Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 3929–3936

BIOORGANIC & MEDICINAL CHEMISTRY

Global Optimization of Conformational Constraint on Non-phosphorylated Cyclic Peptide Antagonists of the Grb2-SH2 Domain

Ya-Qiu Long,[a,*] Feng-Di T. Lung[b] and Peter P. Roller[b,*]

[a]*State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China*

[b]*Laboratory of Medicinal Chemistry, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, FCRDC, Building 376, PO Box B, Frederick, MD 21702, USA*

Received 16 April 2003; revised 10 June 2003; accepted 18 June 2003

Abstract—Following our earlier work on a phage library derived non-phosphorylated thioether-cyclized peptide inhibitor of Grb2 SH2 domain, a series of small peptide analogues with various cyclization linkage or various ring size were designed and synthesized and evaluated to investigate the optimal conformational constraint for this novel Grb2-SH2 blocker. Our previous SAR studies have indicated that constrained conformation as well as all amino acids except Leu[2] and Gly[7] in this lead peptide, cyclo(CH_2CO-Glu[1]-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys[10])-amide (termed **G1TE**), was necessary for sustenance of the biological activity. In this study, in an effort to derive potent and bioavailable Grb2-SH2 inhibitor with minimal sequence, we undertook a systematic conformational study on this non-phosphorylated cyclic ligand by optimizing the ring linkage, ring configuration and ring size. The polarity and configuration of the cyclization linkage were implicated important in assuming the active conformation. Changing the flexible thioether linkage in G1TE into the relatively rigid sulfoxide linkage secured a 4-fold increase in potency (**4**, $IC_{50}=6.5$ μM). However, open chain, shortening or expanding the ring size led to a marked loss of inhibitory activity. Significantly, the introduction of ω-amino carboxylic acid linker in place of three C-terminal amino acids in G1TE can remarkably recover the apparently favorable conformation, which is otherwise lost because of the reduced ring size. This modification, combined with favorable substitutions of Gla for Glu[1] and Adi for Glu[4] in the resulting six-residue cyclic peptide, afforded peptide **19**, with an almost equal potency (**19**, $IC_{50}=23.3$ μM) relative to G1TE. Moreover, the lipophilic chain in ω-amino carboxylic acid may confer better cell membrane permeability to **19**. These newly developed G1TE analogues with smaller ring size and less peptide character but equal potency can serve as templates to derive potent and specific non-phosphorylated Grb2-SH2 antagonists.

Introduction

Growth factor receptor-bound protein **2** (Grb2) plays a central role in the protein-tyrosine kinase-dependent signalling.[1,2] Grb2 is an adaptor protein composed of one SH2 domain flanked by two SH3 domains.[3] It binds to a range of tyrosine-phosphorylated targets, such as the epidermal growth factor (EGF) receptor, via its SH2 domain, and to its downstream target, the guanine nucleotide exchange factor, Son of sevenless (Sos), via its SH3 domains.[4] The binding of Grb2 to Sos converts the GDP-bound inactive form of Ras to its GTP-bound active form. Activated Ras triggers the kinase cascade which is critical for both cellular proliferation and differentiation.[5] Actually activated Ras oncogenes have been found in up to 30% of all human tumors analyzed.[6] The involvement of Grb2 in the mitogenically important Ras signal-transduction pathway has rendered this protein an attractive target for the design of inhibitors as anticancer agents. Compounds that prevent the linkage of the Grb2-SH2 domain to phosphorylated receptors are expected to be useful for the treatment and/or prevention of hyperproliferative diseases such as leukemias,[7] breast and ovarian cancers.[8]

Over the past few years, considerable progress has been made in designing antagonists with high affinity for the Grb2-SH2 domain,[9–12] but the cellular activity and selectivity problems still remain unsolved.[13] Our

*Corresponding author. Tel.: +86-21-5080-6876; fax: +86-21-5080-7088; e-mail: yqlong@mail.shcnc.ac.cn (Y.-Q. Long); tel.: +1-301-846-5904; fax: +1-301-846-6033; e-mail: proll@helix.nih.gov (P.P. Roller)

0968-0896/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00411-5

approach to develop bioavailable and specific inhibitors of Grb2-SH2 domain is based on our earlier discovery of a phage library derived peptide. The peptide ligand bound to the Grb2-SH2 protein with 15–25 μM affinity, and was comprised of a 9 a.a. long sequence motif, **E^1-L-Y-E-N-V-G-M-Y^9,** flanked by two terminal disulfide linked cysteines (designated as **G1**).[14,15] This non-phosphotyrosine cyclopeptide specifically binds to the Grb2-SH2 domain, but does not bind to the homologous Src-SH2 domain.[14] Apparently the absence of a phosphate moiety in this phage peptide contributes to its remarkably high specificity. From the phage lead peptide, we developed an equipotent redox-stable thioether cyclized analogue, cyclo(CH_2CO-Glu^1-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys^{10})-amide (termed **G1TE**) which is more physiologically compatible, and effectively inhibits the association of the Grb2-SH2 protein with growth factor receptor, $p185^{erbB2}$ in cell lysates derived from the breast cancer cell line MDA-MB-453.[14]

Significantly, the lack of such a highly charged group as phosphate or its mimics in **G1** and **G1TE** provides a strategy to circumvent the primary drawback of current SH2 domain blockers, namely poor cell penetration. On the other hand, in pTyr or mimics containing agents, the pTyr moiety itself is a major contributor to the binding, which results in less specificity to any given SH2 domain. Without pTyr or its mimics present in **G1TE**, the binding affinity is compensated for by additional well defined binding interactions within the binding pocket of the protein, which might confer a more specific binding conformation. Our initial findings[16–19] have confirmed the multipoint-binding feature inherent in this non-phosphorylated cyclic peptide ligand of Grb2-SH2 domain. And, the molecular modeling and NMR studies have suggested that **G1TE** preferably adopted a β-turn like conformation within the YENV sequence upon binding to Grb2-SH2.[19,20] In this paper, we reported our systematic SAR study on **G1TE** to explore the determining factors in maintaining active conformation and identify the structurally important constituents.

Since **G1TE** and its analogues may form a large circle-like binding surface upon binding to Grb2-SH2 domain, as suggested by molecular modeling and NMR studies, the nature of the cyclization linkage with respect to polarity and configuration would definitely affect the orientation of the cyclic peptide surface. As shown in Figure 1, we incorporated various cyclization linkage into G1TE scaffold, and examined the potency to determine the optimal linkage which favors forming the β-turn like conformation.

The ring configuration and ring size is another important issue in the context of conformational constraint. Changes in the larger peptide structure through cyclization could alter overall binding affinity by modifying the ring conformation and orientations of the side chains of each residue. We designed a series of G1TE analogues with different terminals for ring closure (Fig. 2) to indentify the favorable conformation and active core structure.

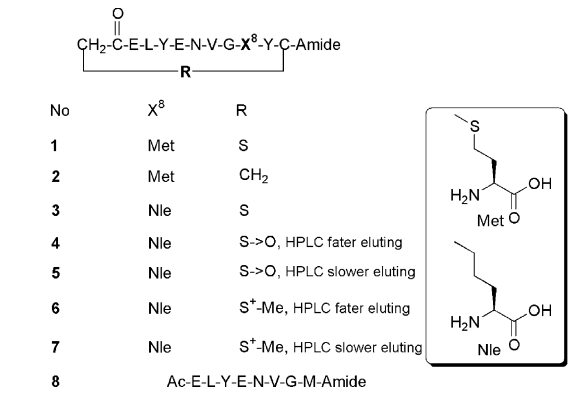


Figure 1. A series of G1TE analogues with variations of the cyclization linkage.

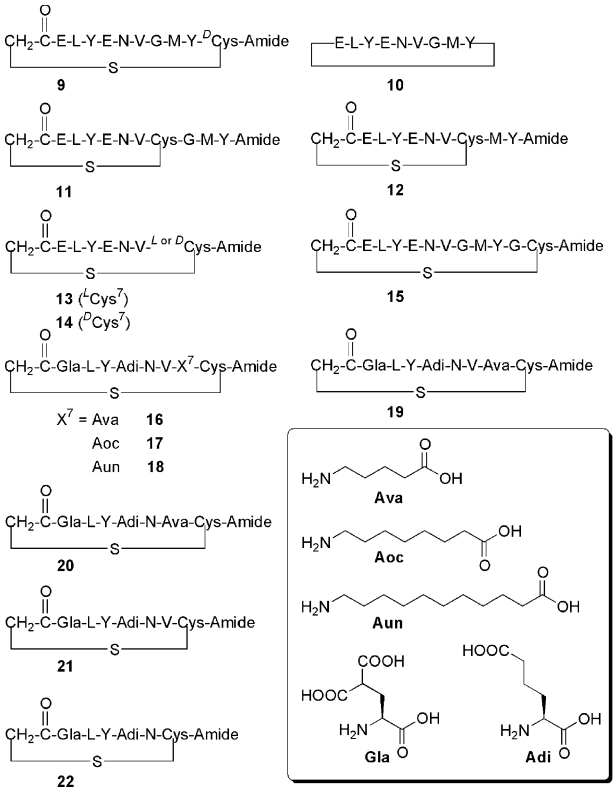


Figure 2. A series of G1TE analogues with alterations in ring size.

By rationally optimizing the nature of the cyclization linkage and the ring size, we obtained an equally potent analogue (peptide **19**) with considerably smaller ring size relative to G1TE. Further 4-fold increase in inhibitory activity is gained by changing the cyclization linkage of thioether to sulfoxide (peptide **4**). Current studies provide a better understanding of how the structure of G1TE confers high binding affinity, and advance the design of less peptidic and highly selective non-phosphorylated Grb2-SH2 antagonists.

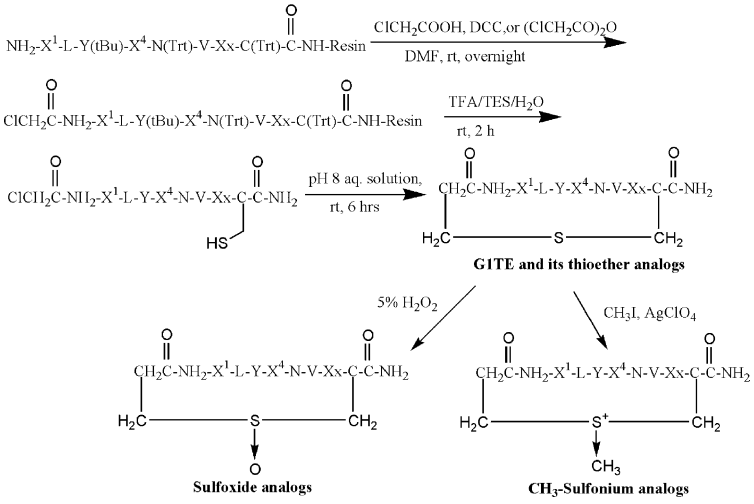
Syntheses

The linear peptides were synthesized by means of solid-phase peptide synthesis in Fmoc chemistry. The Fmoc-PAL resin was employed for establishing the C-terminal

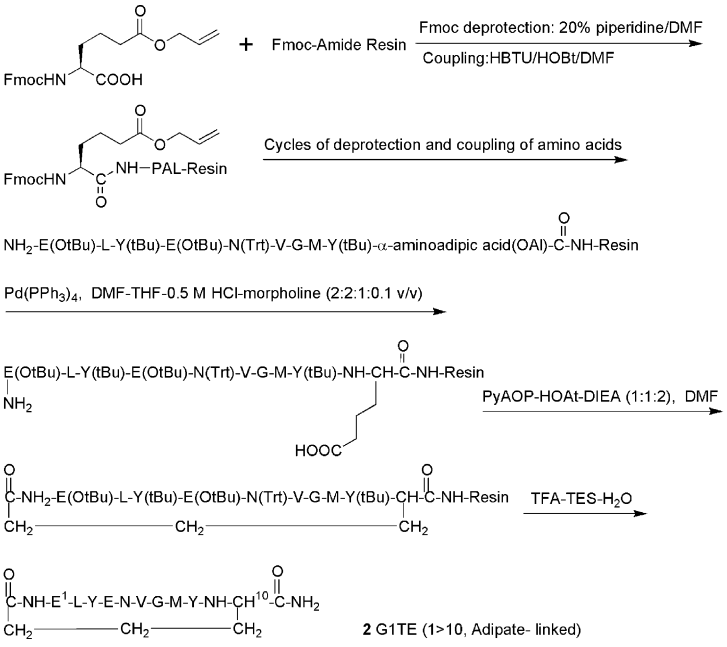
carboxamide for side-chain cyclization, while the acid-labile Rink resin was used for the head-to-tail backbone cyclization.

The syntheses of the thioether, sulfoxide, and methyl-sulfonium bridged cyclic peptides were based on the same protocol as depicted in Scheme 1.[16,17] Sulfoxide linkage can be readily achieved by oxidizing the thioether with 5% hydrogen peroxide. The methyl-sulfonium linkage was synthesized from the thioether counterpart in the presence of methyl iodide and silver perchlorate. Two diastereoisomers were obtained when synthesizing the sulfoxide or methyl–sulfonium analogues, but they were RP-HPLC separable. In order to avoid the simultaneous oxidization of methioline in the backbone while oxidizing the thioether into sulfoxide in the linker, we replaced Met[8] with norleucine (Nle). The hydrophobic Nle has an approximately similar side-chain length as Met. In this manner, we synthesized the Nle[8] containing thioether analogue to specifically determine the effect of the polarity change in the cyclization linker on its binding conformation.

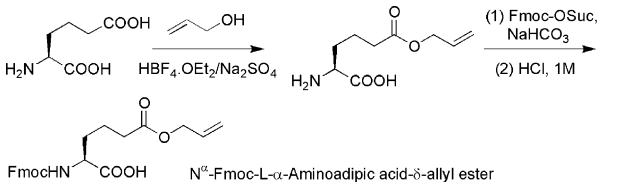
The methylene bridged peptide was synthesized by side-chain cyclization strategy as depicted in Scheme 2. All carboxyl side-chains in the constituent amino acids were protected with TFA-cleavable *tert*-butyl groups, whereas the aminoadipic acid side chain was protected with the $Pd(PPh_3)_4$-cleavable allyl group. The linear protected peptide was assembled on a PAL amide resin with N^{α}-Fmoc-L-α-aminoadipic acid-δ-allyl ester at the C-terminus. The allyl group was selectively removed by $Pd(PPh_3)_4$ and the deprotected side chain of aminoadipic acid was coupled with the free N-terminus group of the resin-bound, side-chain-protected peptide. The cyclization was accomplished using the coupling



Scheme 1. The synthesis of thioether, sulfoxide, and methyl-sulfonium bridged cyclic peptides.



Scheme 2. Synthesis of cyclic peptide G1TE (Adipate linker).



Scheme 3. Synthetic route for N^{α}-Fmoc-L-α-aminoadipic acid-δ-allyl ester.

Table 1. The binding affinity of the non-phosphorylated cyclic peptides to Grb2-SH2 domain; variations in cyclization linkage[a]

Compd	Peptide analogues	IC_{50} (μM)
1	G1TE	25±5
2	G1TE-methylene linkage	35
3	G1TE(Nle^8)	26.5±3.5
4	G1TE(Nle^8)-sulfoxide linkage HPLC faster eluting	6.5±2.5
5	G1TE(Nle^8)-Sulfoxide linkage HPLC slower eluting	70
6	G1TE(Nle^8)-methyl sulfonium linkage HPLC faster eluting	390
7	G1TE(Nle^8)-methyl sulfonium linkage HPLC slower eluting	750
8	Open chain	810±10

[a]The experiments were performed on a BIAcore 2000 instrument by the method described previously.[14] The results represent mean value of at least two independent experiments and are expressed as the concentration at which half-maximal inhibition (IC_{50}) of binding of Grb2-SH2 to biotinylated DDPSpYVNVQ was observed. IC_{50} of reference SHC (pY317) peptide: 1.0±0.2 μM.

reagents of PyAOP-HOAt-DIEA in DMF. The simultaneous removal of the side chain-protecting groups and cleavage from the resin ($TFA/H_2O/HSiEt_3$) afforded the methylene-bridged cyclic peptide **2**.

The building block, N^{α}-Fmoc-L-α-aminoadipic acid-δ-allyl ester was prepared as outlined in Scheme 3. L-α-Aminoadipic acid was selectively allyl esterified with allyl alcohol on the carboxyl group of the side chain, in the presence of 2 equivalent $HBF_4{\cdot}Et_2O$. The resulting L-α-aminoadipic acid-δ-allyl ester was efficiently protected on the N-terminus with Fmoc, which is suitable for solid phase peptide synthesis.

Results and Discussion

The nature of the cyclization linkage is an important determining factor for the binding conformation of the non-phosphorylated cyclic ligand binding to Grb2-SH2

As our earlier results have indicated,[14] for the non-phosphorylated library based peptide, the cyclized constrained structure is necessary for retention of significant Grb2-SH2 binding affinity. Consistently, the open chain analogue of G1TE exhibited negligible binding affinity (**8**, IC_{50} = 810 μM). Along with this, the polarity and configuration of the cyclization linkage were disclosed to exert a subtle influence on the binding conformation. In current study, we replaced the thioether linkage of G1TE with sulfoxide, sulfonium or methylene moieties, and examined the effect on their Grb2-SH2 binding potency. As shown in Table 1, the analogues with polar sulfoxide linkage produced the most active agents, while analogues with less polarity linker resulted in less potency, for example, the methylene linkage analogue showed reduced binding affinity (**2**, IC_{50} = 35 μM). However, increasing the polarity didn't increase the binding affinity proportionally when the configuration of the cyclization linkage is involved. Only one of the diastereoisomers of the sulfoxide analogues (and $Met^8 -> Nle^8$) displayed a 4-fold enhancement in potency (**4**, IC_{50} = 6.5 μM) relative to its thioether counterpart, G1TE(Nle^8) (**3**, IC_{50} = 26.5 μM). The other less polar sulfoxide analogue (**5**, IC_{50} = 70 μM) and both diastereoisomers of sulfonium analogues (**6**, IC_{50} = 390 μM; **7**, IC_{50} = 750 μM) exhibited much less binding affinity. These results indicate that a specific constrained conformation is required for the cyclic ligand binding to Grb2-SH2. The natural or synthetic phosphopeptide ligands that bind Grb2-SH2 domain are required to adopt a β-turn conformation, which has been demonstrated by both NMR and X-ray crystal structure.[21,22] For non-phosphotyrosine ligands, previous molecular modeling[19] suggested that G1 has the capacity to assume a β-turn structure similar to the β-turn of the natural phosphopeptide, and NMR study[20] confirmed that this cluster of conformations are the preferred conformation of G1TE in solution. Recently the incorporation of a turn-inducing amino acid, such as 1-amino-1-cyclohexylic acid (Ach) in the position 4 of G1TE remarkably enhanced the potency.[23] Consequently, we hypothesized that the variations in the cyclization linkage that favors the β-turn-like conformation of the **ELYEN** motif in G1TE will facilitate the Grb2 binding. Binding assay results indicate that sulfoxide linker is such an optimal cyclization motif.

Incorporation of ω-amino carboxylic acid linker in place of three C-terminal amino acids (Gly, Met, Tyr) in G1TE leads to a new family of Grb2-SH2 antagonists with smaller ring size and less peptide character but equal binding affinity and predicted better cell permeability

In early studies,[17–19] we have identified that residues E^1, Y^3, E^4 and N^5 in G1TE play a key role in the Grb2-SH2 binding via a side-chain interaction. Since some residues in G1TE do not participate in any specific interactions with Grb2 SH2, we undertook the optimization on the ring size of G1TE to determine the minimal core structure binding to Grb2 SH2. We synthesized a series of modified analogues of G1TE with variations in ring size and ring configuration, and evaluated their binding affinities with Biacore 2000 SPR methodology.[14]

As shown in Table 2, the inversion of the configuration of the C-terminal cysteine reduces the binding affinity by 10-fold (**9**, IC_{50} = 160 μM), showing the preference for L- over D-stereochemistry of the cyclization in adopting the active conformation. Polyamide backbone cyclization whithin the same peptide sequence also eliminates the binding affinity (**10**, 40% inhibition at 1500 μM), indicating that the rigid cyclization constraint conferred by the backbone cyclization disfavors either the formation of β-turn conformation or the optimal

Table 2. The binding affinity of the non-phosphorylated cyclic peptide on Grb2-SH2 domain. Alterations in the ring configuration and size

Compd	Peptide analogues	IC_{50} (μM)
9	G1TE(^{D}Cys^{10})	160±60
10	G1HT	At 1500 μM, 40% inhibition
11	G1TE(E^{1}->C^{7})-GMY	At 1500 μM, 10% inhibition
12	G1TE(E^{1}->C^{7})-MY	At 1500 μM, 22% inhibition
13	G1TE(E^{1}->C^{7})	At 1000 μM, 14% inhibition
14	G1TE(E^{1}->$^{D}C^{7}$)	At 1000 μM, 23% inhibition
15	G1TEG	81.5±27.5
16	G1TE(E^{1}->C^{8}, Ava^{7})	246.7±113.3
17	G1TE(E^{1}->C^{8}, Aoc^{7})	167.5±37.5
18	G1TE(E^{1}->C^{8}, Aun^{7})	87.5±12.5
19	G1TE(Gla^{1}->C^{8}, Adi^{4}, Ava^{7})	23.3±2.7
20	G1TE(Gla^{1}->C^{7}, Adi^{4}, Ava^{6})	82.5±2.5
21	G1TE(Gla^{1}->Cys^{8}, Adi^{4})	410.0±10.0
22	G1TE(Gla^{1}->Cys^{7}, Adi^{4})	At 1000 μM, 20% inhibition

orientation of the side chains of essential residues. Still keeping the whole amino acid sequence the same as in G1TE, incorporating Cys in the middle of the sequence, the resulting thioether-bridged analogue with shortened ring size exhibits complete loss of the binding affinity (**11**, 10% inhibition at 1500 μM). Not surprisingly, all other analogues with smaller ring size and deleted residues are essentially inactive (**12**, **13**, **14**). However, moderate lengthening of the ring size by one glycine still maintains moderate inhibitory activity (**15**, IC_{50} = 81.5 μM). These results indicate that those amino acid residues in G1TE whose side chains are not involved in the direct interactions with the binding pocket of the protein still have a function to induce the required conformation via their backbone.

Therefore, we introduced an ω-amino carboxylic acid linker to replace the three C-terminal amino acids (Gly, Met, Tyr) in G1TE in order to maintain the specific conformation with fewer residues. We found that 8-aminooctanoic acid (Aoc) can compensate for the loss of bond number, resulting from the deletion of the three residues of Gly, Met and Tyr in the backbone of G1TE. Its incorporation remarkably improves the binding affinity (**17**, IC_{50} = 167.5 μM) compared to its inactive parent analogue **13**. Subsequently, reduction (5-aminovaleric acid, Ava) or extension (11-aminoundecanoic acid, Aun) of the linker by three methylene groups results in corresponding decrease or increase of potency respectively (**16**, IC_{50} = 246.7 μM; **18**, IC_{50} = 87.5 μM) which is partly due to the difference in the conformational constraint. This indicates that the longer, thus more flexible, linker is favored for adapting a required conformation for the binding. In order to develop small non-peptide analogues of G1TE with high-affinity Grb2 binding and good cell permeability, we conducted further modification on the smallest linker (Ava) incorporated analogue **16**. Further efforts in optimization of structure was based on our recent discovery of the significant overlapping roles of Glu^{1} and the $pTyr^{3}$ side chains in peptide **1**.[17] Moreover, extending the side chain of Glu^{4} by one CH_2 group benefited the interactions of G1TE with the binding pocket of Grb2-SH2.[24] Consistently, using similar substitutions of Gla for Glu^{1} and of Adi for Glu^{4} in **16**, we achieved 10 times increase in the binding affinity (**19**, IC_{50} = 23.3 μM). The same substitution was applied to a smaller cyclic peptide analogue with five amino acid residues and turned out a moderate inhibitory activity (**20**, IC_{50} = 82.5 μM). The results indicate that the substitutions might stabilize the preferred binding structure of G1TE or facilitate the direct contacts of essential residues with the binding pocket of Grb2 SH2 protein. Nevertheless, the substitutions of Gla for Glu^{1} and of Adi for Glu^{4} alone cannot effectively make up the loss of potency as the result of the loss of ring size, which was embodied on the seven-residue (**21**, IC_{50} = 410 μM) and 6-residue cyclic analogues (**22**, 20% inhibition at 1000 μM) without ω-amino carboxylic acid linker.

Conclusions

In this study, by optimizing the cyclization linkage, ring configuration and ring size, we demonstrated that a specific constrained conformation is required for this non-phosphorylated cyclic peptide ligand binding to Grb2-SH2 domain. The specific mode of cyclization, ring size and amino acid configuration as well as cyclization are the important determining factors. Variations that favor the β turn-like conformation of the **ELYEN** motif in G1TE will facilitate the binding.

The polarity and configuration of the cyclization linkage were found to modulate the binding affinity by affecting the conformation of the ligand. Only one of the diastereoisomers of the sulfoxide analogues exhibited improved binding affinity (**4**, IC_{50} = 6.5 μM).

The open-chain analogue or shortening or expanding the ring size result in loss of potency. The introduction of lipophilic ω-amino carboxylic acid linker leads to a new category of small non-phosphorylated cyclic ligand of Grb2-SH2. The lipophilic chain of ω-amino carboxylic acid would probably confer a better biocompatibility. Replacement of three C-terminal residues (Gly, Met, Tyr) with 5-aminovaleric acid linker in G1TE reduces the binding affinity, but further substitutions of Gla for Glu^{1} and Adi for Glu^{4} in this six-residue cyclic peptide has a compensatory role and results in similar potency (**19**, IC_{50} = 23.3 μM) relative to G1TE, the latter being a nine-residue ring structure. The conformational optimization on this non-phosphorylated cyclic ligand binding to Grb2-SH2 domain brings us closer to our objective of identifying a Grb2-SH2 antagonist suitable for pharmacological investigations, and suggests a new strategy for designing potent small non-phosphorylated inhibitor of Grb2-SH2 as potential anticancer drugs.

Experimental

Binding affinity measurements using surface plasmon resonance (SPR)

The competitive binding affinity of ligands for the Grb2-SH2 protein was assessed using Biacore SPR methods on a BIAcore 2000 instrument (Pharmacia Biosensor,

Uppsala, Sweden). IC_{50} values were determined by mixing the inhibitor with recombinant GST-Grb2 SH2 protein and measuring the amount of binding at equilibrium to an immobilized SHC(pTyr-317) phosphopeptide, that is, biotin-DDPSpYVNVQ in a manner similar to that reported previously.[14] The biotinylated phosphopeptide was attached to a streptavidin coated SA5 Biosensor chip, and the binding assays were conducted in pH 7.4 PBS buffer containing 0.01% P-20 surfactant (Pharmacia Biosensor).

Peptide synthesis and characterization

Solid phase peptide synthesis. The routine part of the synthesis was carried out with an ABI 433A peptide synthesizer using Fmoc chemistry. The Pal amide resin and Fmoc derivatives of standard amino acids were obtained from Perkin-Elmer/Applied Biosystems Division (Foster City, CA, USA). Side-chain protections are as follows: Glu(*t*-Bu), Tyr(*t*-Bu), Asn(Trt), Cys(Trt). Fmoc-γ-carboxy-L-Glu(O*t*Bu)$_2$-OH [Fmoc-Gla(O*t*Bu)$_2$-OH] and Fmoc-L-α-aminoadipic acid-δ-*t*-butyl ester [Fmoc-Adi(O*t*Bu)-OH] were purchased from BACHEM (Torrance, CA, USA). Fmoc-11-aminoundecanoic acid [Fmoc-Aun-OH], Fmoc-8-aminooctanoic acid [Fmoc-Aoc-OH], and Fmoc-5-aminovaleric acid [Fmoc-Ava-OH] were purchased from Advanced Chemtech (Louisville, KY, USA). Diisopropylcarbodiimide (DIPCDI), 4-dimethylamino-pyridine (DMAP), pepridine, trifluoroacetic acid (TFA), chloroacetic acid and triethylsilane (TES) were purchased from Fluka (Ronkonkoma, NY, USA). *N*-Methylmorpholine (NMM) was purchased from Sigma (St. Louis, MO, USA). 1,3-Dicyclohexylcarbodiimide, allyl alcohol, iodomethane, silver perchlorate and tetrakis(triphenylphosphine) palladium(0) were purchased from Aldrich Chem Co. (Milwaukee, WI, USA). 7-Azabenzotriazol-1-yloxytris(pyrrolidino)-phosphonium-hexafluoro-phosphate (PyAOP) was purchased from PerSeptive Biosystems Inc. (Hamburg, Germany). HBTU/HOBt/DIEA activation of N^{α}-protected amino acids was employed for coupling and 20% piperidine/DMF was used for Fmoc deprotection. HATU/HOAt/DIEA in DMF was used for backbone cyclization. PyAOP/HOAt/DIEA in DMF was used for G1TE(Adipate) cyclization. TFA/TES/H_2O (9.5:0.25:0.25) was used for the resin cleavage and side-chain deblocking. The crude peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography (RP-HPLC). HPLC conditions: Vydac C18 column (10×250 mm) or Vydac C4 column (20×250 mm); solvent gradient, A, 0.05% TFA in water; B, 0.05% TFA in 90% acetonitrile in water with gradient indicated below; flow rate, 2.5 mL/min for C18 column, 10 mL/min for C4 column; UV detector, 225 nm. FAB-MS (unit resolution, glycerol matrix) was performed on a VG Analytical 7070E-HF mass spectrometer. The purity of products was characterized by analytical HPLC. Amino acid analysis (6 N HCl, 110 °C, 24 h) was carried out at the Protein and carbohydrate Structure Facility (University of Michigan, Ann Arbor, MI, USA).

Synthesis of L-α-Aminoadipic acid-δ-allyl ester. Under N_2, 20 mL of allyl alcohol (294 mmol) and 2.2 mL of tetrafluoroboric acid–diethyl ether complex (85%, 24.8 mmol) were injected into the mixture of L-α-aminoadipic acid (2 g, 12.4 mmol) and Na_2SO_4 (3.0 g) in a 100-mL round-bottom flask. The reaction mixture was stirred at rt overnight, then added 80 mL of anhydrous tetrahydronfuran, and filtered through a pad of activated carbon. The clear solution was treated with triethylamine (36 mL, 26 mmol) and concentrated by rotavapor. The residue was treated with 100 mL of ethyl acetate, and the white precipitate was isolated by suction via a fine filter and washed with AcOEt, dried in vacuum. 2.39 g white solid was obtained in yield of 95.6%. ^{1}H NMR (DMSO-d_6, 250 MHz): δ ppm 8.04–7.10 (br, 2H), 6.06–5.69 (m, 1H), 5.44–5.05 (dt, 2H), 4.76–4.38 (dt, 2H), 3.18–2.99 (t, 1H), 2.86–2.66 (t, 2H), 1.85–1.44 (m, 4H).

Synthesis of N^{α}-Fmoc-L-α-aminoadipic acid-δ-allyl ester. 2.38 g of L-α-aminoadipic acid-δ-allyl ester (11.8 mmol), 4.0 g of 9-fluorenylmethyl succinimidylcarbonate (11.8 mmol) and 3.0 g of sodium bicarbonate (35.7 mmol) were mixed in 100 mL of H_2O/dioxane (1:1), stirred at rt overnight. Then the mixture was poured into 100 mL of ice cold 1 N HCl aq solution, and extracted with AcOEt (40 mL×3). The combined organic phases were dried over anhydrous Na_2SO_4, then evaporated to remove the solvent. The crude product was purified by recrystallization with 10 mL of $CHCl_3$ and 50 mL of hexanes. 4.55 g of white solid was obtained in yield of 91%. ^{1}H NMR (DMSO-d_6, 250 MHz): δ ppm 12.70 (br, 1H), 7.89 (d, 2H, J=8.3 Hz), 7.73 (d, 2H, J=7.8 Hz), 7.69 (d, 1H, J=9.3 Hz), 7.42 (dt, 2H, J=7.6 Hz, J=1.0 Hz), 7.32 (dt, 2H, J=7.3 Hz, J=1.0 Hz), 5.90 (m, 1H), 5.24 (m, 2H), 4.54 (dt, 2H, J=5.4 Hz, J=1.5 Hz), 4.27 (m, 3H), 3.93 (m, 1H), 2.35 (t, 2H, J=6.7 Hz), 1.59 (m, 4H). FAB-MS: m/z 423.7 (MH^+) (calcd 424.17).

Synthesis of cyclo(ELYENVGM-α-Aminoadipic acid) [G1TE(1>10, Adipate-linked), 2]. After the linear peptide E(O*t*Bu)LY(*t*Bu)E(O*t*Bu)N(Trt)VGM-Y(*t*Bu)-Adi(OAl)-NHResin was assembled by standard solid phase methods (using the ABI 433A peptide synthesizer and FastMoc chemistry). Then 0.292 g of the peptide-resin (0.083 mmol) was suspended on 10 mL of DMF/THF/0.5 M HCl/morpholine (2:2:1:0.1 v/v) and treated with $Pd(PPh_3)_4$ (0.593 g, 0.513 mmol) for 2.5 h under N_2. The resin then washed with THF (3×2 min), DMF (3×2 min), DCM (3×2 min), DIEA/DCM (1:19) (3×2 min), DCM (3×2 min), sodium diethyldithiocarbamate/DMF (5 g/L; 3×15 min), DMF (5×2 min), and DCM (3×2 min). The head-to-tail side-chain cyclization of the resin bound peptide was performed with PyAOP (458 mg, 0.88 mmol), HOAt (120 mg, 0.88 mmol), and DIEA (306 mL, 1.76 mmol) in dry DMF at rt overnight. After resin cleavage and side chain deprotection with TFA/TES/H_2O (9.5:0.25:0.25, v/v), the crude peptide was purified by RF-HPLC, R_t=13.5 min (gradient 20–70% B over 30 min on C18 column). FAB-MS $(M+H)^+$ 1241.2 (calcd 1241.5).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Nle-Tyr-Cys)-amide [**G1TE(Nle^8)**, **3**]: Using the

ABI 433A peptide synthesizer and fluorenylmethoxycarbonyl (Fmoc) protection strategy, the linear side-chain protected peptide ELYENVG-Nle-YC was synthesized on the PAL amide resin (0.189 g, 0.1 mmol, 0.53 mmol/g) coupled with the respective amino acids, and the N^{α}-Fmoc group in the N-terminal of the resin-bound protected peptide was removed with 20% piperidine/DMF (12 min). Then the resin-bound protected peptide was N-terminally chloroacetylated by $(ClCH_2CO)_2O$ which was prepared by mixing 0.5 M $ClCH_2COOH$/DCM (48 mg, 0.5 mmol, 1.0 mL) and 0.5 M DCC/DCM (52 mg, 0.25 mmol, 0.5 mL) for 0.5 h at rt. The precipitated DCU was filtered off and the filtrate was added into the peptide-resin which was swollen in 1 mL of DMF. The resulting reaction mixture was shaken at rt for 6 h, Ninhydrin Test was negative. The peptide was cleaved from the resin by using TFA containing 2.5% each (v/v) of triethylsilane (TES) and deionized water (2 h). For isolation of the product, two-thirds of the cleavage reagent mixture was evaporated under N_2 and the mixture triturated with ice-cold ether. The precipitated crude peptide was dissolved in 50 mL of water and added dropwise into 100 mL of H_2O solution, which was adjusted to pH 8 ~ 9 with triethylamine, repeatedly. Under the basic conditions the *N*-chloroacetylated linear peptide cyclized spontaneously by intramolecular nucleophilic displacement of the chloro group by cysteine thiol. the cyclization process was monitored by HPLC. After 6–7 h at rt, the solution was acidified with 30% AcOH aq solution and lyophilized. The product was purified by RP-HPLC (gradient 20–65% B over 25 min on C18 column, $R_t = 12.3$ min) to provide G1TE (Nle^8) in overall yield of 50%. FAB-MS $(M+H)^+$ 1242.1 (calcd 1242.5).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Nle-Tyr-Cys)-amide sulfoxide [G1TE(Nle^8, sulfoxide) 4 and 5]. Cyclic thioether G1TE (Nle^8) was oxidized by 5% H_2O_2 aq solution (1 mg/mL), stirring at rt for 6 h. The oxidization process was monitored by HPLC. After lyophilizing, the crude product was purified by RP-HPLC, two diastereoisomers were obtained with R_t of 13.0 and 14.2 min, respectively (gradient 25–40% B over 30 min on C18 column). The faster eluting fraction: FAB-MS $(M+H)^+$ 1258.1 (calcd 1257.5); Asp 0.90(1), Val 1.03(1), Leu 1.09(1), Glu 1.79(2), Gly 0.93(1), Tyr 1.12(2)*, Nle 1.04(1). The slower eluting fraction: FAB-MS $(M+H)^+$ 1258.1 (calcd 1257.5); amino acid analysis: Asp 0.79(1), Val 1.03(1), Leu 1.12(1), Glu 1.64(2), Gly 1.01(1), Tyr 0.85(2)*, Nle 1.08(1).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Nle-Tyr-Cys)-amide methyl-sulfonium [G1TE(Nle^8, Me-sulfonium), 6 and 7]. 10 mg cyclic thioether G1TE (Nle^8) was dissolved in 0.5 mL of a 50:50 mixture of formic acid and acetic acid containing 50 μL of iodomethane. An 83 mg portion of silver perchlorate dissolved in 0.83 mL of 50:50 formic acid/acetic acid (10% W/V) was then added. The reaction mixture was stirred at rt in the dark for 24 h. the precipitated AgI was removed by centrifugation. The clear solution was diluted in water and lyophilized. The crude product was purified by RP-HPLC. Two diastereoisomers were obtained, with R_t of 12.1 and 13.1 min, respectively (HPLC gradient 25–35% B over 40 min on C18 column). FAB-MS: (M^+) the faster eluting fraction, peptide **6**, 1255.6; the slower eluting fraction, peptide **7**, 1255.6 (calcd 1255.6).

Synthesis of open-chain CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Nle-Tyr-amide [Ac-G1TE(no Cys^{10})-9mer, 8]. Using the ABI 433A peptide synthesizer and fluorenylmethoxycarbonyl (Fmoc) protection strategy, the PAL amide resin (0.189 g, 0.1 mmol, 0.53 mmol/g) was coupled with the respective amino acids, and the N^{α}-Fmoc group in the N-terminal of the resin-bound protected peptide was removed with 20% piperidine/DMF (12 min). The resin-bound side-chain protected peptide ELYENVGMY-NHResin was N-terminally acetylated with 1-acetylimidazole (6 equiv in DMF). The mixture was shaken at rt overnight, and Ninhydrin test was negative. The peptide was cleaved from the resin using 95%TFA containing 2.5% each (v/v) of TES and water (1.5 h, rt). The crude peptide was purified by RP-HPLC, $R_t = 16.0$ min (gradient 10–70% B over 25 min on C18 column). FAB-MS $(M+H)^+$ 1158.3 (calcd 1158.5). Amino acid analysis: Asp 1.30(1), Val 1.26(1), Leu 1.18(1), Glu 2.10(2), Gly 1.17(1), Tyr 1.41(2), Met 0.60(1).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Nle-Tyr-$\underline{D}$Cys)-amide [G1TE($\underline{D}Cys^{10}$), 9]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 12.8$ min (gradient 20–80% B over 30 min on C18 column). FAB-MS $(M+H)^+$ 1260.2 (calcd 1259.5).

Synthesis of cyclo(Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr) [G1HT, 10]. The head-to-tail backbone cyclized peptide **10** was synthesized in a manner described previously.[16] RP-HPLC, $R_t = 11.1$ min (gradient 20–70% B over 20 min on C18 column). FAB-MS $(M+H)^+$ 1099.4 (calcd 1099.3). Amino acid analysis: Asp 0.94(1), Glu 1.99(2), Gly 1.00(1), Leu 1.00(1), Met 0.5(1), Tyr 1.70(2), Val 1.05(1).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Cys)-Gly-Met-Tyr-amide [G1TE(E^1->C^7)-GMY, 11]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 14.2$ min (gradient 20–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 1260.3 (calcd 1259.5).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Cys)-Met-Tyr-amide [G1TE(E^1->C^7)-GM, 12]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 14.3$ min (gradient 20–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 1203.0 (calcd 1202.5).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Cys)-amide [G1TE(E^1->C^7), 13]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 14.8$ min (gradient 10–90% B over 30 min on C18 column). FAB-MS $(M+H)^+$ 908.4 (calcd 908.4). Amino acid analysis: Asp 1.02(1), Val 0.98(1), Leu 1.00(1), Glu 2.05(2), Tyr 0.97(1).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-^{D}Cys)-amide [G1TE(E^1->$^{D}C^7$), 14]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 13.6$ min (gradient 10–70% B over 24 min on C18 column). FAB-MS $(M+H)^+$ 907.8 (calcd 908.4). Amino acid analysis: Asp 1.02(1), Val 0.98(1), Leu 1.00(1), Glu 2.02(2), Tyr 0.98(1).

Synthesis of cyclo(CH_2CO-Gly-Glu-Leu-Tyr-Glu-Asn-Val-Cys)-amide [G1TEG, 15]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 15.0$ min (gradient 10–70% B over 24 min on C18 column). FAB-MS $(M+H)^+$ 1316.2 (calcd 1316.5). Amino acid analysis: Asp 1.03(1), Val 1.01(1), Leu 1.01(1), Glu 2.06(2), Gly 1.97(2), Tyr 1.94(2), Met 0.98(1).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Ava-Cys)-amide [G1TE(Ava^7), 16]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 11.4$ min (gradient 20–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 1007.5 (calcd 1007.4). Amino acid analysis: Asp 1.07(1), Val 0.77(1), Leu 1.14(1), Glu 2.01(2), Tyr 0.92(1).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Aoc-Cys)-amide [G1TE(Aoc^7), 17]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, Rt = 16.8 min (gradient 10–80% B over 28 min on C4 column). FAB-MS $(M+H)^+$ 1049.8 (calcd 1049.5).

Synthesis of cyclo(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Aun-Cys)-amide [G1TE(Aun^7), 18]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 18.3$ min (gradient 20–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 1091.7 (calcd 1091.5). Amino acid analysis: Asp 1.08(1), Val 0.76(1), Leu 1.13(1), Glu 2.16(2), Tyr 0.87(1).

Synthesis of cyclo(CH_2CO-Gla-Leu-Tyr-Adi-Asn-Val-Ava-Cys)-amide [G1TE(Gla^1, Adi^4, Ava^7), 19]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 16.4$ min (gradient 10–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 1065.8 (calcd 1065.5). Amino acid analysis: Asp 1.07(1), Adi 1.08(1), Val 0.75(1), Leu 1.11(1), Glu 1.03(1), Tyr 0.94(1).

Synthesis of cyclo(CH_2CO-Gla-Leu-Tyr-Adi-Asn-Ava-Cys)-amide [G1TE(Gla^1, Adi^4, Ava^6), 20]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 14.4$ min (gradient 10–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 966.5 (calcd 966.4).

Synthesis of cyclo(CH_2CO-Gla-Leu-Tyr-Adi-Asn-Val-Gly-Cys)-amide [G1TE(Gla^1, Adi^4, Gly^7), 21]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 15.5$ min (gradient 10–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 1023.6 (calcd 1023.4).

Synthesis of cyclo(CH_2CO-Gla-Leu-Tyr-Adi-Asn-Val-Cys)-amide [G1TE(Gla^1, Adi^4), 22]. The peptide was synthesized analogously to peptide **3**. RP-HPLC, $R_t = 15.3$ min (gradient 10–70% B over 27 min on C4 column). FAB-MS $(M+H)^+$ 966.2 (calcd 966.4).

Acknowledgements

The financial supports from Chinese Academy of Sciences (Hundred Talent Program) and Ministry of Personnel of China are greatly appreciated.

References and Notes

1. Lowenstein, E. J.; Daly, R. J.; Batzer, A. G.; Li, W.; Margolis, B.; Lammers, R.; Ullrich, A.; Skolnik, E. Y.; Bar-Sagi, D.; Schlessinger, J. *Cell* **1992**, *70*, 431.
2. Fry, M. J.; Panayotou, G.; Booker, G. W.; Waterfield, M. D. *Protein Sci.* **1993**, *2*, 1785.
3. Downward, J. *FEBS Lett.* **1994**, *338*, 113.
4. Buday, L.; Downward, J. *Cell* **1993**, *73*, 611.
5. Lewis, T. S.; Shapiro, P. S.; Ahn, N. G. *Adv. Cancer Res.* **1998**, 7449.
6. Bos, J. L. *Cancer Res.* **1989**, *49*, 4682.
7. Pendergast, A. M.; Quilliam, L. A.; Cripe, L. D.; Bassing, C. H.; Dai, Z.; Li, N.; Batzer, A.; rabun, K. M.; Der, C. J.; Schlessinger, J. *Cell* **1993**, *75*, 175.
8. Janes, P. W.; Daly, R. J.; de Fazio, A.; Sutherland, R. L. *Oncogene* **1994**, *9*, 3601.
9. Cody, W. L.; Lin, Z.; Panek, R. L.; Rose, D. W.; Rubin, J.R. *Curr. Pharm. Des.* **2000**, *6*, 59 and references therein.
10. Sawyer, T. K. *Biopolymers (Peptide Sci.)* **1998**, *47*, 243.
11. Ettmayer, P.; France, D.; Gounarides, J.; Jarosinski, M.; Martin, M.-S.; Rondeau, J.-M.; Sabio, M.; Topiol, S.; Weidmann, B.; Zurini, M.; Bair, K. W. *J. Med. Chem.* **1999**, *42*, 971.
12. Liu, W.-Q.; Vidal, M.; Gresh, N.; Roques, B. P.; Garbay, C. *J. Med. Chem.* **1999**, *42*, 3737.
13. Shakespeare, W. C. *Curr. Opin. Chem. Biol.* **2001**, *5*, 409.
14. Oligino, L.; Lung, F.-D. T.; Sastry, L.; Bigelow, J.; Cao, T.; Curran, M.; Burke, T. R., Jr; Wang, S.; Krag, D.; Roller, P. P.; King, C.R *J. Biol. Chem.* **1997**, *272*, 29046.
15. Amino acid residues in the peptides are numbered from N-terminal to C-terminal residues, excluding the cysteines.
16. Lung, F.-D. T.; King, C. R.; Roller, P. P. *Lett. Peptide Sci.* **1999**, *6*, 45.
17. Long, Y.-Q.; Voigt, J. H.; Lung, F.-D. T.; King, C. R.; Roller, P. P. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2267.
18. Long, Y.-Q.; Yao, Z.-J.; Voigt, J. H.; Lung, F.-D. T.; Luo, J. H.; Burke, T. R., Jr.; King, C. R.; Yang, D.-J.; Roller, P.P *Biochem. Biophys. Res. Commun.* **1999**, *264*, 902.
19. Lung, F.-D. T.; Long, Y.-Q.; King, R. C.; Varady, J.; Wu, X.-W.; Wang, S.; Roller, P. P. *J. Pep. Res.* **2001**, *57*, 447.
20. Lou, Y.-C.; Lung, F.-D. T.; Pai, M.-T.; Tzeng, S.-R.; Wei, S.-Y.; Roller, P. P.; Cheng, J.-W. *Arch. Biochem. Biophys.* **1999**, *372*, 309.
21. Rahuel, J.; Gay, B.; Erdmann, D.; Strauss, A.; Garcia-Echeverria, C.; Furet, P.; Caravatti, G.; Fretz, H.; Schoepfer, J.; Grutter, M. J. *Nat. Struct. Biol.* **1996**, *3*, 586.
22. Ogura, K.; Tsuchiya, S.; Terasawa, H.; Yuzawa, S.; Hatanaka, H.; Mandiyan, V.; Schlessinger, J.; Inagaki, F. *J. Mol. Biol.* **1999**, *289*, 439.
23. Li, P.; Peach, M. L.; Zhang, M.; Liu, H.; Yang, D.; Nicklaus, M.; Roller, P. P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 895.
24. Roller, P.P.; Long, Y.-Q.; Lung, F.-D T.; Voigt, J.H.; King, C.R. In *Peptides 1998, Proceedings of the 25th European Peptide Symposium*; Bajusz, S., Hudecz, F., Eds.; Akademiai Kiado: Budapest, Hungary, 1999; p 706.