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Design and analysis of EphA2-SAM peptide ligands: A multi-disciplinary screening approach

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ABSTRACT

EphA2 receptor plays a critical and debatable function in cancer and is considered a target in drug discovery. Lately, there has been a growing interest in its cytosolic C-terminal SAM domain (EphA2-SAM) as it engages protein modulators of receptor endocytosis and stability. Interestingly, EphA2-SAM binds the SAM domain from the lipid phosphatase Ship2 (Ship2-SAM) mainly producing pro-oncogenic outcomes. In an attempt to discover novel inhibitors of the EphA2-SAM/Ship2-SAM complex with possible anticancer properties, we focused on the central region of Ship2-SAM (known as Mid-Loop interface) responsible for its binding to EphA2-SAM. Starting from the amino acid sequence of the Mid-Loop interface virtual peptide libraries were built through *ad hoc* inserted mutations with either L- or D- amino acids and screened against EphA2-SAM by docking techniques. A few virtual hits were synthesized and experimentally tested by a variety of direct and competition-type interaction assays relying on NMR (Nuclear Magnetic Resonance), SPR (Surface Plasmon Resonance), MST (Microscale Thermophoresis) techniques. These studies guided the discovery of an original EphA2-SAM ligand antagonist of its interaction with Ship2-SAM.

1. Introduction

EphA2 (Erythropoietin-Producing Hepatoma Kinase A2) receptor belongs to the Eph family of receptor tyrosine kinases and is over-expressed in several types of tumors [1,2]. Although it is considered a drug target in anticancer drug-discovery [3], EphA2 function in cancer onset and progression is indeed very controversial as it can work as either an oncogene or a tumor suppressor through balancing of ligand-independent or -dependent signaling [2].

EphA2 contains, within its cytosolic portion, a SAM (Sterile Alpha Motif) domain whose function has started to be clarified only recently and is possible linked to tumor progression and metastatic spread [4–6]. Recent studies in different cancer cell lines have also pointed out that the SAM domain in EphA2 (EphA2-SAM) lowers receptor oligomerization and decreases kinase activity [4,5].

EphA2-SAM is able to interact with SAM domains from other proteins including the lipid phosphatase Ship2 [7] and the adaptor Odin [8]. Structural studies indicated that these interactions occur through a canonical Mid-Loop (ML)-End-Helix (EH) SAM-SAM interaction model [7–14]. Within this binding topology the central region of one SAM domain (i.e., ML interface) interacts with the EH interface of an adjacent SAM domain, constituted by the C-terminal helix and adjacent loops. EphA2-SAM generally provides the EH interface which dimerizes with the ML surfaces of Ship2-SAM [7] and the first SAM domain of Odin [8]. These SAM-SAM interactions are highly driven by electrostatic contacts between the EH interface of EphA2-SAM, which is enriched in positively charged residues, and the negatively charged surface of Ship2-SAM and Odin-SAM1 [7–10]. Interestingly, the heterotypic interaction between EphA2-SAM and Ship2-SAM is linked to a negative regulation of receptor endocytosis [15] and should mainly produce pro-oncogenic outcomes [9].

During the last few years, to identify potential anti-cancer agents, we designed and evaluated through different strategies peptide antagonists of SAM-SAM interactions in which EphA2-SAM is involved [16–19].

In the current study we describe a complementary computational and experimental approach to identify peptide ligands of EphA2-SAM based on the ML interface of Ship2-SAM.

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2. Results and discussion

EphA2 plays a pivotal role in anti-cancer drug discovery, and thus far the largest efforts to find novel therapeutics have been spent to target its ligand-binding and kinase domains with both peptides and small molecules [20–27]. Recently, a screening approach of a library made up of macrocyclic peptidomimetics led to the identification of a novel potent ligand of the EphA2 cytosolic portion able to antagonize EphA2 pathway [28]; later, a novel peptide based on the amino acid sequence of the EphA2 transmembrane region, capable of modulating receptor activation, has also been reported [29].

The SAM domain represents an interesting alternative target as it is the EphA2 site where protein regulators of receptor endocytosis and stability are recruited trough heterotypic SAM-SAM associations [6].

2.1. Peptide design and virtual screening

In this study, we focused our design on the ML interface of Ship2-SAM and explored the possibility of mutating a few amino acids within this region to increase the affinity towards EphA2-SAM.

To this aim, two different Ship2-SAM fragments were employed for peptide design leading to two analogues series denominated "SML" and "ShipH", respectively (Fig. 1). The "SML" sequences are all constituted by 22 residues, and the wild-type starting sequence encompasses completely the binding site of Ship2-SAM for EphA2-SAM, as identified in the NMR structure of the Ship2-SAM/EphA2-SAM complex [9] (Fig. 1a). Previous structural studies [7,9] pointed out the most crucial residues for the interaction of Ship2-SAM with EphA2-SAM, that were kept as fixed positions. "SML" peptide libraries were thus generated by mutating each residue, recognized as less relevant for the SAM-SAM association, with L- and D- amino acids (Fig. 1a). The conformation employed for each peptide within the "SML" series closely resembles that assumed by the ML region in the intact protein (Fig. 1a). The acronym "**SML**" derives from "**S**hip2 **M**id-**L**oop".

"ShipH" series comprise peptides composed of 17 amino acids; the wild-type sequence encompasses partly the ML interface of Ship2-SAM (Fig. 1b). This sequence is derived from the so called "Shiptide" peptide, which we previously described, and whose conformational properties were largely investigated in aqueous buffer alone and in presence of the folding co-solvent 2,2,2-trifluoroethanol (TFE) [30]. The Shiptide is disordered in merely aqueous buffer while presenting essentially an α-helical conformation in solution in presence of 70% TFE [30]. Thus, "SML" peptides have all helical conformations and were built starting from the helical region in the NMR structure of the Shiptide (Fig. 1b)

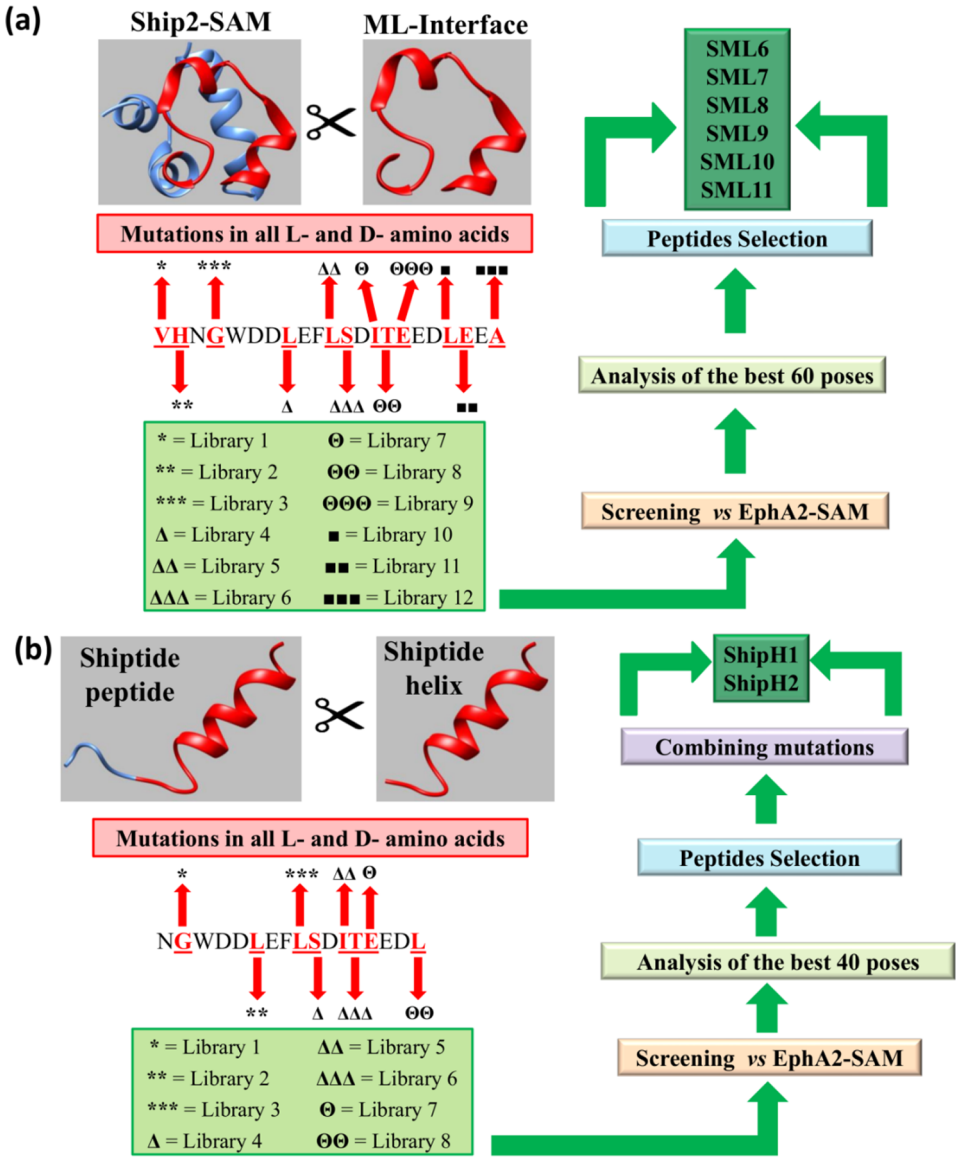

Fig. 1. Design of peptide libraries and virtual screening approach for "SML" (a) and "ShipH" (b) series. (a) The Ship2-SAM NMR structure (first conformer, pdb code: 2KSO) is shown on the top along with the ML interface (outlined in red), that represents the starting point for design of "SML" peptides. The amino acid sequence of the ML region is reported as well and mutated residues are colored red and underlined. (b) The NMR structure of the Shiptide (first conformer), which was used to generate "ShipH" peptides, the corresponding sequence after cutting of the disordered *N*-terminal tail and residue mutations are shown. In addition, in both panels (a) and (b) the number of generated libraries and analysis of docking results are schematically indicated.

Table 1
Peptide sequences. Underlined residues are those mutated with respect to the wild-type sequence (SML in first line, residues from 1218 to 1239 of human Ship2-SAM-Uniprot code: O15357); D-amino acids are reported with the lower case letters. "Ac" and "NH_2" indicate *N*-terminal acetylation and C-terminal amidation, respectively. CTRL is a *N*-terminal elongated native sequence (residues from 1215 to 1239 of human Ship2-SAM-Uniprot code: O15357). Peptides that were synthesized and experimentally tested are reported in bold.

Peptide	Sequence
SML	Ac-VHNGWDDLEFLSDITEEDLEEA-NH_2
SML6	Ac-VHNGWDDLEF**f**SDITEEDLEEA-NH_2
SML7	Ac-V**E**NGWDDLEFLSDITEEDLEEA-NH_2
SML8	Ac-VHNGWDDLEF**W**SDITEEDLEEA-NH_2
SML9	Ac-VHN**y**WDDLEFLSDITEEDLEEA-NH_2
SML10	Ac-VHNGWDDLEF**Q**SDITEEDLEEA-NH_2
SML11	Ac-VHNGWDDLEFLSDITEEDL**n**EA-NH_2
ShipH1a	Ac-NGWDDLEFLSDI**w**EEDL-NH_2
ShipH1b	Ac-NGWDDLEFL**E**DITEEDL-NH_2
ShipH1	Ac-NGWDDLEFL**E**DI**w**EEDL-NH_2
ShipH2a	Ac-NGWDD**n**EFLSDITEEDL-NH_2
ShipH2b	Ac-NGWDDLEF**d**SDITEEDL-NH_2
ShipH2	Ac-NGWDD**n**EF**d**SDITEEDL-NH_2
CTRL	Ac-EGLVHNGWDDLEFLSDITEEDLEEA-NH_2

[30]. "ShipH" stands for "**Ship**tide **H**elix".

Different "ShipH" peptide libraries were built by mutating single residues, dispensable for the EphA2-SAM/Shiptide interaction [30], following an identical approach to that already described for the "SML" series.

"SML" and "ShipH" peptide libraries were virtually screened against the EH region of EphA2-SAM with AutoDock Vina [31] (Figs. 1 and S1).

2.2. Analysis of docking results and peptides selection

At the end of each screening, the best 5 poses of each library (i.e., the ones with the most negative values of Vina scores) [31] were visually inspected with the softwares AutoDock Tools (ADT version 1.5.6) [32] and Chimera (version 1.10.1) [33] to analyze inter-molecular contacts (Supplementary Tables S1–S13). Among these best 100 solutions, including 60 hits from "SML" screening and 40 others from "ShipH" screening, 8 peptides were selected to be synthesized and experimentally tested (Tables 1 and Supplementary S14). Final peptide sequences were selected among those provided with good docking scores (Supplementary Table S14) using first of all a "diversity criterion". In major details, great attention was put to choose compounds provided with different types of mutations and consequently dissimilar physical-chemical properties, in order to allow decent variability and increase the chance to find out an EphA2-SAM ligand. Thus, solutions with conservative mutations with respect to the wild-type sequences (for example replacements of Ile with L-/D-Leu or Val, or of Ser in L-/D-Thr) were given low priority. Moreover, as the EH surface of EphA2-SAM is rich in positively charged amino acids and electrostatic contacts with negatively charged residues in Ship2-SAM appear crucial for EphA2-SAM/Ship2-SAM complex [7], poses enriched in either D- or L-Arg, Lys and His were discarded. On the contrary, mutations inserting aromatic residues that could perform additional interactions with the EH interface in EphA2-SAM were prioritized as well.

2.2.1. SML peptides

With respect to the native peptide (SML in Table 1), whose Vina score is −4.5 kcal/mol, docking poses of selected mutated peptides present a better score (Supplementary Table S14). Screening results indicate that for the interaction with selected "SML" peptides, residues Y66, L69, and R63 on the EH interface of EphA2-SAM play a pivotal role (Fig. 2 and Supplementary Tables S1–S7). In addition, Q74, positioned at the C-terminal end of the α5-helix, and S21-I22 in the α1-α2 loop of EphA2, are also involved in contacts with different residues in several poses (Fig. 2 and Supplementary Tables S1–S7).

2.2.2. ShipH peptides

From the 40 best ranked docking solutions of "ShipH" peptides, 4 sequences were first selected (ShipH1a, ShipH1b, ShipH2a, ShipH2b) (Tables 1 and Supplementary S8-S14). Similarly to "SML" series results, a few EphA2-SAM residues perform dominant interactions in analyzed docking poses: Y66, R63 and Q74 (Fig. 3 and Supplementary Tables S8–S11). To maximize potential advantageous interactions of mutated residues, mutations of ShipH1a and ShipH1b were combined together in the ShipH1 peptide (Tables 1 and Supplementary S14), similarly ShipH2 peptide contains both mutations present in ShipH2a and ShipH2b (Tables 1 and Supplementary S14). ShipH1 and ShipH2 sequences were generated by using the identical protocol adopted for all screened compounds, and were virtually screened against EphA2-SAM with AutoDock Vina [31] (Fig. 1 and Supplementary Tables S12, S13).

The peptides SML6, SML7, SML8, SML9, SML10, SML11, ShipH1 and ShipH2 were synthesized along with the CTRL Ship2-SAM native sequence (Table 1). CTRL includes, in addition to identical SML region, also the N-terminal "EGL" motif that is contained in the "Shiptide" peptide (within blue region in Fig. 1b) but was excluded in docking studies due to its large flexibility (Fig. 1b). All peptides were obtained with purities superior to 95%.

2.3. SPR and NMR screening

Selected peptides were analyzed through a SPR (Surface Plasmon Resonance) based screening in order to compare their abilities to bind EphA2-SAM [19] (Fig. 4).

The histograms of Response Unit (RUmax) evaluated at 100 and 300 μM peptide concentrations (Fig. 4), show that the ShipH1 peptide gives the higher signal variation upon binding to EphA2-SAM with respect to other generated sequences including the native peptide (CTRL).

To corroborate these data, a NMR (Nuclear Magnetic Resonance) based analysis of the same sequences was performed as well by means of [^{1}H-^{15}N] HSQC experiments and ^{15}N labeled EphA2-SAM [19,34] (Supplementary Figs. S2 and S3). HSQC spectra were recorded for the protein alone and after addition of each peptide. A few peptides of the "SML" series (such as SML8, SML10, SML11) and CTRL were able to produce very small changes in the spectrum of the protein (Supplementary Fig. S2). More changes in the HSQC spectrum of EphA2-SAM were generated by addition of ShipH1 clearly indicating, in line with SPR results, that ShipH1 is an EphA2-SAM interactor (Supplementary Fig. S3).

2.4. Conformational studies of ShipH1 peptide

Since ShipH1 appears as the best EphA2-SAM ligand in the preliminary screenings, additional conformational investigation of this peptide was conducted by NMR and CD (Circular Dichroism) spectroscopies (Fig. 5). CD spectra recorded in 10 mM sodium phosphate buffer at pH = 7.2 indicate that the peptide is disordered and only in presence of high TFE percentages − 80 and 90% – an enlargement of helical content can be detected (Fig. 5a). NMR chemical shift analysis (Tables S15, S16 and S17) and mainly, comparison of Hα chemical shifts with respect to random coil values (CSD) [35–37] (Fig. 5b), further confirms CD results. In details, in PBS solution ShipH1 CSD present absolute values lower than 0.1 ppm (Fig. 5b) and no NOEs typical of regular secondary structure elements are revealed from analysis of NOESY spectrum (Fig. 5c and Supplementary S4). The NOESY spectrum is in fact dominated by sequential inter-proton contacts of the type $H\alpha_i$-HN_{i+1} that are canonical of random coil species [38] (Fig. 5c). In presence of 50% and 80% TFE NMR spectra show an increase of signal dispersion that, along with appearance of several H_N-H_N contacts in the 2D NOESY spectrum, point out peptide increase of order

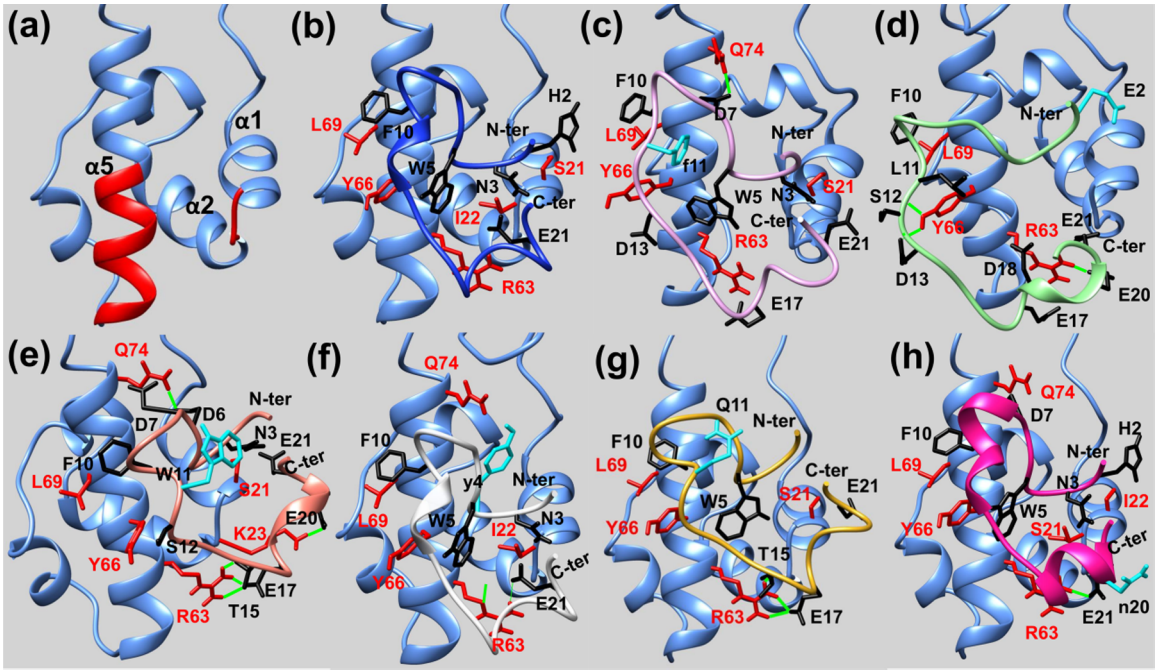

Fig. 2. (a) The SAM domain of EphA2 (pdb code: 2E8N) with regions encompassing the End-Helix surface (I22-M24 in the α1-α2 loop and P58-Y66 in the α5 helix) colored red. (b, c, d, e, f, g, h) AutoDock Vina models of EphA2-SAM/SML (b), EphA2-SAM/SML6 (c), EphA2-SAM/SML7 (d), EphA2-SAM/SML8 (e), EphA2-SAM/SML9 (f), EphA2-SAM/SML10 (g), EphA2-SAM/SML11 (h) complexes. Residues of EphA2-SAM and "SML" peptides, that are mainly involved in intermolecular-interactions, are highlighted in red and black, respectively, with side chains shown in a line representation with heavy atoms and polar hydrogens. Mutated peptide residues are colored cyan, hydrogen bonds are outlined with green lines.

(Supplementary Fig. S4). CSD values lower than −0.1 ppm outline an enlargement of helical structuration in the stretch E7-E10 and I12-E14 (Fig. 5b). Helical content estimated from CSD is 34% and 43% for ShipH1 samples in presence of 50% and 80% TFE, respectively. NOEs pattern evaluated in presence of 80% TFE shows that the peptide assumes a well organized helical conformation, due to the occurrence of several Hα(i)-Hβ(i + 3) and a few Hα(i)-H_N(i + 3) [38] contacts in the peptide region W3-I12 (Fig. 5c and Supplementary S4). A complete 3D structure calculation was conducted for ShipH1 in solution containing 80% TFE and confirmed an α-helix structural organization in the stretch from W3 to I12 and a more disordered structure at the C-terminal region (Supplementary Fig. S5).

2.5. The ShipH1/EphA2-SAM association: binding affinity and structural details

NMR experiments indicate that the addition of ShipH1 peptide to ^{15}N labeled EphA2-SAM produces a few changes in the [^{1}H-^{15}N] HSQC spectrum of the protein and point out a weak interaction (Fig. 6a). Observed changes in the spectrum of EphA2-SAM in presence of ShipH1 (at protein : peptide ratio 1 : 21) were mapped on the 3D structure of EphA2-SAM (pdb code: 2E8N) (Fig. 6b, c). Results show that the peptide mainly targets the EH interface of EphA2-SAM and close regions (Fig. 6c); a displacement-like experiment confirms this evidence and further demonstrates that ShipH1 is a weak antagonist of the EphA2-SAM/Ship2-SAM association as it can be gradually replaced in the complex with EphA2-SAM by unlabeled Ship2-SAM (Supplementary Figs. S6 and S7).

Differently from EphA2-SAM, the peptide ShipH1 is not able to produce changes in the HSQC spectrum of ^{15}N labeled Ship2-SAM at a protein : peptide molar ratio equal to 1 : 16 (Supplementary Fig. S8) thus pointing out that binding to EphA2-SAM is rather specific.

A dissociation constant K_D for the interaction EphA2-SAM/ShipH1 was obtained by MST experiments (Fig. 7) and resulted equal to 72.4 ± 0.5 μM. On the contrary, MST titration experiments with EphA2-SAM and increasing amounts of other different peptides (i.e., CTRL, SML8, SML10, SML11) did not produce an effective binding curve and consequently did not allow to estimate a dissociation constant K_D (Supplementary Fig. S9).

2.6. Serum stability

To test ShipH1 peptide ability to resist to enzymatic degradation and evaluate its possible future employment in cell-based assays, stability in FBS (Fetal Bovine Serum) was evaluated [39]. ShipH1 contains a D-Trp (Table 1) and it is well recognized that D- amino acid replacement is a potential route to lower peptides susceptibility to proteases attack [40,41]. Thus, for comparison purpose, the stability of the native peptide CTRL (Table 1) was also monitored. As it can be seen in Fig. S10, the shorter sequence in ShipH1 along with the presence of a D-Trp (Table 1) is indeed increasing the serum stability of this peptide, in fact after 24 h CTRL is fully degraded while ShipH1 is only 50% cleaved.

3. Conclusion

Molecules able to inhibit SAM-SAM associations in which EphA2-SAM is involved may act as novel anti-cancer compounds or be employed to advance knowledge of EphA2-SAM role within receptor signaling and are thus, attractive in the drug discovery field [6]. However, identification of such molecules is particularly challenging as SAM-SAM binding interfaces are flat and dynamics and thus far, at the best of our knowledge, no high affinity inhibitors of these interactions have been described in literature [42].

Herein, we report on a dual computational and experimental approach to identify peptide ligands of EphA2-SAM based on the interaction surface of Ship2-SAM. In details starting from the amino acid sequence of the ML interface of Ship2-SAM and the conformation assumed by this region in (a) the intact protein or (b) in the corresponding isolated peptide fragment in presence of the folding agent TFE, we designed virtual libraries of peptides containing L- and D- amino

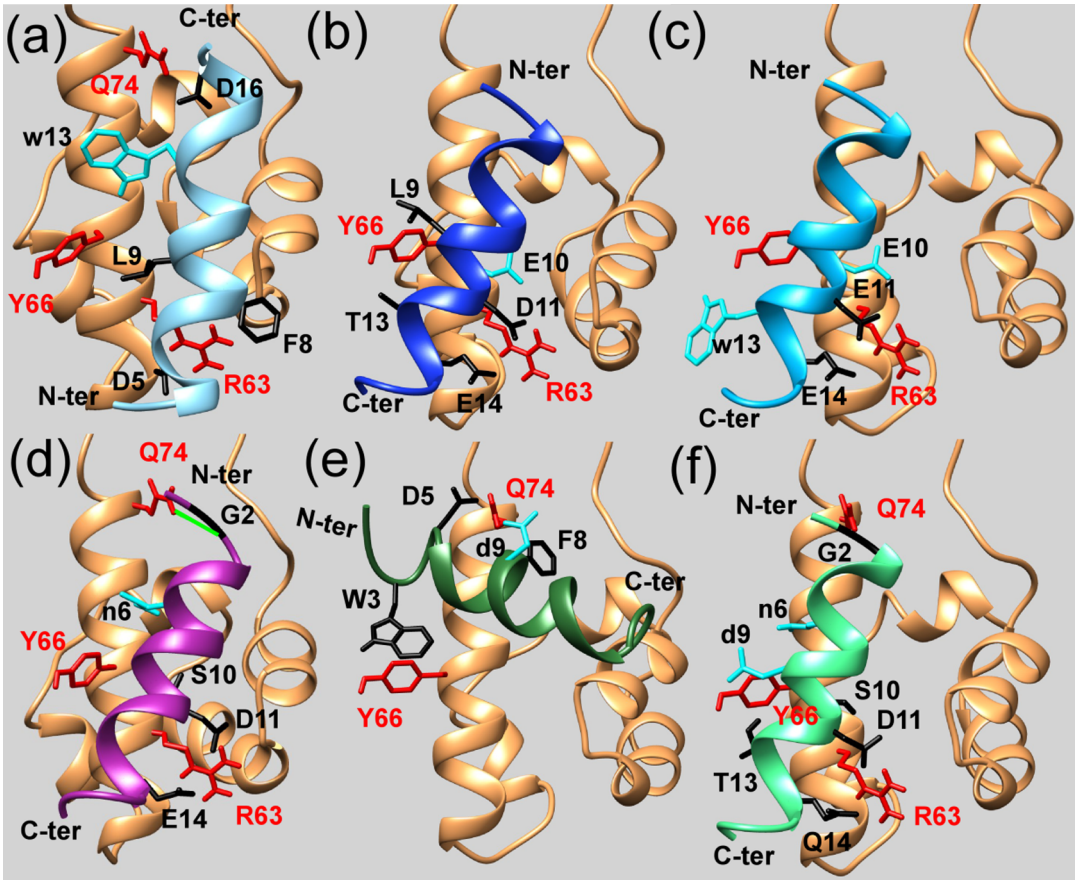

Fig. 3. AutoDock Vina models of EphA2-SAM/ShipH1a (a), EphA2-SAM/ShipH1b (b), EphA2-SAM/ShipH1 (c), EphA2-SAM/ShipH2a (d), EphA2-SAM/ShipH2b (e), EphA2-SAM/ShipH2 (f) complexes. Residues belonging to EphA2-SAM and ShipH peptides, that provide intermolecular interactions, are highlighted in red and black, respectively, and corresponding side chains are reported in a line style with heavy atoms and polar hydrogens. Peptides mutated residues are colored cyan, hydrogen bonds are shown with green lines.

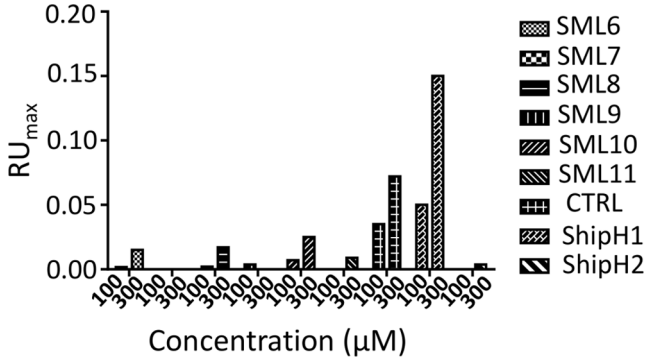

Fig. 4. RU_{max} values from SPR experiments to monitor binding of different peptides to immobilized EphA2-SAM. Values refer to sensorgrams recorded at two different concentrations (i.e., 100 and 300 μM). Signals were normalized for the molecular weight of each peptide.

acids. These peptide libraries were virtually screened against EphA2-SAM, the best *in silico* hits were analyzed and a few peptides were synthesized. These compounds were later experimentally tested in interaction assays through a variety of techniques such as NMR, SPR, MST. This study led to identification of a novel EphA2-SAM ligand with a dissociation constant in the high micromolar range which possesses improved serum stability with respect to the wild-type sequence.

Based on these results, in the close future we are planning to conjugate our novel ligand (ShipH1) to cell cargo systems and to test it in cell-based assays. The anticancer potential of SAM targeting-peptides could be explored by monitoring their ability to modulate EphA2 receptor activation, internalization and cell migration in cancer cells [20,29]. Noticeably, the discovery of the ShipH1 sequence paves the way for the design of novel peptides, provided with at least two aromatics residues, one of which in a D-configuration, and enriched in negatively charged amino acids, which could result more efficient EphA2-SAM interactors.

In conclusion, this work shed further light on the "druggability" of SAM domains and potential approaches to target their interactions, and add another tile to the complex route to obtain a potent antagonist of EphA2-SAM mediated associations with good drug-like properties.

4. Material and methods

4.1. Design of virtual peptide libraries

Peptide sequences to be used as starting point for the mutagenesis approach were selected with two different strategies. For the design of the "SML" peptides we started from the NMR structure of Ship2-SAM in complex with EphA2-SAM (pdb code: 2KSO [9]) and selected the Ship2-SAM fragment corresponding to the whole ML interacting region for EphA2-SAM (Uniprot code for human Ship2-SAM: O15357, residues from 1218 to 1239 corresponding to segment V46 to A67 in the pdb file 2KSO [9]). For the design of the "ShipH" peptides we started from a shorter Ship2-SAM segment including most of the ML interface (Uniprot code for human Ship2-SAM: O15357, fragment from 1215 to 1236 corresponding to segment E43-L64 in the pdb file 2KSO [9]) that was previously characterized in detail and whose NMR structure obtained in 70% TFE has already been reported by us [30]. This peptide fragment

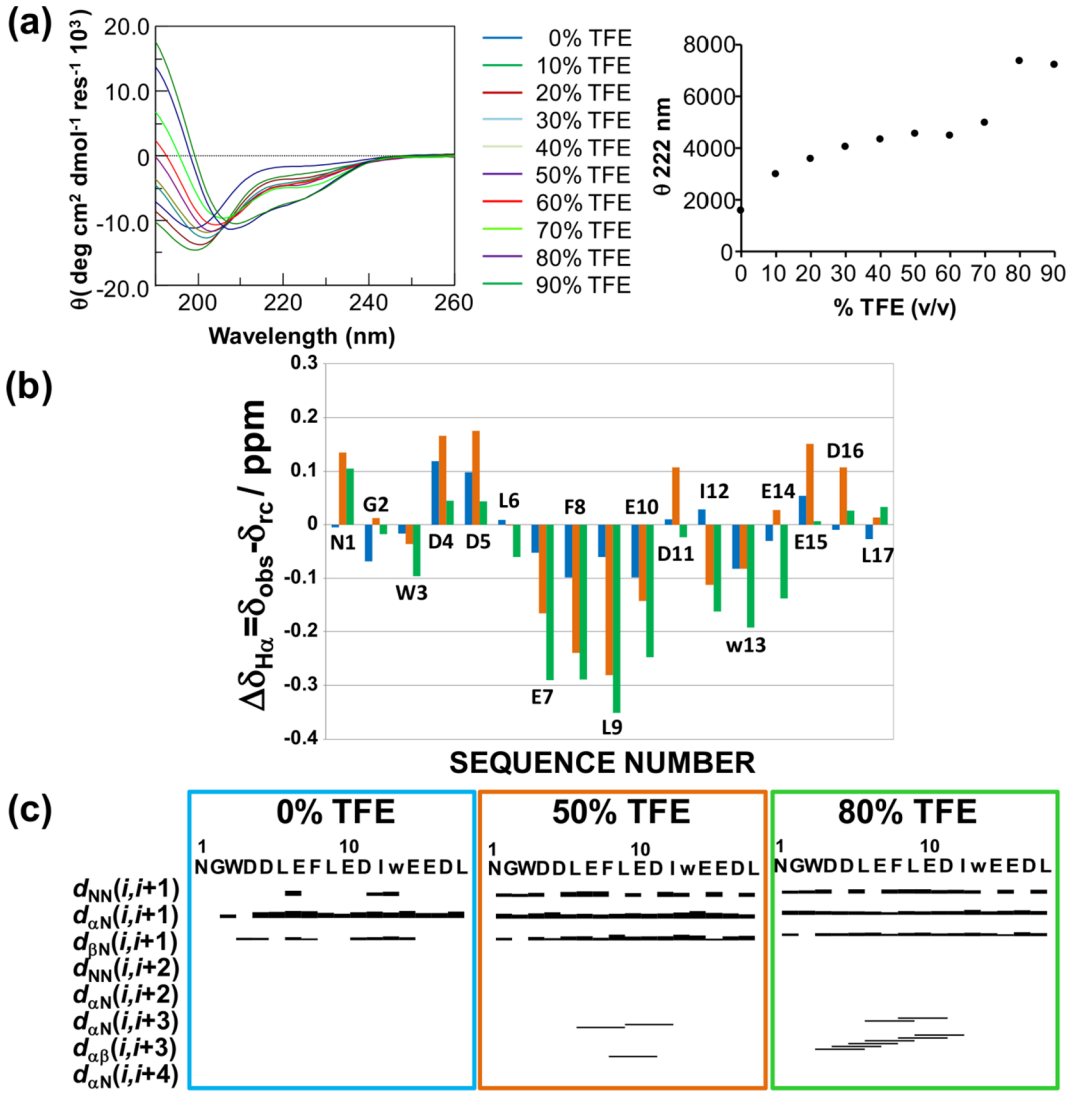

Fig. 5. Conformational analysis of ShipH1 by NMR and CD. (a) Superposition of CD spectra of ShipH1 recorded at increasing percentages of TFE are shown in the left panel; the right panel reports Θ_{222} nm absolute values as function of increasing TFE amounts. (b) Chemical shift deviations of observed Hα chemical shifts (δ_{obs}) with respect to random coil values (δ_{rc}), evaluated in 91.4/7.9/0.7 PBS/D_2O/DMSO v/v/v (blue), in 49.5/49.5/1 PBS/TFE/DMSO v/v/v (orange) and 19.5/79.5/1 PBS/TFE/DMSO v/v/v (green). (c) NOEs patterns of ShipH1 peptide calculated from NOESY spectra acquired in 91.4/7.9/0.7 PBS/D_2O/DMSO v/v/v, 49.5/49.5/1 PBS/TFE/DMSO v/v/v, and 19.5/79.5/1 PBS/TFE/DMSO v/v/v.

(i.e., "Shiptide" [30]) in presence of TFE is composed of an α-helix and a small unstructured region at the N-terminus (segment E43-H47), that was excluded in the following structure editing steps.

A few residues of the two selected Ship2-SAM starting fragments were then mutated. Fixed amino acid positions were established by considering results from previous studies (mutagenesis and/or docking data), obtained for the EphA2-SAM/Ship2-SAM [7,9] and EphA2-SAM/Shiptide [30] complexes, that indicated the putative relevance of a few Ship2-SAM amino acids for these interactions.

Virtual peptides were built with Chimera (version 1.10.1) [33]. For "SML" peptides the first conformer of the NMR structure of Ship2-SAM (pdb code: 2KSO [9]) was implemented, and protein regions outside the ML portion were deleted. Similarly, for "ShipH" peptides, the N-terminal disordered region was cut from the NMR structure of the Shiptide (first conformer) [30]. Next, acetyl and amide groups were added to the N- and C- termini of the starting structures, respectively.

Residues to be mutated (i.e., V1, H2, G4, L8, L11, S12, I14, T15, E16, L19, E20, A22 and G2, L6, L9, S10, I12, T13, E14, L17 for "SML" and "ShipH" series respectively) were individually selected and changed in each L- amino acid, or in its D- stereoisomer, with the structure editing tool of Chimera [33]. The resulting peptides were energy minimized with the MMTK (Molecular Modelling Toolkit) minimization module of Chimera [43]. Minimization runs included at least 1000 steps of steepest descendant and 3000 steps of conjugate gradients [43]. During the minimization step of "ShipH" peptides the backbone of the segment from residue number 6 to residue number 14 was kept fixed to avoid peptide helix unfolding. This protocol led to building a library of 39 peptides for each mutated residue (in detail, 12 libraries for "SML" peptides and 8 libraries for "ShipH" peptides). Peptides were initially generated in .pdb format and next converted in .pdbqt format, required for AutoDock Vina screening [31], with AutoDock Tools (ADT) [32]. During the conversion the backbone bonds of the peptides were set as non-rotatable.

4.2. Virtual screening

Virtual screening was performed using AutoDock Vina (version 1.1.2) [31] on an IBM IntelliStation Z Pro workstation equipped with 4 cpu. The first conformer of the EphA2-SAM NMR structure (pdb code: 2E8N) was used as receptor entity and treated as rigid in all virtual

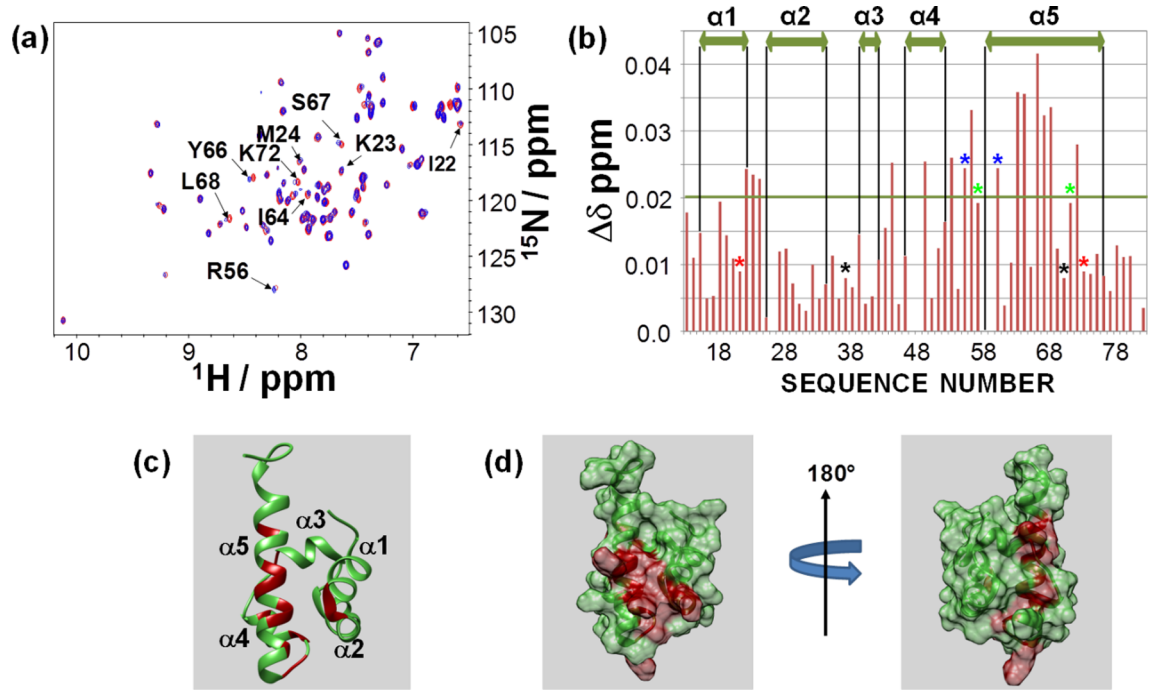

Fig. 6. (a) Overlay of [^{1}H-^{15}N] HSQC spectra of EphA2-SAM (25 μM concentration) in its free form (red) and after addition of the ShipH1 peptide (520 μM concentration) (blue). Residues undergoing the largest chemical shifts variations are indicated. (b) Histogram showing chemical shift deviations ($\Delta\delta = [(\Delta H_N)^2 + (0.17 * \Delta^{15}N)^2]^{1/2}$) versus residue numbers. Δδ values were set equal to zero for P58 and P81 along with Q26, N47, D48, G59 whose peaks are not visible under the experimental conditions used to run the experiments. Asterisks indicate pairs of residues with ambiguous assignments due to spectral overlaps (i.e., S21 with D73, T37 with G70, V55 with H60, L57 with L71). (c, d) The 3D solution structure of EphA2-SAM (conformer number 1, pdb code: 2E8N) is reported in ribbon (c) and surface (d) representations and the amino acids with $\Delta\delta \geq 0.02$ ppm (i.e., I22, K23, M24, Q44, D49, I53, V55, R56, H60, R63, I64, Y66, S67, L68, K72) are highlighted in red.

screening runs. The EphA2-SAM .pdb file was converted in .pdbqt file with ADT [32]. The Vina grid was designed on the EH region of EphA2-SAM (See Supplemental Fig. S1) [7]. In detail for both "SML" and "ShipH" peptides, a first screening was conducted with a wide grid centered on His 60 that belongs to the C-terminal EphA2-SAM helix in the EH surface [7]. Next, the best solution of each screening was used to set the final grid that was centered on the ligand and enlarged of 10 Å in all directions [31,44]. By default, AutoDock Vina performs calculations with a randomized seeding, consequently dissimilar binding poses can be generated by repeating the same calculation on an identical protein-peptide complex [45]. Thus, to guarantee reproducibility of our solutions, the number of seed was fixed in all screening runs to 297,061,376 [45]. The value of the configuration parameter "exhaustiveness", that regulates how many times the same run needs to be repeated, was set to 4 [45]. The maximum number of output binding poses was fixed to 1 [45]. Details about local optimization algorithm and scoring function implemented in AutoDock Vina can be found in Ref. [31].

The best solutions in terms of Vina scores (i.e., the ones with the lowest binding energies) [31] were visually analyzed with Chimera [33] and ADT [32].

4.3. Peptide synthesis

Peptide syntheses were achieved with standard solid phase methodologies with a fully automated multichannel peptide synthesizer Syro I (Multisynthech, Germany). Reagents needed for peptide synthesis

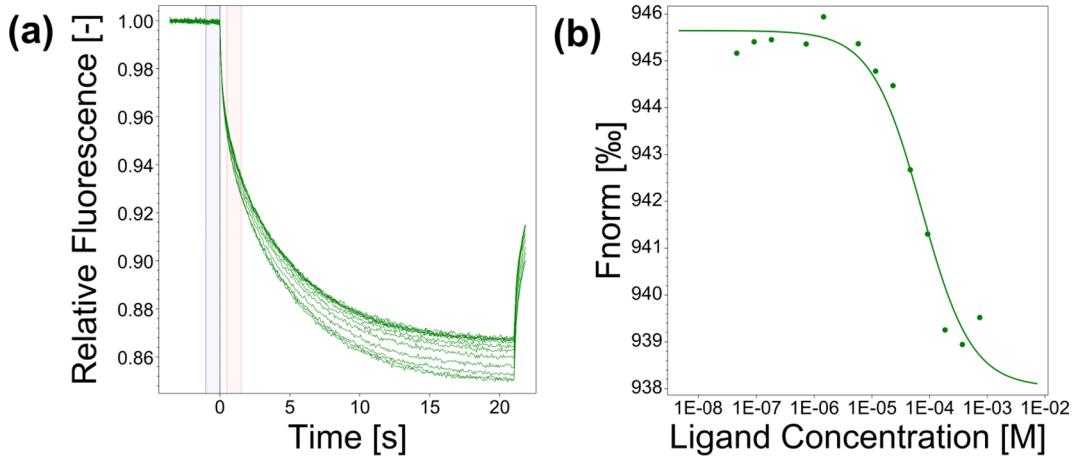

Fig. 7. MST experiment for the interaction of ShipH1 peptide with EphA2-SAM. MST traces and plot of normalized fluorescence from EphA2-SAM binding experiment versus ShipH1 peptide at different concentrations (4000 μM–120 nM) are presented in panels (a) and (b), respectively.

(i.e., Fmoc-protected amino acids, resins, activation, and deprotection reagents) were obtained from Calbiochem-Novabiochem (Laufelfingen, Switzerland). Solvents for synthesis and HPLC (High Performance Liquid Cromatography) analyses were purchased from Romil (Dublin, Ireland).

"SML" (SML6, SML7, SML8, SML9, SML10, SML11), "ShipH" (ShipH1 and ShipH2) and CTRL peptides were synthesized on Rink Amide MBHA resin (0.7 mmol g^{-1} substitution; 50 μmol scale), which allows to recover the peptide sequences amidated at the C-terminus. Peptide *N*-terminus was acetylated by treatment with a mixture of acetic anhydride (4.7%) and pyridine (4%) in DMF for 10 min. The cleavage from the solid support and the simultaneous deprotection of all side chains were performed by suspending the fully protected compound-resins in TFA/H_2O/TIS (97: 2: 1) for 3 h. The peptides were isolated by precipitation into cold diethyl ether and centrifuged to form a pellet.

Analytical RP (Reverse Phase)-HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex (Torrance, California) Kinetex column (5 μm C18 100 A− 60 × 4.60 m) with a flow rate of 1.0 mL min^{-1} and a linear gradient starting from 5% to 70% B in 10 min; preparative RP-HPLC was carried out on HP Agilent Series 1200 apparatus using a Phenomenex (Torrance, California) Gemini column (5 μm NX-C18 110 Å− 150 × 21.2 mm, AXIATM) with a flow rate of 15 mL min^{-1} and a linear gradient starting from 5% to 70% B in 20 min. LC-ESI-TOF-MS analyses was performed with an Agilent 1290 Infinity LC System coupled to an Agilent 6230 TOF LC/MS System (Agilent Technologies, Cernusco Sul Naviglio, Italy).

4.4. Protein expression and purification

Recombinant EphA2-SAM and Ship2-SAM were expressed in *E. coli* and purified according to protocols that we have reported before [7,19]. Unlabeled protein samples were produced in LB (Luria-Bertani) broth; ^{15}N-labeled samples were produced in M9 minimal medium containing 1 g/L of $^{15}NH_4Cl$. Bacteria growth were conducted at 37 °C till a cell optical density OD_{600nm} = 0.6. The induction step was achieved at 25 °C overnight by implementing isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM concentration. His-tagged Ship2-SAM and EphA2-SAM were purified through a Nickel column by affinity chromatography with an Akta Purifier FPLC System (GE Healthcare, Milano, Italy). In the end, proteins were dialyzed against Phosphate Buffer Saline (PBS, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, from Sigma-Aldrich, Milan-Italy) pH 7.4.

4.5. NMR experiments

NMR experiments were recorded on a Varian Unity Inova 600 MHz spectrometer equipped with a cold probe at 298 K. For the preliminary NMR screening all peptides (roughly 2 mg each) were dissolved in 500 μL of PBS pH 7.4 to obtain concentrated stock solutions. Due to the poor solubility of a few peptides, after dissolving them they were centrifuged at room temperature and the precipitate was discarded, the exact concentrations in the stock solutions were measured by a NanoDrop 2000c spectrophotometer (Fisher Scientific) and were the following: SML6-931 μM; SML7-700 μM; SML8-914 μM; SML9-514 μM; SML10-1.1 mM; SML11-1 mM; CTRL-760 μM; ShipH1-367 μM; ShipH2-1.5 mM. NMR samples for binding studies consisted of a total volume equal to 500 μL, including 200 μL of peptide stock solutions and ^{15}N labeled EphA2-SAM (25 μM concentration). Moreover, DMSO (Dymethyl-Sulfoxide-D6, 99.9% D, Sigma-Aldrich, Milan) (1% v/v) was added to each NMR sample to overcome possible solubility issues and avoid peptide precipitation during acquisition of NMR experiments along with 10% D_2O (Deuterium Oxide 98% D, Sigma-Aldrich, Milan). Control experiments with samples containing only ^{15}N labeled EphA2-SAM protein and 1% DMSO were recorded as well. The pH of the samples containing protein and peptides was checked and eventually adjusted to 7.4 upon addition of a few drops of a concentrated NaOH stock solution.

Displacement experiments were conducted by adding to a sample containing ^{15}N labeled EphA2-SAM (25 μM) and the ShipH1 peptide (520 μM), unlabeled Ship2-SAM at increasing concentrations (i.e., 6.2 μM, 12.5 μM, 25 μM, 50 μM and 75 μM). Identical spectra were acquired with ^{15}N labeled EphA2-SAM (25 μM) after addition of Ship2-SAM at increasing concentrations in absence of ShipH1 peptide.

In addition, a 2D [^1H, ^{15}N] HSQC spectrum was acquired with ^{15}N labeled Ship2-SAM (25 μM) and the ShipH1 peptide (400 μM).

NMR conformational studies of ShipH1 were performed by recording spectra of the peptide in 91.4/7.9/0.7 PBS/D_2O/DMSO v/v/v, in 49.5/49.5/1 PBS/TFE/DMSO v/v/v, and in 19.5/79.5/1 PBS/TFE/DMSO v/v/v.

2D [^1H, ^1H] TOCSY (Total Correlation Spectroscopy) [46], NOESY (Nuclear Overhauser Enhancement Spectroscopy) [47], and DQFCOSY (Double Quantum-Filtered Correlated Spectroscopy) [48], acquired for these studies were normally obtained with 16–64 scans, 128–256 FIDs in t1, 1024 or 2048 data points in t2. TOCSY experiments were recorded with 70 ms mixing time, NOESY experiments with 200 and 300 ms mixing times. Water suppression was achieved by *Excitation Sculpting* [49]. The Wüthrich protocol was used to obtain proton resonance assignments [38]. TSP (Trimethylsilyl-3-propionic acid sodium salt-D4, 99% D, Armar Scientific, Switzerland) was used as internal standard for chemical shifts referencing (0.0 ppm).

Spectra were processed with VNMRJ 1.1D (Varian, Italy) and analyzed with the software NEASY [50] contained in CARA (http://www.nmr.ch/).

Chemical shift deviations from random coil values for Hα protons (CSD) were calculated with the protocol suggested by Kjaergaard and collaborators [36,37] assuming equal random coil chemical shift values for D-Trp and L-Trp; random-coil chemical shift reference values were estimated (http://www1.bio.ku.dk/english/research/bms/research/sbinlab/groups/mak/randomcoil/script/) at T = 25 °C, pH 7 for ShipH1 in PBS, pH 5 for ShipH1 in 49.5% v/v aqueous TFE, and pH 2.8 for ShipH1 in 79.5% v/v aqueous TFE. The percentage of helical population was estimated with the equation: [ΔδHαaverage/(−0.39)] × 100] where $\Delta\delta H\alpha = \delta H\alpha_{observed} - \delta H\alpha_{random\text{-}coil}$ was averaged over residues in a helical conformation (i.e., the ones with negative CSD values) [51].

4.6. Circular dichroism

CD spectra were registered on a Jasco J-810 spectropolarimeter (JASCO Corp, Milan, Italy) as previously reported [16,17,19,30]. They were obtained by averaging three scans, subtraction of blanks and conversion of the signal to mean residue ellipticity (deg × cm^2 × $dmol^{-1}$ × res^{-1}). CD spectra of ShipH1 were recorded in 0.1 cm path-length quartz cuvette at 43 μM concentrations in phosphate buffer 10 mM pH 7.4. A TFE titration (from 0 to 90% TFE) was conducted as well with a ShipH1 sample (43 μM).

4.7. Microscale thermophoresis

MST experiments were performed on a Monolith NT 115 system (Nano Temper Technologies) provided with 100% LED and 40% IR-laser power. The His-Tag labeling Kit RED-tris-NTA was used for protein labeling. EphA2-SAM was used at a concentration equal to 100 nM in labeling buffer (Nano Temper Technologies) [16,19], the dye concentration was adjusted to 100 nM as well. Both protein and fluorescent dye solutions were incubated for 1 h at room temperature in the dark and centrifuged after incubation. To check binding of ShipH1 to EphA2-SAM, a 16-steps serial dilution (1:1) was prepared (final concentration range 4000 μM–120 nM); a concentration range of 150 μM − 4.5 nM, 420 μM–128 nM, 365 μM–111 nM and 380 μM–115 nM for SML8, SML10, SML11 and CTRL were explored, respectively. Each peptide

sample was inserted into standard treated capillaries. Experiments were carried out at 25 °C in PBS supplemented with 1% DMSO, 0.05% Tween-20. In order to fit normalized fluorescence values at different concentrations of ligands, an equation executed by the software MO-S002 MO Affinity Analysis, supplied by the manufacturer, was implemented.

4.8. Surface plasmon resonance

SPR experiments were conducted as previously reported [8,16,17]. Briefly, a Biacore 3000 SPR with associated reagents (GE Healthcare) was employed. EphA2-SAM domain was immobilized on a CM5 sensor chip at a concentration equal to 10 μg/mL in 10 mM acetate buffer pH 5.0, for 7 min at a flow-rate of 5 μL/min reaching an immobilization level of 1867 RU.

For SPR screening, different peptides were dissolved in PBS buffer with 1% DMSO and tested at 100 and 300 μM concentrations.

SPR assays were carried out with following parameters: (i) flow: 20 μL/min, (ii) contact time: 4.5 min, (iii) running buffer: HBS (HEPES (10 mM), NaCl (150 mM), EDTA (3 mM), pH 7.4) and reference channel signals were subtracted as blanks.

4.9. Serum stability

Serum stability assay was conducted as already reported [39]. Briefly peptides were dissolved at 1 mg/mL and incubated in 25% of Fetal Bovine Serum (FBS) at 37 °C for 32 h. Equal peptides aliquots were taken at different times and mixed with 25 μL of a 30% trichloro-acetic acid (TCA) solution by diluting peptides and TCA of a 5 and 2-fold factors, respectively. In order to allow precipitation of plasma proteins, following incubation at 2 °C for 15 min, samples were centrifuged (10,000 rpm for 10 min). RP-HPLC was next carried out on an Agilent Technologies 1200 series system employing an elution gradient from 5 to 70% in 20 min with buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile).

Conflict of interest

The authors report no declarations of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.12.009.

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