A *De Novo* Designed Library of Linear Helical Peptides: An Exploratory Tool in the Discovery of Protein-Protein Interaction Modulators

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ABSTRACT. Protein-protein interactions (PPIs) have emerged as important targets for pharmaceutical intervention because of their essential role in numerous physiological and pathological processes, but screening efforts using small-molecules have led to very low hit rates. Linear peptides could represent a quick and effective approach to discover initial PPI hits, particularly if they have inherent ability to adopt specific peptide secondary structures. Here, we address this hypothesis through a linear helical peptide library, composed of four sub-libraries, which was designed by theoretical predictions of helicity (Agadir software). The 13-mer peptides of this collection fixes either a combination of three aromatic or two aromatic and one aliphatic residues on one face of the helix (Ac-SSEE$\mathbf{X^5}$ARN$\mathbf{X^9}$AA$\mathbf{X^{12}}$N-NH_2**)**, since these are structural features quite common at PPIs interfaces. The 81 designed peptides were conveniently synthesized by parallel solid-phase methodologies, and the tendency of some representative library components to adopt the intended secondary structure was corroborated through CD and NMR experiments. As proof of concept in the search for PPI modulators, the usefulness of this library was verified on the widely studied p53-MDM2 interaction and on the communication between VEGF and its receptor Flt-1, two PPIs for which a hydrophobic α–helix is essential for the interaction. We have demonstrated here that, in both cases, selected peptides from the library, containing the right hydrophobic sequence of the hot-spot in one of the protein partners, are able to interact with the complementary protein. Moreover, we have discover some new, quite potent inhibitors of the VEGF-Flt-1 interaction, just by replacing one of the aromatic residues of the initial $F^5Y^9Y^{12}$ peptide by W, in agreement with previous results on related antiangiogenic peptides. Finally, the HTS evaluation of the full collection on thermoTRPs has led to a few antagonists of TRPV1 and TRPA1 channels, which open new avenues on the way to innovative modulators of these channels.

INTRODUCTION

Protein-protein interactions (PPIs) are ubiquitous in biological systems and play pivotal roles in key biological processes.[1] Therefore, while aberrant, inappropriate or poorly regulated interactions might cause various diseases, the ability to interfere with specific PPIs can provide attractive opportunities for therapeutic intervention.[2-4] Progress in recent years has shown that protein-protein interfaces are more tractable than had initially been thought and diverse PPI inhibitors have been reported.[5-8] In this regard, the pioneering work of Clackson and Wells, defining the "hot spots" as bimolecular interface residues that contribute mostly to the free binding energy of PPIs, has been a major conceptual advance.[9] On this basis, disruptors or stabilizers of PPIs do not need to mimic the entire protein surface, but rather a smaller subset of key residues and, consequently, peptides, peptidomimetics and small molecules could be used for modulating these interactions. An attractive strategy is the design and synthesis of compounds able to imitate the conformation and electronic properties of the functional epitopes at native protein interfaces, mimicking elements of protein secondary structures.[11,12] In this sense, conformationally defined peptide libraries have successfully been used to identify new PPI modulators,[13,14] and constituted the first step toward non-peptide secondary structure mimetics.[15-18]

α-Helices, the largest class of secondary structure elements of proteins, mediate a plethora of highly specific protein-protein interactions.[19] In some of these protein complexes, just one face of the α-helix is involved in binding, with important residues for affinity displayed at *i,* i+3(*i+4*) and *i+7* relative disposition.[20] Quite frequently, the key contacts between these helical motifs and the complementary binding sites are mediated by hydrophobic residues at the indicated positions. For instance, the 19-25 peptide fragment of the pro-apoptotic protein p53 forms an

amphipathic α-helix in its interaction with MDM2, being Phe^{19}, Trp^{23} and Leu^{26} crucial interacting residues.[21] HIV-1 envelope glycoprotein gp41 displays a challenging intramolecular protein–protein interaction that is mainly mediated by the Trp^{628}, Trp^{631}, and Ile^{635} residues on an α-helix of the CHR domain.[22] Within the 17–25 VEGF-A fragment, a Phe^{17} residue has been identified as key for the interaction of this growth factor with its Flt-1 and KDR receptors (VEGFR1 and VEGFR2), while Tyr^{21} and Tyr^{25} seem to be important for the stabilization of the α-helical structure.[23] In epoxide hydrolase oligomers, clusters of aromatic residues commonly participate in the protein–protein interactions, as deduced from computational analysis.[24]

PPIs are also essential in ion channels, where multimeric transmembrane-spanning proteins interact to form the channel pore, and diverse accessory proteins contribute to regulate their function.[25,26] In addition, ion channels are substrates for different protein kinases and phosphatases that regulate channel gating, bind G protein β,γ-heterodimers, or interact with Calmodulin (CaM), an ubiquitous calcium sensor protein, among many others. Recent structural studies reveal a central role of α-helix secondary structures, as well as of particular hydrophobic interactions, in the formation and stabilization of protein-protein complexes involving ion channels.[27-31]

Thermosensory channels (thermoTRPs), belonging to the family of transient receptor potential (TRP) channels, are activated by a variety of environmental temperatures, from noxious (<15°C, TRPA1) to innocuous cold (<28°C, TRPM8), and to injurious heat (52°C, TRPV1), and contribute to the thermal hypersensitivity under painful conditions.[32,33] Additionally, thermoTRPs are also implicated in the physiopathology of several diseases, like inflammation, asthma, and certain cancers.[32-34] All this facts have trigger intensive drug discovery programs aimed at developing new modulators of these channels, especially of TRPV1.[33,35] However, after

remarkable investment and a plethora of molecules, the clinical trials with TRPV1 antagonists have mostly been disappointing due to unpredicted side effects, while the development of modulators of other TRP channel is still in its infancy.[33] Therefore, there is a need for new approaches to TRP channel modulators with alternative mechanisms of action. In this respect, we have recently described short peptides (TRPducins), mapping the sequence of TRPV1 TRP domain, which were able to block selectively the channel, probably by disrupting protein-protein interactions at the cytosol.[36]

General chemical strategies for finding PPI modulators include α–helix-derived constrained peptides (through covalent disulfide, lactam bridges, hydrocarbon stapling and HSB cyclization methods), foldamers (β- and mixed α/β-peptides) and proteomimetics (terphenyl, and their related heterocyclic and amide α–helix-mimetics).[12-19,37,38] All these strategies, which usually require elaborated chemistry, have been successfully applied to PPIs with known interfaces.[37] Within this context, linear peptide-based libraries have been much less explored, because these peptides generally adopt random conformations that could prevent their binding to protein partners.[39-41] However, if linear peptides had a certain tendency to be helical, they would be able to adopt the right 3D structure by an induced fit mechanism during the interaction. This, combined with the inherent degree of flexibility, makes linear peptides ideal for the discovery of primary modulators of topographically diverse, unknown PPIs.

In view of the considerations above, we proposed the preparation of a linear peptide library as a versatile tool towards PPI modulators. Peptides within this library should show a tendency to adopt helical conformations, and to have at least three hydrophobic amino acids conveniently located at one side of the helical structure. This article provides details on the *de novo* design of this library, its solid-phase parallel synthesis and the conformational characterization of a few

components. Moreover, the ability of some components of this library to modulate protein-protein interactions has been validated in a couple of known PPIs, p53-HDM2 and VEGF-VEGFR-1. In both PPI systems the existence of a hydrophobic face on one helix is crucial for the molecular recognition by the target protein. Finally, the evaluation of the full library on thermoTRPs has permitted the identification of a few peptides able of inhibiting the activation of TRPV1 and TRPA1 channels.

RESULTS AND DISCUSSION

De Novo Design. PPIs are mainly driven by the hydrophobic effect,[42] and quite frequently the α-helix secondary element is involved within protein-protein interfaces. Therefore, helical peptides with appropriately located aromatic and aliphatic hydrophobic residues could serve to discover new PPI modulators. To guarantee at least three points of interaction, peptides containing three aromatic or two aromatic and one aliphatic residues were designed. The length of the peptide should assure the possibility of three turns for situating key residues on the same helix face (i, i+4, i+7, relative positions). Using Agadir software,[43] we started by predicting the helicity of the 12-mer peptide Ac-AAA**F**^{4}AAA**F**^{8}AA**F**^{11}A-NH_2 (11.9% helicity) and four analogues containing E(D) and K(R) at positions 2 and 6 to stabilize the helix through saline bridges (opposite face to the aromatic F residues). Peptide Ac-A**E**A**F**^{4}A**R**A**F**^{8}AA**F**^{11}A-NH_2, with the highest helicity (24.8%), was selected as the starting point to study the best residues for the non-highlighted positions. With this aim, we generated virtual libraries in which each Ala residue was replaced by the other 19 proteinogenic amino acids. Computational predictions of helicity on each library component allowed the selection of those residues that lead to higher helicity than Ala (see supporting information for details), which were then considered and combined for the next generation of virtual libraries. Since the solubility of peptides over six residues is normally a

limiting factor, theoretical solubility (calculated with ALOGPS[44]) was also taken into account to discriminate among certain amino acids. Finally, the addition of an additional residue, either at *N*- or *C*-terminal, was considered to balance helicity and solubility. This process resulted in the final design of 13-mer peptide sub-libraries **L1-L4**, as candidates for synthesis (Table 1). Library **L1** is the result of all possible combinations of the three aromatic amino acids (Phe, Trp, Tyr) at positions 5, 9 and 12, while collections **L2-4** mix up two aromatic (from Phe, Trp, Tyr) and one aliphatic (from Leu or Ile) residues at these positions. Ranges of helicity predicted for these libraries are included in Table 1.

Table 1. Designed sub-libraries of helical peptides

Library (#Compd)	Sequence[a]	% Helicity[b]
L**1** (27)	SSEEAr^{5}ARNAr^{9}AAAr^{12}N	35-65
L**2** (18)	SSEEAl^{5}ARNAr^{9}AAAr^{12}N	27-55
L**3** (18)	SSEEAr^{5}ARN Al^{9}AAAr^{12}N	32-46
L**4** (18)	SSEEAr^{5}ARNAr^{9}AA Al^{12}N	33-58

[a] All peptides are acetylated at N-terminal and amidated at C-terminal. Ar = aromatic amino acids F, W, and Y; Al = aliphatic amino acids I, and L. [b] Calculated by Agadir.

Synthesis of Peptide Libraries. Sub-libraries L**1**-L**4** (81 peptides) were individually prepared using parallel solid-phase methodologies (Fmoc/tBu strategy). Peptides were elongated on a

Rink amide resin, either manually or in a microwave-coupled peptide synthesizer, using HCTU and HBTU/HOBT, respectively, as coupling agents. Peptides were acetylated at N-terminal before cleavage from the resin with TFA/EDT/H_2O/TIPS (94:2.5:2.5:1). After precipitation with ethyl ether and liophylization, compounds obtained with purity lower than 80% were purified by MPLC on reverse-phase cartridges or by semipreparative RP-HPLC-MS in difficult cases. Yield of L**3** compounds were lower than those of the other sub-library components, due to unexpected difficult couplings of the Arg residue, resulting in mixtures of the desired peptide and Arg-deletion analogues. All final peptides were characterized by MS spectrometry, and a few components also through exact mass measurements (Tables S1-S4).

Conformational Studies. Several peptides were randomly selected for conformational studies in solution to evaluate their ability to adopt helical structures.

CD Experiments. The predisposition of the chosen peptides to adopt helical conformations was first checked by circular dichroism (CD). All CD spectra displayed the helix-characteristic double minimum at 208 and 222 nm (Figures 1 and S1-S5). As shown in Table 2, selected peptides showed significant helix populations in aqueous solution (≥20%). This percentage was considerably increased in the presence of the structure-inducing cosolvent TFE (up to 47%), symptomatic of their high propensity to be helical in a suitable environment.

Table 2. Experimental CD helicity of selected peptides Ac-SSEEX^5ARNX^9AAX^{12}N-NH_2

Compd. [a]	% Helicity	
$X^5X^9X^{12}$	H_2O	30% TFE
FWF	22	40
LWW	19	41
IWY	23	28
FIY	26	38
WIW	28	44
YWL	30	47

[a] The notation indicates residues at positions 5, 9 and 12 of the peptide sequence.

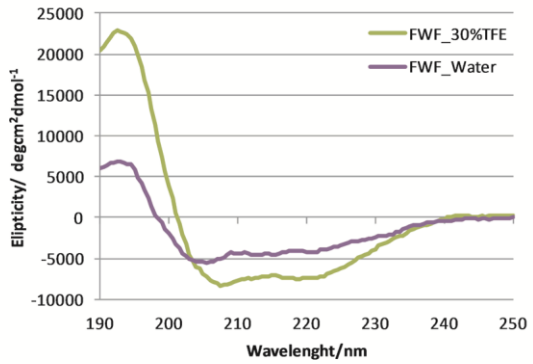

Figure 1. CD spectra of peptide $F^5W^9F^{12}$ in H_2O and 30% TFE/H_2O at pH 5.5 and 5ºC.

NMR studies. Four of the randomly selected peptides were shown to adopt helical structure by 1H NMR in TFE/H_2O solutions. It is known that he ^{1}Hα chemical shifts depend on the ϕ and ψ dihedral angles,[45] and negative values of conformational shifts (Δδ□= δobserved – δrandom coil,[46] ppm) are characteristic of helical structures. In agreement with that, all peptides showed the expected profile for the Hα protons Δδ, with negative values for almost all residues in the sequence, except for the first Ser residue (Figure 2, and S6-S8).

As frequently found in linear helical peptides, Δδ absolute values of central residues are larger than those at the *N*-terminal position, indicating that the helices are slightly less coiled at this end (Figures 2 and S6-S8). Further NMR studies with peptide $I^5W^9Y^{12}$, namely ^{13}C- and NOESY experiments, definitely corroborated the α-helix conformation. Thus, we obtained positive values for the Δδ of C_α chemical shifts (Figure S9), and a set of non-sequential NOEs ($d_{\alpha N(i,\, i+3)}$, $d_{\alpha N(i,\, i+4)}$ and $d_{\alpha\beta(i,\, i+3)}$, Figure 2, and S10-S11), both characteristic of helical structures. Using the intensities of the non-sequential NOEs as a restriction, we performed molecular dynamic calculations (CYANA program) to elucidate the structure of this peptide in solution, which adopts a well defined α-helix 3D conformation, with residues 5, 9, and 12 aligned along a single face of the helix (Figures 3 and S12).

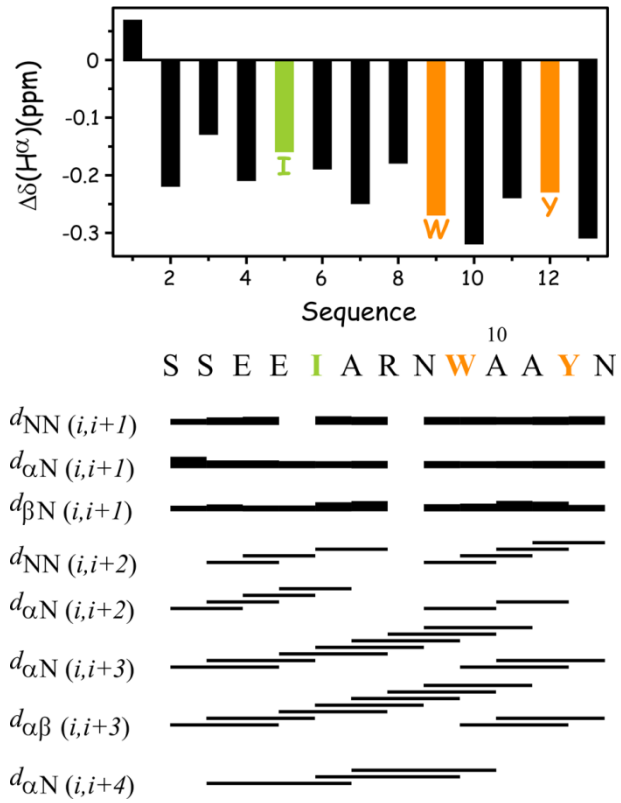

Figure 2. Histogram showing the ΔδHα values ($\Delta\delta H\alpha = \delta H\alpha^{observed} - \delta H\alpha^{RC}$, ppm) as a function of sequence number, and NOE summaries of peptide $I^5W^9Y^{12}$ (30% TFE at 25ºC). N- and C-

termini are, respectively, in acetylated and amidated form, respectively. The thickness of the lines indicates the intensities of the sequential NOEs, i.e., strong, medium, weak.

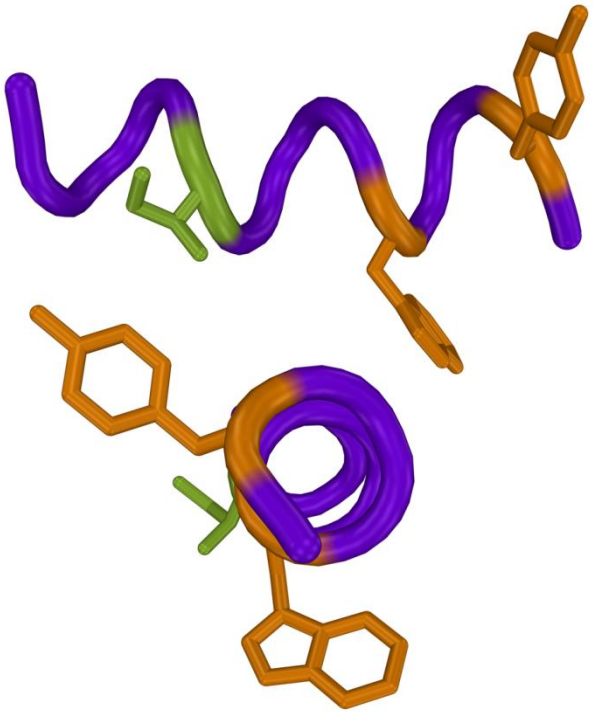

Figure 3. Solution NMR structure of the peptide $I^5W^9Y^{12}$, side view (up) and viewed from above (down). Residues at positions 5, 9 and 12 are colored in green and orange. Hydrogen atoms and other side-chains are not shown.

Validation as PPI modulators. Before exploring the usefulness of our library in the search for new inhibitors of poorly documented PPIs, we considered pertinent to validate it on well known models. For this purpose, we selected p53-MDM2 and VEGF-VEGFR1, because aromatic and aliphatic residues within a helical structure have been defined as key for the recognition process.

p53-MDM2. The activity of p53, a tumor suppressor protein, is regulated by interaction with other proteins, including the ubiquitin ligase MDM2.[47] This system has extensively been studied, and a number of structurally diverse molecules are able to inhibit the p53-MDM2 interaction, triggering either cell-cycle arrest or apoptosis in cancer models, without affecting healthy cells.[48-50] MDM2 binds p53 mainly through hydrophobic interactions, with a short helix of p53

positioned in the MDM2 binding cleft. Phe[19], Trp[23], and Leu[26], located on the same side of the amphipathic p53 helix, are crucial contributors to the free energy of interaction, making direct, deep contacts in the binding cavity.[51] Here we were not looking for new inhibitors, since several potent α- and β-peptide MDM2 antagonists (linear and stapled) have already been described.[52-55] Our aim was the use of the p53-MDM2 system as a proof of the validity of our library in the search of PPI modulators. Thus, peptide $F^5W^9L^{12}$, which is supposed to align the key residues of p53 along one face of the helical structure, was selected to evidence its potential interaction with MDM2. Saturation transfer difference (STD) NMR experiments were performed to this end.[56,57] For comparison, some other peptides, namely $F^5L^9W^{12}$, $W^5L^9F^{12}$, and $W^5F^9L^{12}$, having scramble disposition of key residues, were also evaluated. As shown in Figure 4, peptide $F^5W^9L^{12}$ was able to interact with the HDM2 protein. In addition, peptide $W^5L^9F^{12}$ showed also some STD effects, but weaker than the p53-related $F^5W^9L^{12}$ (relative STD effect, η_{sat}, received by the two peptides was 7.9 and 5.1 %, respectively). On the contrary, $F^5L^9W^{12}$ and $W^5F^9L^{12}$ analogues elicited lower STD effects upon saturation of the protein ($\eta_{sat} < 5\%$). The STD spectrum of the FWL-MDM2 complex allows the qualitative identification of protons from the peptide at the MDM2 interface. Thus, the signals corresponding to some protons of aromatic residues (7.0-7.5 ppm region) and those of Leu methyl groups (< 1 ppm) can clearly be distinguished, among other protons of $F^5W^9L^{12}$ peptide. This result suggests the direct implication of Phe, Trp and Leu residues of our peptide on the interaction with the protein, probably mimicking the p53 helix and occupying the same pocket.

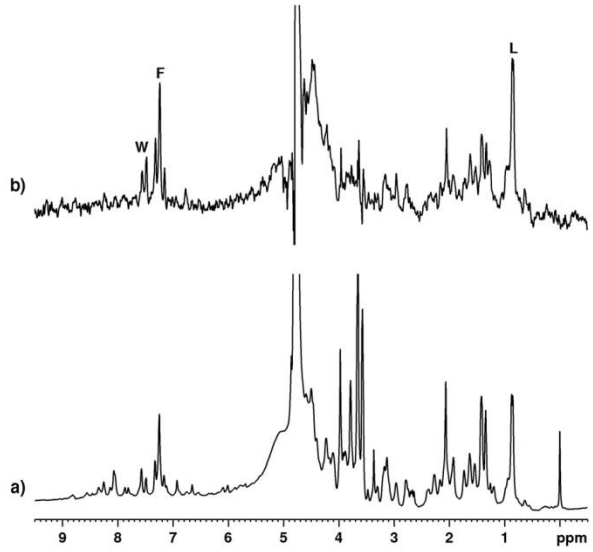

Figure 4. 1D proton spectrum of peptide $F^5W^9L^{12}$ (0.5 mM)/MDM2 (0.025 mM) complex (a). The corresponding STD NMR spectrum recorded upon saturation at –1 ppm (b).

VEGF-VEGFR1. Vascular Endothelial Growth Factor (VEGF), has different isoforms. Among them, VEGF-A, a key protein in promoting angiogenesis, exerts its biological actions through binding to specific receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR).[58] The α–helical fragment $VEGF_{17-25}$, located at the *N*-terminal part of VEGF, has been identified as a main hot-spot for the interaction with VEGFR-1. Within this helix, Phe^{17} residue seems crucial for the molecular recognition between the two proteins, while Tyr^{21} and Tyr^{25} appear more important for the stabilization of the secondary structure.[59] In a recent paper, we described that 13-mer linear and cyclic peptides derived from the 17-25 VEGF fragment, and structured as α-helices in solution, were able to bind the Flt-1 receptor with micromolar affinity.[60] Related longer helical peptides, in which the Phe residue has been substituted by Trp, have been described by D'Andrea et al. to show either pro- or anti-angiogenic properties.[61,62] Since peptide $F^5Y^9Y^{12}$ from sub-library **L1** has a similar arrangement of key aromatic residues than $VEGF_{17-25}$, its

hypothetical interaction with Flt-1 could represent an additional validation of our library. Although the third aromatic residue in our peptides is not exactly at the same relative position than in p53, the three aromatic amino acids are on the same face of the helix and could imitate the helical fragment in the native protein. Furthermore, we have already shown that cyclic peptides with only two aromatic residues YY or Y/Aromatic motif, without any amino acid between the two aromatic residues, are also able to inhibit the VEGF-Flt-1 interaction.[63] Therefore, peptide $F^5Y^9Y^{12}$, and some analogues containing a single point mutation at 5, 9, or 12 position, were evaluated for their ability to displace biotinylated $VEGF_{165}$ bound to the extracellular domain of recombinant Flt-1 (Table 3). In this assay, all peptides with three aromatic residues at the indicated positions (100 μM) inhibited the binding of btVEGF165 to Flt-1 receptors. The model FYY peptide, with a displacement of about 30% at 100 μM, was the weaker modulator of the VEGF-Flt-1 interaction, while the substitution of some of its key residues by Trp afforded more potent inhibitors of this PPI (45-50% inhibition at 100 μM). In particular, FWY and FYW peptides are among the most potent Flt-1 peptide binders described to date (Table 3, Figure 5).[63-65] Moreover, the change of the central Tyr^9 by a Phe residue slightly improve the inhibitory capacity with respect to the model peptide, while replacement of Tyr^{12} residue by an aliphatic isoleucine led to an inactive peptide. For those peptides with an inhibition percentage greater than 35% at 100 μM, we perform experiments of dose-response relationships, observing that the magnitude of the response (the efficacy) is always comprised between 60 and 70 % (Fig. 5). The relative IC_{50} values corroborated the initial results and confirm the beneficial effects of the substitution of individual Tyr residues of $F^5Y^9Y^{12}$ peptide by Trp (Table 3).[63-65] These data confirmed the interaction of FYY peptide with Flt-1, giving support to the general interest of the library described here. In addition, the evaluation of some analogues of sub-library

L1 has uncovered novel potent modulators of this receptor, which deserve further pharmacological characterization, and could be the starting point to develop α–helical mimetics to interfere with the VEGF-Flt-1 interaction.

In is worth noting that although all assayed peptides have a hydrophobic side, not all were able to interact with Flt-1 and not all are recognized by the same extent, indicating certain selectivity. Therefore, this kind of library could be of interest to study PPIs for which key interacting residues are unknown, and could provide valuable clues regarding important side-chains at PPI interfaces.

Table 3. Inhibitory potency of selected peptides on Flt-1. Displacement assays.

Compd. $X^5X^9X^{12}$	% Inhibition[a]	IC_{50}[b](μM)
FYY	29 ± 3	ND[c]
FYW	48 ± 6	29 ± 5
FWY	50 ± 5	23 ± 4
WYY	45 ± 4	>100
FFY	39 ± 6	>100
YYY	33 ± 4	ND
FYI	0	ND
QK[d]	69 ± 3	32 ± 8[d]

[a] Activity corresponds to the percentage of biotinylated $VEGF_{165}$ displaced by a 100 μM concentration of peptide on the whole extracellular domain (ECD, D1-D7) of Flt-1 (this study). [b]Relative inhibitory concentration 50 (concentration required to bring the curve down to point half way between the top and bottom plateaus of the curve). [c]ND = Not determined. [d]Described Flt-1-binder peptide, Ac-KLTWQELYQLKYKGI-NH_2 (from references 60 and 63).

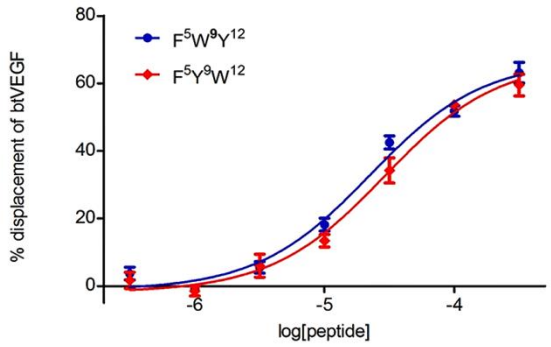

Figure 5. Dose-response curves for $F^5W^9Y^{12}$ and $F^5Y^9W^{12}$ peptides on Flt-1 displacement assays. Curves were fitted with log(Inhibitor) vs. response method using GraphPad Prism. When hill slope is fixed at 1, r^2 are respectively 0,956 and 0,945 for $F^5W^9Y^{12}$ and $F^5Y^9W^{12}$ peptides.

Effects on ThermoTRP Channels. From our previous work,[36] we knew that the most active TRPducing peptide, TRP-p5, has a sequence that resembles that of amphipathic helical peptides. Additionally, the TRP domain of the different transient potential receptor channels contains various sets of hydrophobic residues, some forming the coiled-coil necessary for channel assembly, and others supposedly implicated in channel activation.[66,67] Site-directed mutagenesis studies have also revealed the functional importance of aromatic amino acid clusters at different locations of channel monomers.[68]

Inspired by the results of TRPducings,[36] and by the above structural and functional facts, we decided to evaluate our library of helical peptides on TRPV1, TRPM8 and TRPA1 channels in the search for allosteric modulators. We were interested in exploring protein-protein interactions occurring during channel formation and/or gating. Thus, all components of our library (L**1**-L**4**) were assayed on these three channels, stably expressed in the appropriate cell lines (see SI for details). The agonist-induced intracellular Ca^{2+} signals were measured by microfluorography, using a fluorescence plate reader. While most library components, added at a 5 μM concentration, did not display blockade activity (<15%) at any of the assayed TRP channels, a

few Tyr-containing peptides blocked TRPV1 and/or TRPA1 activity in a significant manner (>20%, Table 4). None of the peptides showed activity at TRPM8 channels. Note that the best TRPV1 blockers have the aliphatic amino acid in the central position, but these peptides did not show selectivity with respect to TRPA1 receptors, being almost equipotent in both channels. Curiously, just displacing the aliphatic residue toward *C*-terminus resulted in TRPA1 selective compounds. Notably, peptides $W^5Y^9I^{12}$, $Y^5F^9L^{12}$ and $Y^5Y^9I^{12}$ showed >50% blockade activity at 5 μM. These peptides could serve as model for the development of peptidemimics able to reproduce the 3D disposition of the two aromatic and the aliphatic side chains within the helical structure.[37] Since these peptides do not share either the structural requirements found in pore blockers, normally positively charged compounds,[69] or the chemical composition of competitive small-molecule antagonists,[35] it is possible that they act at PPIs related to channel formation or modulation by other proteins.

Table 4. Peptides able to block significantly TRPV1 and TRPA1 channels

Compd. $X^5X^9X^{12}$	TRPV1 blockade (%)[a,c]	TRPA1 blockade (%)[b,c]
FLF	23,93 ± 3.86	26,05±5,96
YLF	25,61 ±5,6	24,03±10,93
YIY	24,09 ±3.56	17,95±3,06
YLY	38,03 ±4,39	37,34±5,71
WYI	28,18 ±4,23	55,51±11,29
YFL	19,77 ±3,71	51,18±10,21
YYI	18,63 ±3,33	51,38±9,80

[a] Blockade of capsaicin-induced Ca^{2+} entry trough TRPV1 channel by peptides. [b] Blockade of allyl isothyocyanate-induced Ca^{2+} entry trough TRPA1 channel by peptides. [c] Peptides were assayed at 50 and 5μM.

CONCLUSIONS

Since PPIs are usually mediated by interactions between defined elements of peptide secondary structure, the search for modulators of these interactions could be speeded by testing libraries of easily prepared linear peptides, provided they are able to adopt these particular conformations. In this respect, we have used theoretical predictions to design a collection of linear peptides with a priori intrinsic ability to adopt helical conformations. In addition, they possess aromatic or combinations of aromatic/aliphatic residues conveniently located at one face of the intended helix, since this arrangement is quite common in PPI interfaces. After solid-phase synthesis, CD and NMR studies demonstrated the ability of these peptides to adopt the anticipated helical conformation in solution.

Notably, specific member of the library were able to interact with proteins implicated in well known PPIs of therapeutic importance, thus validating the utility of the library in the search for PPI modulators. Thus, STD NMR experiments confirmed that peptide $F^5W^9L^{12}$ associates with HDM2, one partner of the pro-apoptotic protein p53, implicated in tumor cell suppression. The interaction was tighter for the indicated peptide, which contains the same disposition of the hydrophobic residues as the p53 hot spot responsible for HDM2 binding, than for related analogues with a changed arrangement of these residues. In the same way, peptide $F^5Y^9Y^{12}$, imitating key residues of an α–helix crucial for the molecular recognition between VEGF and its receptor Flt-1, was able to bind to this receptor. Remarkably, some Trp-containing analogues from sub-library **L1** are among the most potent Flt-1 peptide binders reported to date. All these findings emphasize the usefulness of these linear helical peptides in the search for PPI modulators.

The library was also assayed for activity at three TRP channels, namely TRPV1, TRPM8 and TRPA1 thermoTRPs, given that certain domain segments of these channels are involved in several PPIs that participate in their regulation. Some peptides were able to inhibit the activation of TRPV1 and especially TRPA1 ($W^5Y^9I^{12}$, $Y^5F^9L^{12}$ and $Y^5Y^9I^{12}$). Although the exact mechanism of action of these peptides remains to be determined, the TRPA1-interacting peptides described here could represent a first step toward the development of innovative non-peptide TRPA1 antagonists.

We expect that this library of linear helical peptides could have broad application as tool to interrogate pharmacologically relevant PPI targets. Further studies on other known and unrevealed PPIs will show whether this library of easily prepared peptides could have wider practical application. In addition, the rational of design used here could also serve to fine-tune the potential of helical peptides implicated in PPIs, when extracted from their native proteins, and to ideate new libraries bearing other combinations of amino acids.

EXPERIMENTAL PROCEDURES

Parallel Synthesis of Peptides. Peptides were prepared starting from a Rink amide resin (0.34 g/mol) following the Fmoc/ ^{t}Bu strategy.

Manual synthesis was performed repeating the cycle described below for each amino acid:

First, resin was swelled with DMF and DCM (1ml/100mg of resin, 30 seconds x 4). Fmoc deprotections were performed with 20% piperidine in DMF (1 mL/100 mg of resin, one wash for 1 min and three for 10 min). Coupling reactions were carried out with HCTU (2 equiv), DIEA (2 equiv) and the corresponding Fmoc amino acid (2 equiv) in DMF for 1 h to obtain the peptides. Each coupling was checked by the Kaiser test and repeated if necessary.

Automated synthesis was performed using a peptide synthesizer coupled to a microwave heater (Cem Liberty1): resin was swelled with DCM for 10 min. Fmoc deprotections were performed with 20% piperidine in DMF in two steps. The first step was performed at 40ºC for 30 sec. and the second at 75ºC for 5 min. Coupling reactions were performed at 75ºC using Fmoc amino acids in DMF (5 equiv related to the resin), HBTU/HOBT in DMF (5 equiv) and DIEA in NMP (10 equiv).

Acetylation reactions were performed with DIEA (20 equiv) and Ac_2O (20 equiv) in DMF for 1 h or using an Ac_2O/DIEA/DMF (1:1:1) solution (4x10 min).

Cleavage of peptides from the resin, and concomitant side chain deprotection were performed using TFA/EDT/H_2O/TIPS (94:2.5:2.5:1) (1 ml, 100 mg of resin) at room temperature for 3 h. The resin was filtered off and crude products were precipitated with cold Et_2O. The resulting solid was centrifuged, washed twice with ethyl ether, and then lyophilized.

Peptides with <80% purity were purified by MPLC using SNAP 12g KP-C18-HS cartridges in an ISOLERA ONE (BIOTAGE). A gradient of CH_3CN:H_2O (0.05% TFA) from 0:100 to 30:70 over 60 min as mobile phase, and a flux of 5 mL/min were used. Some peptides were purified by semipreparative RP-HPLC-MS (Waters 2545) coupled to a mass spectrometer 3100 detector, using a SUNFIRE™ column C18 (5µ, 10x150 mm) and a flux of 8 mL/min using the methods described in Tables S1-S4. Peptide purity was analyzed using an analytical HPLC: Waters (model 2690) with a SUNFIRE™ column C18 (3.5m, 4.6 x 50 mm) at 1 mL/min with a 5 to 50% gradient of CH_3CN (0.08% HCO_2H):H_2O (0.01% HCO_2H) in 15 min as mobile phase or Agilent (model 1120 Compact LC) with Eclipse Plus column C18 (4.6 x150 mm) at 1.5 mL/min with a 5 to 50: gradient of CH_3CN :H_2O (0.05% TFA) in 20 min as mobile phase. Characterization of

the products was performed by HPLC-MS (Waters) coupled to a single quadrupole ESI-MS (Micromass ZQ 2000).

Characterization data (HPLC t_R and MS) of all library peptides are recorded in Tables S1-S4.

Detailed experimental procedures for the conformational analysis (CD and NMR), and the biological experiments are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information. Virtual libraries used for design, and Agadir prediction of helicity. Library description: HPLC and MS characterization of library components, and relevant results of CD and NMR experiments. Experimental methods for biological evaluation (STD experiments, binding to Flt-1 and Ca^{2+}-influx test). This material is available free of charge via the Internet at http://pubs.acs.org.

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R.G.-M., and M.M.-M. idea and design of peptides; R.G.-M., M.J.P.V., O.M., I.G.M., A.C., M.V., and A.F. designed research; M.A.B., B.B., B.L.-M., D.B., L.M.P., M.R.-S., N.G.-E., and R.T. performed research; R.G.-M., M.J.P.V., O.M., A.C., M.V., and A.F.-C. supervised

experiments and analyzed data; R.G.-M., M.M.M., M.J.P.V., and M.T.G.-L. wrote the paper; O.M., I.G.M., A.C., M.V., A.F.-C., and A.F. corrected the manuscript.

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Notes

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In Memoriam, dedicated to our colleague and friend Prof. Enrique Pérez-Payá.

ABBREVIATIONS

Flt-1: vascular endothelial growth factor receptor 1; KDR: vascular endothelial growth factor receptor 2; MDM2: murine doble minute 2; PPI: protein-protein interaction; STD: saturation transfer difference; TRP: transient receptor potential channel; TRPV1: transient receptor potential vanilloid receptor type 1; TRPM8: transient receptor potential melastatin 8; TRPA1: transient receptor potential ankyrin 1; VEGF: vascular endothelial growth factor.

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