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Toward an HDAC6 inhibitor: synthesis and conformational analysis of cyclic hexapeptide hydroxamic acid designed from α-tubulin sequence

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Abstract—A cyclic hexapeptide hydroxamic acid inhibitor for HDAC6 has been designed and synthesized on the basis of the facts that α-tubulin is the substrate of HDAC6 and of the excellent inhibitory activity of cyclic tetrapeptide hydroxamic acids (CHAPs) for HDACs. Unexpectedly, cyclic hexapeptide hydroxamic acid showed very low HDAC inhibitory activity. To explain the low activity, we have carried out conformation analysis and compared it to the crystal structure of α-tubulin. The conformation around the acetylated lysine of the cyclic hexapeptide substrate or the aminosuberate hydroxamic acid [Asu(NHOH)] of cyclic hexapeptide inhibitor is different from that around α-tubulin's lysine-40. The difference in the conformation seems to cause some steric hindrance at the capping site resulting in poor binding capacity.

1. Introduction

The reversible acetylation of histone has a critical role in transcriptional regulation and the reversible acetylation of non-histone substrates is also important for other cellular processes.[1–3] Acetylation and deacetylation of histones are catalyzed by specific enzyme families, histone acetyl transferases (HATs) and deacetylases (HDACs) respectively. HDACs are integral nuclear isozymes that modulate the deacetylation of specific acetylated lysine residues. An increasing number of HDACs are being identified in different species, which is classified into three distinct classes.[4,5] Class I HDACs are related to yeast Rpd3 and include HDAC1, HDAC2, HDAC3 and HDAC8. Class II HDACs are related to yeast Hda1 and include HDAC4, HDAC5, HDAC6 and HDAC7. Class III HDACs are NAD dependant and related to yeast silencing protein Sir2. A number of HDAC inhibitors have been identified which include natural product trichostatin A (TSA),[6] naturally occurring cyclic tetrapeptides trapoxin (TPX),[7] Cyl-1,[8] apicidin,[9–11] FK228,[12–14] synthetic compounds such as butyrate,[15] valproate,[16] suberoyl anilide hydroxamic acid,[17] analogues of TSA[18–20] and benzamide derivatives of MS-275.[21,22] We have reported synthetic cyclic tetrapeptide inhibitors (CHAPs) having inhibitory activity in nanomolar range.[23,24]

Inhibitors of HDACs possess a Zn binding functionality and a cap substructure that interacts with the rim of *N*-acetyl lysine binding channel.[25] Of the reported HDAC inhibitors, TSA can inhibit HDAC1 and HDAC6 in almost equal intensity. Synthetic inhibitors with cyclic tetrapeptide cap and hydroxamic acid functional group (CHAP) and the natural compound TPX are less potent toward HDAC6 in comparison with HDAC1. This difference in the inhibitory activity may be attributed to the variation in the cap group, which is due to the different extent of interaction with the rim of active site of HDACs. Unlike other HDACs, HDAC6 have two catalytic domains and both the catalytic domains contribute independently to the overall activity.[26] Further, HDAC6 has many deviations from the other HDACs in the rim region of the catalytic domain. Recent reports showed that HDAC6 is a tubulin deacetylase.[27,28] This reversible acetylation of α-tubulin is associated with

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microtubule stability. α-tubulin is modified by the acetylation of a lysine residue at position 40.[29,30] Schreiber et al. synthesized a hydroxamic acid inhibitor, which has some selectivity for HDAC6 over HDAC1 using the combinatorial approach.[31,32] We attempted to design a new inhibitor for HDAC6 based on the fact that α-tubulin is the target of HDAC6. The proposed inhibitor (Fig. 1) has a hydroxamic acid functional group, and the same chain length as in CHAP1. The cap group is cyclic hexapeptide derived from α-tubulin's amino acid residues of position 38 to 43, the lys-40 being replaced by aminosuberic hydroxamic acid (Asu(NHOH)). Here we describe the synthesis, activity and conformation studies on the cyclic hexapeptide.

2. Results and discussion

2.1. Chemistry

From the crystal structure data of α-tubulin, it was found that the β-turn around the residues from 38 to 42 is type II′ β-turn.[30] To verify the conformation of the active sequence of cyclic hexapeptide to α-tubulin sequence, we initially synthesized a substrate, *cyclo*(-Ser-Asp-Lys(Ac)-Thr-Ile-Gly-) (**3**). This compound was prepared using solid phase peptide synthesis method with Kaiser's oxime resin by Boc strategy with benzyl protected side chains. The linear peptide was cyclized in DMF using HATU and the side chain protections were removed by catalytic hydrogenation. The cyclic hexapeptide **3** was purified by HPLC and characterized by NMR and HR-FABMS.

Next we synthesized cyclic hexapeptide inhibitor *cyclo*-(-Ser-Asp-Asu(NHOH)-Thr-Ile-Gly-) (**4**). A protected linear hexapeptide was synthesized using Barlos resin preloaded with Fmoc-glycine on a synthesizer by Fmoc strategy. Fmoc amino acids used were, Ile, Thr(^{t}Bu), Asu(OBzl), Asp(O^{t}Bu) and Ser(^{t}Bu), where Asu is aminosuberic acid. The linear hexapeptide was cleaved from resin using acetic acid and cyclized in DMF using HATU as coupling agent and purified by column chromatography to yield the cyclic hexapeptide. The solubility problem of the linear hexapeptide was overcome by using one mM solution in DMF. The side chain carboxyl group of Asu was deprotected by catalytic hydrogenation and then coupled with hydroxylamine. Other side chain protections of cyclic peptide were removed by treatment with TFA and the compound **4** was purified by gel filtration (Sephadex LH-20, DMF). The cyclic hexapeptide was further purified by HPLC and characterized by NMR and HR-FABMS.

The HDAC activity assay of compound **4** was carried out and the activity is in millimolar order (Table 1). The

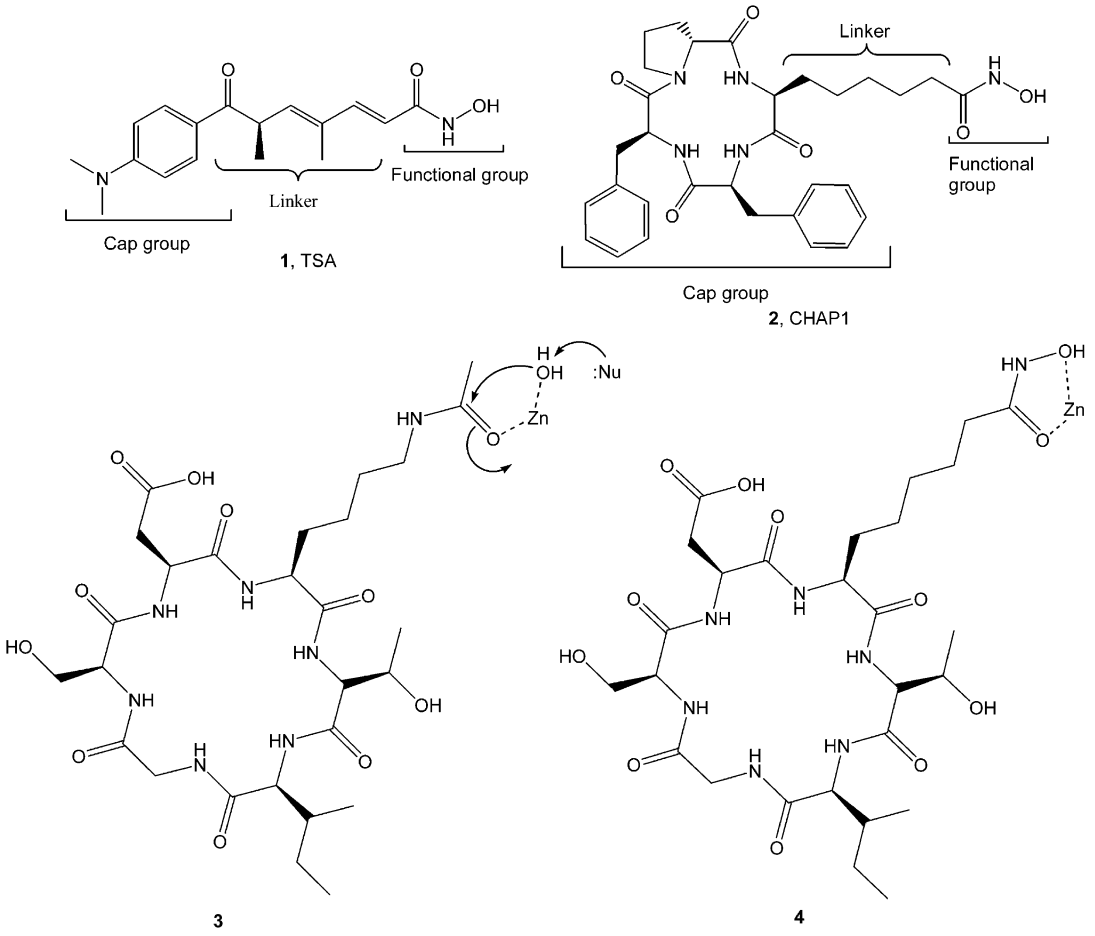

Figure 1. Reported and proposed HDAC inhibitors and substrate.

Table 1. HDAC inhibitory activity

Compd	HDAC1[a] (nM)	HDAC6 (nM)
TSA	22	28
CHAP1	20	190
4	>100,000	>100,000

[a] HDACs prepared from 293T cells.

Table 2. NMR data for compounds **3** and **4**

Residue	δ (ppm at RT)		$J_{NH-C^{\alpha}H}$ (Hz)[a]		$\Delta\delta/\Delta T$ ($\times 10^{-3}$)	
	3	**4**	**3**	**4**	**3**	**4**
Ser	8.28	8.27	7.94	7.93	−3.86	−3.80
Asp	8.22	8.21	7.93	7.93	−2.36	−2.70
Lys/Asu	7.97	7.96	7.93	7.93	−3.25	−3.80
Thr	7.38	7.37	7.32	7.32	−1.14	−1.01
Ile	7.59	7.60	—	—	−0.232	−0.463
Gly	8.87	8.87	—	—	−5.45	−5.96

[a] IleNH and GlyNH protons were observed as singlets at 300 K.

unexpected low activity of the inhibitor **4** forced us to investigate the reason and therefore we carried out molecular modeling studies of the substrate **3** and inhibitor **4** and compared it with the X-ray crystal structure of α-tubulin.[30] HDAC6 have a unique structure with the internally duplicated catalytic domains. However, it is still unclear that both the domains are involved in the α-tubulin deacetylation. Therefore we speculate that the comparison of conformation of inhibitor **4** with that of α-tubulin give a better explanation for the resistance of **4**.

Conformation analyses of the cyclic hexapeptide substrate **3** and inhibitor **4** were studied using ^{1}H NMR in DMSO-d_6. Complete assignments of the chemical shifts were made using COSY, HOHAHA and NOESY spectra. Table 2 shows the chemical shift values for NH Protons and the temperature coefficients of compound **3** and **4**. $J_{NH-C^{\alpha}H}$ coupling constants were used to calculate the ϕ angles. The NMR data of compounds **3** and **4** are very similar and suggest that the backbone conformations of these two compounds are not considerably different. Although the NOE patterns of **3** and **4** are different, the H-bonding between the residues in both compounds are found as the same. For compound **3**, medium NOEs are observed between ThrNH and LysNH, LysNH and AspNH, and Ser NH and GlyNH. Low temperature coefficients of the IleNH protons and ThrNH protons suggest that the compound **3** adopts a type I β-turn. For compound **4**, the NOEs are between ThrNH and AspNH, ThrNH and IleNH and ThrNH and SerNH. Low temperature coefficient values of IleNH protons and ThrNH protons suggest that the compound **4** also adopts a type I β-turn.

Table 3. Structural comparison of α-tubulin sequence with compounds **3** and **4**

Residue	Natural sequence in α-tubulin		**3**		**4**	
	ϕ	ψ	ϕ	ψ	ϕ	ψ
Ser	−85.9	−172.9	−90.9	−45.3	−90.6	−72.5
Asp	148.5	163.0	−89.0	−138	−90.5	−123
Lys/Asu	58.3	−133.4	−89.4	36.5	−89.8	53.2
Thr	−73.0	−9.0	−90.6	−49.8	−91.1	−47.2
Ile	88.1	−4.8	−91.0	−49.2	−89.2	−66.7
Gly	−73.0	−45.0	164	−27	172	−30

The structures of cyclic hexapeptides **3** and **4** having minimum energy configuration were generated using CHARMM program of Insight II. The values of ϕ from NMR results were applied to calculate the ψ values of **3** and **4**. These values and the values for α-tubulin around Lys-40 are given in Table 3. The dihedral angle values of compounds **3** and **4** confirmed that these compounds have type I β-turn. Molecular modeling studies showed that the conformation around the Lys residue of α-tubulin is different from that of the synthesized cyclic hexapeptides **3** and **4** as shown in Figure 2. The carbonyl of Lys in α-tubulin and that in **3** or carbonyl of Asu in **4** is lying in the opposite plane. This might be the reason for the low inhibitory activity of **4**, eventhough, it contains a hydroxamic acid functional group and the optimum spacer length of five methylene units.

In conclusion, we synthesized a cyclic hexapeptide inhibitor for HDAC6 based on the fact that HDAC6 is tubulin deacetylase. Biological assay showed that the cyclic hexapeptide hydroxamic acid **4** is a very weak

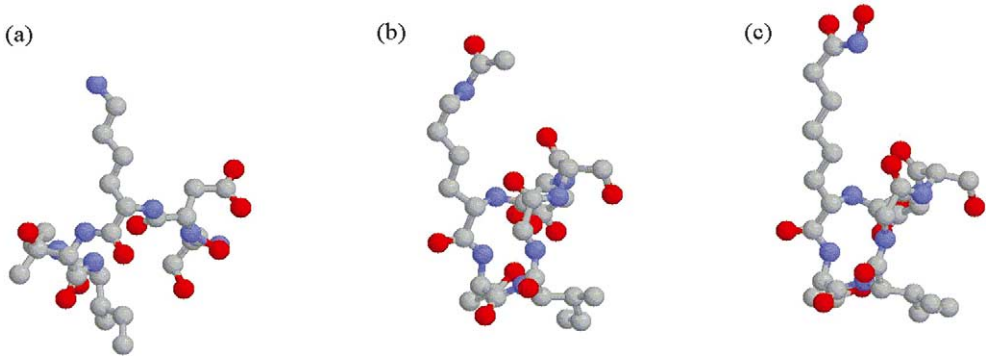

Figure 2. (a) X-ray structure of α-tubulin fragment (-Ser- Asp -Lys^{40}- Thr- Ile-Gly-); (b) Calculated conformation of compound **3**. (c) Calculated conformation of compound **4**.

inhibitor for HDAC. Molecular modeling studies revealed that the configuration around the Lys-40 of α-tubulin and the cyclic hexapeptide **4** were different and therefore the interaction of the cyclic hexapeptide scaffold to the rim of the binding pocket of the enzyme is limited. Further modification according to this result can generate specific inhibitor for HDAC6.

3. Experimental

3.1. General methods

All compounds were routinely checked by thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC). Analytical HPLC were performed on a Hitachi instrument equipped with a chromolith performance RP-18e column (4.6×100 mm, Merck). The mobile phases used were A: H_2O with 10% CH_3CN and 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A to B over 15 min with detection at 220 nm with a flow rate of 2 mL/min. FAB-Mass spectra and high-resolution mass spectra (HR MS) were measured on a JEOL JMS-SX 102A instrument. Amino acids were coupled with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), or *O*-(7-azabenzotriazoyl-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagents.

3.2. Synthesis of *cyclo*(-Ser-Asp-Lys(Ac)-Thr-Ile-Gly-) (3)

Synthesis was performed on Kaiser's 4-nitrobenzophenone oxime resin starting from glycine by the application of standard Boc methodology. Boc-Gly-OxR (2g) was deprotected with 25% TFA in CH_2Cl_2 (1×1 min, 1×30 min), washed with CH_2Cl_2 (2×), *i*-PrOH, CH_2Cl_2 (3×) and DMF and the deprotection was confirmed by Kaiser's test. A solution of Boc-Ile-OH (404 mg, 1.68 mmol), HBTU (636 mg, 1.68 mmol) HOBt (258 mg, 1.68 mmol) and DIEA (0.5 mL, 2.8 mmol) in DMF (30 mL) were added to the resin and shaking was maintained for 30 min. After this period the resin was washed with DMF (3×), CH_2Cl_2 (3×) and the completeness of coupling was confirmed by Kaiser's test. Deprotection and coupling were repeated as described above for the amino acids Boc-Thr(Bzl)-OH, Boc-Lys(Ac)-OH, Boc-Asp(OBzl)-OH and Boc-Ser(Bzl)-OH, respectively. The protected hexapeptide, Boc-Ser(Bzl)-Asp(OBzl)-Lys(Ac)-Thr(Bzl)-Ile-Gly-OH was obtained from Boc-Ser(Bzl)-Asp(OBzl)-Lys(Ac)-Thr(Bzl)-Ile-Gly-OxR by shaking with *N*-hydroxypiperidine (HOPip) (226 g, 2.24 mmol) in DMF (30 mL) for 30 h. The DMF solution was collected and the resin was washed with DMF (30 mL×3) and collected. DMF (120 mL) was evaporated in vacuo and the Boc-Ser(Bzl)-Asp(OBzl)-Lys(Ac)-Thr(Bzl)-Ile-Gly-OPip was dissolved in acetic acid (10 mL) and sodium dithionate (487 mg, 2.8 mmol) was added. The solution was stirred for 1 h at rt and the completion of the reaction was monitored by HPLC. After removal of acetic acid, water was added to precipitate the Boc-Ser(Bzl)-Asp(OBzl)-Lys(Ac)-Thr(Bzl)-Ile-Gly-OH. It was filtered from water and dried over P_2O_5 (640 mg, 0.623 mmol, 110%). The hexapeptide Boc-Ser(Bzl)-Asp(OBzl)-Lys(Ac)-Thr(Bzl)-Ile-Gly-OH (640 mg, 0.623 mmol) was deprotected using TFA (10 mL) in an ice bath for 30 min. TFA was evaporated and the peptide was solidified as TFA salt by adding ether and filtered from ether (650 mg, 0.623 mmol, 100%). The linear hexapeptide trifluoroacetate (600 mg, 0.576 mmol) was cyclized in DMF (125 mL) under a high dilution condition. For this, one-fifth of the hexapeptide, HATU (total 328 mg) and diisopropylethylamine (total 0.4 mL) were added portionwise in 20 min intervals at room temperature with stirring. The reaction was monitored by HPLC. DMF was removed in vacuo and the residue was dissolved in ethyl acetate and washed with 10% citric acid, 4% sodium bicarbonate and brine respectively. Ethyl acetate was evaporated and the cyclic hexapeptide was purified using silica gel column chromatography using 10% methanol in chloroform as eluent. (287 mg, 0.316 mmol, 51%). FAB MS gave peaks of $(M+H)^+$ and $(M+Na)^+$ at m/z 914 and 936 respectively. The cyclic hexapeptide was dissolved in acetic acid (5 mL) and 5% Pd/C (100 mg) was added and hydrogenated at rt under ordinary pressure. Acetic acid was removed in vacuo and the cyclic hexapeptide was lyophilized from water. The cyclic hexapeptide thus obtained was further purified by RP-HPLC using 5-30% CH_3CN gradient containing 0.1%TFA over 30 min. HPLC: 2.9 min (chromolith, 10–100% CH_3CN gradient containing 0.1%TFA over 15 min), FABMS gave peaks of $(M+H)^+$ and $(M+Na)^+$ at m/z 644 and 666 respectively. HR-FABMS $(M+H)^+$ 644.3216 for $C_{27}H_{46}O_{11}N_7$ (calcd 634.3255).

3.3. Synthesis of *cyclo*(-Ser-Asp-Asu(NHOH)-Thr-Ile-Gly-) (4)

The linear hexapeptide, Ser(^{t}Bu)-Asp(O^{t}Bu)-Asu(OBzl)-Thr(^{t}Bu)-Ile-Gly was synthesized using peptide synthesizer (PE ABI 433A) by Fmoc strategy using Balros resin (0.25 mmol) and cleavage from the resin using acetic acid in a mixture of dichloromethane and trifluoroethanol (1:8:1) at rt yield 230 mg (100%, 0.25mmol) of protected linear peptide. The linear peptide (230 mg) was dissolved in DMF (250 mL) by sonication and stirring. HATU (144 mg, 0.38 mmol) and DIEA (0.17 mL, 0.1 mmol) were added to the solution and stirred for 3 h at rt. The reaction was monitored by HPLC. Solvent was removed in vacuo and the residue was dissolved in ethyl acetate and washed with 10% citric acid, 4% sodium bicarbonate and brine respectively. Ethyl acetate was evaporated to give cyclic hexapeptide *cyclo*(-Ser(^{t}Bu)-Asp(O^{t}Bu)-Asu(OBzl)-Thr(^{t}Bu)-Ile-Gly-) (113 mg, 0.125 mmol, 50%). HPLC: 11.8 min (chromolith, 10–100% CH_3CN gradient containing 0.1%TFA over 15 min), FABMS gave peak of $(M+H)^+$ and $(M+Na)^+$ at m/z 903 and 925 respectively. The protected cyclic hexapeptide (113 mg, 0.125mmol) was dissolved in methanol (5 mL) and catalytically hydrogenated with Pd-C (50 mg) at rt under ordinary pressure to remove the benzyl ester group. The reaction was completed in 5 h, which was monitored by

HPLC. Pd-C was filtered off and methanol was removed to give *cyclo*(-Ser(^{t}Bu)-Asp(O^{t}Bu)-Asu(OH)-Thr(^{t}Bu)-Ile-Gly-) (100 mg, 0.123 mmol, 98%). HPLC: 8.6 min (chromolith, 10–100% CH_3CN gradient containing 0.1%TFA over 15 min). The *cyclo*(-Ser(^{t}Bu)-Asp(O^{t}Bu)-Asu(OH)-Thr(^{t}Bu)-Ile-Gly-) (100 mg, 0.123 mmol) was dissolved in DMF (2 mL). Hydroxylamine hydrochloride (43 mg, 0.62 mmol), HOBt.H_2O (95 mg, 0.62 mmol) BOP (274 mg, 0.62 mmol) and Et_3N (0.18 mL, 1.24 mmol) were added to the solution at 0 °C. The reaction mixture was stirred for 3 h and monitored by HPLC. DMF was removed in vacuo and the residue was treated with TFA (2 mL) at 0 °C for 3 h. TFA was evaporated in vacuo at 0 °C and the residue was applied to a column of Sephadex LH-20 with DMF. The fractions containing the desired cyclic hexapeptide hydroxamic acid were analyzed by HPLC and collected. DMF was removed and the residue was lyophilized from water to give *cyclo*(-Ser-Asp-Asu(NHOH)-Thr-Ile-Gly-) (81 mg, 0.123 mmol, 100%). The product was further purified by HPLC. HPLC: 2.8 min (chromolith, 10–100% CH_3CN gradient containing 0.1% TFA over 15 min). FABMS gave peaks of $(M+H)^+$ and $(M+Na)^+$ at *m*/*z* 660 and 682 respectively. HR-FABMS $(M+H)^+$ 660.3207 for $C_{27}H_{46}O_{12}N_7$ (calcd 660.3204).

3.4. NMR spectroscopy and structure calculation

NMR spectra were recorded on a JEOL spectrometer operating at 500 MHz in DMSO-d_6 using TMS as internal standard. Spectra were recorded at variable temperatures such as, 298, 303, 313, 323, and 333 K respectively. Assignments of proton resonances were confirmed, when possible, by COSY, HOHAHA and NOESY experiments. Mixing times for NOESY experiments were 200 and 400 ms. Number of scans varied between 32 and 64, and the number of points in t_2 dimension was 512. ^{1}H resonances were assigned using standard procedures. $^3J_{NH-C^{\alpha}H}$ coupling constants for non-overlapping signals were determined from 1D spectra with high digital resolution.

3.5. HDAC inhibitory activity assay

The preparation and assay of the enzymes were performed as described in ref 14.

3.6. Molecular modeling studies

All calculations were performed on a Silicon Graphics computer. The distance geometry program was used to generate structures consistent with the distance constraints derived from the NOEs. Temperature coefficient of NH protons indicating hydrogen bonds and ϕ angles calculated from $J_{NH-H^{\alpha}}$ were used to filter out structures that did not meet the experimental data. An error of $\pm 30°$ was tolerated for the ϕ angles calculated from $J^{\alpha}_{NH\text{-}H}$ at this stage of refinement. Energy minimization and molecular dynamics calculation were carried out using the CHARMm program of Insight II using CHARMm forcefield. α-Tubulin structure was used from Protein Data Bank.[29]

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