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Novel peptidomimetics related to Gonadotropin releasing hormone (GnRH) †

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Novel GnRH I and II analogues were designed and synthesized by Solid Phase Peptides Synthesis (SPPS), since GnRH has antiproliferative property, but poor metabolic stability. To rationalize synthetic difficulties, molecular dynamics simulations were performed, showing the conformational behavior of three derivatives. Among the two peptidomimetics series (**Ie,f** and **IIe,f** , GnRH I and GnRH II analogues respectively) several compounds (**Id-f** and **IIc-e**) showed a significant binding affinity. In particular, derivative **Ie** has an increased metabolic stability with respect to the physiological ligand (**Ie** $t_{1/2}$= 3.96 h *versus* GnRH I $t_{1/2}$ = 2.63 h).

Introduction

The steroid hormones, estrogens and androgens, are key drivers of several types of cancer, such as that of prostate, one of the most common invasive cancer in men. At present, hormone therapies demonstrate success on this type of tumor, highlighting its dependence on gonadal. Although most prostate tumors initially respond to hormone therapy[1] by inhibition of their growth, others stopped respond to the treatment, indicating the need to develop novel therapeutic approches.

Prevoius research[2] has shown that GnRHs (GnRH I and II) and their receptors are expressed in cancer cells and the activation of GnRH receptors negatively regulates their proliferation for instance in prostate cancer.

GnRH I is a hypotalamic decapeptide (pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2, Figure 1) able to bind specific receptors on pituitary gonadotrope cells to modulate the synthesis and secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH)[3] that, in turn, regulate gonadal steroidogenesis and gametogenesis. Numerous experimental data showed an overexpression of GnRH I and its receptor in human malignant tumors, such as prostate, breast, uterine, ovarian cancers and also in the non-reproductive ones.

In addition to its direct action on cancer cells, GnRH I affect their growth by triggering the secretion of steroid hormones from the gonads.

Moreover, the antiproliferative activity of GnRH analogues have been proven through several studies.[3]

Furthermore a second form of GnRH (GnRH II, Chart 1) has been identified in humans.[4] It is a decapeptide, encoded by a diverse gene, differing from the classical GnRH (GnRH I, Chart 1) in three amino acids . The ability of GnRH II to inhibit selectively the growth of cancer cells has been recently discovered.[2] This occurs through a different mechanism of action than the classic GnRH agonists one, which involves the suppression of secretion of pituitary hormones and hence production of sex hormones[1]

Although GnRH antagonists (which can be considered as a new generation of male and female contraceptives with add-back gonadal steroids[3]) have found increasing application in the therapy of prostate cancer,[5a] however recent studies on the activation of the receptors by GnRH agonists also showed a significant reduction of the proliferation of prostate cancer cells both *in vitro* and *in vivo*[5b] (when inoculated into nude mice). Moreover, GnRH agonists are able to decrease the invasive behavior[6,7] of androgen-dependent and independent prostate cancer cells,[8-10] although in this case the administration may be used as an adjuvant therapy. Preliminary experimental data indicate that GnRH agonists increase expression of genes related to apoptosis and develop a cytostatic action rather than cytotoxic.[11] They can sensitize prostate cancer cells to the activity of pro-apoptotic agents, which can be considered as a possible targeted therapy, in combination with traditional chemotherapy, with the aim to increase antiproliferative activity and possibly reduce undesired side-effects .[12]

As many hormones of peptidic nature, GnRH I and II are highly flexible molecules, existing in solution as a mixture of different conformers in *equilibrium*. GnRH is characterized by the presence of a β-turn in position Gly[6]-Leu[7], as proven through the substitution of Gly[6] [13] with D-amino acids, Leu[7] with N-methyl-leucine and the introduction of a lactam bridge, able to force the angles Ψ of Gly[6] and Φ of Leu[7] [14] favoring a β-turn conformation.[15] All these modifications resulted to give the maintenance or improvement of biological activity.

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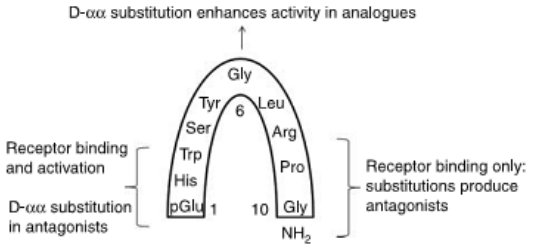
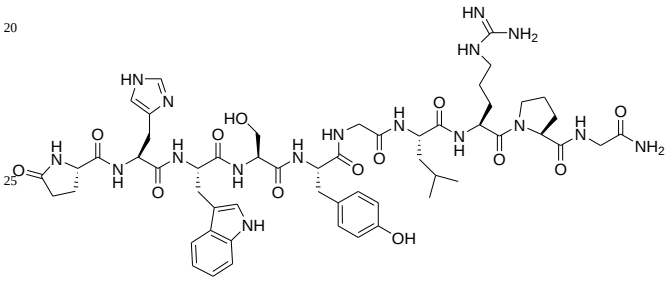


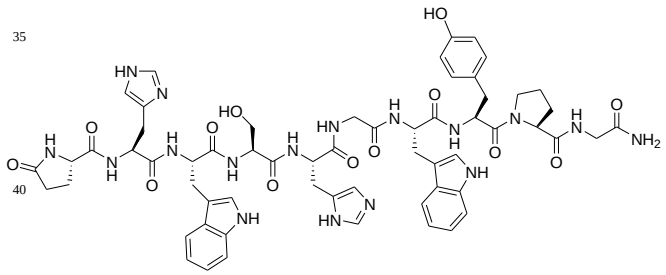
Figure 1.[16] Schematic representation of GnRH I in the folded conformation in which it is bound to the GnRH pituitary receptor.

Since the β-turn present in the GnRH-I and II seems to be essential for the biological activity of these hormones, a large number of cyclic peptides related to GnRH-I have been designed to maintain this property.



pE	H	W	S	Y	G	L	R	P	G-NH$_2$

GnRHI



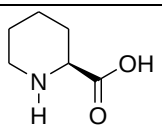
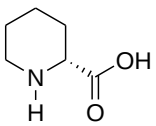
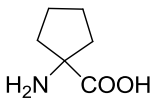
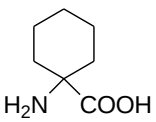
pE	H	W	S	H	G	W	Y	P	G-NH$_2$

GnRHII

Chart 1

The replacement of the glycine residue at position 6 has been shown to stabilize of the folded conformation and decreases the metabolic clearance.[17] All the analogues, agonists and antagonists, have been synthesized with this structural modification (for instance, among the agonists, Leuprolide has a D-Leu residue,[8a] while Goreselin a D-Ser[18-20] at position 6). In order to identify new potential peptidomimetic analogues of GnRH, with potential biological activity, we focused our attention on the synthesis of two novel series (**I** and **II**, GnRH I and GnRH II analogues, respectively) by SPPS methodology.[21,22] We considered the introduction of non natural amino acids at position 6, such as those with cycled structures, which are known to favour the interaction between the receptor and the ligand, because of their β-turn inducer nature (**I-II a-e**, Table 1). Of note, the steric hindrance and the minor peptidic character of the novel analogues could reduce the metabolic cleavage, occurring at position 6-7, and increase their biological half-life.[23-24]

Table 1. GnRH I and II analogues **I-II a-e**.

Analogue	Non natural aa at position 6	Name	Chemical Structure
I-IIa	L-Pip-OH	2*S*-piperidine-2-carboxylic acid	
I-IIb	D-Pip-OH	2*R*-piperidine-2-carboxylic acid	
I-IIc	AC4C-OH	1-aminocyclobutancarboxylic acid	
I-IId	AC5C-OH	1-aminocyclopentan carboxylic acid	
I-IIe	AC6C-OH	1-aminocyclohexan carboxylic acid	

Furthermore in attempt to improve the metabolic stability, few other derivatives (Table 2) of GnRHI (**I f-g**) and GnRH II (**II f-g)** have been prepared, using **Cptd** (3-carboxymethyl-1-phenyl-1,3,8-triazaspiro [4.5]decan-4-one. The introduction of this latter could reduce the peptidic character of the new derivatives. The **Cptd** moiety replaces a variable number of amino acids in the GnRH structure, in particular in compounds **I-IIf** it has approximately the same length of the three amino acids replaced. Instead, compounds **I-IIg** are shorter than the natural GnRH.

This paper describes the synthesis of compounds **I-II a-g**, molecular dynamics simulations performed on some of them, to explain some synthetic problems, as well as the human GnRH binding assay data. Finally, preliminary metabolic stability tests of the most interesting compounds are also reported.

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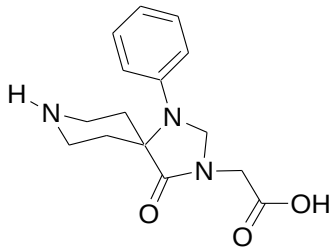
Table 2. GnRH I and II analogues **I-II f-g**.



Cptd

Analogue	Sequence
If	pE-H-W-Cptd-L-R-P-G-NH_2
Ig	pE-H- Cptd-R-P-G-NH_2
IIf	pE-H-W-Cptd-W-Y-P-G-NH_2
IIg	pE-H-Cptd-Y-P-G-NH_2

Chemistry

Solid Phase Peptides Synthesis (SPPS) of GnRH I and GnRH II analogues

The peptidomimetics were synthesized by the general SPPS procedure reported in Scheme 1.

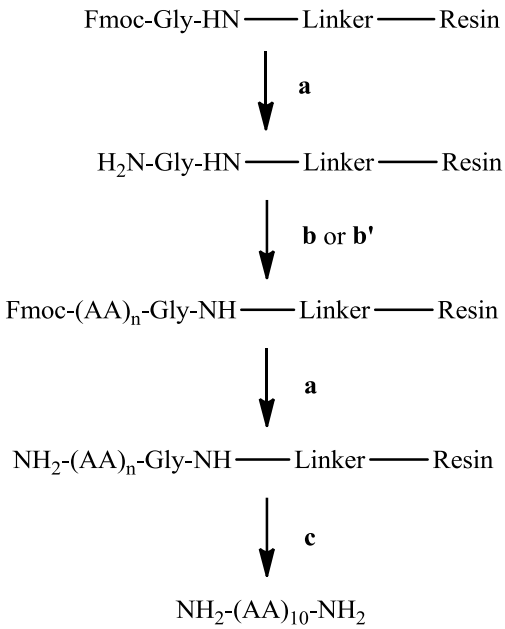
Series **I a**,**b**,**d-g** and **II a-g** were obtained on solid phase using the resin Rink amide MBHA (4-methyl-benzhydryl-hydroxy-ammine), that provides peptides carboxy amidated, and Fmoc (fluorenyl-methoxycarbonyl group) to protect the N-terminal end. The support consists in a modified Rink amide linker, bound through a Norleucine residue to the MBHA resin. Glycine, Proline, Leucine and Pyroglutamic acid did not require the use of protecting groups.

Peptide synthesis begins with deprotection of Fmoc group from the Rink Amide MBHA resin (activation of the resin) using Piperidine in DMF.

In coupling reaction for amino acids and non natural amino acids **(**L-Pip-OH, D-Pip-OH, AC4C-OH, AC5C-OH, AC6C-OH and pGlu), TBTU and HOBt were used as coupling reagent in alkaline conditions (DIEA).This reactive is able to generate a benzotriazole ester, characterized by excellent activity and chiral stability. Instead, in coupling reaction with **Cptd**, HATU was used as coupling reagent, because of the required stronger conditions, due to the steric hindrance of the compound.

The synthesis occurred very quickly, without side reactions. The first amino acid Glycine was attached to the resin by the C-terminal end, while the N-terminal end was deprotected before the following coupling.

After the complete synthesis, the peptide was washed with DCM and cleaved with TFA and a scavenger cocktail. The latter consists in a mixture of thioanisole, to accelerate the removal of some protecting groups in TFA, phenol, and triisopropylsilane (TIS), very effective in the case of peptides containing Arg (Pmc) and Trp (Boc) and for the elimination of free cations, formed by the cleavage of Trt resin and Rink Amide linker. Finally, peptides were purified by HPLC. All the designed compounds were obtained according to this scheme with the exception of **Ic**, since the shorter derivative **D-Ic**, precursor of **Ic** lacking of pyroglutamic acid (pGlu), was achieved.



Scheme 1. SPPS procedure for the synthesis of GnRH I and GnRH II analogues. **a**) 20% piperidine in DMF; **b**) Fmoc-aa, TBTU, HOBt, DIEA, DMF (for natural Fmoc amino acids L-Pip-OH, D-Pip-OH, AC4C-OH, AC5C-OH, AC6C-OH and pGlu); **b'**) HATU, DMF (for Cptd); **c**) TFA, water, phenol, thioanisole, triisopropylsilane.

Molecular Dynamics Simulations

Molecular dynamics (MD) simulations were performed on the nonapeptide His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly **1** (derivative of GnRH I without pGlu) and derivatives **D-Ic**, **D-Id**, **D-Ie** (Figure 2), which are the corresponding analogues of products **Ic**, **Id**, **Ie**, lacking pGlu. The MD simulations were used to properly compare the behaviour of these analogues in order to understand the unsuccessful coupling among pGlu and the nonapeptide **D-Ic**, last intermediate of **Ic** synthesis. The N-terminus residue was protected as ACE (acetyl), while the C-terminus as NME (N-methyl).

All the calculations were carried out using the SANDER module of the AMBER 10 package[25] along with the ff03 force field for **1** and the general amber force field (gaff) for **D-Ic**, **D-Id**, **D-Ie** and RESP atomic charges. The TIP3P model[26] was employed to explicitely represent water molecules. The polypeptides were immersed in a box containing about 1300 water molecules. At first, the energy of the water molecules was minimized, keeping the atoms of the polypeptides frozen.

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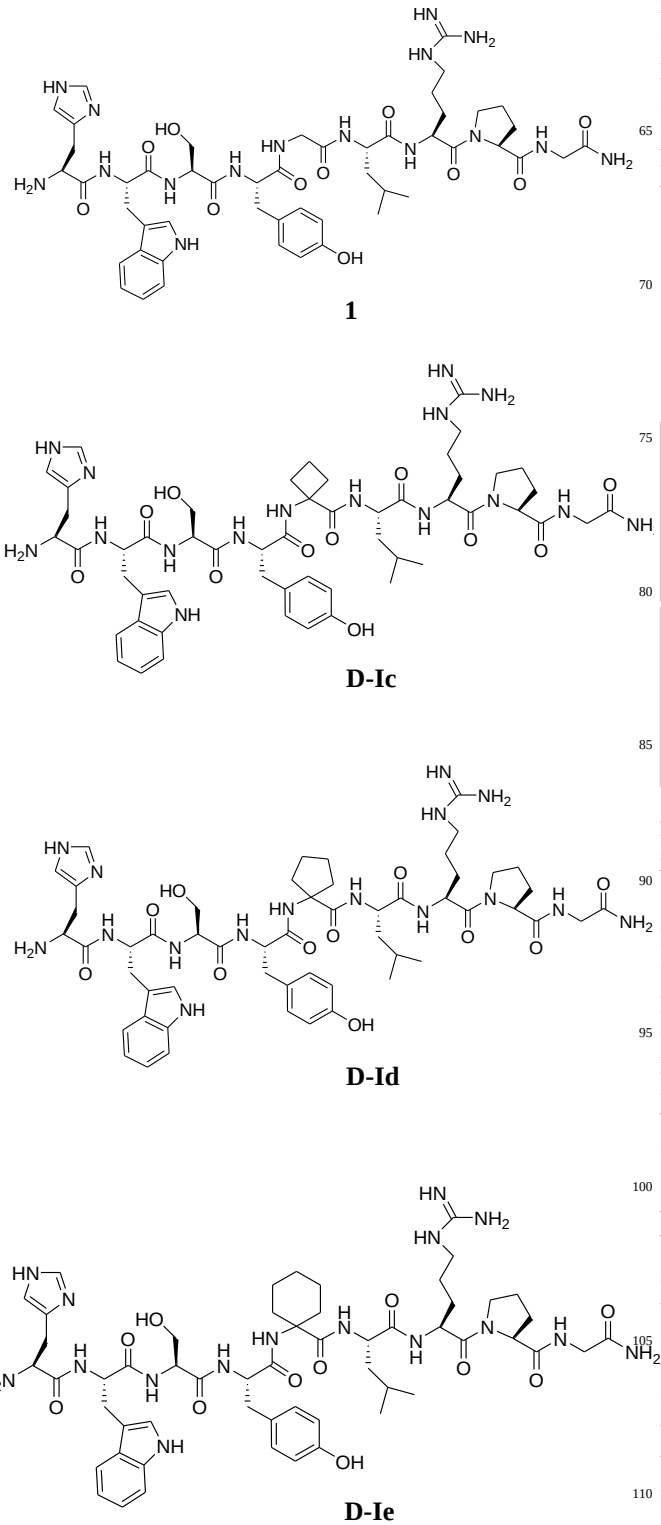

Figure 2. Nonapeptidic analogue of GnRH I and its derivatives **D-Ic**, **D-Id**, **D-Ie**.

Then, a minimization of the whole system was performed by setting a convergence criterion on the gradient of 10^{-4} kcal mol^{-1} Å^{-1}. Prior to starting the MD simulations, the system was equilibrated for 40 ps at 300 K in isocore conditions (NVT). Subsequently, 5 ns of MD simulations in isothermal-isobaric ensemble were carried out at 300 K with a 2 fs time-step (NPT). In the production runs, the systems were performed in periodic boundary conditions. Van der Waals and short-range electrostatic interactions were estimated within a 8 Å cutoff. SHAKE algorithm[27] was applied to all bonds involving hydrogen atoms. VMD 1.8.7 was used for molecular visualization and for animating trajectory data.[28]

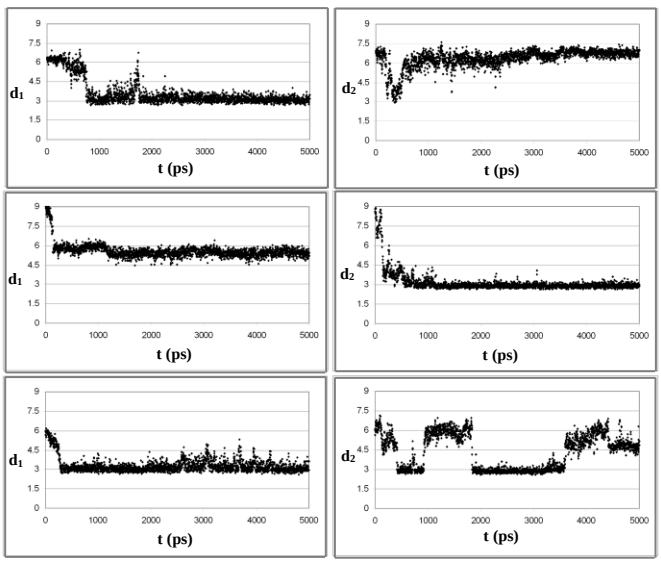

Figure 3. History of d_1 (left) and d_2 (right) in the MD simulation starting from a linear arrangement of peptides **D-Ic-e**, from top to bottom.

Particular attention was focussed on the possibility of β-turn formation. In fact, it is known that the presence of a β-turn in position Gly^6-Leu^7 of GnRH I is essential for biological activity. During the MD simulation, in the case of **1**, the presence of two β-turns, already known in literature was detected: the first one between Tyr and Arg, the second one between Ser and Leu.[23] Previous structure-function studies showed that the substitution of Tyr to eliminate the postulated β-II turn with Arg led to a reduction in the binding affinity, while the substitution of Leu to remove the envisaged H bond between the C=O of Ser and the NH of Leu did not reduce activity. So, MD trajectories were examined in order to assess the maintenance of these turns and the distances d_1 between C(Tyr)-N(Arg) and d_2 between C(Ser)-N(Leu). In Figure 3 the trajectories of d_1 and d_2 for peptides **D-Ic**, **D-Id** and **D-Ie** are shown, respectively, from top to bottom. In the case of **D-Ic**, the β-II turn between Tyr and Arg is present during about all the MD simulation, while the other one (Ser-Leu) tries to form at the beginning of the simulation but breaks almost immediately and C and N atoms keep themselves at a distance of about 6 Å. On the contrary, in the case of peptide **D-Id**, the Tyr-Arg β-turn never forms and the distance between C and N is about 5 Å, while the Ser-Leu β-turn is present for about all the simulation. Finally, as expected on the base of the geometry of tetrahedral C atom of hexacyclic ring, peptide **D-Ie** shows the most similar behaviour to the reference compound **1**. In fact, the presence of both β-turn was detected during the simulation.

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The different conformational preferences of **D-Ic**, and, in particular, the complete lack of the β-turn between Ser-Leu, present in all the other peptides, seem to explain the synthetic difficulties found to bind the last monomer to its polypeptidic chain. More precisely, the absence of the second β-turn is supposed to lead to an unfavourable orientation of the peptidic chain for introducing the N-terminal amino acid.
Moreover, the MD simulations show that the peptidomimetic **D-Ie** presents the most similar behavior to the reference.

Biological evaluation

Compounds **I a,b,d-g**, **D-Ic**, **IIa-g** were pharmacologically evaluated for their ability to bind to GnRH receptor. Before determining the binding affinities of these compounds their ability to inhibit specific binding of [^{125}I]-DTyr^6-His^5-GnRH to human type I GnRH receptor was tested, at the single concentration of 500 nM, under equilibrium conditions (Figure 4), since it is known that GnRH II binds the type I GnRH receptor in different cell lines (e.g. prostate and ovarian cancer cells)[2]. The Leuprolide (500 nM) was used as a reference compound. The radioligand binding experiments were performed on membrane homogenates from HEK 293 cells stably expressing human GnRH receptor. In this initial screening compounds with poor ability to inhibit [^{125}I]-DTyr^6-His^5-GnRH specific binding were not further pharmacologically evaluated. Binding affinities (-LogIC$_{50}$) for the human GnRH receptor were determined for the most interesting compounds **Id-f** and **IIc-e** (Table 3) in radioligand competition binding experiments.

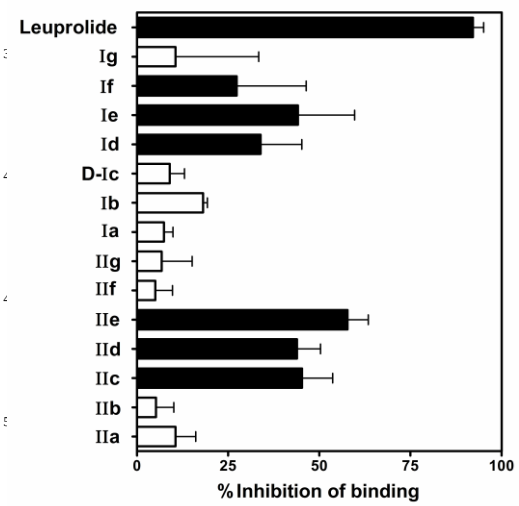

Figure 4. Inhibition of [^{125}I]-DTyr^6-His^5-GnRH specific binding by the GnRH analogues on membrane homogenates from HEK 293 cells stably expressing the human type I GnRH receptor. The bars represent the % inhibition of radioligand specific binding by 500 nM of the GnRH analogues, **I a,b,d-g**, **D-Ic**, **II a-g (**or Leuprolide as control), determined from 2-3 experiments (with their means and S.E.).

Table 3. Binding affinity values of GnRH analogues **Id-f**, **IIc-e**.

Compound	-LogIC$_{50}$± S.E (N=2-3)
Id	6.23 ± 0.03
Ie	6.26 ± 0.26
If	6.14 ± 0.28
IIc	6.72 ± 0.28
IId	6.55 ± 0.13
IIe	6.39 ± 0.40
Leuprolide	9.54 ± 0.31

Competition of [^{125}I-D-Tyr^6, His^5] GnRH specific binding by increasing concentrations of the GnRH analogues **Id-f**, **IIc-e** was performed on membrane homogenates from HEK 293 cells stably expressing the human type I GnRH receptor. The mean values and S.E. are shown from 2–3 different experiments. The data were fit to a one site competition model by nonlinear regression and the -LogIC$_{50}$ values were determined as described in the Experimetal part.
Compounds **Id-f** and **IIc-e** bound to human type I GnRH receptor in a concentration dependent manner (Figure 5).

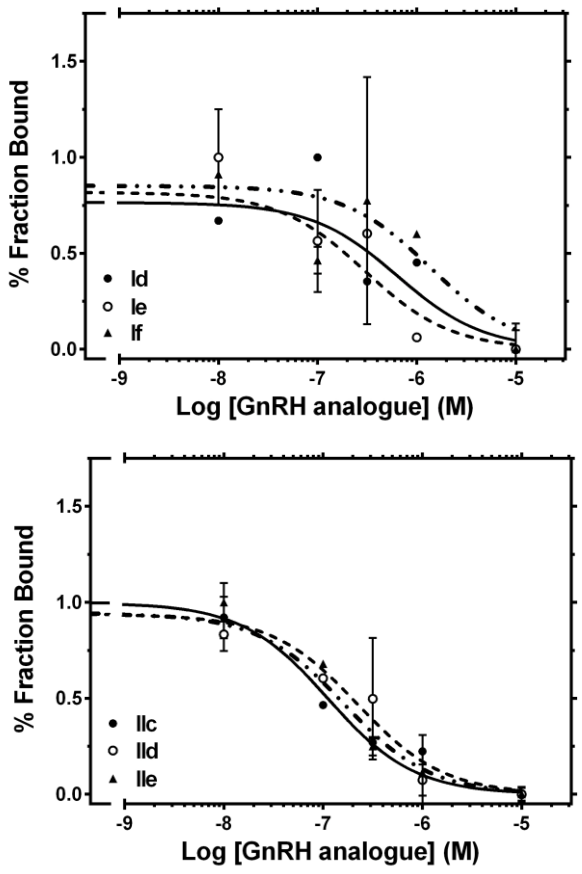

Figure 5. Competition binding isotherms of GnRH analogues **Id-f**, **IIc-e** to human type I GnRH receptor.

Since GnRH II binds the human type I GnRH receptor mediating the

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antiproliferative activity[2], compound **IIe** was choosen as reference for a colorimetric MTT assay. The latter is based on the ability of living cells to reduce the yellow MTT to purple formazan and it is widely used to quantitate cell viability and cytotoxicity of various drugs. The results indicate that similar to Leuprolide, the compound **IIe** decreased the growth of HEK 293 cells expressing the type I GnRH receptor (Figure 6). However, the antiproliferative efficacy of compound **IIe** (as determined by its absorbance ratio versus the control) was smaller than that of Leuprolide (Figure 6), in agreement with the ability of this compound to bind to type I GnRH receptor although with a smaller affinity than that of Leuprolide (Figure 5).

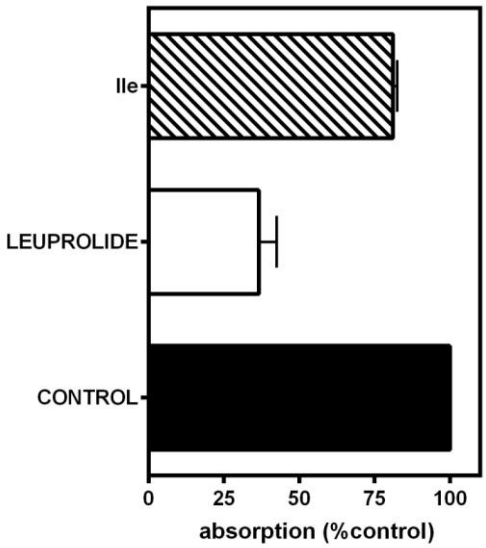

Figure 6. Effect of compound IIe on cell proliferation. Antiproliferative effect of **IIe** compound and Leuprolide were determined by their abilities to inhibit the growth of HEK 293 cells stably expressing the type 1 GnRH receptor following continuous 4 days exposure of cells to drugs. The antiproliferative effect was estimated by the MTT assay, as described in the Experimental section. The antiproliferative efficacy of **IIe** was determined by the % ratio of its normalized absorbance value versus that of the untreated cells (control). Normalization was performed by subtracting the absorbance values of wells containing only medium (control-blank) from those of sample and control. Each bar represents the mean ± standards error value for 2 independent experiments.

Metabolic stability

To evaluate the metabolic stability of GnRH I, GnRH II and their analogues **Ie** and **IIe**, that show the most interesting biological results, a preliminary assay using rat plasma was performed.

The results (Figure 7) showed that in compounds **Ie** and **IIe** the substitution of Gly6 with the non natural amino acid AC6C-OH results to improve their stability by 15-20 % after four hours when compared to GnRH I and GnRH II, respectively.

In particular, product **Ie** showed a significant increase of half life time with respect to GnRHI (**Ie** $t_{1/2}$= 3.96 h *versus* GnRHI $t_{1/2}$ = 2.63 h) (For further detail see ESI).

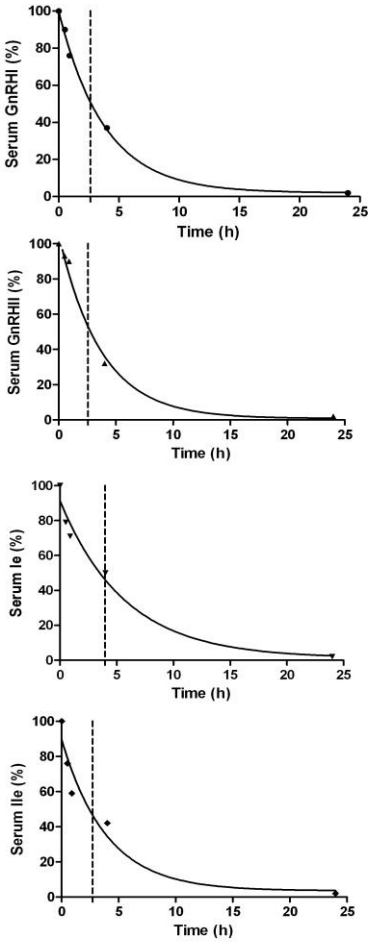

Figure 7. Metabolic stability of GnRHI-II and their derivatives **Ie** and **IIe**.

Conclusions

Our research aimed to synthesise novel peptidomimetics as analogues of GnRH I and II, in order to identify new agonist for the type I GnRH receptor with potential antiproliferative activity.

All derivatives have been synthesized by solid phase peptide synthesis with the exception of **Ic**. MD simulations, performed on peptidomimetics **Ic-e** deprived of the last residue (**D-Ic-e**), explained the difficulty in the synthetis of **Ic**, probably due to a different conformational behaviour of this analogue and in particular to the complete lack of the β-turn between Ser-Leu, present both in **D-Id** and **D-Ie**. Moreover, calculations showed that **D-Ie** might have the most similar behaviour to the reference compound **1**.

The synthesized products were tested in binding assays on human type I GnRH receptor. Compounds **Id-f** and **IIc-e** exhibited considerable binding affinities, supporting the assumption that the introduction of β-turn inducers in GnRH I and II structures were essential for the binding to GnRH receptor and confirming the fundamental role of the β-turn presence for the activity. Biological data showed that compounds **I-IIe** may be considered the most active in their series (**I** and **II** respectively), giving experimental support to the modeling hypothesis.

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Finally, preliminary metabolic stability assays revealed that the two derivatives **Ie** and **IIe** were more stable than GnRH I and GnRH II, due to their reduced peptidic character. These results will be useful for the development of novel GnRH analogues with improved stabilities and antiproliferative activities.

Experimental Part

Chemistry

Resin, Fmoc amino-acids, solvents and reagents were purchased from IRIS Biotech GmbH (Schnelldorf, Germany) and were used without further purification. The peptides were synthesized manually using polypropylene syringes (10 mL) with a porous polyethylene disc purchased from Grace. The analytical HPLC was performed using apparatus Elite La Chrome. The preparative HPLC was performed using apparatus Waters 3000 System Controller. The spectra of high-resolution ESI-MS were recorded using a mass spectrometer LTQ-Orbitrap XL.

General procedure. The synthesis of peptides was performed on solid phase starting from 290 mg of Rink amide resin (130 mmol, loading: 0.46 mmol /g).

Swelling. Resin was weighted in a test tube. N-methylpyrrolidone (NMP, 1 mL for 130 mmol of resin) and dichloromethane (DCM, 2 mL) were added. The suspension was allowed to sonicate for 2-3 minutes and then poured into the reactor, washing the tube with DCM in order to recover the resin. The liquid was filtered to obtain the dry resin.
The Fmoc-protected Rink-amide resin was treated with 20 % piperidine in DMF for 5 minutes, then the solution was filtered and the resin was treated again with 20% piperidine in DMF for 15 minutes. After the solution was filtered, the resin was washed 6 times with DMF.

Coupling. The Fmoc-amino acid (4 equivalents) and non-natural amino acids were dissolved in a solution of TBTU / HOBt (0.45 M in DMF, 4 eq.) Then N,N-diisopropylethylamine (DIEA, 8 eq.) was added. The solution was poured into the reactor containing the resin and the reaction mixture was left under stirring for 50 minutes, after which the solution was filtered and the resin washed with DMF (6 times). Fmoc-Cptd was dissolved in a solution of HATU (0.45 M in DMF, 4 eq.) and the followed procedure was identical to that described above. Standard conditions: 130 mmol of resin, 500 mmol Fmoc-aa, 500 mmol activator TBTU solution 0,45 M in DMF, DIEA 170 μL (1 mol). Standard conditions for Cptd: 130 mmol of resin, 250 mmol Fmoc-Cptd, 1 mmol activator HATU solution 0.45 M in DMF.

Fmoc cleavage. A solution of 20 % piperidine in DMF was added to the resin. The reaction mixture was left under stirring for 5 minutes, after which the solution was filtered and piperidine solution was added to the resin again. The reaction mixture was left under stirring for 15 minutes, after which the solution was filtered and then the resin washed with DMF (6 times). Subsequently, the resin was subjected to a new coupling process or, if the sequence was finished, washed with DCM for five times and dried under nitrogen flow.

Resin cleavage and purification. A solution of TFA/H_2O/TIS/thioanisole/phenol (10: 0.5: 0.25: 0.5: 0.75) was added to the resin. The reaction mixture was left under magnetic stirring for 2 h. Then in a test tube it was prepared a mixture of tert butylmethyl ether and petroleum ether (1:1), in which the contents of the reactor was filtered to allow the precipitation of the peptide. The peptide precipitate was washed with t-butyl methyl ether and petroleum ether (1:1), centrifuging and removing the supernatant each time. At the end of the precipitate was dissolved in the solution of eluents A (0.1% TFA, water / acetonitrile 97:3) and B (0.1% TFA, water/acetonitrile 30:70) (1:1).

Peptide purification. The peptides were purified by preparative reverse phase HPLC using an appropriate gradient of eluents A and B (see above) with a flow of 14 mL/min, and the detection was performed at 230 and 280 nm. The purified peptide fractions were concentrated under vacuum and to the acqueous was added a drop of 1M HCl. The purified peptides were finally freeze-dried. Each final product was checked for purity by analytical HPLC (C18 column) using the following gradient: 0-14 min from 100 % A to 70 % B (flow 1.0 mL/min). The survey was carried out at 230 and 280 nm.

Synthesis of peptides Ia,**b**, **Id-g and IIa-g**. Peptides **Ia**,**b**, **Id-g**, and **IIa-g** were synthesized by coupling of Fmoc-Gly-OH (4 eq., 150 mg) to Rink amide resin (203 mg, 0.64 mmol/g) using TBTU /HOBT (4 eq., 1.1 mL, 0.45 M DMF) and DIEA (10 eq., 170 μL). The products were obtained following the general procedures for synthesis, cleavage and folding described above.
Ia: white solid (7 mg, 4.35 %) MS (ESI) m/z: $[M+H]^+$ Measured 1235.55; Calculated: 1235.62
Ib: white solid (5 mg, 3.07 %) MS (ESI) m/z: $[M+H]^+$ Measured 1236.70; Calculated: 1235.62
Ic: = not obtained
Id: white solid (7 mg, 4.3 %) MS (ESI) m/z: $[M+H]^+$ Measured 1236.50; Calculated: 1235.62
Ie: white solid (21 mg, 12.9%) MS (ESI) m/z: $[M+H]^+$ Measured 1250.68; Calculated: 1249.64
If: white solid (20 mg, 20.07%) MS (ESI) m/z: $[M+H]^+$ Measured:1146.47; Calculated: 1145.59
Ig: white solid (12.6 mg, 10.8 %) MS (ESI) m/z: $[M+H]^+$ Measured:847.22; Calculated: 846.42
IIa: white solid (68 mg, 40.5%) MS (ESI) m/z: $[M+H]^+$ Measured: 1290.80; Calculated: 1289.57
IIb: white solid (75 mg, 44.6%) MS (ESI) m/z: $[M+H]^+$ Measured: 1290.80; Calculated: 1289.57
IIc: white solid (30 mg, 18.07%) MS (ESI) m/z: $[M+H]^+$ Measured: 1276.80; Calculated: 1275.56
IId: white solid (31 mg, 18.4%) MS (ESI) m/z: $[M+H]^+$ Measured: 1291.60; Calculated: 1290.39;
IIe: white solid (30 mg, 17.6 %) MS (ESI) m/z: $[M+H]^+$ Measured: 1304.60 ; Calculated: 1303.59
IIf: white solid (19.7 mg, 11.6%) MS (ESI) m/z: $[M+H]^+$ Measured:1226.51; Calculated: 1225.55
IIg: white solid (35 mg, 20.6 %) MS (ESI) m/z: $[M+H]^+$ Measured 854.60; Calculated: 853.39
HPLC chromatograms and mass spectra of each compound were reported on ESI.

Molecular dynamics simulations

All the calculations were carried out using the SANDER module of the AMBER 10 package[23] along with the ff03 force field for **1** and the general amber force field (gaff) for **D-I c**, **D-I d**, **D-I e** and RESP atomic charges. The TIP3P model[24] was employed to explicitely represent water molecules. The polypeptides were immersed in a box containing about 1300 water molecules. At first, the energy of the water molecules was minimized, keeping the atoms of the polypeptides frozen. Then, a minimization of

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the whole system was performed by setting a convergence criterion on the gradient of 10^{-4} kcal mol^{-1} $Å^{-1}$. Prior to starting the MD simulations, the system was equilibrated for 40 ps at 300 K in isocore conditions (NVT). Subsequently, 5 ns of MD simulations in isothermal-isobaric ensemble were carried out at 300 K with a 2 fs time-step (NPT). In the production runs, the systems were performed in periodic boundary conditions. Van der Waals and short-range electrostatic interactions were estimated within a 8 Å cutoff. SHAKE algorithm[25] was applied to all bonds involving hydrogen atoms. VMD 1.8.7 was used for molecular visualization and for animating trajectory data.[26]

Binding Studies

The binding experiments were performed using as radioligand the [^{125}I]-DTyr^6-His^5-GnRH and membrane homogenates from human embryonic kidney 293 (HEK 293) cells stably expressing the GnRH receptor, which were prepared as previously described.[29] Radioiodination of DTyr^6-His^5-GnRH was performed according to the method of Laimou et al. [^{125}I]-DTyr^6-His^5-GnRH (100,000-120,000 cpm) was added into membrane homogenates and the mixtures were incubated as previously described.[29] Briefly, aliquots of diluted membrane suspensions (50 µL) and radioligand were added into tubes containing buffer B (25 mM HEPES containing 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5% BSA, pH 7.4 at 4ºC) with or without GnRH-analogues at the single concentration of 500 nM or with increasing concentrations of these analogues (competition binding experiments) in a final volume of 0.2 mL. The mixtures were incubated at 4°C for 16-19 h and then filtered (Brandel cell harvester) through Whatman GF/C glass fiber filters, presoaked for 1 h in 0.5 % polyethylenimine at 4 °C. The filters were washed four times with1.5 mL of ice-cold 50 mM Tris-HCl, pH 7.4 at 4 °C. Filters were assessed for radioactivity in a gamma counter (LKB Wallac 1275 minigamma, 80% efficiency). The amount of membranes used was adjusted to ensure that the specific binding was always equal to or less than 10% of the total concentration of the added radioligand. Specific [^{125}I]-DTyr^6-His^5-GnRH binding was defined as total binding less nonspecific binding in the presence of 1000 nM Triptorelin (Bachem, Germany). Data for competition binding were analyzed by nonlinear regression analysis, using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). The -LogIC_{50} values were obtained by fitting the data from competition studies to a one-site competition model.

Colorimetric MTT assay

HEK 293 cells stably expressing the type I GnRH receptor were grown in Dulbecco's modified Eagle's medium/F-12 (1:1) (DMEM/F-12) containing 3.15 g/L glucose and 10% bovine calf serum at 37°C and 5% CO_2. The cells were plated in 96 well plates at a density of 2000-2500 cells/well and the plates were incubated at 37°C and 5% CO_2 for 16 hours. At the end of the incubation, the medium was replaced by DMEM/F-12, containing 3.15 g/L glucose and 5% bovine calf serum, and the cells were incubated in a total volume of 100 µL, in the absence (untreated cells) or presence of 1µM Leuprolide or 1µM compound **IIe**, at 37°C and 5% CO_2. Controls included both wells with untreated cells (control) and wells containing only medium (control-blank). The medium with or without peptides was changed every 24 h. After 4 days of treatment, the medium was replaced with a fresh one without peptides and containing 0.5 mg/mL MTT and the plates were incubated for 4 hours at 37°C and 5% CO_2. At the end of the incubation 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the insoluble purple MTT-derived formazan crystals into a colored solution. After 1 hour incubation at 37°C, the absorbance values at 595 nm were determined on a Bio-Rad Elisa microplate reader. The experiments were repeated twice in quadruplicates.

Metabolic stability studies

A solution of the peptide 100 µM in was prepared; then sample extraction was performed by adding
90µL of rat plasma to 10 µL of the solution 100 µM, vortexing and incubating at 37.4°C for respectively 0 minutes, 30 minutes, 60 minutes, 4 hours and 24 hours. The proteins were precipitated by addition of 100 µL of a solution of perchloric acid 0.1 M; samples were left at 0°C for 10 minutes and centrifugated at 1400 rpm for 10 minutes; to 180 µL of surnatant 180 µL of mobile phase were added; the solution is filtered into the analysis vial.
A Surveyor system equipped with a binary pump, autosampler, vacuum degasser, and temperature-controlled column compartment was used for analyte separation by High Performance Liquid Chromatography (HPLC). The mobile phase consisted of solvents A (100% water, 0.1% formic acid) and B (100 % acetonitrile, 0.1 % formic acid). A Synergi 4µ-POLAR-RP 80A 150 x 2.00 mm column (Phenomenex) was used at a flow rate of 0.2 mL/min. A linear gradient (run time of 20 min) from 95% A- 5% B to 15% A- 85% B over 10 min; 15% A- 85% B up to 5 min was used for the chromatographic separation of peptides of interest.
Mass spectrometry was performed on a LCQ Advantage system fitted with a ElectroIonSpray source and a triple quadrupole mass spectrometer (Thermofinnigan, Rodano, Milano). The instrument was operated in positive ion mode under the following conditions: IonSpray voltage, 4000 V; source temperature, 250°C; sheat gas (nitrogen) at 29 (arbitrary units); auxiliary gas 9 (arbitrary units).
GnRH I (1182.60 g/mol) was monitored by using the multiplereaction monitoring (MRM) transitions of m/z 1182.60 $\rightarrow$ 742.27 + 1011.4 m/z
GnRH II (1236.70 g/mol) was monitored by using the multiplereaction monitoring (MRM) transitions of m/z 1236.70 $\rightarrow$ 902.27 + 1065.27 m/z
In vitro peptide stability was determined as a function of time; using the corresponding MRM transitions for each analyte, the peak areas for GnRH I, GnRH II, (peptide concentration of 500 ng/mL) at t = 0 were set as 100 %. Those peak areas were compared to the peak areas derived from samples at t = 30 min, 1 h, 4 h, 24 h. In addition, analysis of blank samples confirmed the absence of interference and established the selectivity of the assay. The experimental data were analyzed by the exponential decay regression model, using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA) and the $t_{1/2}$ values were obtained (ESI).

Abbreviations

Fmoc: Fluorenylmethoxycarbonyl group
TBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
HATU: *O*-(7-Azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HOBt: 1-Hydroxybenzotriazole
DIEA: *N,N*-Diisopropylethylamine
TFA: Trifluoracetic acid
DMF: N,N-dimethylformamide
DCM: Dichloromethane
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMEM: Dulbecco's Modified Eagle Medium

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† Electronic Supplementary Information (ESI) available: HPLC chromatograms, mass spectra of each product, metabolic stability data regression analyses and CD spectra. See DOI:10.1039/b000000x/

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