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Probing Protein Surfaces: QSAR Analysis using Helix Mimetics

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Abstract

α -helix mediated protein-protein interactions (PPIs) represent important targets for small molecule inhibition, however generic approaches to inhibitor design are in their infancy and would benefit from QSAR analyses to rationalize the non-covalent basis of molecular recognition by designed ligands. In this manuscript using a helix mimetic based on an oligoamide scaffold we utilize the power of a modular synthesis to access compounds which can readily be used to understand the non-covalent determinants of hDM2 recognition by this series of cell active p53/hDM2 inhibitors.

Keywords

helix mimetics; protein-protein interactions; foldamers; p53/hDM2; chemical biology

Introduction

The design and synthesis of small molecule inhibitors of protein-protein interactions (PPIs) represents an ongoing challenge in chemical biology.^{1–6} These bio-molecular interfaces have historically been considered “undruggable” in comparison to traditional targets for small molecule probe- and drug-discovery (e.g. kinases, GPCRs and enzymes).⁷ However, the perception has been effectively challenged over the last decade through discovery of numerous inhibitors, particularly, for helix mediated PPIs⁸ (whereby a helix from one protein binds in a complementary cleft on its partner),⁹ leading to a number of small molecules in clinical trials.^{10–12} In this respect notable examples include inhibitors of p53/hDM2(X)¹³ and Bcl-2 family¹⁴ interactions. Many of these compounds have been discovered using conventional methodologies e.g. high-throughput screening (HTS), Fragment Based Drug Discovery (FBDD) and Computer Aided Drug Design (CADD);⁸ such compounds are

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In Memory of Dr Julie Fisher, friend and colleague

typically complex and this can make analysis of the determinants of molecular recognition challenging. In contrast biomimetic ligands e.g. foldamers^{15–21} and helix mimetics,^{22–34} as a consequence of the modular approaches for their assembly, should readily yield to conventional physical organic approaches (i.e. quantitative structure activity relationships – QSAR)^{35, 36} used to assess these parameters.

Our group have developed a series of aromatic oligoamide small-molecule helix mimetics **1–4**^{28, 31, 34} that effectively present functional groups in a spatial orientation so as to mimic that presented by the *i*, *i* + 3/4 and *i* + 7 side chains of an α -helix (Fig. 1). These scaffolds can be readily synthesized using conventional Fmoc based solid-phase methods^{37, 38} and have been shown to act as inhibitors of the HIF-1 α /p300 interaction²⁸ and potent cell-permeable dual inhibitors of the p53/*h*DM2 and NOXA-B/Mcl-1 interactions.³⁴ We further elaborated our set of helix mimetic scaffolds through development of a hybrid structure comprising a central α -amino acid monomer; this allowed us to demonstrate that the selectivity of inhibition (*h*DM2 v Mcl-1) was influenced by the amino-acid stereochemistry.³¹ Although helix mimetics have been shown to vary in their inhibitory potency with stereochemistry, such enantiomeric discrimination between proteins is unprecedented, suggesting these ligands are capable of a high degree of structural complementarity towards their target proteins. In the current manuscript, using this hybrid scaffold and p53/*h*DM2 as a model PPI, we outline, to the best of our knowledge, the first QSAR analysis of an α -helix mimetic: in doing so we provide valuable insights on the nature of molecular recognition in the *h*DM2 helix binding cleft.

Results and Discussion

The p53/*h*DM2 interaction involves the binding of the p53 transactivation domain to a cleft on *h*DM2 with Phe19, Trp23 and Leu26 representing a minimal pharmacophore for recognition (Fig. 2a-b). Although additional side chains within p53 contribute towards binding specificity and selectivity, many of the known p53/*h*DM2 inhibitors reproduce the minimal pharmacophore to achieve inhibition.^{39, 40} Of these, multiple ligands (including the p53 sequence)⁴¹ have been shown to have increased inhibitory potency when the functional group mimicking the tryptophan bears a chloride group (Fig 2c-d).^{12, 42} Such improved potency might derive from better steric complementarity between protein and ligand, increased hydrophobicity or increased polarization on the aromatic ring bearing the chloride group. Whereas highly optimized small-molecule inhibitors assembled using multi-step syntheses might not readily succumb to studies geared toward dissecting these influences, our helix mimetics due to the modular nature of their synthesis are perfectly suited to this purpose.

Helix mimetic **1a** (scheme 1) was previously shown to act as a low μ M p53/*h*DM2 inhibitor (*(S)* = 11.9 ($\pm$ 0.6) μ M and (*R*) = 9.2 ($\pm$ 0.4) μ M) in a competition fluorescence anisotropy assay (Fig. 3 and Table 1). ¹H-¹⁵N HSQC perturbation shift analyses supported the hypothesis that this compound functions by binding to the p53 binding site on *h*DM2, whilst a requirement for all three side-chains and a role for side-chain spacing in determining molecular recognition were all established. Likewise, the tryptophan bearing analogue **1b** was also shown to act as a low μ M p53/*h*DM2 inhibitor (Table 1), data that are reinforced

by the results for the previously unpublished regioisomer of **1b** i.e. **5a** (see Scheme 1 for synthesis). The utility of these compounds as bioactive helix mimetics is demonstrated by a cellular assay for p21 which is induced downstream in response to active p53. p21 has emerged as a key mediator of p53-dependent growth arrest, and its measurement acts as an indicator of p53 activation rather than expression. Thus we were able to measure whether our panel of compounds were causing p53 transactivation in cells. Similarly to Nutlin-3a (a known p53/*h*DM2 inhibitor), compounds **1a-b** and **5a** were all shown to increase p21 levels in contrast to DMSO and a negative control compound **6a** (Fig. 4). Consistent with our earlier work³⁴ correlation between biophysical and cellular effect is difficult to evaluate and influenced by multiple other factors e.g. inhibitor uptake; this will be studied in more detail in due course.

To perform QSAR analysis, helix mimetics '**1a-I**' were synthesized as shown in Scheme 1 (see ESI for spectra and further characterization). The central amino acid was varied so as to permit analyses of the tryptophan mimicking residue.

Compounds were tested in a previously described fluorescence anisotropy competition assay with the inhibitory potencies (IC_{50}) varying between ~10 and >100 μ M (Fig. 3 and Table 1). For comparative purposes Nutlin-3a gave an $IC_{50} = 0.50 (\pm 0.02) \mu$ M in this assay.³¹ Four different properties were considered to investigate the effect of the *p*-substituent on the benzyl ring of Phe: (i) hydrophobicity of the substituents, expressed as π values;³⁶ (ii) electronic effects related to the electron withdrawing properties of the substituents, expressed as σ_p Hammett constants;³⁶ (iii) steric effects of the substituents, expressed as molar refraction (MR) values;³⁶ (iv) H-bonding interactions, expressed as α^H (donor) or β^H (acceptor) values;⁴³ The logarithm of the IC_{50} values for compounds the (*S*) isomers of **1a** and **1c-h** was plotted against all four parameters and the result is shown in Fig. 5a-e. The H-bonding ability of these substituents did not show a direct correlation with the activity. The steric effect seemed to have a linear correlation when considering the halo-substituted derivatives, but the trend was not consistent throughout the series. Important in differentiating between hydrophobicity and electronic effects were the substituents with uncorrelated π and σ_p values (e.g. *p*-nitrile: $\pi = -0.57$, $\sigma_p = 0.66$; *p*-methyl: $\pi = 0.56$, $\sigma_p = -0.17$).³⁶ Partial correlation was observed with σ_p value but not for the nitrile which would be anticipated to be more potent than other substituents. This allowed us to surmise that the dominant contribution to inhibitory potency derives from the hydrophobicity on the Trp mimicking substituent as full correlation was observed with π value, which is consistent with the hydrophobic nature of the protein binding cleft. To ascertain the extent to which the cellular behavior of this series of compounds mirrors the parent compounds **1a-b**, the most potent (**1e**) was also tested for p21 induction with positive results (Fig. 4)

Conclusions

In this paper we illustrated that foldamers comprising 2- and 3-*O*-alkoxyaminobenzoic acid and α -amino acid building blocks act as effective α -helix mimetics and elicit a cellular response consistent with inhibition of the p53/*h*DM2 protein-protein interaction. These "hybrid" helix mimetics are amenable to QSAR analyses, which, reveal that molecular recognition of *h*DM2 is governed by hydrophobicity in the central α -amino acid side chain

as opposed to several other parameters assessed, notably steric and electronic factors. This observation may be useful in the design of small molecule p53/hDM2 inhibitors. Our future studies will center on extending this analyses to a broader array of substituents so as to optimize binding potency and, on repurposing this helix mimetic scaffold to target other helix mediated protein-protein interactions

Experimental Section

General methods

Unless otherwise stated, all the chemicals and reagents were obtained commercially from Sigma-Aldrich, Fisher Scientific, Alfa Aesar or Merck and used without further purification. Amino acid derivatives, coupling reagents and resins were purchased from Merck Millipore. All solvents used were HPLC grade. Analytical Thin Layer Chromatography was performed on precoated silica gel plates (Kieselgel 60F254, Merck). Organics. NMR spectra were recorded in DMSO- d_6 , unless otherwise stated, on an AV 500 MHz Bruker NMR spectrometer. All chemical shifts are reported in δ ppm downfield of TMS and peak multiplicities as singlet (s), doublet (d), quartet (q), quintet (quin), septet (sep), doublet of doublets (dd), broad singlet (bs), and multiplet (m). Signal assignment, where possible/necessary, was made with the help of 2D-NMR techniques (COSY, HMQC, HMBC, and NOESY). High resolution mass spectra (HRMS) were obtained using either a Waters GCT Premier mass spectrometer, using electron impact (EI), a Bruker micrOTOF using electrospray ionisation (ESI), or a Bruker MaXis Impact, using electrospray ionisation (ESI). Liquid chromatography mass spectra (LC-MS) were run on an Agilent 1200 LC system equipped with a Phenomenex Luna C18(2) 50×2 mm column, 5 mm particle size, on an acetonitrile/water gradient (5-95% acetonitrile, 0.1% formic acid, over 3 minutes) and a Bruker Daltonics HTCUltraTM system equipped with an Ion trap MS detector. Mass-directed HPLC purifications were run on an Agilent 1260 Infinity Preparative HPLC system equipped with a Waters XBridgeTM Prep C18 19×100 mm column, 5 mm particle size, on an acetonitrile or methanol/water gradient (5-95% acetonitrile or methanol over 8 minutes) and an Agilent 6120 Quadrupole system equipped with a quadrupole MS detector, using electrospray ionisation (ESI). Infra-red (IR) analyses were performed using a Perkin Elmer FT-IR spectrometer or a Bruker Platinum-ATR system equipped with an Alpha FT-IR spectrometer and the samples were analysed as solids. Optical rotations were recorded on a Schmidt Haensch Polatronic H 532 polarimeter using the sodium D line (589 nm). $[\alpha]_D$ are reported in units of 10^{-1} deg dm^2 g^{-1} .

Synthetic procedures: Solid phase synthesis of hybrid α -helix mimetics

Compounds **1a-b**, **1i** and **6a** were synthesized as described previously as were the monomers required for synthesis of the novel helix mimetics described herein.³¹ A generic procedure was followed by adapting the previously reported solid phase synthesis of 3-*O*-alkylated oligobenzamides. The reactions were all carried out on a CEM Liberty[®] automated microwave assisted peptide synthesiser. The procedure is described briefly below:

Resin preparation

127 mg of Fmoc-Gly-Wang resin (0.79 mmol g^{-1} , 100-200 mesh; carrier: polystyrene, crosslinked with 1% DVB) were used for hybrid mimetics **1-9**. The resin was swelled in Corning® tubes in DMF for at least 30 minutes prior to coupling. Standard washing and deprotection cycles were carried out on the synthesiser. A filtered drain took place in between every wash as follows: wash the reaction vessel from the top ('DMF top', 5 mL), then from the bottom ('DMF bottom', 7 mL), then 'DMF top' (5 mL).

Fmoc deprotection

Before coupling of each monomer and after the last coupling reaction, two deprotection cycles were carried out on the CEM synthesiser using 6 mL of a 25% piperidine solution in DMF, under microwave heating at 75 °C. The initial deprotection lasted 30 seconds and was followed by a 'DMF top' wash and filtered drain. After a second addition of deprotection mixture, the deprotection was maintained under microwave heating for 3 minutes, and was again followed by DMF washes.

Coupling of 2-O-alkylated monomers to the resin

Prior to the reaction, each fully protected monomer (1.5 eq.) was dissolved in anhydrous DMF (2.5 mL) in a dry 50 mL Corning® tube (stored in an oven at 60 °C for at least 24 hours), and pre-activated with HATU (1.9 eq.) and DIPEA (3.8 eq.) at room temperature. A

Coupling of middle amino acids

Prior to the reaction, each fully protected amino acid (2.5 eq. per coupling) was dissolved in anhydrous DMF (2.5 mL per coupling) in a dry 50 mL Corning® tube (stored in an oven at 60 °C for at least 24 hours), and pre-activated with HATU (3 eq. per coupling) and DIPEA (5 eq. per coupling) at room temperature for 1-3 hours under a nitrogen atmosphere. For natural amino acid derivatives, a double coupling of 30 minutes was carried out under microwave heating at 60 °C. For non-natural amino acids, a triple coupling of 30 minutes was carried out under microwave heating at 60 °C.

Coupling of 3-O-alkylated monomers

Prior to the reaction, each fully protected monomer (1.5 eq. per coupling) was dissolved in anhydrous DMF (2.5 mL per coupling) in a dry 50 mL Corning® tube (stored in an oven at 60 °C for at least 24 hours), and pre-activated with HATU (3 eq. per coupling) and DIPEA (5 eq. per coupling) at room temperature. A double coupling method of 30 min was carried out under microwave heating at 60 °C.

Cleavage

The cleavage step was carried out manually, in 3 mL 'Extract-Clean' polypropylene reservoirs fitted with 20 µm polyethylene frits, both available from Alltech. The resin was transferred to a reservoir then washed with dichloromethane ($2 \times 3 \text{ mL} \times 2 \text{ min}$) and diethylether ($2 \times 3 \text{ mL} \times 2 \text{ min}$). 3 mL of a 1:1 mixture of TFA in dichloromethane was added and the mixture was allowed to stir at room temperature for 40 minutes. The content of the reservoir was collected and the procedure was repeated. The combined solution was

then concentrated under reduced pressure and the resulting oil was washed with hexane and diethylether to afford a solid.

Purification

The hybrid mimetics were purified using mass-directed preparative HPLC (0.1% formic acid water/methanol or acetonitrile; 5-95% gradient) to afford the desired products with purity higher than 90-95%.

H₂N-[O-Bn-(3-HABA)]-4-F-Phe-[O-*i*Pr-(2-HABA)]-Gly-CO₂H **1c** (**S**)

Colourless solid, > 95% pure by NMR; isolated yield: 12.5 mg, 19%; *R_f* 0.10 (SiO₂, 20% methanol in acetonitrile); $[\alpha]_D^{27.1} = +140.6^\circ$ (c 0.005 g mL⁻¹, methanol); ¹H NMR (DMSO-d₆, 500 MHz) d: 10.44 (s, 1H, 1-Gly-CO₂H), 8.47 (t, *J* = 5 Hz, 1 H, 1-Gly-NH), 8.34 (d, *J* = 8 Hz, 1 H, 3-(4-F)-Phe-NH), 7.90 (d, *J* = 9 Hz, 1 H, 2-H6), 7.62 (s, 1 H, 2-H3), 7.51 (d, *J* = 7 Hz, 2 H, 4-HAr2), 7.44 (s, 1 H, 4-H2), 7.43-7.39 (m, 5 H, 3-(4-F)-Phe-HAr2, 4-HAr3, 4-HAr4), 7.35 (s, 1H, 2-NH), 7.33 (d, *J* = 8.5 Hz, 1 H, 4-H5), 7.25 (d, *J* = 9 Hz, 1 H, 2-H5), 7.10 (t, *J* = 9 Hz, 2H, 3-(4-F)-Phe-HAr3), 6.66 (d, *J* = 9 Hz, 1 H, 4-H5), 5.34 (br, 2H, 4-NH₂), 5.16 (s, 2 H, 4-Ha), 4.81-4.76 (dd, *J* = 9.5, 5 Hz, 1 H, 3-(4-F)-Phe-Ha), 4.74-4.70 (quin, *J* = 6 Hz, 1 H, 2-Ha), 4.06 (d, *J* = 5 Hz, 2 H, 1-Gly-H1), 3.14-3.07 (dd, *J* = 9.5, 5 Hz, 2 H, 3-(4-F)-Phe-Hb), 1.43-1.41 (dd, *J* = 12, 6 Hz, 6 H, 2-Hb) ppm; ¹³C NMR (DMSO-d₆, 126 MHz) d: 171.2 (1-Gly-CO), 166.4 (4-CO), 163.9 (2-CO), 161.9-160.0 (d, *J* = 242 Hz, 3-Phe-CAr4), 156.2 (3-Phe-CO), 144.1 (4-C3), 143.0 (2-C2), 141.6 (4-C4), 137.2 (2-C4), 134.3 (3-(4-F)-Phe-CAr1), 131.9 (2-C6), 131.0 (4-CAr1), 128.3 (4-CAr3, 4-CAr4), 127.7 (4-C2), 127.4 (3-(4-F)-Phe-CAr2, 4-CAr2), 121.7 (4-C6), 120.8 (4-C1), 116.6 (2-C1), 114.8-114.7 (d, *J* = 20 Hz, 3-Phe-CAr3), 112.3 (4-C5), 111.3 (2-C5), 104.7 (2-C3), 71.9 (2-Ca), 69.4 (4-Ca), 55.9 (3-(4-F)-Phe-Ca), 41.6 (1-Gly-C1), 36.2 (3-Phe-Cb), 21.6 (2-Cb) ppm; IR (neat) $\tilde{\nu}$ (cm⁻¹): 3358, 2975, 2931, 1690, 1596, 1496, 1260, 1216, 754; ESI-MS found: 643 (M+H)⁺; HRMS *m/z* calculated for C₃₅H₃₅FN₄NaO₇ (M+Na)⁺: 665.2382; Found: 665.2386.

Cellular Analyses

U2OS cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin and 2 mM L-glutamine. Compounds were added as 50 μM stocks in serum-free media (DMEM) and the cells were incubated for 4 hours. Cells were lysed with 100 μl of lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5 % Nonidet P-40) and the lysate was analysed by SDS PAGE electrophoresis and probed with antibodies against p21 (C-19, Santa Cruz, sc-397), and GAPDH (G-9, Santa Cruz, sc-365062).

Proteins expression and purification

hDM2 (17-126) L33E was expressed in *E.coli* BL21 (DE3) GOLD and purified following previously published methods. The purified hDM2 (17-126) L33E was concentrated (typically to ~ 100 μM) and stored at -80 °C.

Fluorescence anisotropy assays

WT-p53(15-31) transactivation domain peptide and its fluorescein-labelled analogue p53 (15-31) Flu were purchased from Peptide Protein Research Ltd and used without further purification. Fluorescence anisotropy assays were performed in 384-well plates (Greiner Bio-one). Each experiment was run in triplicate and the fluorescence anisotropy measured using a Perkin Elmer EnVision™ 2103 MultiLabel plate reader, with excitation at 480 nm (30 nM bandwidth), polarised dichroic mirror at 505 nm and emission at 535 nm (40 nM bandwidth, *S* and *P* polarised). All experiments were performed in assay buffer: 40 mM phosphate buffer at pH 7.50, containing 200 mM NaCl and 0.02 mg mL⁻¹ bovine serum albumin (BSA) and data analysed following previously published methods.

Competition assays

Stocks of hybrid α -helix mimetics (400 μ M in 90:10 (v/v) assay buffer: DMSO) were used to prepare serial dilution across the plate (starting point: 100 μ M; 18-points, 3/4 serial dilution). p53 (15-31) Flu and hDM217-126 L33E were then added to each well to give a final concentration of 54.5 nM and 154.2 nM, respectively. For control wells the tracer peptide was replaced with an identical volume of assay buffer. The total volume in each well was 60 μ L. The data for both the *P* (perpendicular intensity) and *S* (parallel (same) intensity) channels, resulting from this measurement and corrected by subtracting the corresponding control wells, were used to calculate the intensity and anisotropy for each well following Equations S 1 and S 2:

$$I = (2PG) + S \quad \text{Equation 1}$$

$$r = \frac{S - PG}{I} \quad \text{Equation 2}$$

Where *I* is the total intensity, *G* is an instrument factor which was set to 1 for all experiments and *r* is the anisotropy.

The average anisotropy (across three replicates) and the standard deviation of these values were then calculated and fit to a sigmoidal logistic model (Equation 3) using OriginPro 9.0.

$$y = r_{\max} + \frac{r_{\min} - r_{\max}}{1 + \left(\frac{x}{x_0}\right)^p} \quad \text{Equation 3}$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of previously described aromatic oligoamide α -helix mimetics 1-4.

Figure 2.

Structural studies on the p53 binding domain of *hDM2* (a) p53/*hDM2* X-ray crystal structure (PDB ID: 1YCR) (b) p53 transactivation domain excised from *hDM2* illustrating key side chains required for binding (c) structure of a cyclic β -hairpin containing a chloro-tryptophan bound to *hDM2* (PDB ID: 2AXI) (d) structure of AMG232 containing a chlorophenyl group bound to *hDM2* (PDB ID: 4OAS)

Figure 3. Fluorescence anisotropy (FA) competition assay data for inhibition of p53/hDM2 interaction with hybrid helix mimetics **1a-I** ((*S*) isomers)

Figure 4.

Cellular response to mimetics; U2OS cell lines were incubated with mimetics (50 μ M) or Nutlin-3a (10 μ M) for 4 h and lysates analysed by western blotting for p21 and GAPDH.

Figure 5.

QSAR analyses for compounds (*S*) **1a** and (*S*) **1c-h** (a) hydrophobicity (π) (b) electronic withdrawing capability (σ_p) (c) steric bulk (molar refraction – MR) (d) hydrogen-bond acidity (α_H) (e) hydrogen-bond basicity (β_H) (f) correlation between hydrophobicity (π) and electronic (σ_p) values

Scheme 1.

Solid phase synthesis of hybrid α -helix mimetics a. Loading: resin (1 eq.) swelled in anhydrous DMF and deprotected with 20% (v/v) piperidine/DMF (two cycles of 3 min at 75 °C), 2-*O*-alkylated monomer (1.5 eq.) in anhydrous DMF pre-activated with HATU (1.9 eq.) and DIPEA (3.8 eq.), single coupling method (30 min at 50 °C); b. Deprotection: 20% (v/v) piperidine/DMF (two cycles of 3 min at 75 °C); c. Coupling: amino acid (2.5 eq. per coupling) or 2-*O*-alkylated monomer (1.5 eq. per coupling) in anhydrous DMF pre-activated with HATU (3 eq. per coupling) and DIPEA (5 eq. per coupling), double or triple coupling

method (2 or 3×30 min at 60 °C); d. Manual cleavage: 50% (v/v) TFA/CH₂Cl₂ (two cycles of 40 min at r.t.).

Table 1
IC₅₀ values for inhibitors 1a-i and 5a as determined by FA competition assay.

Compound	R ²	IC ₅₀ in μM ($\pm$)
(<i>S</i>) 1a	(<i>S</i>) phenyl	11.9 (0.6)
(<i>R</i>) 1a	(<i>R</i>) phenyl	9.2 (0.4)
(<i>S</i>) 1b	(<i>S</i>) 2-indolyl	11.5 (0.3)
(<i>R</i>) 1b	(<i>R</i>) 2-indolyl	24.1 (1.4)
(<i>S</i>) 1c	(<i>S</i>) 4-F-phenyl	24 (0.9)
(<i>S</i>) 1d	(<i>S</i>) 4-Cl-phenyl	13.5 (1.3)
(<i>S</i>) 1e	(<i>S</i>) 4-Br-phenyl	9.0 (0.4)
(<i>S</i>) 1f	(<i>S</i>) 4-NC-phenyl	>100
(<i>S</i>) 1g	(<i>S</i>) 4-CH ₃ -phenyl	16.7 (0.9)
(<i>S</i>) 1h	(<i>S</i>) 4-OH-phenyl	>100
(<i>S</i>) 1i	(<i>S</i>) imidazolyl	>100
(<i>S</i>) 5a	(<i>S</i>) 2-Indolyl	20.3 (2.1)
(<i>R</i>) 5a	(<i>R</i>) 2-Indolyl	13.8 (0.6)
(<i>S</i>) 6a	(<i>R</i>) phenyl	>100