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Rigid Linkers for Bioactive Peptides

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Abstract

Rigid linkers of variable length were used to connect two high-affinity Nle⁴-D-Phe⁷- α -melanocyte stimulating hormone (NDP- α -MSH) or two low-affinity MSH(4) ligands. The linked peptides were synthesized by solid-phase methods. Control experiments indicate there is little or no effect of these linkers on NDP- α -MSH or MSH-(4) binding to the human melanocortin 4 receptor (hMC4R).

Tethering two high-affinity ligands gave no binding enhancement, while tethering two low-affinity ligands resulted in binding enhancement that decreased with increased linker length. Furthermore, for the low-affinity ligands, the enhancement of affinity is inversely proportional to the estimated molecular moments of inertia. These results are consistent with a model wherein binding is enhanced when the rate of ligand reattachment to the receptor is fast relative to the rate of ligand diffusion.

INTRODUCTION

Early diagnosis is critical to the successful treatment of many human cancers. Noninvasive methods for early detection depend on the availability of nontoxic reagents that can seek out and selectively bind to cancer cells and report their existence and location by molecular imaging (1,2). We are engaged in a program to develop reagents that target human cancers that presently are difficult to detect, such as melanoma and pancreatic cancer. Our strategy involves linking together reporter moieties and ligands that bind to cell surface receptors that are expressed in cancer cells (3). The resulting multivalent molecules could display enhanced affinity for the targeted cells (4,5).

Factors that must be considered in designing linkers for bioactive multimeric ligands include the distance that must be spanned, availability, and ease of use. The distance that must be spanned between targeted receptors on the cell surface will vary depending on the number, kind, and mobility of the receptors, but it is expected to greatly exceed the typical dimensions of small molecules (6). Several small molecule linkers connected in series will therefore be necessary to span the distance between receptors. For peptide ligands, linkers should terminate in amine and/or carboxylic acid functional groups to facilitate incorporation by solid-phase peptide synthesis. Both flexible linkers (7,8) and rigid linkers (9) are of interest. Each new linker must be validated as noninterfering with the binding of the ligand. We report herein the use of rigid linkers **1–4** (Figure 1), which are of variable length (10) to join two “high-affinity” or two “low-affinity” α -melanocyte stimulating hormone (α -MSH) peptide ligands (11–16), and evaluations of the bioactivities of these linker-spliced peptides.

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Supporting Information Available: Additional synthetic schemes and details of the solid-phase synthesis and compound characterization. This material is available free of charge via the Internet at <http://pubs.acs.org/BC>.

EXPERIMENTAL PROCEDURES

Materials and Methods

The *N*-9-fluorenylmethyloxycarbonyl (Fmoc) derivative **5** (Figure 1) was purchased from Torviq (Niles, MI). The *N*-trifluoroacetamide (TFA) derivatives **6–8** (Figure 1) were prepared as previously described (17). Other *N*- α -Fmoc-protected amino acids and Rink amide resin were purchased from SynPep (Dublin, CA) or from Novabiochem (San Diego, CA). *O*-[1*H*-6-Chlorobenzotriazol-1-yl](dimethyl-amino)methylene]uranium hexafluorophosphate *N*-oxide (HCTU), 1-hydroxybenzotriazole (HOBt), and 6-chloro-1-hydroxybenzotriazole (HOCT) were purchased from IRIS Biotech (Marktredwitz, Germany). For the *N*- α -Fmoc-protected amino acids, the following side-chain protecting groups were used: Arg(*N*^γ-2,2,4,6,7-pentamethyldihydrobenzofuran-5-yl sulfonyl); Glu(*O*^γ-*t*Bu); His(*N*^{im}-triphenylmethyl); Lys(*N*^ε-*tert*-butyloxycarbonyl); Ser(*O*-*t*Bu); Trp(*N*^l-*tert*-butyloxycarbonyl). Peptide synthesis solvents, reagents, and acetonitrile for HPLC were reagent grade, were acquired from VWR (West Chester, PA) or Sigma-Aldrich (Milwaukee, WI), and were used without further purification unless otherwise noted. Solid-phase synthesis was performed in fritted syringes using a Domino manual synthesizer obtained from Torviq (Niles, MI).

Purification of peptide products was achieved on a Waters 600 HPLC using a reverse-phase column (Vydac C-18, 15–20 m, 22 × 250 mm). Peptide products were eluted with a linear gradient of CH₃CN/0.1% CF₃CO₂H at a flow rate of 5.0 mL/min. Separation was monitored at 230 and 280 nm. The purity of the final peptide products was checked by TLC and by analytical PR-HPLC using a Waters Alliance 2695 separation model with a Waters 2487 dual wavelength detector (220 and 280 nm) on a reverse-phase column (Waters Symmetry 4.6 × 75 mm, 3.5 μm). Peptide products were eluted with a linear gradient of 60% aqueous MeCN/0.1 M NH₄OAc or MeCN/0.1% CF₃CO₂H at a flow rate of 1.0 mL/min. Size exclusion chromatography was performed on a borosilicate glass column (2.6 × 250 mm, Sigma, St. Louis, MO) filled with medium-sized Sephadex G-25 or G-10. The compounds were eluted with an isocratic flow of 1.0 M aqueous AcOH.

Structures were characterized by ESI (Finnigan, Thermoquest LCQ ion trap instrument) or MALDI-TOF (Bruker Rexlex-III, α -cyanocinnamic acid as a matrix). For internal calibration, an appropriate mixture of standard peptides was used with an average resolution of 8000–9000.

Solid-Phase Synthesis (18–19)

Rink resin was washed with DMF, and the *N*-Fmoc protecting group was removed with 50% piperidine in DMF (1 × 2 min and 1 × 20 min). The resin was washed successively with DMF, 1.0 M HOBt in DMF, and DMF, and the next *N*- α -Fmoc amino acid was coupled using preactivated 0.3 M HOCT ester in THF (3 equiv of *N*- α -Fmoc amino acid, 3 equiv of HOCT, and 3 equiv of DIC). The resin slurry was stirred for 2 h or until the bromophenol test became negative. If the test failed, the resin was washed with DMF, and the amino acid was coupled again by the HCTU/2,4,6-lutidine procedure (0.3 M solution of 3 equiv of *N*- α -Fmoc amino acid, 3 equiv of HCTU, and 6 equiv of 2,4,6-lutidine in DMF) for 3 h or until the Kaiser test was negative. If a positive Kaiser test was observed after the second coupling, the resin was washed with DMF and any reactive amine groups capped by treatment with 50% Ac₂O in pyridine for 10 min.

After the first peptide segment had been assembled on the solid support (e.g., see Scheme 1), an *N*-protected linker (**5**, **6**, **7**, or **8**) was attached to the free *N*-terminal amine using the coupling methods previously described. The resin was then split into two portions. The first portion of the resin was treated either with 50% piperidine in DMF to remove the Fmoc protecting group or with 15% anhydrous N₂H₄ plus 15% MeOH in THF at room temperature for 6 h to remove

the trifluoroacetyl protecting group, from the N-terminus of the resin-bound linker. The conditions for TFA cleavage were defined by an empirical study (see Table 1 and the accompanying discussion). The free amine was acetylated using 50% Ac₂O in pyridine for 10 min and the [peptide + linker] cleaved off the resin. A cleavage cocktail (10 mL per 1 g of the resin) consisting of CF₃CO₂H (91%), H₂O (3%), HSCH₂CH₂SH (3%), and PhSMe (3%) was injected into the resin, and the mixture was stirred at room temperature for 3 h. The solution was filtered off, the resin was washed with CF₃CO₂H (2 × 3 min), the liquid phases were concentrated under a stream of nitrogen, and the product was precipitated using cold Et₂O. The peptide pellets were washed three times with cold Et₂O, lyophilized, purified, and characterized as described above. For the second portion of the resin, removal of the N-terminal Fmoc or TFA was carried out, and assembly of the second peptide segment was completed using the methods previously described. Following incorporation of the last amino acid, the N-terminal protecting group was removed and the free amine acetylated using 50% Ac₂O in pyridine for 10 min. The resin was then thoroughly washed with DMF and CH₂Cl₂, the [peptide + linker + peptide] was cleaved off the resin using the cleavage cocktail, and the product purified as described above. Mass spectral and HPLC characterization data for the peptide products are given in Table 2 and are discussed later.

Biological Assays

Ligand binding was evaluated using a previously described lanthanide-based binding assay (20). HEK293 cells overexpressing the human melanocortin 4 receptor (hMC4R) were used to assess ligand binding (21). The coding region of the hMC4R gene was expressed in pcDNA3.1 (Invitrogen, Carlsbad, California). HEK293/hMC4R cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were plated in Black & White Isoplates (Wallac, 1450-583) at a density of 12 000 cells/well and were allowed to grow for 3 days. On the day of the experiment, media was aspirated from all wells. Ligands of interest were diluted in binding buffer [DMEM, 1 mM 1,10-phenanthroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, 0.3% bovine serum albumin (BSA)] to result in final dilutions ranging from 10 μM to 4 pM. Eu-labeled NDP-α-MSH was used at a final concentration of 10 nM. 50 μL of the ligand of interest and 50 μL of Eu-NDP-α-MSH were added to each well, and plates were incubated for 40 min at 37 °C. Following the incubation, cells were washed four times with Wash Buffer (50 mM Tris-HCl, 0.2% BSA, 30 mM NaCl) using Molecular Devices SkanWasher. Enhancement solution (Perkin-Elmer, 1244-105) was added (100 μL/well), and the plates were incubated for 30 min at 37 °C prior to reading. The plates were read on a Wallac VICTOR³ instrument using the standard Eu time-resolved fluorescence (TRF) measurement (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm). Competition curves were analyzed with GraphPad Prism software using the sigmoidal dose-response classical equation for nonlinear regression analysis. Each data point represents the average of at least four samples, with the error bars indicating standard error of the mean.

RESULTS

Solid-Phase Synthesis

The synthesis of compounds **9–11** consisting of two linked “high-affinity” NDP-α-MSH ligands connected in a head-to-tail fashion by linkers **1–3** is depicted in Scheme 1. Also depicted is the synthesis of control compounds **12–14** which are composed of a single “high-affinity” NDP-α-MSH ligand and an N-terminally acylated linker.

The tridecapeptide NDP-α-MSH was constructed on PS-Rink resin (**15**, initial loading 0.17 mmol/g, 1% DVB). Resin **16** retained all side-chain protecting groups. The N-protected linkers **5–7** were coupled to the N-terminus of **16**, giving resins **17–19**. The N-Fmoc group of **17** was

cleaved by treatment with piperidine, and the *N*-TFA groups of **18** and **19** were cleaved by treatment with 15% hydrazine and 15% methanol in THF. The resulting resins **20–22** were each split into two portions. For the synthesis of **9–11**, the free amine groups of resins **20–22** were coupled with Fmocvaline and solid-phase peptide synthesis continued to completion of the second NDP- α -MSH sequence, giving resins **23–25**. The peptide was then capped by *N*-acetylation, giving resins **26–28**. Simultaneous side-chain deprotection and cleavage of the peptides from the Rink resin was effected using a mixture of trifluoroacetic acid, 1,2-ethanedithiol, thioanisole, and water (91/3/3/3) that produced the desired compounds **9–11**. For the synthesis of **12–14**, resins **20–22** were terminally *N*-acetylated to give resins **29–31**. Treatment of these resins with the CF₃CO₂H/HSCH₂CH₂SH/PhSMe/H₂O cocktail effected simultaneous side-chain deprotection and cleavage of the peptides from the resin, producing compounds **12–14**. Compounds **32** and **33** (Figure 2) that incorporate the phenylnaphthyl linker **4** were similarly prepared (see Scheme S1 in the Supporting Information).

N-TFA derivatives **6–8** were employed instead of the corresponding Fmoc derivatives of **2–4**, since this enhanced the solubility of these more hydrophobic linkers under the conditions employed for solid-phase synthesis.

The intermediate resin **34** was used to establish conditions for the cleavage of the TFA protecting group from resin-bound linkers (Table 1). Published conditions (22) for *N*- ϵ -trifluoro-acetyl group deprotection in peptides (aqueous piperidine) gave poor results (Table 1, entry 1). DMF suppressed the cleavage of the *N*- ϵ -trifluoroacetyl group (entries 1, 3, and 4). Optimal conditions developed for the removal of the TFA protecting group from the resin-bound linker were treatment with 15% N₂H₄ and 15% MeOH in THF for 5 h at room temperature (entry 5).

A similar synthetic strategy was employed to synthesize molecules **37–40** containing linked “low-affinity” MSH(4) ligands (His-D-Phe-Arg-Trp; see Schemes S2 and S3 in the Supporting Information) and the corresponding controls **41–44** (Figure 3).

All products from solid-phase syntheses were purified by reverse-phase C₁₈ preparative HPLC and were characterized by analytical HPLC, ESI-MS, and/or MALDI-TOF. Characterization data are listed in Table 2.

Biological Assays

Shown in Figure 4 are representative results from a competition binding assay, described in Handl et al. (20). This figure shows the reduction of Eu-NDP- α -MSH binding in the presence of increasing concentrations of compounds **10** and **13**. From these data, IC₅₀ values were calculated as the concentration of test ligand that reduced Eu-NDP- α -MSH binding by 50%. These values can be quantitatively related to *K_d* by correcting for the ratio of the Eu-NDP- α -MSH concentration relative to its *K_d* (11). Similar data sets were obtained multiple times for all compounds. Table 3 lists the IC₅₀ values (averaged over *n* experiments) for the dimeric and monomeric complexes of NDP- α -MSH and MSH(4). Compounds **9–11** and **32** contained dimeric NDP- α -MSH ligands, compounds **37–40** contained dimeric MSH(4) ligands, and compounds **12–14**, **33**, and **41–44** were the corresponding monomeric ligand controls. This table also shows the fold increase in avidity as the ratio of IC₅₀ for the dimeric ligands relative to the corresponding monomeric ligands.

DISCUSSION

The IC₅₀ values for the [linker + NDP- α -MSH] control compounds **12**, **13**, and **33** were virtually the same as the IC₅₀ for the parental NDP- α -MSH ligand. Similarly, the IC₅₀ values for the [linker + MSH(4)] control compounds **41**, **42**, and **44** were very close to the IC₅₀ for the parental

MSH(4) ligand. These results indicate that attachment of the phenyl, biphenyl, and phenylnaphthyl linkers to the NDP- α -MSH and MSH(4) ligands had little or no effect on the binding of either ligand to hMC4R. In contrast, control compounds **14** and **43** that incorporate the terphenyl linker showed modest (3-fold and 2-fold, respectively) increases in IC₅₀. These results indicate that attachment of the terphenyl linker diminishes slightly NDP- α -MSH and MSH(4) ligand binding affinity to hMC4R.

The expected statistical halving of the IC₅₀ for compounds that contain two ligands was observed only for terphenyl-containing NDP- α -MSH compounds **11** and **14** and for MSH-(4) compounds **39** and **43**. This is consistent with the observation that terphenyl-containing compounds bind less well to hMC4R. Tight binding in the cases of **9**, **10**, and **33**, presumably due to slow off-rates for the NDP- α -MSH ligand, effectively removes tethered, unbound ligands from the ligand pool (Figure 5), implying that states B, C, and D are insignificant. Poorer binding of the terphenyl-containing compounds **11** and **14** permits observance of statistical halving of the IC₅₀ for these compounds (Figure 5), implying that states B, C, and D are significant.

A greater than stoichiometric increase in affinity for the hMC4R is observed relative to controls for compounds **37**, **38**, and **40** that contain two of the more weakly binding MSH(4) ligands joined by noninterfering linkers. This increase in affinity is inversely correlated (Figure 6) with an estimation of the molecular moments of inertia (I_M) calculated using eq 1 where M is the compound molecular weight and R is the linker length measured from one benzylic carbon to the other.

$$I_M = MR^2/2 \quad (1)$$

Compound **39** that incorporates the terphenyl linker is displaced from the best-fit line, possibly due to its inherently poorer binding to hMC4R. The existence of this correlation is consistent with a competition between diffusion of the compound away from the receptor (to give state E in Figure 5) and rotation of the compound necessary for presentation of the second ligand to the receptor (equilibration of states B and C). The greater the moment of inertia, the longer this rotation will take, leading to an increased probability of diffusion away from the cell surface before rebinding (to states A and D) can occur.

CONCLUSION

Low- and high-affinity forms of melanocortin ligands were attached to rigid linkers in either monomeric or dimeric forms. Binding affinities of these ligands were tested in whole cells expressing the hMC4 receptor using a sensitive competition assay based on detection of lanthanide (europium) with time-resolved fluorescence. Consistent with previous results, addition of linkers resulted in a reduction in binding affinity that was dependent on the size of the linker. Notably, the short phenyl and biphenyl linkers did not adversely affect binding affinities of monomeric ligands. Also consistent with previous results, there was an increase in binding affinity in dimers compared to the corresponding monomers, and the increase was inversely related to the calculated moment of inertia for the molecules. We interpret these results to indicate that the increased affinity of dimers was due to increased local concentrations of ligand, also known as the statistical effect of Kiessling (5). Somewhat surprisingly, this enhancement of dimers was only observed for the low-affinity MSH(4) ligands and not for the high-affinity NDP- α -MSH ligands. We interpret this to suggest that the statistical effect enhancement is critically dependent on a low-affinity ligand, with potentially faster on-rates relative to the rate of diffusion.

Acknowledgments

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Figure 1.
Rigid amino acid linkers **1–4** and *N*-protected derivatives **5–8**.

Figure 2.
Dimeric and monomeric NDP- α -MSH compounds **32** and **33**.

Figure 3.
Dimeric and monomeric MSH(4) compounds **37–44**.

Figure 4. Representative ligand binding curves generated through competitive ligand binding analysis compare the binding affinities of ligands **10** (□) and **13** (■). The calculated IC_{50} for **10** is 8 nM with an R^2 value = 0.91 and for **13** 10 nM with an R^2 value = 0.93.

Figure 5.

Binding of Linked MSH Ligands to hMC4R. **State A:** L₁ is bound, L₂ is unavailable for binding. **State B:** L₁ and L₂ are unbound, L₁ is positioned for binding. **State C:** L₁ and L₂ are unbound, L₂ is positioned for binding. **State D:** L₂ is bound, L₁ is unavailable for binding. **State E:** Compound is dissociated from cell surface (not shown).

Figure 6.
Inverse correlation of compound binding affinity and linker length.

Scheme 1. Solid-phase Synthesis of Compounds 9–11 Containing Linked NDP- α -MSH Ligands and Control Compounds 12–14

Reagents and conditions: (a) piperidine. (b) Fmoc/tBu solid-phase synthesis (Refs 18 and 19). (c) *N*-protected linker (5,6, or 7), HOCT-DIC, DMF. (d) THF/MeOH/N₂H₄ (70/15/15). (e) pyridine/Ac₂O (50/50). (f) CF₃CO₂H/HSCH₂CH₂SH/PhSMe/H₂O (91/3/3/3).

Table 1

Optimization of *N*-TFA Cleavage Using Resin 34^a

entry	conditions	time (min)	yields (%) ^b		impurity ^c
			35	36	
1	10% piperidine, 10% H ₂ O in DMF	30	0	100	0
2	0.6 M potassium silanolate in THF	30	0	100	0
3	15% N ₂ H ₄ in DMF	75	9	91	0
4	15% N ₂ H ₄ in DMF	180	34	64	2
5	15% N ₂ H ₄ , 15% MeOH in THF	300	100	0	0
6	15% N ₂ H ₄ , 15% MeOH in THF	180	99	>1	0
7	15% N ₂ H ₄ , 15% MeOH in THF	75	78	22	0
8	15% N ₂ H ₄ , 15% MeOH in THF	10	15	85	0
9	3% N ₂ H ₄ , 15% MeOH in THF	75	3	97	0
10	15% 0.5 M LiOH, 15% MeOH in THF	180	49	0	51
11	15% 0.5 M CsOH, 15% MeOH in THF	180	40	0	60

^a NDP-α-MSH Rink resin **34** (30 μL of resin slurry in THF) was placed into a small fritted 2 mL syringe and washed with an *N*-TFA cleavage solvent mixture. After the treatment, the resin was washed with THF and CH₂Cl₂, and the peptide was cleaved off the resin and side-chain protecting groups removed as described in the Materials and Methods section. The peptide was then dissolved in 30% aqueous MeCN and analyzed by HPLC using a linear MeCN/0.1% CF₃CO₂H aqueous gradient (10% to 40% in 30 min) at a flow rate of 1.0 mL/min.

^b Yields were determined as ratios of peak areas at 280 nm on HPLC analyses; product **35** had a retention time of 16.1 min, and product **36** had a retention time of 24.2 min.

^c Unidentified impurities.

Table 2

Analytical Data for Synthetic Compounds

Ac-NDP- α -MSH-linker-NDP- α -MSH-NH ₂				Ac-linker-NDP- α -MSH-NH ₂			
compd	mass spectrum ^a			mass spectrum ^a			HPLC ^b
	ion	calcd	obsd	ion	calcd	obsd	
9	(M + 3) ³⁺	1122.896	1122.90	(M + 2) ²⁺	890.4446	890.40	15.11
10	(M + 2) ²⁺	1721.860	1721.81	(M + 2) ²⁺	995.4462	995.40	22.69
11	(M + 3) ³⁺	1173.592	1173.50	(M + 2) ²⁺	993.4618	993.91	27.11
32	(M + 4) ⁴⁺	873.9417	873.94	(M + 3) ³⁺	653.9693	653.98	25.25

Ac-MSH(4)-linker-MSH(4)-NH ₂				Ac-linker-MSH(4)-NH ₂			
compd	mass spectrum ^a			mass spectrum ^a			HPLC ^b
	ion	calcd	obsd	ion	calcd	obsd	
37	(M + 1) ¹⁺	1445.705	1445.911	(M + 1) ¹⁺	819.3976	819.40	7.05
38	(M + 1) ¹⁺	1521.737	1522.176	(M + 1) ¹⁺	895.4289	895.41	9.65
39	(M + 1) ¹⁺	1597.768	1597.910	(M + 1) ¹⁺	971.4603	971.65	13.13
40	(M + 1) ¹⁺	1571.752	1571.833	(M + 1) ¹⁺	945.4445	945.48	11.66

^aMS found molecular peaks (M + 1)¹⁺, (M + 2)²⁺, (M + 3)³⁺, and (M + 4)⁴⁺. One diagnostic peak is listed here for each compound.

^bPeptides were eluted with a linear MeCN/0.1% CF₃CO₂H aqueous gradient (10% to 40% in 30 min) at a flow rate of 1.0 mL/min.

Table 3
Competitive Binding of Linked NDP- α -MSH and MSH(4) Ligands to hMC4R

compound	monomer/dimer	linker ^a	IC ₅₀	<i>n</i> ^b	fold increase
NDP- α -MSH		none	5.6 $\pm$ 0.2 nM	3	
9	D	Ph	3.5 $\pm$ 2.2 nM	4	1.4
12	M	Ph	5.0 $\pm$ 1.2 nM	4	
10	D	BP	5.6 $\pm$ 3.5 nM	5	0.9
13	M	BP	5.2 $\pm$ 5.2 nM	5	
11	D	TP	8.4 $\pm$ 2.9 nM	5	2.0
14	M	TP	16.5 $\pm$ 14.7 nM	5	
32	D	PN	3.7 $\pm$ 0.9 nM	5	1.1
33	M	PN	3.9 $\pm$ 1.2 nM	4	
MSH(4)		none	2.1 $\pm$ 0.8 μ M	3	
37	D	Ph	0.1 $\pm$ 0.05 μ M	4	22.9
41	M	Ph	1.8 $\pm$ 0.2 μ M	4	
38	D	BP	0.6 $\pm$ 0.2 μ M	4	4.8
42	M	BP	2.8 $\pm$ 1.1 μ M	4	
39	D	TP	2.6 $\pm$ 1.0 μ M	4	1.9
43	M	TP	5.0 $\pm$ 3.6 μ M	5	
40	D	PN	0.5 $\pm$ 0.2 μ M	4	3.2
44	M	PN	1.5 $\pm$ 0.7 μ M	4	

^aPh = phenyl linker **1**; BP = biphenyl linker **2**; TP = terphenyl linker **3**; and PN = phenyl/naphthyl linker **4**.

^bThe IC₅₀ value given is the average of *n* independent binding experiments, each done in quadruplicate.