eighties/early nineties. The first involves the tremendous amount of knowledge that has been gained from structural, biochemical and functional studies of GH-GHR interaction, which led among others to the development of GHR antagonists [35]. The second is based on structure-function studies of natural and engineered variants of PRL [3]. In general, the major mechanisms that were discovered for the activation of the GHR could be successfully extrapolated to the PRLR, although both systems present hormone-specific features [3].

The initial model of receptor activation proposed for the GHR (and later for the PRLR) suggested that receptor homodimerization was achieved upon ligand binding, and occurred in a two-step (or sequential) process [36] (left panel of Fig. (1)). First, one molecule of hormone binds to one molecule of receptor through its binding site 1, of high surface and affinity, leading to the formation of an intermediate and inactive complex of 1:1 stoichiometry. Then, the hormone involved in this intermediary complex recruits a second (identical) receptor molecule through its binding site 2, of lower surface and affinity, leading to the formation of an active trimeric 1:2 complex, in which the receptor is homodimerized. Recent studies came to the conclusion that these receptors are actually pre-dimerized at the cell membrane (right panel of Fig. (1)), suggesting that receptor clustering *per se* is not induced by the ligand [37-40]. Our current understanding of receptor activation is thus more in favor of a conformational change induced by the ligand. The group of M. Waters has recently proposed that a relative rotation of both receptor chains would be the mechanism involved for the GHR [37,38].

Whether receptor dimerization *per se*, or a conformational change within pre-formed receptor homodimers is the actual mechanism of receptor activation, a critical role is attributed to the binding site 2 of the hormone, since this is the region that interacts with the second receptor chain once interaction involving site 1 has occurred. This is the reason why altering the intrinsic structural features of binding site 2 has been the rationale for the design of the majority of competitive GH or PRL receptor antagonists developed to date. These engineered variants are described and discussed in the next sections.

### 2.2. Steric Hindrance in Binding Site 2 by Mutation of the Helix 3 Glycine

#### 2.2.a. G120R-hGH

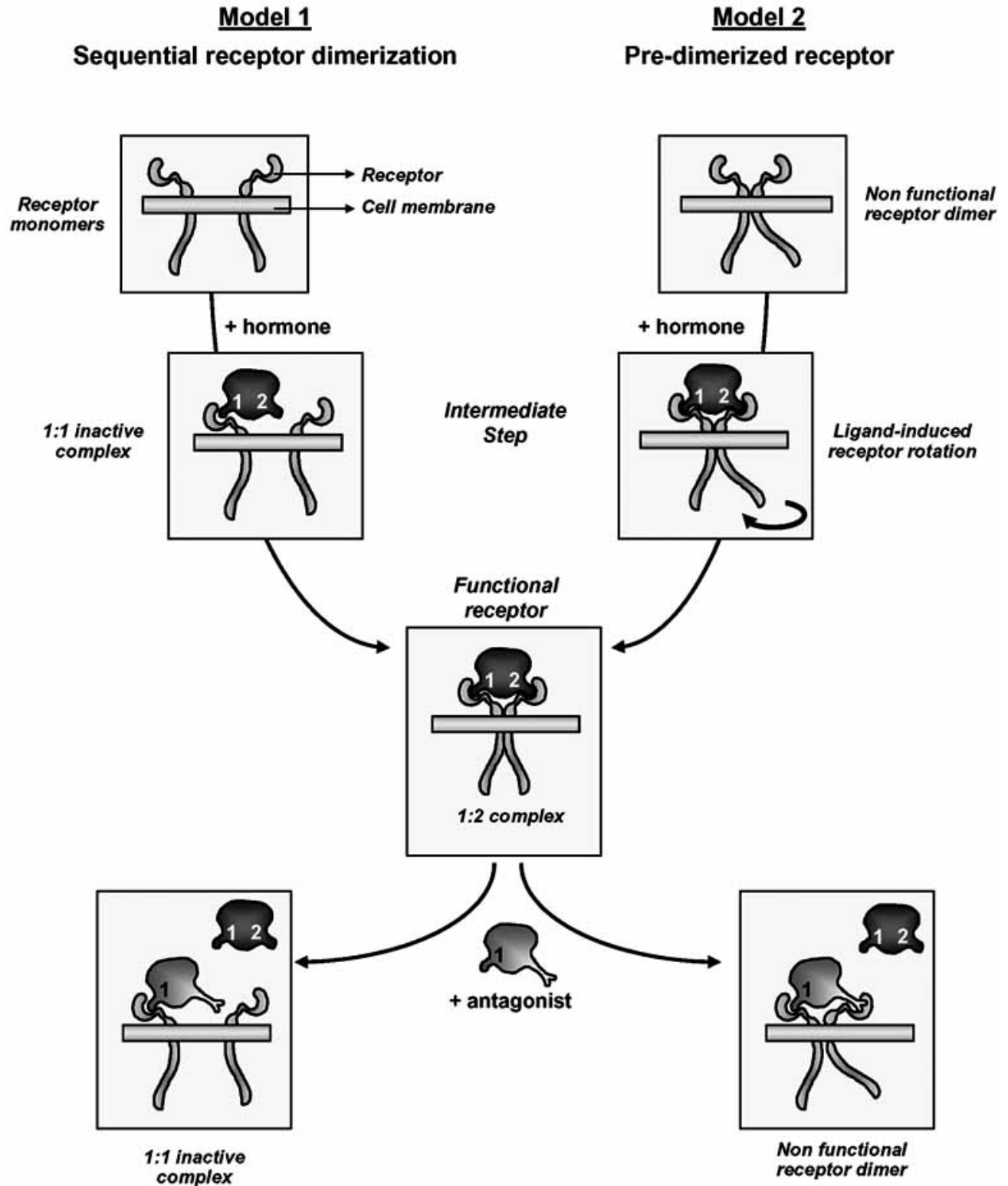
The paradigm mutation for shifting PRL/GH hormones from agonists to antagonists was discovered (and patented) more than 15 years ago by the group of John Kopchick in studies involving bovine GH [35,41]. Soon after, similar findings were patented for human GH, based on different

sets of data obtained at Genentech [42]. As demonstrated by the crystal structure of the hGH-hGHbp (bp = binding protein) complex, the helix 3 of GH is involved in the receptor binding site 2 of the hormone [43]. This helix 3 contains a glycine residue (G120 in hGH) that maintains a cleft into which a tryptophane residue of the GHR (Trp 104) docks upon binding. Substitution of any residue (other than Ala) for this glycine prevents correct docking of the tryptophane. As a result, the interaction of GH with the second receptor via site 2 is impaired, while hormone-receptor interaction involving site 1 persists. Large residues such as arginine (G120R) or lysine (G120K) have been described as the preferred substitution of the helix 3 glycine to generate potent antagonists.

The helix 3 glycine is conserved in all the hormones belonging to the PRL/GH/PL family [44]. Based on the conventional model of sequential receptor dimerization, the intellectual property of Genentech patents partly relies on the ability of modified analogs (such as hGH-G120R) to impair receptor homodimerization, i.e. to form only inactive 1:1 complexes with the receptor [45]. Recent evidence that the membrane GHR is pre-dimerized suggests that glycine mutants actually interact with a receptor dimer, but are unable to induce the conformational change required to induce receptor activation (Fig. 1). Although the mechanism of action of these analogs at the receptor level remains to be elucidated, receptor activation is impaired, while binding persists. G120R-hGH is thus a competitive antagonist of the GHR. Although this falls beyond the scope of this study, we have to mention that further development of this molecule led to the development of pegvisomant (Somavert), which is now used to treat some acromegalic patients [46].

In agreement with the fact that in humans, hGH is a natural ligand of the PRLR, hGH-G120R was shown to be also an antagonist of this receptor [47]. It was further shown to reduce the growth of breast cancer cell lines *in vitro* [48], suggesting that hGH-G120R may be considered as a potential drug for PRL-responsive pathologies such as breast cancer [49]. It is of note that inhibition of GHR signaling has also been reported to reduce the growth of mammary and prostate cancers in animal studies [50,51]. The ability of G120R-hGH to inhibit the activation of two distinct receptors (GHR and PRLR) could represent an advantage when both systems act in concert to promote the development of a disease, as this is perhaps the case in breast cancer. However, such duality of targets is also a pharmacological limiting factor with respect to the requirement of specificity. Indeed, there is presumably no advantage to target the GHR in pathologies in which inhibiting PRLR-mediated effects is the primary goal. This is likely the case for dopamine-resistant prolactinoma, which is a typical PRL-related pathology in which there is no physiological reason to inhibit GHR actions.

In addition to pharmacological limitations, the real ability of G120R-hGH to abolish PRLR actions should be addressed, since this analog seems to display imperfections similar to those we reported for the homologous PRL mutant, G129R-hPRL (see below). Indeed, G120R-hGH was shown to exhibit significant agonism in sensitive bioassays



**Fig. (1).** Activation of the PRL receptor.

The two models of interaction between PRL/GH hormones with their receptors. In model 1 (left panels), the hormone induces sequential receptor dimerization via interactions involving binding site 1 first, then binding site 2. In such a model, competitive receptor antagonists that are mutated at site 2 are supposed to be unable to induce functional receptor dimerization. In model 2 (right panels), the receptor is pre-dimerized at the membrane and its activation is achieved by conformational changes (rotation) induced upon ligand binding. In such a model, receptor antagonists are supposed to prevent the right conformational change to occur. This model has been recently proposed for the GHR, and remains to be established for the PRLR.

[52-55], clearly indicating it is not a pure antagonist. The ability of G120R-hGH to efficiently inhibit PRLR-mediated signaling in animal models has also been questioned in at least two reports. The first suggested that it may actually activate, rather than antagonize the PRLR in rats [56]. The second showed that dwarf transgenic females expressing G120R-hGH remained fertile [57], while infertility is the main phenotype of PRLR-deficient mice [58]. This strongly suggests that, at least in rodents, this analog is unable to fully abolish PRLR-mediated signals. This issue remains unknown in humans, since pegvisomant - the commercial hGHR antagonist that was developed based on the G120R-hGH molecule - contains 8 additional mutations in site 1 that prevent binding to the PRLR, thereby ensuring specificity towards the sole GHR [35].

Last but not least, the agonism/antagonism ratio displayed by G120R-hGH towards the PRLR is highly dependent on zinc concentrations [47,59]. It is thus likely that individual variations of zinc concentrations will get the prediction of the ultimate action of G120R-hGH in patients more complicated. A recent patent application proposed that this feature may be an opportunity to increase the efficacy of G120R-hGH towards the PRLR by co-administering zinc to the putative patients [60]. However, such an approach remains totally speculative at this time, since it is not supported by any published experimental observation.

### 2.1.b. G129R-hPRL

A few years after the discovery of the GHR antagonist by the Kopchick group, our laboratory developed the homologous molecule based on the hPRL core, namely G129R-hPRL. The molecular basis of this PRL analog is exactly the same, meaning that the Gly to Arg mutation is anticipated to interfere with the docking of a Trp in the PRL receptor (Fig. (2)). We did not fill a patent application for this molecule. One of the reasons is that the first results regarding its properties in the reference cell bioassay for lactogens (Nb2 cell proliferation) were disappointing [55]. In this assay, G129R-hPRL appeared to be a partial agonist and a very poor antagonist. Since these initial studies, we and others have extensively characterized the biological properties of this analog [9]. However, some controversy remains with respect to the results obtained. Our data clearly showed that, depending on the intrinsic features of cell bioassays, the ability of G129R-hPRL to compete with hPRL for receptor binding was variable and resulted in more or less pronounced antagonism [54,55,61]. In contrast, others claimed that G129R-hPRL intrinsically induces apoptosis by exerting effects opposite to those of hPRL on the expression/activation of anti-apoptotic (bcl-2 and TGF $\beta$ ) or pro-apoptotic (TGF $\beta$  1, caspase-3 and bax) proteins, leading to the inhibition of breast cancer cell growth [62-64]. Since these effects were not observed in the context of a competition with hPRL in the assays, this suggests that G129R-hPRL activates specific signaling cascades resulting in biological effects opposed to those of hPRL. This is in contradiction with our data which indicate that when G129R-hPRL activates the PRLR, it exerts PRL-like rather than anti-PRL actions [55,65]. Results obtained using transgenic mice expressing G129R-hPRL are also unclear, since others reported versatile phenotypes reflecting either agonistic or

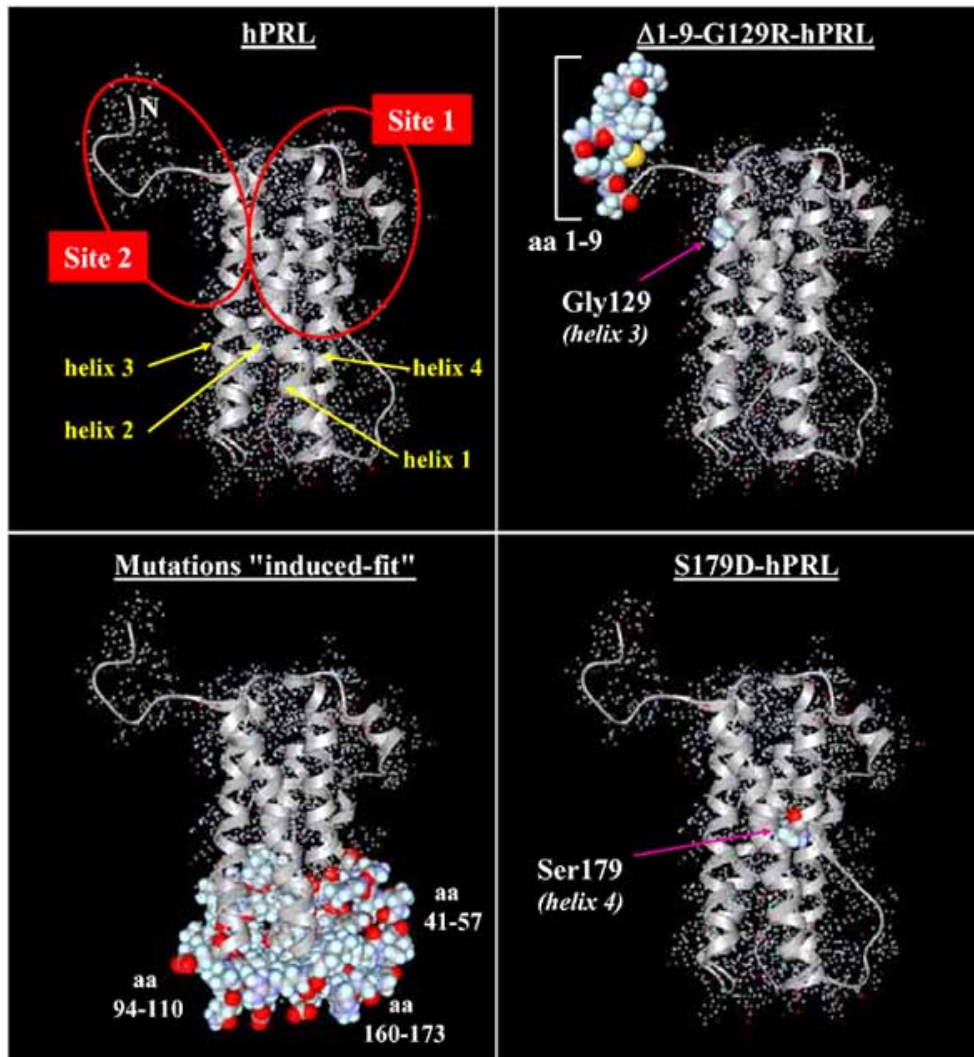
antagonistic activity depending on the age of the animals [9,64]. In contrast, our ongoing studies reveal that over-expression of G129R-hPRL in transgenic mice leads to various phenotypes reminiscent of those exhibited by PRL-transgenic animals [13,28], including prostate hyperplasia and mammary over-development. The biological properties of this analog are thus far to be unambiguously delineated.

One of the major weakness of G129R-hPRL is that the glycine mutation drops the global affinity for the receptor by one log unit, which is presumably due to alteration of site 2 affinity [61]. As a result, efficient competition with WT hPRL requires the analog to be used in at least 10-fold excess. Despite the intrinsic limits of G129R-hPRL as an antagonist, the growth of breast cancer cells xenografted in immunodeficient mice was shown to be reduced in the animals treated chronically by G129R-hPRL [66]. Similar inhibitory effect on mammary tumor rate was observed in G129R-hPRL transgenic mice treated with DMBA (a potent carcinogen) compared to control animals [67].

In summary, even if conflicting data have been obtained regarding the antagonistic activity of G129R-hPRL, and the mechanisms underlying this property, it is clear that this molecule is not a pure antagonist, as it displays residual agonism in many situations. Its ultimate activity (agonist versus antagonist) is directed by various parameters, including assay sensitivity, cell responsiveness, species specificity and probably many others as well [9]. This presumably makes this molecule a poor candidate for drug development.

### 2.1.c. 1-9-G129R-hPRL

Recent studies have shown that the N-terminus of ovine placental lactogen (oPL) plays a critical role in the binding site 2 of this hormone [68]. Since PL and PRL are homologous proteins that activate the same receptor, this prompted us to perform various deletions of the N-terminal region in G129R-hPRL (Fig. (2)). The resulting analogs were named 1-9-G129R-hPRL and 1-14-G129R-hPRL depending whether 9 or 14 N-terminal residues were removed [69]. Similarly to G129R-hPRL, these mutants exhibit 10-fold reduced affinity for the human PRLR compared to WT hPRL [70]. With respect to *in vitro* cell bioassays, N-terminal deletions did not markedly improve the antagonistic properties of these new analogs compared to G129R-hPRL. In sharp contrast, whereas the latter displayed agonistic activities in many cell bioassays, the truncated analogs failed to stimulate the receptor at a detectable level in any of the assays that we used [71-73]. Thus, the absence of residual agonism confers to N-terminal truncated G129R analogs the great advantage of acting as pure PRLR antagonists. Treatment of mice with very high doses of these new antagonists failed to reveal any detectable agonism, in contrast to G129R-hPRL. Accordingly, transgenic mice over-expressing 1-9-G129R-hPRL do not develop prostate hyperplasia, further confirming *in vivo* the absence of residual agonism. The intellectual property of our patent application is based on that unique property among PRLR antagonists [69]. The molecular mechanism underlying the abolition of the residual agonistic activity after removal of the N-terminus are under investigation.



**Fig. (2).** Three-dimensional representation of the residues that were mutated to generate some of the prolactin receptor antagonists discussed in this article.

The 3D structure of WT hPRL is represented in the top left panel, using one of the 20 nuclear magnetic resonance structures of human prolactin [78]. The four alpha-helices are represented, and the 2 binding sites are located on the protein. The pure antagonist 1-9-G129R-hPRL (top right) was obtained by truncating the 9 N-terminal residues (represented in CPK) and by substituting an arginine for the natural glycine 129 (represented in CPK), which impairs binding site 2. In contrast, serine 179 is far from site 2 and is buried inside the hydrophobic core of the protein (bottom right). The mechanism by which its replacement by an aspartate (S179D-hPRL) results in an alternate agonist remains unknown. Finally, the bottom left panel displays the three stretches of residues that were proposed to play a role in the PRL induced-fit mechanism; this representation highlights that they are located opposite to both binding sites.

Antagonistic properties were further assessed *in vivo* by co-injecting hPRL and the analog in a 1:50 molar ratio to compensate the lower affinity of the antagonist, which showed abolition of all PRL-mediated signals investigated (activation of MAP kinase in mammary tissue, of Stat3 and Stat5 in the liver) [71]. Using transgenic mice that develop prostate hyperplasia due to local PRL overexpression [14], we have demonstrated that 1-9-G129R-hPRL was able to inhibit various constitutively activated PRLR signaling pathways (MAPK, Stat5), while G129R-hPRL was much less efficient. These results indicate that 1-9-G129R-hPRL is able to exert some inhibition on the biological effects of paracrine/autocrine PRL in prostate tumors [71,74]. It is noteworthy, however, that efficient inhibition of autocrine

PRL in prostate-specific PRL transgenics required an elevated circulating concentration of 1-9-G129R-hPRL (1-2  $\mu$ M). This is presumably due to both very high local PRL concentration in the prostate, and low affinity of the antagonist for the mouse PRLR. Ongoing studies are aimed at demonstrating whether 1-9-G129R-hPRL can prevent or delay tumor appearance/growth with long-term treatment. The first approach involves the use of double transgenic mice expressing autocrine PRL in the prostate, and systemic antagonist. The second involves xenografts of human prostate and breast tumor cells in immunodeficient mice. Based on the results published for G129R-hPRL in such models [66,67], 1-9-G129R-hPRL should be at least equally efficacious, if not more so.

In the family of monomeric human PRL/GH ligands harbouring mutation of the helix 3 glycine, 1-9-G129R-hPRL is the only one targeting a single receptor (PRLR) and exhibiting no residual agonistic activity. This mutant is thus the best candidate in this class of molecule for drug development. Since two regions of 1-9-G129R-hPRL are mutated (N-terminus deletion and the helix 3 glycine), it is possible that anti-antagonist antibodies could be produced in humans. This situation was also met in a few patients treated with pegvisomant (carrying 9 mutations), although antibody production did not seem to be a major problem. We must await clinical trials to see if this occurs, and in this case if it impairs the actions of PRLR antagonists.

## 2.2. Mutations Interfering with the Potential Induced-Fit

Another way to generate potential PRLR antagonists has been proposed more recently. It is based on the concept that the binding of lactogens to the PRLR could occur in a sequential and cooperative manner. This means that the ligand would undergo conformational changes upon binding, that are required to achieve receptor activation. Following this model, called "induced-fit", the first step of hormone-receptor interaction (via site 1) would induce conformational changes within site 2, which would be necessary for the latter to interact with the second receptor molecule. The hypothesis is that mutations preventing these conformational changes will convert PRL from an agonist to an antagonist. This concept of induced-fit has been well documented for hGH binding to the PRLR. The comparison of free and receptor-bound structures of hGH showed that two regions of the ligand adopt a different conformation upon binding. The first involves aa 38-49, which belong to the loop just downstream helix 1; upon receptor binding, this stretch of amino acids adopts a mini-helical structure. The second involves amino acids 90-99, which encompasses the small loop linking helices 2 and 3; upon binding, this loop folds in a helical structure, thereby extending helix 2 by a few amino acids [75,76]. These changes lead to the repositioning of helix 3, which allows the interaction of site 2 (involving surfaces of helices 1 and 3) with the receptor. Various intramolecular hydrophobic interactions within GH have been suggested to be responsible for these conformational changes [75,77].

A similar mechanism has been proposed by Brooks and colleagues for hPRL binding to the PRLR. However, the arguments are more sparse, among others because there is currently no receptor-bound 3D structure for PRL to be compared to free hPRL structure [78]. An alternative approach involving surface plasmon resonance (Biacore) has been used to investigate whether receptor binding of PRL sites 1 and 2 were independent or, in contrast, coupled [70]. Briefly, when hPRL is immobilized on the chip via site 1 (which then becomes inaccessible), no interaction occurs between site 2 and the PRLbp. In contrast, when the hormone is immobilized via site 2, site 1 remains able to interact with the soluble receptor. These data were interpreted as an evidence that the binding capacity of site 2 requires initial receptor binding at site 1. Structural changes inside the ligand are also supported by changes of tryptophan fluorescence upon receptor binding, although this approach could not identify the residues involved in these structural

readjustments [70]. The intellectual property of the patent application filed by Brooks and colleagues is based on the introduction of mutations in regions of hPRL that are outside receptor binding sites 1 and 2, but are assumed to play a role in the induced-fit mechanism [79]. By homology to hGH, the 41-57 region of hPRL (mapping approximately aa 32-46 in hGH) is proposed to be involved in this process. Other regions involve aa 94-110 (loop connecting helices 2 and 3), and aa 160-173 (N-terminus to helix 4) of hPRL (Fig. 2).

In the current state of the art, the strength of this strategy to generate PRLR antagonists remains questionable. First, the induced-fit theory is not unanimously accepted, since RMN studies of PRL failed to display any significant structural change in the hormone whether it was analyzed in the absence or in the presence of meaning bound to the soluble PRLR [78]. This would argue against the induced-fit mechanism. Second, and more importantly, we are not aware of any data demonstrating that the introduction of mutations in abovementioned regions conferred antagonistic properties in any cell system. Mutants harboring replacements or deletions within the regions of interest have only been characterized in agonistic assays - i.e. when each analog is tested alone, without PRL to be competed, which clearly highlighted the detrimental effect of some of these mutations on agonistic properties [70,76,77]. However, to assess their ability to antagonize PRL actions, competition assays showing dose-response inhibition of PRL activity by any of these analogs is awaited.

## 2.3. S179D-hPRL, an Alternative Agonist

Based on the observation that naturally-occurring phosphorylated rat PRL is an antagonist of PRL actions in proliferation assays [80], a molecular mimic of phosphorylated human PRL was generated by substituting an aspartate for the natural serine 179 (Fig. 2), since the latter was proposed to be the phosphorylated residue in the human sequence [81].

The mechanism of action of S179D-hPRL remains partly unclear, although it is now well established that, in contrast to PRL/GH antagonists harboring a mutation of the helix 3 glycine, it is intrinsically a receptor agonist [52]. It has been reported to be a growth antagonist, including in the context of tumors [82,83], in part because it inhibits signaling triggered by PRL [84,85], and in part because it induces its own intracellular signal cascades [86]. These effects would favor cell differentiation (at the expense of proliferation) [87], cell cycle arrest and apoptosis [88,89]. Recently, it has also been reported as an inhibitor of angiogenesis [90], and an anti-inflammatory agent [91]. In terms of biological activities, however, both PRL-like and anti-PRL actions were attributed to S179D-hPRL [9]. Since S179D-hPRL was reported to regulate PRLR expression, part of its specific properties may result from changes in the ratio of the various receptor isoforms, which are known to differ in their ability to activate signaling pathways [1]. However, a clear picture is still lacking, since S179D-hPRL was shown to induce expression of long PRLR isoforms in human breast cancer cells MCF-7 [84], and otherwise to up-regulate expression of the short receptor in normal mouse mammary cells HC11 [86].

It is clear that the mechanism of action of S179D-hPRL is non conventional. At the molecular level, the mechanisms underlying both competitive antagonistic properties and alternate signalling remain to be elucidated. The affinity of S179D-hPRL for the human PRLR is >1 log lower than that of hPRL [52], which correlates with dose-response curves displaced to the right in several bioassays [52,65,92]. Therefore, it is surprising that very low concentrations (0.1 nM) significantly inhibit the growth of human prostate tumor cell lines, even more when the inhibition is supposed to target autocrine PRL, which is known to be very difficult to inhibit [83]. Understanding the specific features of the molecular interaction between S179D-hPRL and the human PRLR is also a missing information. Serine 179 belongs to helix 4 [93], which contains several binding site 1 determinants [3]. However, this residue points towards the inside of the helix bundle (Fig. (2)), therefore it is not anticipated to be involved in a direct interaction with the receptor. Mutation of serine into an aspartate may also result in local disturbance of binding site 1, as perhaps natural phosphorylation of serine 179 does in the physiological context. How such potential structural changes lead to a ligand exhibiting properties so different from WT hPRL remains to be elucidated.

One important issue relates to the production of this compound by recombinant technology. We and others [52,92] have shown that S179D-hPRL is one of the rare hPRL mutant engineered to date that strongly aggregates during the refolding step when produced in bacteria. The eventual homogeneity of these aggregates is not established, which could partly explain the controversies between the different groups that have studied S179D-hPRL. In addition, this may render difficult the production of protein batches exhibiting reproducible properties. Mammalian systems of recombinant expression seem to solve the problem [92], since there is no re-folding step, but the yields are far lower than prokaryotic systems, which may not be compatible with the development of a drug.

## 2.4. Dual Receptor Targets

### 2.4.a. Co-Administration of Anti-Tumor Drugs

Current anti-hormonal approaches of breast and prostate cancers essentially involve the use of molecules leading to the inhibition of estrogen and androgen secretion or actions. For example, Tamoxifen, an estrogen receptor antagonist, has been confirmed to be effective anti-tumor drug [94]. One of the most interesting molecules that recently appeared in this field is Herceptin, a humanized monoclonal anti-HER-2 antibody, which led to very promising results in subsets of metastatic breast cancer patients which over-express HER-2 receptor [95]. PRLR antagonists should be viewed as one new class of compounds to be added to the panel of hormone/growth factor receptor blockers, such as Tamoxifen and Herceptin.

Since tumor growth depends on many different factors/stimuli, the combination of two or more anti-hormone drugs is naturally anticipated to be more effective than a treatment involving a single drug. In agreement, G129R-hPRL and Tamoxifen were shown exert additive inhibitory on the *in vitro* growth of human breast cancer cells [96]. Recently, a

patent application was filed for the combination of G129R-hPRL and Herceptin [97]. The concept is based on an earlier report showing that autocrine PRL secreted by breast cancer cell lines stimulated tyrosine phosphorylation of HER-2 via PRLR-activation of the tyrosine kinase Jak2 [98]. Another argument is that the relative expression of PRLR and HER-2 in breast cancer cell lines seems to be correlated, highlighting potential relationships between the two systems. The combination of G129R-hPRL and Herceptin was claimed to have synergistic effect on the inhibition of cell growth and intracellular signaling of breast cancer cells, although close analysis of experimental data suggests that in some instance, the effect is more additive than synergistic.

Such strategy is certainly promising for treating breast cancer patients. However, Herceptin is a new therapy for the management of HER-2/neu-overexpressing metastatic breast cancer, which accounts for not more than 20-25% of human breast cancers. The status of PRLR signalling in *in situ* metastatic cancers remains unknown, therefore any benefit of PRLR antagonists is currently difficult to predict. Highly invasive, low differentiated breast cancer cell lines (e.g. MDA-MB231) are known to express virtually no PRLR, suggesting that the effect of PRLR antagonists may be very limited in dedifferentiated cells. In addition, a recent study [99] has suggested that PRL may actually act as a brake of the epithelial-mesenchymal transition preceding metastases [100], and that blocking PRLR signalling activates prometastatic signalling pathways. In conclusion, although the combination of Herceptin and PRLR antagonist is in theory attractive, its benefits need to be better evaluated in terms of efficacy and potential patient subsets.

### 2.4.b. Bi-Functional Molecules

Another way to combine two anti-tumor drugs is to link them within the same molecule (fusion protein). This strategy has been used to develop a new type of compounds, combining an endocrine-based entity, or apoptosis-promoting domain (G129R-hPRL), with a component regulating cell processes external to the tumor, but influencing its growth, e.g. immune responses or angiogenesis [101]. The rationale is thus to target more than one cell type using bi-functional compounds.

The first example combines G129R-hPRL and IL-2 [102]. The role of this cytokine, as a positive immunomodulator domain, is to induce tumor-specific recruitment and enhancement of T lymphocyte cytotoxicity. *in vitro* Analysis of this fusion protein showed that it was able to bind to both PRLR and IL-2 receptor. *In vivo*, it partly inhibited the growth of breast cancer xenografts slightly more efficiently than G129R-hPRL alone. One of the advantages claimed by the inventors is that, *in vivo*, the receptor-antagonizing domain (i.e. G129R-hPRL) will localize the effects of the positive immunomodulator domain (IL-2) to the diseased tissue. This statement remains to be demonstrated, however, since the PRLR is ubiquitously expressed in the body, and not specifically in the tumor. One mechanism of action that has been proposed for G129R-hPRL involves the inhibition of basal or PRL-induced Stat5 activation in human breast cancer cells, leading to apoptosis. In competition experiments, the chimaeric protein was shown to decrease PRL-induced Stat5 phosphorylation in T-47D

breast cancer cells, although less efficiently than G129R-hPRL itself. This presumably reflects steric hindrance of the IL-2 subunit towards G129R-hPRL binding site 1. IL-2R-mediated cell proliferation of T cells induced by the chimaeric protein did not appear to be altered compared to IL-2. The fusion protein, as IL-2 or G129R-hPRL tested individually, were shown to not induce Stat5 activation in breast cancer cells. Since IL-2 is known to activate this transcription factor, this finding suggests that the cell lines used did not express the IL-2 receptor. In contrast, the development of breast tumour in patients has been associated with an increased expression of the IL-2 receptor complex [103]. Thus, it is not perfectly clear how the G129R-IL-2 fusion will guarantee the inhibition of Stat5 activation in the tumor. Finally, another drawback of such class of molecules is the apparent difficulties to produce reasonable amounts of recombinant product [101], with yields that may reveal to be incompatible with production up-scaling required for drug development. In conclusion, although the increase of the molecular mass should confer to such fusion proteins an increased half-life *in vivo*, this type of compound present some weakness that should not be ignored.

The second example of bi-functional molecules combines G129R-hPRL and antiangiogenic entities [104]. Endostatin and angiostatin are potent inhibitors of angiogenesis, that have been shown to prevent tumor growth/occurrence in many studies [105]. G129R-endostatin fusion protein bound the PRLR expressed in breast cancer cells with similar affinity compared to G129R-hPRL. Binding of G129R-endostatin to the putative endostatin receptor expressed in endothelial cells was also assessed, but the qualitative data of the method used (immunofluorescence) prevented direct evaluation of its affinity compared to endostatin [106]. Antagonistic activity of the fusion protein were also assessed towards i) PRL-induced Stat5 phosphorylation and proliferation of breast cancer cells, and ii) endothelial tube formation, with no major change compared to G129R-hPRL or endostatin alone, respectively. One theoretical advantage of this type of fusion protein is the increased molecular mass (43 kDa versus 23 kDa for G129R-hPRL). This is close to the passive cut-off of glomerular filtration (45-50 kDa), which probably accounts for the significantly increased half-life of the fusion protein compared to PRLR antagonist or endostatin alone [106]. However, experiments investigating the growth rate of breast cancer xenografts in nude mice were partly disappointing, since G129R-endostatin was not significantly more efficient than endostatin alone or endostatin and G129R-hPRL co-injected. The advantages of this fusion protein over its individual components will have to be confirmed in further studies.

### 3. CURRENT & FUTURE DEVELOPMENTS

Mutation of the helix 3 glycine is the paradigm for converting PRL/GH hormones from agonists to antagonists [9]. However, this mutation alone is not sufficient to fulfill all requirements for generating a drug. In hPRL even more than in hGH, this mutation has a detrimental effect on global affinity for the receptor. Since the mechanism of action of these analogs is a competition with WT hormones, it is clear that affinity loss goes the wrong way. We have tried various strategies to improve the affinity of G129R-hPRL, without

success [107]. Alternative strategies or mutations will have to be investigated to solve this issue. Another important point relates to the ability of the glycine mutation to completely abolish the agonistic properties. In the family of molecules harbouring this prototype mutation, 1-9-G129R-hPRL is the only one exhibiting no detectable residual agonistic activity. This property is important since it suggests that this compound could be used at high concentration, in order to compensate its low affinity, without any risk to activate the receptor. Last but not least, it targets a unique receptor, namely the PRLR, which is interesting pharmacologically speaking. Mutations other than the helix 3 glycine have also been developed. The best characterized is S179D-hPRL. This analog is a very unusual molecule, since its antagonistic properties seem to result from alternate agonistic pathways. This renders the evaluation of its ultimate effects more difficult than for classical competitive antagonists. In addition, one key question still remains unanswered: what differs between WT hPRL and S179D-hPRL at the level of receptor binding/activation to explain that this PRLR agonist activates specific intracellular signaling? This missing information is probably a pre-requisite to the development of this molecule as a drug. Finally, mutations affecting the induced-fit mechanism have been proposed to generate PRLR antagonists, but this strategy is still at the level of hypotheses, and it awaits stronger experimental evidence.

All these hormone analogs present a common weakness, which is short half-life *in vivo*. Due to their small molecular mass (22-23 kDa), they are eliminated from the circulation at similar rate than unmodified PRL/GH (15-30 minutes). Rapid elimination combined with low affinity could impose these antagonists to be used in high amount to be effective. This issue was partly solved for the GHR antagonist (pegvisomant) by conjugating the GH mutant to 5 polyethylene (PEG) moieties. This modification increases half-life up to 70 hours, but it also reduces biological potency due to steric hindrance of PEG entities. PEGylation, or any other mean to prolong half-life *in vivo*, will have to be tested for PRLR antagonists. An alternative way to increase the mass of the antagonists is to generate fusion proteins. From the strict pharmacokinetic point of view, fusion proteins stay longer in the circulation, which is clearly an advantage. However, the benefits of targeting other compartments than the tumor itself (immune system, vasculature) needs to be confirmed, since data currently available do not argue for a clear improvement of the anti-tumor potency of the fusion proteins over their individual components. Beyond the intrinsic properties of these various PRLR antagonists, defined to date using cell or animal models only, clinical trials will be necessary to evaluate their effectiveness and safety in treating PRL-related pathologies, including hyperprolactinemia (whatever its origin) and benign or malignant tumors affecting target tissues of PRL, the best characterized of which involve the breast and the prostate.

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