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Spantide II, an effective tachykinin antagonist having high potency and negligible neurotoxicity

(neuropeptide analogue design/substance P/histamine release/nociceptor flexor)

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ABSTRACT **Spantide (D-Arg[1]-Pro[2]-Lys[3]-Pro[4]-Gln[5]-Gln[6]-D-Trp[7]-Phe[8]-D-Trp[9]-Leu[10]-Leu[11]-NH_2) was introduced as a tachykinin antagonist in 1984 and has served as a starting point in the design of new antagonists that have proven to be more effective and have exhibited no neurological side effects. The most remarkable and unpredictable structural change that significantly increased potency was deletion of a methylene group by changing Gln[6] to Asn[6]. On the basis that D-Arg[1] and Lys[3] of spantide contribute to neurological side effects, many new designs led to D-Lys(Nic)[1]-Pro[2]-Pal(3)[3]-Pro[4]-D-Phe(Cl_2)[5]-Asn[6]-D-Trp[7]-Phe[8]-D-Trp[9]-Leu[10]-Nle[11]-NH_2 [spantide II, where D-Lys(Nic) is N^{ϵ}-nicotinoyllysine, Pal(3) is 3-(3-pyridyl)alanine, D-Phe(Cl_2) is 3,4-dichloro-D-phenylalanine, and Nle is norleucine], which is a potent antagonist without neurotoxicity. Spantide II, an undecapeptide, has a total of seven substitutions in the sequence of substance P, consisting of two natural L amino acids, and one unnatural L amino acid, and four unnatural D amino acids. The π- and σ-bond amino acid substituents of substance P and spantide II are compared toward a future understanding of the essential substituents for mechanism and inhibition binding. Spantide II has five π-bond and six σ-bond amino acid moieties, and substance P has two π-bond and nine σ-bond moieties.**

During the last decade, increasingly potent tachykinin antagonists have become available (1, 2). By 1984, the substance P (SP) analogue [D-Arg[1],D-Trp[7,9],Leu[11]]SP was introduced; it had a pA_2 value (negative logarithm of antagonist concentration producing a 2-fold shift of the agonist concentration–activity curve) of 7.1 and was named spantide (3). It was used by many pharmacological investigators as a model antagonist in the periphery. Also, spantide served as a reference antagonist for the chemical design of new analogues to provide even more effective inhibitors. By 1986, these efforts had led to the replacement of Gln[5] with aromatic D amino acids (4), and the most potent analogue was [D-Arg[1],D-Phe(Cl_2)[5],D-Trp[7,9],Nle[11]]SP [where D-Phe(Cl_2) is 3,4-dichloro-D-phenylalanine and Nle is norleucine]. In 1987 it was found that introduction of Asn[6] in place of Gln[6] gave rise to a considerable and unpredicted increase in potency (5). Presumably, the increase may be based upon the precision of conformation and of antagonist–receptor binding. The analogue [D-Arg[1],D-Phe(Cl_2)[5],Asn[6],D-Trp[7,9],Nle[11]]SP had a pA_2 value of 7.7. Replacement of Lys[3] with 3-(3-pyridyl)alanine [Pal(3)] gave a derivative (spantide II) with a pA_2 value of 8.1.

SP and many SP analogues release histamine from mast cells (6). The presence of the strongly basic amino acids arginine and lysine has been suggested to explain why such peptides readily release histamine from mast cells (6). This activity is in analogy with the activities of other lysine/arginine-containing peptides—e.g., luteinizing hormone-releasing hormone (LHRH) (7), somatostatin analogues (8), neuropeptides (9), and gastrin—which are known to induce release of histamine from human cutaneous mast cells (10). For example, in analogues of LHRH, hydrophobic amino acids at the N terminus and the strongly basic residues D-Arg[6] and Arg[8] were implicated in the release of histamine from mast cells.

If antagonists of LHRH and of SP are to be used therapeutically, it is essential that they have little or no histamine-releasing activity and do not produce neurotoxic effects (11). Karten *et al.* (7) and Ljungqvist *et al.* (12) designed and synthesized LHRH antagonists that emphasized acylated lysine [N^{ϵ}-nicotinoyllysine, Lys(Nic)] residues, Lys(Nic)[5] and D-Lys(Nic)[6], frequently associated with alkylated lysine [N^{ϵ}-isopropyllysine, Lys(iPr)] or arginine in position 8. This approach to eliminate histamine-mobilizing activity was successful with antide [Ac-D-Nal[1]-D-Phe(pCl)[2]-D-Pal(3)[3]-Ser[4]-Lys(Nic)[5]-D-Lys(Nic)[6]-Leu[7]-Lys(iPr)[8]-Pro[9]-D-Ala[10]-NH_2, where D-Phe(pCl) is 4-chloro-D-phenylalanine]. Antide and related antagonists of LHRH are long-acting and even have oral activity (13).

Based on this strategy of design to achieve antagonism without side effects, as exemplified by antide to function as an antagonist of LHRH, we have synthesized SP antagonists that contain D-Lys(Nic), D-Lys(iPr), or N^{δ}-(4,6-dimethyl-2-pyrimidyl)-D-ornithine (D-Dpo) in position 1 (Table 1). One of these new analogues, designated as spantide II (peptide **1**), has been shown to be a good tachykinin antagonist and to have no side effects (14–17).

On the basis that both D-Arg[1] and Lys[3] of spantide may contribute to its side effects, the new design required the replacement of these residues with D-Lys(Nic)[1] and Pal(3)[3].

EXPERIMENTAL AND RESULTS

Materials. The natural amino acids were purchased from Peninsula Laboratories. The α-amino functions were protected by the *tert*-butoxycarbonyl (Boc) group. Boc-Pal(3), Boc-D-Lys(Nic), Boc-D-Phe(Cl_2), and Boc-D-Lys(iPr,Z) (where the secondary amine function of the alkylated lysine is protected by benzyloxycarbonyl, Z) were synthesized by standard procedures. Benzhydrylamine resin hydrochloride was obtained from Beckman. The dicyclohexylcarbodiimide was distilled before use. The dichloromethane was distilled from sodium carbonate. All other reagents were reagent grade.

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Abbreviations: SP, substance P; LHRH, luteinizing hormone-releasing hormone; Dpo, N^{δ}-(4,6-dimethyl-2-pyrimidyl)ornithine; Lys(iPr), N^{ϵ}-isopropyllysine; Lys(Nic), N^{ϵ}-nicotinoyllysine; Nle, norleucine; Pal(3), 3-(3-pyridyl)alanine; Phe(Cl_2), 3,4-dichlorophenylalanine.

Table 1. Peptide structures and biological assay data

Peptide	pA_2 vs. exogenous SP (taenia coli)	pIC_{50} vs. electrical nerve stimulation (iris sphincter)
[D-Arg[1],D-Trp[7,9],Leu[11]]SP (spantide)	7.1 ± 0.4	5.2 ± 0.4
1 [D-Lys(Nic)[1],Pal(3)[3],D-Phe(Cl_2)[5],Asn[6],D-Trp[7,9],Nle[11]]SP (spantide II)	7.7	6.0
2 [D-Lys(iPr)[1],Pal(3)[3],D-Phe(Cl_2)[5],Asn[6],D-Trp[7,9],Nle[11]]SP	—	—
3 [D-Dpo[1],Pal(3)[3],D-Phe(Cl_2)[5],Asn[6],D-Trp[7,9],Nle[11]]SP	—	—

pA_2 and the slope of the Schild plot for analogue were calculated as described (14).

Peptide Synthesis. The peptides were synthesized by the solid-phase method on a Beckman model 990 peptide synthesizer (18). After attachment of the first amino acid was completed, the resin was acetylated by a 25% (vol/vol) acetic anhydride solution in dichloromethane/pyridine. The peptides were cleaved from the resin with concomitant deprotection by treatment for 45 min at 0°C, with doubly distilled HF containing anisole and thioanisole, as described (19).

Purification and Characterization. The crude peptides were first purified by chromatography on silica gel with the solvent system 1-butanol/acetic acid/water, 4:1:2 (vol/vol), or on Sephadex LH-20 (Pharmacia) with the solvent system 1-butanol/acetic acid/water/methanol, 10:10:90:15 (vol/vol). Then they were further purified over a column of Sephadex G-25 (2.5 × 100 cm) with 6–10% acetic acid as the eluant. The peptides were examined for purity on silica gel TLC plates (Merck). At least four different solvent systems were used with the chlorine/*o*-toluidine test, and the R_f values of the peptides are listed in Table 2. The purity of the peptides was determined by HPLC on a column of μ-Bondapak C_{18} (3.9 mm × 30 cm). The equipment for HPLC was from Waters. The solvent system employed buffer A (0.01 M KH_2PO_4, pH 3.0) and buffer B (20% buffer A in acetonitrile). A gradient programmer was used to produce a linear gradient with buffer B in 15 min at a flow rate of 2 ml/min. The data on purity and retention time of the peptides are in Table 2.

Amino Acid Analyses. Analyses were performed on a Beckman 118CL automatic amino acid analyzer equipped with a Hewlett–Packard 3390A integrator. The peptides (0.5 mg) were hydrolyzed with constant-boiling HCl in an evacuated tube for 24 hr at 110°C. Tryptophan and the unnatural amino acids were qualitatively determined. The amino acid analytical data are in Table 3.

Bioassay. The specificity and potency of the analogues to inhibit tachykinin-evoked contraction of the guinea pig taenia coli and to suppress the contractile response of the rabbit iris sphincter evoked by electrical stimulation of sensory nerve fibers were tested (results are summarized in Table 1), and their activity to release histamine was studied with rat peritoneal mast cells, as described (3, 14). The activity of spantide II was tested on the rat spinal cord to determine whether it could antagonize the action of SP and whether it had neurotoxic side effects (15, 16).

Table 2. Analytical data on spantide II (peptide **1**) and related analogues

Peptide	R_f values* A	B	C	D	HPLC retention time, min	Purity, %
1	0.39	0.77	0.59	0.75	10.2	99
2	0.31	0.69	0.55	0.70	9.2	98
3	0.37	0.74	0.58	0.72	10.0	99

*From TLC in four different solvent systems: A, 1-butanol/acetic acid/water, 4:1:2 (vol/vol); B, 1-butanol/pyridine/acetic acid/water, 5:5:1:4; C, 1-butanol/ethyl acetate/acetic acid/water, 1:1:1:1; D, 1-butanol/pyridine/acetic acid/water, 5:3.3:1:4.

DISCUSSION

The first four or five N-terminal amino acid residues of SP are not required for biological activity (20, 21). The N-terminal portion of the molecule appears to contribute to binding and to receptor recognition. Histamine release from mast cells by SP analogues requires the presence of a charged basic amino acid residue, arginine or lysine, at the N terminus (6). Consequently, within the general structure of new antagonists following spantide (3)—Pal(3) and asparagine in positions 3 and 6, respectively, lipophilic D amino acids in positions 5, 7, and 9, and Nle in position 11—substitution of D-Lys(Nic) for D-Arg[1] gave spantide II (peptide **1**, Table 1). Toward a definition of theory(ies) on the direct design of a peptide antagonist of a peptide agonist, it is noted that SP has two amino acid moieties that contain carbon–carbon π bonds and six amino acid moieties that contain only σ bonds between carbons. In contrast, spantide II has five π-bond amino acid moieties and six σ-bond amino acid moieties. The two π-bond moieties of SP, Phe[7] and Phe[8], are changed to the σ-bond structure of Asn[6] and the D configuration of a π-bond substituent (D-Trp) in position 7, which may possibly be critical to diminution or elimination of the structure(s) of SP that is required for mechanism. The additional three π-bond structures of spantide II may be significant for receptor binding but not for mechanism.

The potency of spantide II with regard to SP antagonism is quite good, but even more important was the finding that it is really less effective than previously tested analogues in releasing histamine from mast cells (14). Antinociceptive and SP antagonistic effects of intrathecally injected spantide II in rats were demonstrated with no signs of motor impairment or neurotoxicity (15). These results indicate that spantide II is an effective tachykinin antagonist in the central nervous system and that it antagonizes tachykinins without causing neural damage. Thus, spantide II effectively antagonizes the SP-induced facilitation of the nociceptive flexor reflex (16). The facilitation of the nociceptive flexor reflex by C-fiber conditioning stimulation in the rat was antagonized by span-

Table 3. Amino acid analyses of spantide II (peptide **1**) and related analogues

Peptide	No. of residues per mole Asp*	Pro	Leu	Phe	Lys	Pal(3)	Trp	Nle	Phe(Cl_2)	Lys(iPr)	Dpo
1	0.61	2.16	1.12	1.08	0.95	1.08	++	+	+		
2	0.53	2.19	0.98	1.03		1.26	++	+	+	+	
3	0.68	2.16	1.09	0.99		1.09	++	+	+		+

*When Asn[6] is followed by D-Trp[7], the Asp value is always low, 50–60% of theory. The reason for this is unclear but may involve acid-catalyzed interaction between the $CONH_2$ group of Asn and the indole moiety of D-Trp (4).

tide II (17). SP mediates spinal reflex facilitation, but not after section of primary afferents (22).

In conclusion, spantide II appears to be a relatively potent and quite safe tachykinin antagonist; it may serve as a useful model for further pharmacological and exploratory chemical investigations.

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