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An on-bead assay for the identification of non-natural peptides targeting the Androgen Receptor–cofactor interaction

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ABSTRACT

An efficient and rapid on-bead screening method was established to identify non-natural peptides that target the Androgen Receptor–cofactor interaction. Binding of the Androgen Receptor ligand binding domain to peptide sequences displayed on beads in a One-Bead-One-Compound format could be screened using fluorescence microscopy. The method was applied to generate and screen both a focussed and a random peptide library. Resynthesis of the peptide hits allowed for the verification of the affinity of the selected peptides for the Androgen Receptor in a competitive fluorescence polarization assay. For both libraries strong Androgen Receptor binding peptides were found, both with non-natural and natural amino acids. The peptides identified with natural amino acids showed great similarity in terms of preferred amino acid sequence with peptides previously isolated from biological screens, thus validating the screening approach. The non-natural peptides featured important novel chemical transformations on the relevant hydrophobic amino acid positions interacting with the Androgen Receptor. This screening approach expands the molecular diversity of peptide inhibitors for nuclear receptors.

1. Introduction

Nuclear receptors (NRs) are transcription factors which typically bind small hydrophobic ligands in their Ligand Binding Domain (LBD).[1] Depending on the molecular characteristics of these ligands, the LBD[2] changes its conformation and binds to cofactor proteins. The cofactor proteins are typically corepressors when the ligand is an antagonist and coactivators in case the ligand is an agonist.[3] The interaction of the LBD with protein coactivators, inducing gene transcription, occurs via leucine-rich motifs in the coactivator proteins. This motif has the form of a short amphiphatic helix with a characteristic LXXLL[4] peptide sequence for almost all nuclear receptors, but the Androgen Receptor (AR) preferentially binds cofactor proteins with a FXXLF motif.[5,6] This protein–protein interaction constitutes an attractive interface, possibly amendable by synthetic inhibitors.[7] Synthetic compounds inhibiting NR–coactivator interactions have been described in the last years, both based on peptides[8,9] and small molecules.[10] Peptide based screening methods have been based on large and diverse libraries such as those obtained via phage display techniques[9] and on smaller focussed libraries obtained via synthetic techniques and allowing for the incorporation of non-natural amino acids.[8] New rapid screening techniques of synthetic peptide libraries targeting the NR–coactivator interaction are highly desirable. This will enable the screening of new peptide inhibitor motifs for the NR–coactivator interaction, the incorporation of non-natural amino acids, and possibly the generation of peptide binders targeting other NR surfaces. Using the AR[11] as a model system, we show here that an on-bead screening of One-Bead-One-Compound (OBOC) libraries[12,13] against agonist-liganded AR LBD provides an entry to generate such new peptide binders for NRs (Fig. 1). Beads amendable to organic synthetic modifications and compatible to protein screening conditions were modified in a combinatorial fashion with a specific peptide library leading to OBOC peptide libraries. Incubation of these libraries with glutathione-*S*-transferase (GST)-tagged AR LBD and GST-antibody tagged quantum dots (Qdot) enabled detection via fluorescence microscopy (Fig. 1). The approach was applied both for the screening of a focussed peptide library around an established FXXLF motif and for the screening of a random peptide library and provides entries to identify new peptide binders for AR–coactivator interaction.

2. Results and discussion

2.1. Library design

To evaluate the potential of an OBOC approach to screen for AR binding peptides, first an OBOC library was generated based on a strong AR peptide binding motif. One of the best known AR peptide binders (SSR**F**ES**LF**AGEKESRG),[6] found via phage display, was selected as starting point for the focussed peptide library. This peptide sequence features the characteristic FXXLF motif. Amino

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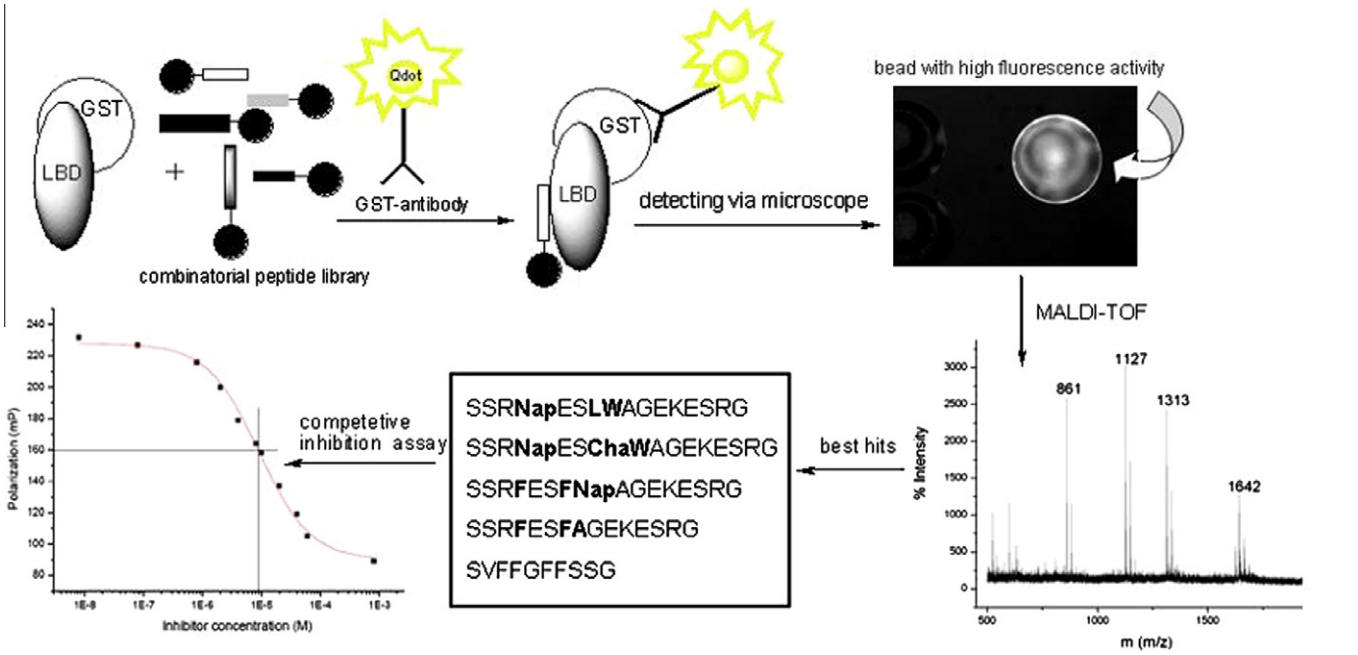

Figure 1. OBOC screening approach for peptides targeting the AR–cofactor interaction. GST-tagged AR is incubated with beads displaying peptides, binding is detected using GST-antibody-tagged Qdots. Hits are identified via mass spectrometry, resynthesized and evaluated in a fluorescence polarization based competition assay.

acid mutations of the three hydrophobic F and L positions would result in a library that allows for evaluation of the hydrophobic groove on the AR with respect to recognition motif and steric adaptability. The amino acids in these three important positions were randomized in the library with nine natural amino acids of diverse nature (A, F, H, L, M, P, Q, W, Y) and the three non-natural hydrophobic amino acids β-cyclohexylalanine (Cha), β-(2-naphthyl)alanine (Nap) and 4-chlorophenylalanine (PhCl) (Fig. 2). The hydrophobic nature of the non-natural amino acids was specifically selected, because of its documented importance to confer high binding affinity of peptides to the AR.[5,6]

2.2. Library synthesis

The peptide-bead library was synthesized on a glycine-preloaded TentaGel MB HMB resin using an automatic peptide synthesizer via standard solid phase Fmoc-chemistry with HOBt/DIC as coupling reagents. This specific resin has good swelling characteristics in both aqueous and organic solvents. This allows peptide synthesis with concomitant on-bead evaluation, while featuring good mechanical stability and low non-specific protein binding. The permutation steps were performed in accordance with the split and pool synthesis methodology. After the incorporation of random amino acids in positions +5, +4 and +1 a partial end-capping of the peptide sequence was performed after each of the coupling steps using 10% of acetylated alanine as an endcapper. This capping step was essential to simplify the analysis of the peptide sequence on a single bead. As a result, each bead is not completely covered with one peptide sequence anymore, but also contains capped, truncated peptides. Even though these might bind in the initial screen, the subsequent control experiments will evaluate each complete peptide for binding. The complete synthesis resulted in an OBOC library with a theoretical size of $12E^3$ different peptide sequences. For control experiments a batch of beads was prepared displaying only the SSR**F**ES**LF**AGEKESRG[6] sequence.

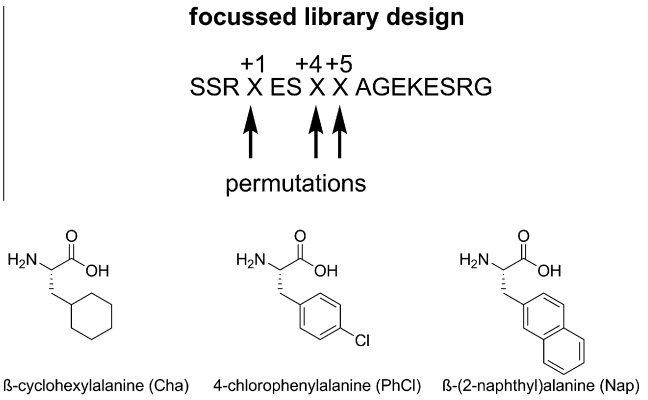

Figure 2. Library design of a focused combinatorial peptide library targeting the AR–coactivator interaction. The three positions in the peptide marked X were randomized using nine natural amino acids (A, F, H, L, M, P, Q, W, Y) and three non-natural amino acids (β-cyclohexylalanine (Cha), 4-chlorophenylalanine (PhCl), β-(2-naphthyl)alanine Nap)).

2.3. Protein expression

The LBD of the AR was expressed in *Escherichia coli* as a fusion protein with an N-terminal Glutathione-*S*-Transferase (GST), in the presence of the endogenous agonist dihydrotestosterone (DHT) and purified via glutathione affinity chromatography.[14] Initially, the protein was labelled with amine reactive texas red dye for fluorescence microscopy visualization. However, this resulted in rapid protein aggregation and denaturation. Therefore, an alternative labelling strategy was developed using the already incorporated GST-tag of the protein. The GST-tag was used to detect beads to which the fusion protein was bound by a subsequent incubation with quantum dots (Qdot) functionalized with a GST-antibody. The large wavelength difference between the excitation and emission spectrum of the Qdot (λ_{ex}: 405 nm; λ_{em}: 655 nm) has the additional advantage that unfavourable autofluorescence (intense and broad) of the polystyrene-based resin is not hampering the fluorescence microscopy evaluation of the protein binding to the beads.[13]

2.4. On-bead screening of a focused library

The on-bead screening was performed by preblocking the peptide beads with bovine serum albumin (BSA) in HEPES-buffer

for 24 h at 4 °C. After removal of this solution, the beads were incubated with the GST-tagged AR LBD bound to dihydrotestosterone (DHT) in the presence of BSA for 4 h at room temperature. Finally, for monitoring, the library was washed and incubated with a 0.01 μM solution of Qdot-labelled *anti*-GST-antibody (Qdot® 655 goat *anti*-glutathione-*S*-transferase, Invitrogen) for 2 h at room temperature. After the supernatant solution was removed and the beads were washed, the binding of the AR to the beads was screened using a fluorescence microscope. Beads binding the labelled AR protein by virtue of their attached peptides are expected to strongly fluoresce at 655 nm. On the other hand beads binding the AR weakly or not are expected to show little or no fluorescence intensity at that wavelength (Fig. 3). Firstly, both positive and negative reference beads were analyzed in the absence and presence of AR-GST and the anti-GST Qdot, in order to evaluate the range of bead intensities to be expected. Unmodified TentaGel beads (Fig. 3a) or beads known to carry a non-binding peptide sequence exhibited almost no background fluorescence, prior or after exposure to the protein and Qdots. Beads functionalized solely with the strongly binding peptide reference sequence[6] showed high fluorescence upon incubation with the AR protein and the Qdot-labelled antibody (Fig. 3b).

The focused library was screened in exactly the same way as the reference beads (Fig. 3c). The brightest beads observed under the fluorescence microscope were isolated from the library using a micropipette. Beads were considered as positive hits when the average minimum intensity of the bead, as measured across the bead diameter, was higher than the intensity of the reference beads with the FXXLF motif. Single beads visually identified as hits were incubated with a 1% SDS-solution at 99 °C for 30 min to denature the bound protein and subsequently washed. The cleavage of the peptide from the single bead was achieved by treatment of the single bead with a 1 M NaOH solution, which was afterwards neutralized with an equimolar amount of a 1 M HCl solution and formic acid. The sequences of the cleaved peptides were determined using MALDI-TOF mass spectrometry. Table 1 shows an overview of selected peptide sequences with their corresponding bead fluorescence intensities (additional bead sequences can be found in Supplementary Table 1). The hit sequences contain various amino acids, both of natural and of non-natural nature, at the randomized positions.

The affinity of the peptide hits for the AR coactivator binding pocket was determined using a fluorescence polarization competition assay. The peptide hits were resynthesized on solid-phase Rink amide resin using standard Fmoc-chemistry and HOBt/DIC as coupling reagents. Peptides were cleaved under acidic conditions and purified by reversed phase preparative high performance liquid chromatography. The affinity constants K_i of the peptide inhibitors were calculated from the observed IC_{50} values (see Supplementary data). The resulting K_i values are listed in Table 1.

Interestingly, most of the identified peptide sequences contained one or two non-natural amino acids at the three mutated amino acid positions and a few selected natural amino acids were incorporated. The most frequently encountered natural amino acids at the mutated positions were F, W and L. There is a preference of the leucine for the +4 position, in line with the natural motif. The high occurrence of phenylalanines is similarly in line with its high occurrence in the natural FXXLF motif. The frequent occurrence of tryptophan nicely complements previous reports from Fletterick and co-workers who reported similar results from peptides isolated via phage display.[6] Especially the high affinity of hit 10 with the FXXWF motif is striking in this respect. All three non-natural amino acids were frequently encountered in the peptide hits at all positions. The hydrophobic/aromatic nature of these non-natural amino acids apparently makes them good replacements for the natural hydrophobic and aromatic amino acids phenylalanine and leucine encountered normally at these positions. The bead with the highest fluorescence intensity and binding affinity (entry 11) featured the sequence NapESChaW, thus displaying none of the naturally found F and L amino acids. Picked beads with fluorescence intensities lower than that the FXXLF reference peptide, in general featured peptide sequences which included natural, small or polar amino acids, like alanine, methionine or histidine (see Supplementary material). These results thus confirm the importance for large, hydrophobic amino acids, frequently of aromatic nature, at the +1, +4 and +5 positions in the peptides for strong affinity to the AR. Also the results show the validity of the on-bead assay in identifying relevant AR peptide binders.

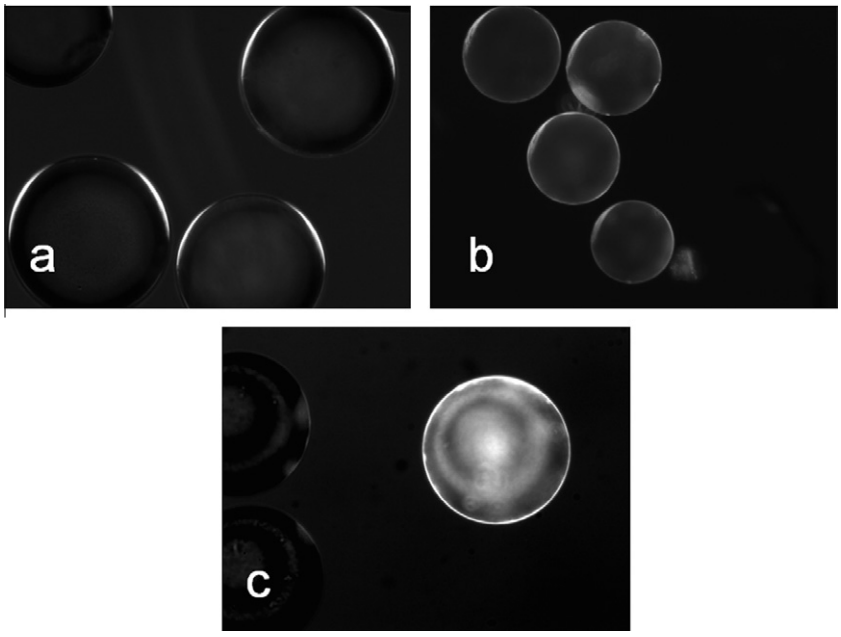

Figure 3. Representative fluorescence microscopy images recorded under identical conditions from GST-AR-LBD and anti-GST-Qdot incubated with: (a) unmodified TentaGel beads; (b) beads with a strongly binding reference peptide sequence; (c) bead from a combinatorial library featuring both high and low fluorescence intensity.

Table 1
Peptide sequences SSRXESXXAGEKESRG of selected bead hits, the fluorescence intensity of the bead and the affinity of the resynthesized peptide for the AR

Entry	∼X	ES	X	X∼	Intensity[a]	K_i (μM)
1	∼P	ES	F	Nap∼	500	–[c]
2	∼W	ES	W	F∼	550	82.9
3	∼W	ES	F	Cha∼	550	2.44
4	∼L	ES	W	PhCl∼	600	166
5[b]	∼F	ES	L	F∼	600	0.76
6	∼Nap	ES	L	PhCl∼	650	38.5
7	∼F	ES	F	Nap∼	700	8.82
8	∼H	ES	Cha	H∼	750	–
9	∼Nap	ES	L	W∼	800	3.68
10	∼F	ES	W	F∼	800	0.94
11	∼Nap	ES	Cha	W∼	900	0.47

[a] Minimum intensity of the beads as measured along the diameter.
[b] Reference peptide.[6]
[c] No affinity determinable.

The beads that showed high fluorescence intensities under the microscope generally carried peptides that also demonstrated high binding affinities in the fluorescence polarization competition assay. There is a correlation between bead intensity and peptide affinity. In cases where a distinct discrepancy was found such as for bead entries 1 and 8 (Table 1), the peptides featured amino acids that are not supporting helix formation, such as proline, or are of polar nature, like histidine. For these peptides the high bead fluorescence could not be confirmed in the solution assay. Either these peptides featured strong unspecific binding to the GST-AR-LBD, or they bind at different sites on the AR surface and are signal silent in the solution assay. In general the small and polar amino acids (A, H, M, P, Q, Y) in the library did not occur in peptide hits. The strongest peptide sequence (entry 11) featured a K_i = 0.5 μM. This is slightly stronger than the already very strongly binding reference peptide, obtained out of a large phage display library.[6] The possibility to incorporate non-natural amino acids, thus allows for screening of libraries chemically more diverse than natural peptide libraries, with possibly novel properties such as protease stability.[9] The design of the current peptide library was rather similar to the starting sequence. The amino acids surrounding the hydrophobic motif are most probably already favourable for obtaining a good binding affinity of peptides for the AR.[6] Nevertheless, by varying only three positions within the peptide remarkable changes in the affinity for the target protein could be observed. Also it could be shown that the hydrophobic groove on the AR surface is adaptable to large hydrophobic side chains such as those from β-cyclohexylalanine (Cha) and β-(2-naphthyl)alanine (Fig. 2).

2.5. On-bead screening of a random library

We subsequently applied the developed on-bead screening approach to evaluate a random peptide library (XXXXXXXSSG). This library contained a random seven amino acid stretch. To ensure water solubility and facilitate peptide sequence analysis, the peptide additionally featured three hydrophilic amino acids at the C-terminus (SSG). Seven natural amino acids (S, Y, L, F, G, V and W) were selected for the random positions X in the peptide. The library was synthesized in a similar approach as described above for the focussed library and subsequently evaluated for its affinity to the AR LBD. Microscopy evaluation of this library also showed a number of brightly fluorescent beads. However, the overall 'hit-rate' of this library as observed by the eye was significantly lower than for the first focussed library. The random character of this library thus apparently results in many peptide sequences without significant affinity. Hits were picked, their sequences analyzed, the peptides resynthesized and evaluated in the fluorescence polarization competition assay for binding to the AR coactivator interaction site (Table 2).

Table 2
Peptide sequence XXXXXXXSSG of picked hits, the fluorescence intensity of the bead and the affinity of the resynthesized peptide for the AR

Entry	XXXXXXX*SSG*	Intensity[a]	K_i (μM)
12	LLGLWV*SSG*	500	–
13	SVFFGFF*SSG*	800	5.8
14	LVYLYLV*SSG*	400	–
15	YSVFVLY*SSG*	650	44.9
16	VGFWGWW*SSG*	600	–

[a] Minimum intensity of the beads as measured along the diameter.

For this series, some of the peptide hits were badly soluble, which prevented the determination of their AR binding affinity (Table 2, entries 12 and 16). Entry 14 in Table 2 did not show a displacement of the fluorescent peptide in the competitive fluorescence polarization assay. This could result from either unspecific binding or from binding to an allosteric site on the AR surface, or from simply weak affinity. The two beads that showed the highest fluorescence intensities (Table 2, entries 13 and 15) also featured peptides that demonstrated high binding affinities in the fluorescence polarization competition assay. This points to a favourable correlation between bead intensity and peptide affinity. Hit 13 exhibited the highest fluorescence intensity in the on-bead assay and also showed a strong binding affinity to the AR–coactivator site. After resynthesis, a K_i value of 5.8 μM was determined. This peptide contains a high number of phenylalanines and a so-called FXXFF motif. The same motif has also been established in peptides screened against the AR with phage display and shown to bind to the adaptive surface of the AR.[6] Even though the size of the random library was rather limited, also this chemically randomized approach yields peptide hits in line with biological approaches.

3. Conclusions

An on-bead OBOC library was set-up and shown to be successfully used for screening peptides targeting the AR–coactivator interaction. Hits could be selected based on the bead fluorescence and the identity could be determined with mass spectrometry. The AR affinity of the isolated hits correlated nicely with the bead fluorescence intensity. The screening approach could both be applied to focused and diverse peptide mutations. Peptide hits with a high affinity for the AR featured bulky, hydrophobic amino acids at the characteristic FXXLF positions, known from natural proteins. Especially large aromatic and non-natural hydrophobic amino acids turned out to be preferred candidates for optimal AR affinity. In both screening approaches, focused or randomized, peptides hits were found with natural peptide sequence motifs, like FXXWF and FXXFF, also encountered in biological screens.[5,6] The synthetic libraries thus yield relevant peptide hits, in line with biological screening, but with the potential to insert amino acids or chemical moieties not accessible via biological approaches. The use of non-natural amino acids at the critical interaction positions opens up the opportunity to increase binding affinity, NR selectivity and membrane permeability.[8] Additionally, such synthetic peptides could provide design parameters for the generation of small molecules targeting AR–coactivator interactions.[10] The developed on-bead screening of non-natural peptide inhibitors for the AR–cofactor interaction could generate a set of molecular tools for the biological evaluation of this important protein–protein interaction.

4. Experimental procedures

4.1. General information

Rink Amide MBHA resin with an initial loading of 0.72 mmol/g was purchased from Novabiochem. Fmoc-protected amino acids

were purchased from MultiSyntech and Novabiochem in their appropriately protected form. All other reagents were purchased from Aldrich–Sigma, Fluka and Acros. All automated peptide syntheses were performed on a Syro II automated peptide synthesizer (MultiSynTech GmbH) using standard solid phase Fmoc-chemistry. The on-bead libraries were synthesized automatically (MultiSynTech GmbH) by the pool and split synthesis and standard solid phase Fmoc-chemistry using TentaGel Macrobeads HMB (200 μm diameter, 0.26 mmol/g, Rapp Polymere Germany) as solid-phase support.

LCMS experiments were performed on an Agilent 1100 series HPLC system connected to a Thermo LCQ Advantage mass spectrometer equipped with an electrospray ion source. Analytical chromatography separations were performed using a C18 Nucleodur gravity column (125 × 4 mm, 3 μm particle size, Macherey–Nagel). Material was eluted using a gradient of acetonitrile and water containing 0.1% formic acid and a flow rate of 1 mL/min. Preparative HPLC was performed on a Agilent Series 1100 system equipped with a C18 Nucleodur gravity column (125 × 21 mm, 5 μm particle size, Macherey–Nagel) using a gradient of acetonitrile and water each containing 0.1% trifluoroacetic acid and a flow rate of 25 mL/min.

MALDI-TOF mass spectra were recorded on a Voyager DE Pro MALDI-TOF instrument equipped with a LeCroy Digitizer and an internal nitrogen laser using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix.

Image acquisition was performed with an Axiovert 40 CFL fluorescence microscope (Carl Zeiss). Quantum dots (Qdot® 655 goat anti-glutathione-*S*-transferase, Invitrogen) were excited at 405 nm and images were taken with a camera with an exposure time of 100 ms.

4.2. Bead library synthesis

Loading of the resin: TentaGel MB HMB resin (1 g, 0.26 mmol/g initial loading) was swollen first in dry dichloromethane (10 mL) for 30 min. A solution of Fmoc-glycine (3 equiv) and DIC (1.5 equiv) in dry dichloromethane (2 mL) stirred for 20 min at room temperature. Then DMAP (1.5 equiv) was added to the resin and the suspension was stirred at room temperature overnight. The resin was washed with dichloromethane and washed with diethyl ether and dried under vacuum. Fmoc-quantification was determined by UV-spectroscopy at 301 nm with a small amount of resin (5 mg) and allowed the calculation of the initial resin loading.

The 'One-Bead-One-Compound' combinatorial library was prepared by a 'spilt and pool-synthesis' approach using a glycine-preloaded TentaGel MB HMB resin and standard Fmoc-chemistry with HOBt/DIC as coupling reagents. The first coupling step on the preloaded resin was performed routinely by double coupling cycles and all other coupling steps were performed as single coupling cycles. Each of the amino acids which were used after the permutation steps contained 10% of acetylated alanine as an end-capper. In the case of peptide libraries, the resin was first split into as many aliquots as there were different amino acids for the permutation, after which a fourfold molar excess of each individual Fmoc-amino acid, together, with the coupling reagents was added. After the coupling reactions were complete, the samples were washed, mixed together and split into aliquots again and made ready for the next cycle of couplings. After the permutation steps of the synthesis, all aliquots were mixed together and the common successive couplings were completed to achieve the complete sequence. After that, the resin was washed carefully with dichloromethane and diethyl ether, dried under high vacuum over night and stored at −20 °C.

4.3. Peptide hit synthesis and purification

All sequences were synthesized from C- to N-terminus on solid support, using an automatic solid-phase synthesizer on a 144 μmol scale (200 mg of Rink Amide MBHA resin, loading of 0.72 mmol/g). The coupling of amino acids was carried out following standard Fmoc-chemistry, using HOBt (4 equiv) as amino acid activation, DIC (4 equiv) as coupling reagent, DMF as solvent and 4 equiv of the protected Fmoc-amino acids. The resin was first swollen in DMF (1 × 30 min) and the Fmoc protecting group was removed by treatment with piperidine/DMF (2/3, 1 × 3 min; 1/4, 1 × 10 min), then washed with DMF (6 × 1 min). One cycle of peptide elongation consisted of the following steps. First, the deprotected resin was treated for 50 min with a mixture containing the appropriate amino acid (4 equiv, solution 0.3 M in DMF) with an equimolar addition of HOBt/DIC (4 equiv, solution 0.3 M in DMF) and DIPEA (4 equiv). After washing the resin with DMF (4 × 1 min), the Fmoc protecting group was removed by treatment with piperidine/DMF (2/3, 1 × 3 min; 1/4, 1 × 10 min). After deprotection, the resin was again washed with DMF (6 × 1 min). These steps were repeated until the desired peptide sequence was complete. After the completion of the sequence, the resin was washed with DMF (5 × 30 s), CH_2Cl_2 (5 × 30 s) and Et_2O (5 × 30 s) and dried under vacuum for 2–3 h.

Simultaneously, cleavage and side chain deprotection was carried out by treatment of the resin for 2 h with a mixture containing TFA/H_2O/EDT/TIS (96:2:1:1). The cleaved resin was washed with TFA (2 × 2 mL) and the cleaved peptide was collected, concentrated into less than 1 mL solution and precipitated by addition of cold Et_2O (30 mL). The mixture was cooled, centrifuged (4000 rpm, 5 min, 4 °C) and the Et_2O was decanted from the pellet. Cold Et_2O was added again and the procedure was repeated twice. The crude peptide obtained was dissolved in H_2O/CH_3CN and lyophilized to dryness.

The crude mixture of each peptide hit was analyzed by LCMS and purified by reversed phase preparative HPLC on Nucleodur C18 Gravity column with a gradient of A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN) with a flow rate of 25 mL/min. After purification, all peptides were lyophilized and stored at −20 °C.

4.4. Protein expression and purification

The protein GST_*h*AR_LBD (residues 664–919) was expressed from *E. coli* BL21(DE3). A 200 mL overnight culture was used to inoculate in 2.5 L of TB media containing 150 μM ampicillin and 10 μM DHT. After being induced with 30 μM IPTG at OD_{600} = 1 the cells were grown in the presence of 10 μM DHT and 150 μM ampicillin overnight at 17 °C. The cells were centrifuged at 4500 rpm for 20 min and lysed with a microfluidizer (7 passes at 600 kPa) in buffer (50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 100 μM DHT, 100 μM PMSF and 10 mM DTT) and centrifuged at 20.000 rpm for 30 min. The soluble fraction was filtered (Schleicher & Schnell, Whatman FP 30/0.45 μM CA-S) before isolating GST-*h*AR-LBD using FPLC (Amersham Bioscience, Äkta FPLC) and a GSTrapFF 5 mL column from GE Health. The soluble cell lysate was immobilized on a glutathione Sepharose 4 Fast Flow affinity matrix, washed with buffer (50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT and 1 mM DTT) and eluted with elution buffer (50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT and 1 mM DTT) containing 15 mM glutathione. The fractions containing the fusion protein were combined and desalted on a Sephadex G25 PD-10 column (Amersham Biosciences) and preequilibrated with buffer (50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT and 1 mM DTT). The concentration of the protein was determined using the Nano-Drop (Peqlab, Nanodrop ND-1000) machine.

4.5. On-bead screen

The on-bead screen was performed using following procedure:

1. Bead swelling in DMF for 2 h at room temperature.
2. Bead washing with buffer A (containing 50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT), 3 × 5 min.
3. Bead swelling in buffer A (containing 50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT).
4. Bead blocking with 75 μM BSA overnight at 4 °C.
5. Bead washing with buffer A (containing 50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT), 3 × 5 min.
6. 0.5 μM GST-h-LBD binding for 2 h at room temperature.
7. Bead washing with buffer A (containing 50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT), 5 × 5 min.
8. Bead incubation with 0.01 μM Qdot® goat-anti-glutathione-*S*-transferase for 4 h at room temperature.
9. Bead washing with buffer A (containing 50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol, 10 μM DHT), 3 × 5 min.

The beads were subsequently imaged and characterized using a fluorescence microscope using 405 nm excitation and 655 nm emission wavelengths and an exposure time of 100 ms. Each picked bead carrying bound protein were then treated with 1% of a SDS-solution at 99 °C for 30 min and washed to remove the attached protein. After transferring the beads into a new vial, the cleavage of each single peptide from the resin was performed using 4.4 μL of 1 M NaOH. After shaking the solution for 15 min at room temperature, the solution was neutralized by 4.4 μL of 1 M HCl and 4.4 μL formic acid.[13] The resulting solution was then analyzed via MALDI-TOF using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix.

4.6. Fluorescence polarization assay

The fluorescence competitive assay was performed using 0.1 μM fluorescein-labelled peptide Fl-CSSRFESLFAGEKESR and a 1 μM solution of purified GST_*h*AR_LBD in assay buffer (50 mM HEPES, pH 7.3, 300 mM NaCl, 5 mM EDTA, 10% glycerol and 10 μM DHT). The competition assays were performed in 384-well plates (Perkin–Elmer, Optiplate-384 F) by adding 40 μL of the protein–peptide mixture to 10 μL of solution with increasing concentrations of inhibitor (diluted in assay buffer). Wells not containing inhibitor as well as wells only containing Fl-labelled peptide in assay buffer were used as controls. The plates were then incubated at 4 °C for 1 h. The fluorescence polarization was measured at 23 °C using black 384-well plate (Perkin–Elmer, Optiplate-384 F) with 490 nm as excitation and 530 nm as emission wavelength using a Tecan Safire[2] plate reader with fluorescence polarization option. The K_i values were determined following a published procedure (Vaz et al.).[8]

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Supplementary data

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References and notes

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