Journal of Medicinal Chemistry

Article

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.7b01280 • Publication Date (Web): 07 Nov 2017

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

One-Bead-Two-Compound Thioether Bridged Macrocyclic γ-AApeptide Screening Library against EphA2

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Abstract

Identification of molecular ligands that recognize peptides or proteins is significant, but poses a fundamental challenge in chemical biology and biomedical sciences. Development of cyclic peptidomimetic library is scarce and thus discovery of cyclic peptidomimetic ligands for protein targets is rare. Herein we report the unprecedented One-Bead-Two-Compound (OBTC) combinatorial library based on a novel class of the macrocyclic peptidomimetics γ-AApeptides. In the library, we utilized the coding peptide tags synthesized with Dde-protected α-amino acids, which were orthogonal to solid phase synthesis of γ-AApeptides. Employing the thioether linkage, the desired macrocyclic γ-AApeptides were found to be effective for ligand identification. Screening the library against the receptor tyrosine kinase EphA2 led to the discovery of one lead compound which tightly bound to EphA2 (K_d: 81 nM) and potently antagonized EphA2-mediated signaling. This new approach of macrocyclic peptidomimetic library may lead to a novel platform for biomacromolecular surface recognition and function modulation.

Introduction

Chemical biology and biomedical sciences are undergoing a new era of vigorous development due to the rapid discovery of new protein targets and unveiling of their biological importance. Consequently, it is an unmet need to identify ligands that recognize peptide or protein targets with high specificity and affinity,[1] since such an effort could help understanding function of proteins and lead to potential therapeutic agents for diagnosis and treatment. Combinatorial chemistry which serves as a powerful tool for ligand screening in basic medicinal chemistry, has emerged to meet this challenge.[2] The diverse library of compounds provides concurrent but independent chances for target screening. Since it provides the possibility to yield important therapeutic agents from various target proteins, the progress of drug discovery has been largely accelerated.[3] As peptides are versatile molecules, which have modular chemical diversity and favorable binding activity, they are natural building blocks for combinatorial libraries for screening against various targets. Among them, macrocyclic peptides which have enhanced conformational constraint and binding affinity are widely recognized for exploring ligand-receptor interactions. Several general methods have been successfully developed to construct the macrocyclic ring systems.[4-5, 8-12]

Recent efforts have contributed to the creation of non-natural sequence-specific peptidomimetics.[13] These peptidomimetics are developed based on the mimicry of peptide primary structure and possess a modified peptide backbone for the introduction of diverse functional side chains. Compared with natural peptides, peptidomimetics possess improved protease-resistance, chemodiversity, and bioavailability.[14] The past decade has witnessed noteworthy progress in the development of biomimetic oligomers, including β-peptides,[15-17, 19]

peptoids,[18, 20-23] α-aminoxy-peptides,[24] α/β-peptides,[25,26] azapeptides,[27] and others. However, to date only a handful of peptidomimetic combinatorial libraries were systematically investigated for protein ligand identification.[28] The development of the macrocyclic peptidomimetic combinatorial library is even rarer.[29-31] Motivated by these findings, we have recently developed the new class of peptidomimetics, γ-AApeptides (oligomers of γ-substituted-*N*-**a**cylated-*N*-**a**minoethyl amino acids), which were inspired by the backbone structure of the chiral PNA.[32,33] γ-AApeptides are highly resistant to proteolytic degradation and possess cellular translocation capability and have enhanced chemodiversity, making them ideal candidates as molecular probes or therapeutic agents.[34] Indeed, these features have been demonstrated by our previously developed one-bead-one-compound (OBOC) linear γ-AApeptides-based combinatorial libraries capable of inhibiting Aβ aggregation and disrupting STAT3/DNA interaction.[35,36] We thus envisioned that the macrocyclic combinatorial library of γ-AApeptides would be endowed with enhanced conformational rigidity and constraints compared with the linear counterparts, could be even more beneficial in the identification of bioactive ligands.

Results and Discussion

Design of the cyclic Library. The thioether moiety is widely found in nature. For instance, methionine has a thioether side chain and is virtually present in all proteins. Other natural products such as antibiotic lanthipeptides directly utilize thioether bridges to form multimacrocylic ring structures. Heinis et al also adopted the thioether linkage to develop phage-display mediated bicyclic peptide libraries.[4-7] This thioether-bridge mediated cyclization has proven to be highly efficient in cyclization, leading to cyclic peptides with not only rigidified conformational freedom but also enhanced metabolic stability. These features make them promising affinitive candidates for biological applications. By employing a thioether-bridge

approach, a novel cyclic γ-AApeptide library could be synthesized and tested against target proteins. To test our hypothesis, we embarked on the design of the cyclic γ-AApeptide library by introducing a Dmt (4,4′-dimethoxytrityl) protected mercaptoethyl carbonyl group to the secondary amine in the first γ-AApeptide building block (Figure 1) on the solid phase. After another three γ-AApeptide building blocks were assembled subsequently, the 4-(bromomethyl)benzoyl group was attached to the N-terminal amino group of the sequence. Next, the Dmt group was removed, the γ-AApeptide could be cyclized under nearly neutral condition with virtually quantitative yield due to the high efficiency of sulfur-mediated S_N2 reaction.

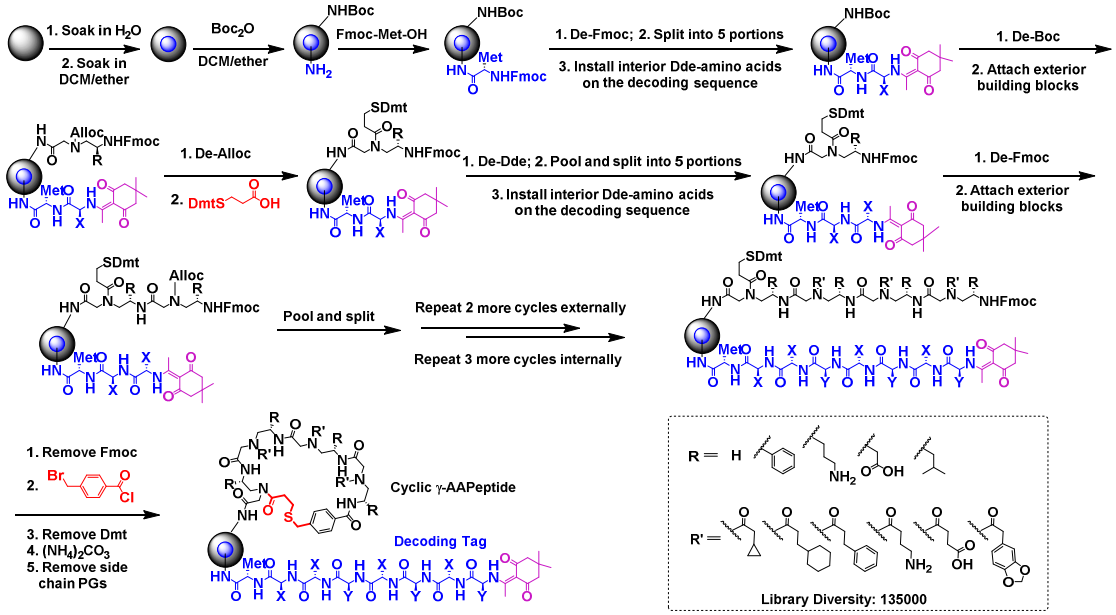


Figure 1. Synthesis of the thioether bridged *o*ne-bead-two-compound macrocyclic γ-AApeptide library. Please see supporting information for details.

Molecular Dynamics (MD). In order to assess the potential conformational rigidity of this class of cyclic γ-AApeptides, we conducted molecular dynamics (MD) simulations of the cyclic backbone and linear backbone in explicit water (Supporting information, Figure S5). Consistent with our hypothesis, MD simulation (Supporting information, Figure S5) suggested the backbone

of thioether bridged cyclic γ-AApeptides is much more conformationally rigid that of the linear one, leading to possibility of identification of novel ligands with high affinity.

Library Diversity and decoding. To ensure the development of relatively unbiased cyclic library, a diverse set of hydrophobic, cationic and negatively charged sidechains were chosen. Briefly, due to the structural nature of γ-AApeptides, 4 chiral side chains came from the side chains of 5 different *N*-Alloc protected γ-AApeptide building blocks (R, Figure 1). The other three side chains were introduced by acylating the secondary amino group with 6 different carboxylic acids or acyl chlorides after deprotection of the alloc group (R', Figure 1). Thus, by using the split and pool method, the theoretical diversity of the library was expected to be 135000 (Figure 1), and 405000 beads were used for the preparation of the library.

Unlike linear γ-AApeptide library,[35,36] the direct sequencing of cyclic γ-AApeptides was no longer feasible using MS/MS. Instead, the strategy of a one-bead-two-compound (OBTC) library containing both ligands and analyzable coding sequences on the same bead was desired.[37,38] Peptides consisted of α-amino acids could serve well as coding sequences since the MS/MS pattern of amino acid fragments is unambiguous. To this end, we developed a highly effective approach to synthesize coding peptides by using Dde ((1-(4,4-dimethyl-2,6-dioxacyclohexylidene) ethyl) protected α-amino acids. The deprotection of Dde was very mild using NH_2OH•HCl and imidazole[39] and fully orthogonal to chemistry engaged in the synthesis of thioether-bridged cyclic γ-AApeptides.

Synthesis of the Library. To prepare these desired OBTC cyclic γ-AApeptide combinatorial library, TentaGel beads (200-250 μm; 1.5 nmol/bead)) were soaked overnight in water and then exposed to 1:1 (v/v) DCM/Et_2O containing 0.5 equiv di-tert-butyl decarbonate (Boc_2O) (Figure 1).[40,41] This was expected to possess Boc protection of amino groups on the outer surface of the

beads since the interior of the beads still remains in water. After washing with DMF, the interior of the beads was allowed to react with the Met, the amino acid facilitating coding peptide cleavage upon cyanogen bromide (CNBr) treatment. After removal of the Fmoc protecting group, the beads were split into 5 equal aliquots and reacted with 5 different Dde protected amino acids respectively, so as to establish a coding tag representing the first γ-AApeptide building block on the outer layer. Subsequently, the Boc group of the outer layer was removed by TFA, followed by the attachment of 5 Alloc γ-AApeptide protected building blocks. Next, the Alloc group was removed by $Pd(PPh_3)_4$ and $Me_2NH{\cdot}BH_3$, and the Dmt protected 3-mercaptopropanoic acid was added to react with the secondary amino group. The beads were pooled and split into 5 aliquots again. After the Dde group was removed, the second set of the Dde protected amino acids was added to introduce the coding tag for the second γ-AApeptide building block on the outer layer of the beads. These steps were repeated 3 more times. Compared with the cycle of the first building block, the only difference in the subsequent synthetic process on the outer layer was that after the Alloc group was removed, the N group of the γ-AApeptide building block were reacted with a different carboxylic acid or acyl chloride to introduce diverse side chains. Since each γ-AApeptide building block bears two side chains, two Dde protected amino acids were used to code each building block (Figure 1). Finally, the 4-(bromomethyl)benzoyl chloride was used to cap the N-terminus of γ-AApeptides on the outer layer, followed by selective removal of the Dmt group on the thiol linker with 2% TFA in DCM. The cyclization of γ-AApeptides was achieved in the presence of the ammonium carbonate ($(NH_4)_2CO_3$), which occurred on the surface of the beads only due to the lack of cyclization linker in the interior of the beads. The deprotection of side chain protecting groups was finally conducted in 94% TFA, 2% Triisopropylsilane, 2% water and 2% Thioanisole (v:v:v:v). Quality

of the beads was excellent, as evidenced by the fact that 8 out 10 randomly selected beads showed unambiguous MS/MS fragmentation patterns by MALDI and were able to provide information of the coding peptide sequence almost instantly.

Library Screening and Binding Affinity. With the success of obtaining the cyclic γ-AApeptide combinatorial library, we moved forward to examine its potential for identification of valuable biological ligands. EphA2 (Ephrin type-A receptor 2) was chosen due to its prominent role in the pathogenesis of various tumors.[42] EphA2 (Figure 2) belongs to the family of Eph receptor tyrosine kinases which regulate tissue development, patterning of the visual system, and play a critical role in mediating the cell-cell communication and angiogenesis.[43,44] Recent findings suggest that EphA2 overexpression is a key factor contributing to multiple cancers such as melanoma, ovarian, lung, and breast.[45-47] Therefore, EphA2 is a promising target for cancer therapeutic development. Considerable efforts have started to be made to identify inhibitors that block the capability of EphA2 for the phosphorylation of its downstream protein substrates, thereby dampening EphA2 mediated cell signaling. However, to date only limited success was achieved.[48] Thus, it is compelling to identify ligands from our macrocyclic γ-AApeptide library that bind to EphA2 with high affinity.

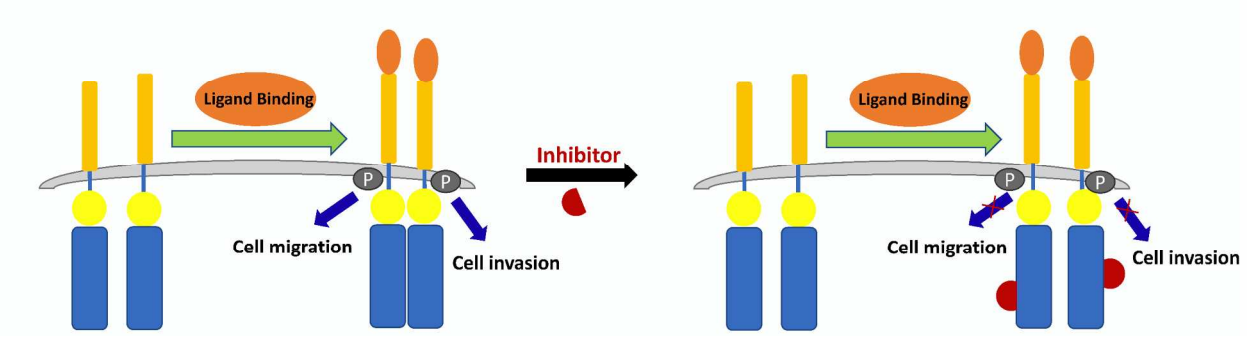


Figure 2. EphA2 signalling and inhibition pathway.

To conduct the library screening, we first incubated beads with GST tagged intracellular domain of EphA2 protein (Figure 3A and supporting information). After the thorough wash and the following incubation with the mouse anti-GST antibody, the beads were washed again and treated with the Alexa Flour 594 labeled goat-anti mouse secondary antibody. Ten beads emitting red fluorescence were identified under the fluorescence microscope and picked up as the putative positive hits. Subsequently, these beads were incubated with guanidium chloride (GdmCl) to denature any potential proteins stuck on the surfaces of the beads. The coding peptides in the inner layers of the beads were then cleaved off the beads by treatment with CNBr, and subsequently sequenced by tandem MS/MS of MALDI. Among the ten hits, seven different peptide structures could be determined unambiguously (The rest three hits had poor fragmentation which were very difficult for us to work out the structures. This may be caused by the cleavage step.). Next, the corresponding sequences of the seven cyclic γ-AApeptides conjugated with fluorescein isothiocyanate (FITC) labels were resynthesized and measured for their binding affinity toward EphA2 by fluorescence polarization (FP) assay. Fortunately, the most potent hit, AApeptide **2** (FITC labeled AApeptide **1**) (Figure 3B), exhibited excellent binding affinity to EphA2 with a K_d value of 81 nM (Figure 3C). The potential binding to the GST tag was excluded as **2** only showed negligible binding affinity to GST protein with a K_d of 14.2 μM, which is ~170-fold weaker than that of EphA2 binding (Figure 3D). Besides, the GST protein, another two kinases AKT1-PKBα and NFKBIA were chosen to test the selectivity of the **1**, which showed K_d of 3.62 μM and 0.741 μM (Supporting information, Figure S8), respectively, demonstrating the binding of **1** toward EphA2 is selective. Moreover, we treated C13 cells with 1 μM and 5 μM **2**, and the diffuse fluorescence was clearly visible in 1 h (Supporting information, Figure S4), suggesting the compound is cell permeable.

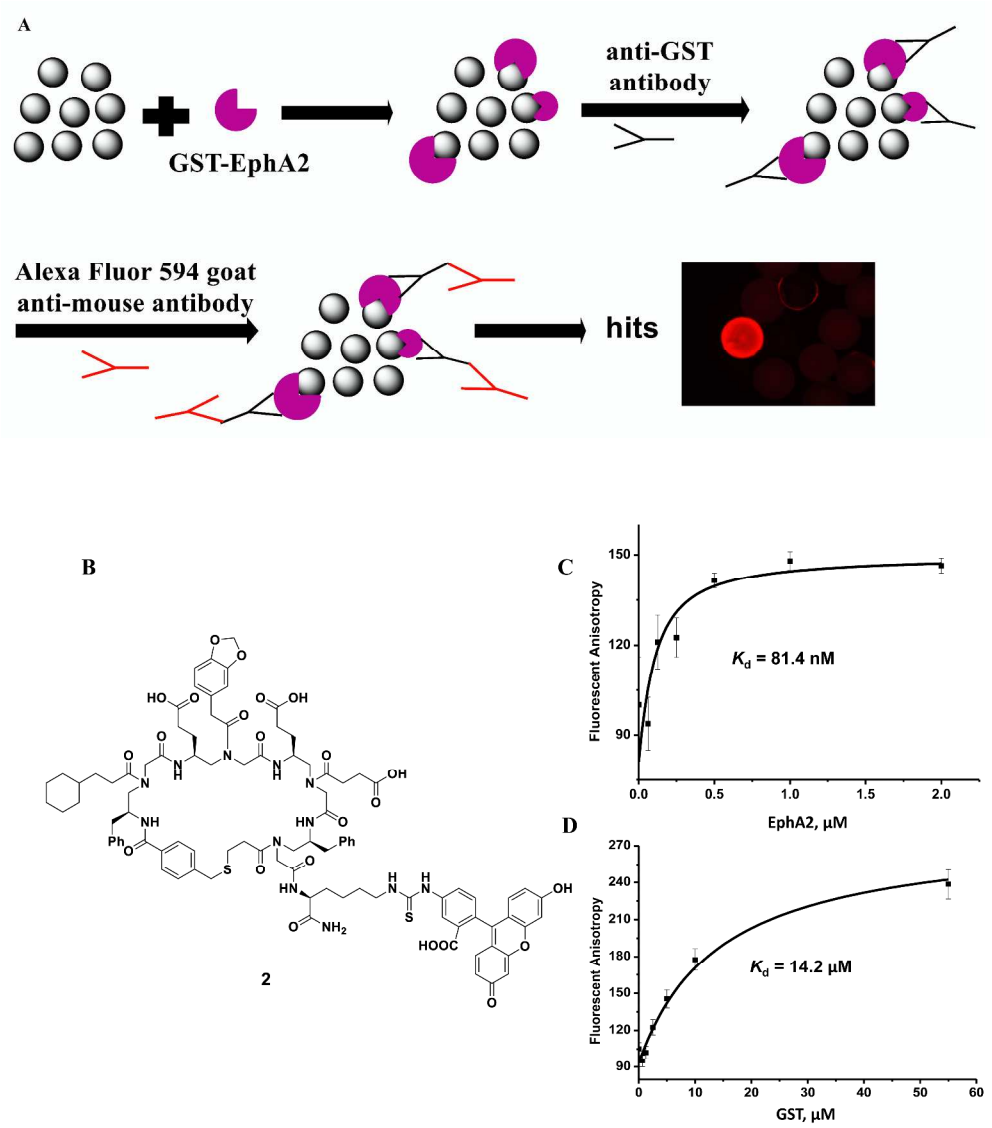


Figure 3. A). Screening of the γ-AApeptide library. B). Structure of **2** (FITC labeled lead compound **1**). C). The K_d of the compounds to the EphA2. D). The K_d of the compounds to the GST.

Inhibition Kinase activity of EPhA2. Given the strong binding affinity of **1** (Figure 4A) toward EphA2, we next performed assays to evaluate its biological activity. The initial *in vitro* kinase assay (Figure 4B) show that 2 μM **1** could completely inhibit EphA2 kinase activity by

preventing phosphorylation of its substrate poly(Glu-Tyr). Another compound **9** (Figure S1, supporting information), with the same molecular scaffold, failed to inhibit activity of EphA2, suggesting that recognition of **1** toward EphA2 was specific. At the same concentration, **1** also greatly suppressed the auto-catalytic activity of EphA2 (Figure 4C). Both abovementioned *in vitro* kinase assays suggested **1** is a potent inhibitor of EphA2 kinase activity. Inspired by the results, we further investigated the ability of **1** for the regulation of catalytic activity in an ovarian cancer cell line C13 cells which displays high expression of EphA2.[49] As shown in Figure 4D, the phosphorylation level of EphA2 was inhibited on a dose-dependent fashion with the increased concentrations of **1**. The IC_{50} of **1** for the inhibition of EphA2 inhibition was approximately 5 μM.

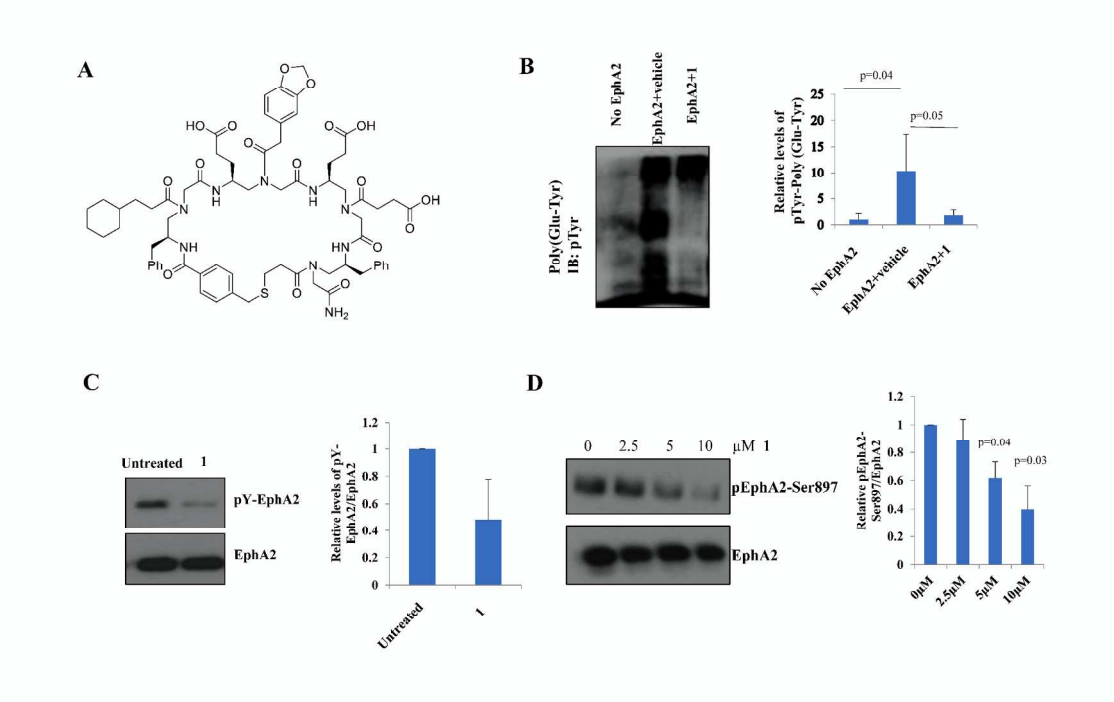
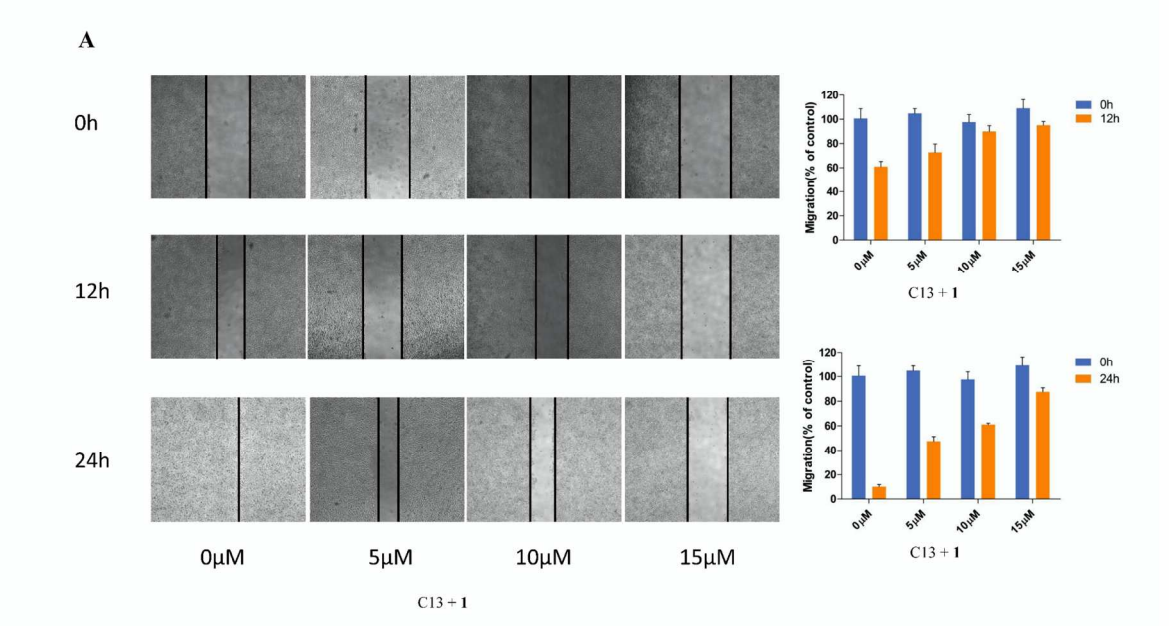


Figure 4. A). Structure of lead compound **1**. B). in vitro kinase assay was performed using 50 ng EphA2 and 50 μg Poly(Glu-Tyr) as a substrate containing 2μM **1**. Quantification of substrate phosphorylation was shown. C). Auto phosphorylation of EphA2 was detected by incubating 50 ng EphA2 with 2μM **1** in the kinase assay buffer. Immunoblot analysis with pTyr antibody was performed. D). C13 cells were treated with the indicated

concentrations of **1** for 24 hours. Immunoblot analysis of EphA2-Ser897 was performed. Total EphA2 was used as a control. The extent of phosphorylation was quantified using ImageJ software. Densitometric analysis was performed by SPSS

Cell Migration and Invasion. Since EphA2 was shown to play a critical role in cell migration,[50] we next tested the effect of **1** on cell migration and invasion. **1** was applied to C13 cells and analyzed using a scratch-wound motility assay. The data in Figure 5A showed a time and dose-dependent downregulation of cell migration in response **1** treatment, as shown by as much as 70% delay in wound closure at 24 hours post-treatment with the highest dose of **1** (Figure 5A). Similar results were seen in Matrigel-coated transwell assays, treatment of 10 μM **1** led to around 70% and 80% decrease in cell migration (Figure 5B) and invasion (Figure 5C), respectively, suggesting EphA2 mediated cell signaling was significantly suppressed. The inactive compound **9** was also tested for comparison (Supporting information, Figure S2), which as expected did not show capability to prevent migration. A linear compound AApeptide **10** was also tested (Figure S3), and the result was consistent with the MD simulation because **10** was much less potent. Taken together, the ability of **1** for the strong inhibition of EphA2 activity in cells suggests that cyclic γ-AApeptides possess excellent cell permeability, augmenting their future development in biomedical sciences.



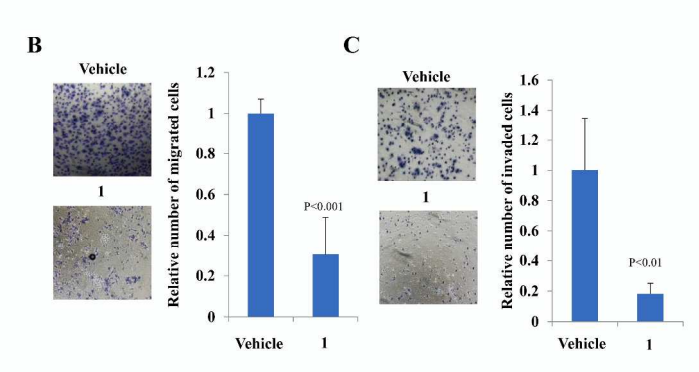


Figure 5. A). Wound-healing assay. Images were taken 0, 12 and 24 hours after wound formation. Data are presented as mean ± SD of triplicate experiments and using image J software. C13 cells treated with 10 μM **1** or vehicle were subjected to migration B) and invasion C) assays. Transwell data was determined by the number of the migrated cells and the value from parental cells was arbitrarily set at 100%.

Conclusion

In summary, a new class of macrocyclic peptidomimetic combinatorial library has been developed. With the unique γ-AApeptide backbone, which ensures the chemodiversity in the library, this new method has great potential to be a rich source in protein/peptide ligands identification. In addition, compared with previous linear peptide library developed by our group, the thioether bridged macrocyclic γ-AApeptide library has the dual advantages of significantly enhanced conformal rigidity of the backbones and cell permeability. This led to higher promise in the identification of more potent and useful ligands/molecular probes. Furthermore, the new encoding approach of Dde peptide tags also greatly increases the possibility and ease of the structural elucidation of putative hits. The promise of this macrocyclic library was manifested by the identification of potent ligands that specifically target the EphA2 receptor tyrosine kinase. With a K_d value of 81 nM, the compound **1** was found to be a potent inhibitor of the EphA2 signaling in both in vitro and cellular assays. Currently, the library is

being used as a new platform for screening against various targets in our group, and the in vivo study of compound **1** is underway.

Experimental Section

General Information. Fmoc-protected amino acids were purchased from Chem-impex (Wood Dale, IL). TentaGel resin (0.23 mmol/g) was purchased from RAPP Polymere (Tuebingen, Bermany). Rink Amide-MBHA resin (0.55 mmol/g) was purchased from GL Biochem (Shanghai, China). 1-Hydroxybenzotriazole wetted with no less than 20% wt. water (HOBt), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 5,5-Dimethyl-1,3-cyclohexanedione and 4,4'-Dimethoxytrityl Chloride were purchased from Oakwood Chemical (Estill, SC). 4-(Bromomethyl)benzoic acid was purchased from AK-Scientific (Union City, CA). 3-Mercaptopropionic Acid was purchased from TCI (Tokyo, Japan). Fluorescein isothiocyanate (FITC) was purchased from Chemodex (Gallen, Switzerland). Solid phase synthesis was conducted in peptide synthesis vessels on a Burrell Wrist-Action shaker. γ-AApeptides were analyzed and purified on a Waters Breeze 2 HPLC system, and then lyophilized on a Labcono lyophilizer. The purity of the compounds was determined to be >95% by analytical HPLC. Masses of γ-AApeptides and the MS/MS analysis were obtained on an Applied Biosystems 4700 Proteomics Analyzer. ^{1}H NMR spectra were recorded at 500 MHz using TMS as the internal standard. ^{13}C NMR spectra were recorded at 125MHz using TMS as the internal standard. The multiplicities are reported as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m). Coupling constants are reported in Hertz (Hz). High resolution mass spectra were obtained on an Agilent 6220 using electrospray ionization time-of-flight (ESI-TOF). Cell culture media was purchased from Gibco (Rockford, IL), fetal bovine serum (FBS) was purchased from Peak Serum (Fort Collins, CO), penicillin-streptomycin was purchased from

Invitrogen (Carlsbad, CA). GST-EphA2 recombinant protein, Poly(Glu-Tyr) and Alexa Fluor 594 goat anti-mouse antibody were purchased from Life Technologies (Carlsbad, CA) and Sigma-Aldrich (St. Louis, MO) respectively. Phospho-Tyrosine HRP conjugated antibody (pTyr) was purchased from R&D Biosystems (Minneapolis, MN). pEphA2-Ser897 and EphA2 antibodies were purchased from Cell signaling technologies (Danvers, MA). Anti-GST antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). All solvents and other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) and were used without further purification.

Synthesis of the Dmt protected mercaptopropionic acid (11). 4,4'-Dimethoxytrityl Chloride (6.38g, 18.82 mmol) was dissolved in 40 mL CH_2Cl_2 containing 3-Mercaptopropionic Acid (1.64ml, 18.82 mmol) at room temperature. The Triethylamine (3.93, 22.58 mmol) was slowly added to the above solution. The solution was stirred at room temperature for 4 h to 6 h. After that the mixture was evaporated under reduced pressure, and the residue was washed with saturated citric acid and extracted with ethyl acetate (30 mL ×3). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified by flash column chromatography (Hexane/Ethyl acetate 1:1) to afford the desired product as a light yellow solid (80% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.42-7.44 (d, J = 8.00 Hz, 2H), 7.34-7.35 (d, J = 9.00 Hz, 2H), 7.29(t, J = 7.50 Hz, 2H), 7.21 (t, J = 7.50 Hz, 1H), 6.84-6.82 (d, J = 9.00 Hz, 2H), 3.79 (s, 6H), 2.49 (t, J = 7.50 Hz, 2H), 2.30 (t, J = 7.50 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.2, 158.1, 145.2, 137.0, 130.7, 129.4, 127.9, 126.6, 113.2, 66.0, 55.2, 33.5, 26.6.

Synthesis of the 4-(bromomethyl)benzoyl chloride (12). The 4-(bromomethyl) benzoic acid (5g, 23.25 mmol) was dissolved in 10 mL thionyl chloride and reflux for 5 h. The excess thionyl

chloride was removed under reduced pressure to afford the desired product as a white solid and directly use without purification (85% yield).[51] ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.06 (d, J = 5.00 Hz, 2H), 7.52 (d, J = 10.00 Hz, 2H), 4.50 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.7, 145.3, 132.9, 131.8, 129.6, 31.4.

Synthesis of 2-acetyl-5,5-dimethylcyclohexane-1,3-dione (13). To a 100 mL round bottom flask was added 5,5-dimethylcyclohexane-1,3-dione (10 g, 71.34 mmol), *N*, *N*-Diisopropylethylamine (14.91mL, 85.6 mmol), 4-Dimethylaminopyridine (435.76mg, 3.57 mmol) and 50 mL DCM. The mixture was stirred in an ice bath to which acetyl chloride (6.08 mL, 85.6 mmol) was added. The reaction was warmed up to room temperature and allowed to stir for 8 h. The solvent was evaporated and the residue was washed with 1M HCl then extracted with ethyl acetate (30 mL × 3). The organic layer was dried over anhydrous Na_2SO_4 and then removed in vacuo. The residue was purified by flash column chromatography (Hexane/Ethyl acetate 1:1) to afford the 2-acetyl-5,5-dimethylcyclohexane-1,3-dione as a yellowish solid (11 g, yield 85%).[52] ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.54 (s, 3H), 2.48 (s, 3H), 2.3 (s, 2H), 1.01 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 202.3, 197.8, 195.1, 112.3, 52.4, 46.8, 30.6.

Synthesis of Dde protected amino acids. The L-Amino acid (1 equiv) was suspended in a solution of the 2-acetyl-5,5-dimethylcyclohexane-1,3-dione (1.3 equiv) in absolute ethanol (ca. 50 mL). Triethylamine (1.5 equiv) was added and the reaction mixture was refluxed for 18 h. The resulted yellow solution was cooled and concentrated under reduced pressure. The residue was dissolved in CH2Cl2 (50 mL), and washed with 1 M HCl (50 mL × 2). The organic layer was dried over Na_2SO4, filtered and concentrated in vacuo. Addition of Et2O (ca. 40 mL) to the residue resulted in immediate white precipitate, which was filtered and washed with cold Et2O to afford the title compound as an off-white crystalline solid (~ 70 %).[53]

Dde-Ala-OH (14). White solid**.** ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.51 (d, J = 5.00 Hz, 1H), 4.61 (t, J = 5.00 Hz, 1H), 2.48 (s, 3H), 2.27 (s, 4H), 1.41 (d, J = 5.00 Hz, 3H), 0.92 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 197.4, 172.9, 172.5, 107.6, 52.8, 51.6, 30.3, 28.3, 19.1, 18.1. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{13}H_{20}NO_4$: 254.1392, found: 254.1396.

Dde-Val-OH (15). White solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 13.6 (d, J = 5.00 Hz, 1H), 10.97 (s, 1H), 4.61 (t, J = 5.00 Hz, 1H), 2.5 (s, 3H), 2.39 (s, 4H), 2.36 (m, 1H), 1.08 (d, J = 5.00 Hz, 3H), 1.04 (d, J = 5.00 Hz, 3H), 1.0 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.3, 171.6, 107.9, 62.3, 51.9, 31.1, 30.1, 28.1, 19.1, 18.7, 17.0. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{15}H_{24}NO_4$: 282.1705, found: 282.1717.

Dde-Phe-OH (16). White solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 13.71 (d, J = 5.00 Hz, 1H), 7.18-7.27 (m, 5H), 4.57-4.61 (m, 1H), 3.05-3.09 (m, 2H), 2.36 (s, 4H), 2.20 (s, 3H), 1.00 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 198.1, 173.6, 171.0, 135.5, 129.4, 128.6, 127.4, 107.9, 58.3, 52.4, 45.5, 39.3, 30.1, 28.0, 18.1, 8.5. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{19}H_{24}NO_4$: 330.1705, found: 330.1714.

Dde-Leu-OH (17). White solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 13.60 (d, J = 10.00 Hz, 1H), 10.01 (s, 1H), 4.57-4.61 (m, 1H), 2.50 (s, 3H), 2.37 (s, 4H), 1.82 (m, 2H), 1.77 (m, 1H), 0.99 (s, 6H), 0.95 (d, J = 5.00 Hz, 3H), 0.89 (d, J = 5.00 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 198.9, 173.9, 107.9, 54.9, 52.3, 45.6, 41.3, 30.1, 28.2, 24.8, 22.7, 21.7, 18.7, 8.4. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{16}H_{26}NO_4$: 296.1862, found: 296.1873.

Dde-Glu(OBn)-OH (18). Pale yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.77 (d, J = 5.00 Hz, 1H), 10.50 (s, 1H), 7.33 (s, 5H), 5.10 (s, 2H), 4.55 (m, 1H), 2.53-2.59 (m, 2H), 2.51 (s, 3H), 2.39 (s, 4H), 2.21-2.25 (m, 2H), 1.01 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.3, 171.9,

171.2, 135.4, 128.6, 128.4, 128.3, 128.2, 66.7, 55.4, 52.3, 30.2, 29.6, 28.2, 27.7, 18.7. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{22}H_{28}NO_6$: 402.1917, found: 402.1925.

Dde-Asp-OH (19). White solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.56 (d, J = 10.00 Hz, 1H), 4.84 (m, 1H), 2.90 (dd, J = 15.00, 5.00 Hz, 1H), 2.78 (dd, J = 15.00, 5.00 Hz, 1H), 2.46 (s, 3H), 2.27 (s, 4H), 1.36 (s, 9H), 0.92 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.3, 170.7, 168.7, 107.7, 81.5, 52.5, 38.2, 30.1, 38.3, 28.0, 17.9. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{18}H_{28}NO_6$: 354.1917, found: 354.1929.

Dde-Glu-OH (20). White solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.54 (d, J = 10.00 Hz, 1H), 4.26 (q, J = 5.00 Hz, 1H), 2.43 (s, 3H), 2.28 (s, 4H), 2.24-2.26 (m, 2H), 1.90-2.10 (m, 2H), 1.36 (s, 9H), 0.93 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.7, 171.8, 107.4, 80.3, 54.2, 52.1, 45.2, 30.8, 30.1, 28.3, 28.1, 27.8. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{19}H_{30}NO_6$: 368.2073, found: 368.2075.

Dde-Lys-OH (21). White solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 13.69 (m, 1H), 10.24 (s, 1H), 4.80 (m, 1H), 4.40 (m, 2H), 3.09 (m, 2H), 2.50 (s, 3H), 2.34 (s, 4H), 1.95 (m, 2H), 1.50 (m, 2H), 1.40 (s, 9H), 1.01 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.3, 172.9, 172.0, 171.6, 155.9, 107.6, 80.9, 78.9, 60.1, 56.1, 40.9, 39.8, 32.1, 30.3, 29.1, 27.9, 21.6, 18.4, 13.9. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{21}H_{35}N_2O_6$: 411.2495, found: 411.2497.

Fmoc-Lys(Dde)-OH (22).[54] Pale yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 13.29 (s, 1H), 7.73-7.74 (d, J = 5.00 Hz, 2H), 7.58 (t, J = 5.00 Hz, 2H), 7.37 (t, J = 5.00 Hz, 2H), 7.27 (t, J = 5.00 Hz, 2H), 5.79 (d, J = 10.00 Hz, 1H), 4.42-4.46 (m, 1H), 4.35-4.37 (d, J = 10.00 Hz, 2H), 4.18 (t, J = 5.00 Hz, 1H), 3.38-3.39 (m, 2H), 2.53 (s, 3H), 2.35 (s, 4H), 1.78-1.80 (m, 2H), 1.70-1.72 (m, 2H), 1.47-1.57 (m, 2H), 1.00 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 198.5, 174.7,

174.3, 156.2, 143.8, 143.7, 141.3, 127.7, 127.0, 125.1, 119.9, 107.8, 67.1, 53.4, 52.2, 47.1, 43.4, 31.9, 30.2, 28.3, 28.2, 22.4, 21.1, 18.3, 14.2. HRMS (ESI) ($[M+H]^+$) Calcd. for $C_{31}H_{37}N_2O_6$: 533.2652, found: 533.2661.

Synthesis of Cyclic γ-AAPeptides Hits. After structures of putative hits were determined by MALDI MS/MS, the hits and its FITC labeled analogues were re-synthesized on the Rink Amide resin and confirmed by Applied Biosystems 4700 Proteomics Analyzer (Scheme S2). For the synthesis of the fluorescent cyclic peptide, the Fmoc-Lys(Dde)-OH was first attached to the Rink amide resin. The Fmoc protection group was then removed, followed by the desired building blocks needed for the sequence synthesis. After the γ-AApeptides were cyclized, the Dde group was removed. Then FITC (2 equiv.) and DIPEA (6 equiv.) in DMF were added to the resin and shaken for 12 h at room temperature. The FITC labeled cyclic γ-peptide was cleaved by 1:1 (v/v) DCM/TFA containing 2% triisopropylsilane. The crude was purified by the Waters HPLC system with flow rate of 0.8 mL/min with a linear gradient from 5% to 100% (CH_3CN in water) in 40 min (Figure S5).

Fluorescence Polarization (FP). The binding affinity (K_d) of the hits was obtained by fluorescence polarization (FP). The FP experiment was performed by incubating 50 nM FITC labeled AApeptide with EphA2 (0.0625 to 2 μM) in 1×PBS. The binding affinity of the lead compound to the GST protein (K_d) was obtained by incubating 50 nM FITC labeled AApeptide in GST ranging from 0.3125 to 55 μM. Dissociation constants (K_d) were determined by plotting the fluorescence anisotropy values as a function of protein concentration, and the plots were fitted to the following equation (Figure S7-8).[37] The L_{st} is the concentration of the AApeptide and the x stands for the concentration of the protein. The experiments were conducted in triplicates and repeated for three times.

$$Y = [\text{FPmin} + (FPmin - FPmin)]\frac{(Kd + \text{L}_{st} + \text{x}) - \sqrt{(Kd + L_{st} + x)^2 - 4L_{st}x}}{2L_{st}}$$

in vitro **Kinase assays and immunoblotting.** in vitro kinase assay to evaluate EphA2 kinase activity was performed as described before.[46] Briefly, 50µl kinase reaction containing 50 ng recombinant EphA2, 50 µg Poly(Glu-Tyr) and 200 µM ATP and 2 µM of **1** and **9** (negative control) were incubated at 30 ^{o}C for 40 min. The reaction was stopped by addition of gel loading buffer. The samples were boiled for 5 min and SDS PAGE was performed. Immunoblotting with pTyr-HRP antibody (1:2000 dilution) was performed to detect substrate phosphorylation. The extent of phosphorylation was quantified using ImageJ software. For studying the activity of the compounds in cells, C13 cells were treated at indicated doses for 24 h. The cells were lysed in lysis buffer and immunoblot analysis was performed. Densitometric analysis was performed by SPSS. The experiments were conducted in triplicates and repeated for three times.

Scratch-wound motility assay. C13 cells were trypsinized and 10^5 cells were reseeded on a twelve-well tissue culture plate. After 12 h, the attached cells were scratched with a 200ml pipette tip and cell migration was observed for up to 24h. 0 h images were captured using a Nikon ECLIPSE microscope. The plates were placed back at 37 ºC and 5% CO_2 for 24h, and another set of images were captured of the same wounds. The wound widths were measured by ImageJ (version 1.50). The experiments were conducted in triplicates and repeated for three times.

Matrigel-coated transwell assays. The assays were performed by transfected the C13 cell line with control EphA2 for 48 h. After that the cells were plated into the top chambers and treated with either vehicle or 10µM **1**. 24 hours later cells in top chamber were scraped and migrated cells were fixed with crystal violet staining. Transwell data was determined by the number of the

migrated cells and the value from parental cells was arbitrarily set at 100%. The experiments were conducted in triplicates and repeated for three times.

ASSOCIATED CONTENT

Supporting Information

Procedures for library preparation, library screening, the bioactivity assay, cell permeability test, molecular dynamics simulations; the list of hits, the determination of decoding sequences, the Kd data of other hits and kinases, ^{1}H and ^{13}C NMR spectra and HPLC traces.

Molecular formula strings (CSV).

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Note

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was generously supported by NSF CAREER 1351265 (JC), NIH 1R01GM112652-01A1 (JC), National Natural Science Foundation of China (No. 81520108031，81473478), Shanghai Outstanding Academic Leaders Plan(No.16XD1403600); The Science Foundation for Shanghai Committee of Science Project (14430722900) Shanghai Outstanding Medical Academic Leader; Shanghai Three-Year Action Plan of Traditional Chinese Medicine (No.ZY3-CCCX-3-3012, No.ZY3-CCCX-2-1003). We thank Ning Ma for technical assistance with the parameterization. Computer time was provided by USF Research Computing, sponsored in part by NSF MRI CHE-1531590.

ABBREVIATIONS USED

EphA2, ephrin type-A receptor 2; DMF, dimethylformamide; DCM, dichloromethane; HOBt, hydroxybenzotriazole; DIPEA, N,N-diisopropylethylamine; DIC, N,N′-diisopropylcarbodiimide; TFA, trifluoroacetic acid; FITC, fluorescein isothiocyanate; K_d, dissociation constant; Tris, tris(hydroxymethyl)aminomethane.

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