Journal of Computer-Aided Molecular Design, 10 (1996) 83–86
ESCOM

J-CAMD 328

Solution conformation by NMR and molecular modeling of three sulfide-free somatostatin octapeptide analogs compared to angiopeptin

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Received 23 November 1995
Accepted 19 December 1995

Keywords: Somatostatin; Angiopeptin; Cyclic peptide; Peptide NMR; Peptide molecular modeling; Peptide pharmacophore; Restenosis

Summary

The conformation in dimethylsulfoxide of the somatostatin derivative angiopeptin and of three disulfide-free analogs was estimated by two-dimensional nuclear magnetic resonance spectroscopy at room temperature. The resulting 3D molecular graphics were compared and shown to reflect the observed differences in the inhibition of restenosis after rat aorta balloon injury by these octapeptide inhibitors. Angiopeptin and its active analog **2** displayed a relatively rigid conformation of the cyclic hexapeptide backbone due to the presence of two well-defined hydrogen bonds, further stabilized by a third hydrogen bond outside the ring. No such constraints were detected for the two biologically inactive analogs, which, compared to **2**, had a two-atom longer or shorter hexapeptide ring. The well-defined structure of compound **2** may serve as an improved pharmacophore for this new class of drugs.

The cyclic disulfide octapeptide angiopeptin, 3-(2-naphthyl)-D-Ala-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2, is a somatostatin analog that was first synthesized by Coy et al. [1]. This compound is currently in clinical trials for the treatment of psoriasis, acromegaly, neuroendocrine tumors and breast, prostate and lung cancers [2]. Angiopeptin prevents restenosis following coronary artery angioplasty in rat [3], rabbit [4] and pig [5], a property shared by only few other minisomatostatins. Recently, we reported on the synthesis and pharmacological evaluation of three analogs of angiopeptin in which the disulfide bond was replaced by an amide bond and in which the ring size was varied [6]. One of these analogs, compound **2** (Table 1, S16077), was able to inhibit neointima formation after balloon injury in the rat aorta as efficiently as angiopeptin, while the two other amide analogs with different ring sizes were practically inactive. Assuming that this intriguing result was due to a conformational constraint of the active analog, we utilized NMR and molecular modeling to investigate the conformation of the analogs in solution. We report here on the 3D features of angiopeptin and its analogs in solution and relate them to the observed biological activity in vivo.

The peptides were synthesized using classical solid phase synthesis and characterized by analytical HPLC, amino acid analysis and FAB mass spectrometry [6]. Assessment in vivo of the neointima formation after balloon injury of the rat aorta was performed, as previously described [6], by measuring [^{3}H]-thymidine uptake and by histomorphometry.

NMR measurements were carried out on a BRUKER AMX spectrometer operating at 500 MHz. Peptide concentrations varied from 10 to 16 mg/ml in DMSO-d_6 and the temperature was kept at 300 ± 0.1 K, or was varied from 295 to 315 K in steps of 5 K for measurement of the temperature coefficients. One-dimensional spectra used 32K data points. Two-dimensional experiments were performed in phase-sensitive mode. The mixing time was 80 ms in HOHAHA experiments, while it was varied from 150 to 550 ms in NOESY experiments. Post-processing was done with the AURELIA software (Bruker) [7] on a Silicon Graphics INDIGO^2 workstation. For HOHAHA and NOESY experiments, spectra were obtained using 1K data points in the F2 and 512 points in the F1 dimension. Zero-filling was applied in the F1 dimension, resulting in a $1K \times 1K$ data point matrix. For double-

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TABLE 1
PERCENT INHIBITION[a] OF [^{3}H]-THYMIDINE UPTAKE AND OF NEOINTIMA FORMATION AFTER BALLOON INJURY IN THE RAT AORTA BY ANGIOPEPTIN AND ANALOGS AT A DOSE OF 100 μg kg^{-1} d^{-1}

Compound	Structure[b]	n[c]	Inhibition (%) of [^{3}H]-thymidine uptake[d]	Inhibition (%) of neointima formation[e]
	1 2 3 4 5 6 7 8			
Angiopeptin	H-nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	20	26.7[f]	35.0[f]
1	H-nal-Asp-Tyr-D-Trp-Lys-Val-Dpr-Thr-NH_2	20	10 (n.s.)[g]	n.d.[h]
2[i]	H-nal-Glu-Tyr-D-Trp-Lys-Val-Dab-Thr-NH_2	22	25.2[f]	52.9[f]
3	H-nal-Glu-Tyr-D-Trp-Lys-Val-Lys-Thr-NH_2	24	n.d.	7.0 (n.s.)

[a] For raw data, see Ref. 6.
[b] nal = D-2-naphthylalanine, Dpr = 2,3-diaminopropionic acid, Dab = 2,4-diaminobutyric acid.
[c] Number of ring atoms.
[d] Measurement 3 days after injury.
[e] 14 days after injury.
[f] $p < 0.05$.
[g] n.s. = not significant.
[h] n.d. = not determined.
[i] Company code: S16077.

quantum-filtered COSY experiments, 8K data points were used in the F2 dimension. Zero-filling resulted in a final matrix of 8K × 1K data points. The final resolution in the F2 dimension was 1 Hz, thus leading to a precision of ± 0.5 Hz for the coupling constants. NMR experiments [8] included COSY, HOHAHA and NOESY to complement the one-dimensional spectrum for the assignment of all proton resonances. The coupling constants from the COSY spectrum were then used to obtain the torsion angles ϕ and χ according to the equations of Bystrov [9] and DeMarco and Wüthrich [10]. The temperature coefficients were estimated for the NH protons, in order to detect intramolecular hydrogen bonds. Finally, key interatomic distances were determined from NOESY measurements as a function of the mixing time and from the previously obtained correlation time. The H2-H7 proton distance of tryptophan was taken as a reference for calibration.

Molecular modeling of the molecules was carried out using standard bond lengths and angles, with the SYBYL program [11] (v. 6.1a) running on a Silicon Graphics INDIGO^2 R4400 Extreme workstation. The geometries were adjusted and minimized using the Powell [12] algorithm, and the energy calculations were based on the Tripos force field [13]. Globally neutral molecules were considered, using Gasteiger and Marsili electrostatic point charges [14], with the electrostatic terms of the Tripos force field, and a dielectric constant value of 4 was employed. The Kollman force field [15] was not used because of the presence of the nonnatural amino acids naphthylalanine, diaminobutyric acid and diaminopropionic acid. Two types of NMR-compatible parameters, H-bond lengths and NOE-derived distances, were introduced as distance-range constraints within SYBYL, using a k force constant varying between 50 and 100 kcal/mol Å^2 depending on the temperature coefficients, and between 1 and 20 kcal/mol Å^2 depending on the NOE intensity. The conformation of the side chains was determined, whenever possible, from the coupling constants between the protons, adjusted manually and then minimized. However, the exact conformations of the side chains could only be determined in a few cases.

The biological activity of the peptides is reported in Table 1. Obviously, only angiopeptin and compound **2** displayed a significant inhibitory potency against both [^{3}H]thymidine uptake and neointima formation after balloon injury of the rat aorta, while compounds **1** and **3** had no significant effect under the same conditions.

One- and two-dimensional ^{1}H NMR experiments led to the assignment of all proton resonances of angiopeptin and its analogs, thus providing a firm ground for the conformational study. Exploitation of the NMR data and their translation to a 3D structure by molecular modeling resulted in well-defined cyclic backbones and less well defined spatial orientations of the side chains. The $^3J_{NH\text{-}H^\alpha}$ coupling constants were compatible throughout with trans peptide bonds in angiopeptin and its three analogs. The other main conformational features of angiopeptin and its analogs are assembled in Table 2.

The most striking feature of the modeled 3D structures is the presence of three clearly defined hydrogen bonds between valine-NH and tyrosine-CO, valine-CO and tyrosine-NH, and naphthylalanine-CO and threonine-NH, respectively, both in angiopeptin and in compound **2**. The two intracyclic hydrogen bonds are located nearby in the two molecules, while the hydrogen bond outside the cycle is more distant (Fig. 1A). In contrast, only one hydrogen bond is seen between naphthylalanine-CO and diaminopropionic acid-N$^\alpha$H in compound **1**, and no hydrogen bond at all is detectable in compound **3**. The similarity of the cyclic peptides is further quantified by calculating the root mean square (rms) of the distances between all the common backbone nonhydrogen atoms (21 atoms, including O atoms in peptide bond C=O) of compound **2** and those of any of the other cyclic analogs in the best possible superimposition. The results (column 6 of Table 2) confirm the expected greater similarity of **2** with angio-

TABLE 2
CONFORMATIONAL FEATURES OF ANGIOPEPTIN AND ITS ANALOGS

Compound	Ring atoms[a]	Hydrogen bonds[b]			Coupling constants $^3J_{NH\text{-}C\alpha H}$ (Hz)					Rms[d]
		Total	Endocyclic	Exocyclic	2[c]	Tyr	D-Trp	Lys	7[c]	
Angiopeptin	20	3	2	1	9.3	5.8	7.7	8.6	9.2	0.82
1[e]	20	1	1	0	6.8	1.8	1.9	9.7	9.5	1.33
2[f]	22	3	2	1	8.4	5.7	8.4	8.2	9.1	0
3	24	0	0	0	7.9	8.4	7.4	7.4	7.4	1.18

[a] Number of atoms in cyclic chain.
[b] Number of hydrogen bonds according to NMR.
[c] NMR coupling constant $^3J_{NH\text{-}C\alpha H}$ of residues in positions 2 and 7, respectively.
[d] Root mean square of distances between the common nonhydrogen atoms of the backbone of **2** (reference compound) and each of the other analogs.
[e] Three-bond coupling constants for **1** are calculated from the molecular model.
[f] Company code: S16077.

peptin (in spite of the fact that they have a different number of atoms in the cyclic chain). This similarity of angiopeptin and compound **2** is also testified by the comparable values of the coupling constants $^3J_{NH\text{-}C\alpha H}$ of the backbone protons in the residues in positions 2 to 7, in contrast to those of the analogs **1** and **3** (Table 2).

Superimpositions of angiopeptin and compound **2** in Fig. 1A show that the side chains, in particular the aromatic ones, can occupy the same spatial region without extensive distortion of the energy-minimized structures, in contrast to the less easy spatial fitting of the side chains in Figs. 1B and C.

The four investigated peptides are chemically well-characterized compounds, which, due to the presence of a bridge between the side chains of residues 2 and 7, are constrained in the cyclic backbone. In contrast, the spatial orientation of the side chains and of the N- and C-terminal residues with respect to the ring can vary at relatively low energy cost (not shown).

The conformation found for angiopeptin is very similar to that obtained by Van Binst and Tourwé [16] from measurements at low temperature (−30 to 10 °C) in water/DMSO mixtures, except for the position of the disulfide bridge on the opposite side of the ring. This difference does not suppress the antiparallel β-pleated sheet structure of the hexapeptide ring (also observed in compound **2**).

The optimized conformations are compatible with the NOEs observed with NMR and violations of the observed distances do not exceed the experimental error (0.4 to 0.5 Hz). However, significantly different conformations of the more flexible analogs **1** and **3** may coexist with the most probable retained one.

The superimposition process was greatly facilitated by the common part of the cyclic chains, which could be used as a starting point for the function MATCH of SYBYL [11], thus leading to optimized fitting (Fig. 1).

The two bioactive compounds (angiopeptin and **2**) display not only a similar 3D structure, but also a more stabilized structure, which strengthens the probability of a well-defined receptor complementarity. This bioactive conformation cannot easily be reached by the two inactive analogs (**1** and **3**), in spite of their higher flexibility.

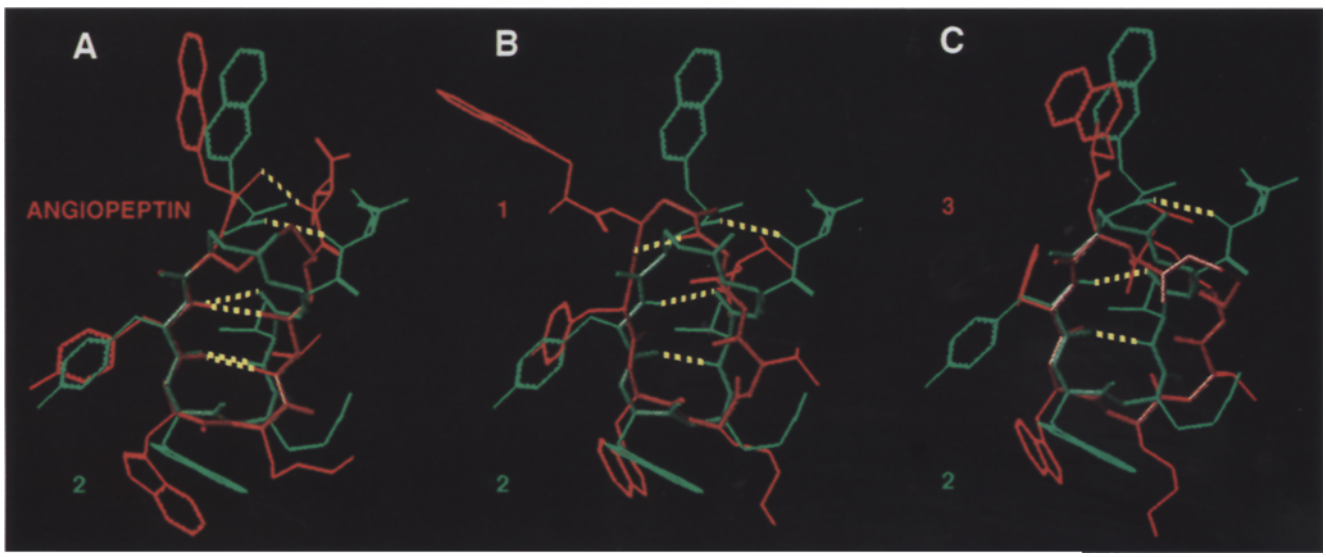

Fig. 1. Paired superimpositions of the NMR-defined molecular model of compound **2** (green) with angiopeptin (A, red) and its analogs **1** (B, red) and **3** (C, red).

We conclude that the observed differences in the potency of angiopeptin and compound **2** to inhibit restenosis, compared to compounds **1** and **3**, may have a structural foundation and that the constrained structure of **2** may represent an improved pharmacophore for this class of drugs in this therapeutic indication.

Acknowledgements

Thanks are due to Karine Simonet for her participation in the NMR experiments.

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