Research Article

Probing the GnRH receptor agonist binding site identifies methylated triptorelin as a new anti-proliferative agent

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Abstract

D-amino acid substitutions at glycine postion 6 in GnRH-I decapeptide can possess super-agonist activity and enhanced *in vivo* pharmacokinetics. Agonists elicit growth-inhibition in tumorigenic cells expressing the GnRH receptor above threshold levels. However, new agonists with modified properties are required to improve the anti-proliferative range. Effects of residue substitutions and methylations on tumourigenic $HEK293_{[SCL60]}$ and WPE-1-NB26-3 prostate cells expressing the rat GnRH receptor were compared. Peptides were ranked according to receptor binding affinity, induction of inositol phosphate production and cell growth-inhibition. Analogues possessing D-Trp^6 (including triptorelin), D-Leu^6 (including leuprolide), D-Ala^6, D-Lys^6, or D-Arg^6 exhibited agonist and anti-proliferative activity. Residues His^5 or His^5,Trp^7,Tyr^8, corresponding to residues found in GnRH-II, were tolerated, with retention of sub-nanomolar/low nanomolar binding affinities and EC_{50}s for receptor activation and IC_{50}s for cell growth-inhibition. His^5D-Arg^6-GnRH-I exhibited reduced binding affinity and potency, effective in the mid-nanomolar range. However, all GnRH-II-like analogues were less potent than triptorelin. By comparison, three methylated-Trp^6 triptorelin variants showed differential binding, receptor activation and anti-proliferation potency. Significantly, 5-Methyl-DL-Trp^6-Triptorelin was equipotent to triptorelin. Subsequent studies should determine whether pharmacologically enhanced derivatives of triptorelin can be developed by further alkylations, without substitutions or cleavable cytotoxic adducts, to improve the extent of growth-inhibition of tumour cells expressing the GnRH receptor.

Introduction

Gonadotropin releasing hormone (GnRH) agonists can elicit cancer cell growth inhibition via activation of the GnRH receptor (Franklin *et al*. 2003). Cell growth-inhibition usually occurs provided the receptor expression levels exceed a certain threshold at the cell surface (Franklin *et al*. 2003; Morgan *et al*. 2008 and 2011) but the extent of growth inhibition may be confounded by other factors including the particular organization or mutation of the intracellular signaling pathways (Morgan *et al*. 2011). Consequently, there is a need to develop new GnRH agonists that are able to improve experimental tumour eradication and to impact upon cells expressing sub-threshold levels of the receptor; or to affect certain cell types currently resistant to the anti-proliferative effects of GnRH receptor activation.

Different approaches to the development of GnRH agonists have been used in the past. Historically, structure-function studies using synthetic peptide analogues of GnRH (Karten & Rivier 1986) led to the identification of super-agonists containing D-amino acid substitutions (Coy *et al*. 1976). A D-amino acid residue at position 6, replacing glycine in native GnRH -I (pyroGlu^1,His^2,Trp^3,Ser^4,Tyr^5, **Gly^6**,Leu^7,Arg^8, Pro^9,Gly^{10}-NH_2) increases receptor binding affinity and lowers the dose required to reach half-maximal receptor activation (Excitatory Concentration, EC_{50}) (Clayton & Catt 1980). The D-amino acid also increases the resistance of synthetic peptides to enzymatic degradation by peptidases and slows clearance *in vivo*, contributing to an increased pharmacokinetic half -life (Barron *et al*. 1982, Ezan *et al*. 1986, Redding & Schally 1981). A modified D-amino acid residue, containing a benzene- or a di-cyclic aromatic ('naphthyl')

moiety can be incorporated at position 6, with the bulkiest side-chain reported being iso-butyl-[6-(3-(2-naphthyl)-D-alanine)]-GnRH-I. This analogue was estimated to be slightly more potent than the D-Trp^6-GnRH-I super-agonist, triptorelin (Ho *et al*. 1984, Nestor *et al*. 1982 and 1984) in bioassays available at that time (Clayton & Catt 1980, Loumaye *et al*. 1982). Subsequently, an extended side-chain at position 6 was used to introduce cytotoxic GnRH conjugates such as D-Lys^6-doxorubicin (Janáky *et al*. 1992) and D-Lys^6-curcumin (Aggarwal *et al*. 2011). However, a drawback associated with cytotoxic GnRH conjugates is their cleavage by serum enzymes prior to GnRH receptor-mediated cell targeting (Nagy *et al*. 2000).

An alternative drug development strategy would be to seek to improve the anti-proliferative efficiency by developing new GnRH receptor agonists without the addition of cleavable cytotoxic adducts. Several studies have investigated how GnRH-II (pyroGlu^1,His^2,Trp^3,Ser^4, Hisr^5, **Gly^6**,Trp^7,Tyr^8, Pro^9,Gly^{10}-NH$_2$) interacts with the type I GnRH receptor (Lu *et al*. 2005, Millar *et al*. 2008, Pfleger *et al*. 2002 and 2008) and suggested that GnRH-II-like ligands may elicit cancer cell growth inhibition more potently than GnRH-I analogues (Lopez de Maturana *et al*. 2008).

In this study, we compared the properties of nine GnRH analogues possessing substitution of specific residues or side-chain methylation. Screening was performed using two human cell lines which stably express high levels of the rat GnRH receptor and which are reproducibly growth inhibited following receptor activation (Morgan *et al*. 2008 and 2011).

Three analogues possessed a D-amino acid at position 6 (either D-Trp^6, -Lys^6 or -Arg^6) and a His residue at position 5. A further three analogues possessed an additional dual substitution at positions 7 and 8, thus incorporating His^5,Trp^7,Tyr^8 to generate GnRH-II-like peptides. Finally, three methylated -DL-Trp^6 GnRH-I peptides, modified by alkyl moiety addition to the tryptophan side-chain benzene ring positions 4, 5 or 6, were compared to the super-agonist triptorelin since previous studies of side-chain alkylation have only addressed position Trp^3 but not the critically important position 6 (Yabe *et al*. 1979, Deghenghi, 1997).

Interestingly, the GnRH-II residue substitution analogues were less potent than triptorelin whilst one of the methylated triptorelin analogues exhibited high potency, indicating scope for the generation of new triptorelin-based drugs which may possess improved pharmacological or pharmaco-dynamic properties for tumour growth inhibition.

Materials and Methods

Reagents

HPLC purified synthetic peptides were purchased from Sigma, UK or were custom synthesized by EZ Biolabs, USA or Eurogentech, Germany. GnRH analogues were selected to enable examination of primary sequence differences between GnRH-I and GnRH-II. Methylated triptorelin isoforms were synthesised using readily available methylated-DL-tryptophan isomers.

Cell culture

HEK293$_{[SCL60]}$ cells were maintained in DMEM with 10% fetal bovine serum containing G418. All assays using these cells required plates coated with matrigel. WPE-1-NB26-3 cells were grown in keratinocyte medium supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Invitrogen kit, UK). Human prostate cancer cell lines PC3, LNCaP and DU145 were grown in DMEM with

Table 1. Receptor binding and activation properties of GnRH analogues.

Cell Type	HEK293$_{[SCL60]}$		WPE-1-NB26-3	
	Binding IC$_{50}$, (nM)	Inositol phosphate EC$_{50}$, (nM)	Binding IC$_{50}$, (nM)	Inositol phosphate EC$_{50}$, (nM)
Peptide				
D-Trp^6 GnRH	0.5 ± 0.2	0.14 ± 0.01	0.9 ± 0.3	0.2 ± 0.03
His^5 D-Trp^6 GnRH	0.8 ± 0.5	0.6 ± 0.2	0.3 ± 0.1	0.4 ± 0.1
His^5 D-Trp^{6}Trp^{7}Tyr^8 GnRH	3.4 ± 0.5	5.9 ± 0.9	2.6 ± 0.3	3.9 ± 0.5
His^5 D-Lys^6 GnRH	3.0 ± 0.5	12.5 ± 1.7	5.1 ± 0.3	6.2 ± 0.2
His^5 D-Lys^{6}Trp^{7}Tyr^8 GnRH	0.8 ± 0.2	1.7 ± 0.06	0.7 ± 0.2	0.8 ± 0.2
His^5 D-Arg^6 GnRH	22.3 ± 2.3	45.0 ± 6.9	27.5 ± 4. 9	21.2 ± 6.9
His^5 D-Arg^{6}Trp^{7}Tyr^8 GnRH	0.5 ± 0.1	1.6 ± 0.2	0.9 ± 0.03	0.9 ± 0.2

Figure 1

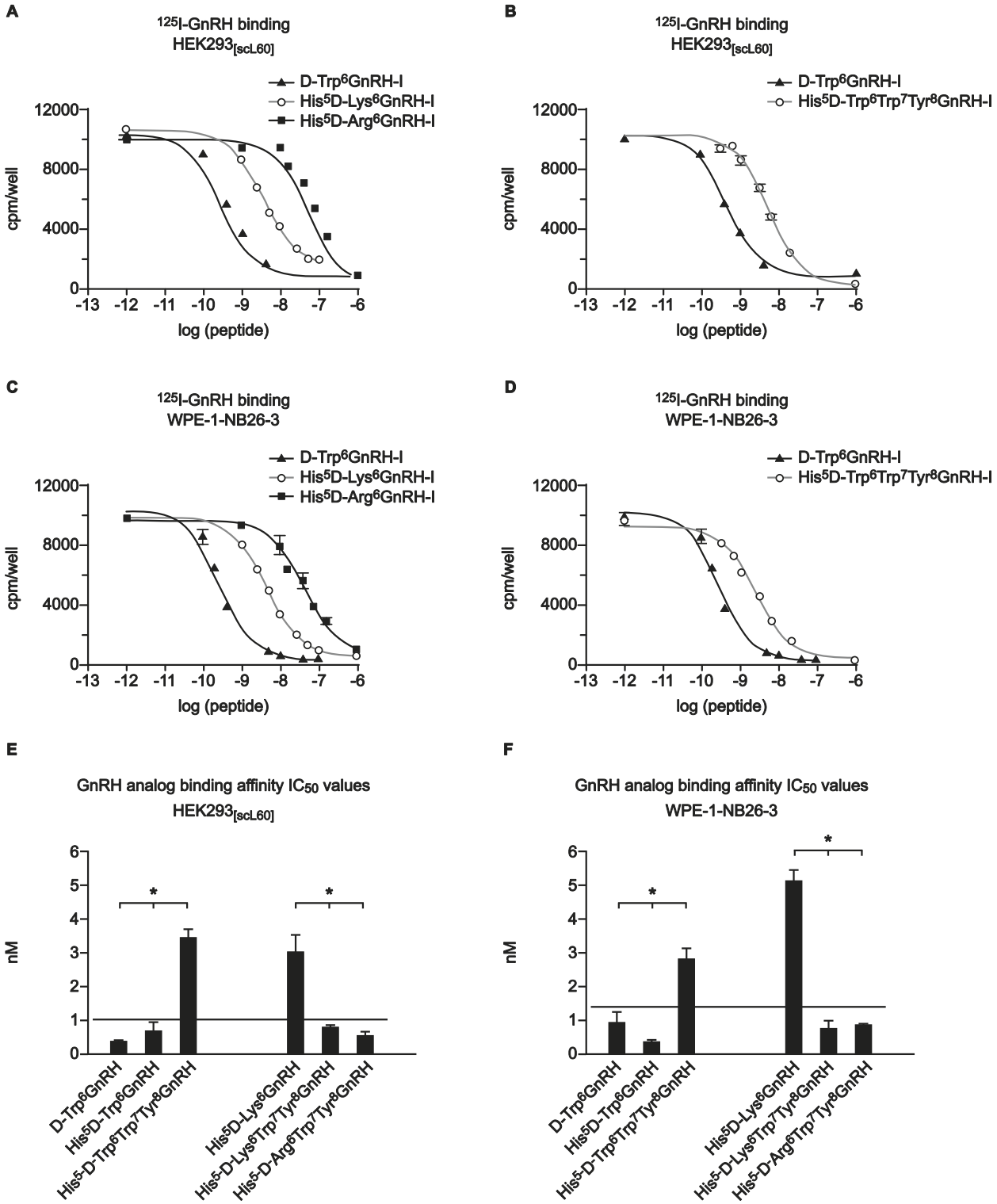

Figure 1. A-D. Receptor binding assays for D-^{6} GnRH analogues. Displacement of ^{125}I-His^{5}D-Tyr^{6} GnRH-I bound to the surface of cells grown in multi-well plastic culture dishes by co-incubation with different concentrations of unlabelled GnRH analogue enabled generation of curves and estimation of relative binding affinities. Examples of binding of four GnRH ligands to two cell lines expressing rat GnRH receptor are presented. Binding was determined in triplicate and on separate occasions for each data-point. E-F. Bar charts for binding IC_{50} values. Individual IC_{50} calculations were performed using the Prism software (Graphpad, USA), assuming a single binding site. The dashed line distinguishes sub-nanomolar values from nanomolar values. Error bars indicate mean $\pm$ SEM, p values derived using T-test.

10% fetal bovine serum.

Whole cell binding assay

Cells were plated in 12-well or 24-well plates for the ^{125}I-GnRH binding assay, using ^{125}I-labeled His^{5}D-Tyr^{6}GnRH-I as a tracer (Flanagan *et al*. 1998). The method has been described previously (Morgan *et al*. 2008 and 2011). Peptide dilutions were prepared in HEPES-DMEM containing 0.1% BSA. Specific binding was determined in triplicate by counting gamma-ray radiation following washing and lysis of cell monolayers. Inter-plate comparisons were made by counting the number of cells per well in plates prepared in parallel.

Inositol phosphate assay

Cells were plated into 24-well plates for the ^{3}H-myoinositol assay, performed as described previously (Morgan *et al*. 2008 and 2011). Peptide dilutions were prepared in HEPES-DMEM containing 10 mM LiCl and 0.1% BSA. Receptor stimulation was performed at 37^{0}C for 60 minutes.

Cell growth assay

Cells grown in 12-well plates were treated with GnRH receptor analogues and growth was analysed by sulforhodamine B staining and spectrophotometric quantification, as described previously (Morgan *et al*. 2008, 2011).

Statistical analysis

Analyses of quantitative data were performed using online tools available at http://easycalculation.com/statistics/standard-deviation.php and http://www.quantitativeskills.com/sisa/ or using Microsoft Excel (T Test). All data were generated in triplicate on at least two separate occasions. Graphs were plotted using calculations of mean ± standard error of mean (SEM) and analyzed by non-linear regression modeling using the Prism software (GraphPad, USA). T-Test P values < 0.05 were considered to be statistically significant.

Results

All of the GnRH analogues studied exhibited specific binding to the rat GnRH receptor that was stably expressed in human embryonic kidney cells (HEK293 $_{[SCL60]}$) or immortalized human prostate epithelial cells (WPE-1-NB26-3) (see Figure 1 and Table 1 for examples). Displacement of ^{125}I-GnRH bound to the cell surface with different concentrations of unlabelled GnRH analogues enabled calculation of approximate binding affinities (IC_{50}s, Table 1).

In HEK293$_{[SCL60]}$ and WPE-1-NB26-3 cells, D-Trp^6 GnRH-I exhibited the highest binding affinity compared to all residue substitution analogues tested. Replacement of Tyr^5 by His^5 had a modest effect on binding (Table 1). Replacement of Leu^{7}Arg^8 by Trp^{7}Tyr^8 to generate 'D-Trp^6-GnRH-II' (His^5 D-Trp^{6}Trp^{7}Tyr^8 GnRH) resulted in significantly poorer ^{125}I-GnRH tracer displacement. Differences between binding affinities for certain peptides were statistically significant (see Figure 1 and Table 1).

Introduction of a positively charged D-residue side chain at position 6 (D-Lys^6 or D-Arg^6) interfered with ligand binding in the D-6 substituted GnRH-I analogues but not in the D-6 substituted GnRH-II analogues (compare results for His^5 D-Lys^6 GnRH or His^5 D-Arg^6 GnRH with His^5 D-Lys^{6}Trp^{7}Tyr^8 GnRH and His^5 D-Arg^{6}Trp^{7}Tyr^8 GnRH in Table 1).

Comparison of GnRH analogues revealed statistically significant differences in the potency of agonists to stimulate production of inositol phosphates. D-Trp^6 GnRH-I was the most potent agonist compared to the substitution variants (Figure 2 and Table 1). D-Trp^6

Table 2. Cell growth inhibition potencies of GnRH analogues.

Cell Type	HEK293$_{[SCL60]}$	WPE-1-NB26-3
	Cell growth inhibition IC_{50}, (nM)	Cell growth inhibition IC_{50}, (nM)
Peptide		
D-Trp^6 GnRH	0.4 ± 0.06	0.17 ± 0.05
His^5 D-Trp^6 GnRH	1.2 ± 0.5	0.4 ± 0.1
His^5 D-Trp^{6}Trp^{7}Tyr^8 GnRH	0.5 ± 0.06	2.3 ± 0.9
His^5 D-Lys^6 GnRH	24.8 ± 17.6	3.1 ± 1.8
His^5 D-Lys^{6}Trp^{7}Tyr^8 GnRH	6.5 ± 2.8	1.1 ± 0.8
His^5 D-Arg^6 GnRH	35.9 ± 14.8	20.9 ± 11.8
His^5 D-Arg^{6}Trp^{7}Tyr^8 GnRH	7.5 ± 2.3	0.9 ± 0.4

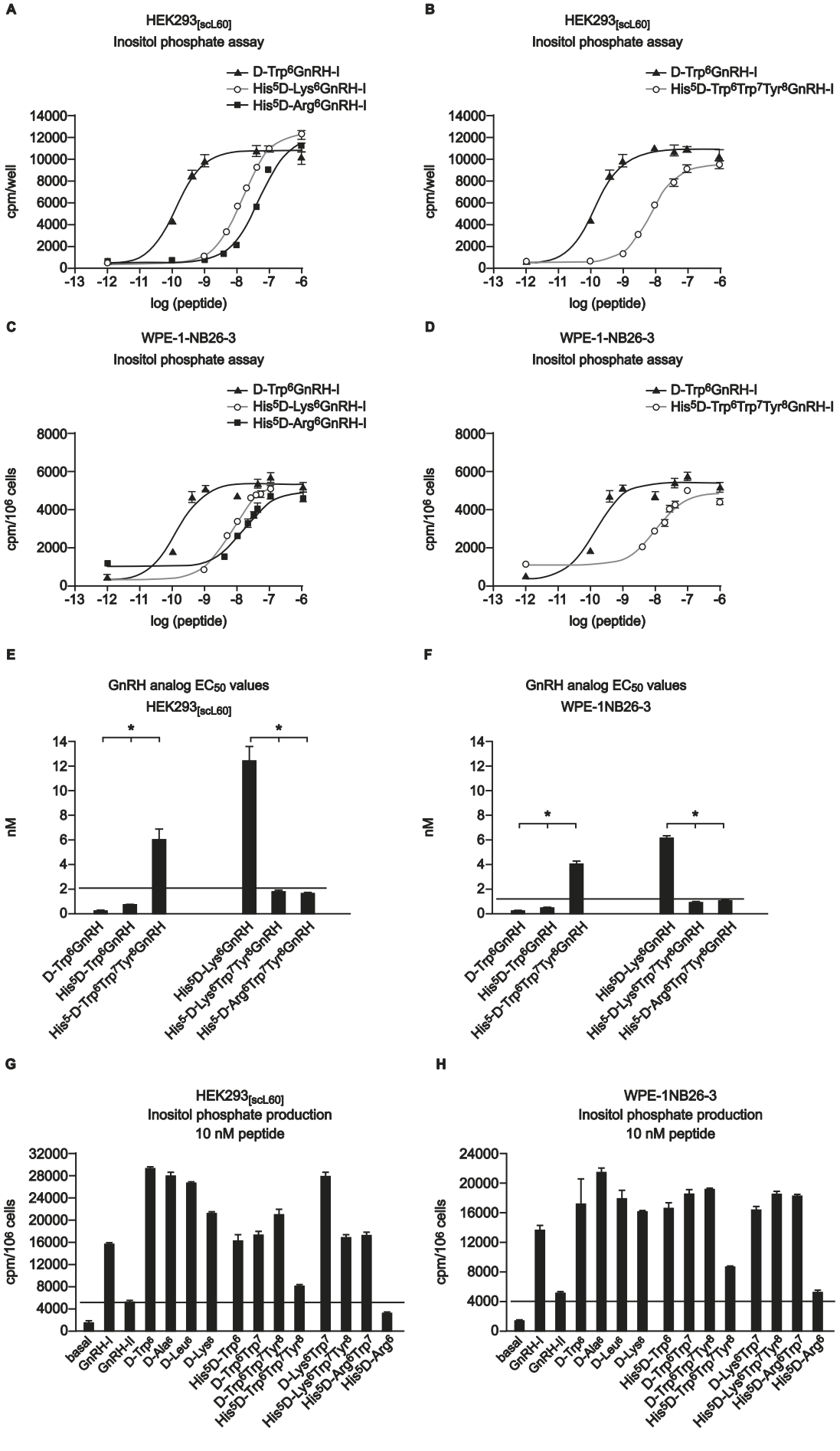

Figure 2. Inositol phosphate assays. Titrations were performed to enable calculation of EC_{50}s (A-D). Bar chart representation of receptor activation EC_{50} values, where dashed lines emphasize the salient differences between GnRH analogues (E-F). GnRH analogues were screened for receptor activation using stimulation with a single dose of peptide (10 nM), enabling rapid comparisons to be made prior to titrations (G-H). Error bars indicate mean $\pm$ SEM, p values derived using T-test.

GnRH-I behaved as a super-agonist relative to native GnRH-I at the rat GnRH receptor expressed in $HEK293_{[SCL60]}$ and WPE-1-NB26-3 cells. Decreased relative potency in the other GnRH analogues correlated with lower binding affinity.

Dose-response analyses of the effects of GnRH analogues on cell growth were performed (Figure 3), enabling calculation of IC_{50}s (Table 2). The anti-proliferative potency of GnRH analogues paralleled binding affinities and EC_{50}s (Tables 1 and 2).

Methyl adducts were incorporated into three separate locations within the benzene ring of the Trp^6 side-chain of DL-Trp^6 GnRH-I. These peptides were synthesized as DL epimer mixtures (50:50% abundance confirmed by HPLC analysis). Each mixture exhibited differential properties for binding, receptor activation, and inhibition of cell growth (Figure 4 and Table 3).

Methylation at position 5 of the benzene-ring of DL-Trp^6 GnRH-I did not adversely affect the *in vitro* binding, receptor activation or cell growth inhibitory properties of the peptide relative to triptorelin (D-Trp^6 GnRH-I). Thus 5-methyl-DL-Trp^6 GnRH retained high potency. Methylation at position 4 of the benzene -ring of DL-Trp^6 GnRH-I modestly reduced binding affinity, receptor activation and cell growth inhibitory potency. The IC_{50} values for binding, EC_{50} values for receptor activation and IC_{50} values for growth inhibition were statistically different relative to D-Trp^6 GnRH-I and 5-methyl-DL-Trp^6 GnRH using both cell lines. Methylation at position 6 of the benzene-ring of DL-Trp^6 GnRH-I dramatically reduced binding affinity, receptor activation and cell growth inhibitory potency. Growth of the prostate cell line was more sensitive to the methyl-DL-Trp^6 GnRH analogues than observed for $HEK293_{[SCL60]}$ cells.

None of the GnRH analogues used in this study exhibited specific binding, inositol phosphate signalling or significant cell growth inhibition in non-transfected HEK293, WPE-1-NB26, PC3, LNCaP or DU145 cell lines (data not shown).

Discussion and Conclusions

Using appropriate *in vitro* tools it has become possible to explore GnRH analog structure-function relationships with increasing depth and application (Beckers *et al*. 1995, Coetsee *et al*. 2008, Flanagan *et al*. 1994 and 2000, Fromme *et al*. 2001, Karten & Rivier 1986, Lopez de Maturana *et al*. 2008, Lu *et al*. 2005 and 2007, Marheineke *et al*. 1998, Millar & King 1983, Millar *et al*. 1986, 2004 and 2008, Pfleger *et al*. 2002 and 2008, Sealfon *et al*. 1997, Söderhäll *et al*. 2005). One challenge is to use this technology to develop GnRH agonists that are more effective than triptorelin in tumor growth inhibition, ultimately aiming to enable growth inhibition of cells with moderate to low levels of GnRH receptor or altered intracellular signalling pathways.

We found that new anti-proliferative agonists may be identified by screening for subtle changes in GnRH peptide structure which serve to probe the ligand binding site, such as side-chain alkylation. The latter may result in modified binding and receptor acti-

Table 3. Receptor binding, activation and cell growth inhibitory properties of methylated triptorelin derivatives.

Cell Type	$HEK293_{[SCL60]}$		WPE-1-NB26-3	
	Binding IC_{50}, (nM)	Inositol phosphate EC_{50}, (nM)	Binding IC_{50}, (nM)	Inositol phosphate EC_{50}, (nM)
Peptide				
D-Trp^6 GnRH	0.5 ± 0.2	0.14 ± 0.01	0.9 ± 0.3	0.15 ± 0.01
6Me DL-Trp^6 GnRH	2.3 ± 0.2	1.70 ± 0.4	2.7 ± 0.3	1.05 ± 0.05
5Me DL-Trp^6 GnRH	97.7 ± 17.0	0.30 ± 0.03	0.7 ± 0.3	0.17 ± 0.01
4Me DL-Trp^6 GnRH	0.6 ± 0.07	36.6 ± 5.10	138.5 ± 26.0	11.90 ±1.40
Cell Type	$HEK293_{[SCL60]}$		WPE-1-NB26-3	
	Cell growth inhibition $IC_{50,}$ (nM)		Cell growth inhibition $IC_{50,}$ (nM)	
Peptide				
D-Trp^6 GnRH	0.4 ± 0.06		0.17 ± 0.05	
4Me DL-Trp^6 GnRH	4.5 ± 0.6		0.8 ± 0.20	
5Me DL-Trp^6 GnRH	0.5 ± 0.06		0.1 ± 0.01	
6Me DL-Trp^6 GnRH	64.4 ± 4.0		10.1 ± 0.10	

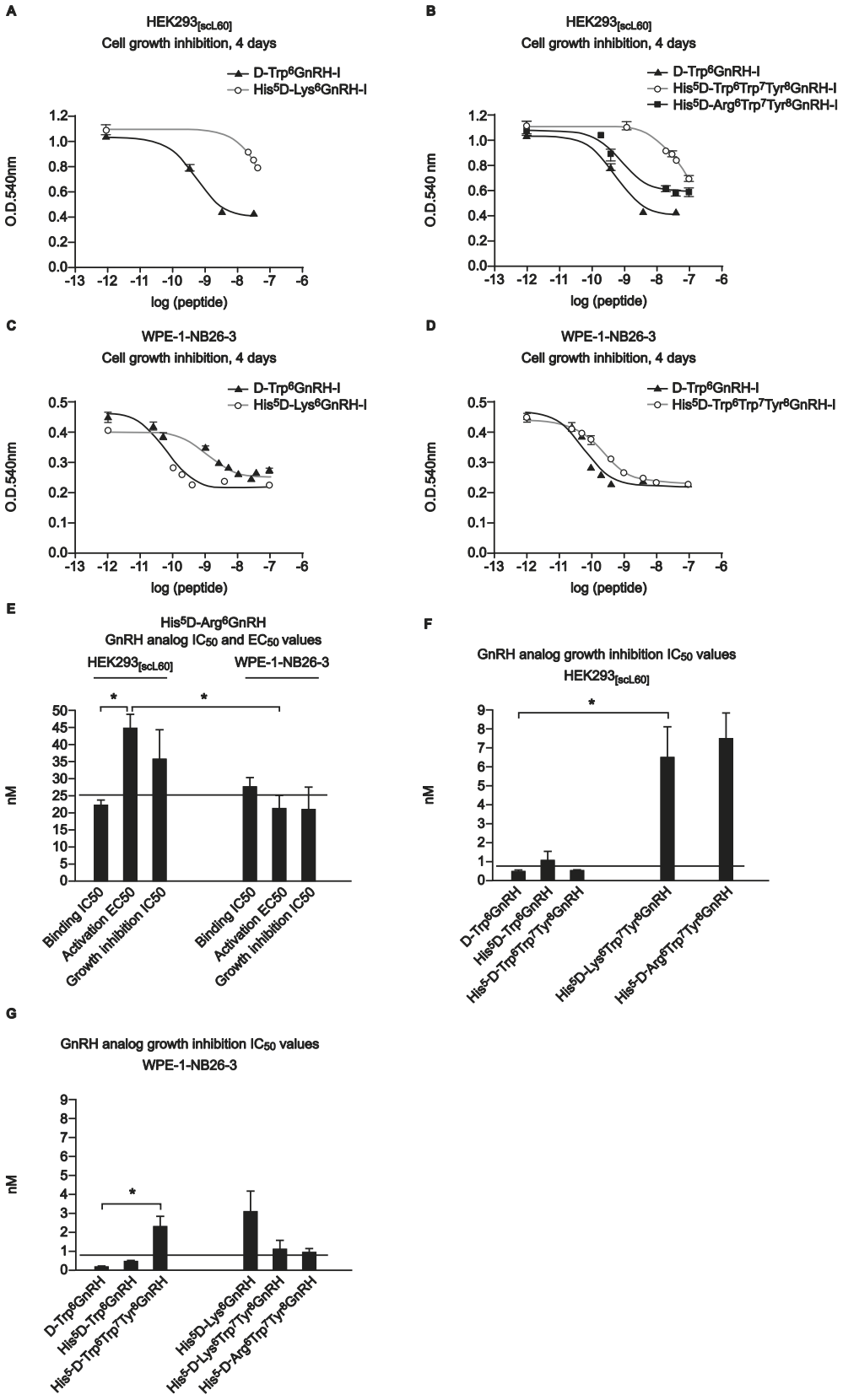

Figure 3. Examples of GnRH analog dose-responses associated with cell growth-inhibition (A-D). Note that WPE-1-NB26-3 cells grow more slowly than HEK293$_{[SCL60]}$ cells over four days, hence the difference in the scale bar for optical density at 540 nm (O.D. 540 nm) between the two cell lines. Values for IC_{50} calculations are presented as bar graphs (E-G) and dashed lines emphasise differences in potencies. His^{5}D-Arg^6 GnRH exhibited the lowest ability to inhibit cell growth. Error bars indicate mean $\pm$ SEM, p values derived using T-test.

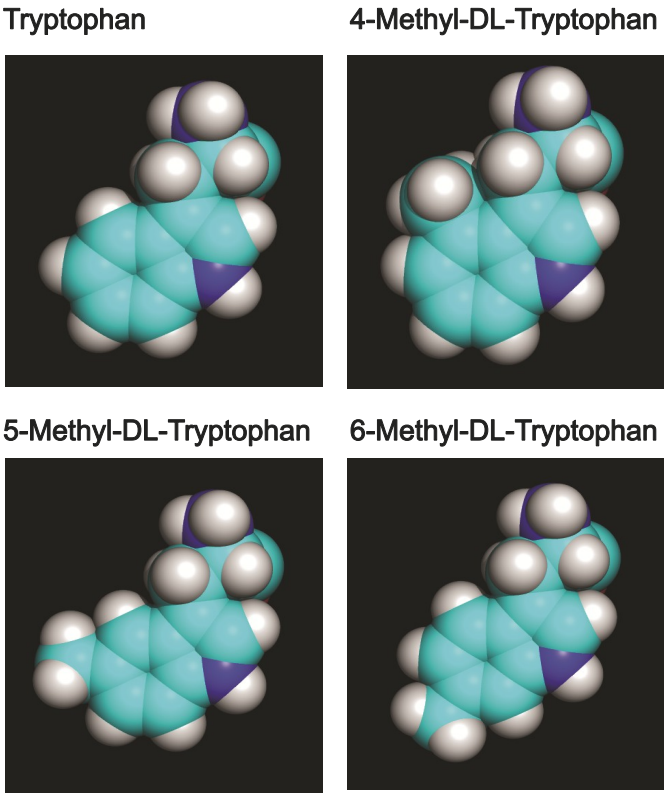

Figure 4. Location of methyl-DL-Trp^6 side-chain positions 4, 5 and 6 on the benzene ring of the indole moiety in space-filling depictions of methylated tryptophan.

vation dynamics and altered pharmacodynamics. This approach is distinctly different to the development of GnRH conjugates containing cleavable toxic adducts (Nagy *et al*. 2000), such as GnRH-doxorubicin or GnRH-curcumin (Aggarwal *et al*. 2011), in which the adduct moiety is positioned on a linker to avoid contact with the ligand-receptor binding site.

We compared the properties of GnRH analogues containing a D-amino acid residue at position 6 and combinations of residues found in GnRH-II to the effects of methylation of residue 6 using two human cell lines expressing high levels of rat GnRH receptor (HEK293$_{[SCL60]}$ and WPE-1-NB26-3 cells). New insights into GnRH agonist structure-function were obtained.

Changing the D-amino acid residue at position 6 and the flanking residues at position 5 and positions 7 and 8 in a range of analogues did not improve binding affinity relative to triptorelin, (D-Trp^6 GnRH-I) (Table 1).

The GnRH-II-like peptides His^{5}D-Arg^{6}Trp^{7}Tyr^8- GnRH-I and His^{5}D-Lys^{6}Trp^{7}Tyr^8-GnRH-I, possessed binding affinities similar to D-Trp^6 GnRH-I (Table 1). Interestingly, they bind better than His^{5}D-Trp^{6}Trp^{7}Tyr^8-GnRH-I, probably due to the way in which complex charge and steric interactions between side-chains affect peptide conformation and docking to the receptor.

Peptide-receptor binding affinity generally correlated with estimates of the EC_{50} for receptor activation, although EC_{50}s were poorer than expected (when compared to binding IC_{50}) for GnRH analogues possessing weaker binding (namely, His^{5}D-Trp^{6}Trp^{7}Tyr^8 GnRH, His^{5}D-Lys^6 GnRH and His^{5}D-Arg^6 GnRH, Table 1 and Figures 1 and 2). Perhaps analogues with poor binding affinity are unable to induce the correct receptor conformation for optimal activation.

We found that the GnRH receptor in the prostate cells appeared to be more efficiently coupled to inositol phosphate production. This phenomenon may be due to higher receptor levels, 1.7 fold higher in prostate cells relative to HEK293$_{[SCL60]}$ (Morgan *et al*. 2011).

Peptide binding and receptor activation relationships were consistent with analyses of cell growth inhibition (Table 2 and Figure 3), indicating that the level of GnRH receptor activation correlates with the extent of anti-proliferation in both of these cell types.

Incorporation of D-residues into GnRH-II-like analogues did not enhance binding, receptor activation or anti-proliferative properties relative to triptorelin (D-Trp^6 GnRH-I). Hence our results negate the hypothesis that GnRH-II agonists may be more effective anti-proliferative agents than GnRH-I agonists (Lopez de Maturana *et al*. 2008). Furthermore, corroborative data generated in the current study indicate that at least two cell models may be required to properly assess the anti-proliferative properties of GnRH analogues, rather than examination of HEK293$_{[SCL60]}$ alone, as used previously (Lopez de Maturana *et al*. 2008). We note that HEK293$_{[SCL60]}$ cells adhere to matrigel extracellular matrix more efficiently than to poly-L-lysine coated culture plates. This difference, coupled with the more accurate sulphorhodamine B growth assay rather than reliance on trypsin/manual cell counting, may account for inconsistencies between new data and previous studies. It is also possible that the use of GnRH analogues possessing D-amino acid residues alters the half-life of synthetic peptides *in vitro* and that this makes comparisons of anti-proliferative potency with native GnRH-II-like analogues more difficult to interpret. Further investigations into the *in vitro* stability of GnRH analogues are required in this respect.

Since triptorelin was the most potent agonist amongst the substitution ligands studied, further analyses of the interaction of this peptide with the receptor were performed. The binding affinity of triptorelin to the rat GnRH receptor is sub-nanomolar, similar to other mammalian GnRH receptors (Beckers *et al*. 1995) although some estimates place it at around 2 nM

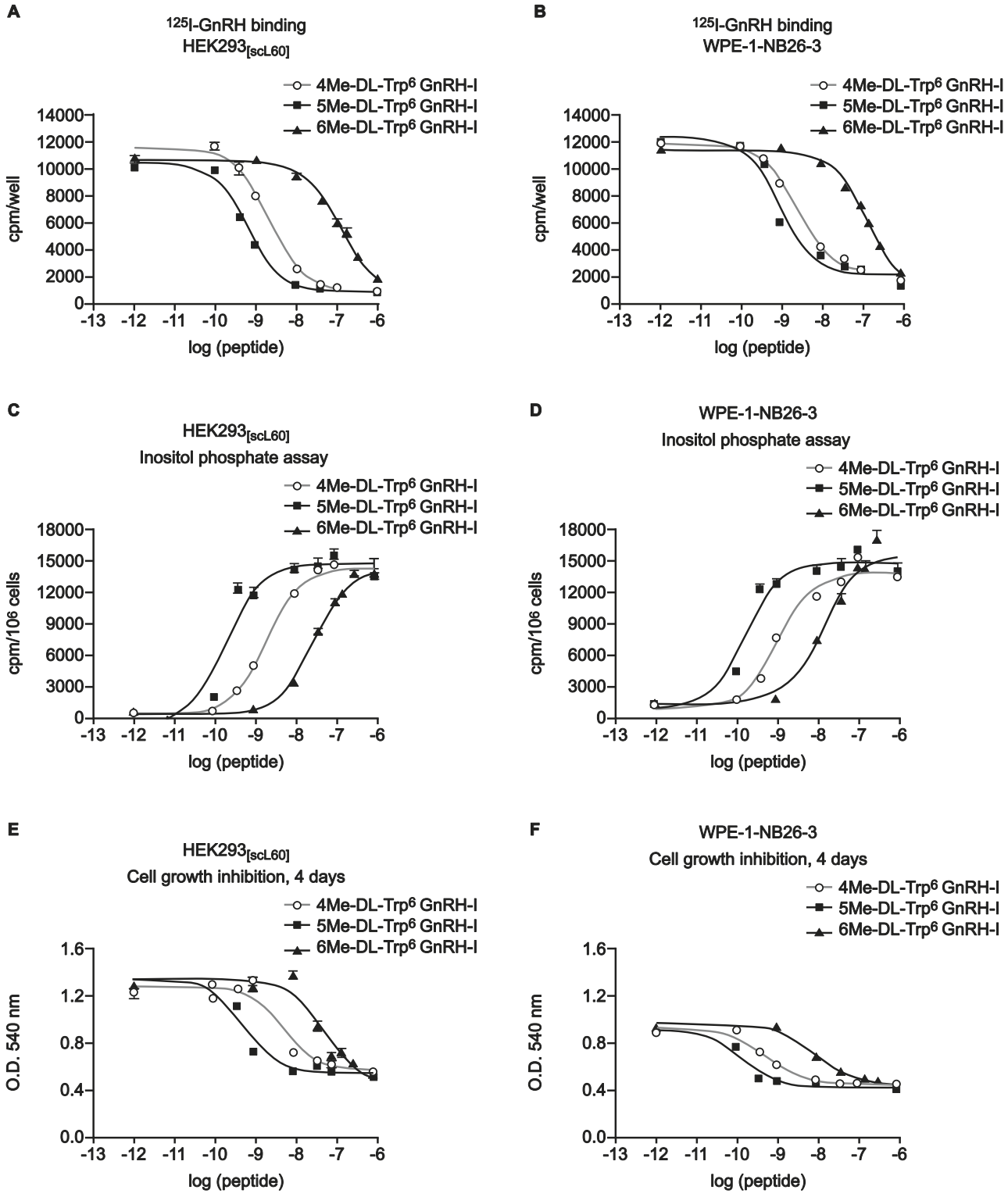

Figure 5 (continued on the next page).

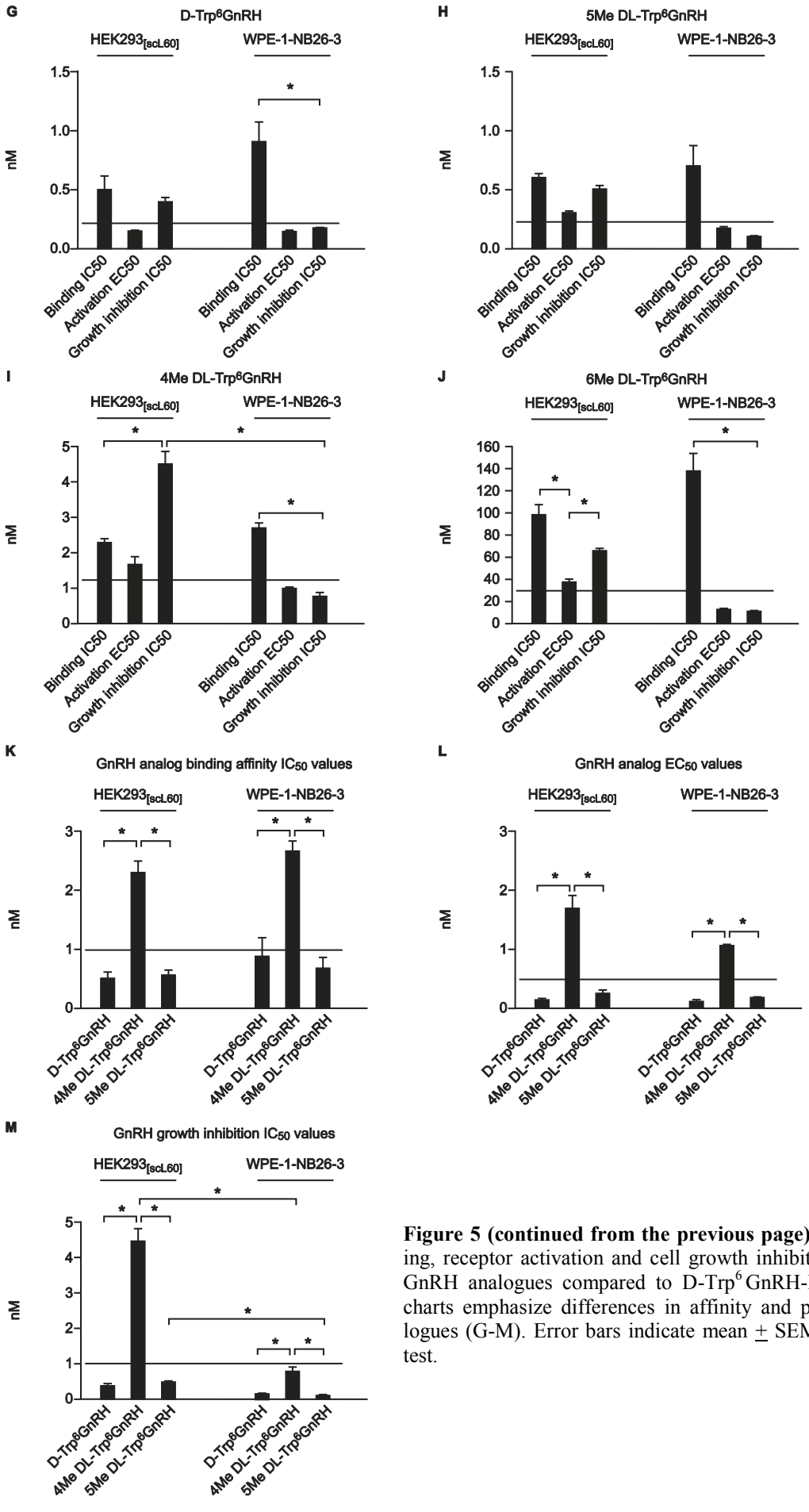

Figure 5 (continued from the previous page). Graphical analyses of binding, receptor activation and cell growth inhibition for methylated DL-Trp[6] GnRH analogues compared to D-Trp[6] GnRH-I (A-F; previous page). Bar charts emphasize differences in affinity and potency for the various analogues (G-M). Error bars indicate mean ± SEM, p values derived using T-test.

(Pfleger *et al*. 2002 and 2008), probably due to less physiological experimental conditions (isolated cell membrane suspensions in which structural integrity of receptors is not biologically maintained by cellular homeostasis). Estimates of GnRH analogue binding affinity can vary according to the experimental assay and methodology employed. Levels of GTP and the status of receptor sulfhydryl moieties have been suggested to influence estimation of GnRH ligand binding affinity (Hazum 1981). Other factors, including the relative abundance of G proteins and their regulator proteins may conceivably affect the GnRH receptor conformation through formation of multi-protein macromolecular protein complexes involving the intracellular face of the receptor.

A modified version of triptorelin, 5-methyl-DL -Trp^6 GnRH-I exhibited sub-nanomolar binding affinity and potency, whereas 6-methyl-DL-Trp^6 GnRH-I bound less well to the receptor and was much less potent (Figure 4 and Table 3). We hypothesise that hydrophobic and steric constraints conferred by a methyl adduct on the Trp^6 benzene ring affect the opportunities for hydrogen bonding between the Trp^6 indole nitrogen atom and the receptor according to the precise location of the adduct. The methyl moiety in 4- and 6-methyl-DL-Trp^6 GnRH-I may cause steric clash.

These are significant discoveries, building on previous reports documenting the high potency of isobutyl-naphthyl-alanine at position 6 (Nestor *et al*. 1982 and 1984). The possible effects of further methylations of Triptorelin on its pharmacological properties are intriguing.

Although methyl-DL-Trp^6 GnRH-I enantiomeric mixtures were used in our studies (50:50 w/w, confirmed by HPLC-UV detector analysis), it is known that large L-amino acid side chains are not tolerated at position 6. Substitution of Gly^6 with D-Ala^6 increased the potency of the GnRH analog to 350-400% that of native GnRH-I, while a corresponding L-Ala^6 substitution resulted in a peptide with a potency of only 4% of native GnRH-I (Karten & Rivier 1986). As such, we would expect the EC_{50} of L-Trp^6 GnRH-I to have a potency of only 1% of D-Trp^6 GnRH-I and a similar relationship is likely for methyl-L- and methyl-D- Trp^6 GnRH-I analogues, although this remains to be formally confirmed.

Likewise, the relatively bulky L-Asp^6 side chain in lamprey GnRH-III results in less than 0.0003% potency relative to mammalian GnRH-I at the human GnRH receptor (Neill 2002) and Ciona GnRH isoforms possessing an L-amino acid residue at position 6 bind poorly to mammalian GnRH receptor (Barran *et al*. 2005). Binding and receptor activation can be increased ten-fold by substitution with Gly or a D-amino acid at Ciona GnRH position 6 (Barran *et al*. 2005).

Significantly, since 5-methyl-DL-Trp^6 GnRH-I retained properties similar to triptorelin, further alkylations of the position 6 side chain may be valuable in attempts to improve the anti-proliferative capacity of GnRH receptor super-agonists. For example, introduction of hydrophobic moieties may significantly affect the *in vivo* pharmacodynamics of triptorelin derivatives. These observations indicate that site-specific ligand methylation/alkylation may be a superior strategy compared to whole residue substitutions or addition of cleavable cytotoxic adducts for the identification of new potent anti-proliferative GnRH super-agonists in future studies. Ligand and receptor structure-function studies and molecular modelling are required to investigate how the methylated triptorelin docks into the agonist binding site in the GnRH receptor.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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